THE SUPREMACY OF NATURAL/HERBAL-BASED ANTIBIOTICS AND INVESTIGATION OF THE SYNERGISTIC ACTIVITY OF FOENICULUM VULGARE, PIPER BETEL, ABELMOSCHUS ESCULENTUS AND ALLIUM SATIVUM AGAINST STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI

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Abstract:-

In the past few years, there has been a rising concern regarding the excessive utilization and resistance of synthetic antibiotics in treating bacterial infections. The excessive use of these antibiotics has led to a decline in their efficacy against harmful human pathogens. As a result, there has been a greater emphasis on natural antibiotics due to their lower toxicity and reduced side effects. Natural antibiotics not only function as antibacterial agents but also contribute to the development of natural immunity within the body. One strategy to enhance antibacterial activity involves the combination of extracts from natural sources, which can lead to synergistic effects. This synergistic activity has the potential to overcome the increasing antibiotic resistance. The aim of the current research is to assess the effectiveness of different natural sources, specifically *Foeniculum vulgare*, *Allium sativum*, *Piper betel*, and *Abelmoschus esculentus*, in combating *Staphylococcus aureus* and *Escherichia coli* by the agar disc diffusion method.

These particular bacterial strains were chosen due to their significant role in causing bacterial infections and their increasing resistance to antibiotics. The components of these natural sources were extracted using various methods and subjected to initial phytochemical tests. The antimicrobial activity was then evaluated through a microbial assay. The findings of our study provide new insights that the combined effect to use *Foeniculum vulgare, Allium sativum, Piper betel,* and *Abelmoschus esculentus* as potential plant sources for controlling pathogenic bacteria and potentially considered as cost-effective in the management of diseases and to the threat of drug resistance phenomenon. Consequently, these natural sources have the potential to serve as viable alternatives to synthetic antibiotics for the treatment of bacterial infections.

Key words: - Bacterial Inhibition, *Escherichia coli,* Resistance, Synergistic, *Staphylococcus aureus.*

1. Introduction

Antibiotics are medicines that fight bacterial infection in people and animals. They work by killing the bacteria or by making it hard for the bacteria to grow and multiply. Natural antibiotics are substances that occur in nature such as a plant, microbe that possess an activity to either harm or kill the microorganisms like bacteria. Natural products have served as a potential therapeutic agent against pathogenic bacteria since the golden age of antibiotics of the mid-20th century. In 90 years since the discovery of penicillin, natural products have provided a major foundation for the development of antibiotic drugs. Natural antibiotics historically have a crucial importance in the identification and development of antibacterial agent. The rapid emergence of antibiotic resistance had led a route to identify novel chemical skeleton with antibacterial activity for elaboration in drug development. As combinatorial approach has not benefited much in yielding effective drugs, the development of new antibiotics from natural scaffolds is the best short-term solution to the rising crisis of antibiotic resistance. Recent approaches had reengineered natural products into potent antibiotics. [(1)]

Natural antibiotics are not synthesized in the laboratory like other antibiotics prescribed by physician. Natural antibiotic plant and substances are being used for centuries by many other cultures worldwide, but the first natural antibiotic used is penicillin which is a by-product of the by-process of the mould *penicillium notatum*. The Scottish bacteriologist Alexander Fleming discovered the antibiotic- *penicillin* when a culture of the bacterium *Staphylococcus aureus* was inhibited by a mould grown on it. [(2)]

Plants produce complex compounds such as secondary metabolites that are necessary for enhancing the likelihood of survival. Plants are capable of producing structurally diverse compounds that are useful for the plant to defence against phytopathogens and also against human pathogens. There are four main groups of antimicrobial compounds produced by plants such as polyphenols and phenolics, terpenoids, essential oils and alkaloids. These compounds act by different mechanisms such as inactivation of proteins and enzymes to act against the pathogens. Plants are one of the unique and underexploited sources of bioactive compounds and ethnobotanical research tools to guide the future research efforts. [(3-4)]

Certain studies by scientists have proved that the concept of synergistic action of multiple natural products are more effective and can overcome resistance note in mono therapy models. Synergistic activity of certain plant extracts may provide a potential antibacterial activity to overcome the growing antibiotic resistance.

1.1. Types of antibiotics based on the source:

Most of the antibiotics in the clinical use are of bacterial or fungal origin such as streptomycin, penicillin, erythromycin, neomycin. There are also antibiotics derived from non-microbial sources such as plant source, animal source, marine source and so.

1.1.1 Natural source of antibiotics:

1.1.1.a. Plant source [5-6]

Source	Part of Plant used	Chemical constituents	Uses	ectrum of action
Garlic	Clove	Allicin, Alin, Diallylsulfide, diallyl trisulfide ajoene, and S-allyl-cysteine	Wards off cough and cold, boost immunity, has antibiotic properties,prevents cancer and peptic ulcer	*Staphylococcus aureus*, *E.coli*
Goldenseal	Roots and Leaves	Berberine, Hydrastine, canadine, Hydrastinine	Dietary supplement for colds and other RTI, allergic rhinitis, ulcers, diarrhea, and Constipation	*hylococcus aureus*
Tea tree	Leaves	Terpinen-4-ol,Terpinene,Limonene,Eucalyptol	Used for acne, athlete's foot, lice, nail, fungus, mite infections at base of eyelids	*oli*, *Candida albicans*
Oregano	Leaves	Carvacrol, Thymol, Caryophyllene, p-Cymene	arasite infections,Wound healing	*hylococcus aureus*
Aloe barbadense (Aloe vera)	Dried juice of leaves	Reducing sugar,tannins,anthraquinone,glycoside,saponins, Flavonoid	Antibacterial, anti-inflammatory, anti-diabetic, antioxidant	*S.aureus MRSA.*, *K.pneumonia*
Solanum nigrum (Black nightshade)	Plant	alkaloids, cardiacglycosides, flavonoids, saponins, tannins, volatileoils	Treats bacterial infections, indigestion and cough	*E. coli*, *P.aeruginosa*, *S.typhi.*
Cuminum cyminum L.	Seed	Essential oils, aldehydes	ntimicrobial, nsecticidal, antioxidant	*Bacillus cereus*, *hylococcus aureus*

ryophyllum nnatum (Bryophyllum)	Leaves	Reducing sugar, tannins, glycosides, saponins, flavonoids, steroids, terpenoids	Treats respiratory tract infections, dissolves kidney stones.	*E.coli, S.aureus*

Table: 1 Plant sources with antibacterial sources

1.1.1.b. Microbialsource[7]:

