Study on Natural Antimicrobial Compounds against Bacterial Isolates

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By

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UNDER THE GUIDANCE OF

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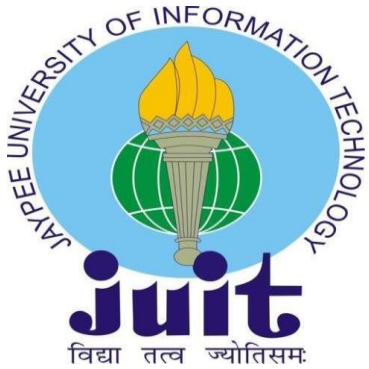

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

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CERTIFICATE

This is to certify that the work which is being presented in the title of **"Study on Natural Antimicrobial Compounds against Bacterial Isolates "** for the end semester of B. Tech. in Biotechnology and submitted in the department of Biotechnology and Bioinformatics, **Jaypee University of Information Technology, Waknaghat** is an authentic record of work carried out by Sakshi (181806) during the period of July 2021 to May 2022 under the supervision of **Dr. Jitendraa Vashistt,** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat , Solan (H.P.)

The above statement made is correct to the best of my knowledge.

Date : May 2022

_____

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Also, I am extremely grateful to my parents and my family for their constant encouragement and for providing me with all the necessary resources required for the successful completion of the project.

DECLARATION

I hereby declare that the present work on "**Study on Natural Antimicrobial Compounds against Bacterial Isolates "** is a record of original work done by me under the guidance of **Dr. Jitendraa Vashistt** at **Jaypee University of Information Technology , Waknaghat , Solan (H.P.)** , from July 2021 to May 2022 at Microbiology lab in the Department of Biotechnology and Bioinformatics , Jaypee University of Information Technology , Solan .

I also Declare that no part of this report has previously been submitted to any University for acquiring any degree.

Date : Sakshi (181806)

Place : B.Tech Biotechnology

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ABSTRACT

Microbes are present everywhere in the environment and that can be disease causing or non disease causing . The chemical antimicrobials used commonly have several side effects on human health as well as on the environment and also the microbes are developing resistance against the present chemical antimicrobials that is why the use of herbal compounds as antimicrobials have great importance . In this study we used three herbal compounds that are *Terminilia Arjuna* , *Tamarindus Indica* and *Withania somnifera* and checked there antimicrobial activity against the bacterial isolates , isolated from environment of three different areas from the university ,using the antimicrobial assay with extracts prepared from these compounds by cold maceration method in which methanol was used as an extraction solvent . The biofilms of these bacterial isolates were grown to check the biofilm forming potential of these compounds. The results of this study showed that the herbal compounds used have a significant amount of antimicrobial activity at higher concentration . Also the bacterial isolates used have great potential to form biofilms and the antibiofilm activity of these compounds can also be checked . In future the antimicrobial and antibiofilm activity against more bacterial isolates with more herbal compounds can also be checked .

OBJECTIVE & RATIONAL

Objective of the project -

The key points of the objective of the project are -

1. To understand the basic concept of antimicrobial compounds .
2. To learn the preparation of micro dilutions and biofilms.
3. To understand the concept of Antimicrobial susceptibility test and Minimum Inhibitory Concentration test .
4. To learn herbal extract preparation and check their antimicrobial property .
5. To Check and Analyze the antimicrobial and antibiofilm properties of *Terminalia arjuna , Tamarindus indica and Withania somnifera* against bacterial Isolates.

Rationale of the project -

Our environment has countless microbes always present around us in the air, in the soil , in the water and everything else. Some of them are pathogenic and some are not . We have many types of chemical and other antimicrobials available but most of the chemical antimicrobials have many side effects . Herbal compounds have a great significance in the history of ayurveda and they are in use from ancient times , they have minimal or almost negligible side effects. Microbes acquire some resistance against the chemical compounds and they also survive in the form of Biofilms that help them survive in the extreme conditions that also helps them in developing resistance against certain antimicrobials . Biofilms are microbial sessile communities, which help microorganisms become much more resistant to antimicrobial treatments, harsh environmental conditions and to the host immunity. Biofilm greatly increase microbial pathogen survival in hosts, so the antibiofilm components have great significance and in today's world that is full of chemical components which have several side effects, we can use the herbal components as an antibiofilm component with less or no side effects and with good antibiofilm properties. Also, we can compare the antimicrobial and antibiofilm properties of these herbal and chemical components and see what is the difference between them in their properties and their effectiveness.

CHAPTER I

INTRODUCTION

I. INTRODUCTION

Microorganisms can be found all over the environment and are involved in a wide range of natural activities. Microorganisms control the basic drug cycles to provide nutrients to plants via the response of organic matter in soil. Because greenhouse gasses are released into the atmosphere simultaneously during these processes, microbes play an important role in climate and climate change. Microorganisms have a lot of potential usage in add-on to performing their routine function in the environment. Biocontrol organisms like bacteria and fungi suppress the growth of hazardous microorganisms. Technical enzymes for food production can be isolated from bacteria, yeasts, and fungi, which are one of the key producers of medications like antibiotics also. These bacterias and other organisms sometimes lead to the diseases also and because of their pathogenic nature their elimination is also important and for that the antimicrobial agents are used. Antimicrobials are natural, semisynthetic, or synthetic agents or substances that kill or inhibit the growth of microbiological organisms. Antibacterial, antiviral, antifungal, and antiprotozoal agents are all included. The organisms develop resistance against these antimicrobials. Antimicrobial resistance is the ability of microorganisms such as bacteria, fungi, viruses, and protozoans to develop despite being exposed to antimicrobial compounds that are supposed to stop them from growing . The bacteria also develop the biofilms to survive in the harsh environment . Biofilms are complex systems composed of microbial communities that are attached to biotic or abiotic surfaces and are enclosed in a matrix containing self-produced, or self-generated, extracellular polymeric substances that are EPSs. The herbal compounds have a great history in the ayurveda and are of several advantages over chemical , similarly in the case of herbal antimicrobial compounds , the herbal antimicrobial compounds have several advantages like lesser or almost no side effects on human health , so that is why the herbal antimicrobial compounds have great importance especially in today's world where chemical antimicrobial compounds are used in heavy amount and have several side effects on human health and environment.In this study we used 3 herbal compounds and tested them against 3 different bacterial isolates that we collected as environment culture from the 3 different areas of the university . Isolate 1 from the microbiology lab that was gram gram positive bacteria , Isolate 2 was from the Washing area that was also gram negative bacteria , Isolate 3 was from the Green house area that was gram positive bacteria . The isolates were cultured and the the antimicrobial activity of the herbal compound's (*T. arjuna , T. indica and W. somnifera*) extract were tested , that was prepared by cold maceration method using 100% methanol as solvent for extract preparation . The

results of the Antimicrobial activity of the compounds against the isolates showed that at higher concentration these compounds show a significant amount of antimicrobial activity . Then the biofilm forming potential of these bacterial isolates were also tested and the results showed that the bacterial isolates have significant potential of biofilm formation .

CHAPTER II

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE -

II.I. Environment Microbe

Materials cycle through the lithosphere (rocks), atmosphere (air), hydrosphere (water), and biosphere (organisms) in the Earth's "closed system". They combine to form all life and nonliving components of our world. Earth produces everything it requires for its inhabitants' existence and growth. The environment is referred to as the situation(s) that surround an individual or group of organisms. Environmental research requires an understanding of living processes at the microscopic and ecological levels right from species to whole ecosystems. Species is a group of creature(s) which are genetically close enough to reproduce in the wild and produce live viable progeny. The population is made up of all members of the same species that cohabitat in the same place at the same time. A biological community is made up of all the population groups of organisms that live and interact in a specific area. Biological community and its physical environment make up an ecological system or ecosystem. Environment is made up of abiotic factors such as climate, water, minerals, sunshine; biotic factors such as organisms, their products and their effects in a given area.

II .II. Environment Microbes and Resulting Diseases

Bacteria are frequently rejected as disease-causing "germs." Bacteria are single-celled tiny creatures that lack a complete nucleus. Bacteria assist humans with a wide range of tasks, including the production of vitamins, the breakdown of some types of waste, and the maintenance of the environment. Everything from sugar and starch to sunlight, sulphur, and iron is consumed by them. Some bacteria are harmful, while many are beneficial. Bacteria multiply quite quickly. Two can rapidly turn into four, eight, and so on. They also make up a huge group of prokaryotic creatures. Bacteria are single-celled microbes with only one cell. When viewed under a microscope, bacteria appear as balls, rods, or spirals. Bacteria are the tiniest and most numerous organisms on the planet. The structure of bacterial cells is more complicated. Outside of its cell wall, it possesses a secondary membrane-like coating. It also has a lot of variety in terms of structure and metabolism. Bacteria produce a variety of dangerous human diseases, some of which result in millions of human lives lost every year. Bacteria reproduce quickly and take nutrients from their surroundings. Bacterial infections are also introduced to new places by contaminated seed or transplants. Bacteria are transmitted via rain, water runoff, wind-driven rain or mists , machinery, insects, and people working near the botanical fauna and floura once they have established themselves. Bacteria (singular bacteria)

are self-replicating unicellular creatures that lack both an organised nucleus (which distinguishes prokaryotes from other cells) and organised intracellular organelles. Bacterias possess plasmid; a single circular chromosome of double-stranded DNA (dsDNA), some additional chromosomal DNA, and the polymer peptidoglycan in their cell walls. There are no introns and only codons in the sequence of bacterial DNA . If the bacteria's bases alter, they create various proteins. Recombination is the term for such alterations. Gram positive bacteria (which stain purple after a Gram stain) peptidoglycan wall is multilayered while gram negative bacteria such as salmonella, Shigella, and Escherichia coli, have a comparatively thinner coating of this polymer but have an extra outer layer of lipopolysaccharide (LPS) which is often endotoxic (able to initiate inflammation and cell-mediated immune responses). Bacteria are further divided depending upon shape. Various bacterial shapes are: bacillus- rod-shaped, coccus-spherical shaped, spirillum- spiral shaped, vibrio- comma shaped, cocco-bacillus- ovoid shaped, and other combinations. Depending upon whether they require oxygen (aerobic) or not (anaerobic) to extract energy from a chemical compound; their mode of reproduction; genus; and species among other ways. Human bacterial diseases can be classified according to how they are acquired and transmitted. Bacterial airborne infections affect the respiratory system and are spread by direct contact of aerosols. Bacterial infections affect the skin (all layers), mucous membranes, and the tissue beneath them. Foodborne and waterborne bacterial infections are contracted by ingesting contaminated food or water. Infections and intoxications are the two main forms of diseases. Some of the bacterias are protective and helpful to mankind. Some thrive in our bodies naturally, especially in the colon and vaginal area. These protective bacterias serve to protect the host from illnesses. Infections with specific germs, on the other hand, can result in catastrophic illnesses like meningitis, pneumonia and tuberculosis that may be even fatal. A course of antibiotics can be used to treat a bacterial illness.

II. III. ANTIMICROBIALS

Antibiotics, which are antimicrobial compounds that inhibit the growth (bacteriostatic) or kill (bactericidal) microorganisms. Antimicrobials are widely used in the treatment of infection caused by bacteria in humans and animals. Antimicrobials are also used for nonmedical purposes. Antibiotic production worldwide is expected to be between 100 and 200 thousand tonnes per year, with more than one billion tones produced since 1940. Irrational use of antibiotics has caused a significant rise in antibiotic excretion and release into the environment which is further adding to the spread of drug resistance in various bacterial strains.

