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ESSENTIAL OILS FROM AEGLE MARMELOS, ARISTOLOCHIA INDICA, AND PIPER NIGRUM ROOTS AND ANTIBACTERIAL ACTIVITY AGAINST DRUG RESISTANT MICROORGANISMS

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ABSTRACT

This research investigates the antimicrobial and antioxidant properties of three essential oils (EO): Aegle marmelos, Aristolochia indica, and Piper nigrum roots. The antimicrobial effectiveness of the EOs was demonstrated against four bacteria and a fungus strains. Antimicrobial activity was evaluated by measuring the size of the inhibition zone, as well as determining the minimum inhibitory concentration and minimum bactericidal concentration. Upon analyzing the inhibition zone diameters, we noted an increased effectiveness of A. indica, which displayed the highest values across all microbial species tested. Antifungal activity of essential oil was tested against the selected C. albicans. A. marmelos, A. indica, and P. nigrum essential oils showed 11 mm, 12 mm and 12 mm zone of inhibition against C. albicans. A. indica essential oil showed maximum activity against E. coli (15 mm zone of inhibition) than P. nigrum essential oil (18 mm zone of inhibition), and A. marmelos essential oil (16 mm zone of inhibition). The minimum inhibitory concentration of essential oils were determined against various bacterial pathogens. Essential oil was highly active against Streptococcus sp. and E. coli than K. pneumoniae and S. aureus.

Keywords: Essential Oils, Medicinal Plants, Antibacterial, Antifungal

1. INTRODUCTION

Plants possess a variety of potential antimicrobial compounds that could contribute to the creation of new medications. It is essential to assess medicinal plants known for their strong antimicrobial effects against specific microorganisms to determine their biological efficacy. Recently, there has been a growing interest in traditional medicine to discover new compounds and formulate effective treatments for microbial and viral infections. Furthermore, the prolonged use of synthetic drugs leads to increased resistance in pathogenic bacteria. Consequently, the quest for new antibiotics derived from medicinal plants persists. India is home to two major biodiversity hotspots for plant species: the Western Ghats and the North-eastern Himalayan region. Indian forests are generally rich in various medicinal plants that exhibit a wide range of biological functions, including antibacterial, antifungal, and antioxidant effects. As a result, the traditional healthcare system in India has flourished due to the abundance of medicinal plant varieties. The diversity of medicinal plants in India is primarily found in the Western Ghats, North-eastern India, the Himalayan region, and the

Andaman and Nicobar Islands. More than 80% of the world's medicinal plant biodiversity is reported in these areas [1, 2].

The Indian subcontinent is significantly enriched with around 45,000 plant species, 18,000 of which are flowering plants. This extensive plant diversity is strongly linked to variations in altitude and diverse ecosystems. In India, agro-biodiversity is chiefly distributed across 15 agro-ecological regions and eight distinct phyto-geographical areas. The range of these plants varies significantly, from the alpine scrubs of the Himalayas and the wet evergreen forests of the Western Ghats to the arid desert of Rajasthan and coastal mangrove forests. The indigenous variety of medicinal plant species in the Ganges swamps is notably high in aromatic properties. Over 1,500 species of medicinal plants have been documented and are commonly utilized in traditional medicinal systems like Siddha, Ayurveda, and Unani. Additionally, more than 3,000 plant species are recorded from traditional knowledge and serve as ethnomedicine. Over 700 species of medicinal plants have been chemically analyzed and are widely employed in contemporary medicine [3]. Nevertheless, understanding the extent of medicinal plant species diversity is crucial for overseeing their use in pharmaceuticals, alongside management and conservation efforts. Data on species diversity can facilitate the sustainable use of medicinal plants within an ecosystem. Measuring species diversity is a vital approach to gauge the extent of diversity present in an ecosystem. In India, there are about 141 endemic genera and over 2,500 endemic species of medicinal plants found predominantly in the Himalayan region, with 1,788 species identified in the peninsular region and 185 species recognized in the Andaman and Nicobar Islands. However, accurately determining the number of medicinal plants in specific ecosystems is quite challenging, especially due to the difficulty of linking local vernacular names from traditional medicine to their respective scientific names. This issue is particularly common in a multilingual country like India [4]. These medicinal plants are often utilized as treatments for various health issues, such as asthma, respiratory and gastrointestinal problems, cardiovascular and liver diseases, skin conditions, and urinary tract infections. Additionally, the concentration and composition of bioactive compounds in medicinal plants can vary significantly based on soil types and plant species. Moreover, the production of secondary metabolites in medicinal plants is heavily influenced by the associated microbial communities. The secondary metabolites derived from medicinal plants exhibit diverse biological activities [5].

