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Antimicrobial Activity of Essential Oils from Rosemary, Mentha, Basil and Ruta on Drug Resistance Bacterial Strains from Clinical Samples

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Abbreviations

AMR	Antimicrobial Resistance
EO	Essential Oil
WHO	World Health Organization
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
AHRI	Armauer Hansen Research Institute
MRSA	Methicillin-Resistant Staphylococcus aureus
UTI	Urinary Tract Infection
MHA	Mueller-Hinton Agar

Abstract

Background: Antimicrobial resistance (AMR) is a growing global health concern, particularly in clinically pathogens like *E. coli* (ESBL), *S. aureus* (MRSA) and *K. pneumonia*. Essential oils (EOs) such as Rosemary, Basil, Mentha and Ruta have known antimicrobial properties because of the bioactive they contain compounds like terpenes and phenolics. These compounds are known to disrupt bacterial memebranes and affect cellular process, meaning Eos are a potential alternative treatment for drug resistant infections.

Objective: The main objective of this study was to evaluate the antimicrobial activity of selected essential oil on selected drug resistant clinical isolates.

Methods: Plant materials were collected from Wondo Genet Agricultural Research Center, and the essential oils were extracted by hydrodistillation. An experimental study was then conducted. Six clinical isolates and the test strains were collected from Wudassie Medical Laboratory and subcultured for identification and resistance profiling. Antibacterial activity was evaluated against the clinical isolates and standard strains of *E. coli*, *S. aureus*, and *K. pneumoniae* (ATCC 25922, ATCC 25923, and ATCC 13883), respectively. The agar well diffusion method was used to determine the zones of inhibition, while broth microdilution was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results: Tested strains demonstrated varying degrees of susceptibility to the Eos. Antimicrobial activity increased along with concentration of the oils. Rosemary and Mentha oils showed comparatively stronger antibacterial activity than Basil and Ruta overall. Gram positive isolates (MRSA) were more susceptible than the Gram negatives. Mentha oil showed zone of inhibiton of 19.33±1.15 mm. MIC values ranged from 0.5 mg/ml to >16 mg/ml. Rosemary oil showed mean MIC value of 6.667 mg/ml against MRSA 1 and Mentha oil showed MIC values ranging from 4 – 16 mg/ml against several isolates. Most recorded MBC values were >16mg/ml. Stronger activity was seen on the ATCC strains used in the study with the highest zone of inhibition being observed with Ruta oil on ATCC *K. pneumonia*. (25.67±0.58) mm .

Conclusion: The tested Eos showed notable antibacterial activity. These findings suggest that the tested EOs may have potential as sources of antimicrobial agents against drug resistant bacterial pathogens; however, further studies are needed to establish their efficacy, safety, and clinical applicability.

Key Words: Essential oils, antimicrobial, agar well diffusion

1. Introduction

1.1 Background

Essential oils are aromatic compounds extracted from plants. They have been used for thousands of years for many reasons including for their medicinal, cosmetic, and aromatic properties (1). They are volatile oils, and they are often called the essence of the plant. They are typically obtained by distillation or cold pressing of various parts of the plant (2). Historically, essential oils have always played a big role in the healing practices of many ancient civilizations. From the Egyptians to the Chinese, Greeks, and even Romans, essential oils have been highly valued for their therapeutic properties, especially in medicine (3).

Compounds like terpens, phenolcs and aldehydes are present in essential oils. They play a signinifcan role in the biomedicine value of the plants. They are effective in eliminating various viral, fungal and bacterial pathoges. Additonally the insect repellent and antioxidant properties of essential oils are established (4). Many studies have indicated the antimicrobial and antioxidant activities of essential oils. These are the two most common biological effects known. The antimicrobial properties mechanisms of actions include disruption of the cell membrane and cell wall, inhibition of enzymatic activity and damage to intracellular structures. Additionally, studies also indicate that combining essential oils can enhance their biological effectiveness (5).

Antimicrobial resistance (AMR) is currently a pressing global health concern. The overuse and misuse of modern antibiotics have led to this problem. This is a significant public health challenge. This indicates the need to consider alternative means of treatment besides modern antibiotics, such as natural compounds from plants (6).

They work through the disruption of the cell membranes of the pathogen. This is related to the inhibition of the efflux pumps that are responsible for antibiotic resistance in Gram negative bacteria. Other widely documented mechanisms of action include inhibition of peptidoglycan layer synthesis in the bacterial cell wall by binding to penicillin binding proteins in Gram positive bacteria (7,8). The antimicrobial properties of essential oils are tied to specific compounds. These include terpenes (such as thymol, carvacrol, and limonene), phenolics (such as eugenol), and aldehydes (such as cinnamaldehyde). They can target multiple sites within the bacteria cell making it difficult for the pathogen to develop resistance. For example, phenolic

compounds are known to affect the lipid bilayer of the cell membrane. This results in leakage of cellular content and eventually cell death. On the other hand terpenes disrupt celluar respiration and increase permeability. This will further inhibit bacterial growth. The synergistic interaction between these compound is what enhances the microbial activity of essential oils (9).

Many studies indicated the antimicrobial potential of essential oils against both Gram positive and negative bacteria, including drug resistant strains. MDR pathogens of *E. coli*, *S. aureus* and *Klebsiella* species are responsible for a wide range of infection from urinary tract infections (UTI) to pneumonia and even skin infections and sepsis. Gram negative bacteria like *E. coli* and *Klebsiella* species have an outer membrane that serves as a barrier against many antibiotics. And Gram positive bacteria *S. aureus* is notorious for its methicillin resistant strain MRSA (10,11).

Essential oils offer a promising alternative treatment because of their ability to penetrate bacterial membranes and disrupt critical cellular processes. There are studies that have shown rosemary, clove and lemongrass oil exhibit strong antimicrobial activity. Including drug resistant strains of these pathogens (12)

1.2 Statement of the problem

Antimicrobial resistance (AMR) is a major public health challenge in Ethiopia, where bacterial infections remain an important cause of morbidity and mortality. The increasing resistance of bacterial pathogens to commonly used antimicrobial agents has reduced available treatment options and complicated the management of infections. This problem is particularly concerning for clinically important pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella* species, which are frequently associated with community- and healthcare-associated infections and may exhibit resistance to multiple classes of antibiotics (13).

In the cases of low to middle income countries. Studies show that up to 80% of antibiotics used in these countries are consumed without prescriptions. This makes the problem even more challenging (14). The burden of AMR in Ethiopia is further complicated by the increasing occurrence of multidrug-resistant (MDR) organisms, including extended-spectrum beta-lactamase (ESBL)-producing *E. coli* and carbapenem-resistant *Klebsiella pneumoniae*. Resistance to commonly used antibiotics has been reported among bacterial pathogens causing diverse infections in Ethiopia, with studies demonstrating approximately 30–85% resistance to key antimicrobials included in the Ethiopian Standard Treatment Guidelines, and resistance reaching 100% for some antibiotics, including penicillin, ampicillin, and amoxicillin. Such resistance can result in treatment failure, prolonged illness and hospitalization, increased healthcare costs, and increased risk of transmission of resistant organisms (15) (16).

The increasing prevalence of antimicrobial-resistant pathogens highlights the need to investigate alternative or complementary sources of antimicrobial agents. Essential oils (EOs) derived from medicinal and aromatic plants have demonstrated antibacterial activity against a range of pathogenic microorganisms and contain bioactive compounds that may interfere with bacterial growth through different mechanisms. However, evidence regarding their activity against clinical isolates of drug-resistant bacterial pathogens in Ethiopia remains limited. Therefore, there is a need for laboratory-based studies evaluating the antibacterial activity of selected EOs against clinically relevant drug-resistant bacterial pathogens.

