Antimicrobial, Antiviral Activity and GC-MS Analysis of Essential Oil Extracted from Achillea fragrantissima Plant Growing In Sinai Peninsula, Egypt

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Abstract
Medicinal plants are considered new resources for producing agents that could act as alternatives to antibiotics in treatment of antibiotic-resistant bacteria. The present study aimed to evaluate the efficacy of essential oil extracted from Achillea fragrantissima plant growing in Egypt for antimicrobial, antiviral activities and chemical composition analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). We also performed determination of essential oil antimicrobial activity by agar desk diffusion method and Minimal Inhibitory Concentration (MIC). Also, antiviral activity on ORF virus by pock reduction test was performed. It was reduced virus titer from 5.9 to 1.00 at 180 minutes. Detection of beta lactams resistant bacteria (Gram-positive bacteria Staphylococcus aureus, Staphylococcus epidermidis (MRSA) and Gram-negative bacteria Escherichia coli) by PCR with primers of mecA gene and Bela gene. The essential oil obtained by hydrodistillation was analyzed by GC-MS. The major components were found to be Santolina triene (1.97%), 2,5,5-trimethyl-3,6-heptadien-2-ol (8.23%) Eucalyptol 8.17, trans-2,7-Dimethyl- 4,6-octadien-2-ol (24.40%), 1,5-Hepadien-4-one-3,5-trimethyl (7.65%), Artemisia alcohol (3.49%), à Thujone (33.97%), Casabinol (1.92%), Lavandulol (0.71%), 2-Octen-4-ol, 2-methyl (2.02%), 3-Cyclohexen1ol,4-methyl1 (1 methylthethyl) (CAS) (2.15%), à terpineol ( 0.05% ), Estragole (0.71%) Lavandulyl acetate (0.49%), Sabinyl acetate (2.12 %), Germacrene (0.94%). Finally, our study proved that the essential oil has bactericidal effect on some bacterial resistant antibiotic (Gram-positive bacteria Staphylococcus aureus, Staphylococcus epidermidis (MRSA) and Gram-negative bacteria Escherichia coli ) as well as antiviral activity on ORF virus but it is still need further extensive studies for safety and drug interaction.

Keywords: Achillea fragrantissima; Essential oil; Antimicrobial activity; GC/MS; Antiviral; PCR

Introduction
Medicinal plants still constitute one of the major source of drugs in modern as well as traditional medicine throughout the world [1,2]. Since a long period of time, plants have been a valuable source of natural products for maintaining animals and human health [2,3]. The development of multidrug-resistant(MDR) bacteria takes place because of the accumulation of different antibiotic resistance mechanisms inside the same strain which able to live in the presence of antibiotics drugs so that standard treatments become ineffective [4,14-16]. Although, the pharmacological companies have produced a number of new antibiotics recently, but even then microorganism resistance to antibiotics increased throughout the world [5,7]. This situation has attract attention of researchers towards herbal products for developing and improve the antibacterial drugs quality [6,7].

Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms for treatment of infectious diseases [5,9]. Medicinal plants contain active principles which can be used as alternative effective herbal drugs against common bacterial infections [10]. They would be the best source to obtain a variety of drugs and active compounds. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [11]. Essential oils are commercially used in different industries such as pharmaceutical, agronomic, food, sanitary, cosmetic and perfume [12]. In medicine they are used antioxidant, antitumor and antifungal [13-16].

The genera Achillea belongs to Family Asteraceae are widely distributed in the Middle East countries [17]. Ancient people had been used Achillea species in traditional medicine to alley pain, spasms and inflammation [18,3,4]. In Arabian countries all Achillea species were referred to locally names as Qaysum, Gesoom or Bu’eithraan [20,42]. Numerous studies describe the various pharmacological effects of the Achillea fragrantissima hydrodistilled volatile oils in management of several diseases, used topically and orally [18-21].

