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Synergistic antimicrobial action of antibiotics and plant extracts on pathogens

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Abstract
Plants such as Neem, Clove, Cinnamon, Aloe vera, Ginger are used as medicinal plants since long time. They produce secondary metabolites such as alkaloids glycoside etc, which are responsible for the antimicrobial activity of the plants. The ethanolic extracts of these plants produce lysosome which is capable of killing pathogens. The antimicrobial activity of these plant extracts is checked on pathogens such as *E. coli, S. aureus, P. aeruginosa*. Agar well diffusion technique was used for checking the antimicrobial activity of plants extract with synergy of antibiotics penicillin, ampicillin and streptomycin. 90µl of extract was mixed with 10 µl of antibiotic solution and the plates were observed for inhibition zone. The most inhibition zone was observed for Aloe vera, Clove and Neem and least was observed for Cinnamon and Ginger.

Keywords: Synergy, well diffusion, plant extracts, antibiotics

Introduction
Medicinal plants are the richest bio-resource of drugs for traditional system of medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. In more than 80% of developed countries, plant have been used as traditional medicine as they are the good source of compound derivation, Therefore, plants are investigated for better understanding of their properties, safety and efficacy. For thousands of years, plants have played an important role in maintaining human health and improving the quality of human life, as well as serving as valuable components in medicines [1].

Many plants have been used for its antimicrobial traits, which are chiefly due to the synthesis of secondary metabolites such as glycoside, terpenoids, alkaloids, phenol and flavonoids. and its inhibitor effect against the growth of pathogens. Plants extract have both phytochemical and antimicrobial properties and can be of great significance in therapeutic treatments [2].

In recent times the research for potent antimicrobial agents has been shifted to plants. Most plants are medicinally useful and in most of cases the antimicrobial efficacy value attributed to some plants is beyond belief. The advent of science into the search for antibiotics largely depends on some of these medicinal plants as a raw material. According to WHO, a medicinal plant is any plant that contains substances in one or more of its organs that can be used for therapeutic purposes or that are precursors to the synthesis of useful drugs [3].

Syzygium aromaticum (Cloves), a spice used in Ayurveda, is a source of antimicrobial agent against oral bacteria that are commonly associated with dental caries and periodontal disease. It was also reported that *S. aromatica* have been successfully used for Asthma and various allergic disorders by oral administration. Clove is directly applied to gums for toothache for pain control during dental work and for complication of tooth extraction called dry socket [4].

Azadirachta Indica (Neem) belongs to the family *Meliaceae*, commonly known as neem. It is used in traditional medicine as a source of many therapeutic agents. It used for leprosy, eye disorders, bloody nose, intestinal worms, stomach upsets, loss of appetite, diseases of heart and blood vessels [5].

Zingiber Officinale (Ginger) is a medicinal plant that has been widely used all over the world since antiquity, for a wide array of unrelated ailments including arthritis, colds, nausea, migraines and hypertension [6]. *Aloe barbadensis* (Aloe vera) is a succulent plant from the *Liliaceae* family. It has a whorl of elongated, pointed leaves.

The species is frequently mentioned as being used in herbal medicine. It has antioxidant and antibacterial properties, aids in wound healing, reduces dental plaque, and treats cancer sores [7]. *Cinnamomum Verum* (Cinnamon) is a spice obtained from inner bark of several tree species from the genus *Cinnamomum.* Cinnamon bark is used for gastrointestinal upset, diarrhea and gas. It is also used for stimulating appetite for infections caused by bacteria and parasitic worms and for menstrual cramps and common cold [8].

Pseudomonas aeuriginosa is a common gram-negative, rod-shaped bacterium. *P. aeuriginosa* is an unusual cause of diseases in humans, usually affects patients with compromised immune systems (e.g. Patients on cancer treatment) [9]. *Staphylococcus aureus* is a gram-positive, round shaped bacteria that is the member of the farmicutes, and is frequently found in the nose, respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infection such as a skin abscess, respiratory infection such as sinusitis, food poisoning [8]. *E. coli (Escherichia coli)* is a gram-negative, rod-shaped bacteria. It is a resident of gut of certain animals. Many strains are however pathogenic to animals. They are facultative aerobes with both aerobic and anaerobic growth [10].