Source	Part of microbe Used	Chemical constituent	Uses	Spectrum of action
Penicillium chrysogenum	Mycellium or spore of fungi	β-lactamring, thiazolidine ring and side chain	Used to treat bacterial infections like respiratory tract infection, urinary tract infection, skin infection.	Gram positive bacteria like *Staphylococcus aureus*
Cephalosporium acremonium	The mycelium of filtrate of The fungus.	β-lactamring, dihydrothiazine ring, and a side chain	Cephalosporins are used to treat bacterial infections, caused by gram-negative bacteria	*Escherichia coli, Proteus mirabilis, Klebsiella pneumonia*
Streptomyces griseus	The filtrate of bacteria	Amino glycoside antibiotic	Streptomycin is used to treat tuberculosis and other bacterial infections caused by gram-negative bacteria	Active against *Staphylococci, Streptococci, Pneumococci.*
Saccharopolyspora erythraea	The filtrate of the Bacteria	Macrolide antibiotic	It is used to treat respiratory tract infections, skin infection,sexually transmitted infections	*Staphylococcus aureus, Streptococcus, Legionella pneumophila*
Streptomyces venezulae	The filtrate of bacteria	Chloramphenicol molecule	Chloramphenicol is used to treat meningitis, Ricketts	Gram negative bacteria like *E. Coli*

Table: 2 Microbial sources with antibacterial sources

1.1.1.c. Marine source[8-10]

Organism	Geographical Location	Compound	Antimicrobial activity Against
Marinactinospora thermotolerance	South China sea	Marthiapeptide A	*Micrococcus leuteus, Staphylococcus aureus, Bacillus subtilis*

Streptomyces scopuliridis	South China sea	Desotamide B	*Staphylococcus aureus, Streptococcus pneumonia*
Streptomyces Drozdowiczii	South China sea	Marfomycins A,B, E	*Micrococcus luteus*
***Streptomyces* sp.**	South China sea	Lobophorin F	*Staphylococcus aureus*
***Streptomyces* sp.**	South China sea	Lobophorin H	*Bacillus subtilis*
***Verrucosispora* sp.**	Japanese sea	Abyssomicin C	MRSA, vancomycin Resistant *Staphylococcus aureus*
***Streptomyces* sp.**	Saharan debris flown earth Canary Islands	Caboxamycin	*Bacillus subtilis, Staphylococcus lentus, Staphylococcus epidermidis*
***Emericella* sp.**	South China sea	Emerixanthones A,B,C, D	*E.coli, Staphylococcus aureus, E.faecalis*
Engyodontium Album	SouthChinasea	Engyodontiumone H	*E.coli, Bacillus subtilis*
***Spiromastix* sp.**	South Atlantic Ocean	SpiromastixonesA,B, C, D, E, F, G, H,K,L,M, N, O	*Staphylococcus aureus, Bacillus subtilis, E.faecalis,* MRSA
***Penicillium* sp.**	Chinesesea	Penicyclones A,B,C,D, E, F	*Staphylococcus aureus*

Table: 3 Different Types of Marine Sources Possessing Antibacterial Activity

1.2. Introduction to Bacteria:

1.2.a. *Staphylococcus aureus* [11-13]

Staphylococcus aureus is a gram-positive bacterium which is a major bacterial human pathogen that causes a wide variety of clinical manifestations. Infections caused by the bacteria are common in both community-acquired as well as hospital-acquired settings. The treatment of these infections remains challenging to manage due to the emergence of multi-drug resistant strains such as MRSA (Methicillin-Resistant *Staphylococcus aureus*). The bacteria cause potentially serious infections when it enters into the bloodstream and internal tissues. Transmissions mostly from direct contact; however, some infections involve other transmission methods.

Etiology:

Staphylococcus aureus is a gram-positive bacteria (stains purple by gram stain) that are cocci-shaped and are described as “grape-like” bacteria. These organisms can grow aerobically and anaerobically (facultative) at temperatures between 18°C and 40°C.

MRSA strains carry a mec gene on the bacterial chromosome, which is component of the larger *Staphylococcus chromosomal cassette* mec (SCCmec) region, conferring to resistance to multiple antibiotics depending on the SCCmec type. This gene encodes the protein PBP-2a (penicillin binding protein 2a) which is an essential enzyme that catalyses the production of peptidoglycan in the bacterial cell wall. PBP-2a has less affinity to bind to beta-lactams than PBPs, so PBP-2a continue to catalyse the synthesis of bacterial cell wall even in the presence of many antibiotics. Thus, *Staphylococcus aureus* strains that synthesizes PBP-2a are resistant to many antibiotics. MRSA strains tend to be resistant to methicillin, nafcillin, oxacillin and cephalosporins.

Epidemiology:

Staphylococcus aureus are found on the skin and mucous membranes, and humans are the major reservoir for these organisms. Infection can be transmitted person to person by direct contact.

Treatment or management:

Treatment of infection depends upon the type of infection as well as presence or absence of resistant strains. In general, penicillin is the drug of choice if the isolates are sensitive and vancomycin for MRSA strains. MRSA infections are emerging as serious pathogens in both the hospital and the community settings.

1.2. b. *Escherichia Coli* [14-16]

Escherichia coli is a gram-negative bacterium which is a part of normal intestinal flora but can also be a cause of intestinal and extra intestinal illness in humans.

Etiology:

E.coli is a gram-negative bacteria in the human gastrointestinal tract. The bacteria can cause urinary tract infections (UTI), pneumonia, bacteria and peritonitis. It can also be found in soil, on vegetables, and in water as well as in undercooked meats. Pathogenic strains cause intestinal illness in humans.

Epidemiology:

• The intestinal illness caused by one of the five subtypes of E. coli and they are identified according to their O and H antigens. The O antigens determined by repeating polysaccharide chain present in the lipopolysaccharide outer membrane and H antigen is determined by the flagellum.

• Extra intestinal illness is caused due to translocation of gut bacteria into other parts of the body or the environmental spread in hospital and long-term care facilities. It can cause urinary tract infection, abdominal and pelvic infection, pneumonia, bacteremia and meningitis.

Treatment or management:

• Rehydration - oral fluids or IV fluids

• Anti-motility - bismuth salicylate, loperamide and antibiotics like fluoroquinolones, azithromycin.

• Extra-intestinal illness

• Beta-lactam antibiotics - cephalosporins, carbaphenams, monobactams.

• Nitrofurantoin

• Fluoroquinolones and Fosfomycin

• Trimethoprim-sulfamethoxazole.

1.3. Synthetic antibiotics [17-18]:

Synthetic antibiotic chemotherapy as a science and development of antibacterial began in Germany with Paul Ehrlich in the late 1880s. Antibiotics are the chemotherapeutic agents that are used to eradicate bacterial infections or restrain bacterial reproduction. They exhibit antibacterial activity as bactericidal or bacteriostatic or both. Antibiotic agents can either isolated from the natural sources or synthetically produced or semi-synthetic antibiotics. Synthetic antibiotics are chemically produced antibiotics in the laboratory. Certain examples of synthetic antibiotics are amoxicillin, ampicillin, azithromycin and so.

1.3.1. Drawbacks:

The most common side effects caused by synthetic antibiotics are Change of sense of taste, nausea, vomiting, loss of appetite, sources, ulcers on lips and mouth, skin rashes.

Resistance developed by synthetic antibiotics: Due to the mass production and usage of antibiotics lead to the development of microbial resistant to the antibiotics. The antibiotic effect has been decreased than its earlier effect due to over-usage and development of resistance to the antibiotics by the bacteria. Resistance arises when bacteria acquire drug resistance genes or mutations in genes that alter their sensitivity to an antibiotic. Organisms develop resistance through different mechanisms such as limiting the uptake of drug, modifying drug target, inactivating the drug, active drug efflux. The microorganism develops resistance to a particular antibiotic on its continuous usage. There are also chances for cross-resistance where the bacterium develops survival methods that are effective against different types of antimicrobial molecules with similar mechanism of action.