Researchers studied antibacterial properties of various plants against Gram-negative as well as Gram-positive bacterial strains but only few reports on drug resistant bacteria and antiviral activity. Our study was aimed to evaluate in vitro antibacterial of essential oil extracted from Achillea fragrantissima plant against Gram-positive bacteria Staphylococcus aureus, Staphylococcus epidermidis (MRSA) and Gram-negative bacteria Escherichia coli isolated from bovine mastitis as well as antiviral activity against ORF virus isolated from skin lesion of human and animals.

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Materials and Methods

Plant material and extraction

The aerial parts with leaves and flowers of Achillea fragrantissima plant were collected from Saint Catherine, South Sinai, Egypt in November 2013. Authentication of the plant was performed by the group of Genetics and Breeding of Medicinal and Aromatic plants at the Department of Genetics and cytology, Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt. These samples were air-dried in shade at room temperature until constant weight (about 20 days). The dried aerial parts of the plant materials were grounded with a blender. The aerial plant parts powder was stored at 4°C in a tightly covered bottle. One hundred grams of plant powder was subjected to hydrodistillation in a microscale (v/w) return flow distillation apparatus according to [25]. The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4°C until used.

Gas chromatography and mass spectrometry (GC-MS) analysis

The essential oil was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (NRC-GC/El-MS Lab), according to method [25-27] column with 0.25 μm film thickness, Helium was used as carrier gas, the flow through the column was 1.4 ml/min. The column temperature was programmed from 40 to 85°C at 20°C/min, increased from 85 to 300°C at rate of 5°C/min and finally held for 10 min. The MS operating parameters were as follows: ionization voltage, 70 V; ionization current, 2 A; ion source temperature, 200°C, resolution, 1000. Mass unit were monitored from 30 to 450 m/z. Determination and identification of each components in the oil was based on RI relatives to n-alkanes and computer matching with library search report, as well as by comparison of the fragmentation patterns of mass spectra [27].

Bacterial strain

Microbial strains: Gram-positive bacteria Staphylococcus aureus, Staphylococcus epidermidis (MRSA) and Gram-negative bacteria Escherichia coli were isolated from bovine mastitis provided by Escherichia coli and Gram-negative bacteria Staphylococcus aureus (MRSA) and Gram-negative bacteria Escherichia coli were previously isolated from bovine mastitis by Agar diffusion methods and Minimal Inhibitory Concentration (MIC).

Antimicrobial activity

Essential oils were individually tested against Gram-positive bacteria Staphylococcus aureus, Staphylococcus epidermidis (MRSA) and Gram-negative bacteria Escherichia coli were previously isolated from bovine mastitis by Agar diffusion methods and Minimal Inhibitory Concentration (MIC).

Agar diffusion method: These methods were performed according to Forbes et al. [31]. Bacteria cultures were diluted with sterile physiological saline solution with reference to the Mc Farland 0.5 standard to achieve inoculums of approximately 10^6 CFU/ml. A suspension was swab in three directions on 4 mm thick Mueller Hinton Agar (MHA) (Oxoid) with a cotton swap. Modified discs of 6 mm were prepared using a Whitman filter paper. 100 discs were obtained by punching and putting in vials-bottles and sterilizing in an oven at 170°C for 30 min. The discs were impregnating with 20 μl of essential oil. Prepared discs containing the various essential oil were carefully placed on the inoculated plates using a sterilized forceps in each case [31]. The disc with solvent alone used as negative control and antibiotic discs as control positive. The plates were then turned upside-down and incubated at 37°C for 24 h in an incubator by the same manner used rosette with 6 well puncture the agar and 50 μl each well by essential oil. Tween 20 with saline and saline only as control negative. The results were taken by measuring the Diameter of the Inhibition Zone (DIZ) around the disc or well. This was repeated thrice and mean ± SD was calculated.

