Evaluation of Antibacterial Activity and Phytochemical Screening of *Pimpinella anisem*'s Essential Oil

Azadeh Foroughi¹, Pouya Pournaghi², Fariba Najafi³, Akram Zangeneh¹,³, Mohammad Mahdi Zangeneh¹,³, Rohallah Moradi³,⁴.

¹Microbiology section, Pathobiology & Basic sciences department, Veterinary faculty, Razi University, Kermanshah, Iran.
²Department of Biology, Payame Noor University, Iran.
³Department of Dermatology, School of Medicine, Kermanshah University of Medical Science, Kermanshah, Iran.
⁴Department of Chemistry, Kermanshah Center of Payame Noor University, Kermanshah, Iran.

ABSTRACT
Medicinal plants are considered new resources for producing agents that could act as alternatives to antibiotics in treatment of antibiotic-resistant bacteria. The aim of the study was to evaluate the chemical composition and antibacterial effect of essential oil of *Pimpinella anisum* against *Pseudomonas aeruginosa* and *Bacillus subtilis*. Gas chromatography mass spectrometry was done to specify chemical composition. As a screen test to detect antibacterial properties of the essential oil, agar disk and agar well diffusion methods were employed. Macrobrot tube test was performed to determinate MIC. The results indicated that the most substance found in *P. anisum* essential oil was Trans-anethole (89.7 %), also the essential oil of *P. anisum* in 0.003 and 0.007 g/ml concentrations has prevented from the growth of the *P. aeruginosa* and *B. subtilis*, respectively. Thus, the research represents the antibacterial effects of the ethnomedical herb against both of bacteria. The results indicate the fact that the essential oil from the plant can be useful as medicinal or preservatives composition. Additional in vivo studies and clinical trials would be needed to justify. Also, further evaluation is necessary on potential of it as an antibacterial agent in topical or oral applications. Fractionation and characterization of active molecules will be the future work to investigate.

Keywords: *Pimpinella anisum*, Essential oil, Chemical composition, Antibacterial effect.

INTRODUCTION
Antibiotics provide the primary basis for the treatment of microbial (bacterial and fungal) infections. Since the detection of these antibiotics and their use as chemotherapeutic agents, there was a belief in the medical fraternity that this would cause to the presumptive eradication of infectious diseases. But overuse of antibiotics has become the main factor for the emergence and dissemination of multi-drug resistant strains of different groups of microorganisms¹. Nowadays, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in treatment of infectious diseases². Herbs and spices are invaluable resources useful in daily life as food additives, flavors, fragrances, pharmaceuticals, colors or directly in medicine. These plants contain medicinal properties which make them potent to cure or prevent diseases³. Some medicinal plants used in traditional Iranian medicine are efficient in treating diverse ailments caused by bacterial and oxidative stress⁴. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant⁵. Phytochemicals such as vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes and minerals that have antimicrobial and antioxidant activity⁶. The specific function of many phytochemicals is still unclear; however, a considerable number of studies have shown that they are involved in the interaction of plants/pests/diseases. Phytochemical studies have attracted the attention of plant scientists due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry⁷. Essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. The main constituents of essential oils –mono- and sesquiterpenes including carbohydrates, phenols, alcohols, ethers, aldehydes and ketones are responsible for the biological activity of aromatic and medicinal plants as well as for their fragrance. Due to these properties, spices and herbs have been added to food since ancient time, not only as flavouring agents but also as preservatives⁸. Plants and their essential oils are potentially useful sources of antimicrobial compounds.

⁸Author for Correspondence: akramzangeneh@yahoo.com
Antimicrobial screening of plant essential oils and phytochemicals, then, represents a starting point for antimicrobial drug discovery. Essential oils are effective on a wide range of Gram-negative and positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* O157:H7. *P. anisum* (Anise, also called aniseed), a plant belonging to the Umbelliferae family, is one of the oldest medicinal plants. It is an annual grassy herb with 30–50 cm high, white flowers, and small green to yellow seeds, which grows in the Eastern Mediterranean Region, West Asia, the Middle East, Mexico, Egypt, and Spain. This plant is primarily grown for its fruits (aniseeds) that harvested in August and September. Its flavor has similarities with some other spices, such as star anise, fennel, and licorice. It was reported that *P. anisum* had several therapeutic effects such as neurologic, digestive, gynecologic, fungal disease, and respiration disorders. The most substance found in *P. anisum* essential oil is Trans-anethole. The aim of this study was to screen the in vitro antibacterial activity of the plant essential oil against some bacteria including *P. aeruginosa* and *B. subtilis*.