Antimicrobial resistance is a global issue and a major threat to human health. Almost a century has passed since Fleming's discovery of penicillin as a magical remedy in 1928. Despite an aggressive quest for newer and effective antibacterial medications, humanity may be once again confronted with the lack of an efficient weapon for fighting against fatal bacterial infections. Antimicrobial resistance is predicted to cause 10 million deaths per year by 2050 if allowed to go unchecked at current pace (L. Sarwacinski, 2020). The rapid spread of antibiotic-resistant bacteria (ARB), including resistance to newer antimicrobials including antibiotics of last resort therapy, has piqued interest in this area over the last few decades. This interest stems from growing concern among the medical and scientific communities about the spread of bacterial resistance in the natural environment. Resistant strains of bacteria cause difficult to treat illnesses which necessitate the use of high end antimicrobial therapy which may be more toxic and expensive. Bacteria have developed resistance to all known antibiotics in some circumstances. Widespread use of antibiotics not only for medical and veterinary therapeutic purposes but also for causes like to stimulate cattle growth exacerbates the problem of drug resistance. Antibiotic usage and misuse on such a large scale accelerate the evolution of ARB (Antibiotic Resistant Bacteria) and ARG (Antibiotic Resistance Genes) in the environment, posing threat of human transmission of the environmental resistome. Pathogen infections have increased as a result, and the issue of ARB's has now become a challenge to global public health. This hazard has higher mortality and morbidity rates than HIV, prostate cancer, breast cancer combined together. For years, the main focus areas of research were the clinical elements of bacterial antibiotic resistance propagation; however, in the last decade an focus has diverted towards studies that investigate the quantitative and qualitative features of antibiotic release into the environment. Antibiotics and its active metabolites can infiltrate numerous environmental compartments through a variety of routes some of which include urban wastewater and fertilisation with antibiotic-polluted sewage sludge, manure mixing with water and food source, sediment, or biosolids in agro-ecosystems. These ecotoxic chemicals are rapidly transmitted to aquatic and soil ecosystems due to their high water solubility. Their buildup poses a significant risk to the quality of natural resources like water including groundwater and soil ecosystems. In addition to antibiotic residues, ARB and ARG have been identified as new environmental contaminants with the potential to spread globally. To overcome this issue, close international coordination is required. A comprehensive approach to cater to this issue would be a preferable option, more specifically the one that considers the lack of new antibiotics with novel mechanisms of action, the irrational use (overuse and

underuse) of existing antibiotics, and the spread of antibiotics into the environment. If implemented properly, this strategy could provide sustainable and chronic benefits.

II. IV. ANTIMICROBIAL RESISTANCE

AMR develops when bacteria acquire or develop the ability to bypass the antimicrobial's actions. Antibiotic-resistant pathogen infections are difficult to treat, have higher risk of relapse causing significantly higher morbidity and mortality. Over the last decade the prevalence of Antibiotic-resistant bacterias has increased. According to the Centre for Disease Control and Prevention (CDC), at least 2 million people in the United States are infected with antibiotic-resistant bacteria each year causing at least 23,000 deaths annually. AMR is responsible for 700,000 deaths annually globally and this figure is expected to climb upto 10 million by 2050. Antimicrobial-resistant bacteria infections such as those caused by multidrug-resistant Acinetobacter baumannii, Klebsiella pneumoniae, and methicillin-resistant Staphylococcus aureus (MRSA) result in longer duration of hospital stays and significantly increase financial burden on national healthcare systems across the globe. AMR which is linked to irrational antibiotic use in humans, veterinary, agriculture, farming and industry sectors may be tackled by One-Health strategy. As human health is intimately tied to animal health and ecosystem sustainability One-Health strategy may be the most useful tool to cater the emerging problem of AMR. This study attempts to highlight the key antimicrobial resistance mechanisms as well as illustrate their mechanism and evolution over time. A greater knowledge of the evolutionary drivers of antibiotic resistance could lead to new ideas for combating this serious public health problem. (E. Christaki et.al. 2019)

II.V. WHY IS RESISTANCE A CONCERN?

There are multiple reasons for physicians to get concerned about bacterial resistance. Resistant strains of bacterias specially staphylococci, enterococci, Klebsiella pneumoniae, and Pseudomonas spp 2-7 are becoming ubiquitous in healthcare settings. Bacterial resistance often results in treatment failure that may have serious consequences more so in critically ill patients. Irrational empiric antibacterial therapy (the initial use of an antibacterial agent to which the causative pathogen may/may not be susceptible) has been associated with higher mortality rates in patients with bloodstream infections caused by resistant strains of P. aeruginosa, S. aureus, K. pneumoniae, E. coli, Enterobacter spp, coagulase-negative

staphylococci and enterococci. 8,9 Prolonged therapy with high end antibiotics like vancomycin and/or linezolid may also lead to the development of low-level resistance that compromises therapeutic benefits although the same may not be detected by routine susceptibility testing methods used commonly in hospital microbiology laboratories.10 Resistant bacteria may spread not only to healthcare settings but also in community as well and may pose a greater healthcare challenge. Clinically important bacteria like methicillin-resistant S aureus (MRSA) 3,11 and extended-spectrum -lactamase (ESBL)–producing E coli, 12,13 are increasingly observed in the community. Infected individuals more specifically vulnerable population like children often lack identifiable risk factors for MRSA and appear to have acquired the infections in a variety of community settings.14,15 Community-associated MRSA strains are generally less resistant to antimicrobial agents than nosocomial (hospital acquired) MRSA, but are more likely to produce Panton–Valentine leukocidin and other toxins .14 The spread of resistant bacteria within the community poses obvious additional challenge of infection control in a broader group of population. Controlling such a situation is a tough task which is further complicated by the increased mobility of our population. Lastly, with respect to the cost-containment constraints of today's healthcare environment, antibacterial drug resistance puts an additional financial burden on healthcare costs,16 although its full economic impact remains to be determined. (F. C. Tenover et.al., 2006)

II.VI. HERBAL PLANTS AS ANTIMICROBIAL AGENTS -

II.VI.i History of Herbal antimicrobials -

Medical plants have been traditionally used for centuries to treat a variety of diseases in the Indian subcontinent. Ayurveda is the branch of science that deals with plants and their therapeutic effects. Ayurveda is a major medical and medicinal therapy system in India. Many Ayurvedic medicines' pharmacologically active constituents have now been identified, and their utility in medication therapy is being investigated. Nearly 3,000 of the 17,000 species discovered are believed to be useful in medicine. Ayurveda has been practised in India for thousands of years, according to legend. It uses a variety of approaches to treat ailments. Ayurveda relies entirely on herbal plants and their derivatives. As per World Health Organization (WHO) medicinal plants are the finest source for innovative herbal medications. Traditional medicine, which contains substances derived from medicinal plants, is used by almost 80% of people in developed countries. As a result, such plants should be studied further to learn more about their qualities, safety, and efficacy. Plant extracts and phytochemicals, both of which are known to have antibacterial characteristics, can be very useful in therapeutic

treatments. Large number of studies have been conducted across the globe in recent times to demonstrate this efficiency. Because of their antibacterial properties, many plants have been employed (R. Tyagi and G. Sharma , 2016). People from all over the world use plant products for healing. For example, hollyhock was evidently used by Neanderthals who lived 60,000 years ago in what is now Iraq, and these plants are still widely employed in ethnomedicine all over the world. Hippocrates mentioned the therapeutic effects of approximately 300-400 plants in the late fifth century B.C.. Antibacterial chemicals can be found in a variety of plants. Plant-derived compounds including emetine, berberine, and qunine are highly efficient against infectious microorganisms. There are about 3,000 different plant species on the planet, yet only around 2% of them have been tested for antibacterial characteristics. Plant extracts with putative antibacterial activities have been described from over 157 plant groups. Higher plants are the source of around 1/4th to 1/2th of the medications distributed in the United States (US). Taxol, camptothecin (anticancer), and artemisinin are examples of medicines that were once produced from natural resources (antimalarial). These and many other medications demonstrate that plants can still be used as a source of medicine(M. Ahmad et. al. 2013)

II.VI. ii. Plants used as Antimicrobial Agents -

Because of their diverse capabilities, plant-derived substances have become increasingly important. Medicinal plants are the most abundant source of all types of medications, including ancient and modern medicines, nutraceuticals, and new chemical leads. Approximately 14-28% of higher plants are utilised as medicines, with 74 percent of plant medicines deriving from ethnomedicinal use. The procedure for introducing new products. The discovery of novel chemical entities was the first step in developing pharmacological active drugs. evaluation of its pharmacological action, formulation of dosage forms, and lastly implementation of pharmacokinetic research, and the same is true for medicinal compounds derived from plants. In 75-80 percent of the population, mostly in underdeveloped nations, herbal medicines are the mainstay of treatment. The reason for this is that they are more acceptable due to cost and have no or few adverse effects. Herbal medications have recently experienced an increase in popularity in developed countries. Because of the growth of antimicrobial resistance and the negative effects associated with synthetic or semi synthetic antimicrobial drugs, we are currently concentrating our efforts on plant derived extracts or crude plant products. The below table contains the list of plants which have proven antimicrobial activity , the table is opted from M. Ahmad et. al. 2013.

Table 1 . List of plants which have proven antimicrobial activity

No.	Plant name	Part Used	Extract	Microorganisms used	Reference
1	*Achyranthus aspera, Cynodon dactylon,Lanata camara & Tagetes patula*	Leaves and shoots	Ethanolic	*Bacillus subtilis, Staphylococcus aureu, Pseudomonas aeroginosa*	Narayan, *et al.*, 2010.
2	*Quercus infectoria*	Galls	Aqueous and acetone	*Staphylococcus aureu, Staphylococcus epidermidis, Bacillus subtilis, Escherichia coli, Sallmonella typhimurium, & Pseudomonas aeruginosa*	Basri, and Fan, 2005.
3	*Coccinia grandis L.*	Leaves and stem	Water and ethanol	*Bacillus cereus, Corynebacterium diptheriae, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi and Shigella boydi*	Farrukh, *et al.*, 2008.

4	*Acacia nilotica, Sida cordifolia, Tinospora cordifolia, Withania somnifer and Ziziphus mauritiana*	Root / Bark	Methanol	*Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus and Xanthomonas axonopodis pv. malvacearum* Fungus *Aspergillus flavus, Dreschlera turcica and Fusarium verticillioides*	Mahesh and Satish., 2008.
5	*Polygonum aviculare*	Whole plant	Acetone, ethanol, chloroform and water	*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, S. paratyphi and Shigella flexneri, Staphylococcus aureus, Bacillus subtilis, and Streptococcus pyogenes, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger and C. albicans.*	Salama, and Marraiki., 2010.
6	*Heliotropium strigosum*	Whole plant	Ethyl acetate, n-hexane, chloroform and water	*Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia*, *Staphylococcus epidermidis*, *Bacillus subtilis* and methicillin resistant *Staphylococcus aureus, Aspergillus*	Hussain, *et al.,* 2010.

				niger, Aspergillus fumigates, Fusarium solani and Aspergillus flavus	
7	*Woodfordia fruiticosa*, *Adhatoda vasica*, *Chenopodium ambrosoides*, *Viburnum cotinifolium*, *Euphorbia hirta*, *Vitex negundo*, *Peganum harmala*, *Broussonetia papyrifera*, *Taraxacum officinale*, *Urtica dioica*, *Verbascum thapsus*, *Caryopteris grata* and *Mimosa rubicaulis*	Different parts	Methanol	*Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Klibsella pneumonia, Vibrio Cholera, Enterobacter coccus, Aspergilus niger, Aspergilus flavus, Aspergilus fumigates and Rhyzoctonia solani*	Khan, *et al.*, 2011.
8	*Satureja hortensis L., Stachys woronowii R. Mill, Ziziphora clinopodioides Lam. Origanum vulgare L. Letswaart and Sideritis montana L.*	Different parts	Methanol	*Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Bacillus megaterium, Candida albicans , Candida glabrata , Epidermophyton sp. and Trichophyton sp.*	Kursat and Erecevit., 2009.
9	*Sapindus emarginatus, Hibiscus rosa-sinensis, Mirabilis jalapa, Rheo discolor, Nyctanthes arbortristis, Colocasia esculenta, Gracilaria corticata, Dictyota spps., and Pulicaria wightiana*	Leaves or whole plant	Water and methanol	*Pseudomonas testosteroni, Staphylococcus epidermidis, Klebsiella pneumoniae, Bacillus subtilis, Proteus morganii, and Micrococcus flavus*	Nair, *et al.*, 2005.