1.3 Significance of the study

Investigating the antimicrobial activity of essential oils can provide a natural and cost effective alternative to modern antibiotics. The findings of this study can contribute to development of new treatment options. This can also help by reducing the dependency on synthetic antibiotics. It can also slow the rate of progression of AMR. This study holds significant importance in addressing the growing challenge of antimicrobial resistance. Specifically, targeting drug-resistant strains of E. coli, S. aureus, and Klebsiella species.

With the prevalence of drug-resistant infections rising and healthcare resources being limited, essential oils offer a promising therapeutic option. Although it is rooted in traditional knowledge it should be supported by modern science. The research outcomes may help in filing existing gaps in scientific knowledge about the efficacy of essential oils but also inform public health strategies aimed at mitigating AMR in Ethiopia and other low resource settings.

1.4 Objective

1.4.1 General objective

To evaluate the antimicrobial activity of selected essential oils against selected drug-resistant bacterial strains from clinical isolates.

1.4.2 Specific objective

- To determine the antibacterial activity of selected essential oils against drug resistant clinical isolates of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella* pneumonae
- To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the selected essential oils against the tested bacterial isolates.

2. Literature Review

A study done in Iran on the antimicrobial properties of Rosemary (*Rosmarinus Officinalis*), specifically on *Staphylococcus Aureus* and *Escherichia Coli*, found that rosemary essential oil has remarkable antibacterial activity and can be used as a suitable replacement for synthetic antibiotics. The MIC and MBC were found to be 0.625 and 1.25 respectively for *S. aureus* and on the other hand they were found to be 1.25 and 2.5 for *E.coli* (17). The antibacterial effect of this plant stands out even on drug resistant bacteria. Zones of inhibition ranged from 16 to 28 mm against all the test isolates which were MRSA (18).

Another study done by Denisa in Romania showed that fresh rosemary oil showed strong antimicrobial property on *S. aureus, S. Pyogenes* and *E. faecalis*, with inhibition zones of 18 mm $\pm$ 0.3, 18 mm $\pm$ 0.7 and 17 mm $\pm$ 0.8 respectively. (19) It's important to note that when compared with other essential oils like Clove oil, Rosemary Oil shows overall better antimicrobial properties (20).

A study by Hassan on the antioxidant and antibacterial properties of Rosemary (*Rosmarinus officinalis*) essential Oil, found 30 compounds in the oil mainly consisting of Cineole (21.24%), Camphor (10.81%), and α - pinene (7.41%). The oil exhibited effective antimicrobial activity against all tested microbial strains, with MIC values ranging from 1.56 to 25 mg/L for different test microbes. This study also reported that Rosemary oil showed greater zones inhibition on *Pseudomonas aeruginosa* when compared to *Staphylococcus aureus* (21).

In another study Monika tested the effectiveness of Rosemary and Basil oils against standard strain *Escherichia coli* (ATCC 25922) as well as 60 other clinical multidrug resistant strains of *Escherichia coli* (isolated from patients with infections of the respiratory tract, abdominal cavity, urinary tract, skin and from hospital equipment). The result showed MIC values for Basil oil of 8.0 µL/mL for the standard strain and between 8.0 µL/mL to 11.5 µL/mL for the sixty tested clinical strains. On the other hand, reported MIC values were between 18.0 and 20.0 µL/mL for the Rosemary oil (22). Another study done by Kariminik in Iran has also also reported the MIC and MBC for Rosemary oil to be 2.5 and 5 respectively, against *S. aureus* (23). MIC and MBC levels were determined to be 0.125 µL/mL and 0.1µL/mL for Basil against *E.coli* and with an inhibition zone of 15 mm. This study reported that the inhibition zone of Rosemary oil on the same test microbe (*E. coli*) was 25mm (24). Amir in another study has reported that Basil oil

inhibition zones for *S. aureus* were between 29.20 mm and 30.56 mm, *E. coli* showed inhibition zones between 17.48 mm and 23.58 mm. The minimum inhibitory concentrations (MICs) were found to be 18 µg/mL for *S. aureus* and *E. coli* (25).

Basil oil also showed strong antimicrobial properties against drug resistant bacteria strains of *S. aureus* and *E.coli* with inhibition zones of 20±2.58 mm and 20±1.83 respectively. It has also been reported to have strong antimicrobial activity against gram negative bacteria such as *K. pneumoniae* (26). A study done in our country by Getachew showed strong antibacterial activity with maximum zone of inhibition 15.47 mm, and MIC of 0.09 µg/ml, and corresponding MBC level of 0.19 µg/ml against *S. aureus* (27). Basil oil shows significant antimicrobial effects, especially against Gram-positive bacteria such as *Staphylococcus aureus* due to its main components like linalool and estragole. However, it is less effective against Gram-negative bacteria like *E. coli* and *Klebsiella* due to their protective outer membranes, which limit the penetration of hydrophobic compounds found in essential oils. Studies have shown that the Minimum Inhibitory Concentration (MIC) of basil oil against *S. aureus* can range from 512 to 1024 µg/mL, and the Minimum Bactericidal Concentration (MBC) is often above 1024 µg/mL. Against *E. coli*, basil oil MIC values are reported to be higher, reflecting its reduced efficacy against Gram-negative bacteria (28,29).

A study by Sienkiewiez done in Poland showed that basil oil exhibited significant antibacterial effects, particularly against *E. coli* strains, including extended-spectrum β-lactamase (ESBL)-producing strains. The minimal inhibitory concentration (MIC) values for basil oil ranged from 0.025% to 0.5% (v/v), and the oil demonstrated a higher inhibitory effect compared to rosemary oil in this case. In another study, basil oil was tested alongside other essential oils, including oregano and thyme, on human pathogens such as *S. aureus* and *Klebsiella pneumoniae*. The MIC values were determined using micellar and aqueous extracts. The results showed basil oil as having promising antibacterial activity, though its performance was lower than highly active oils like oregano (30).

The Basil oil has been shown to inhibit the growth of both standard and multidrug-resistant strains of *E. coli*. MIC values for basil oil against clinical strains of *E. coli* have been reported around 0.25–1.42% (v/v), with higher efficacy at lower concentrations in some studies. The oil's antimicrobial effect is primarily attributed to its high content of oxygenated monoterpenes like

linalool and 1,8-cineole. These compounds disrupt bacterial cell membranes, leading to leakage of essential cell contents. The unique blend of compounds in basil oil makes resistance development by pathogens less likely compared to synthetic antimicrobials (31).

Mentha piperita essential oil contains various bioactive compounds, including menthol, menthone, and 1,8-cineole, which contribute to its antimicrobial effects against a range of pathogens (32,33). Several studies have demonstrated the effectiveness of *Mentha piperita* oil against Gram-positive bacteria, particularly *Staphylococcus aureus*. A study that tasted the antimicrobial properties of Mentha piperita on MDR clinical isolate of *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* revealed that the MIC was lower for the first three strains (20 mg/dl) compared to the others (40 mg/dl) (33). A similar study performed by Pramila that the plant extract showed antimicrobial activity against clinical isolates of *Escherichia coli* and *Staphylococcus aureus,* with *E. coli* showing more sensitivity compared to *Staphylococcus aureus* (34). On the other hand in another study the minimum inhibitory concentrations for the bacterial species ranged from 0.4% to 0.7%, and the lowest MIC values were found for the *K. pneumoniae* strain tested. MIC and MBC values of essential oil of *M. piperita* indicate that *S. aureus* is more susceptible than *E. coli* (35).

