Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(11): 1459-1463

ISSN- 0975 1556

Research Article

Assessment of Chemical Composition and Antibacterial Effects of Anethole-Rich Hydroalcoholic Extract of *Pimpinella anisum*

Azadeh Foroughi[1], Pouya Pournaghi[2], Reza Tahvilian[3], Mohammad Mahdi Zangeneh[1,3], Akram Zangeneh[1,3*], Rohallah Moradi[3,4]

[1]*Microbiology section, Pathobiology & Basic sciences department, Veterinary faculty, Razi University, Kermanshah, Iran.*
[2]*Department of Biology, Payame Noor University, Kermanshah, Iran.*
[3]*Research Pharmaceutical Center, School of Pharmacy, Kermanshah University of Medical Science, Kermanshah, Iran.*
[4]*Department of Chemistry, Kermanshah Center of Payame Noor University, Kermanshah, Iran.*

Available Online: 15th November, 2016

ABSTRACT
Increasing microbial resistance to chemical antibiotics and their probabilistic side effects cause popularity of medicinal plants, so there is an instantaneous and steady need for novel antimicrobial compounds from plants. As we know, there is no documented proof on antibacterial effects of *Pimpinella anisum* (PA) hydroalcoholic extract in west of Iran. Gas chromatography mass spectrometry was done to determine chemical composion. As a screen test to discover antibacterial properties of the extract, agar disk and agar well diffusion methods were employed. Macrobroth tube test was performed to specify MIC. The findings show that the most substance found in PA was anethole. The results indicated the MIC and MBC values was 0.031 g/ml for PA except in case of *B. subtilis* which was 0.062 g/ml. Thus, the present research demonstrates the antibacterial effects of the medical plant on *E. coli*, *S. aureus* and *B. subtilis*, suggesting to use as antibacterial supplement in the developing countries towards the development of new therapeutic agent.

Keywords: *Pimpinella anisum*, Hydroalcoholic extract, 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]. 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. They have been screened for their potential uses as alternative remedies for the treatment of several infectious diseases[2]. Some medicinal plants used in traditional Iranian medicine are efficient in treating diverse ailments caused by bacterial and oxidative stress[3]. There is growing concern in correlating phytochemical constituents of plant with its pharmacological activity. In herbal medicines, raw plant extracts in the form of infusion, decoction, tincture or herbal extract are traditionally consumed by the population for the treatment of diseases including infectious diseases. Plant-derived products have a major variety of phytochemicals such as phenolic acids, flavonoids, tannins, lignin, and other small compounds.

The antibacterial properties of extracts have been identified for many years, and their rudiment have found applications as naturally occurring antimicrobial agents in the field of pharmacology, pharmaceutical botany, phytopathology, medical and clinical microbiology, food maintenance, etc. There are reports of the active principles of extracts from different plants with antifungal or antibacterial effect. Herbal extracts have antimicrobial activity on a wide number of bacteria, and most of these compounds have phenolic groups in their structure. The original benefit of natural factors is that they do not increase the "antibiotic resistance", an event usually encountered with the long-term use of synthetic antibiotics; because they have a significant role in the defense system of the plant to microbial diseases due to their intrinsic anti-oxidative and anti-microbial properties[4]. The compounds of plant extracts contain numerous health-related effects such as antibacterial, antimutagenic, anticarcinogenic antithrombotic and vasodilatory activities[5]. PA (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

**Author for Correspondence akramzangeneh@yahoo.com:*

harvested in August and September. Its flavor has similarities with some other spices, such as star anise, fennel, and licorice[6,7]. It was reported that PA had different therapeutic effects such as neurologic, digestive, gynecologic and respiration disorders. Moreover, it was demonstrated that Anise showed ovicidal activity to stored-product insects[8]. The chemical constituents of aniseed (PA seeds) extract (picked in kermanshah) analyzed by GC/MS. The main compound was anethole. The aim of this study was to screen the in vitro antibacterial activity of the plant hydroalcoholic extract against some bacteria including *E. coli, S. aureus, and B. subtilis.*

MATERIALS AND METHODS

Plant sample collection

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

Preparation of hydroalcoholic extract

Successive solvent extraction was performed for aniseed. Seeds were washed, air dried for 7-8 days, and ground into powder before they were placed into the flask of the Soxhlet apparatus for extraction using 70% ethanol with increasing order of polarity to extract the phytoconstituents separately at 20ºC for 3-4 h (The ethanol used was HPLC grade obtained from Sigma-Aldrich, Germany). Whitman filter papers No.1 were then applied to filter the extract. After that, reduced pressure was applied to evaporate and dry the filtrates which were stored at -20ºC in labeled, sterile, screw capped bottles.