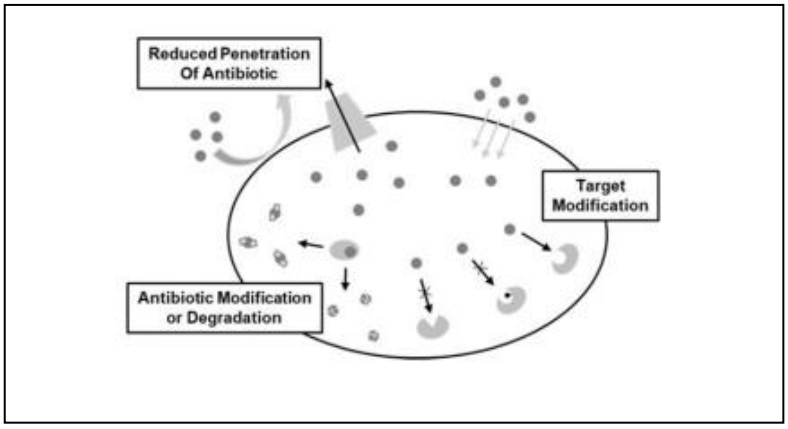


Figure 1: Mechanisms through Which Bacteria Develops Resistance

Major factors contributing to the development of resistance are - the frequent prescription against infection of anti-bacterial nature, such as viral infections, and unregulated usage, which can lead to the sub-lethal doses, permitting resistance to spread rapidly.

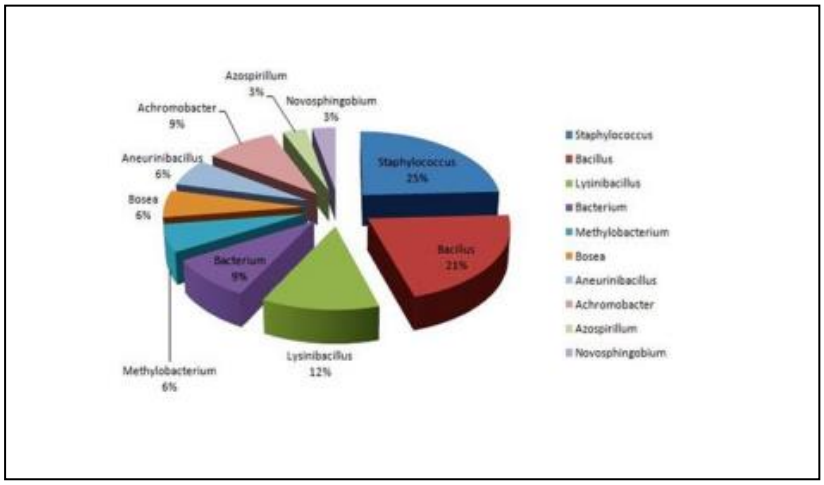


Figure 2: Pie-chart showing the percentage of antibiotic resistant bacteria

Antibiotic resistant bacteria	Percentage
Staphylococcus	**25%**
Bacillus	**21%**
Lysinibacillus	**12%**
Bacterium	**9%**
Achromobacter	**9%**
Methylobacterium	**6%**
Bosea	**6%**
Aneurini bacillus	**6%**
Azospirillum	**3%**
Novoshingobium	**3%**

Table 4: Showing the percentage of antibiotic resistant bacteria

Centers of disease control (CDC) have stated that antibiotic resistance had led to the millions of illnesses and thousands of deaths. Previously treatable infections are now serious concerns due to lack of effective antibiotics. Even with the developing the potential new antibiotic isolates and scaffolds, we must take care to preserve the efficacy of drugs currently prescribed. We should educate the people about causes and persistence of antibiotic resistance.

Microbial assay of antibiotics: [19] Microbial assays are a technique to assess a compound's potency or the concentration. Microbial assay is carried out by exposing the microorganisms to the compound and determining the effect of the compound on the microorganism. Microbial assays are required for the antibiotic assay in the US Pharmacopoeia and the British Pharmacopoeia. These official public medicinal drugs, their effects, and directions for their use, Microbial assays provide the information about the effect of antibiotic in inhibiting the growth of microorganisms. It is a comparative method. This method is also used to study the effect of other compounds such as vitamins and amino-acids. Measured concentrations of the target compound are compared with the standard concentration of a particular compound with known activity, providing information on the compound under investigation.

Microbial assays are used for many purposes such as antibiotic sterility testing, testing the muta genicity or carcinogenicity of chemicals, disease diagnosis and monitoring using immobilized enzymes. They are also used to evaluate the Pharmacokinetic properties of drugs for human and animal use.

1.4.1. Methods used in microbial assays :[20]

Different methods used in microbial assay are disc diffusion method (cylindrical cup plate method), the tube assay (turbidimetric method), the urease assay, the luciferase assay, radio-enzymatic assay.

1.4.1.a. The cylindrical cup plate method

Cylindrical cup plate method involves using circular plates of nutrient agar inoculated with a susceptible test organism. The cup plate method includes the selection of inoculant concentration to obtain suitable dose-response and sharp inhibition zones at different concentration of the standard, keeping plates on a flat surface to ensure equal distribution of inoculant and incubating plates at a suitable temperature over a time.

The microbial assay of antibiotics is done by comparing the zone of inhibition formed by the microorganisms to a specific concentration of antibiotics having known activity. There are different types of methods for microbial assay of antibiotics such as cup plate method and disc diffusion method.

Figure: 3 Cup plate

In the cup plate method, the antibiotic containing cylinder is diffused into the agar layer containing microorganisms. The zone is formed around the cylinder.

Requirements:

Test organism, Nutrient agar medium, Antibiotics Apparatus- borer, petri plates.

Procedure:

Prepare nutrient agar plates inoculated with test organism, with a depth of 4-5mm and then allow it to solidify. Divide the nutrient agar plates into four equal portions. Then with the help of a sterile borer make four cavities in each portion. Then fill three cavities with antibiotic solution and one cavity with the standard solution. Slowly incubate the plates at 37^0C for 24hours.After incubation measure the zones of inhibition.

Table (1.5) consists of components of nutrient agar medium, their functions and pH of the medium along with the temperature in degree Celsius (^{0}C).

Ingredients	Purpose
0.5% Peptone	Provides organic nitrogen
0.3% Beef extractor yeast extract	The water soluble contents provide vitamins, carbohydrates, nitrogen and salts.
1.5% Agar	It gives mixture solidity
0.5% Sodium chloride	Mixture is similar those found in the cytoplasm of most of organisms.
Distilled water	Serves as transport medium
pH	Neutral (6.8) at 25^0C

Table 5: Showing the Composition of Nutrient Agar Medium

1.4.1. b. Turbidimetric method:

Includes the controls that are not inoculated with the target compound are used to set the optical apparatus used to measure the growth of microorganism. When the tubes are incubated, growth is arrested by adding formaldehyde R, or the opacity of tubes can be measured at a set incubation time. Appropriate statistical methods are used to calculate the potency of the compound.

