Antibacterial activity of four herbal extracts against Methicillin resistant *Staphylococcus aureus* Strains isolated from patients in Almadinah hospital, Saudi Arabia.

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**ABSTRACT**

The purpose of this study was to explore antimicrobial effect of four plant extracts *Mentha cervina*, *Mentha longifolia*, *Ocimum basilicum* and *Origanum vulgare* against multi-drug resistance (MDR) *Staphylococcus aureus* isolated from wound infections in Almadinah Almouwrah, Saudi Arabia. MRSA is resistant to not only methicillin and other β-lactam antibacterial agents but also other antibacterial agents; therefore new agents are needed to treat MRSA. Antibacterial activity of the medicinal plant extract was determined using well diffusion assays and paper disc diffusion method. The medicinal plant extract exhibited antibacterial activity against multi-drug resistance (MDR) *Staphylococcus aureus*. The synergetic effect was clearly observed with *Mentha longifolia* were combined with *Ocimum basilicum*, and followed by *Mentha longifolia* + *Origanum vulgare*, *Mentha longifolia* + *Mentha cervina* and *Ocimum basilicum* + *Mentha cervina* then, *Origanum vulgare* + *Ocimum basilicum* and *Origanum vulgare* + *Mentha cervina* and showed the strongest antimicrobial activity. Our result confirmed that ethanolic extracts, could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms from community as well as hospital settings. In addition ethanolic extracts of our medicinal plants may have the potential to restore the effectiveness of β-lactam Antibacterial agents against MRSA.

**Keywords:** Herbal extract, antibacterial, *Mentha longifolia*, *Mentha cervina*, *Ocimum basilicum*, *Origanum vulgare*.

**INTRODUCTION**

Antibacterial agents provide the main basis for the therapy of microbial infections. Their use would lead to the eventual eradication of infectious diseases. However, overuse of antibacterial agents has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms [1, 2, 3].

The extensive use of antibacterial agents over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. *Staphylococcus aureus* is one of the most important pathogens that can cause suppuration, abscess formation, a variety of pyogenic infection and even fatal septicemia in human beings. MRSA is still constitute an emerging pathogen and public health threats result from the spread of hospital-acquired as well as community-acquired MRSA [4].

MRSA is resistant not only methicillin and other β-lactams antibacterial agents but also other antibacterial agents; [5] which resulted in severe consequences including increased cost of medicines and mortality of patients. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. Researchers are turned their attention to herbal products, looking for new leads to develop better drugs against MDR microbe strains [6]. Many plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown...
to inhibit growth of pathogenic bacteria. Traditional herbal medicine practice has been known for centuries in many parts of the world for the treatment of various human ailments. The use of antibacterial agents has revolutionized the treatment of various bacterial infections. However, their indiscriminate use has led to an alarming increase in antibiotic resistance among microorganisms, thus necessitating the need for development of novel antimicrobials. Recent years have witnessed a renewed interest in exploring natural resources for developing such compounds. Medicinal plants are relied upon by 80% of the world’s population [7-13].

Traditional healers have long used plants to prevent or cure infectious conditions; Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial, anti viral anti parasites and anti cancers properties [14-18]. Accordingly, the present work aimed at studying the antibiotic effect of the Four plant extracts Mentha cervina, Mentha longifolia, Ocimum basilicum and Origanum vulgare against clinical isolates of Staphylococcus aureus at the purpose of overcoming its resistant infection spreading in the Saudi society. Also create directly comparable, quantitative, antimicrobial information generate recent data for plant extract applicable in the antimicrobial field.

MATERIAL AND METHODS

Specimens: isolates

In our present study, 80 clinical samples (wound infection) were collected from patients attending Ohoud Hospital, Al Madinah, Saudi Arabia. Out of them 30 were positive for staphylococcus aureus, only 10 MRSA isolates of them (isolated from wound infections) were used in our susceptibility testing study.