Gas chromatography mass spectrometry (GC/MS)

PA hydroalcoholic extract was analyzed using GC/MS (GC 7890N, AGILENT and MS 5975C, MODE EI) with two fused silica capillary column HP-5MS (30 m, 5 mm l.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 perfused 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 used as carrier gas (1 ml/min). The relative amount of individual components of the total extract is expressed as percentage peak area relative to total peak area. Qualitative reconnaissance of the several constituents was accomplished by comparison of their relative retention times and mass spectra with those of authentic reference compounds and mass spectra.

Source of microorganisms

Three bacterial species namely *Escherchia coli O157:H7* (ATCC No. 25922), *Staphylococcus aureus* (ATCC No. 25923) and *Bacillus subtilis* (ATCC No. 21332) were procured from Veterinary school of Tehran University 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 and agar well diffusion were used as screen tests to evaluate antibacterial property of PA based on standard protocol. The solution of the PA 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 in order. After a period of 24 hours incubation, the diameters of growth inhibition zones around the disks were measured. Distillated water was used as negative control whereas kanamycin and cephalexin were used as positive controls in case of *E. coli* and *S. aureus*/*B. subtilis*, 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 this 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[9].

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 PA was anethole. In contrast, *p*-anisaldehyde was the least constituent discovered in PA. Composition of the plant using Gas chromatography mass spectrometry method can be perceived in table 1.

Agar disk diffusion test

About PA, the most sensitive bacterium was *E. coli* by developing the halo around which in 10 mm in diameter in dilution 0.25 g/ml. There was no inhibition zone in *S. aureus* due to dilution 0.031 g/ml whereas the other two bacteria showed sensitivity in this amount. Growth inhibition zones due to different dilutions are listed in table 2. No inhibition zone was observed due to distillated water.

Agar well diffusion test

In regard to PA, the widest zone was seen in 0.031 g/ml, due to *E. coli* (19 mm). It was no growth inhibition in 0.002 g/ml and less for all bacteria. The data are discoverable in table 3.

MIC determination

The least and the most values for MIC were acquired in 0.031 g/ml for *E. coli*/*S. aureus* and 0.062 g/ml for *B. subtilis* in occasion of PA (Table 4).

MBC ascertaining

The values of MBC are 0.031 g/ml for *E. coli* and *S. aureus* but 0.062 g/ml for *B. subtilis* in PA (Table 5). As the tables showed, PA have prevented the growth of *E. coli*, *S. aureus* and *B. Subtilis*. Also, by increasing the concentration of PA, 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 PA. In other words, the most sensitivity was observed in *E. coli*.

DISCUSSION

The type and level of biological effect exhibited by any plant material depends on different factors, including the plant part, geographical source, soil conditions, harvest time, humidity content, drying method, storage conditions, and post-harvest processing. For example, the relatively high temperatures that can be generated during tissue grinding can denature chemical constituents and the extraction solvent, time period, and temperature can touch the level and composition of secondary metabolites extracted from plant tissues. Because of their safety and low cost as well as their impact on a wide number of microbes, medicinal plants may have the potency to treat bacterial resistance to different types of antibiotics[10]. The antimicrobial effects of plant extracts from a vast number of plants have been appraised and reviewed[11,12], and the mechanisms that enable the natural components of herbs and spices to resist microbes have been discussed[13]. The results show that these mechanisms vary greatly depending on the components of the plant[14,15]. Since the antibacterial effectiveness of medicinal plants destabilized significantly depending on the phytochemical characteristics of plan families and subfamilies, it is not surprising to note the difference in this efficacy even when using samples taken from the similar plant, but from two various regions[16]. *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[6,7]. The medicinal use of aniseed is largely due to antispasmodic, secretolytic, secretomotor and antibacterial effects of its plant[8].

Yield and analysis of PA.

In this study, Presence of anethole, *cis*-pseudoisoeugenyl 2-methylbutyrate, γ-himachalene, methylchavicol, *trans*-pseudoisoeugenyl 2-methylbutyrate, and *p*-anisaldehyde were identified in the composition of the obtained PA using mass gas-chromatograph. The most substances found in PA was anethole (90%). In contrast, *p*-anisaldehyde (1.0%) was the least constituent discovered in PA. Anethole ((*E*)-1-methoxy-4-(1-propenyl) benzene) is an organic compound that is widely used as a flavoring substance. It is a derivative of phenylpropene, a type of aromatic compound that occurs widely in nature, in essential oils and extracts. Also, anethole is a clear and colorless to pale-yellow liquid with freezing and boiling points of 20 °C and 234 °C, respectively[17]. Anethole has potent antimicrobial properties, against bacteria, yeast, and fungi[18,19]. Reported antibacterial properties include both bacteriostatic and bactericidal action against Salmonella enterica[20], but not when used against Salmonella via a fumigation method[21].