Antibiotics are the chemical that kill or inhibit the growth and are used to treat bacterial infection. Antibiotics are essential for control of bacterial diseases. Most of the antibiotics are used for clinical uses. The antibiotic kills the bacteria by disintegrating the cell wall. Effect arising between two or more agents, entities, factors or substances that produces an effect greater than the sum of their individual effects is synergistic effect. Synergy is the creation of a whole that is greater than the sum of the individual effects. The screening of crude extract for synergistic interaction with standard antibiotics against the resistant bacteria as this world pave the way for possible isolation of antibiotic-resistant inhibitors [11, 12].

Materials and Methods

Experimental material and experimental site

The medicinal plants were collected from local areas and used as experimental materials. The experiment was performed in MGM Institute of Biosciences, Chh. Sambhajinagar.

Experimental Details

The work was undertaken to study the antibacterial activity of the ethanol extract, of *Aloe vera, Zingiber Officinale, Azarirchta Indica, Syzygiun Aromaticum, Cinnamaomum Verum*. The extracts are added with antibiotics such as Ciprofloxacin, Chloramphenicol, Erythromycin, Gentamycin and Streptomycin in order to improve the effect of antibiotics against the phathogens: *Pseudomonas aeuriginosa, Styphylococcus aureus and Escherichia coli*.

Media Preparation: Mueller-Hinton agar, which is required for microorganism growth, was prepared. The medium was prepared by combining 2 gm beef extract, 17.5 gm casein hydrolysate, 1.5 gm starch, and 17 gm. Agar is dissolved in 1000 mL of distilled water in conical flasks. The required petri plates, glassware, and media are autoclaved at 121ºC for 20 minutes at 15 psi pressure. The media is then poured into sterile petri plates under aseptic conditions for future use [13].

Culture Selection and Inoculation

The bacterial cultures of *Pseudomonus aeuriginosa, Staphylococcus aureus and Escherichia coli* were collected from the MGM Institute of Bioscience and Technology Aurangabad. The bacterial culture of subcultured on Mueller Hinton agar media and the culture plates were maintain at 37ºC for 24 hrs [14].

Preparation of Ethanoic Extract

Medicinal plants (*Aloe vera, Zingiber Officinale, Azarirchta Indica, Syzygiun Aromaticum, and Cinnamomum Verum*) were gathered from the surrounding area and market. The plant parts were washed with tap water and air-dried at room temperature A dried plant weighing 8 gm was ground to produce a fine, homogeneous mixture. The mixture was soaked in 30 ml of 95% ethanol at room temperature for 72 hours in the dark. The solution was then filtered using Whatman filter paper. The medicinal plant extract was filtered and stored at 20 ºC for future use [13].

Preparation of antibiotics solution

A stock solution was prepared by dissolving 200 mg of antibiotic in 2 ml of distilled water. Dilute 100 µl of antibiotic stock solution with 1 ml of double distilled water to create a working solution of 10 mg/ml.

Test for secondary metabolites

The secondary metabolite tests in the project were conducted to detect the presence of alkaloids, glycosides, flavonoids, terpenoids, and phenols in the medicinal plant extracts. The methods used for each test are as follows:

- **Alkaloid Test:** 2 ml of the ethanolic plant extract was mixed with 0.2 ml of dilute HCl, followed by the addition of 1 ml of Mayer's reagent. The appearance of a precipitate indicated the presence of alkaloids.
- **Glycoside Test:** 1 ml of the ethanolic extract was mixed with 1 ml of distilled water and 5 drops of NaOH. The formation of a yellow colour indicated the presence of glycosides.
- **Flavonoid Test:** 1 ml of the plant extract was mixed with 5 drops of concentrated HCl. The development of a red or orange colour indicated the presence of flavonoids.
- **Terpenoid Test:** 0.5 ml of the extract was mixed with 2 ml of chloroform, followed by the addition of 3 ml of concentrated H_2SO_4. The formation of a reddish-brown interface indicated the presence of terpenoids.
- **Phenol Test:** 1 ml of the extract was mixed with 1 ml of distilled water, followed by the addition of 2 drops of ferric chloride. A blue or green colour indicated the presence of phenolic compounds.