10	*Vernonia amygdalina, Eucalyptus citriodora and Phyllanthus amarus*	Different parts	Water and ethanol	*Vernonia amygdalina (Bitterleaf), Eucalyptus citriodora (Eucalypt) and Phyllanthus amarus*	Sule and Agbabiaka, 2008.
11	*Balanites aegyptiaca and Moringa oleifera*	Leaves	Water acetone and ethanol	*Salmonella typhi*	Doughari *et al.*, 2007.
12	*Parthenium hysterophorus*, *Stevia rebaudiana* and *Ginkgo biloba*	Leaves	Methanol, ethanol and dichloro-methane	*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, Enterococcus* spp., and *Staphylococcus aureus*	Fazal *et al.*, 2011.
13	*Acalypha indica, Cassia auriculata, Eclipta alba and Phyllanthus niruri*	Leaves and roots	Water and ethanol	*Escherichia coli, Proteus vulgaris, Staphylococcus aureus* and *Bacillus subtilis*	Chitravadivu *et al.*, 2009.
14	*Prosopis cineraria, Capparis decidua*, *Tinospora cordifolia*, *Carissa carandas* and *Cordia Dichotoma*		Benzene, chloroform, acetone methanol and water	*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Aspergillus niger* and *Candida albicans*	Salar and Dhall, 2010.
15	*Solanum xanthocarpum Schrad. and Wendl.*	Leaves, stem, roots and fruits	Ethanol, benzene, acetone and methanol	*Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger*	Salar and Suchitra, 2009.
16	*Aegle marmelos*	Leaves	Petroleum ether, dichloromethane, chloroform, ethanol and	Micrococcus glutamicus, Lactobacillus bulgaris,	Rajasekaran *et al.*, 2008.

			water	Streptococcus faecalis, Staphylococcus aureus, Bacillus stearothermophilus, Staphylococcus pyogenes, Micrococcus luteus, Bacillus cereus, Escherichia coli and Pseudomonas aeruginosa	
17	*Allium sativum* Linn, *Zingiber officinale* and *Citrus aurantifolia* Linn.	Fruits and rhizome	Water and ethanol	*Bacillus spp., Staphylococcus aureus, Escherichia coli, Salmonella spp.*	Onyeagba *et al.*, 2004.
18	*Ximenia americana*	Leaves	Ethanol	*Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus subtilis, Staphylococcus aureus, Candida albicans*	Ogunleye and Ibitoye, 2003.
19	*Acacia nilotica, Terminalia arjuna, Eucalyptus globulus, Syzygium aromaticum and Cinnamomum zeylanicum*	Leaves and bark	Ethanol	*Escherichia coli, Klebsiella Pneumonia, Candida albicans, Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis, Streptococcus bovis, Pseudimonas aeruginosa, Salmonella typhimurium, Escherichia coli, Klebsiella pneumonia, Candida albicans*	Khan *et al.*, 2009.

20	*Syzyium aromaticum, Cinnamomum cassia, Salvia officinalis, Thymus vulgaris and Rosmarinus officinalis*	Water methanol and ethanol	Seeds, bark and leaves	*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis*	Abu-shanab *et al.*, 2004.

II. VII. BIOFILMS

Biofilms are complex systems composed of microbial communities that are attached to biotic or abiotic surfaces and which are enclosed in a matrix containing self-produced, or self-generated, extracellular polymeric substances that are EPSs. Two distinct life modes are the Nomadic (planktonic) or sedentary (sessile) states of microorganisms. Biofilms have unusual characteristics, such as the presence of phenotypes that are higher in resistance to antibiotics than those of planktonic cells. These organisms can also contribute to the development of various diseases, such as respiratory infections and cystic fibrosis. Biofilms can also cause infections on medical implants. These infections can require surgical removal and can contribute to the high cost of care in the US. That is why more effective and efficient ways of removing biofilm-related infections are needed very much. (Xi-Hui Li et.al.2017). In addition to the growth mode of bacteria in biofilms, another important feature of bacterial biofilms is stagnation of bacteria in the microenvironment as long as the conditions are favorable. As a general rule, the amount of bacteria in biofilms is less than 10% of their dry mass, but the amount of matrix can reach over 90%. The matrix is composed of various types of polymers created by the organisms, including extracellular polymeric substance (EPS). These bacteria behave differently than their planktonic counterparts due to EPS, which allows them to live in close proximity to each other and interact within a colony of bacterias. By forming an extra layer of EPS around the cells, the bacteria within the biofilm can resist adverse conditions like antimicrobials, environmental changes and even evade detection by the host immune system in a better way. This is a matter of concern for industrial as well as clinical settings. (Khan Alama et.al., 2020).

II.VIII. Anti-biofilm agents based on plant Products -

Biofilm generation and development is a multi-stage process that can be prevented by using natural anti-biofilm chemicals.

(1) bacterial cell adhesion to a suitable biotic and/or abiotic surface,

(2) formation of biofilm,

(3) maturation of the biofilms, and

(4) Biofilm dispersion is a well-studied stage in the evolution of biofilms.

The best way to prevent biofilm formation appears to be to target one or both of the first two processes. Cytoskeletal elements (mainly flagella and fimbriae) and lipopolysaccharides play key roles in the attachment stage. The surface signaling/communication of a group of bacteria, known as Quorum Sensing, is critical for the formation of biofilm.

II.VIII.i. Phytochemicals-

Natural chemicals with high anti-biofilm capabilities can be divided into following categories.

1. Phenolics,
2. essential oils
3. terpenoids
4. lectins
5. alkaloids
6. polypeptides and
7. polyacetylenes

Phenolics are a class of chemicals that seven subclasses which are:

A. phenolic acids,
B. quinones,
C. flavonoids,
D. flavones,
E. flavonols,
F. tannins, and
G. coumarins

Tannins, specifically condensed tannins, having anti-biofilm activity.

These chemicals act on biofilm by six different mechanisms:

I. substrate deprivation,
II. membrane rupture
III. binding to the adhesion complex and cell wall;
IV. bind to proteins;
V. interact with eukaryotic DNA; and
VI. prevent viral fusion.

Several solvents were utilised to extract natural chemicals from various sources for anti-biofilm activity, including water, methanol, ethanol, chloroform, ether, dichloromethanol, and acetone. Researchers discovered that water extracts anthocyanins, sugars like tannins, saponins, terpenoids, polypeptides, and lectins in a variety of trials.

Methanol extracts anthocyanins, terpenoids, saponins, tannins, xanthoxyllines, quassinoids, totarol, flavones, lactones, phenones, and polyphenols, whereas ethanol extracts tannins, polyphenols, polyacetylenes, flavonol, terpenoids, sterols, alkaloids, and propolis.

Chloroform extraction yields terpenoids and flavonoids; dichloromethanol extraction provides just terpenoids; ether extraction yields terpenoids, alkaloids, fatty acids, and coumarins; and acetone extraction yields flavonols.

Hydroquinone and caffeic acid methyl ester, obtained from the aqueous extract of Cnestis ferruginea Vahl ex DC., showed promising antibacterial activity against S. aureus. In vitro tests revealed that 12-Methoxy-trans-carnosic acid and carnosol isolated from the methanolic extract of Salvia officinalis L., an Algerian medicinal herb, have antibiofilm efficacy against Candida biofilm. Phytochemicals disrupt quorum sensing inducers as AHL, autoinducers, and autoinducers type 2 to inhibit the quorum sensing mechanism. Garlic extracts are important in inhibiting quorum sensing signalling molecules in biofilms of Pseudomonas and Vibrio spp. Emodin is a strong inhibitor that aids in the proteolysis of transcription factors involved in quorum sensing.

II.VIII.ii. Biosurfactants -

Biosurfactants (BS) are the chemicals that prevent the development of biofilm by altering cell adhesion ability via reducing the cell surface hydrophobicity, rupture of rupture, inhibition of the ETC in mitochondria and reducing cellular energy requirement. Various microorganisms create biosurfactants of various types with anti-bacterial, anti-fungal and anti-biofilm properties. Extensive studies are available on the impact of biosurfactants from Lactobacillus plantarum and Pediococcus acidilactici on quorum sensing signaling molecules and expressions of genes related to biofilm in Staphylococcus aureus. Biosurfactants have been observed to inhibit S. aureus biofilm growth by modulating the expression of biofilm-related genes such as dltB, icaA, cidA, and others in a study. At 12.5 mg/ml, BS from Lactobacillus plantarum dramatically inhibited cidA gene expression. At a dose of 50 mg/ml, biosurfactant Pediococcus acidilactici inhibited autoinducer-2 (AI-2) signaling molecules, staphylococcal accessory regulatory (sar A) gene expression and accessory gene regulator (agr A) expression. Lactobacillus-derived BS loaded liposomes were found to have better anti-biofilm action than

free biosurfactants in inhibiting S. aureus (MRSA) biofilm development and removal in previous investigations. An anionic lipopeptide from Acinetobacter junii was discovered that self-aggregates to produce b sheet–rich BS vesicles along with effects on the growth, development and dispersal of biofilm. This biosurfactant can be utilized as an anti-biofilm agent because it is thermostable and less poisonous.

II.VIII.iii. Antimicrobial Peptides-

Antimicrobial peptides (AMP) are antimicrobials having a broad spectrum activity and are commonly used to treat bacterial and fungal biofilms. AMP break-up biofilms on medical devices like stents, artificial valves, catheters, and dentures. Hospital-acquired infections are most commonly caused by S. aureus, Klebsiella pneumoniae, P. aeruginosa, Enterococcus faecium, Acinetobacter, and Enterobacter spp. (ESKAPE), as well as non-ESKAPE pathogens. AMPs found in humans, animals, plants and microorganisms reduce the chances of bacterial resistance to antibiotics by acting upon bacterial cell membranes. They electrostatically engage with membrane phospholipids, get inserted into the membrane and thus kill the bacteria. Multiple molecular pathways involved in biofilm development have been reported to be inhibited by synergizing AMPs with antimicrobial drugs.

II.VIII.iv. Therapeutic Strategies Using Natural Products-

As traditional antibiotics have failed to act upon, upgrading biofilm treatments is the need of time. Anti-biofilm chemicals found naturally selectively kill persistent biofilms while allowing antibiotics/antimicrobials to diffuse into the biofilm matrix causing the breakdown of biofilm matrix and killing the released cells at various stages of the biofilm cycle. Better anti-biofilm techniques may be developed with a better grasp on knowledge of biofilms mechanisms.

In a recent study, elastin (an anti-biofilm chemical derived from the actinobacteria Streptomyces mobaraensis DSM 40847) was found to degrade the matrix in a multispecies biofilm, making it more susceptible to antibiotics.

II.VIII.v. Extracellular Polymeric Substance (EPS)–Targeting Strategies

EPSs secreted by a wide range of bacteria have polysaccharides, structural proteins and extracellular DNA as the major components. EPS matrix supports microbial attachment to a surface, aggregation in multilayered biofilms, and acting as a 3-D scaffold for hydration, digestion, and protection against antimicrobials and host defense. The EPS matrix can actively change nutritional gradients and alter the pathogenic settings which may affect tolerance and

virulence features. EPS matrix is the target of several therapeutic techniques which aim to remove biofilms, disaggregate bacteria, and disrupt the pathogenic environment by targeting the. Many bacterial enzymes and secondary metabolites interfere with pathogenic bacteria's quorum sensing processes, affecting biofilm development. For example Actinobacillus actinomycetemcomitans, agram-negative periodontal pathogen secretes biofilm matrix grading enzymes beta-N-acetylglucosaminidase and dispersin B which dismantle mature Staphylococcus epidermidis biofilms.