In another study done by Shalayel that seeked to assess the antimicrobial activities of peppermint extracts against 10 multidrug resistant (MDR) pathogenic bacterial clinical isolates showed that there is promising antibacterial activity of *Mentha piperita* extracts, particularly ethyl acetate extracts, against multidrug-resistant *Streptococcus pyogenes, Enterococcus faecalis,* MRSA, and carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* . (36)

Tena Adam (*Ruta chalepensis*), a member of the Rutaceae family, is found in both temperate and tropical regions. Traditionally, decoctions of R. chalepensis have been used for "spiritual cleansings" and to ward off the evil eye. Infusions made from its fresh leaves are commonly used to treat gastric issues, headaches, and rheumatism. The plant is also known for its diuretic, anti-inflammatory, and anti-spasmodic effects. Additionally, R. chalepensis has been utilized as a mosquito repellent, an antidote for snake and scorpion venom, and applied as a poultice for bites and stings. It exhibits potent antibacterial and antifungal activity against a range of pathogens, including Gram-positive and Gram-negative bacteria as well as fungal species. Studies have

reported that the oil is effective against common bacterial pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* (37).

A study performed by Alotaibi to determine the antimicrobial status of the oil collected by different solvents, showed that the methanolic extract of *Ruta chalepensis* exhibited a maximum inhibition zone of 11.1±0.55 mm in diameter against *Staphylococcus aureus. Klebsiella pneumoniae*, *Shigella dysenteriae,* and *Escherichia coli* showed moderate inhibition zones of 9.5±0.72 mm, 9±0.4 mm, and 9±0.3 mm, respectively. Other pathogens, such as *Pseudomonas aeruginosa* (8.8±0.26 mm) and *Salmonella typhi* (7±0.5 mm), also displayed inhibitory activity. The ethanol extract of *R. chalepensis* demonstrated notable antibacterial activity, with the highest zone of inhibition observed against *S. typhi* (15±0.5 mm), followed by *S. aureus* (13±0.11 mm), *P. aeruginosa* (13±0.2 mm), and *E. coli* (12.5±0.5 mm). The lowest inhibition zone was recorded against *K. pneumoniae* (7.9±0.11 mm) (38). The results of this study can also be supported by another research done in Saudi Arabia that revealed similar results (39).

A study done in Ethiopia by Asfere in 2020, that tested the antimicrobial properties of three different common medicinal plants in our country, which included Tena Adam, showed results of the aqueous leaf extracts showing zones of inhibition ranging from 6 to 14 mm. Most of the extracts of R.chalepensis leaf showed a significant growth inhibition against the tested organisms (40). Bekkar stated that the essential oils of this plant demonstrated significant antimicrobial activity against selected multidrug-resistant human pathogens. The strongest effects were observed against *Proteus mirabilis, Salmonella enterica subsp. Arizonae* with growth inhibition zone diameters of 24.06 ± 0.12 mm, 40.1 ± 0.1 mm, and 40.16 ± 0.15 mm, respectively. However there was no reported microbial activity on *S. Pyogens* . (41)

In another study by Habtamu T, the acetone crude leaf extract of R. chalepensis showed a strong zone of inhibition against *K. pneumoniae* (11.1±0.28 mm) and a moderate level zone of inhibition against *S. dysentry, S. typhi* and *S. aureus* with zone of inhibition of 8.2±0.3mm, 7.8±0.26mm, 7.5±0.5mm, respectively. The least antibacterial activity was detected against *Escherichia coli* with a zone size of 5.2±0.43 mm (42).

Overall there are many studies that highlight essential oils, especially those derived from local plants such as rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*), mint (*Mentha piperita*), *and Ruta chalepensis*, offer promising alternatives or complementary treatments to

combat these drug-resistant strains. This is particularly relevant in Ethiopia, where traditional medicine has long utilized plants like *Ruta chalepensis* and *Ocimum basilicum* for their therapeutic properties. The efficacy of these plants against multidrug-resistant bacteria presents an opportunity to integrate traditional knowledge with modern microbiological practices. Such integration could enhance healthcare delivery, particularly in rural areas where access to antibiotics is limited.

3. Materials and Methods

3.1 Study Settings

This study was conducted at the Traditional Medicine Center of AHRI, in Addis Ababa, Ethiopia. The research took place in their microbiology laboratory, which is equipped with facilities for microbial culture, antimicrobial susceptibility testing, and essential oil extraction.

This laboratory conducts research on traditional medicine and traditional medical practices, along with research on modern drugs. Many methods of production of traditional medicines are studied and are proven therapeutic efficacy, safety and quality at this center. There is ongoing development to materialize integration of traditional medicine into the health care service system, and the center also collaborates with concerned organizations for conservation and sustainable use of medicinal plants.

3.2 Study Design and Period

3.2.1 Study Design

This study employed an experimental in vitro design to evaluate the antimicrobial activity of essential oils on clinical isolates.

3.2.2 Study Period

The study was conducted over a period of 6 months, from January 2026 to May 2026.

3.3 Study Variables

3.3.1 Dependent Variables

Efficacy of essential oils (Eos) on test organisms, MIC and MBC of Eos on test organisms

3.3.2 Independent Variables

Type of Eos, Concentration of Eos

3.4 Plant Material Collection

The plant materials that were used in this study are basil (*Ocimum basilicum*) Besobila in amharic, rosemary (*Rosmarinus officinalis*) Siga Metbesha in amharic, mint (*Mentha piperita*)

and Tena adam (*Ruta Chalepensis*). The plant materials were collected from the Wondogenet Agricultural Research Center field. The Center is located in the SNNPR Regional State (7º19′ N 38º, 38’E).



Figure 1: Collection of Plant materials at Wondogenet Agricultural Research Center (Date: January 4, 2026)

3.5 Bacterial Strains

The bacterial strains used in this study were clinical isolates and standard microbes of *Escherichia coli, Staphylococcus aureus,* and *Klebsiella pneumoniae.* These isolates were collected from the Wudassie Diagnostic Center. All bacterial isolates are drug-resistant strains, as identified by standard clinical microbiology procedures at the laboratory. And the reference standard strains of the test microbes were obtained from AHARI for *E. coli, S.aureus* and *K. pneumonia.* (ATCC 25922, ATCC 25923, ATCC 13883)

3.6 Laboratory Analysis

3.6.1 Oil Preparation

Hydrodistillation was the method of choice for the extraction of the Eos. It uses steam to extract essential oils from plant material. The plant material was placed in a flask with water, and heat was applied to generate steam. The steam penetrates the plant material and vaporizes the essential oils. These vapors are condensed back into liquid form, which separates into essential oils and water in the collection vessel. The essential oils are then separated, dried, and stored. The hydrodistillation apparatus consists of a round-bottom flask, a condenser, and a collection vessel to capture the essential oils. (43)

Fresh plant materials were washed under tap water and air dried. The plant material was cut or shredded into small pieces to increase the surface area for extraction. The plant material was

placed into the round-bottom flask. Enough water was then added to the flask to submerge the plant material. The condenser was attached to the top of the flask, ensuring proper sealing. It was then connected to a water source to maintain a continuous flow of cold water during the distillation process. Following this heat was applied to the flask, causing the water to boil. The steam was passed through the plant material, carrying volatile essential oil compounds with it. The vapor was then condensed back into a liquid, which separates into oil and water. The condensed distillate consisted of two layers: the water layer and the essential oil layer (Figure 2). Essential oils are less dense than water and float on top of the water layer. A separating funnel was carefully used to separate the essential oil from the water. The mixture was poured into the funnel, allowing the layers to separate, and then the water layer was drained from the bottom. Essential oils are sensitive to light and heat, so proper storage is important to maintain their quality. The oils were transferred into dark amber glasses to protect it from light and stored in a cool, dark place.