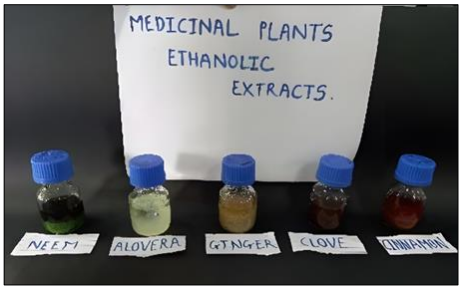

Fig 1: Medicinal Plant Extracts

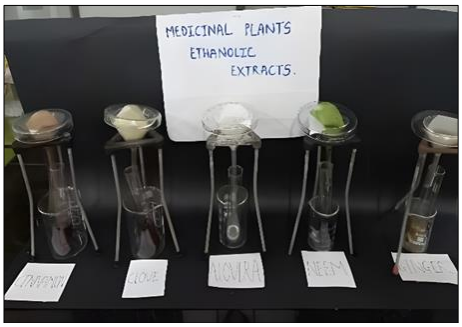

Fig 2: Filtration of Exrtracts

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was used to separate and analyze the bioactive compounds in the medicinal plant extracts. Four different solvent systems were employed to evaluate the relative mobility of these compounds, expressed as RF values. The plant extracts (Neem, Clove, Cinnamon, and Ginger) were applied on TLC plates, and the solvent systems-benzene and ethanol; ethyl acetate, butanol, and water; ethyl acetate, formic acid, and glacial acetic acid; and toluene and ethyl acetate-were used to separate the compounds based on their polarity.

Gram Staining

Gram staining of bacterial cultures was performed to confirm the presence of bacteria. First the smear of *E. coli* culture was made on grease free slide and it was allowed to dry. Primary stain crystal violet was applied for about 1 min. It was washed with grams iodine and after it with 95% ethanol. After that it was washed with distilled water. Then secondary stain saffranin was applied on the smear for 1min and it washed with distilled water. Extra stain was blotted with filter paper and smear was observed under 100X resolution under a microscope. This procedure was repeated for *S. aureus* and *P. aeuriginosa.*