Minimal inhibitory concentration (MIC) using microdilution method

It was done according to [32,33] guidelines for determination of Minimum Inhibitory Concentration (MIC). The MIC was defined as lowest concentration of essential oils that inhibiting visible bacterial growth after incubation for 20 h at 37°C. Into each well of microplate 100 μl of Mueller-Hinton Broth (Oxoid) inoculated with the bacteria strain. An aliquot (100 μl) of the essential oil was added in first well serial dilutions of the essential oils were prepared in a 96 well micro titer plate, including one growth control and one sterility control. The contents of the wells were mixed and micro plates were incubated...
at 37°C for 24 h. Micro titer plates were incubated at 37°C for 24 h. The activity was measured as a function of turbidity. Lack of turbidity was further confirmed by pouring suspension aliquot of 0.1 ml into pre-sterilized Petri dishes with nutrient agar medium. The tests were conducted in triplicate.

Estimation of the number of the viable bacterial cells

The time that taken by essential oil to kill staphylococcus and E. coli were evaluated by the time-kill assay method. The Time-kill curves were performed in tube containing nutrient or Muller Hinton broth, using inoculums density of approximately (10⁶ CFU/mL) in the presence of essential oil. The tubes were continuously shaken and incubated at 37°C. Samples were obtained at 0, 2, 4, 8 and 16. At each sample time, they were taken and serially diluted log 2 2, 4, 8, 16, 32. 100 µl of undiluted and diluted samples were inoculated on nutrient agar or Muller Hinton agar. The plates were incubated at 37°C for 24 h. Lack of turbidity or growth of the microorganisms tested. It was especially active against Staphylococcus aureus and MRSA and E. coli.

Polymerase chain reaction (PCR)

Detection of the mec A and Bela A Genes for Staphylococcus epidermidis strains and E. coli were accomplished using PCR amplification. Cells were suspended in a lysis buffer containing 1 M Tris HCl, 5 M NaCl, and 0.1 M EDTA, which was incubated at 95°C for 10 minutes. After incubation, the suspension was centrifuged at 23000 rpm for 5 min. The supernatant was used as a template in PCR. PCR assay was carried out as described by [34]. Using primers Beta-lactams gene resistant MRSA Mec A F (GTGAAAGATATACCAAGTGATT) and R (GTGGAAGATATACCAAGTGATT) gave 147 bp PCR product of mecA gene from strains Staphylococcus epidermidis and also using primer Beta-lactams gene resistant for E. coli bla C (F) TGGCCAGAAGTCGACGGCAA (R) TTTCCTCCTGAACGTGGGCTGGC 462 [35,34]. The final PCR products were visualized using UV-trans-illuminator after electrophoresis on 1.5% agarose gel containing 50 µg/mL EtBr.

Statistical analysis

All data were subjected to statistical analysis including the calculation of the mean and standard Deviation. Significance between data of control and infected groups was evaluated by the Student t-test at level P<0.05 according to [36] using SPSS for windows version 15 computer program.

Results

The volatile oil or (essential oil) was extracted from aerial parts, leaves and flowers of A. fragrantissima by conventional hydro distillation, which gave liquid oil, ranged pale yellow oil to yellow with a strong penetrating pleasant odor characteristic of each plant.

The identified compounds, together with the Retention Indices (RI) of the compounds are shown in Table 1. The main chemical compounds of the essential oil extracted from A. fragrantissima were Santolina triene (1.97%), 2-Octen4ol, 2-methyl (2.02%), 3 Cyclohexen1ol,4methyl1(1methylethyl) (CAS) (2.15%), à TERPINEOL (1.92%),-Lavandulol (0.71%), 2-Octen-4-ol, 2-methyl (2.02%), 3 Cyclohexen1ol,4methyl1(1methylethyl) (CAS) (2.15%), à terpineol (0.05%), Estragole (0.71%) Lavandulyl acetate (0.49%), Sabinyl acetate (2.12%), Germacrened (0.94%) as in (Table 1 and Figures 1 and 2).