Urease assay is used to assay antibiotics that inhibit the protein synthesis, as these antibiotics also inhibit urea production. The urease production is indicated with increase in the media's pH. Certain microbes such as Proteus mirabilis can be used as they produce the enzyme urease.

Luciferase assay uses the enzyme luciferase to detect the presence of ATP in a microbial sample. This is detected by bioluminescence. Antibiotics such as aminoglycosides inhibit ATP production, and this assay can be used to analyze the potency of the drugs.

Radio enzymatic assay, a radio labelling element is used to analyze the potency of the test compounds. The radio labelling agent such as 14C carbon isotope (as co-factor of 1-14C acetyl coenzyme A)

1.5. Microbes used in the microbial assay:

There are different types of microbes used in the microbial assay. The selection of microbial strain depends upon the antibiotic being selected for testing. These include *Staphylococcus aureus, Bacillus pumilus, Staphylococcus epidermis and Klebsiella pneumoniae*

1.5.1. Culture media:

Different culture media are used in the microbial assay. The type of media selected depends upon the microbe being used in the assay such as anaerobic bacteria, aerobic bacteria or fungi. A fluid thioglycolate medium is used for anaerobic bacteria and also aerobic bacteria. Soybean-casein digest is a culture medium for aerobic bacteria and fungi. Culture media must be produced under strict conditions to ensure sterility so that the results obtained by the microbial assay on the potency of target compounds are error-free. The first liquid artificial culture medium was created by Louis Pasteur in 1860. A culture medium is essentially composed of basic elements (water, nutrients) to which must be added different growth factors that will be specific to each bacterium and necessary for their growth. The first solid media was developed by Koch. The main gelling agent used in solid culture media is agar. An enriched culture medium consists of the elements essential for the growth of bacteria.

For the growth of bacteria minimum nutrients are required such as water, carbon source, nitrogen source and some mineral salts.

Water: Water plays an important role in solubilizing nutrients, transporting them and promoting hydrolysis reactions. If evaporation occurs during incubation, results in the loss of water, decreasing colony size and inhibiting the bacterial growth.

Carbon source: Carbon is the most abundant element in the bacteria. It is essential for bacteria to produce carbon molecules such as fats, carbohydrates, proteins and nucleic acids.

Nitrogen source: Nitrogen allows bacteria to synthesize their proteins. It is available in both inorganic form as nitrates and organic form as protein-hydrolysates.

Energy sources: There are two types of bacteria such as *Thiocapsa roseopersicina*, which uses light as an energy source by transforming it into electrochemical gradient of protons, and chemotrophic bacteria that use energy of oxidation of minerals or organic compounds as energy sources.

Growth factors: It is sometimes necessary to add growth factors to culture medium to boost the multiplication of bacteria. Growth factors are required in small quantities in culture medium.

Purine and pyrimidine bases: These are necessary for the synthesis of nucleic acids.

Amino acids: Amino acids are also growth factors that are used for protein synthesis.

Vitamins: A vitamin is an organic substance, necessary in small quantities for the metabolism of a living organism, which cannot be synthesized in sufficient quantity by that organism.

Antioxidants: Antioxidants are added to the culture media to allow the culture of strict anaerobic bacteria under anaerobic conditions.

The table (6) consists of the ingredients used in the preparation of fluid thio glycolate medium along with their quantities in grams (g) and milliliters (ml).

Ingredients	Composition
L-cysteine	0.5g
Sodium chloride	2.5g
Dextrose monohydrate	5.5g
Granular agar	0.75g
Yeast extract	5.0g
Pancreatic digest of casein	15.0g
Sodium thio glycolate	0.5g
Resazurin sodium solution	1.0ml
Distilled water	1000ml
pH of the medium after sterilization	7.1+/-0.2

Table 6: Fluid- thio glycolate medium

The table (7) consists of ingredients used in the preparation of soya bean- casein digest medium with their quantities in grams (g) and milliliters (ml)

Ingredients	Composition
Pancreatic digest of casein	17.0g
Papaic digest of soya bean meal	3.0g
Sodium chloride	5.0g
Di potassium hydrogen phosphate	2.5g
Dextrose monohydrate	2.5g
Distilled water	1000ml
pH of the medium after sterilization	7.3+/-0.2

Table: 7 Soya bean –Casein Digest Medium

2. Methodology:

- Collection of plant materials
- Extraction of plant extracts.
- Preliminary phyto chemical screening of the extracts.
- Microbial assay of the extracts against *E.coli* and *Staphylococcus aureus*.

2.1. Collection of plant parts

Different plant parts are collected from various sources such as:

The fresh leaves of *Foeniculum vulgare* are obtained from well-grown plants from the local market of Shapur Nagar, Hyderabad. The leaves are air-dried for one week to completely remove the moisture from the fresh leaves.

The fresh cloves of *Allium sativum* are bought from a local market near Gandimaisamma, Hyderabad. The fresh cloves are made into a rough paste using motor and pestle.

The pods of *Abelmoschus esculentus* are collected from the local market of Shapur Nagar, Hyderabad, and shade-dried for about 5 days until the pods are completely dried without any moisture left in them. The dried seeds are ground into a rough powder.

The fresh leaves of Piper betel are collected from the market of Kukatpally, Hyderabad, and subjected to drying for about 5 days. The completely moisture-free leaves are ground into a fine powder.

2.2. Extraction of plant materials

Powdered plant material was subjected to extraction process, the soxhlation and maceration.

2.2.a. Soxhlation[21]

Extraction procedure

The 2g of powdered plant material is taken to carry out Soxhlet extraction process. Plant material is subjected to soxhlation using methanol solvent and up to solvent is colorless. After extraction, the solvent is evaporated in a water bath, yielding the extracted compound and their percentage yield is calculated respectively.

$$\text{Percentage of extract (\%)} = \frac{\text{Weight of extract}}{\text{Total weight of the compound to be extract}} \times 100$$

2.2. b. Maceration

Extraction procedure:

This extraction procedure is carried out to extract three plant extracts:

• 100g of *Allium sativum*, 19g of Piper betel, and 7.61g of *Abelmoschus esculent* are taken to carry out maceration process.

• Maceration is carried out using methanol as solvent for continuous stirring for 3 hours and left for 5 days without disturbance.

• After extraction, the solvent is separated from marc by filtration and the extract is then separated from menstruum by evaporation on the top of the water bath.

2.3. Preliminary phyto chemical screening of extracts

2.3.a. Detection of Alkaloids

Dragendorff's test: To 1ml of test filtrate, two drops of Dragendorff's reagent (potassium bismuth iodide solution) was added and observed for the formation of prominent reddish-brown precipitate.

Mayer's test: 1ml of test filtrate was taken into a test tube and added two drops of Mayer's reagent (potassium mercuric iodide solution) along the sides of the test tube and observed for the white or creamy precipitate.

