INTRODUCTION: Occurrence of multidrug resistant microorganism is the result of irrational use of antibiotics. Recently essential oil, an important constituent of therapeutic and scented plant has been explored for their antimicrobial activity. OBJECTIVES: In this study, antibacterial activities of rosemary essential oil (REO) alone and also in conjunction with beta lactam antibiotic cefuroxime (CXM) were assessed. The purity of REO was checked by gas chromatography / mass spectrophotometry method (GC-MS). METHODOLOGY: By agar well diffusion method for REO and its diluents with dimethyl sulfoxide (DMSO) and tween 80, the zone of inhibition (ZOI) against four Gram positive bacterial strains of Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, and Staphylococcus epidermidis and three Gram negative bacterial strains of Pseudomonas aeruginosa (1:2,1:10, 1:20, 1:40 and 1:50). E. coli as bacterial and DMSO as solvent were used as a control. RESULTS: Antimicrobial activity was found to be highest against Staphylococcus aureus with ZOI 17.56mm and the lowest against Pseudomonas aeruginosa with ZOI 5.53 mm. The combination of REO and CXM indicated synergistic effects against E. coli, Staphylococcus aureus, Klebsiella pneumonia and Proteus mirabilis and antagonistic effect against Pseudomonas aeruginosa and indifferent effects against Bacillus subtilis and bacillus epidermidis based on fractional inhibitory concentration (FIC) values. The t test was applied to FIC values that showed significant p value (< 0.5). CONCLUSION: In conclusion, the antibacterial activity of the REO and CXM was enhanced when administered in combination, with the exception of Pseudomonas aeruginosa. In future this study may lead the way to the development of new drug combinations and dosage forms for increased antimicrobial and a reduced resistance against common pathogens.

KEYWORDS: Rosemary essential oil, Cefuroxime, Zone of inhibition, Fractional inhibitory concentration.
INTRODUCTION

The demand for new antimicrobial entities has been augmented with the passage of time because of the emergence of resistant bacteria to presently available antibiotics (1). Some of the presently available drug therapies have lost their therapeutic effectiveness. In addition, use of synthetic precursors for the treatment of infectious disorders is associated with systemic adverse effects, drug-drug interactions medication-food interactions and carcinogenic effects (2). Considering the limitations associated with synthetic drugs, researches are keen to exploit alternative therapies with promising therapeutic efficacy accompanied by lesser unwanted effects and resistance. In this situation, using essential oils to manage multi-drug resistant pathogenic bacteria in epidemics can help fight many infectious diseases (3).

Cefuroxime is a 2nd generation semisynthetic drug, use for its antimicrobial activity. As compare to other beta lactam Cephalosporin is stable to hydrolysis due to presence of methoxyimino group at position 7. Structure of cefuroxime is presented in figure 1 (4) (5).

![Figure 1. Structure of cefuroxime](image)

Figure 1. Structure of cefuroxime

Essential oils are mixtures of a variety of volatile substances, including aliphatic, aromatic, and terpenoids substances (2). Traditional medicine has employed essential oils to treat a number of infectious disorders. Many kinds of essential oils, such as lemon, eucalyptus, clove, and cinnamon oils, have been employed as antibacterial agents (6). The leaves of the fragrant perennial flowering shrub rosemary resemble hemlock needles (6). Pharmacologically, it is used to treat flatulence, gastritis, constipation, bloating, and stomach pains (7).

The combination therapy against multidrug resistant bacteria may open up the gate to counter increasing incidence of resistance to antimicrobial therapy. Combination therapy's synergistic effects may cause a decrease in the lowest effective dose of antibiotics, which would reduce adverse effects and broaden the antibacterial range (8) (7).

The purpose of this study is to assess the antibacterial activity of essential oils alone against dangerous bacterial strains that are multidrug resistant. Also, this study is committed to evaluating the antibacterial efficacy of essential oil in combination with antimicrobial medications against numerous different resistant bacterial strains.