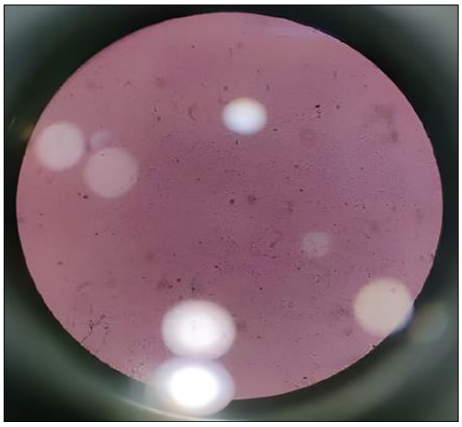

Fig 3: *E. Coli*

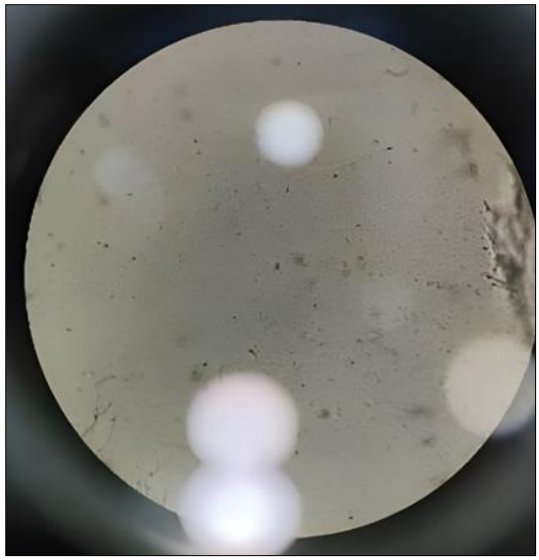

Fig 4: *S. aureus*

Synergistic Antimicrobial Activity

The test organisms were inoculated onto Muller-Hinton agar plates and incubated at 37°C for 24 hours to allow for bacterial growth. After incubation, wells were made in the centre of the plates, and varying concentrations of medicinal plant extracts along with antibiotics were placed in the wells. The plates were left to settle for 10-15 minutes to allow proper diffusion of the extracts and antibiotics into the agar. The plates were then incubated again at 37°C for an additional 24 hours.

Measurement of Zone of Inhibition

To assess antimicrobial effectiveness, the clear zones around the wells were measured using a ruler. These zones represented areas where bacterial growth had been inhibited due to the action of the plant extracts, antibiotics, or their combination. The diameter of each inhibition zone was carefully measured to quantify the antimicrobial activity. Larger zones indicated stronger bacterial inhibition, reflecting either the individual effect of the plant extract or antibiotic, or the synergistic action of both. This measurement was crucial for evaluating the interaction between the plant extracts and antibiotics.

Results

The antimicrobial activity of five medicinal plants (*Aloe barbadensis, Zingiber Officinale, Azadirachta Indica, Syzygium Aromaticum, and Cinnamomum Verum*) was tested in combination with three antibiotics (Ampicillin, Penicillin, and Streptomycin) against bacterial pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

Secondary Metabolite Tests

The secondary metabolites, including alkaloids, glycosides, flavonoids, terpenoids, and phenols, were tested in five medicinal plants: *Aloe barbadensis* (Aloe vera), *Zingiber officinale* (Ginger), *Azadirachta indica* (Neem), *Syzygium aromaticum* (Clove), and *Cinnamomum verum* (Cinnamon). These metabolites are essential for antimicrobial activity as they disrupt bacterial cells and inhibit growth. Alkaloids were present in all plants except Aloe vera, with Clove, Ginger, and Neem showing particularly strong reactions. Glycosides were detected in Neem, Clove, and Cinnamon,

while Ginger and Aloe vera tested negative. Flavonoids were found in Neem, Ginger, and Clove, but absent in Cinnamon and Aloe vera. All plants, except Aloe vera, tested positive for terpenoids, with high activity observed in Cinnamon, Clove, and Neem. Phenols were detected in all the plants, though Aloe vera showed a weaker reaction. This analysis highlights that the presence of multiple bioactive compounds in Neem, Clove, and Cinnamon likely contributes to their stronger antimicrobial properties compared to Aloe vera.

Table 1: Result of secondary metabolites test

Sr. No.	Test	Chemicals	Sample	Results
1.	Alkaloid	2 ml Ethanoic extract + 0.2 ml dilute HCL +1 ml Meyer's Reagent.	Neem	Positive
			Ginger	Positive
			Clove	Positive
			Cinnamon	Positive
			Aloe vera	Negative
2.	Glycoside	1 ml Ethanoic extract + 1 ml of H2O + 5 Drops NaOH	Neem	Positive
			Ginger	Negative
			Clove	Positive
			Cinnamon	Positive
			Aloe vera	Negative
3.	Flavonoids	5 Drops Concentrated HCL + 1 ml of Ethanoic extract	Neem	Positive
			Ginger	Positive
			Clove	Positive
			Cinnamon	Negative
			Aloe vera	Negative
4.	Terpenoids	2ml of Chloroform + 0.5 ml of Ethanoic extract + 3 ml of Concentrated H2SO4	Neem	Positive
			Ginger	Positive
			Clove	Positive
			Cinnamon	Negative
			Aloe vera	Negative
5.	Phenols	1 ml of Ethanolic extract + 1 ml of Water + 2 Drops Ferric Chloride	Neem	Positive
			Ginger	Positive
			Clove	Positive
			Cinnamon	Positive
			Aloe vera	Positive