Wagner's test: 1ml of test filtrate was taken into a test tube, added two drops of Wagner's reagent (iodide-potassium iodide solution) along the sides of the test tube and observed for reddish-brown precipitate.

Hager's test: To 1ml of filtrate, two drops of Hager's reagent (picric acid) was added and observed for prominent yellow precipitate.

2.3. b. Detection of Carbohydrates

Molisch's test: 1ml of the test solution was taken and two drops of alcoholic solution of α-naphthol (Molisch's reagent) was added. The mixture was shaken and 1ml of conc. H_2SO_4 was added slowly from the sides of the tube. The test tube was cooled in ice water and allowed to stand. Then the test tubes were observed for violet ring formation at the junction.

Fehling's test: 1ml of the test filtrate was boiled on a water bath with a mixture of 1ml each of Fehling's solutions A and B and allowed to boil for 1 minute and observed for the formation of red precipitate.

Benedict's test: To 0.5ml of the filtrate, 0.5ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes and observed for the formation of yellow, green, or red-colour precipitate.

Barfoed's test: To 1 ml of test filtrate, 1ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes and observed for the formation of red precipitate.

2.3. c. Detection of Proteins and Amino acids

Biuret test: To 3ml of test filtrate, two drops of 4% NaOH was added and treated with two drops of 1% $CuSO_4$ solution and observed for the formation of pink colour.

Ninhydrin test: To 3ml of test filtrate, three drops of 5% Ninhydrin reagent was added and heated in boiling water bath for 10 minutes and observed formation of a characteristic purple colour.

Salkowski test: To the test filtrate, 2ml of chloroform and 2ml of concentrated sulphuric acid were added, shaken well and observed the coloration of chloroform and acid layers. Chloroform layer is red in colour and acid layer is greenish yellow fluorescence.

Liebermann-Burchard test: To the test filtrate, 2ml of acetic anhydride, 2ml of chloroform were added and heated to boiling and cooled. Then 1ml of concentrated sulphuric acid was added along the sided of the test tube and observed for the formation of colour at the junction.

Ferric chloride test: The test filtrate was taken and added two drops of neutral 5% ferric chloride solution and observed for blue, green or violet colour.

Lead acetate test: The test filtrates were taken and to this 3ml of 10% lead acetate solution were added and observed for formation of bulky white precipitate.

Bromine water test: The test filtrate was taken and 1ml of bromine water was added and observed for discolouration of bromine water.

Cardiac glycosides test: The test filtrates were taken and added few drops of pyridine and 1drop of 2% sodium nitro prusside and a drop of 20% sodium hydroxide solution was added and observed for formation of deep

Keller-Killiani test: The test filtrate was taken and added 2ml of glacial acetic acid and two drops of 5% ferric chloride solution and mixed. Then 1ml of sulphuric acid was added. Reddish brown colour appears at the junction of the two liquid layers and upper layer appears bluish green colour.

Foam test: Filtrates were taken and 20ml of distilled water was added and shaken for 15 minutes in a graduated cylinder and observed for the formation of a layer of stable foam.

Tests for Fats and Fixed Oils (petroleum ether extract): Fixed oils and fats can be confirmed by chemical test for glycerine, which is produced by hydrolysis.

Saponification test: Add few drops of 0.5N alcoholic potassium hydroxide to a small quantity of various extracts along with a drop of phenolphthalein separately and heat on a water for 1-2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Treat 5 drops of sample with 1ml of 1% copper sulphate solution. Then add 10% sodium hydroxide solution. A clear blue solution is obtained which shows glycerine is present in sample. The cupric hydroxide formed in the reaction does not precipitate out as it is soluble glycerine.

To the 5 drops of sample add a pinch of sodium hydroxide sulphate, pungent odour emanates, indicating presence of glycerine.

Microbial assay: The antibacterial activity of the test compound against *E.coli* and *Staphylococcus aureus* was assayed using cup plate method.

Cup plate method:

Principle: In cup plate method, antibiotic containing cylinder is diffused into the agar layer containing the microorganisms. The zone is formed around the cylinder.

Requirements: Organism-Test microorganism, DMF solvent, antibiotics, nutrient agar medium, antibiotic disc.

Procedure: All glass wares required for the microbial assay are sterilized in the hot air oven for 10 to 15 minutes. The required quantity of nutrient agar is taken into a conical flask and dissolved in distilled water. The conical flask is placed into an autoclave at 121°C temperature and 15lbs pressure for sterilization of media. After sterilization, 20ml of nutrient agar media is poured into each petri plates near the flame and allowed the media to solidify. After the solidification of media, the microorganism is spread throughout the petri plates using L-shape rod and the entire process is carried out near the flame to contamination mediation other microorganisms. The cups are made using a sterile borer and the sample is poured into the cups using micro pipette. The agar plates containing extracted sample inoculated with the bacteria are incubated in the incubator at 37°C (99°F) for 24 hours. After incubation, the plates are removed and the zone of inhibition is measured using a scale.

Microbial assay is carried out in two ways: Microbial assay of single extract where only single plant extract is assayed for antibacterial activity and Microbial assay of combination of extracts. The antibacterial activity of combination of the extracts will be assayed for their synergistic activity.

Results and Discussion:

Foeniculum vulgare is subjected to soxhlation and Piper betel, *Abelmoschus esculentus*, Allium sativum are subjected to maceration using methanol as solvent. The results of percentage yield, phytochemical screening and biological activities of the extracts are shown below in respective tables.

Physical status of methanolic extracts of *Foeniculum vulgare*, *Piper betel*, *Abelmoschus esculentus* and *Allium sativum*.

S.no	Plant extract	Method of extraction	Colour	Weight	Yield (%)
1.	*Foeniculum vulgare*	Soxhlation	Green	2g	45
2.	*Allium sativum*	Maceration	Light yellow	100g	45
3.	*Piper betel*	Maceration	Green	7g	28.57
4.	*Abelmoschus esculentus*	Maceration	Light yellow	5g	20

Table: 8 Showing percentage yield of methanolic extracts of *Foeniculum vulgare, Abel moschusesculentus, Allium sativum* and *Piper betel.*

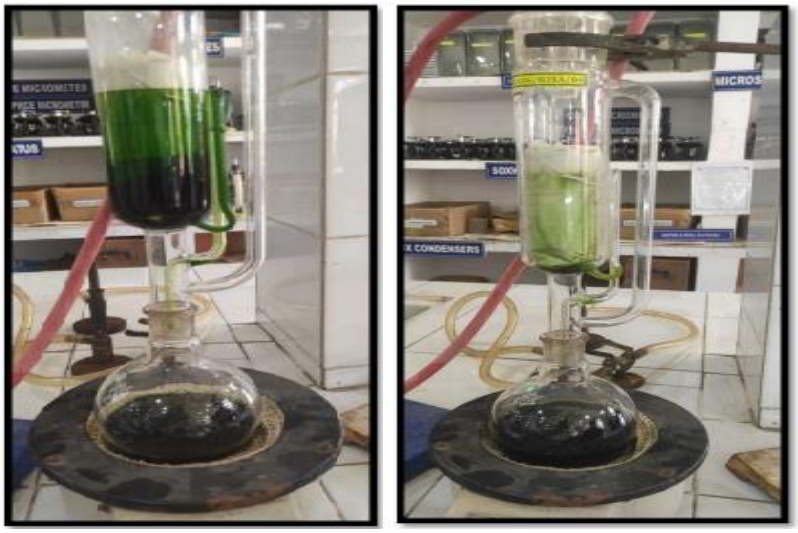


Figure: 4 Extract of Fennel leaves

Figure : 5 Extract of Okra Seeds , Betel Leaves, Garlic

3.2: Preliminary phyto chemical screening of methanolic extracts of leaf Foeniculum vulgare, Piper betel, cloves of Allium sativum, and seeds of Abelmoschus esculentus.