II.VIII.vi. Quorum Sensing Targeting Strategies

Quorum sensing (the prevention of intracellular communication) is an effective technique for preventing biofilm formation. Metalloprotein AHL-lactonase found in the cell-free extract of endophytic Enterobacter species degrades N- AHLby preventing Aeromonas hydrophila from forming biofilms. Lactobacillus crustorum ZHG 2-1, a novel quorum-quenching bacteria, degrades N- 3-oxododecanoyl-dl-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-dl-homoserine lactone (C4-HSL). In a study by Cui et al it was found to act as an anti-biofilm agent against P. aeruginosa (Cui et al., 2020). Several reports are available in literature on quorum quenching (QQ) enzymes and substances; the majority of which come from natural/plant sources. QS inhibitory activity against P. aeruginosa biofilm has been detected recently from ethyl acetate extracts from cell-free supernatants and cells of the Natrinemaversi forma. Multiple QS inhibitors derived from plant-based natural compounds have been detected and proposed as potential biofilm targeting agents. The first section of the review discusses the role of natural anti-biofilm agents in inhibiting QS molecules. Here attempt is to explain the impact of QS targeting molecules on the breakdown of the QS mechanism in this paper. These anti-biofilm compounds work in two ways to disrupt quorum-sensing systems:

(1) inhibiting and degrading signal molecules, and

(2) simulating signal molecules to prevent them from attaching to matching receptors.

On the other hand quorum quenchers are usually species specific; thus, to eradicate mixed-species biofilms generally a mixture of quenchers is required. Ajoene, a sulfur-rich compound found in garlic, reduces the expression of small regulatory RNAs (sRNAs) in both Gram-negative (P. aeruginosa) and Gram-positive (S. aureus) bacterias. Ajoene is the first chemical to target broad-spectrum quorum sensing inhibitors, lowering the expression of sRNA.

II.VIII.vii. Phage Therapy-

Lytic bacteriophages have been proven to be an effective option to remove biofilm cells. Recently vB SauM ME18 lytic phage and vB SauM ME126 lytic phage were detected as potential natural antimicrobials that suppress MDR S. aureus biofilm. Phage-derived enzymes like polysaccharide depolymerase or peptidoglycan degrading enzymes are some of the promising anti-biofilm options. Trials for the first ever phage treatment approved by the US FDA in 2019 were held at the School of Medicine, University of California San Diego (UCSD) phage therapy center. As there are multiple challenges right from the formation of phage banks with well-characterized phages; safety issues, stability at variable environment conditions, maintaining the quality of phage preparations throughout manufacture and the ever evolving resistance of bacteria to antimicrobials hence only a few nations employ phage therapy..

II.VIII.viii. Combination Therapy-

Sensitized by natural anti-biofilm chemicals antibiotics have proven to be more effective when used in combination. For example using sodium houttuyfonate and levofloxacin together inhibits biofilm development more effectively. Sodium houttuyfonate, a plant-derived anti-neuropeptide, effectively disrupted the biofilm by P. aeruginosa. Naringin, a flavanone glycoside derived from citrus and grapefruits was found to be more effective against P. aeruginosa biofilms compared to individual treatment with the marketed antibiotics like ciprofloxacin and tetracycline. Naringin lowers pellicle development and slows the flagellar motility of bacteria on catheter surfaces by depleting biofilm EPS and facilitating antimicrobial diffusion. The effect of hordenine, a polyphenolic substance derived from barley, on biofilm development was investigated separately and in combination with netilmicin, an aminoglycoside antibiotic. The results were positive, with a combination of hordenine and netilmicin reducing P. aeruginosa PAO1 biofilms by up to 88 percent, which was significantly better than any of the individual therapies. It suggests that drug–herb combination therapy could be investigated for anti-biofilm formulation possibilities. The biofilm layer's thickness was reduced, and its architecture was disrupted, according to the SEM analysis. The study's findings also demonstrated that hordenine inhibits biofilm growth by downregulating the expression of quorum-sensing regulatory genes, particularly lasR. Actinobacterial substances from several microbial species have also demonstrated potential anti-biofilm activity against pathogenic bacteria by disrupting the cell surface and cell-cell contact. Studies combining multiple natural antibiofilm compound/s from various sources or acting on distinct phases of

biofilm development will aid in the creation of more effective biofilm-targeting agents. Furthermore, choosing a more effective molecule is important because natural compounds' efficacy against biofilm growth varies depending on the bacteria type.

II.VIII.ix. Anti-biofilm Biomaterial Therapy-

Biofilm-associated pathogenic organisms' adhesion to implant surfaces limits their therapeutic utility. Numerous attempts have been made to cover biomaterial as a prophylactic measure against pathogenic microorganisms. Natural polymer-based surface coatings such as anti-adhesive coating obtained from algal polysaccharide dextran, ulvan, and dermatan sulphate; also antimicrobial-releasing polysaccharide coatings are now first choice antibiofilm therapy. Anti-adhesive CyanoCoating (a coating from the marine cyanobacterium Cyanothece sp. CCY 0110) has been used as a defensive tactic against a broad spectrum of microorganisms (particularly Proteus mirabilis, E. coli, and Candida albicans biofilms) in Catheter Associated Urinary Tract Infections (CAUTI). The hydrophilic polysaccharides generate a hydration layer on the surface, which functions as a physical barrier and hinders cell adherence to the surface (Damodaran and Murthy, 2016). Calcium phosphate cement and hydroxyapatite are calcium phosphate materials used as a bone coating to prevent biofilm infections, however they have a number of drawbacks in clinical trials). Chitosan hydrogel coatings, which limit bacterial adhesion and biofilm formation due to membrane leaching, can help prevent implant-related illnesses. Natural polymers were utilized as drug transporters in the form of fibers, strips, gels (Badam gum, Karaya gum, chitosan), films (chitosan), nanoparticles, and microparticles to assist deliver antibiotics to periodontal biofilm-forming microorganisms. Nisin, an FDA-approved AMP for methicillin-resistant Staphylococcus aureus, Streptococcus pneumoniae, Enterococci, and Clostridium difficile by acting as an anti-biofilm agent in combination with conventional antibiotics. Nisin in combination with a biocompatible polysaccharide gellan gum may be a promising therapy in biomaterial research.

II.IX . Herbal Antibiofilm compounds -

Biofilm antimicrobial tolerance has arisen as a key concern for medical scientists working in a variety of healthcare settings. Synthetic medications/antimicrobials, various combinational therapies and hybrid antibiotics may not be able to achieve and provide the anticipated benefits. Focus of the scientific community is towards innovative natural anti-biofilm agents as part of the search for novel antimicrobials in the face of the drug resistance crisis. Literature is available regarding various natural products for fighting biofilm-forming microorganisms,

concise information on existing constraints and recent developments in the modification of various natural anti-biofilm agents to sharpen their effectiveness as potential drug candidates for use by clinicians. The most common mechanisms of AMR are the glycocalyx, outer membrane structure, efflux pumps, heterogeneity in growth rate, genetic adaptation, metabolic state, and metabolism of cells within a biofilm. The mode of biofilm formation in multiple infections in human beings and mechanism of drug resistance have been widely described, reviewed and plotted. The mechanism of tolerance development is biofilm can be demonstrated under three sections:-

(1) Physical tolerance: excessive EPS synthesis prevents antimicrobials from penetrating and diffusing into the biofilm, giving biofilm cells extra time for being forbearance. EPS generation increases antimicrobial tolerance, according to similar findings.Salmonella Typhimurium's isogenic 1csgD mutant (EPS deficient mutant) is highly vulnerable to hydrogen peroxide and ciprofloxacin. As a result, therapeutic techniques that destabilize and inhibit EPS are the most effective anti-biofilm strategies for inhibiting biofilms and reducing antimicrobial resistance concerns. Deokar made a similar observation, stating that EPS inhibition affects cell adhesion as well as drug tolerance in biofilms.

(2) Passive tolerance: Antimicrobial compounds are inactivated by enzymes of the biofilm matrix. The catalase enzymes are responsible for Staphylococcus epidermidis biofilm tolerance to various physicochemical agents.

(3) Physiological tolerance: Cells present in the deeper layers of biofilms and are dormant metabolically adapt stress responses mechanisms that control biofilm tolerance to diverse antibiotics. Due to various adaptation responses lik phenotypic and reversible changes generated by starvation, ecological conditions, SOS and stringent response, persister cells develop further tolerance from multiple antimicrobials. Slowly dividing bacterial cells resistant to antibiotics are known as persister cells. Persister cells play an important role in biofilm re-establishment. Functions like upregulation of the expression of different toxin-antitoxin genes, inhibiting translation and lowering down the cell metabolic functions, making their survival possible in the hostile environment like presence of antibiotics is mediated by persister cells. After the antibiotic treatment, these cells revert to vegetative dividing cells, allowing infection to recur.

Because persister cells exhibit the toxin-antitoxin system, the antibiotic target is inhibited by the toxin module, these cells have been isolated from antibiotics. P. aeruginosa is more resistant to several antibiotics due to a lack of oxygen and limited metabolic activity in biofilms. Sudden pH shifts between layers in a biofilm contribute to the accumulation of organic acids, which

deactivates penetrating chemicals. The formation of oxygen, pH, nutrients, and electron acceptor gradients throughout the biofilm creates microenvironments in which cells respond by changing their gene expression. Antibiotic resistance is higher in complex (polymicrobial) biofilms made up of multiple species than in biofilms made up of a single species. Antibiotic resistance of biofilms is largely determined by cell diversity and metabolic circumstances.

II.X.Extract preparation of herbal components

Plant extracts are one of the most important sources of biomolecules. Extraction of biomolecules from medicinal plants is mediated by a variety of solvents and extraction methods. Filtration, Soxhlet, and Serial exhaustive techniques are common methods for extracting plant extracts/biomolecules from plants. For a decent quantity of extracts and biomolecules with sufficient and outstanding antimicrobial properties, successful extraction utilizing multiple solvents of varying polarities like polar to nonpolar may be required. Ethyl-acetate, hexane, dicholro-methane, chloroform, acetone, butanol are some of the best solvent systems for extracting plant extracts. High Performance Liquid Chromatography (HPLC) is an analytical technique for the separation and detection of active biomolecules that is generally used in conjunction with other chromatographic systems such as gas chromatography mass spectrometry (GC-Ms) and liquid chromatography mass spectrometry (LC-Ms). The GC-Ms has a lot of power when it comes to isolating and categorising volatile chemicals from complex combinations. The LC-Ms is used to analyse and classify nonvolatile substances in small amounts. Thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) are technologies employed further in the characterisation and confirmation of biomolecules contained in plant extracts. biomolecules derived from plants have been demonstrated anticancer, antiarrhythmic, antifungal, antioxidant, antibacterial, antidiabetic and anti-inflammatory capabilities; hence considered as the best alternatives to pharmaceutical and chemical antimicrobials.(A. Abdullahi et,al,, 2022).