Figure 2: Rosemary essential oil before being separated from the water layer

3.6.2 Isolation and Identification

Clinical isolates of drug-resistant *Staphylococcus aureus* (MRSA), ESBL, and MDR *Klebsiella pneumoniae* were collected from Wudassie Diagnostic Center. These clinical isolates were stored in sterile containers and transported on ice to the laboratory, where they were subcultured onto selective media MacConkey agar and Mannitol Salt agar for Gram negative and Gram positive bacterial strains respectively. After a 24-hour incubation at 35-37°C, standard biochemical tests were performed to confirm the identity of the bacterial species.

3.6.3 Antimicrobial Property of Eos

The antimicrobial activity of the test essential oils was first evaluated using the agar well diffusion method. For this, essential oil concentrations of 5%, 10%, and 20% were prepared using Tween 80 as an emulsifying agent to improve the solubility and uniform dispersion of the oils in aqueous media. All prepared concentrations were stored in sterile labeled containers and used during the antimicrobial susceptibility testing procedure.

3.6.3.1 Agar Well Diffusion Method

The antibacterial activity of the prepared essential oil concentrations was evaluated using the agar well diffusion technique, as previously described by Asfaw Mueller–Hinton agar plates were prepared according to the manufacturer's instructions and sterilized before use. Fresh bacterial colonies were suspended in sterile normal saline and adjusted to match the 0.5 McFarland turbidity standard. A sterile cotton swab was used to evenly inoculate the bacterial suspension onto the surface of the Mueller–Hinton agar plates. After inoculation, sterile cork borers were used to punch wells of approximately 8 mm diameter into the agar medium. The wells were properly labeled according to the concentration of the essential oil to be tested.

Using a micropipette, 100 µL of each prepared oil concentration (5%, 10%, and 20%) was carefully dispensed into the respective wells. Tween 80 was used as the negative control, while Ciproflaxin 5 µg/ml was used as positive control. The inoculated plates were allowed to stand for a short period to permit diffusion of the solutions into the agar and were then incubated at 37°C for 18–24 hours. Following incubation, the antibacterial activity was determined by measuring the diameter of the zones of inhibition around each well using a ruler. The results were recorded in millimeters and interpreted as evidence of antibacterial activity of the essential oils against the tested bacterial isolates. Results were read as weak (10 - 13.9 mm), moderate (14 -18 mm), or strong (>18 mm). (43)

3.6.3.2 Determination of MIC and MBC

The MIC of the antimicrobial agents was evaluated by micro-broth dilution method according to Clinical and Laboratory Standards Institute. A 100µl of Muller Hinton broth was added into each well of the 96-well micro-plates. 100µl of the extract, positive and negative (5% Tween 80) controls were added on the first row in different wells. The final column, containing only media,

functioned as a sterility control. Two-fold serial dilutions with multichannel micropipettes were made in MHB across the microtiter plate. Then, the bacterial suspension containing approximately 5× 10^5CFU/ml respectively was prepared from a refreshed culture. A 100 µl aliquot of this suspension was inoculated into each well, excluding the sterility control. The plates were incubated at 37°C for 18-24 hours. After incubation, 40µl of a 0.4 mg/ml solution of 2,3,5-Triphenyltetrazolium chloride was added to each well as an indicator of microbial growth and incubated at 37 °C for 30 minutes. After incubation, the MIC values were visually determined by observing the presence or absence of purple color. The lowest concentration of each extract displaying no visible purple color was recorded as the MIC. For the MBC, samples from wells showing no growth was plated onto fresh Mueller-Hinton agar and incubated for 18-24 hours. The MBC was the lowest concentration at which no bacterial colonies grow (43).

3.7 Data Quality Assurance

3.7.1 Pre-analytical Phase

Efforts were made to ensure proper sample collection, handling, and storage. Samples were collected using sterile techniques to avoid contamination. Each sample was labeled accurately and recorded with complete information. Transportation and storage conditions were carefully monitored to preserve the integrity of the samples, ensuring they are kept under appropriate temperature and conditions prior to analysis.

3.7.2 Analytical Phase

All laboratory procedures were conducted in accordance with standard operating protocols (SOPs) to minimize variability and ensure reproducibility. Equipment were calibrated and maintained regularly, and reagents were checked for expiration and proper storage. Quality control strains and control antibiotics were included in each batch of tests to monitor the accuracy and precision of the results. Each essential oil concentration was tested in triplicate against each bacterial isolate using the agar well diffusion method to reduce random error, and any outliers were carefully reviewed and re-tested where necessary. Additionally, qualified personnel handled all microbiological testing to ensure that techniques such as agar well diffusion and MIC/MBC determination are executed properly.

3.7.3 Post-analytical Phase

Data from the experiments was carefully recorded and cross-checked to prevent transcription errors. All results were reviewed for consistency and accuracy, and any discrepancies were addressed before final data analysis. Data was entered into statistical software (SPSS) with double-checking to avoid entry mistakes. Furthermore, appropriate statistical methods were applied to ensure that the findings are interpreted correctly and to ensure the validity of conclusions drawn from the results.

3.8 Data Analysis and Interpretation

Data analysis was conducted using SPSS 2017, version 25 software to ensure robust statistical evaluation of the antimicrobial activity of the essential oils against clinical isolates. The inhibition zone were recorded, and their mean and standard deviation were calculated to improve the reliability of the results to summarize the zone of inhibition measurements from the disc diffusion tests. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values are presented as medians. Comparative analyses were performed to evaluate the effectiveness of each essential oil.

3.9 Operational Definitions

Essential Oils: Concentrated hydrophobic liquids containing volatile chemical compounds extracted from plants, in this study specifically referring to basil (Ocimum basilicum), rosemary (Rosmarinus officinalis), Mentha (Mentha piperita) and Ruta/Tena adam (Ruta Chalepensis)

Minimum Inhibitory Concentration (MIC): The lowest concentration of an essential oil that inhibits visible growth of a bacterial isolate in broth after a specified incubation period.

Minimum Bactericidal Concentration (MBC): The lowest concentration of an essential oil that kills 99.9% of the bacterial population, as determined by plating aliquots from wells showing no visible growth on agar media.

Zone of Inhibition: The area surrounding an essential oil-impregnated disc on an agar plate where bacterial growth is inhibited, measured in millimeters, indicating the effectiveness of the essential oil.

Clinical Isolates: Bacterial strains obtained from clinical specimens (e.g., urine, blood, or wound swabs) from patients diagnosed with infections, specifically focusing on drug-resistant strains of *E. coli*, *S. aureus*, and *Klebsiella* species.

Disc Diffusion Method: An antimicrobial susceptibility testing technique where paper discs impregnated with essential oils are placed on an agar plate inoculated with bacteria to assess the antimicrobial activity based on the zone of inhibition.

Hydrodistillation: A method of extracting essential oils from plant materials by distilling them with water, allowing the volatile compounds to evaporate and then condense into a liquid form.

3.10 Ethical Considerations

All clinical isolates were obtained from the participating laboratory with prior wavered consent. Relevant stakeholders including the participating medical lab (Wuddassie Diagnostic Center) and laboratory personnel at AHARI, were fully informed about the purpose of the study, procedures involved, potential risks, and benefits. The research protocol was submitted for ethical review and approval by the review board at Addis Ababa University, on 27/12/2024 under reference number MLS/780/24.