MATERIALS & METHODS

Chemicals

Rosemary essential oil (REO), Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Ethanol and Dimethyl Sulfoxide (DMSO) by Sigma-Aldrich, Cefuroxime (CXM) obtained from (GSK).

Bacterial strains

Microorganisms were gathered from the Combined Military Hospital (CMH), Lahore's cultural collection section of the microbiology lab. At 4°C, Mueller Hinton slants were used to maintain the bacterial
cultures. The gram positive and gram-negative bacteria strains listed below were employed. Three-gram negative bacterial strains, *Salmonella typhi* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 10031), and *E. coli*, and four-gram positive bacterial strains, *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), and *Staphylococcus epidermidis* (ATCC 3521).

**Methods**

Different types of culture media, including growth media, nutrient broth (9), and Mueller Hinton agar media, and McFarland standard were prepared (2). Further, solutions of essential oil (10) and CXM were also prepared (11).

**Assessment of antibacterial activity**

The antibacterial potential of REO and CXM alone and then in combination against the aforementioned multidrug resistant pathogenic bacterial strains was evaluated using the well diffusion method. Inoculum, containing the bacterial colonies was spread with the help of sterile cotton swab on to the surface of MHA media, solidified in petri plates. The solutions of different concentrations of REO and CXM were poured into the 10 mm diameter wells bored with the help of sterile borer. Three bores were created in each plate in order to record antimicrobial activity of each concentration of REO and CXM in triplicate. During the investigation, DMSO (10%) was employed as a negative control. All petri plates were incubated in an incubator at 37°C for 24 hours, and the average zone of inhibitions for REO and CXM were recorded (5).

**Minimum inhibitory concentration (MIC) determination**

Minimal inhibitory concentration (MIC) is calculated using micro dilution method; test tubes shouldn't be used for this. Minimum Inhibitory Concentration (MIC) of REO and CXM, both individually and jointly, was determined using the appropriate equipment. Three runs of each test were completed, and the mean value was calculated (12).

**MIC of Rosemary Essential Oil**

70% REO was serially diluted with DMSO and tween 80 to give final concentrations of 1, 4.67, 5.38, 6.36, 7, 8.75, 11.67, 17.5, 35 mg/ml. From these dilutions (70 μL) was added into test tubes containing equal volume of bacterial suspensions. Then identical tests were conducted for positive and negative control. The tubes were then heated in an incubator overnight at 37°C. MIC was reported at the minimal dilution with no visible turbidity (6).

**MIC of Cefuroxime**

For injection, a stock solution of CXM (10 mg/ml) was produced in sterile water. To obtain concentrations of 1 mg/ml, 0.1 mg/ml, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 mg/ml, this solution was serially diluted. The test tubes were filled with 70 L of each CXM dilution in Mueller-Hinton broth. One pair of tubes received a 70 L aliquot of the test organism and a 70 L aliquot of the control. they were then overnight incubated at 37°C (13).
**Minimum bactericidal Concentration (MBC) determination**

Using the individual minimum inhibitory concentration data for REO and CXM, the Minimum Bactericidal Concentration was calculated. Addition of 10 L of turbidity-free medium from each test tube used for the MIC test (made up of all the cells whose bacterial growth had been stopped) to freshly prepared agar plates without adding drug. MBC concentrations were analyzed as those that did not support bacterial growth (13).

**Calculation of effects of REO and CXM in combination**

In medical laboratories, the fractional inhibitory concentration (FIC) index was employed to assess the antibacterial potency of herbs and drugs (13).

**Calculation of MIC of CXM (constant) in combination with REO**

Aliquots of 100 L of each bacterial culture (105 CFU/ ml) were introduced to Mueller-Hinton broth supplemented with CXM at a concentration comparable to 1/2 MIC with various doses of REO (range from 1/32 MIC to 2 MIC, based on MIC values that had previously been evaluated) (13).