The identification of new antibiotics represents one of the most impactful and significant achievements in contemporary science for managing various infectious illnesses. Nonetheless, the rise in pathogen resistance to commonly used antibacterial drugs has been accelerating in recent years. Frequent antibiotic use is primarily linked to several adverse effects on the host, such as the reduction of beneficial gut bacteria, hypersensitivity, and allergic responses. The number of bacterial species resistant to drugs and the rise of bacterial strains showing decreased susceptibility to numerous standard antibiotics are growing swiftly. This increase in drug-resistant bacteria is largely attributed to the regular use of intravenous catheters, broad-spectrum antibiotics, and immunosuppressive agents. Antimicrobial agents derived from medicinal plants constitute a significant reservoir of phytomedicine. These plant-based agents possess vast potential as drugs since they typically do not induce the side effects commonly linked to synthetic antimicrobial agents. Thus, exploring plant-derived antimicrobial agents presents an alternative strategy for treating various ailments [6]. In recent times, many pharmaceutical firms have directed their efforts toward developing new medications from plants to create cost-effective drug options. It is noteworthy that a majority of the antimicrobial agents currently available in modern medicine were previously utilized in impure forms within traditional medicine systems to address various health issues. Key advantages of plant-based treatments are their affordability. Medicinal plants contain numerous secondary metabolites exhibiting antibacterial and antifungal properties. Thorough screening of these plants may lead to the identification of innovative antimicrobial compounds for different diseases. The global application of antimicrobial agents is on the rise. In recent years, screening plant products and extracts for antimicrobial effectiveness has revealed that medicinal plants are a significant source of novel antimicrobial substances. Secondary metabolites obtained from plant-based antimicrobial agents are employed against various multidrug-resistant bacteria. Numerous studies have been conducted to assess the availability of secondary metabolites with antimicrobial properties. Essential oils from plants consist of highly complex mixtures containing various groups of phytochemicals, such as monoterpenes, biologically related sesquiterpenes, and phenols. Research on specific plants has demonstrated that some essential oils not only eliminate mosquito larvae but also possess contact and hyper-fumigant insecticidal properties against a range of target organisms and pest pathogens [7]. Essential oils exhibit a wide scope of activities, including insect repellent and antimicrobial effects. The primary objective of this study is to investigate the antibacterial and antifungal properties of the essential oil extracts from Aegle marmelos, Aristolochia indica, and Piper nigrum roots.

2. MATERIALS AND METHODS

2.1. SAMPLES

The roots of *Aegle marmelos*, *Aristolochia indica*, and *Piper nigrum* were collected from the Kanyakumari District in India. The roots were separated from their respective plants and dried. They were then ground into a fine powder and utilized for the extraction of essential oils. Extraction of essential oil from *A. marmelos*, *A. indica*, and *P. nigrum* powder Twenty grams of the root powder were combined with a chloroform-methanol solution (v/v, 2:1, 100 mL). Subsequently, the mixture was carefully filtered through filter paper, and 20 mL of 0.9% potassium chloride was introduced. The sample was vigorously agitated for a few seconds and allowed to settle into distinct layers. The lower layer was collected, and the solvent was removed by evaporation. The yields were 19 ± 0.4%, 16.3 ± 1%, and9.5 ± 0.1% for the roots *of A. marmelos, A. indica, and P. nigrum*, respectively.

2.2. SUBCULTURING OF FUNGUS

The fungal strain was subcultured in a nutrient agar medium (g/l) composed of peptic digest of animal tissue (5 g), sodium chloride (5 g), beef extract (1.5 g), yeast extract (1.5 g), and agar (15 g). The fungal culture was incubated at 37 °C for 72 hours. Subsequently, all cultures were stored at 4 °C for additional studies.

2.3. ANTIBIOTICS

Antibiotics were employed as positive controls during antimicrobial activity assessments. These were obtained from Himedia, Mumbai, India. Nystatin (10 µg) served as the positive control.