4. Results

4.1 Antibacterial effect of modern antibiotics

Six multidrug-resistant clinical isolates were included in the study, comprising two methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, designated MRSA 1 and MRSA 2; two extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates, designated ESBL 1 and ESBL 2; and two multidrug-resistant *Klebsiella pneumoniae* isolates, designated MDR Kpn 1 and MDR Kpn 2. The isolates were designated numerically to distinguish individual isolates within each resistance category.

The antibiotic resistance profiles of the multidrug-resistant clinical isolates are presented in Table 1. The two MRSA isolates (MRSA 1 and MRSA 2) showed resistance to cefoxitin, trimethoprim/sulfamethoxazole, ciprofloxacin, ceftazidime, and erythromycin. Both isolates were not tested against amoxicillin/clavulanic acid, ceftriaxone, amikacin, and meropenem. MRSA 1 was susceptible to chloramphenicol, whereas MRSA 2 showed intermediate susceptibility. The two ESBL-producing isolates demonstrated resistance to trimethoprim/sulfamethoxazole, ciprofloxacin, ceftazidime, amoxicillin/clavulanic acid, and ceftriaxone. Amikacin showed susceptibility against ESBL 1 and intermediate susceptibility against ESBL 2, while both isolates were susceptible to meropenem.

The two multidrug-resistant *Klebsiella pneumoniae* isolates showed resistance to ceftazidime and both amikacin and meropenem. MDR Kpn 1 showed intermediate susceptibility to ciprofloxacin, whereas MDR Kpn 2 was resistant to ciprofloxacin. Several antibiotics were not tested against the MDR *K. pneumoniae* isolates. Overall, the findings demonstrate considerable resistance to several commonly used antibiotics among the multidrug-resistant clinical isolates, while susceptibility to selected antibiotics such as chloramphenicol in MRSA, amikacin and meropenem in ESBL isolates indicates variation in antibiotic susceptibility among the tested strains. (Table 1).

Table 1: Antibiotics resistant profile of multidrug-resistant clinical isolates

Modern antibiotics	MRSA 1	MRSA 2	ESBL 1	ESBL 2	MDR Kpn1	MDR Kpn2
Cefoxitin (30 µg)	R	R	N	N	N	N
Triemethprim/ Sulfumenthoxazole (23.75 µg)	R	R	R	R	N	N
Ciproflaxin (5 µg)	R	R	R	R	I	R
Ceftazidime (30 µg)	R	R	R	R	R	R
Chloramphenicol (30 µg)	S	I	N	N	N	N
Erythromycin (15 µg)	R	R	N	N	N	N
Augmentin (10 µg)	N	N	R	R	N	N
Ceftriaxone (30 µg)	N	N	R	R	N	N
Amikacin (30 µg)	N	N	S	I	R	R
Meroenem (10 µg)	N	N	S	S	R	R

Note: R = Resistance, I = Intermidiate S = Susceptiable N = Not done

4.2 Antibacterial effect of essential oils

The inhibitory effects of the oils from Rosemary, Mentha, Basil and Ruta were tested against MRSA, ESBL and MDR *K.pneumoniae* at 5% (50mg/ml), 10% (100mg/ml) and 20% (200mg/ml). Based on this, the antimicrobial effect of the oils increased with increasing concentration (Figure 3). The results indicate that the highest zones of inhibition were observed

at 20% (200 mg/ml) when compared to the lower concentrations.

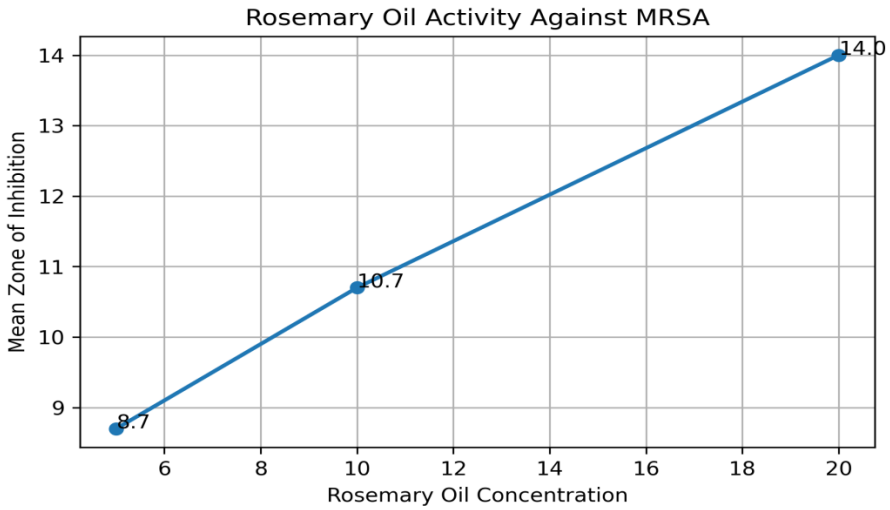


Figure 3: Antimicrobial activity of Rosemary oil against MRSA 2

Among the tested 9 isolates, the resistant clinical strains demonstrated lower susceptibility when compared with the ATCC strains. Nonetheless, the essential oils exhibited varying degrees of antimicrobial activity against both strains. The highest mean zone of inhibition was recorded (25.67±0.58 mm) against ATCC *K.pneumoniae* by essential oil of Ruta at a concentration of 20% (200 mg/ml). (Table 2)

From the tested oils, Mentha and Rosemary oils showed results that were comparatively stronger inhibition activity against most of the tested clinical isolates shown in table 2. Mean inhibition zones remain around 8 mm across the three different concentrations. This suggested limited inhibitory effect under the tested conditions.

Mentha essential oil demonstrated substantial inhibition activity against MRSA 2 at 20% (200 mg/ml) with inhibition zones 19.3±1.15 mm. Rosemary oil also showed moderate inhibitory activity against MRSA 2 with inhibition zone of 14±1 mm at the concentration of 20% (200 mg/ml). Rosemary oil demonstrated strong activity against ATCC *K.pneumoniae* with the mean inhibition zones increasing progressively with concentration and reaching a mean zone of inhibition of 20.3±0.58 mm at 20 % (200 mg/ml). Rosemary oil also demonstrated moderate activity against the ESBL clinical isolates. (Table 2)

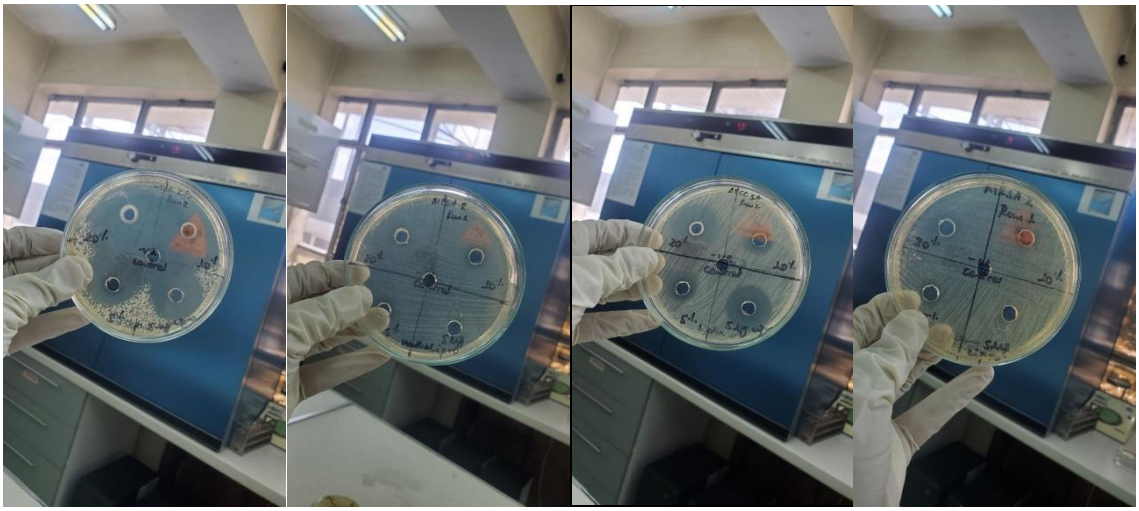


Figure 4: Agar well dissuion assay showing activity of Rosemary and Mentha oils agains test strains (MRSA 2, ATCC K. pneumoniae and ATCC S. aureus)

Ruta oil demonstrated the strongest activity against ATCC *K. pneumoniae* with mean inhibition zones reaching 25.67 ± 0.58 mm at the highest concentration. However ,its activity was relatively low on the resistant clinical isolates. Specifically the ESBL and MDR *K.pneumoniae* showed smaller inhibition zones across most of the tested oils and their concentrations.