Antimicrobial activity by the agar diffusin method

The essential oil extracted from A. fragrantissima inhibited the growth of the microorganisms tested. It was especially active against Staphylococcus aureus, MRSA and E. coli. The Staphylococcus aureus showed the highest sensitivity to essential oil effect ranged from (18-22 mm–24-26 mm) and MRSA from (14-16 mm), (16-18 mm) and followed by E. coli from (8-11 mm) and (12-16 mm) inhibition zone.
The essential oil extracted from *Achillea fragrantissima* plant has been used for many years in traditional medicine in Middle East countries for the treatment of some diseases [37]. Several Achillea plants have been found to possess antiseptic and infection preventing properties [38,39]. They are being used as ingredients in medicinal and cosmetic preparations [40]. Recently, it was documented that the essential oil has anti-inflammatory, antioxidant and anti-proliferative capacities [41]. The essential oil was observed safe, no effect in the nature and color of embryo fluid comparing with control as well as experimental animals [42].

### Discussion

*Achillea fragrantissima* plant has been used for many years in traditional medicine in Middle East countries for the treatment of some diseases [37]. Several Achillea plants have been found to possess antiseptic and infection preventing properties [38,39]. They are being used as ingredients in medicinal and cosmetic preparations [40]. Recently, it was documented that the essential oil has anti-inflammatory, antioxidant and anti-proliferative capacities [41]. The essential oil was observed safe, no effect in the nature and color of embryo fluid comparing with control as well as experimental animals [42].

The essential oil extracted from *A. fragrantissima* plant inhibits growth of microorganism to various degree can determined by disc and well diffusion methods according to diameter of inhibition zone [40]. Recently, it was documented that the essential oil has anti-inflammatory, antioxidant and anti-proliferative capacities [41]. The present study revealed that the essential oil has wide varieties of volatile oil terpene hydrocarbons (aliphatic and cyclic) and their corresponding isoprenoid derivatives and analogues, mixtures of these substances, terpinen-4-01 demonstrated fraction that strongly antimicrobial effect by acting only in eukaryotic bacteria cell as in Table 1 and Figures 1 and 2 and this finding is in agreement with [42].

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The inhibition zone of *Staphylococcus aureus* demonstrated fraction that strongly antimicrobial effect by acting only in eukaryotic bacteria cell as in Table 1 and Figures 1 and 2 and this finding is in agreement with [42].

### Table 2: Determination of the of essential oil antibacterial activity by agar diffusion methods and Minimal Inhibitory Concentration (MIC). MIC: Minimal Inhibitory Concentration; DIZ: Diameter of Inhibition Zone; mm; MBC: Minimal Bactericidal Concentration; DMSO: Dimethylsulphoxide as negative control.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Antibacterial disc diffusion methods DIZ : mm</th>
<th>Antibacterial well diffusion methods DIZ : mm</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Essential oil</td>
<td>Gentamicin</td>
<td>Tetracycline</td>
<td>Essential oil</td>
</tr>
<tr>
<td>Gram positive bacteria</td>
<td>S. aureus</td>
<td>22</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>S.epidermidis</td>
<td>16</td>
<td>1</td>
<td>R</td>
</tr>
<tr>
<td>MRSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram negative bacteria</td>
<td>E. coli</td>
<td>11</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

### Table 3: Antiviral effect of *A. fragrantissima* essential oil on ORF virus titer by pock reduction test.

<table>
<thead>
<tr>
<th>Time</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; ORFV titer kept at 37°C</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; ORFV titer kept at 4°C</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; ORFV titer was treated with essential oil (v/v).</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; titer</th>
<th>Reduction index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>5.9</td>
<td>5.4</td>
<td>5.4-5.9</td>
<td>3.5</td>
<td>2.4</td>
</tr>
<tr>
<td>15 min</td>
<td>5.6</td>
<td></td>
<td></td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td>30 min</td>
<td>5.4</td>
<td></td>
<td></td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>45 min</td>
<td>5.2</td>
<td></td>
<td></td>
<td>1.6</td>
<td>3.5</td>
</tr>
<tr>
<td>60 min</td>
<td>4.8</td>
<td></td>
<td></td>
<td>1.2</td>
<td>4.2</td>
</tr>
<tr>
<td>75 min</td>
<td>4.2</td>
<td></td>
<td></td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>90 min</td>
<td>3.9</td>
<td></td>
<td></td>
<td>0</td>
<td>3.9</td>
</tr>
<tr>
<td>105 min</td>
<td>3.5</td>
<td></td>
<td></td>
<td>0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Staphylococcus aureus (MRSA) and Gram-negative bacteria *Escherichia coli* bacteria may be due to reduction or modified of β-lactamase (methicillin) resistance but the actual mechanism is unknown. However, is likely explained by some structural change in the resistant bacteria. Firstly, volatile oils fraction can be connected with the perturbation of cell membrane, the second mechanism may be inhibited the production of protein binding protein (PBP2) by inhibition of mecA and bel genes expression and final stage involved to subset synthesis of peptidoglycan of cell wall (transpeptidation reaction), which present outside the cell membrane this explanation is agreement with [48,49].