II.X.i. *T. Arjuna*

The plant kingdom is brimming with potential medicines, and the field of herbal medicine has grown at a breakneck pace in recent decades. The Arjun tree (Family-Combretaceae), *Terminalia arjuna* Roxb., is well-known for its medicinal virtues. Terminalia arjuna Roxb, commonly referred to as Arjuna, is a common tree noted for its beneficial phytochemicals. It is a member of the combretaceae family. It has been grown in most parts of India from ancient

times and used in Ayurvedic treatments. *T. arjuna* plant parts such as stem bark, leaves, and fruits are used to cure a number of ailments in traditional medicine. The bark powder of *T. arjuna* has antioxidant, cardioprotective, anti-ischemic, cholesterol lowering, antimicrobial, anti-inflammatory, immunomodulatory, and antinociceptive properties. Obesity, hypertension, and hyperglycemia can all be treated with it. *T. arjuna* stem bark has a higher antioxidant potential due to the presence of more phenolic and flavonoids. Several researchers have found synthetic compounds, natural items, and nanomaterials as broad range antibiofilm agents. Natural products, in particular, are more appealing because of their safety and lengthy history of use. Traditional medicinal plants, which have been used to treat ailments/infections in folklore, might be an excellent location to start exploring for natural goods. The most versatile medicinal plant, Terminalia arjuna (Combretaceae), is often known as Arjuna and possesses a wide variety of biological activities. The bark of *T. arjuna* contains antioxidant, antidysenteric, antipyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypolipidemic, antibacterial, and antiuremic properties. However, no research on the antibiofilm activity of *T. arjuna* stem bark methanolic extract and its constituents has been published to far.(A. Amalraj and S. Gopi, 2016)

II.X.ii. *T. indica*

Tamarindus indica L., sometimes known as tamarind, is a medium-sized tree that belonging to the family Caesalpinaceae. Tamarind has been traditionally used as a medicinal plant for centuries. Its fruits have curative properties for multiple diseases as described in the multiple pharmacopeias. Other plant sections, however, have received less attention. The presence of polyhydroxylated chemicals, many of which are flavonolic in origin, has been linked to hepatoprotective activity in the leaves. The seeds and bark have therapeutic benefits as well. Tamarind leaves have a long history of ethnobotanical use throughout Latin America, Asia and Africa due to its antibacterial, antifungal, and antiseptic properties. Its use is currently limited to a few places, and it is rarely documented in ethnobotanical studies. In a recent study, Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Pseudomona aeruginosa, and Candida albicans were tested microbiologically using decoctions from fresh and sun dried leaves, as well as fluid extracts prepared with 30 and 70 percent ethanol-water and the pure essential oil from tamarind leaves. The total phenols and flavonoids in aqueous and fluid extracts were previously determined using spectrophotometry, while the essential oil was chemically analysed using gas chromatography/mass spectrometry (GC/MS). phenols appear to be potent against B. subtilis cultures, but not against other bacteria, according to research. The essential oil, on the other hand, had a high antibacterial spectrum

when pure, but its low quantities in typical folk remedies prevent any activity in these extracts. (J. Escalona-Arranz, et. al .2010)

II.X.iii. Withania somnifera (Ashwagandha)

Ashwagandha [Withania somnifera] is a medicinal herb used widely as a home treatment in India and other parts of the world for a variety of ailments. In the Vedas, it is described as a herbal tonic and healthy food. It is known as 'Indian Ginseng' in ancient Indian medicine and is listed in the Indian Pharmacopeia as an official drug. Besides its use as a general tonic, ashwagandha has been shown to have immunomodulatory and anticancer properties in recent studies. Furthermore, several portions of the ashwagandha plant may be having antiserotonergic, anticancer, and anabolic qualities which may be effective in the treatment of arthritis, stress, and geriatric issues.The plant has recently been shown to have substantial antifungal activity and to be beneficial in the treatment of mouse aspergillosis.(M. Owais, et.al. 2005). (K. S. Girish , et.al.2006)

CHAPTER III
MATERIAL AND METHODOLOGY

III. MATERIAL AND METHODOLOGY

III.I. Sample preparation -

1.Bark of *T . arjuna ,* Fruit of *T. indica* and stem of *W. somnifera* were collected .

2.All the collected samples were dried @60 degree C for 2-3 days

3. All the dried samples were grinded into fine powder and stored for further use.

III.II. Extract preparation -

1. All the samples were soaked overnight in a shaking incubator in 1:10 with a solvent that is methanol .

2.The overnight methanol and sample mixture were filtered through the Watthman filter paper and the filtrate was collected.

3.Then the collected supernatants are dried in the water bath until it is completely dried and only powder is left .

4.Scrap the powder and weigh the final amount of powder and calculate the percentage productivity of the the extract with formula

Percentage productivity = (Final wt.(eppd.) - Initial wt.(eppd.) /plant material used) X100

5.The final extract powder were dissolved in the autoclaved distilled water and the 10% DMSO.

III.III. GRAM STAINING -

1. On a slide, place a little drop of bacterial sample. Pass the slide through the flame of a Bunsen burner three times to adhere the germs. Too much heat applied for too long can cause the bacteria cell walls to melt, altering their shape and resulting in an incorrect result. The germs will wash off the slide if too little heat is applied during staining.

2. Apply the principal stain (crystal violet) to the slide with a dropper and let it sit for 1 minute. To remove excess discoloration, gently rinse the slide with water for no more than 5 seconds. Too much color can be removed by rinsing too long,while not rinsing long enough can leave too much stain on gram-negative bacteria.

3.Apply Gram's iodine to the slide with a dropper to adhere the crystal violet to the cell wall. Allow 1 minute to pass.

4.Rinse the slide for 3 seconds with alcohol or acetone, then rinse gently with water. Gram-negative cells will fade away, whereas gram-positive cells will remain violet or blue in appearance. If the decolorizer is left on for too long, all of the cells will lose

their color.

5.Allow 1 minute to dry before applying the secondary stain, safranin. Rinse gently with water for no more than 5 seconds. Gram-negative cells should be stained red or pink, and gram-positive cells should remain purple or blue.

6.Using a compound microscope, examine the slide. To determine cell structure and arrangement, a magnification of 10x to 100x may be required.

III.IV. Biofilm staining

1**.** All the content from 96 well plates was discarded .

2. Add 0.1% crystal violet stain and then incubate it for 20 min and after that remove the stain.

3. Remove the excess stain by washing the plate with 1% PBS buffer

4. Add 95% ethanol to the plate and incubate for 20 min .

5. Take the OD at 570 nm .

III .V. Disc - diffusion Method -

1. The MHA media is prepared by following the composition mentioned in appendix

2. All the bacterial isolates were cultured on the media using the cotton swab method of spreading .

3. Paper discs were added and the control antibiotic and the herbal antimicrobial compounds were added to the paper disc .

4. The plates were incubated for 18 hrs at 37 degree C.

III.VI. Minimum Inhibitory concentration test for Antimicrobial testing -

I.Inoculum Preparation for Dilution Tests :

1.Turbidity Standard for Inoculum Preparation -

BaSO4 turbidity standard were used corresponding to 0.5 McFarland standard or its optical equivalent to standardize the inoculum density for a susceptibility test.

2. Inoculum Preparation

i. Direct Colony Suspension Method

1.Inoculum was made by combining isolated colonies from an 18- to 24-hour agar 2. plate in a straight broth or saline solution (use a nonselective medium, such as blood agar).

2. Suspension was adjusted until the turbidity meets the McFarland threshold of 0.5.

For Escherichia coli ATCC®a 25922, this results in a suspension containing around 1 to 2 $\times 10^8$ colony-forming units (CFU)/mL. Use a photometric device or appropriate light to visually evaluate the inoculum tube and the 0.5 McFarland standard against against a card with a white background and contrasting black lines to accomplish this step accurately.

3. Growth Method

1. From an agar plate culture, choose at least three to five well-isolated colonies of the same morphological type. Transfer the growth into a tube containing 4 to 5 mL of an appropriate broth medium, such as tryptic soy broth, by touching the top of each colony with a loop or sterile swab.

2. Incubate the broth culture at 35 2°C until the turbidity of the 0.5 McFarland standard is met or exceeded (usually two to six hours).

3.Adjust the turbidity of the actively growing broth culture with sterile saline or broth until it reaches the turbidity of a 0.5 McFarland standard. For E. coli ATCC® 25922, this resulted in a suspension containing roughly 1 to 2 $\times 10^8$ CFU/mL. Use a photometric device or appropriate light to visually evaluate the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines to accomplish this step accurately.

4. Broth Dilution Procedures (Macrodilution and Microdilution)

1. **Mueller-Hinton Broth**

MHB is indicated as the susceptibility testing medium of choice for commonly isolated, rapidly growing aerobic or facultative organisms because: It has adequate batch-to-batch repeatability for susceptibility testing. It contains few inhibitors that impact sulfonamide, trimethoprim, and tetracycline susceptibility test findings, and it allows most pathogens to thrive well. A substantial amount of information and experience has been acquired from testing conducted using this medium.

1. Cation-adjusted MHB is the preferred medium for regular broth dilution testing (CAMHB).

2. Make sure each batch of MHB has the same pH.

3.Using a standard set of QC organisms, evaluate the MIC performance characteristics of each batch of broth. If a new batch of MHB fails to provide the expected MICs, the cation content, as well as other factors and test components,

should be checked

4.Perform MICs with Enterococcus faecalis ATCC 29212 to determine the appropriateness of the medium for sulfonamide and trimethoprim testing. The last points should be simple to understand (as 80 percent or greater reduction in growth as compared to the control). The medium may be regarded as adequate if the MIC for trimethoprim-sulfamethoxazole is 0.5/9.5 g/mL.

5.When testing oxacillin and staphylococci, 2 percent NaCl must be added to the CAMHB solution.

6. When testing dalbavancin and orita, use 0.002 percent polysorbate-80 (P-80).

II. Macrodilution (Tube) Broth Method

1. Preparing and Storing Diluted Antimicrobial Agents -

1. Conduct the test using sterile 13-100-mm test tubes. If you want to store the tubes for later, be sure they can be frozen.

2.Use loose screw caps, plastic or metal closure caps, or cotton plugs to close the tubes.

3. For each organism examined, use a growth control tube containing broth without an antimicrobial agent.

4. Volumetrically prepare the final twofold (or other) antibacterial agent dilutions in the broth. For the test, each dilution must have a minimum final volume of 1 mL. Measure all diluents using one pipette before adding the stock antimicrobial solution to the first tube. Use a new pipette for each consecutive dilution step.Antimicrobial dilutions are frequently made at double the required final concentration due to a 1:2 5. dilution of the medications when an equivalent volume of inoculum is added.

2. Inoculum Preparation and Inoculation

1. Use either the direct colony suspension or the growth method to make a standardised inoculum.

2. Dilute the adjusted inoculum suspension in broth so that each tube contains approximately 5 105 CFU/mL following inoculation, ideally within 15 minutes of preparation. By diluting the 0.5 McFarland suspension 1:150, a tube containing roughly 1 106 CFU/mL can be obtained.

3.After a 1:2 dilution in step 3, the final inoculum has a concentration of 5 105 CFU/mL.

4.Add 1 ml of the adjusted inoculum to each tube holding 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth) within 15 minutes after the inoculum has been standardised as indicated above, and mix. This results in a 1:2 antimicrobial concentration dilution and a 1:2 inoculum dilution. Subculturing an aliquot of the inoculum suspension onto a nonselective agar plate for simultaneous incubation is recommended as a purity check

3. Inoculum Preparation and Inoculation

1.Use either the direct colony suspension or the growth method to make a standardised inoculum.

2.Dilute the adjusted inoculum suspension in water, saltwater, or broth, ideally within 15 minutes of preparation, so that each well contains approximately 5 $\times 10^5$ CFU/mL after inoculation (range 2 to 8 $\times 10^5$CFU/mL). The dilution procedure for obtaining this final inoculum varies depending on how the inoculum is delivered to individual wells, and it must be calculated for each circumstance. To accomplish this calculation for microdilution testing, the exact inoculum volume delivered to the wells must be known. The 0.5 McFarland suspension (1 $\times 10^8$CFU/mL) should be diluted 1:20 to generate 5 $\times 10^6$CFU/mL if the volume of broth in the well is 0.1 mL and the inoculum volume is 0.01 mL. When 0.01 mL of this suspension is put into the broth, the final test concentration of bacteria is approximately 5$\times 10^5$CFU/mL (or 5 $\times 10^4$CFU/well in the microdilution method).

3.Within 15 minutes after the inoculum has been standardised as stated above, inoculate each well of a microdilution tray with an inoculator device that provides a volume that does not exceed 10% of the volume in the well (eg, 10 L of inoculum in 0.1 mL antimicrobial agent solution). The contents of each well (containing 0.05 mL) are diluted 1:2 using a 0.05-mL pipette, much like in the macrodilution approach.

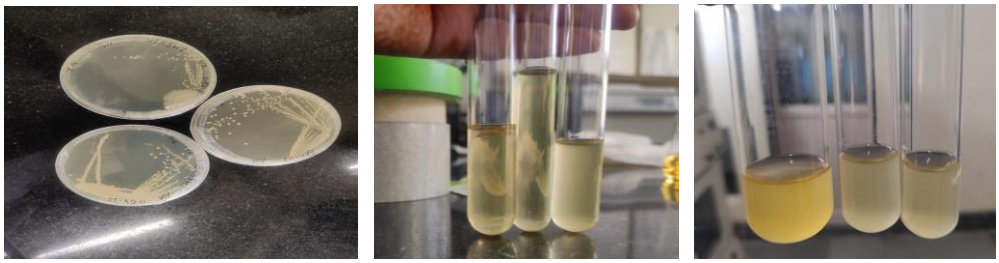
4. Subculturing an aliquot of the inoculum suspension onto a nonselective agar plate and incubating it simultaneously is a purity check.