Table 2 : Average diameters of inhibition zones for the agar well diffusion

		Zone of Inhibition (mean ± SD) (mm)								
Essential Oil	**Concentration(mg/ml)**	**MRSA 1**	**MRSA 2**	**ESBL 1**	**ESBL 2**	**MDR KPn 1**	**MDR KPn 2**	**ATCC S. aureus**	**ATCC E. coli**	**ATCC K. pneumoniae**
Rosemary	50	8±0	8.67±0.58	8±0	8±0	8±0	8±0	8±0	9.67±0.58	11.67±0.58
	100	9±0	10.67±0.58	8±0	8±0	8.33±0.58	8±0	9±0	11.67±0.58	17.67±0.58
	200	9.67±0.58	14±1.0	9.67±0.58	9±0	9±0	9±0	9.67±0.58	12.67±0.58	20.33±0.58
Mentha	50	8±0	9.33±1.15	8±0	8±0	8±0	8±0	10.67±2.08	8±0	8±0
	100	8±0	11.67±0.58	8±0	8±0	8±0	8±0	14.33±1.0	8±0	8±0
	200	8±0	19.33±1.15	9±0	8±0	8.67±0.58	8.67±0.58	21.00±2.89	8±0	8±0
Basil	50	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0
	100	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0
	200	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0	8±0
Ruta	50	8±0	8±0	8±0	8±0	8±0	8±0	10±2	8.33±0.58	25±1
	100	8±0	8±0	8±0	8±0	8±0	8±0	9.67±1.53	9±0	23.67±0.58
	200	8±0	8±0	8±0	8±0	8±0	8±0	10.67±1.53	9.33±0.58	25.67±0.58

The experiment indicatined good reproducibility and consistency of the experimental procedure. The minimum inhibitory concentration (MIC) results demonstrated varying antimicrobial effects among the tested essential oils against the isolates. Lower MIC results were indicatives of

stronger antibacterial activity, while higher MIC values were indicators of reduced susceptibility of the bacterial isolates.

Mentha oil demonstrated strong inhibitory activity against MRSA 1 and ATCC *S.aureus*. The MIC values observed were 3.33±1.15 mg/ml and 4mg/ml respectively. The lowest observed MIC result for Mentha oil was 1 mg/ml against ATCC KPn. (Table 3)

Table 3 : Minimum Inhibitory Concentration (MIC) of the essential oils on the clinical isolates

	Minimum Inhibitory Concentration (mean) (mg/ml)					
Essential oils	**MRSA 1**	**MRSA 2**	**ESBL 1**	**ESBL2**	**MDR KPn 1**	**MDR KPn 2**
Rosemary	6.67	10.67	16.33	16	16	10.67
Mentha	3.33	4	16	16.67	12	3.33
Basil	4	8	8	10.67	6.67	1
Ruta	10.67	>16	>16	>16	>16	>16

Rosemary oil also demonstrated considerable antimicrobial activity against ATCC KPn (2 mg/ml). But, higher MIC values were observed against the ESBL isolates and MDR *K.pneumoniae* isolates with values reaching 16 - 16.33 mg/ml.

Basil oil demonstrated moderate antimicrobial activity against most tested isolates. Markedly, Basil oil showed the lowest MIC value of 0.5 mg/ml against ATCC KPn indicating strong inhibitory activity against this strain. In addition MDR KPn 2 showed high susceptibility to Basil oil with an MIC value of 1 mg/ml. However, higher MIC values were observed against the MRSA and the ESBL strains.

Ruta oil demonstrated generally weaker inhibitory activity against most of the resistant isolates. The MIC values observed for the MRSA, ESBL and MDR KPn strains ranged from 10.67± 4.62

mg/ml to greater than 16mg/ml. This indicates poor inhibitory activity under the tested conditions. It did however, show moderate activity against the ATCC KPn (4 mg/ml).

The minimum bactericidal concentration (MBC) results demonstrated limited bactericidal activity of the tested isolates. The majority of the tested isolate oil combinations showed MBC values greater than 16 mg/ml. This indicated that bactericidal activity was not achieved within the tested concentration range. Among the tested oils, Mentha demonstrated detectable bactericidal activity against MDR KPn 2 at 16 mg/ml. Similarly, Ruta oil demonstrated bactericidal activity against ATCC *S. aureus* (16.67±0.58 mg/ml) while others showed MBC values exceeding 16 mg/ml. Overall, the resistant clinical isolates demonstrated rescued susceptibility to the bactericidal effects of the tested essential oils. And this is evidenced by the high MBC values across most tested concentrations (Table 4).

Table 4: Minimum Bactericidal Concentration (MBC) of the essential oils on the clinical isolates 2026

	Minimum Bactericidal Concentration (mean) (mg/ml)					
Essential oils	**MRSA 1**	**MRSA 2**	**ESBL 1**	**ESBL2**	**MDR KPn 1**	**MDR KPn 2**
Rosemary	>16	>16	>16	>16	>16	>16
Mentha	>16	>16	>16	>16	>16	16
Basil	>16	>16	>16	>16	>16	>16
Ruta	>16	>16	>16	>16	>16	>16

5. Discussion

This study assessed the antimicrobial activity of selected essential oils against drug resistant clinical isolates. The findings demonstrated that Rosemary and Mentha oils exhibited antimicrobial activity against the tested organism. Inhibition zones generally increased as the concentration of the oils increased. Similar findings have also been reported in previous studies done in the Netherlands and Italy investigating the antibacterial properties of essential oils against pathogenic microorganisms. (44,45)

The result showed that the Gram positive isolate (MRSA) was generally more susceptible to essential oils than the Gram negative isolates (ESBL and MDR KPn) which is evident in the larger inhibition zones. Similar observations were reported in a study by Burst and it's also shown in a review article published in 2016 by Swamy (9,44) The resistance may be explained by the structural difference in their cell wall. Gram negative bacteria have an outer membrane that is rich in lipopolysaccharides that is efficient at serving as a barrier against hydrophobic antimicrobial compounds. Gram positive bacteria lack this membrane.

