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Research Article

Antibacterial Effect and Phytochemical Screening of Essential Oil of *Pimpinella anisum* Against *Escherichia coli O157:H7* and *Staphylococcus aureus*

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ABSTRACT
Considering the drug resistance and the side effects of chemical antibacterial drugs, the research approach is increasingly going toward using natural resources. Certainly, using herbal plants is the oldest way of mankind to treat bacterial diseases. The aim of the study was to evaluate the chemical composition and antibacterial activities of essential oil of *Pimpinella anisum* against *Escherichia coli O157:H7* and *Staphylococcus aureus*. 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 *E. coli* and *S. aureus*, respectively. Thus, the research represents the antibacterial effects of the medical herb on test *E. coli* and *S. aureus*. We believe that this article provide support to the antibacterial activities of the essential oil. The results indicate the fact that the essential oil from the plant can be useful as medicinal or preservatives composition. Fractionation and characterization of active molecules will be the future work to investigate.

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

INTRODUCTION

Bacterial diseases are responsible for many deaths each year. Antibiotics are a type of antimicrobial drug used in the treatment and prevention of bacterial infections. They may either kill or inhibit the growth of bacteria. However, only one third of the infectious diseases known have been treated from these synthetic products. Overuse of antibiotics has become the main factor for the emergence and dissemination of multi-drug resistant strains of different groups of microorganisms[1]. The spread of drug resistant pathogens is one of the most serious threats to successful therapy of microbial diseases. Down the ages plants have evoked interest as sources of innate products. According to the World Health Organization plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. They have been screened for their potential uses as alternative remedies for the treatment of several infectious diseases. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens[2]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant[3]. Phytochemicals such as vitamins, carotenoids, terpenoids, flavonoids, alkaloids, tannins, saponins, enzymes and minerals that have antimicrobial activity[4]. Phytochemical studies have attracted the attention of plant scientists due to the development of sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry[5]. 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 medicinal plants as well as for their fragrance[6]. Essential oils are potentially useful sources of antimicrobial compounds. 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[7]. *P. anisum* (Anise,

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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 which grows in the Eastern Mediterranean Region, West Asia and the Middle East[8,9]. This plant is primarily grown for aniseed that harvested in August and September. It was reported that *P. anisum* had several therapeutic effects such as neurologic, digestive, gynecologic, microbial 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 *E. coli O157:H7* and *S. aureus.*

MATERIAL 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 *E. coli O157:H7* (ATCC No. 25922) and *S. aureus* (ATCC No. 25923) 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].

Statistical Analysis

Antibacterial effect was determined by One way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p \leq 0.05$.

RESULTS

Chemical composition

The most substance found in *P. anisum* essential oil was Trans-anethole. In contrast, *sabinene, myrcene, α-Phellandrene,* and *cis-b-ocimene* were the least constituents discovered in the essential oil. Composition of the plant using Gas chromatography mass spectrometry method can be perceived in table 1.

Agar disk diffusion test

In case of *P. anisum*, the inhibitoriest zone of *E. coli* and *S. aureus* was 22 mm in diameter in dilution 0.031 g/ml. Growth inhibition zones due to different dilutions are listed in table 2. No inhibition zone was observed due to DMSO.

Agar well diffusion test

In regard to *P. anisum* essential oil, the widest zone was seen in 0.031 g/ml, due to *E. coli* (15 mm). It was no growth inhibition in 0.001 g/ml and less for both of bacteria. The data are discoverable in table 3.