2.4. INOCULUM PREPARATION

The chosen fungal isolates were cultured separately in the basal medium, which consisted of glucose (0.5 g), yeast extract (0.1 g), peptone (0.25 g), KH2PO4 (0.05 g), MgSO4 (0.01 g), and NaCl (1.0 g) per liter. The pH of the medium was adjusted to 7.0 using HCl or NaOH. The isolates were then inoculated individually and incubated at 37 °C for 24 hours. The fungal growth was measured by monitoring the absorbance at 600 nm with a UV-visible spectrophotometer. The experiment was conducted in 100 mL Erlenmeyer flasks containing 50 mL of the culture medium and incubated at 30 °C for 72 hours. 3.3.5.

2.5. ANTIFUNGAL SCREENING

Essential oil was assessed for antifungal activity against *C. albicans*. Mueller Hinton Agar (g/l) was prepared in an Erlenmeyer flask according to the manufacturer's instructions, containing beef infusion form (300 g), casein acid hydrolysate (17.5 g), starch (1.5 g), and agar (17 g) (Himedia, Mumbai, India). The media, along with pipettes, Petri dishes, and a metallic borer, were sterilized in an autoclave at 121 °C for 15 minutes. The culture media were then poured into Petri dishes under sterile conditions. The essential oil was mixed with dimethyl sulfoxide (DMSO) (100%) to achieve a final concentration of 20 µL/mL. Wells were created in the media, and 20 µL of the sample was added to each well. Nystatin (10 µg) was used as the positive control. All plates were incubated for 72 hours at 37 °C, and the diameter of the zone of inhibition was measured in millimeters (mm).

2.6. ANTIBACTERIAL ASSESSMENT

2.6.1. MICROORGANISMS AND SUBCULTURE

The pathogenic bacteria employed for antibacterial evaluation included *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus mutans*. The bacterial strains were subcultured in nutrient agar medium (g/l) composed of peptic digest of animal tissue – 5 g, sodium chloride – 5 g, beef extract – 1.5 g, yeast extract – 1.5

g, and agar – 15 g. The bacterial culture was incubated for one day at 37°C, while the fungal culture was incubated at 30°C for three days. Subsequently, all cultures were preserved at 4°C for further examination.

2.6.2. CONTROL ANTIBIOTICS

Antibiotics were utilized as a positive control during the antimicrobial activity assessment. These antibiotics were acquired from Himedia, Mumbai, India. Chloramphenicol (10 µg) served as the positive control.

2.6.3. BACTERIAL INOCULUMS PREPARATION

The chosen bacterial isolates (*K. pneumoniae, S. aureus, E. coli, and S. mutans*) were individually cultured in nutrient broth medium, which consisted of (g/l): glucose - 0.5 g; yeast extract - 0.1 g; peptone - 0.25 g; KH2PO4 - 0.05 g; MgSO4 - 0.01 g; and NaCl - 1.0 g. The pH of the medium was adjusted to 7.0 using HCl/NaOH. The isolates were then inoculated separately and incubated at 37°C for 24 hours. The growth of bacteria was assessed by measuring the absorbance at 600 nm using a UV-Visible spectrophotometer. The experiment was conducted in 100 ml Erlenmeyer flasks containing 50 ml of the culture medium, which was incubated at 30 °C for one day. After incubation, the cultures were stored at 2 – 8°C for further analysis. 3.4.5.

2.6.4. ANTIBACTERIAL ACTIVITY

Essential oils were tested for antibacterial efficacy against four bacterial strains: *K. pneumoniae, S. aureus, E. col*i, and *Streptococcus* sp. Mueller Hinton Agar (g/l) composed of beef infusion – 300 g, casein acid hydrolysate – 17.5 g, starch – 1.5 g, and agar – 17 g (Himedia, Mumbai, India) was prepared in an Erlenmeyer flask according to the manufacturer's instructions. The culture media, along with pipettes, Petri dishes, and a metallic borer, were sterilized in an autoclave at 121°C for 15 minutes. The sterile culture media was then poured into Petri dishes under aseptic conditions. The samples were diluted with double distilled water, and 100 µl of the sample was applied to the disc to evaluate activity. Chloramphenicol (10 µg) was utilized as the positive control. All plates were incubated for 24 hours at 37 °C. The diameter of the inhibition zone was measured in millimeters (mm).

2.7. DETERMINING THE MIC AND MBC

The MIC and MBC values of essential oils were assessed using a broth dilution method. Approximately 18-hour broth cultures of the selected bacterial strains were suitably diluted with 0.1% (v/v) peptone water to achieve the final concentration of 10^5CFU/ml. Essential oils were serially diluted accordingly for analysis. The agar plates were incubated for 24 hours for the respective bacterial isolates. The MIC was defined as the lowest concentration of the sample necessary to completely inhibit bacterial growth, while the MBC represented the minimum concentration needed to eradicate the microbes entirely.