Rosemary oil showed inhibitory activity against the tested isolates. This activity may be associated with the presence of bioactive phytochemical constituents such as 1,8-cineole, camphor, α-pinene, and borneol. They are known to interfere with bacterial membrane integrity and permeability. Previous studies have similarly reported antibacterial activity of rosemary oil against resistant strains of S. aureus. (46)

Mentha essential oil also demonstrated considerable antimicrobial activity against the tested bacterial isolates. This effect has also been reported in another study in 2020 by Hina that reported zones of inhibition of 28.5∓3.5 mm for E.Coli. (47,48) Mentha oils are known to contain biologically active compounds such as menthol,menthone, limonene and puegone. These compounds contribute to its antimicrobial activity. The capacity of inhibition produced by Mentha oil varied depending on the concentration used. The difference in activity may be the result of differences in bacterial susceptibility, oil composition, inoculum size, extraction methods or environmental conditions that might be related to the plant growth. Variations like this have been noted in a previous study by Maria where zones of inhibition ranged from 7 to 14 mm. (49)

The MIC results in this study also showed the antimicrobial potentials of Rosemary and Mentha oils. The oils showed inhibitory activity at relatively low concentrations. Similar MIC results

have been reported in other studies as well. This suggests that the oils may possess therapeutic potential. (45)

A study done by Fatima in Algeria evaluated the antibacterial activity of Rosemary oil against MDR clinical isolates including *Klebsiella pneumoniae.* It reported the MIC and MBC at 17.8 µg/ml and 71.4 µg/ml respectively against *Klebsiella pneumoniae*. The result also showed activity in other MDR strains of *E.Coli*. (50) A related study in 2025 reported inhibition zones of 13.6∓1.14 mm for rosemary oil against MRSA. (51) This indicated moderate to strong activity. This result is also similar in the current study for Rosemary oil against MRSA 2 (14∓1 mm). The lack of equal activity on the MRSA 1 strain may be related to genetic resistance pattern and other defensive factors associated with it.

A study done in Romania in 2019 by Muntean on the bactericidal activity of Mentha oil against MDR bacteria reported MIC values for *S. aureus* to be 20 mg/ml and 40 mg/ml for *K. pneumoniae.* (33) The difference in strength of activity when compared with the current study might be explained by difference in oil composition based on geographical origin and different resistance patterns of the isolates used.

The results indicated difference in the antimicrobial susceptibility pattern between clinical isolates and the ATCC strains included in the study. This could arise from differences in their genetic and phenotypic characteristics. ATCC strains are standardized organisms that are genetically stable and usually lack the adaptive resistance mechanisms possessed by clinical isolates. These mechanisms can include efflux pumps, enzymatic degradation, altered target sites and even reduced membrane permeability. Factors such as these can alter the efficacy of antimicrobial agents including essential oils. (52)

The difference in oil activity on the clinical isolates and the standard strains may additionally be related to the history of antibiotic exposure of the clinical isolates. Repeated exposure of organisms to antimicrobial agents especially in a health care setting promotes the emergence of multidrug and cross resistance mechanisms. Although it is known that essential oils have a different mechanism of action than conventional antibiotics these variations can still reduce the susceptibility to plant derived antimicrobials. A study done by Barcerril in Spain also supports this and it had reported that bacterial isolates with repeated exposure to antimicrobial agents showed different susceptibility patterns toward Oregano and Cinnamon essential oils. This is

suggestive of the fact that drug resistance patterns influence bacterial response to essential oil treatment. (53)

One of the most significant clinical implications of this study is the potential use of essential oils as an alternative or adjunct antimicrobial agent against drug resistant bacteria. The results were promising but also indicative of the amount of further research that needs to be done. In the case of MDR bacteria especially, the limited effectiveness of antibiotics contribute to the fast spread of the organisms. (54)

Another important discussion based on the results of this study is the possibility of combining essential oils with conventional antibiotics to improve antimicrobial efficacy. A study by Antonio showed that combining antibiotics with essential oils of known antimicrobial activity enhanced the effectiveness of the antibiotics. (55)

6. Strength and Limitations

Major strength of the study is that it was focused on drug resistant clinical isolates. Unlike studies that only use standard laboratory strains, the inclusion of MDR bacteria increases the clinical relevance of the findings as it is an ongoing global challenge. Studying essential oils as antimicrobial agents is especially helpful in resource limited countries like Ethiopia. Another strength of the study was the inclusion of both gram positive and gram negative bacteria. This had also allowed for the comparisons of susceptibility between the two groups. Additional strength of the study was the use of multiple antimicrobial assessment methods like agar well diffusion, MIC and MBC. Using triplicate testing was also another methodological strength. This insured that the results were reproducible and reliable. So the findings may support future research on locally available medicinal plants.

On the other hand, the study had several limitations including that it was conducted entirely in vitro. The study doesn't account for host immune response, tissue penetration, metabolism, essential oil toxicity and other pharmacokinetics. Another limitation was the limited number of bacterial isolates used especially since resistance patterns can vary from one hospital to another, geographical regions and other similar factors. Overall, limitations related to resources, laboratory equipment may have affected the scope of the study. Despite these limitations, the study successfully showed the antibacterial potential of the essential oils in the study against clinically important pathogens.

7. Conclusion

The study showed that the selected essential oils had measurable antimicrobial activities on both standard and MDR strains of the tested bacteria. The results showed that the activity depended on the type of essential oil, the bacterial strain and the concentration used. Generally, larger zones of inhibition and lower MIC and MBC values were observed against ATCC strains compared to the drug resistant clinical isolates. This difference may be associated with resistance mechanisms such as altered membrane permeability and previous antimicrobial exposure among clinical isolates.

The study showed that increasing oil concentration resulted in increased antimicrobial activity. The use of agar well diffusion along with MIC and MBC methods was able to provide comprehensive evaluation for the oils. Among the tested oils, Rosemary and Mentha showed the strongest activity particularly against MRSA strains.

In conclusion, the results provide evidence that theses oils possess measurable antimicrobial activity against drug resistant bacteria.

8. Recommendations

Additional laboratory and clinical investigations should be performed to understand the therapeutic value of the essential oils in the study for possible application in a clinical setting. Wider range of clinical isolates and drug resistant pathogens should be also be studied. Toxicity and safety evaluations are to be strongly recommended before any pharmaceutical applications for the oils as well.

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Annex

Annex I

Hydrodistillation Essential Oil Extraction Protocol

Plant Material Preparation

Fresh plant materials of rosemary (*Rosmarinus officinalis*), peppermint (*Mentha piperita*), basil (*Ocimum basilicum*), and ruta *Ruta chalepensis* leaves were collected and transported to the processing lab on the day of collection. The plant materials were identified and cleaned thoroughly using distilled water to remove dirt and other contaminants. The cleaned materials were air dried under shade at room temperature for several days to reduce moisture content while preserving volatile compounds. After drying, the plant materials were chopped into small pieces to facilitate efficient extraction of essential oils.

Hydrodistillation Extraction Procedure

Essential oils were extracted using the hydrodistillation method with a Clevenger-type apparatus. Approximately 200 g of dried plant material was weighed using a digital balance and transferred into a 2000 mL round-bottom flask. About 1000 mL of distilled water was added to the flask to completely immerse the plant material.

The flask was connected to the Clevenger apparatus and condenser system. The mixture was heated using a heating mantle until boiling commenced. Hydrodistillation was continued for 3–4 hours to allow the volatile oil components to evaporate together with steam.

The generated steam containing volatile compounds passed through the condenser, where it was cooled into liquid form. The condensed liquid containing water and essential oil was collected in the graduated collection tube of the Clevenger apparatus. Due to differences in density and immiscibility, the essential oil separated from the aqueous layer and accumulated in the collection chamber.

After completion of the extraction process, the essential oil layer was carefully separated using a sterile pipette and transferred into sterile amber-colored glass bottles. The extracted oils were

tightly sealed to prevent evaporation and stored in a refrigerator at 4°C until further antibacterial analysis was performed.