**Calculation of MIC of REO (constant) in combination with CXM**

Aliquots of 100 L of bacterial cultures (105 CFU/ ml) were added to Mueller-Hinton broth that had been supplemented with REO at a concentration corresponding to 1/2 MIC and various concentrations of CXM (ranging from 1/32 MIC to 2 MIC, based on MIC values that had previously been studied). We used the same doses of CXM and REO (3).

**RESULTS & DISCUSSION**

The *in vitro* assessment of the REO's antibacterial effectiveness was made possible by calculating the diameter of inhibitory zones. The top-ranking microbes were *Salmonella typhi* (12.45mm), *Proteus mirabilis* (14.8mm), *Bacillus subtilis* (12.7mm), *Staphylococcus aureus* (17.56mm), *E. coli* (12mm), *Klebsiella pneumonia* (11.8mm), *Staphylococcus epidermidis* (10.4mm), *Bacillus cereus* (9.8mm), and *Pseudomonas aeruginosa* (5.53mm) (Figure 2 and Table 1).

<table>
<thead>
<tr>
<th>Table 1. <em>In vitro</em> assessment of the REO's antibacterial effectiveness</th>
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<tbody>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
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<td><em>E. coli</em></td>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td><em>Staphylococcus epidermidis</em></td>
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<td><em>Klebsiella pneumonia</em></td>
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As the REO was diluted with its diluents, the antibacterial activity (zones of inhibition) decreased for almost all of the bacterial strains studied. When REO and CXM were used in combination the ZOI were increased significantly against all...
bacteria except Pseudomonas aeruginosa as shown in the Figure no. 2.

Figure 2. In vitro assessment of the REO's antibacterial effectiveness

MIC for *Proteus mirabilis*, *K. pneumoniae*, *K. pneumoniae*, *E. coli*, and *S. aureus* when REO and CXM were coupled, *B. cereus* and *Proteus mirabilis* primarily decreased, although MIC increased against *Pseudomonas aeruginosa* (Figure 3 and 4). It demonstrated that REO increased the antibacterial action of CXM and that a lower dose of REO was required to provide the same effect as CXM alone. As a result, the side effects of CXM in patients were reduced, and it also offered a potential answer to the problem of antibiotic resistance.

Figure 3. Comparison of MIC of REO alone and in combination with CXM

Table 2. MIC and FIC values

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC CXM alone (μg/ml)</th>
<th>MIC CXM in combination (μg/ml)</th>
<th>MIC CXM</th>
<th>MIC REO</th>
<th>MIC REO</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. aerogenes</em></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>5.5</td>
<td>0.57</td>
<td>Synergic</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>5.5</td>
<td>2</td>
<td>9</td>
<td>19.9</td>
<td>0.35</td>
<td>Synergic</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>5.5</td>
<td>4</td>
<td>16</td>
<td>10.9</td>
<td>0.75</td>
<td>Synergic</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>7</td>
<td>8</td>
<td>64</td>
<td>10.9</td>
<td>1.64</td>
<td>Indifferent</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>10.9</td>
<td>32</td>
<td>32</td>
<td>21.8</td>
<td>1.5</td>
<td>Indifferent</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>21.8</td>
<td>32</td>
<td>16</td>
<td>21.8</td>
<td>3</td>
<td>Antagonistic</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>10.9</td>
<td>8</td>
<td>8</td>
<td>21.8</td>
<td>1.5</td>
<td>Indifferent</td>
</tr>
<tr>
<td><em>K.</em></td>
<td>5.5</td>
<td>4</td>
<td>16</td>
<td>10.9</td>
<td>0.76</td>
<td>Synergic</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>1.75</td>
<td>2</td>
<td>8</td>
<td>10.9</td>
<td>0.81</td>
<td>Synergic</td>
</tr>
</tbody>
</table>

FIC indexes are additive, at =1. FIC is synergistic: 1. FIC indices were neutral, ranging between 1 and 2. If FIC indices were more than 2, antagonistic (13)

*Cereus* and *Proteus mirabilis* largely decreased when REO and CXM were paired, whereas MIC increased against *Pseudomonas aeruginosa* (Figure 3 and 4). It proved that REO enhanced the bactericidal effects of CXM and that less REO was needed to have the same impact
as CXM by itself. As a result, CXM's adverse effects on patients were diminished, and it also provided a possible solution to the issue of antibiotic resistance (7). The results of FIC index and effects on bacteria are presented in Figure 5 and 6.