3. RESULTS

3.1. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL

Antifungal activity of essential oil was tested against the selected *C. albicans*. *A. marmelos*, *A. indica*, and *P. nigrum* essential oils showed 11 mm, 12 mm and 12 mm zone of inhibition against *C. albicans* (Fig. 1).

Figure 1

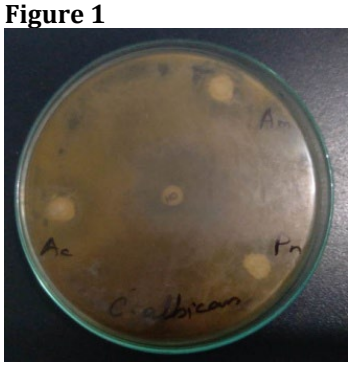

Fig. 1. Antifungal Activity of Essential Oil Against *C. Albicans*. (Am: *A. Marmelos;* Ac: *A. Indica* and Pn: *P. Nigrum*)

3.2. Antibacteiral activity of essential oil

A. indica essential oil showed maximum activity against *E. coli* (15 mm zone of inhibition) than *P. nigrum* essential oil (18 mm zone of inhibition), and *A. marmelos* essential oil (16 mm zone of inhibition) (Fig. 2). *P. nigrum* essential oil showed minimum activity against *S. aureus* (9 mm zone of inhibition) than *A. indica* essential oil (10 mm zone of inhibition), followed by *A. marmelos* essential oil (11 mm zone of inhibition) (Fig. 3). *P. nigrum* essential oil showed maximum activity against *K. pneumoniae* (14 mm zone of inhibition), which was higher than *A. indica* essential oil (17 mm zone of inhibition), followed by *A. marmelos* essential oil (11 mm zone of inhibition) (Fig. 4). In this study, *A. marmelos* essential oil showed least activity against *Streptococccus* sp. (11 mm zone of inhibition), which was lower than *A. indica* essential oil (10 mm zone of inhibition), followed by *P. nigrum* essential oil (12 mm zone of inhibition) (Fig. 5).

Figure 2

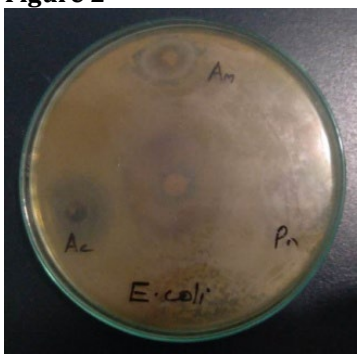

Fig. 2. Antibacterial Activity of Essential Oil Against *E. Coli*. (Am: *A. Marmelos;* Ac: *A. Indica* and Pn: *P. Nigrum*)

Figure 3

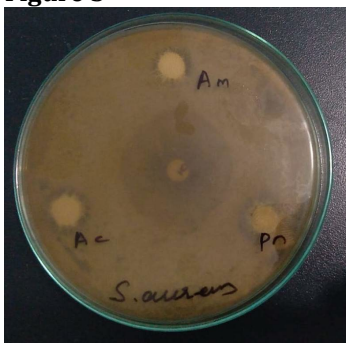

Fig. 3. Antibacterial Activity of Essential Oil Against *S. Aureus*. (Am: *A. Marmelos;* Ac: *A. Indica* and Pn: *P. Nigrum*).

Figure 4

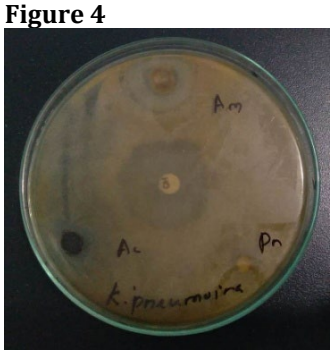

Fig. 4. Antibacterial Activity of Essential Oil Against *K. Pneumoniae*. (Am: *A. Marmelos;* Ac: *A. Indica* and Pn: *P. Nigrum*).

Figure 5

Fig. 5. Antibacterial Activity of Essential Oil Against *Streptococcus* Sp..(Am: *A. Marmelos;* Ac: *A. Indica* and Pn: *P. Nigrum*).

3.2. MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

The minimum inhibitory concentration of essential oils were determined against various bacterial pathogens. Essential oil was highly active against *E. coli* and *Streptococcus* sp. than *K. pneumoniae* and *S. aureus* and the result is depicted in Table 1 to 3. Among the three essential oils, *P. nigrum* was highly active against the selected bacteria and the MIC and MBC values were extremely low than *A. indica* (Table 2) and *A. indica* (Table 1).