Annex II

Clinical Sample Collection and Transportation Procedure

1. Previously identified multidrug-resistant bacterial isolates were obtained from the microbiology laboratory after routine culture and drug susceptibility testing (DST).
2. Selected isolates, including MRSA, ESBL-producing *Escherichia coli*, and *Klebsiella pneumoniae*, were identified based on laboratory antimicrobial susceptibility records.
3. Using a sterile inoculating loop, pure colonies of each isolate were picked from culture plates.
4. The isolates were inoculated into nutrient agar slants.
5. Each transport tube was clearly labeled with the isolate name, organism type, and date of transportation.
6. The inoculated transport media were placed in a cold box to maintain appropriate temperature conditions during transportation.
7. The isolates were transported immediately to the research laboratory for further antibacterial testing.
8. Upon arrival at the research laboratory, the isolates were subcultured onto fresh culture media and incubated for purity checking and subsequent experimental procedures.

Annex III

Biochemical Identification and Drug Resistance Confirmation of Bacterial Isolates

1. Prepare fresh subcultures of bacterial isolates on appropriate culture media and incubate at 37°C for 18–24 hours.

2. Perform catalase and coagulase tests for confirmation of Staphylococcus aureus isolates.

Principle: Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer.

Procedure

1. Pour 2-3 ml of 3% hydrogen peroxide to a test tube
2. Using a sterile wooden stick take the test organism & immerse into theH2 O2 solution.
3. Look for immediate bubbling

Interpretation

Active bubbling . . . . . Positive catalase test

No bubbles . . . . . .…… .Negative catalase test

Principle: Coagulase causes plasma to clot by converting fibrinogen to fibrin.

Procedure

1. Place a drop of physiological saline on two separate slides
2. Emulsify the test organism in each of the drop to make thick suspension
3. Add one drop of plasma to one of the suspensions and mix gently. Look for clumping of the organism within 10 seconds

Interpretation

Clumping within 10 secs . . . . . S. aureus/ S.lugdnesis

No clumping within 10 secs . . . . . No bound coagulase

3. Perform indole, urease, motility, and triple sugar iron (TSI) tests for confirmation of Escherichia coli and Klebsiella pneumoniae isolates.

Indole test Principle The test organism is cultured in a medium which contains tryptophan. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethyl aminobenzaldehyde. This reacts with the indole to produce a red coloured compound. Kovac's

reagent is recommended in preference to Ehrlich's reagent for the detection of indole from enterobacteria.

Method

1. Inoculate the test organism in a bijou bottle containing 3 ml of sterile tryptone water.
2. Incubate at 35–37 O C for up to 48 hr.
3. Test for indole by adding 0.5 ml of Kovac's reagent. Shake gently. Examine for a red colour in the surface layer within 10 minutes.

Interpretation

Red surface layer . . . . . Positive indole test

No red surface layer . . . . . Negative indole test

Urease test (Christensen's (modified) urea broth) Principle: The test organism is cultured in a medium which contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in color of the indicator to pink-red.

Procedure

1. Inoculate heavily the test organism in a bijou bottle containing 3 ml sterile Christensen's modified urea broth.
2. Incubate at 35–37 O C for 3–12 h (preferably in water bath for a quicker result).
3. Look for a pink color in the medium.

Interpretation

Pink color . . . . . Positive urease test

No pink color . . . . . …. . . Negative urease test

Principle of Motility Test: Motility agar was prepared and inoculated with a straight inoculating needle making a single stab about 1-2cm down into the medium. The motility was examined after 35oC for 24hour. Motility was indicated by the presence of diffuse growth (appearing as coloring of the medium) away from the line of inoculation. But if the bacteria are non-motile, the growth of the bacteria was along the stab, diffusion will not occur.

Principle: TSI agar tests are used to determine whether gram negative bacilli utilize glucose and lactose or sucrose fermentative and produce hydrogen sulfide. It contains 1% lactose, 1% sucrose

and0.1% glucose and peptone. Phenol red and ferrous sulphate serves as an indicator for acidification of medium and H2S production.

Procedure

1.Using a sterile inoculating needle, stab the butt of the TSI agar slant twice then streak back and forth along the surface of the agar with the organism.

2. Incubate at 37 O C for 18 to 24 h.

3. Look for the color change and gas production.

Interpretation

If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented.

If alkaline slant–acid butt (red–yellow): glucose fermented only.

If alkaline slant–alkaline butt (red–red): glucose not fermented.

The presence of black precipitate (butt) indicates hydrogen sulfide production, and Presence of splits or cracks with air bubbles indicates gas production.

4. Interpret biochemical reactions according to standard microbiological procedures.

5. Perform antimicrobial susceptibility testing on Mueller-Hinton agar using the Kirby–Bauer disk diffusion method according to CLSI guidelines.

6. Confirm MRSA isolates using cefoxitin (30 µg) resistance testing.

7. Confirm ESBL-producing isolates using ceftazidime and cefotaxime screening methods.

Agar Well Diffusion Procedure

1. Mueller-Hinton agar plates were prepared according to the manufacturer's instructions and allowed to solidify.
2. A bacterial suspension equivalent to 0.5 McFarland standard was prepared from fresh bacterial colonies.
3. Sterile cotton swabs were used to evenly inoculate the bacterial suspension onto Mueller-Hinton agar plates.
4. Wells of approximately 8 mm diameter were made in the agar using a sterile cork borer.
5. Different concentrations of essential oils (5%, 10%, and 20%) were prepared using Tween 80 solution.
6. Approximately 50–100 µL of each oil concentration was dispensed into the respective wells.
7. The inoculated plates were allowed to stand for diffusion of the oils into the agar.
8. Plates were incubated aerobically at 37°C for 18–24 hours.
9. After incubation, the zones of inhibition around each well were measured in millimeters using a ruler or caliper.
10. All tests were performed in triplicate and the average values were recorded.

Minimum Inhibitory Concentration (MIC) Procedure

1. Mueller-Hinton broth was prepared according to the manufacturer's instructions.
2. Different concentrations of essential oils were prepared using serial two-fold dilution methods.
3. A sterile 96-well microtiter plate was labeled appropriately for each bacterial isolate and oil concentration.
4. Approximately 100 µL of Mueller-Hinton broth was dispensed into each well of the microtiter plate.
5. Serial dilutions of the essential oils were prepared across the wells to obtain different concentrations.
6. Fresh bacterial suspensions equivalent to 0.5 McFarland standard were prepared from overnight cultures.
7. A standardized bacterial inoculum was added into each well containing the broth and essential oil dilution.
8. Positive control wells containing broth and bacterial suspension without essential oil and negative control wells containing broth only were included.
9. The microtiter plates were incubated aerobically at 37°C for 18–24 hours.
10. After incubation, the wells were examined for visible turbidity or bacterial growth.
11. The lowest concentration of essential oil showing no visible growth was recorded as the minimum inhibitory concentration (MIC).

Minimum Bactericidal Concentration (MBC) Procedure

1. Samples were taken from broth wells that showed no visible growth during MIC determination.
2. Using a sterile loop, aliquots from the clear were were subcultured onto Mueller-Hinton agar plates.
3. The inoculated plates were incubated aerobically at 37°C for 18–24 hours.
4. After incubation, plates were examined for bacterial growth.
5. The lowest concentration of essential oil showing no bacterial colony growth on agar plates was recorded as the MBC value.
6. All procedures were performed under aseptic conditions according to standard microbiological techniques.

Declaration

The undersigned declares that this thesis complies with the regulations of the University and meets the accepted standards with respect to originality and quality. PI also agrees to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports.

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Date of submission:_ _____

This thesis has been submitted with our approval as advisors.

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