CHAPTER IV
RESULTS AND DISCUSSION

IV.RESULTS AND DISCUSSION

I. Bacterial cultures and growth conditions-

The bacterial cultures were collected from the environment and enriched in nutrient broth at 37 degree C. Successful growth was also obtained on nutrient agar plates. Glycerol stocks were prepared and stored at -80 degree C for future use.

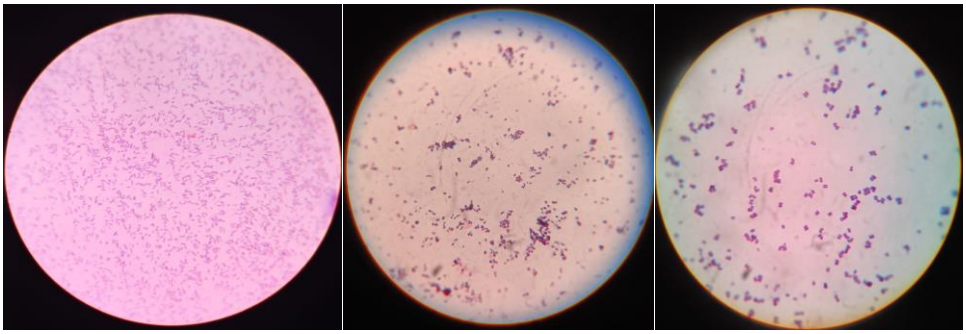


a **b** **c**

Fig 1. Overnight grown bacterial culture - a. Culture plate with Nutrient agar media of the overnight grown culture of isolates 1, 2 and 3 .**b.** Broth Tubes with overnight grown culture in MHA broth . **c.** Sub culture of overnight grown culture in MHA broth

II. Staining of the cultures -

A single colony from nutrient agar plate was taken and fixed on a glass slide and stained by following standard gram staining protocol. Then the microbes were identified as gram positive or negative and the shape of the colonies were also identified . The Results are shown in Fig 2. The morphology of Isolate 1 is gram negative coccobacillus , Isolate 2 was gram positive cocci shaped and Isolate 3 as also gram positive coccus shaped.



a **b** **c**

Fig 2.Staining of the cultures- a. Gram staining of the bacterial culture from the Isolate 2 , shows the gram negative, coccobacillus shaped **b.** Gram staining of the bacterial culture of Isolate 1, shows the gram positive cocci shape. **c.** Gram staining of the bacterial culture of Isolate 3 , shows the gram positive cocci shape

III. Extract preparation -

The final extracts were prepared by cold maceration method by using methanol as a solvent and then finally dissolving the extract powder in the autoclaved distilled water and 10% dmso .

a. **b.** **c.**

Fig 3. Extract preparation- a.*T. arjuna , T . indica and W. somnifera* powder mixed in 100% Methanol. **b.** *T. arjuna , T . indica and W. somnifera* powder after drying the methanol. **c.** *T. arjuna , T . indica and W. somnifera Somnifera* dissolved in autoclaved distilled water concentration of 125mg/ml and 50 mg/ ml

The percentage productivity of the extracts were calculated using the initial weight of powder used to prepare the extract and the amount of final powder obtained after the production of the extract by using the formula

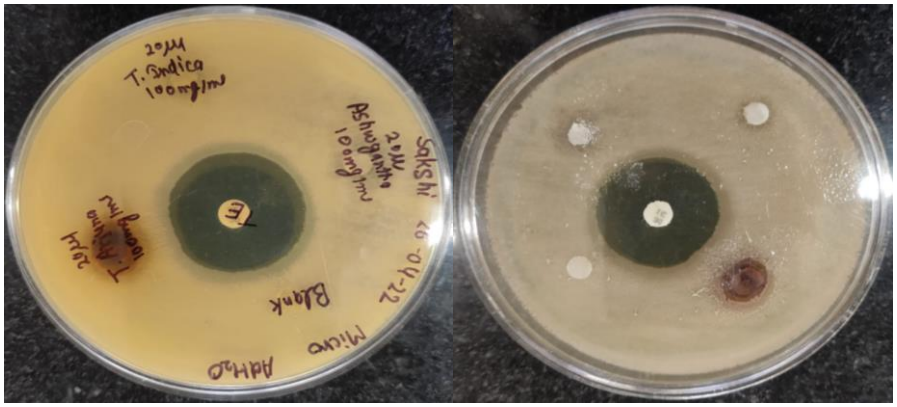
Percentage productivity = (Final wt.(eppd.) - Initial wt.(eppd.) /plant material used) X100

Table2 - Percentage productivity of the extract products

Product	**Percentage Productivity**
T. arjuna	**21.05%**
T. indica	**19.08%**
W. somnifera	**12.05%**

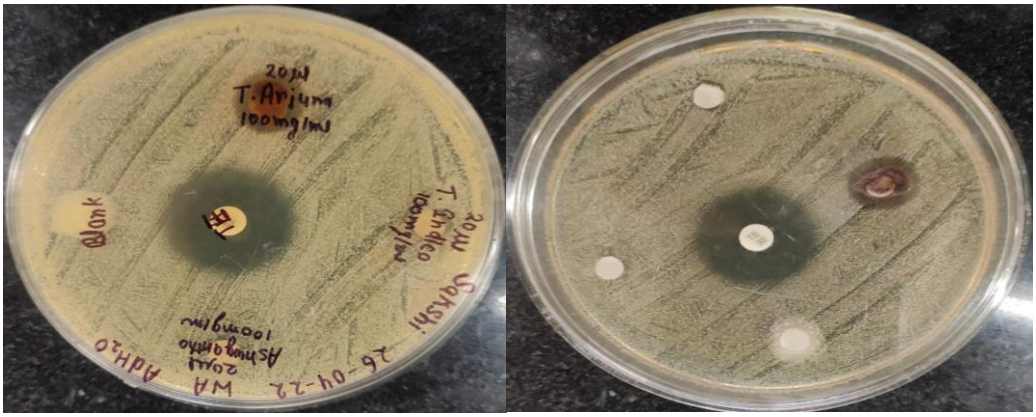
IV. Antimicrobial activity of Herbal compounds -

Antimicrobial activity of the herbal compounds were checked by disc diffusion method. The antimicrobial compounds were loaded on the paper disc and the plates cultured with the bacterial isolates were cultured at 37 for 18 hr .



a **b**

Fig 4. Antimicrobial assay for culture using disc diffusion method of Isolate1 with autoclaved distilled water - a. MHA plate with Isolate 1 and extract in autoclaved distilled water with a concentration of 100 mg/ml and 20microliter of extract per disc were loaded. **b**. Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.



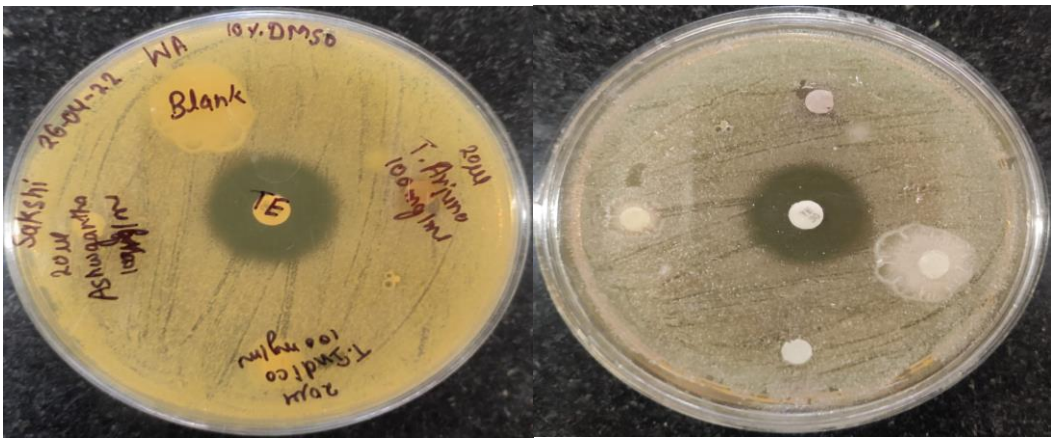
a **b**

Fig 5.**Antimicrobial assay for culture using disc diffusion method of Isolate 2 with autoclaved distilled water - a.** MHA plate with Isolate 2 and extract dissolved in autoclaved distilled water with a concentration of 100 mg/ml and 20microliter of extract per disc were loaded. **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.

a **b**

Fig 6 .**Antimicrobial assay for culture using disc diffusion method of Isolate 3 with autoclaved distilled water - a.** MHA plate with Isolate 3 and extract dissolved in autoclaved distilled water with a concentration of 100 mg/ml and 20microliter of extract

per disc were loaded. **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.



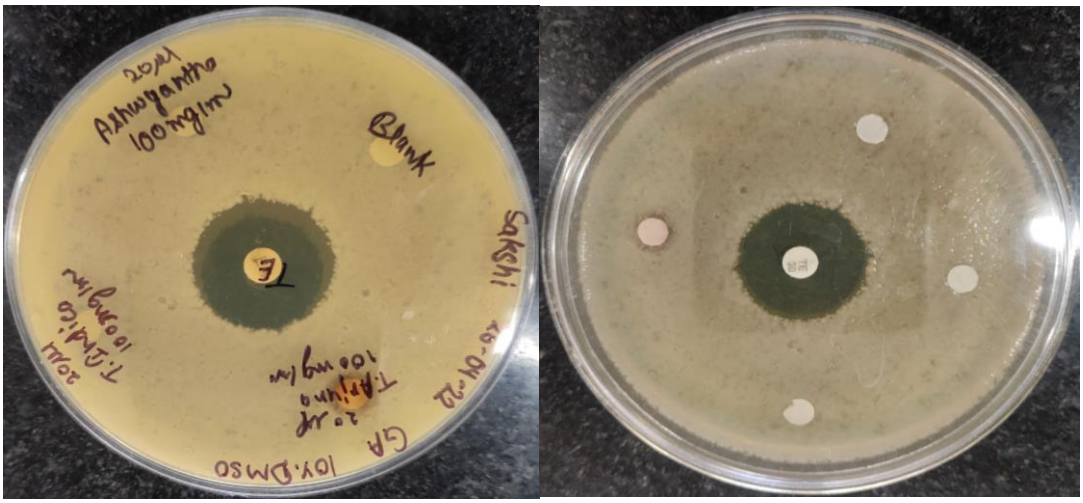
a **b**

Fig 7. Antimicrobial assay for culture using disc diffusion method of Isolate 2 with 10% DMSO - a. MHA plate with Isolate 2 and extract dissolved in 10% DMSO with a concentration of 100 mg/ml and 20 microliter of extract per disc were loaded. **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.

Fig 18 & 19.

Fig 8.Antimicrobial assay for culture using disc diffusion method of Isolate1 with 10% DMSO - a. MHA plate with environment culture from the Microbiology Lab and extract dissolved in 10% DMSO at a concentration of 100 mg/ml and 20 microliter of

extract per disc were loaded. **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.



a **b**

Fig 9 .Antimicrobial assay for culture using disc diffusion method of Isolate3 with 10% DMSO - a.MHA plate with environment culture from the Green House Area and extract dissolved in 10% DMSO with a concentration of 100 mg/ml and 20 microliter of extract per disc were loaded. **b**. Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and no activity by the herbal compounds.