**MATERIALS AND METHODS**

**Plant sample collection**

In the empirical-experimental study, medicine plant collected from Kermanshah. The sample was cleaned from any strange, plants, dust, or any other contaminants.

**Essential oil extraction**

Essential oil from fresh, clean, weighed aerial part *P. anisum* fruits extracted by hydro-steam distillation using the Clevenger apparatus were collected and stored in sterile vials. Briefly, 100 to 150 g of plant was introduced in the distillation flask (1L), which was connected to a steam generator via a glass tube and to a condenser to retrieve the oil. This was recovered in a funnel through a cooling system in order to condense the steam. The hot steam forced the plant material to release the aromatic molecules of the essential oil were released from the plant material and evaporated into hot steam. Then, steam containing the essential oil was yielded in 1g/ml from which six dilutions of the essential oil were done using dimethyl sulfoxide (DMSO).

**Gas chromatography mass spectrometry (GC/MS)**

*P. anisum* essential oil was analysed using GC/MS (Shimadzu capillary GC-quadruple MS system QP 5000) with two fused silica capillary column DB-5 (30 μm, 0.25 mm i.d. film thickness 0.25 μm) and a flame ionization detector (FID) which was operated in EI mode at 70 eV. Injector and detector temperatures were set at 220°C and 250°C, respectively. One microliter of each solution in hexane was injected and analyzed with the column held initially at 60°C for 2 min and then increased by 3°C/min up to 300°C. Helium was employed as carrier gas (1 ml/min). The relative amount of individual components of the total essential oil is expressed as percentage peak area relative to total peak area. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds and mass spectra.

**RESULTS**

**Chemical composition**

Composition of the plant in RI (Retention Index) including α-pinene (0.1%), sabine (0.01%), myrcene (0.01), α-phellandrene (0.01%), p-cymene (0.1%), limonene (0.8%), 1,8-cineole (0.1%), cis-b-ocimene (0.01%), fenchone (4.62%), camphor (0.23%), methyl

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chavicol (2.15%), endo-fenchyl acetate (0.1%), cis-anethole (0.43%), p-anisaldehyde (0.41%), trans-anethole (89.7%), respectively. The results indicate that the most substance found in P. anisum essential oil is trans-anethole, in contrast, sabine, myrcene, α-phellandrene, and cis-b-ocimene are the least constituents discovered in the essential oil.

**Agar disk diffusion test**

P. aeruginosa and B. subtilis were sensitive to P. anisum essential oil. In case of P. anisum, the most sensitive bacterium was P. aeruginosa and B. subtilis by developing the halo around which in 19 mm in diameter in dilution 0.031 g/ml. There was inhibition zone in P. aeruginosa due to dilution 0.002 g/ml whereas there wasn’t inhibition zone in B. subtilis. No inhibition zone was observed due to DMSO. Growth inhibition zones due to different dilutions are listed in table 1.

**Agar well diffusion test**

In regard to P. anisum essential oil, the widest zone was seen in 0.031 g/ml due to P. aeruginosa and B. subtilis (16 mm). It was no growth inhibition in 0.001 g/ml and less for both of bacteria. The data are discoverable in table 2.

**MIC and MBC ascertaining**

The values of MIC are 0.007 g/ml and 0.003 for B. subtilis and P. aeruginosa, respectively. But the values of MBC are same for B. subtilis and P. aeruginosa and they are 0.015 g/ml (Table 3). As the table showed, P. anisum essential oil have prevented the growth of P. aeruginosa and B. subtilis. Also, by increasing the concentration of P. anisum essential oil, the inhibition zone increased (p<0.001). The results determined that in tested bacteria, there was a significant difference (p<0.001) in terms of sensitivity to the essential oil.