Preliminary phytochemical screening includes performing various tests in order to check the presence of certain compounds and to confirm under which class they belong to.

S.no	Chemical constituents	*Piper betel* leaf	*Allium sativum* buds	*Abelmoschus esculentus* seeds	*Foeniculum vulga* leaves
1	Carbohydrates	+ve	+ve	+ve	+ve
2	Proteins	+ve	+ve	+ve	+ve
3	Amino acids	+ve	+ve	+ve	+ve
4	Fats and oils	-ve	-ve	-ve	+ve
4	Phyto sterols	+ve	-ve	+ve	+ve
6	Glycosides	-ve	+ve	-ve	+ve
7	Cardiac glycosides	-ve	+ve	-ve	-ve
8	Alkaloids	+ve	+ve	-ve	+ve
9	Tannins	+ve	-ve	+ve	+ve
10	Phenol	+ve	+ve	+ve	-ve
11	Fixed oil	-ve	-ve	-ve	-ve
12	Saponins	-ve	+ve	-ve	-ve
13	Steroids and terpenoids	-ve	+ve	-ve	+ve

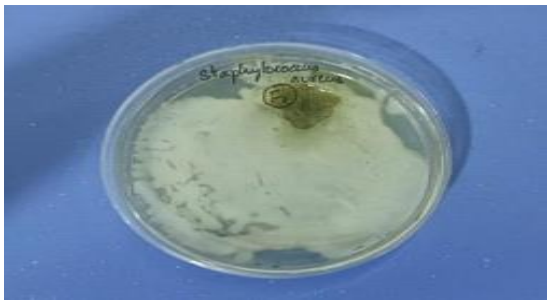
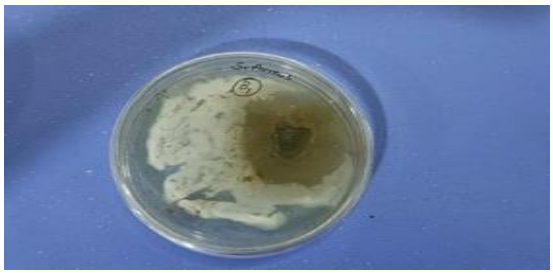
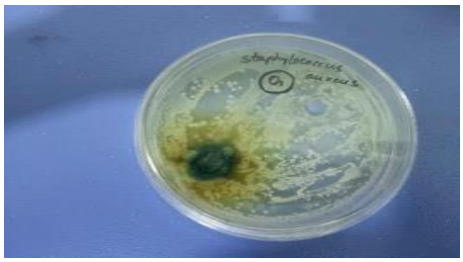
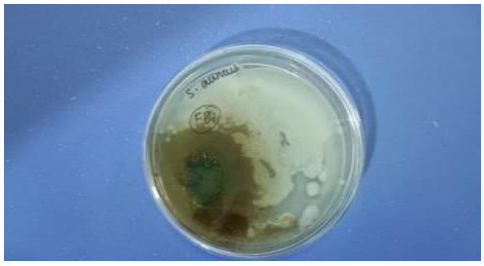
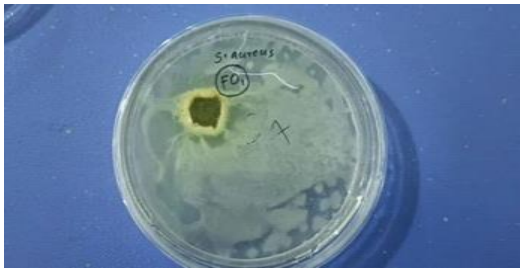
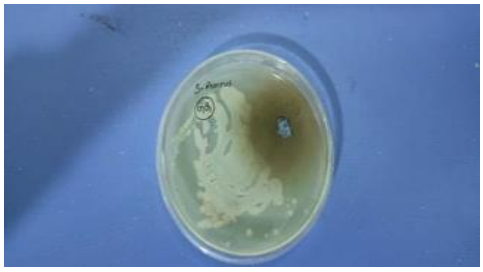
Table: 9 Results of the preliminary phytochemical screening of methanolic extracts of *Piper betel* leaf, *Allium sativum* buds, *Abelmoschus esculentus* seeds, and *Foeniculum vulgare* leaves.

Positive (+ve) and negative (-ve) represents presence or absence of above shown category of phytoconstituents by preliminary phytochemical tests.

3.3 Results of antibacterial activity of the plant extracts against *Escherichia coli* and *Staphylococcus aureus.*

The zones of inhibition is measured for the agar plates containing single extract and the combination of extracts using a scale and the obtained values are as follows:

3.3.1. Zone of inhibition of extracts against *Staphylococcus aureus*

<table>
<tr>
<td>A
 
Fennel (F)zone of inhibition (0.5cm)</td>
<td>B
 
Garlic (G) zone of inhibition (0.5cm)</td>
</tr>
<tr>
<td>C
 
Betel(B) zone of inhibition (4cm)</td>
<td>D
 
Okra(O) zone of inhibition (0.5cm)</td>
</tr>
<tr>
<td>E
 
Fennel and Betel (FB)zone of inhibition (5cm)</td>
<td>F
 
Fennel and Garlic (FG) zone of inhibition (1.2cm)</td>
</tr>
<tr>
<td>G
 
Fennel and Okra (FO) zone of inhibition (0.8cm)</td>
<td>H
 
Garlic and Betel (GB) zone of inhibition (4.5cm)</td>
</tr>
</table>

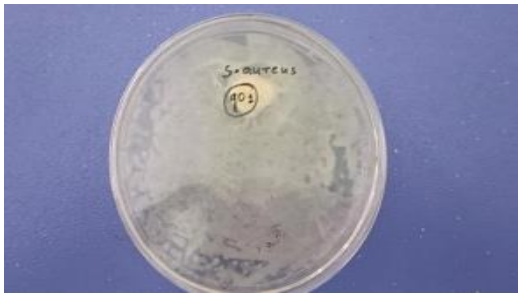
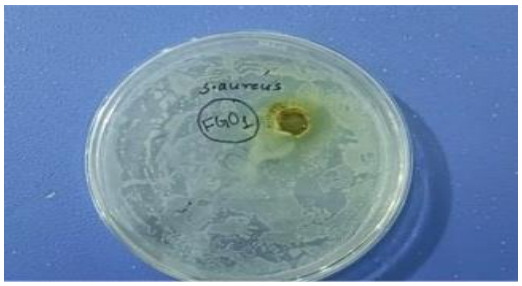
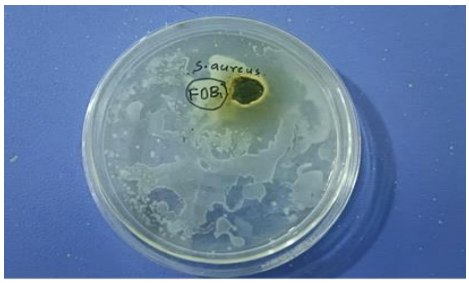
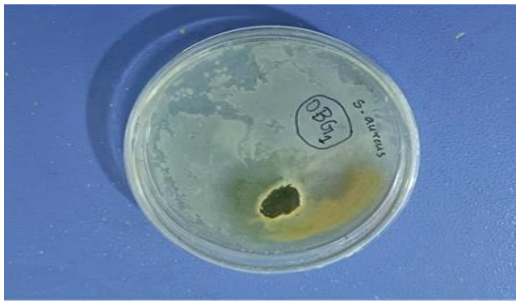
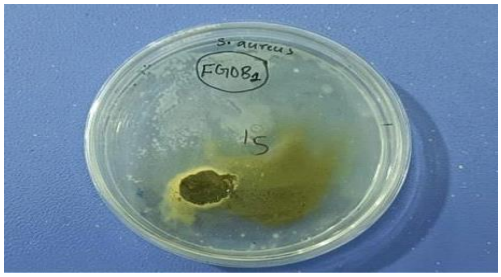
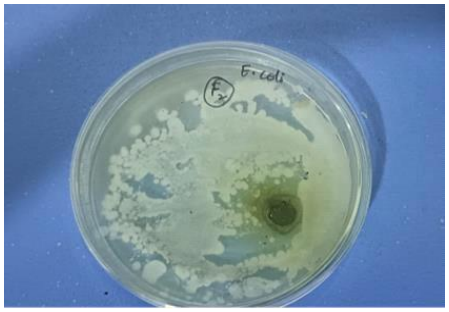
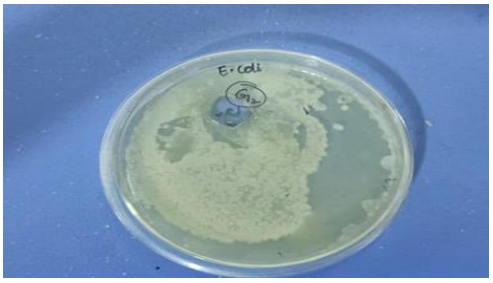
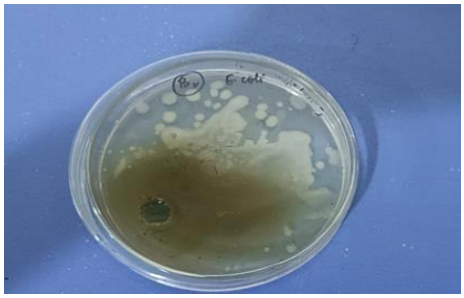
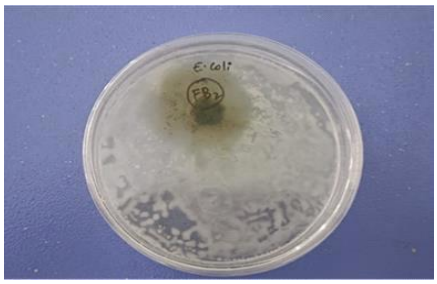
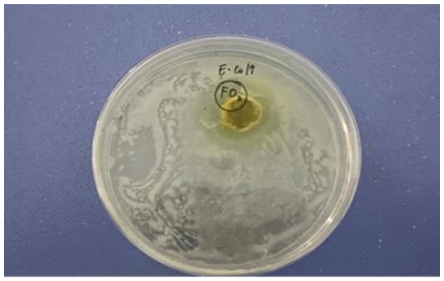
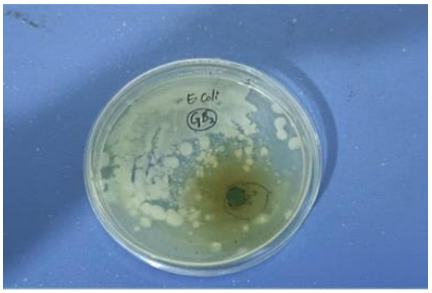
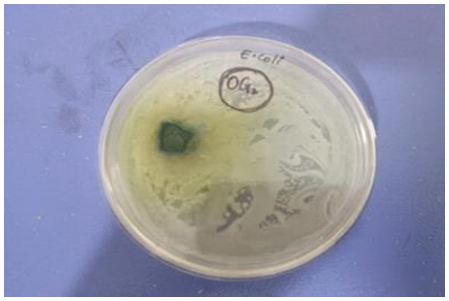
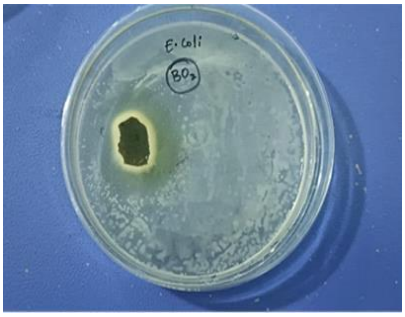
I  **Garlicand Okra(GO)zoneofinhibition(1.1cm)**	J  **BetelandOkra(BO)zoneofinhibition(4.3cm)**
K  **Fennel, Garlic and Okra(FGO) zone of inhibition(2cm)**	L  **Fennel, Okra and Betel(FOB) zone of inhibition(4.8cm)**
M  **Okra, Betel and Garlic(OBG) zone of inhibition(4.2cm)**	N  **Fennel leaves,Okraseeds, Betel leaves and Garlic(FOBG) zone of inhibition(5.5cm)**

Figure: 6 The zone of inhibition measured by using agar plates treated with single extract and the combination of extracts (A–N) against *Staphylococcus aureus* and its area of *inhibition.*

3.3.1. Zone of inhibition of extracts against *Escherichia coli*

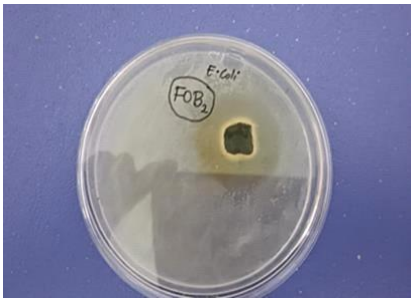
A  **Fennel(F)zone of inhibition(1.8 cm)**	**B**  **Garlic(G) zoneofinhibition(1 cm)**

C  **Betel(B) zoneofinhibition(3.5 cm)**	**D**  **Okra(O)zoneofinhibition(1 cm)**
E  **Fennel and Betel (FB) zone of inhibition (4cm)**	**F**  **Fennel and Garlic (FG) zone of inhibition (2.8 cm)**
G  **Fennel and Okra (FO) zone of inhibition (3 cm)**	**H**  **Garlic and Betel (GB) zone of inhibition (3.4 cm)**
I  **Garlic and Okra (GO)zone of inhibition (1.5cm)**	J  **Betel and Okra (BO)zone of inhibition (3.5 cm)**