Identify organism

API Staph-Idnt strip system. The API Staph. Indent. strips consist of a series of 10 microcups containing dehydrated substrates, nutrient media, or both. The alkaline phosphatase (Phs), β-glucosidase (Gls), and β-glucuronidase (Glc) microcups contain chromogenic substrates and positive reactions are noted by the liberation of p-nitrophenol from them. The urease (Ure) and arginine utilization (Arg) microcups contain phenol red as an indicator to detect alkaline end products. The mannose (Mne), mannnitol (Man), trehalose (Tre), and salicin (Sal) microcups contain cresol red as an indicator to detect acid production. The, 3-galactosidase (Ngp) microcups contain 2-naphthol-13-D-galactopyranoside and requires the addition of STAPH-IDENT reagent (0.35% fast blue BB salt in 2-methoxyethanol) to detect free β-naphthol. Recommended procedures of the manufacturer were followed for the preparation of strips and inoculums and the inoculation, incubation, and reading of strips. Test organisms were removed from an overnight culture inoculated on Trypticase soy agar plates containing 5% sheep blood, using a sterile cotton swab. The inoculated swab was agitated in 2 ml of sterile 0.85% saline so that the final turbidity was approximately that of a no. 3 McFarland (BaSO4) turbidity standard. Each microcups on the strip was filled with three drops (approximately 80 µl) of the above-described bacterial suspension. After inoculation, strips (placed into plastic trays with lids) were incubated for 5 h at 35°C in air and then read. Two drops of STAPH-IDENT reagent were added to the Ngp microcups, and color development was recorded after 30 s. Positive reactions were converted to a four-digit profile for species identification according to instructions of the manufacturer. The following 10 biochemical tests are divided into the four groups arranged in order on the strip: Phs, Ure, and Gls; Mne, Man, and Tre; Sal, Glc, and Arg; and Ngp. A value of one, two, and four is assigned to the first, second, and third positive biochemical test, respectively, in each group. A value of zero is assigned to a negative result. Each resulting profile number represents a unique combination of positive reactions [19].
Plants preparation

Plants: *Mentha Cervina*, *Mentha longifolia*, *Origanum vulgare* and *Ocimum basilicum* were collected from Al Madinah Almounawarah Saudi Arabia. The collected plant was identified by the staff of biology department, faculty of science, Taibah University, Saudi Arabia. Plants was rinsed twice with tap water then, leaves were separated and washed again with tap water to eliminate soil and other surface contaminants, then dried in shade. After shade drying, plant material was pulverized using domestic blender to powder form. Powdered material (100g) was mixed with one liter of 70% methanol and kept 7 days, in closed lab glass Bottles at room temperature. After 7 days, mixture was filtered through whatman filter paper. The filtered extract was subjected to rotary evaporator. The water bath temperature was adjusted to 45°C. The semisolid extract produced was kept in open air at a laminar flow cabinet for 3 days for complete the methanol evaporation [16, 17].

Antimicrobial assay

The antibiotic sensitivity profile of the 10 S. aureus isolates were determined according to the method of Bauer-Kirby [20] The agar disc diffusion method was used to determine the antibacterial activity. Sterile discs (6mm, Hi-media, India) were loaded with 100μl of (100mg/ml) each of four ethanolic medicinal plant leaves extracts dissolved in 5% dimethyl sulfoxide (DMSO) and were left to dry for 18 hrs in sterile condition. Bacterial suspensions were diluted to match the 0.5 McFarland standard scales (approximately 1x10⁸ CFU/ml). Muller Hinton Agar (MHA) was poured into Petri dishes to give a solid plate and inoculated with 100 μl of suspension containing 1.5x10⁸ CFU/ml of bacteria; the discs treated with extracts were placed onto Petri plates. Paper disc treated with DMSO was used as negative control. The plates were then incubated at 37°C for 24hrs; inhibition zones diameter formed around each disc were measured and recorded at the end of the incubation time. Antibiotic susceptibility was determined from the size of the inhibition zone, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012).

Statistical Analysis

All the experimental results were performed in triplicate and the results were expressed as mean ± Standard Deviation (SD) for 4 plant extracts and their combination. Calculation was done using Microsoft Excel 2010 software [21].