ANTIMICROBIAL ACTIVITY RESULTS

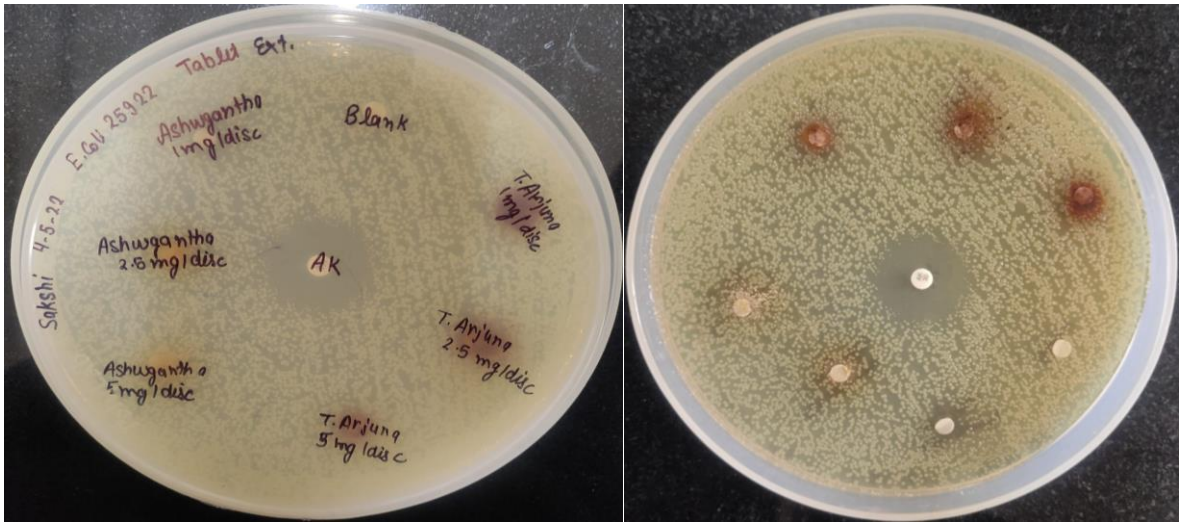
Table 3 - Antimicrobial assay results of all the culture plates

Culture (Bacterial isolates)	Solvent (Extract powder dissolved in)	Concentration 100 mg/ml amount loaded 20 microliter (amount loaded on	*T. arjuna* Activity	*T. indica* Activity	*W. somnifera* Activity	Control (tetracycline) Activity	Blank Activity (Autoclaved distilled water and 10%

		disc)					DMSO)
Isolate 1	Autoclaved distilled water	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity
Isolate 2	Autoclaved distilled water	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity
Isolate 3	Autoclaved distilled water	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity
Isolate 1	10% DMSO	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity
Isolate 2	10% DMSO	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity
Isolate 3	10% DMSO	2 mg	No activity	No activity	No activity	Significant inhibition of microbial growth	No activity

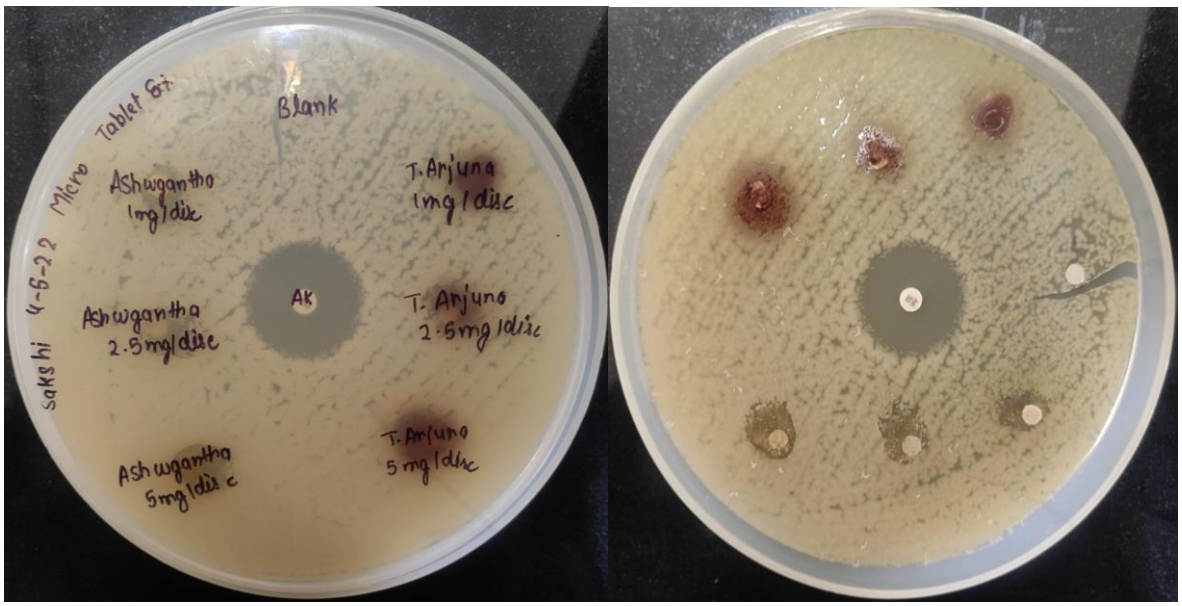
V. Antimicrobial Assay -

The antimicrobial activity of the compounds were rechecked with the higher concentration of the natural extracts of 1 mg per disc , 2.5 mg per disc and 5 mg per disc with bacterial Isolate 1 and E. coli. 25922 strain .



a. **b.**

Fig 10. Antimicrobial assay for culture using disc diffusion method with E. coli. culture and extract in autoclaved distilled water - **a.** MHA plate with E.coli 25922 strain culture and extract dissolved in autoclaved distilled water with a concentration of 100 mg/ml and 1 mg, 2.5 mg and 5 mg of extract per disc were loaded. Control antibiotic used is amikacin . **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and a significant amount of inhibition activity by the herbal compounds.



a . **b.**

Fig 11. Antimicrobial assay for culture using disc diffusion method with Isolate 1 culture and extract in autoclaved distilled water - a. MHA plate with Isolate 1 culture and tablet extract dissolved in autoclaved distilled water with a concentration of 100 mg/ml and 1 mg, 2.5 mg and 5 mg of extract per disc were loaded. Control antibiotic used is amikacin . **b.** Back side of MHA plate showing the growth of culture plate and the inhibition zone of the control antibiotic and a significant amount of inhibition activity by the herbal compounds.

ANTIMICROBIAL ACTIVITY RESULTS

Table 4 . Antimicrobial assay's results of culture plates with higher concentration of abstract

Culture	E. coli. 25922	Isolate 1
Solvent (Extract powder dissolved in)	Autoclaved distilled water	Autoclaved distilled water
T. arjuna Activity (1mg per disc)	4mm (ZOI)	5mm (ZOI)
T. arjuna Activity (2.5mg per disc)	7 mm (ZOI)	9 mm (ZOI)
T. arjuna Activity (5mg per disc)	12 mm (ZOI)	14 mm (ZOI)
W. somnifera Activity (1mg per disc)	3 mm (ZOI)	4 mm (ZOI)
W. somnifera Activity (2.5 mg per disc)	6 mm (ZOI)	8 mm (ZOI)
W. somnifera Activity (5 mg per disc)	13 mm (ZOI)	15 mm (ZOI)
Control	Significant inhibition of microbial growth 16 mm (ZOI)	Significant inhibition of microbial growth 16 mm (ZOI)
Blank	No activity	No activity

When the concentration of the herbal extract were increased from 1mg per disc and from 2.5mg per disc to 5mg per disc the antimicrobial activity of the compounds were significantly increased with the increase in the concentration of the herbal compounds against E.coli. 25922 and microbiology lab culture .

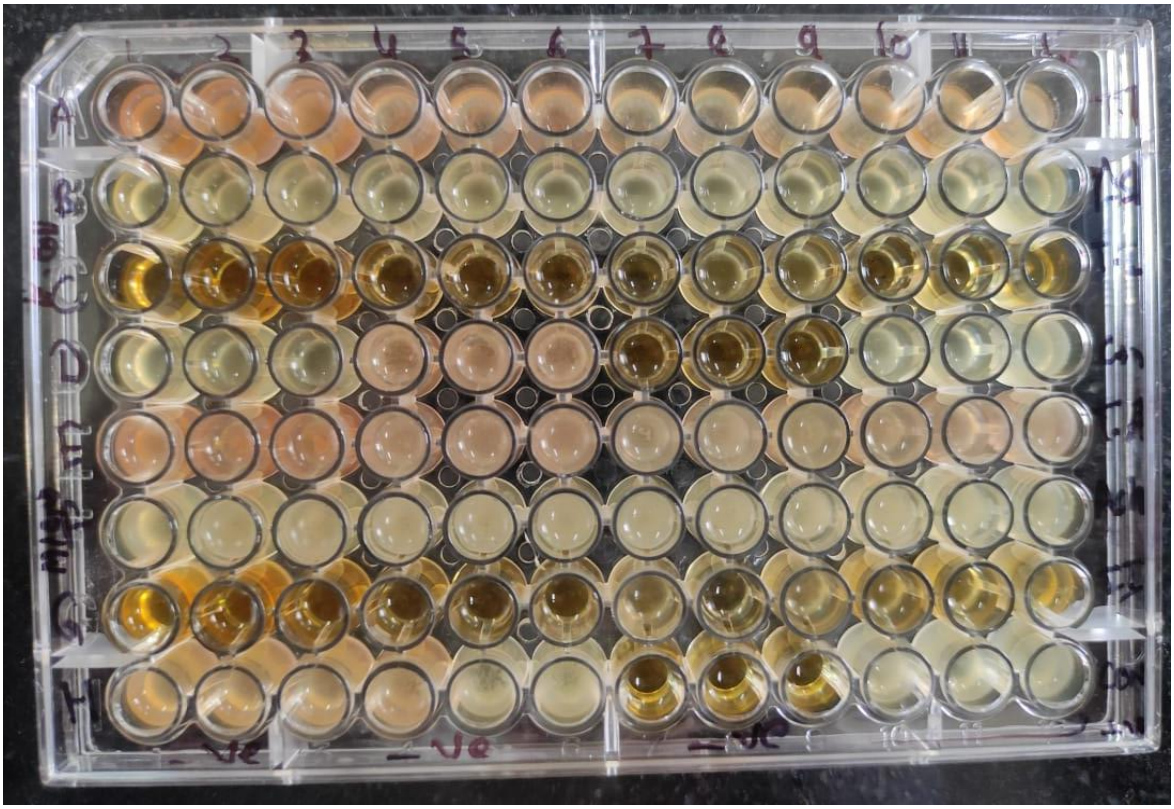


Fig 12.

Fig 12. 96 well plates with different concentrations of antimicrobials and different cultures with broth for MIC Test.

Table 5 - The concentration of the culture , antimicrobial activity and media loaded to each well for the Minimum inhibitory concentration calculation for each herbal compound.

Antimicrobial compound	1	2	3	4	5	6	7	8	9	10	11	12
T.Arjuna	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)
Witania Somnifera	0ul media +100ul antimicrobial compound +100ul culture (E.coli	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	0ul media +100ul antimicrobial compound +100ul culture (E.coli	50ul media +50 ul antimicrobial compound +100ul culture (E.coli	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)