The mecA and Bel gene coding for methicillin resistance via penicillin binding protein 2a (PBP2a) are well established and is considered as “gold standard” for detection of methicillin resistance in comparison with phenotypic methods [46,47]. Our results revealed that the inhibitory effect of essential oils on Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative bacteria *Escherichia coli* bacteria may be due to reduction or modified of β-lactamase (methicillin) resistance but the actual mechanism is unknown. However, is likely explained by some structural change in the resistant bacteria. Firstly, volatile oils fraction can be connected with the perturbation of cell membrane, the second mechanism may be inhibited the production of protein binding protein (PBP2) by inhibition of mecA and bel genes expression and final stage involved to subset synthesis of peptidoglycan of cell wall (transpeptidation reaction), which present outside the cell membrane this explanation is agreement with [50].

The essential oil was observed safe, no effect in the nature and color of embryo fluid comparing with control as well as experimental animals this result is in agreement with [51,52]. It was reduced infectivity titer
of ORF virus from log$_{10}$ 5.9 to 0.00 within 180 minutes. Antiviral effect
of essential oil may be due to block the viral infection by blocking the
cell membrane receptor for ORF virus or induce internal changes in
the host cells, which in turn affect the virus replication cycle or due
to production cytokines which blocked viral infection other health
cells as in Table 3 and Figure 5. This result was in agreement with
[53]. They found the essential oil caused sharp reduction in small pox
viruses infectivity titer. Also, they observed that the concentration of
viruses were reduce log$_{10}$ 5-1 and they infectivity titer were reduced 21
and 25 time more than control group. We found that the essential oil
reduced plaque size and number when mixing with ORF virus before
inoculation on CAM, Finally, Our results proved that the Achillea
fragrantissima essential oil has antiviral and antibacterial activities on
B-lactmase resistant

**Conclusion**

The composition of the essential oil of *Achillea fragrantissima*
growing in Sinai, Egypt has been analyzed by GC-MS and its
antimicrobial activity against Gram-positive bacteria *Staphylococcus
aureus*, *Staphylococcus epidermidis* (MRSA) and Gram-negative
bacteria *Escherichia coli* as well as antiviral activity against ORF virus
reduced titer from 5.9 to 1. Detection of B-lactam antibiotic resistance
bacteria was done by PCR with primers mec A and Bela genes. The
results proved that the essential oil can be used in the treatment
of diseases caused by the drug resistant microorganism. But, still
need further extensive researches are required to prove safety, drugs
interaction and clinical studies are required to prove the safety of the
oil as a medicine.

**Authors' Contributions**

Gamil S.G. Zeedan and Abeer M. Abdalhamed authors’: conception of
the research idea, study design, data collection, main part of
laboratory work and interpret the data and reviewed the manuscript;
Mohmoud E. Ottai and Sobhy Abdelshafy authors’: study design,
collection of plant from Sinai, Egypt, Identification of plant with group
of Genetics and Breeding of Medicinal and Aromatic plants, data
analysis of GC-MS and drafting the manuscript; Eman Abdeen author:
study design and part of laboratory work. All authors have read and
approved the final revised manuscript

**Conflict of Interests**

The authors do not have any conflict of interests regarding the content of the paper.

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