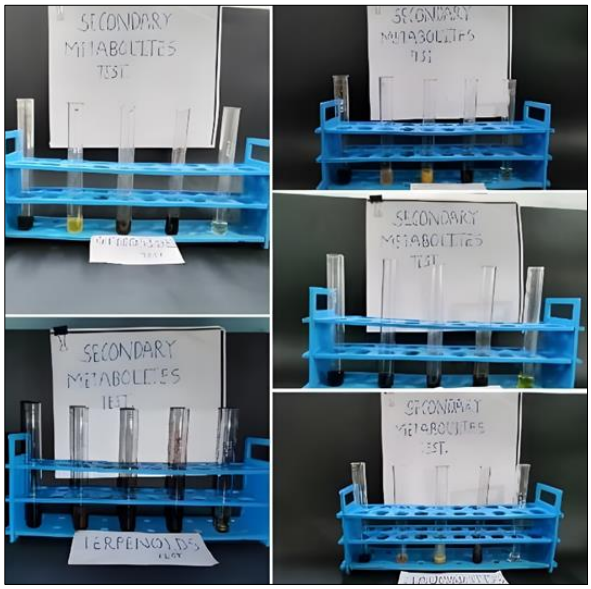

Fig 5: Secondary Metabolites Test

Thin Layer Chromatography (TLC)

Four solvent systems were used to determine the RF values of bioactive compounds in plant extracts, with higher RF values indicating more non-polar compounds and stronger antimicrobial potential. In Solvent System 1 (Benzene and Ethanol), Cinnamon and Ginger showed the highest RF values (0.825 and 0.85), while in Solvent System 2 (Ethyl acetate, Butanol, and Water), Clove and Cinnamon had the highest values (0.93 and 0.94). Solvent System 3 (Ethyl acetate, Formic acid, and Glacial acetic acid) also revealed high RF values for Neem, Clove, and Cinnamon (0.94-0.96), reinforcing their antimicrobial potential. In Solvent System 4 (Toluene and Ethyl acetate), Cinnamon and Clove maintained strong RF values (0.48 and 0.46), showing consistent bioactivity across different systems. Details are as follows:

Fig 6: Thin Layer Chromatography of Plant Extracts

Table 2: Solvent system 1(benzene and ethanol)

Sample	Distance travelled	R_F Value
Neem	3.1cm	0.77
Clove	3.3cm	0.75
Cinnamon	3.4cm	0.825
Ginger	3.0cm	0.85

Table 3: Solvent system 2 (ethyl acetate, butanol and water)

Sample	Distance travelled	R_F Value
Neem	2.6cm	0.78
Clove	3.1cm	0.93
Cinnamon	3.1cm	0.94

Table 4: Solvent system 3 (ethyl acetate, formic acid and glacial acetic acid)

Sample	Distance travelled	R_F Value
Neem	3.3cm	0.94
Clove	3.4cm	0.96
Cinnamon	3.4cm	0.96

Table 5: Solvent system 4 (toluene and ethylacetate)

Sample	Distance travelled	R_F Value
Neem	1.0cm	0.24
Clove	1.9cm	0.46
Cinnamon	2.0cm	0.48

Synergistic Antimicrobial Activity

The study evaluated the synergistic effects of medicinal plant extracts combined with antibiotics against Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus, where the combined effect was greater than that of the individual components. For E. coli, Cinnamon with ampicillin exhibited the highest synergism with a 20 mm inhibition zone, while Ginger and Aloe vera showed moderate effects. Streptomycin combined with Aloe vera demonstrated the strongest effect against P. aeruginosa with a 15 mm inhibition zone, while Cinnamon and Clove also showed strong synergy with ampicillin. Against S. aureus, the highest synergistic effect was seen with Streptomycin and Aloe vera (20 mm), while Cinnamon and Aloe vera showed significant synergy with ampicillin. Clove also enhanced penicillin and ampicillin activity, with Cinnamon displaying broad-spectrum potential across all antibiotics.