![Figure 5. FIC values](image)

![Figure 6. Synergism, Antagonism and Indifferent](image)

When REO and the antibiotic CXM were used against *S. aureus*, there were noticeable synergistic benefits. These outcomes could be the consequence of the active compounds actions or could come as a result of other REO chemicals potentially suppressing one or more bacterial resistance pathways. However, the observed synergistic effects suggest that the active ingredients in the REO may possibly have a different mode(s) of action from the antibiotic under study (Cefuroxime). The current study supports Rosemary's critical contribution to the elevation of beta lactams' susceptibility (14).

Antimicrobial assay findings showed that REO has various antibacterial activity against the various test pathogens. The finding of this study involves that the *S. aureus, P. aeruginosa* and *E. coli* were in agreement with many other research that had been conducted on this plant (2, 15). Our findings regarding *S. aureus* were in line with *Rosmarinus officinalis* essential oil, which inhibited *S. aureus* growth but had no impact on *E. coli* (16). In accordance with our findings, *S. aureus* and *E. coli* growth was suppressed by *Rosmarinus officinalis* essential oil. (17). The genetic discrepancies between the plant and microbial strains utilized in this investigation may be the cause of reported contradictory results. Previous studies have demonstrated that several *Rosmarinus officinalis* extracts have antibacterial activity against a variety of microorganisms (17, 18) is similar to the current study against (*S. aureus, E. coli, K. pneumonia, and P. aeruginosa*) were sensitive to the essential oils of *Rosmarinus officinalis*. Additionally, our findings support previous research that examined the occurrence of Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria in related plants. Rosemary essential oil have shown antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* (19) *Staphylococcus epidermidis, Klebsiella pneumoniae* (20). Essential oil have antimicrobial activity against various bacteria and this activity can be enhanced by using in combination with antibiotics (21).
Future Recommendations
In this research *R. officinalis* have shown antimicrobial activity and its MIC and MBC against selected bacteria have reported, as well as the its synergistic effect with cefuroxime has ever been documented against all of these bacteria. Both gram-positive and gram-negative *Staphylococcus aureus* are effectively treated with rosemary essential oil plus the antibiotic cefuroxime lactam. *E. coli* and Proteus mirabilis work in concern, but the REO and Cefuroxime combination have an antagonistic effect against *Pseudomonas aeruginosa*. Other microorganisms have exhibited a neutral impact. To determine the active constituents, the mechanism of action, and any potential harmful effects in vivo of these compounds, a larger investigation is required. The advantage can be maximized when the pharmacokinetics of the natural ingredient and the antibiotic combination are compatible. It is important to investigate the best ratios and dosing schedules for increased efficacy and diminished toxicity effects.

CONCLUSION
Due to their minimal adverse effects, herbal medicines are widely used in developed countries. As the overuse of antibiotic causes the development of multidrug resistant against microorganisms so herbal medicines always preferred over them. The findings of present study show that REO has excellent antibacterial properties against both Gram positive and Gram-negative microorganisms. And by diluting the REO its antibacterial activity reduced. The results of the study also suggest that essential oils and their combination with antibiotics may be used as novel antimicrobial agents against pathogenic bacteria that are resistant to a number of medications. By minimizing the lowest effective dose of an antibiotic, this combination may reduce any potential negative effects while also slowing the emergence of antibiotic resistance.

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None

DECLARATIONS
Authors’ Contributions
AN contributed to study concept, study design and data collection. SH contributed in data analysis and interpretation. SMA did the literature review and critically reviewed the manuscript. All the authors read and approved the final manuscript.

Conflict of Interest
There is no conflict of interest.

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