MIC and *MBC ascertaining*

The most and the least values for MIC were acquired in 0.007 g/ml for *S. aureus* and 0.003 g/ml for *E. coli* (Table 4). The values of MBC of *E. coli* and *S. aureus* are 0.003 g/ml and 0.007 g/ml, respectively (Table 4). As the table showed, *P. anisum* essential oil have prevented the growth of *E. coli* and *S.aureus*. 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

Plants as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times. Most of modern clinical drugs are of natural product origin[2]. Medicinal plants may have the ability to treat bacterial resistance to many types of antibiotics. The search for such compounds which can be combined with antibiotics in the treatment of drug resistant infections may be an alternative to overcoming the problem of resistance in bacteria. Essential oils of medicinal plants stand out as veritable sources of potential resistance modifying agents. Essential oils 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 properties[12,13]. *P. anisum* is a well-known annual herb with white flowers and small green to yellow seeds which grows in India, Egypt, Turkey, Iran and many other warm areas of the world[8,9]. *P. anisum* is an annual important spice and is considered as a natural raw material and used for pharmaceutics, perfumery, food and cosmetic industries[14]. 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 this plant, and various properties such as antimicrobial, antifungal, and antibacterial. *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 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, there is a partial difference between these results and the similar studies. 15 compounds representing 98.78% of the total essential oil composition of *P. anisum* were identified using mass gas-chromatograph, this compound 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[15,16]. Reported antibacterial properties include both bacteriostatic and bactericidal action against Salmonella enterica[17], but not when used against Salmonella via a fumigation method[18]. In contrast, *sabinene* (0.01)*/ myrcene* (0.01)*/ α-Phellandrene* (0.01)*/ cis-b-ocimene* (0.01) were the least constituents discovered in *P. anisum* essential oil. Findings from the current study revealed that essential oil of *P. anisum* has potential inhibitory effects on *E. coli* and *S. aureus*. In agar disk diffusion test, the maximum activity of *P. anisum* essential oil against *E. coli* and *S. aureus* was 22 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 *E. coli* and *S. aureus*, respectively. In the study, the levels of MBC were observed ranges from 0.003 and 0.015 for *P. anisum*. Thus, the research represents the antibacterial effects of the medical herb on *E. coli* and *S. aureus*. A number of authors have mentioned the antimicrobial activity of *P. anisum*. 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*[19]. 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 effective than methanolic extract, whereas acetone and petroleum ether extracts cannot inhibit the growth of the pathogenic test bacteria[20]. 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

Table 1: Identified main composition of the *P. anisum* essential oil using Gas chromatography mass spectrometry method.

Compounds	RI	MS%
α-Pinene	927	0.1
sabinene	964	0.01
myrcene	983	0.01
α-Phellandrene	998	0.01
p-cymene	1018	0.1
limonene	1023	0.8
1,8-cineole	1026	0.1
cis-b-ocimene	1029	0.01
fenchone	1083	4.62
camphor	1138	0.23
methyl chavicol	1192	2.15
endo-fenchyl acetate	1224	0.1
cis-anethole	1247	0.43
p-anisaldehyde	1253	0.41
trans-anethole	1294	89.7
Total	-	98.78

Table 2: 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	*E. coli*	*S. aureus*
Positive control	22	16
1/32 (0.031)	22	22
1/64 (0.015)	17	17
1/128 (0.007)	12	13
1/256 (0.003)	8	8
1/512 (0.002)	0	0
1/1024 (0.001)	0	0
Negative control	0	0

Table 3: 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	*S. aureus*	*E. coli*
1/32 (0.031)	15	13
1/64 (0.015)	8	10
1/128 (0.007)	8	8
1/256 (0.003)	8	8
1/512 (0.002)	0	0
1/1024 (0.001)	0	0
Negative control	0	0

Table 4: The MIC and MBC of essential oil of *P. anisum*.

Microorganism	*E. coli*	*S. aureus*
MIC (*P. anisum*)	1/256 (0.003)	1/128 (0.007)
MBC (P. anisum)	1/256 (0.003)	1/128 (0.007)

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[21]. 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 activity toward *E. coli* and *S. aureus.* The growth of *E. coli* and *S. aureus* were inhibited by the essential oil tested. These results indicate that each essential oil has its own chemical composition, which may be correlated with its antibacterial properties. *P. anisum* can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agent. 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.

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