**DISCUSSION**

In spite of the current interest in drug discovery by molecular modelling, combinatorial chemistry and other synthetic chemistry methods, plant-derived compounds are still substantiating to be an important source of medicines for human being. The significance and uses of plants in modern drug discovery has been recounted in recent reports. Plant essential oils have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. Essential oils are potential sources of novel antimicrobial compounds, especially against bacterial pathogens. In vitro studies in the work showed that the essential oils inhibited bacterial growth but their effectiveness varied. The antimicrobial activities of many essential oils have been previously reviewed and classified as strong, medium or weak. P. anisum is an annual important spice and medicinal plant belonging to the family Apiaceae and is considered as a natural raw material and used for pharmaceutics, perfumery, food and cosmetic industries. P. anisum is primarily grown for its fruits (aniseeds) that harvested in August and September. Aniseeds contain 1.5–5% essential oil and used as flavouring, digestive, carminative, and relief of gastrointestinal spasms. Consumption of aniseed in lactating women increases milk and also relieves their infants from gastrointestinal problems P. anisum is one of the medicinal plants which have been used for different purposes in traditional medicine of Iran. So far, different studies were performed on the extracts and essential oil of P. anisum to identify the chemical compounds and pharmacological properties of the plant, and various properties such as antimicrobial, antifungal, and antibacterial. The medicinal use of aniseed is largely due to antispasmodic, secretolytic, secretomotor and antibacterial effects of its essential oil. P. anisum extracts and oil as well as some oil components, exhibited in vitro strong inhibitory activities against the growth of a wide spectrum of bacteria and fungi known to be pathogenic for man and other species. Concerning the method of essential oils and preventing from using high temperature to decrease the rate of destruction of effective herbal compound. 15 compounds representing 98.78% of the total essential oil composition of P. anisum were identified using mass gas-chromatograph, these compounds including α-pinene, sabine, myrcene, α-phellandrene, p-cymene, limonene, 1,8-cineole, cis-b-ocimene, fenchone, camphor, methyl chavicol, endofenchyl acetate, cis-anethole, p-anisaldehyde and trans-anethole. The most substance found in P. anisum essential oil was trans-anethole with 89.7 %. Trans-anethole is an alkyl alkyl-phenolether. Both the cis and trans isomers of trans-anethole occur in nature with the trans isomer always being the more abundant. Natural anethole occurs in P. anisum essential oils and in star P. anisum essential oils. It has been shown to block grow of inflammation and carcinogenesis. Anethole has potent antimicrobial properties, against bacteria, yeast, and fungi. Reported antibacterial properties include both bacteriostatic and bactericidal action against Salmonella enterica, but not when used against Salmonella via a fumigation method. Findings from the current study revealed that essential oil of P. anisum has potential inhibitory effects on P. aeruginosa and B. subtilis. In agar disk diffusion test, the maximum activity of P. anisum essential oil against P. aeruginosa and B. subtilis was 19 mm, which is comparable with a zone of inhibition exhibited by kanamycin (22 mm) and cephalxin (16 mm). Also this results indicated that P. anisum essential oil in 0.003 and 0.007 g/ml concentrations has prevented from the growth of the P. aeruginosa and B. subtilis, respectively. In the study, the levels of MBC were same in both of bacteria. Thus, the research represents the antibacterial effects of the medical herb on Gram-negative and Gram-positive pathogenic bacteria. A number of authors have mentioned the antimicrobial activity of P. anisum. The antibacterial activities of the aqueous, 50% (v/v) methanol, acetone and petroleum ether extracts of P. anisum fruits were tested against 4 pathogenic bacteria (Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, and Klebsiella pneumoniae) by disk diffusion method. The results showed that only aqueous and methanol extracts exhibited fair antibacterial activity against all of the test bacteria and the aqueous extract was found to be more
sed especially against multiple bacterial activity. However, bacterial activity was shown to reduce growth of both *P. aeruginosa* and *B. subtilis* were inhibited by the essential oil tested, the results indicate that the essential oil has its own chemical composition, which may be correlated with its antibacterial activity. The essential oil of the plant displayed antibacterial properties, and its activities could be attributed to qualitative and quantitative differences in the chemical constituents of the individual essential oil. It can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agent.