K

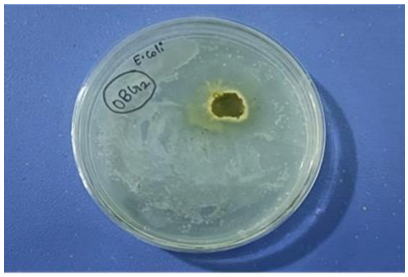
Fennel, Garlic and Okra(FGO) zone of inhibition(3.5 cm)

L



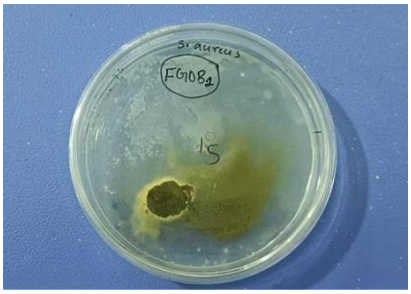
Fennel, Okra and Betel(FOB) zone of inhibition(4.5cm)

M



Okra, Betel and Garlic(OBG) zone of inhibition (4.5cm)

N



Fennel leaves, Okraseeds, Betel leaves and Garlic (FOBG) zone of inhibition (5.8cm)

Figure: 7 The zone of inhibition measured by using agar plates treated with single extract and the combination of extracts (A–N) against ***Escherichia coli*** and its area of inhibition.

Extract	**Zone of inhibition against *Staphylococcus aureus (in cm)***	**Zone of inhibition for against *Escherichia coli (in cm)***
Fennel(F)	0.5	1.8
Garlic(G)	0.5	1
Betel(B)	4	3.5
Okra(O)	0.5	1
Fennel and betel(FB)	5	4
Fennel and garlic(FG)	1.2	2.8
Fennel and okra(FO)	0.8	3
Garlic and betel(GB)	4.5	3.4
Garlic and okra (GO)	1.1	1.5
Betel and okra(BO)	4.3	3.5
Fennel ,garlic and okra (FGO)	2	3.5
Fennel, okra and betel (FOB)	4.8	4.5
Okra ,betel and garlic (OBG)	4.2	4.5
Fennel, garlic, okra and betel (FGOB)	5.5	5.8

Table 10: Zone of inhibition of plant extracts against *Staphylococcus aureus and Escherichia coli*

Discussion:

Individual Extracts effect:

- Betel (B) shows the highest zone of inhibition against both Staphylococcus aureus (4 cm) and Escherichia coli (3.5 cm) among the individual extracts.
- Garlic (G) also demonstrates significant inhibition against Staphylococcus aureus (0.5 cm) but less against Escherichia coli (1 cm).
- Fennel (F) and Okra (O) exhibit minimal inhibition against both bacteria.

Combined Extracts:

- Combinations involving Betel (B) generally enhance the inhibitory effect against both bacteria compared to individual extracts.
- Fennel and Betel (FB) combination show the highest combined inhibition against both bacteria (5 cm and 4 cm, respectively).
- Garlic and Betel (GB), Betel and Okra (BO), and Okra, Betel, and Garlic (OBG) combinations also show considerable inhibition against both bacteria.

Synergistic Effects:

- The combination of Fennel, Garlic, Okra, and Betel (FGOB) demonstrates the highest synergy with significant zones of inhibition against both Staphylococcus aureus (5.5 cm) and Escherichia coli (5.8 cm).
- Other combinations like Fennel and Okra (FO) and Fennel, Okra, and Betel (FOB) also show synergistic effects, particularly against Escherichia coli.

Antagonistic Effect:

- An antagonistic effect occurs if the combination has a smaller effect than the most effective single extract. None of the combinations listed exhibit clear antagonistic effects based on the provided data.

Overall, combinations like FOB and FGOB show promising synergistic effects against both Staphylococcus aureus and Escherichia coli, suggesting potential for enhanced antimicrobial activity compared to individual plant extracts.

CONCLUSION

Due to extensive use of antibiotics, the microorganisms have developed resistance against the antibiotics and thus there is an antibiotic resistance crisis due to overuse and misuse of the medications. Thus, to treat the infections caused by resistant bacteria new compounds need to be developed which should have more efficiency and low toxicity. Natural sources of antibiotics are alternative sources to overcome the resistance. The natural sources, not only have the antibacterial activity but also help to strengthen the immunity to fight against the bacteria. The synthetic antibiotics also show a great consequence to the environment. The excessive use of synthetic antibiotics resulted in the decreased effect of antibiotics against harmful human pathogens and it also led to the contamination of the environment with harmful chemicals. Hence, to overcome these problem natural antibiotics are recommended to treat bacterial infections. Natural source of antibiotics is preferred over synthetic antibiotics because they cause less side effects and they develop immunity in the humans to fight against bacteria. There are many natural sources which have antibacterial activity such plant sources, microbial sources and marine sources.

Here, we have selected plant sources which have antibacterial activity against few elected strains of bacteria (*E. coli & Staphylococcus aureus*). These natural sources of antibiotics are used to treat bacterial infections with less side effects compared to synthetic sources of antibiotics.

The study highlights the potential of these plant extracts, especially in combinations, as natural antibiotics against Staphylococcus aureus and Escherichia coli. Combinations involving Betel (Piper betel) show consistent and significant inhibitory effects, suggesting its potent antimicrobial properties. In both cases, the combinations that include Fennel, Betel, Garlic, and Okra tend to show higher zone of inhibition than individual extracts, suggesting synergistic antibacterial effects. This indicates that these combinations could potentially be explored further for their antimicrobial properties, possibly for therapeutic use against Staphylococcus aureus and Escherichia coli infections. In conclusion, the combinations of fennel with betel, garlic, and okra show notable synergistic activity against both *Staphylococcus aureus* and *Escherichia coli*, indicating potential enhanced antimicrobial properties when these plant extracts are used together.The data also suggests that certain combinations of these plant extracts exhibit synergistic effects against both Staphylococcus aureus and Escherichia coli. Specifically, combinations like Fennel and Betel (FB), Fennel and Garlic (FG), and multiple combinations involving Fennel, Garlic, Okra, and Betel (FGOB) show significantly enhanced zones of inhibition compared to individual extracts. This indicates potential synergistic antimicrobial activity where the combined effect is greater than the sum of individual effects, which could be explored further for their therapeutic potential against bacterial infections. Further research could focus on understanding the mechanisms of synergy among these extracts and their applicability in combating antibiotic-resistant strains of these bacteria.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions in their own way in all aspects for successful completion of research work. **Naveena.R**, **Samyuktha.Metta:** Conceptualization, design, Formal analysis; acquisition of data, or analysis and interpretation of data; Writing-review & editing or revising it critically for important intellectual content **Elakanti Pooja, Golla Nikitha, Podchanpally Vamshi Krishna, Alambaram Vaishnavi:** Funding acquisition; Investigation; Project administration; Visualization, Supervision, Writing-review & editing.

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There is no funding to report.

CONFLICT OF INTEREST

Authors disclose no conflicting interest with current work.

ETHICAL STATEMENT

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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