Table 1: MIC and MBC Values of Essential Oil Extracted from *A. Marmelos* Against Bacterial Pathogens

Microorganisms	MIC (µg/ml)	MBC (µg/ml)
Streptococcus sp.	25	50
K. pneumoniae	100	375
S. aureus	100	250
E. coli	25	100

Table 2: MIC and MBC Values of Essential Oil Extracted From *A. Indica* Against Bacterial Pathogens

Microorganisms	MIC (µg/ml)	MBC (µg/ml)
Streptococcus sp.	50	100
K. pneumoniae	100	250

S. aureus	50	100
E. coli	100	200

Table 3: MIC and MBC Values of Essential Oil Extracted from *P. Nigrum* Against Bacterial Pathogens

Microorganisms	MIC (µg/ml)	MBC (µg/ml)
Streptococcus sp.	100	200
K. pneumoniae	50	100
S. aureus	75	200
E. coli	150	350

4. DISCUSSION

Essential oils comprise a variety of phytochemicals and could be responsible for antifungal properties. Natural products, including essential oils (EOs), flavonoids, alkaloids, and polysaccharides, have garnered significant interest from researchers due to their antimicrobial, antitumor, and anti-inflammatory effects. EOs are intricate blends of volatile compounds derived from plants, with their primary components being aromatic and aliphatic substances that are noted for their low molecular weight [8]. The antifungal mechanisms of citral and geraniol against common Aspergillus species found in grains were explored by assessing cell membrane permeability, changes in cellular ROS, and gene expression related to growth and secondary metabolites. The results indicated that both concentrations of essential oil significantly enhanced cell membrane permeability in A. ochraceus compared to *A. flavus*, resulting in the leakage of intracellular contents. This leakage is likely attributable to the interaction between antimicrobial compounds and the cytoplasmic membrane [9]. The differing changes in relative conductivity observed between *A. ochraceus* and *A. flavus* may be due to the involvement of distinct components and binding sites on the cell membrane when exposed to the same essential oil concentration. Additionally, the electrical conductivity of *A. ochraceus* rose by 28.3% and 28.8% after being treated with citral and geraniol, respectively, suggesting that the cell membrane's integrity was partially compromised. When the cell membrane is damaged, more small molecules like electrolytes are released, leading to increased electrical conductivity and accelerating cell death [10].

In earlier research, the various components of the oils from Aegle marmelos, Azadirachta indica, and Piper nigrum were examined. These essential oils demonstrated antibacterial properties against a range of bacterial pathogens. The minimum inhibitory concentrations of the essential oils were assessed against different bacterial pathogens. The essential oil exhibited stronger activity against Streptococcus species and E. coli compared to K. pneumoniae and S. aureus. These findings indicate that the essential oil has both bacteriostatic and bactericidal properties and shows significant potential as an antifungal agent, exhibiting strong in-vitro fungicidal activity against C. albicans, an opportunistic pathogen responsible for both superficial and systemic fungal infections [11, 12] . Prior studies have noted the antifungal effects of essential oil extracted from the leaves of *Aegle marmelos* (L.) Correa, inhibiting the growth of dermatophytes and *Fusarium* species at a concentration of 500 µg/mL. The essential oil obtained from A. indica displayed moderate activity, which aligns with earlier research findings. The essential oil from *Aristolochia indica*, containing β-caryophyllene and α-humulene as principal components, showed moderate antibacterial activity. Furthermore, the essential oil extracted from *P. nigrum* exhibited bactericidal effects against the tested bacteria. Consistent with our results, several essential oils from plants containing significant amounts of terpenoids have also shown inhibitory effects against various food-borne bacteria [13].

5. CONCLUSION

The unrestricted application of various chemical insecticides in agricultural practices leads to environmental disruption and resistance in microorganisms. Therefore, over the past thirty years, efforts have been made to utilize pesticides derived from natural sources, and the screening of natural pesticides is ongoing. In this context, employing essential oil obtained from roots to eliminate fungal pathogens has proven to be an effective method for controlling both fungi and bacteria. Essential oil extracted from roots demonstrates strong antibacterial and antifungal properties. Such research is valuable for developing effective plant-based antifungal and antibacterial agents for creating green antibiotics and advancing alternative medicine.

CONFLICT OF INTERESTS

None .

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