The detailed results of this study can be summarized as follows:

Table 6: Effect of ampicillin with extracts on *E. coli*

Name of extracts	Inhibition zone
Neem	5 mm
Ginger	10 mm
Clove	5 mm
Cinnamon	20 mm
Aloe vera	10 mm

Table 7: Effect of penicillin with extracts on *E. coli*

Name of extracts	Inhibition zone
Neem	5 mm
Ginger	15 mm
Clove	10 mm
Cinnamon	15 mm
Aloe vera	10 mm

Table 8: Effect of streptomycin with extracts on *E. coli*

Name of extracts	Inhibition zone
Neem	5 mm
Ginger	7 mm
Clove	5 mm
Cinnamon	2 mm
Aloe vera	5 mm

Table 9: Effect of penicillin with extracts on *P. aeuriginosa*

Name of extracts	Inhibition zone
Neem	10 mm
Ginger	5 mm
Clove	5 mm
Cinnamon	10 mm
Aloe vera	10 mm

Table 10: Effect of streptomycin with extracts on *P. aeuriginosa*

Name of extracts	Inhibition zone
Neem	5 mm
Ginger	2 mm
Clove	7 mm
Cinnamon	10 mm
Aloe vera	15 mm

Table 11: Effect of ampicillin with extracts on P. aeuriginosa

Name of extracts	Inhibition zone
Neem	7 mm
Ginger	5 mm
Clove	15 mm
Cinnamon	15 mm
Aloe vera	10 mm

Table 12: Effect of penicillin with extracts on *S. aureus*

Name of extracts	Inhibition zone
Neem	5 mm
Ginger	10 mm
Clove	10 mm
Cinnamon	5 mm
Aloe vera	7 mm

Table 13: Effect of ampicillin with extracts on *S. aureus*

Name of extracts	Inhibition zone
Neem	7 mm
Ginger	5 mm
Clove	10 mm
Cinnamon	15 mm
Aloe vera	15 mm

Table 14: Effect of streptomycin with extracts on *S. aureus*

Name of extracts	Inhibition zone
Neem	10 mm
Ginger	10 mm
Clove	10 mm
Cinnamon	15 mm
Aloe vera	20 mm

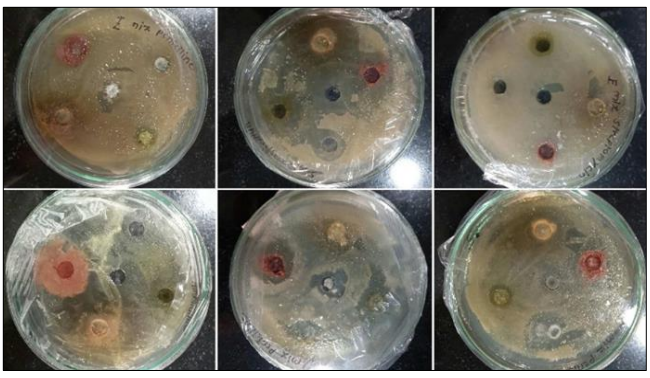

Fig 6: Well Diffusion of the Extracts

Discussion and Conclusion

The results of this study highlight the potential of medicinal plant extracts to enhance the efficacy of conventional antibiotics. The antimicrobial properties of these plants, particularly Clove, Cinnamon, and Aloe Vera, when combined with antibiotics like streptomycin and ampicillin, showed significant inhibitory effects on pathogenic bacteria such as *E. coli*, *S. aureus*, and *P. aeruginosa*. The findings suggest a synergistic relationship between plant extracts and antibiotics, as demonstrated by the increased inhibition zones when both were applied together compared to antibiotics alone. This synergy can be attributed to the bioactive compounds present in the plants, including alkaloids, flavonoids, and phenols, which disrupt bacterial cell walls and enhance antibiotic penetration.

This study confirms that plant extracts have the potential to be used alongside antibiotics to treat bacterial infections. Clove and Cinnamon were the most effective in enhancing the antibiotic effect, especially against *S. aureus* and *E. coli*. This combination approach offers a promising solution to the growing issue of antibiotic resistance, as it can lower the required dosage of antibiotics and prevent the development of resistance.

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