**ACKNOWLEDGMENT**

We, the authors wish to thank Medical Sciences University of Kermanshah, Iran for the financial support of the work. The core idea of this work came from Mohammad Mahdi Zangeneh and Akram Zangeneh, also the experiments, evaluation and Statistical Analysis of antimicrobial activities done by Azadeh Foroughi, Pouya Pournaghi, Fariba Najafi, Akram Zangeneh, Mohammad Mahdi Zangeneh, Rohollah Moradi.

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**Table 1:** The diameters of growth inhibition zones in agar plate diffusion test in different dilutions of *P. anisum* essential oil.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition zone in agar plate diffusion (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>22</td>
</tr>
<tr>
<td>1/32 (0.031)</td>
<td>19</td>
</tr>
<tr>
<td>1/64 (0.015)</td>
<td>19</td>
</tr>
<tr>
<td>1/128 (0.007)</td>
<td>12</td>
</tr>
<tr>
<td>1/256 (0.003)</td>
<td>9</td>
</tr>
<tr>
<td>1/512 (0.002)</td>
<td>8</td>
</tr>
<tr>
<td>1/1024 (0.001)</td>
<td>0</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2:** The diameters of growth inhibition zones in agar well diffusion test in different dilutions of *P. anisum* essential oil.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition zone in agar well diffusion (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>16</td>
</tr>
<tr>
<td>1/32 (0.031)</td>
<td>11</td>
</tr>
<tr>
<td>1/64 (0.015)</td>
<td>8</td>
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<tr>
<td>1/128 (0.007)</td>
<td>8</td>
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<tr>
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<tr>
<td>1/512 (0.002)</td>
<td>0</td>
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<tr>
<td>1/1024 (0.001)</td>
<td>0</td>
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<tr>
<td>Negative control</td>
<td>0</td>
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**Table 3:** MIC and MBC of essential oil of *P. anisum*.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (Dilution g/ml)</th>
<th>MBC (Dilution g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1/256 (0.003)</td>
<td>1/128 (0.007)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1/64 (0.015)</td>
<td>1/64 (0.015)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
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</tr>
</tbody>
</table>

**effective** than methanolic extract, whereas acetone and petroleum ether extracts cannot inhibit the growth of the pathogenic test bacteria. Antimicrobial activity of both water and ethanol extracts of *P. anisum* fructus was tested against *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Citrobacter koseri, Staphylococcus aureus, Streptococcus Pneumoniae, Enterobacter aerogenes, Micrococcus luteus, Staphylococcus Epidermidis and Candida albicans*. Most microorganisms were inhibited, but no not activity of the water *P. anisum* fructus extract was detected against *Pseudomonas aeruginosa and Escherichia coli*. As far as the antibacterial activity of isolated compounds, estragole inhibited all the tested strains; limonene showed an inhibitory activity on *C. jejuni* and *L. monocytogenes* and trans-anethole only inhibited *C. jejuni*. Bactericidal activities of a number of plant essential oils, including the *P. anisum* fruit one, and of their isolated constituents were tested against *Campylobacter jejuni, Listeria monocytogenes* and *Salmonella enterica*. *P. anisum* oil was shown to reduce bacterial activity of all tested bacteria. Bactericidal activities of a number of plant (such as *P. anisum*) essential oils and methanol extracts measured. In this study, Essential oil and methanol extract of these plants exhibited antibacterial activity against most tested pathogens, and the maximum effect was observed against *Bacillus cereus, and Proteus vulgaris*. However, combination of essential oil and methanol extracts of these plants showed an additive effect against most tested bacteria. Finally, our results are in agreement with others who showed that essential oil of *P. anisum* oils produce antimicrobial activity against a broad range of microbes and especially against multiple-antibiotic resistant bacteria. *P. anisum* is an aromatic medicinal plant with antibacterial activities toward *P. aeruginosa* (PTCC No. 1707) and *B. subtilis* (ATCC No. 21332). The growth of both *P. aeruginosa* and *B. subtilis* were inhibited by the essential oil tested, the results indicate that the essential oil has its own chemical composition, which may be correlated with its antibacterial activity. The essential oil of the plant displayed antibacterial properties, and its activities could be attributed to qualitative and quantitative differences in the chemical constituents of the individual essential oil. It can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agent.
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