	25922)		25922)	25922)								
T. Indica	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	0ul media +100ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	50ul media +50 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	75 ul media +25 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)	87.5 ul media +12.5 ul antimicrobial compound +100ul culture (E.coli 25922)
Control	(-ve control) 150ul media + 50 ul antimicrobial compound (Witania Somnifera)	(-ve control) 150ul media + 50 ul antimicrobial compound (Withania Somnifera)	(-ve control) 150ul media + 50 ul antimicrobial compound (Withania Somnifera)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Arjuna)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Arjuna)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Arjuna)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Indica)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Indica)	(-ve control) 150ul media + 50 ul antimicrobial compound (T.Indica)	(+ve control) 100ul broth +100 ul culture	(+ve control) 100ul broth +100 ul culture	(+ve control) 100ul broth +100 ul culture
T.Arjuna	0ul media +100ul antimic	0ul media +100ul antimicr	0ul media +100ul antimicr	50ul media +50 ul antimic	50ul media +50 ul antimicro	50ul media +50 ul antimicro	75 ul media +25 ul antimicrobial	75 ul media +25 ul antimicrobial	75 ul media +25 ul antimicrobial	87.5 ul media +12.5 ul antimicrobi	87.5 ul media +12.5 ul antimicrob	87.5 ul media +12.5 ul antimicro

	robial compo und +100ul culture (microb iology lab environ ment culture)	obial compoun d +100ul culture (microbi ology lab environ ment culture)	obial compou nd +100ul culture (microb iology lab environ ment culture)	robial compo und +100ul culture (micro biology lab environ mnet culture)	bial compoun d +100ul culture (microbi ology lab environm ent culture)	bial compoun d +100ul culture (microbio logy lab environm ent culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	al compound +100ul culture (Microbiolo gy lab environmen t culture)	ial compound +100ul culture (Microbiol ogy lab environme nt culture)	bial compoun d +100ul culture (Microbio logy lab environm ent culture)
Witania Somnifera	0ul media +100ul antimic robial compo und +100ul culture (microb iology lab environ ment culture)	0ul media +100ul antimicr obial compoun d +100ul culture (microbi ology lab environ ment culture)	0ul media +100ul antimicr obial compou nd +100ul culture (microb iology lab environ ment culture)	50ul media +50 ul antimic robial compo und +100ul culture (micro biology lab environ ment culture)	50ul media +50 ul antimicro bial compoun d +100ul culture (microbi ology lab environm ent culture)	50ul media +50 ul antimicro bial compoun d +100ul culture (microbio logy lab environm ent culture)	75 ul media +25 ul antimicrobi al compound +100ul culture (microbiolo gy lab environmen t culture)	75 ul media +25 ul antimicrobi al compound +100ul culture (microbiolo gy lab environmen t culture)	75 ul media +25 ul antimicrobi al compound +100ul culture (microbiolo gy lab environmen t culture)	87.5 ul media +12.5 ul antimicrobi al compound +100ul culture (Microbiolo gy lab environmen t culture)	87.5 ul media +12.5 ul antimicrob ial compound +100ul culture (Microbiol ogy lab environme nt culture)	87.5 ul media +12.5 ul antimicro bial compoun d +100ul culture (Microbio logy lab environm ent culture)
T. Indica	0ul media +100ul antimic	0ul media +100ul antimicr	0ul media +100ul antimicr	50ul media +50 ul antimic	50ul media +50 ul antimicro	50ul media +50 ul antimicro	75 ul media +25 ul antimicrobi al	75 ul media +25 ul antimicrobi al	75 ul media +25 ul antimicrobi al	87.5 ul media +12.5 ul antimicrobi	87.5 ul media +12.5 ul antimicrob	87.5 ul media +12.5 ul antimicro

	robial compo und +100ul culture (microb iology lab environ ment culture)	obial compoun d +100ul culture (microbi ology lab environ ment culture)	obial compou nd +100ul culture (microb iology lab environ ment culture)	robial compo und +100ul culture (micro biology lab environ ment culture)	bial compoun d +100ul culture (microbi ology lab environm ent culture)	bial compoun d +100ul culture (microbio logy lab environm ent culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	compound +100ul culture (microbiolo gy lab environmen t culture)	al compound +100ul culture (Microbiolo gy lab environmen t culture)	ial compound +100ul culture (Microbiol ogy lab environme nt culture)	bial compoun d +100ul culture (Microbio logy lab environm ent culture)
Control					(-ve control) 150ul media + 50 ul antimicro bial compoun d (T.Arjuna)	(-ve control) 150ul media + 50 ul antimicro bial compoun d (T.Arjuna)	(-ve control) 150ul media + 50 ul antimicrobi al compound (T. Indica)	(-ve control) 150ul media + 50 ul antimicrobi al compound (T. Indica)	(-ve control) 150ul media + 50 ul antimicrobi al compound (T. Indica)	(+ve control) 100ul broth +100 ul culture)	(+ve control) 100ul broth +100 ul culture)	(+ve control) 100ul broth +100 ul culture)

Table 6 - Results of the spectrophotometric result of MIC test at wavelength of 620 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unknown 1:1 0.816	Unknown 1:1 0.891	Unknown 1:1 0.877	Unknown 1:1 0.822	Unknown 1:1 0.728	Unknown 1:1 0.763	Unknown 1:1 0.536	Unknown 1:1 0.603	Unknown 1:1 0.687	Unknown 1:1 0.728	Unknown 1:1 0.715	Unknown 1:1 0.965
B	Unknown 1:1 0.535	Unknown 1:1 0.541	Unknown 1:1 0.519	Unknown 1:1 0.424	Unknown 1:1 0.462	Unknown 1:1 0.507	Unknown 1:1 0.596	Unknown 1:1 0.597	Unknown 1:1 0.595	Unknown 1:1 0.588	Unknown 1:1 0.547	Unknown 1:1 0.647
C	Unknown 1:1 0.377	Unknown 1:1 0.330	Unknown 1:1 0.311	Unknown 1:1 0.188	Unknown 1:1 0.192	Unknown 1:1 0.188	Unknown 1:1 0.162	Unknown 1:1 0.346	Unknown 1:1 0.321	Unknown 1:1 0.174	Unknown 1:1 0.180	Unknown 1:1 0.152

D	Blank Assay 0.557	Blank Assay 0.518	Blank Assay 0.477	Blank Assay 0.594	Blank Assay 0.578	Blank Assay 0.561	Blank Assay 0.192	Blank Assay 0.181	Blank Assay 0.200	Blank Assay 0.596	Blank Assay 0.504	Blank Assay 0.706
E	Unknown 2_0 1:1 0.786	Unknown 2_0 1:1 1.359	Unknown 2_0 1:1 1.142	Unknown 2_0 1:1 0.835	Unknown 2_0 1:1 0.652	Unknown 2_0 1:1 0.651	Unknown 2_0 1:1 0.629	Unknown 2_0 1:1 0.623	Unknown 2_0 1:1 0.700	Unknown 2_0 1:1 0.689	Unknown 2_0 1:1 0.670	Unknown 2_0 1:1 0.738
F	Unknown 2_0 1:1 0.503	Unknown 2_0 1:1 0.678	Unknown 2_0 1:1 0.688	Unknown 2_0 1:1 0.680	Unknown 2_0 1:1 0.696	Unknown 2_0 1:1 0.700	Unknown 2_0 1:1 0.693	Unknown 2_0 1:1 0.729	Unknown 2_0 1:1 0.755	Unknown 2_0 1:1 0.814	Unknown 2_0 1:1 0.770	Unknown 2_0 1:1 0.719
G	Unknown 2_0 1:1 0.314	Unknown 2_0 1:1 0.348	Unknown 2_0 1:1 0.300	Unknown 2_0 1:1 0.228	Unknown 2_0 1:1 0.205	Unknown 2_0 1:1 0.196	Unknown 2_0 1:1 0.515	Unknown 2_0 1:1 0.151	Unknown 2_0 1:1 0.520	Unknown 2_0 1:1 0.494	Unknown 2_0 1:1 0.508	Unknown 2_0 1:1 0.734
H	Blank Assay 0.730	Blank Assay 0.740	Blank Assay 0.677	Blank Assay 0.726	Blank Assay 0.541	Blank Assay 0.563	Blank Assay 0.190	Blank Assay 0.195	Blank Assay 0.179	Blank Assay 0.836	Blank Assay 0.547	Blank Assay 0.777

The MIC of the herbal compound is at that concentration which has the absorbance equivalent to the absorbance of blank assay of negative control of that component . So for *T. arjuna* , *T. indica* and *W. somnifera* the absorbance of the concentration which is equivalent to the blank assay are -

Table 7 . MIC concentration of the herbal compound

Herbal compound	**MIC Concentration (microgram/ml) against E. coli.**	**MIC Concentration (microgram /ml) against Microbiology lab culture**
W. somnifera	50	50
T. arjuna	75	75
T. indica	50	75

VI. Biofilms -

Biofilms were grown for the culture for 24 hrs and the stained by 0.1 % crystal violet dye

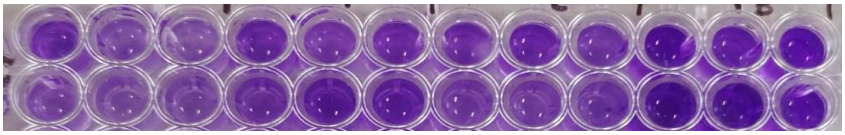


Fig 13.

Fig 13. 96 well plates with biofilm grown and stained .

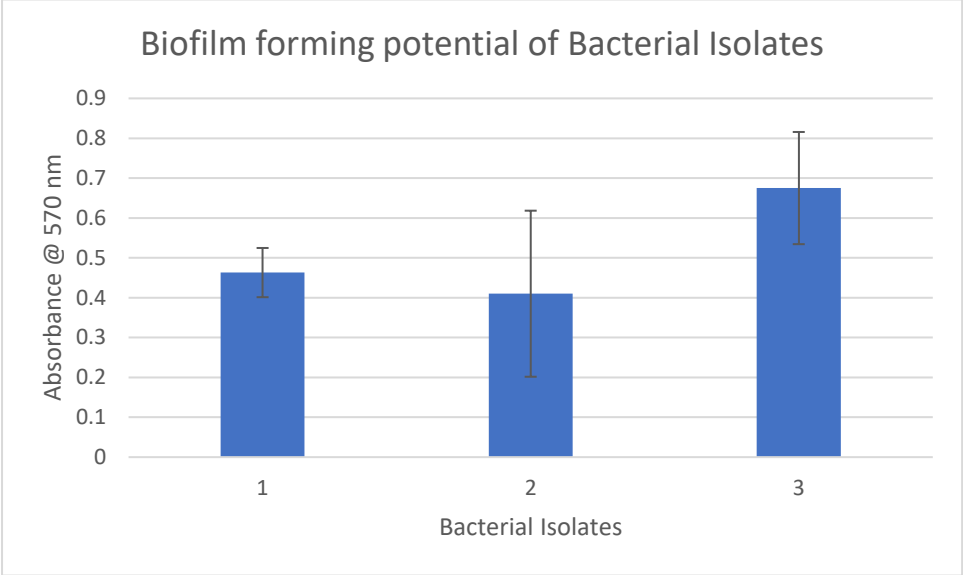


Fig 14. The biofilm-forming capacity of isolates - the 1, 2 and the in the image shows the average of absorbance value of the Isolates 1 , 2 and 3 respectively with the standard deviation

The biofilm-forming capacity of each isolate is summarized in Figure 15 .The OD_{550} values for bacterial isolates 1, 2, 3 and negative control were 0.463 ± 0.0617, 0.41 ± 0.2083 , 0.675 ± 0.1407 and 0.179 ± 0.048 respectively. In total isolates were positive for biofilm formation, Isolates 1 are comparatively weak biofilm former and Isolate 2 and 3 are comparatively strong biofilm former .

CHAPTER V
CONCLUSION

CONCLUSION

The herbal compounds were tested for antimicrobial activity against the bacterial isolates collected from the different areas of JUIT and the results of antimicrobial assay shows that the herbal compounds have a significant amount of antimicrobial activity in them as the increase in the concentration of the herbal compounds shows the increase in the zone of inhibition in the antimicrobial assay . The bacterial isolates also have a great potential for biofilm formation and these compounds can also be tested for the antibiofilm activity .

CHAPTER VI
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APPENDIX I

1. MULLER- HINTON AGAR (MHA)

Composition (1 liter) pH- 7 at 25 degree C

Amount (gm)	Compound
2gm	Beef Extract
17.5 gm	Casein Hydrolysate
1.5 gm	Starch
17.0 gm	Agar

2. NUTRIENT AGAR (NA)

Composition (1 liter) pH- 7-7.2 at 25 degree C

Amount	Compound
3gm	Beef Extract
5.0 gm	Pepton
1000 ml	Distilled water
20.0 gm	Agar

3. LURIA BERTANI BROTH (LB)

Composition (1 liter) pH- 7-7.2 at 25 degree C

Amount	Compound
10 gm	Peptone
6 gm	Sodium Chloride
5 gm	Yeast Extract

4. PBS BUFFER (PBS)

Composition (1 liter) pH- 7-7.4

Amount	Compound

1.44 gm	Sodium Phosphate Dibasic
0.2 gm	Potassium Chloride
8 gm	Sodium Chloride
0.245 gm	Potassium Phosphate Monobasic

5. O.5 McFarland REAGENT

Composition (1OO ml) pH- 7-7.4

Amount	Concentration	Compound
O.5 ml	1.7 %w/v	Barium Chloride
99.5 ml	1%	Sulfuric acid