Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(11); 1886-1890

ISSN: 0975-4873

Research Article

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

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Available Online: 15th November, 2016

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 composion. As a screen test to detect antibacterial properties of the essential oil, agar disk and agar well diffusion methods were employed. Macrobroth 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 microorganisms[1]. Nowadays, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in treatment of infectious diseases[2]. 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 diseases[3]. Some medicinal plants used in traditional Iranian medicine are efficient in treating diverse ailments caused by bacterial and oxidative stress[4]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant[5]. 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 activity[6]. 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 industry[7]. 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 preservatives[8]. Plants and their essential oils are potentially useful sources of antimicrobial compounds.

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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[9]. *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[10]. 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 tube. Aromatic molecules of the essential oil were released from the plant material and evaporated into hot steam. The hot steam forced the plant material to release the essential oil without burning the plant material itself. Then, steam containing the essential oil was passed through a cooling system in order to condense the steam. The steam was applied for 3h. After settling the recovered mixture, essential oil was withdrawn. The supernatant essential oil was filtered through anhydrous Na_2SO_4 to dry the yielded essential oil. Afterward, the essential oil was collected in tightened vials and stored in a refrigerator. For the antimicrobial activity test, several 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-quadrupole 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.

Source of microorganisms

Tow bacterial species namely *P. aeruginosa* (PTCC No. 1707) and *B. subtilis* (ATCC No. 21332) were procured from Iranian Research Organization for Science and Technology as lyophilized. Each bacterial strain was activated on Tryptic Soy broth, constant at 37°C for 18 h. Then 60 μl of the broth was transferred to Nutrient agar and incubated at 37°C for another 24 h; cell concentration was then adjusted to obtain final concentration of 10^8 cfu/ml using Muller Hinton broth.

Culture media

Mueller-Hinton Agar (Müller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing) was prepared according to the manufacturer’s instruction (Oxoid, UK), autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes. Set plates were incubated overnight to ensure sterility before use.

Evaluation of antimicrobial activities

Agar disk diffusion and agar well diffusion were used as screen tests to evaluate antibacterial property of essential oil of *P. anisum* based on standard protocol. The solution of the essential oil was yielded in 1g/ml from which six fold serial dilutions (v/v) were prepared. 60 μl of each dilution was poured on each disk and well in order. After a period of 24 hours’ incubation, the diameters of growth inhibition zones around the disks and wells were measured. DMSO was used as negative control whereas kanamycin and cephalexin were used as positive controls in case of *E. coli* and *S. aureus*, respectively. Minimum inhibitory concentration (MIC) means the lowest concentration of the probable antimicrobial agent which prevents growing of bacteria (regardless of killing the bacteria or stopping the growth of them). The lowest dilution which no gross microbial growth has been seen indicates MIC. Minimum bactericidal concentration (MBC) means the lowest concentration of the agent which causes death to test bacteria. The last can be revealed by pouring 60 μl of MIC tube and six dilutions before contents on agar plate. In the case, after incubation period, the lowest concentration which makes no growth indicates MBC. For determination of MIC value, macrobroth dilution method was applied. Interpretation of the results was done due to national accepted letter[11].

RESULTS

Chemical composition

Composition of the plant in RI (Retention Index) including α-pinene (0.1%), sabinene (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 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, *sabinene, 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 ($p \leq 0.001$). The results determined that in tested bacteria, there was a significant difference ($p \leq 0.001$) 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[12,13]. Plant essential oils have been used for many thousands of years[14], in food preservation, pharmaceuticals, alternative medicine and natural therapies[15,16]. Essential oils are potential sources of novel antimicrobial compounds, especially against bacterial pathogens[17]. *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 has been previously reviewed and classified as strong, medium or weak[18]. *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[19]. *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 reliefs 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[10]. 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, sabinene, 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[20,21]. Reported antibacterial properties include both bacteriostatic and bactericidal action against Salmonella enterica[22], but not when used against Salmonella via a fumigation method[23]. 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 cephalexin (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, Escherchia 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

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

Dilution(g/ml)	Inhibition zone in disk diffusion (mm)	
Microorganism	*P. aeruginosa*	*B. subtilis*
Positive control	22	22
1/32 (0.031)	19	19
1/64 (0.015)	19	17
1/128 (0.007)	12	12
1/256 (0.003)	9	8
1/512 (0.002)	8	0
1/1024 (0.001)	0	0
Negative control	0	0

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

Dilution(g/ml)	Inhibition zone in well diffusion (mm)	
Microorganism	*P. aeruginosa*	*B. subtilis*
1/32 (0.031)	16	16
1/64 (0.015)	11	11
1/128 (0.007)	9	8
1/256 (0.003)	8	8
1/512 (0.002)	8	0
1/1024 (0.001)	0	0
Negative control	0	0

Table 3: MIC and MBC of essential oil of *P. anisum*.

Microorganism	*P. aeruginosa*	*B. subtilis*
MIC	1/256(0.003)	1/128 (0.007)
MBC	1/64 (0.015)	1/64 (0.015)

effective than methanolic extract, whereas acetone and petroleum ether extracts cannot inhibit the growth of the pathogenic test bacteria[24]. 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, Streprococcus 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*[25]. As far as the antibacterial activity of isolated compounds, estragole inhibited all the tested strains; limonene showed an inhibitory activity only on *C. jejuni* and *L. monocytogenes* and trans-anethole only inhibited *C. jejuni*[26]. 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[27]. 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.

AKNOWLEDGMENT

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, Rohallah Moradi.

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