RESULTS

Antibiotic susceptibility test

All 10 isolates were shown to be Multi Drug Resistant (MDR) strains; resistant to at least 5, out of 10 antibacterial agents. Ten different antibiotic patterns were identified (Table-I), three isolates were resistant to all 10 antibacterial agents, 3 to 10 (pattern # 1, 5, 10). Most of wound isolates showed multiple antibiotic resistances in the study area, which may be due to large portion of the bacteria isolate being previously exposed to several antibacterial agents.
Table 1 - Antibiotic resistance profiles index of *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>No. of Sample</th>
<th>Isolates</th>
<th>Class</th>
<th>Antibiotic Resistant Pattern</th>
<th>No. of resistant A.b.</th>
<th>No. of sensitive A.b.</th>
<th>Types of Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Staph.W1</td>
<td>MRSA</td>
<td>G-Of-Cf-R-Va-E-C-P-M-Ox</td>
<td>10</td>
<td>0</td>
<td>MDR</td>
</tr>
<tr>
<td>2</td>
<td>Staph.W2</td>
<td>MRSA</td>
<td>Of-Cf-R- C-P-M-Ox</td>
<td>7</td>
<td>3</td>
<td>MDR</td>
</tr>
<tr>
<td>3</td>
<td>Staph.W3</td>
<td>MRSA</td>
<td>Of-Cf- Va-E- P-M-Ox</td>
<td>7</td>
<td>3</td>
<td>MDR</td>
</tr>
<tr>
<td>4</td>
<td>Staph.W4</td>
<td>MRSA</td>
<td>R-Va-E-P-M-ox</td>
<td>6</td>
<td>4</td>
<td>MDR</td>
</tr>
<tr>
<td>5</td>
<td>Staph.W5</td>
<td>MRSA</td>
<td>G-Of-Cf-R-Va-E-C-P-M-Ox</td>
<td>10</td>
<td>0</td>
<td>MDR</td>
</tr>
<tr>
<td>6</td>
<td>Staph.W.6</td>
<td>MRSA</td>
<td>G-Va-P-M-Ox</td>
<td>5</td>
<td>5</td>
<td>MDR</td>
</tr>
<tr>
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<td>MRSA</td>
<td>Of-Va-C-P-M-Ox</td>
<td>6</td>
<td>4</td>
<td>MDR</td>
</tr>
<tr>
<td>8</td>
<td>Staph.W8</td>
<td>MRSA</td>
<td>Of-Va-P-M-Ox</td>
<td>6</td>
<td>4</td>
<td>MDR</td>
</tr>
<tr>
<td>9</td>
<td>Staph.W9</td>
<td>MRSA</td>
<td>G-R-Va-E-C-P-M-Ox</td>
<td>8</td>
<td>2</td>
<td>MDR</td>
</tr>
<tr>
<td>10</td>
<td>Staph.W10</td>
<td>MRSA</td>
<td>G-Of-Cf-R-Va-E-C-P-M-Ox</td>
<td>10</td>
<td>0</td>
<td>MDR</td>
</tr>
</tbody>
</table>


Table 2 - Susceptibility pattern of crude ethanolic herbal extracts against MDR *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td><em>Mentha longifolia</em></td>
<td>16.5 ± 0.30</td>
</tr>
<tr>
<td><em>Origanum vulgare</em></td>
<td>18.5 ± 0.50</td>
</tr>
<tr>
<td><em>Ocimum basilicum</em></td>
<td>14 ± 1</td>
</tr>
<tr>
<td><em>Mentha cervina</em></td>
<td>15 ± 1</td>
</tr>
</tbody>
</table>

Fig.1 Antimicrobial activity from ethanolic extracts of *Mentha longifolia, Origanum vulgare, Ocimum basilicum* and *Mentha cervina* separated against MDR- *Staphylococcus aureus* strains isolated from Almadinah Hospital In Saudi Arabia.
Table 3: Influence of extract combinations of ethanolic extracts of medicinal plants on MDR staphylococcus aureus.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SD</strong></td>
<td></td>
</tr>
<tr>
<td>Mentha longifolia + Origanum vulgare</td>
<td>26 ± 0.816</td>
</tr>
<tr>
<td>Mentha longifolia + Ocimum basilicum</td>
<td>27.67 ± 0.577</td>
</tr>
<tr>
<td>Mentha longifolia + Mentha cervina</td>
<td>24.33 ± 1.155</td>
</tr>
<tr>
<td>Origanum vulgare + Ocimum basilicum</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Origanum vulgare + Mentha cervina</td>
<td>18.67 ± 1.528</td>
</tr>
<tr>
<td>Ocimum basilicum + Mentha cervina</td>
<td>27.33 ± 0.577</td>
</tr>
</tbody>
</table>

Antimicrobial activity from ethanolic extracts combination of *Mentha longifolia* + *Origanum vulgare*, *Mentha longifolia* + *Ocimum basilicum*, *Mentha longifolia* + *Mentha cervina*, *Origanum vulgare* + *Ocimum basilicum*, *Origanum vulgare* + *Mentha cervina* and *Ocimum basilicum* + *Mentha cervina* against resistant antibiotic *staphylococcus aureus* strains (MRSA) isolated from Almadinah Hospital In Saudi Arabia.

Fig. 2: Effect of Extract Combinations for the four medicinal plants
Antimicrobial activity from ethanolic extracts combination of *Mentha longifolia* + *Origanum vulgare*, *Mentha longifolia* + *Ocimum basilicum*, *Mentha longifolia* + *Mentha cervina*, *Origanum vulgare* + *Ocimum basilicum*, *Origanum vulgare* + *Mentha cervina* and *Ocimum basilicum* + *Mentha cervina* against resistant antibiotic *staphylococcus aureus* strains (MRSA & MDR) isolated from Almadinah Hospital In Saudi Arabia.
The extensive use of antimicrobial agents over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. *Staphylococcus aureus* is one of the most important pathogens that can cause suppuration, abscess formation, a variety of pyogenic infection and even fatal septicemia in human beings. MRSA is still constitute as an emerging pathogen and public health threats result from the spread of hospital-acquired as well as community-acquired MRSA infections [4]. The bacterial resistance to several different antibacterial agents constitute significant problem. Bacteria showing reduced susceptibility to an antibiotic imply that it should not be used on the patient. [22]. Our results showed that all 10 isolates were confirmed to be Multi Drug Resistant (MDR) strains and three of them resistant to all the 10 antibacterial agents used (pattern # 1, 5, 10), (Table 1). Most of wound infections isolates showed multiple antibiotic resistances in the study area, which may be due to large portion of the bacteria isolate being previously exposed to several antibacterial agents. Medicinal plants are the boon of nature to cure a number of ailments of human beings. In many parts of the world, medicinal plants are used against bacterial, viral and fungal infections. [23]. Mint leaves are extensively used as herbal medicine all over the world. Essential oils rich in phenolic compounds found in tested plant extracts are reported to possess high level of antimicrobial activity [24]. It is believed that the phenolic components of essential oils show strongest antimicrobial activity. [25]. In this subject our present study has been showing that, the Antimicrobial activity of ethanol plant extracts of each of *Mentha longifolia*, *Origanum vulgare*, *Ocimum basilicum* and *Mentha cervina* separated against MDR- staphylococcus aureus strains were very weak except *Origanum vulgare* (Table 2 & Fig-1) , but the combination of ethanolic extracts from *Mentha longifolia + Origanum vulgare*, *Mentha longifolia + Ocimum basilicum*, *Mentha longifolia + Mentha cervina*, *Origanum vulgare + Ocimum basilicum*, *Origanum vulgare + Mentha cervina* and *Ocimum basilicum + Mentha cervina* (Table 3- Fig. 2) was able to cause such considerable influence. The synergetic effect was clearly observed with *Mentha longifolia* in combination with *Ocimum basilicum*, and followed by *Mentha longifolia + Origanum vulgare*, *Mentha longifolia + Mentha cervina* and *Ocimum basilicum + Mentha cervina* then, *Origanum vulgare + Ocimum basilicum and Origanum vulgare + Mentha cervina* (Table 3, Fig 2). It has been proposed that the mechanism of the antimicrobial effects involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally ion leakage from the cells [26] The use of plants to heal diseases, including infectious one, has been extensively applied by people all around the world. Data from the literature as well as our results reveal the great potential of plants for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should have their toxicity tested in vivo. Bioassays [27] have demonstrated the toxicity of extracts from different plants. Therefore, our results revealed the importance of plant extracts when associated with antibacterial agents, to control resistant bacteria, which are becoming a threat to human health. Furthermore, in a few cases, these plant extracts were active against antibiotic resistant bacteria under very low concentration, thus minimizing the possible toxic effects. Our results are parallel and in good agreement with the findings of Lodhia [28], who reported that Gram-positive bacteria are more sensitive to plant essential oils than Gram negative bacteria. There is evidence in the literature that the essential oils of some Lamiaceae plants possess a moderate to good antibacterial activities [29] investigated that *M. spicata* oil possessed better antibacterial activity than the standard drugs streptomycin + penicillin. Adilson[30] investigated that antimicrobial activity of *M. spicata* oil. Our results are in good agreement with the findings of Habiba [31] who found the main components of *M. pulegium* essential oil (71.1%) and exhibit a remarkable antibacterial activity against Gram positive bacteria than Gram negative ones. Antimicrobial activity of the essential oils from *M. spicata* and *M. pulegium* was proved. The high concentrations of carvone and pulegone can be used as explanation for traditional uses of the two *Mentha* species for treating microbe related illnesses.
CONCLUSIONS

The ethanolic extracts of the plants extract combinations (Mentha longifolia, Origanum vulgare), (Mentha longifolia, Ocimum basilicum), (Mentha longifolia, Mentha cervina), (Origanum vulgare, Ocimum basilicum), (Origanum vulgare, Mentha cervina) and (Ocimum basilicum, Mentha cervina) could be a good promising source for a new and effective herbal antimicrobial agents to treat infections caused by multi-drug resistant strains of microorganisms and particular isolated from hospitals. However, it is necessary to determine the toxicity of the extracts, their side effects and pharmacy-kinetic properties.

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REFERENCES