Antibacterial activity.

PA in 0.031 g/ml concentration has prevented from the growth of the bacteria. Thus, the research represents the antibacterial effects of the medical herb on *E. coli*, *S. aureus* and *B. Subtilis*. There are correspondences between this result and the similar studies. The antibacterial activities of the aqueous, 50% (v/v) methanol, acetone, and petroleum ether extract of PA fruits were studied by Akhtar et al against 4 pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, and *Klebsiella pneumoniae*). The results indicated that only aqueous and alcoholic extracts exhibited fair antibacterial activity to all of the test bacteria and the aqueous extract was found to be more efficient than methanolic extract, whereas acetone and petroleum ether extracts cannot prevent the growth of the pathogenic test bacteria[22]. In another study, synergic antibacterial effect between *Thymus vulgaris* and PA essential oil and methanol extract was evaluated against 9 pathogenic bacteria by Al-Bayati. Essential oil and methanol extract of these plants exhibited antibacterial effect against most tested pathogens, and the maximum activity was apperceived against *Bacillus cereus* and *Proteus vulgaris*[7]. Antimicrobial activity of both water and ethanol extracts of PA fructus was tested against *Pseudomonas aeruginosa, 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 PA fructus extract was detected against *Pseudomonas aeruginosa*[23]. The results indicated hydroalcoholic extract of PA possess antibacterial effect, and the antibacterial activity of the extract was due to the presence of various active compounds. Hence, the phytochemical compounds responsible for the antibacterial effects of bacteria can be subjected to isolation of the therapeutic antimicrobials. Our results defend the use of the plant in traditional medicine and offer that PA possess compounds with good antibacterial properties. It can be used as antibacterial supplements in the developing countries towards the development of new remedial agent. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of the plant as an antibacterial agent in topical or oral applications.

Table 1: Identified main composition of the PA using Gas chromatography mass spectrometry method.

Compound of PA	Percent
Anethole	90
cis-pseudoisoeugenyl 2-methylbutyrate	3
γ-himachalene	2
methylchavicol	1.5
trans-pseudoisoeugenyl 2-methylbutyrate	1.3
p-anisaldehyde	1
TOTAL	98.8

Table 2: The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of PA.

Dilution(g/ml)	Inhibition zone in disk diffusion (mm)		
Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
Positive control	22	16	22
1/4 (0.25)	10	9	9
1/8 (0.125)	9	8	9
1/16 (0.062)	8	8	8
1/32 (0.031)	8	0	8
1/64 (0.015)	8	0	8
1/128 (0.007)	0	0	0
Negative control	0	0	0

Table 3: The diameters of growth inhibition zones in agar well diffusion test in different dilutions of PA.

Dilution(g/ml)	Inhibition zone in well diffusion (mm)		
Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
1/4 (0.25)	19	17	14
1/8 (0.125)	15	8	9
1/16 (0.062)	10	8	9
1/32 (0.031)	9	0	8
1/64 (0.015)	8	0	8
1/128 (0.007)	8	0	0
Negative control	0	0	0

Table 4: MIC for the PA.

Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
MIC	1/32 (0.031)	1/32 (0.031)	1/16 (0.062)

Table 5. MBC for the PA.

Microorganism	*E. Coli*	*S. aureus*	*B. Subtilis*
MBC	1/32 (0.031)	1/32 (0.031)	1/16 (0.062)

AKNOWLEDGMENT

We, the authors wish to thank Medical Sciences University of Kermanshah, Iran for the financial support of this work.

REFERENCES

1. Punasiya R, Joshi A, Sainkediya K, Tirole S, Joshi P, Das A, Yadav R. Evaluation of Antibacterial Activity of Various Extract of Hibiscus syriacus. *Research Journal of Pharmacy and Technology*. 2011; 4(5): 819-822.
2. Hemalatha M, Arirudran B, Thenmozhi A, Mahadeva Rao US. Antimicrobial Effect of Separate Extract of Acetone, Ethyl Acetate, Methanol and Aqueous from Leaf of Milkweed (Calotropis gigantea L.). *Asian Journal of Pharmaceutical Research*. 2011; 1(4): 102-107.
3. Rangarajan N, Sathiyamoorthy M. Phytochemical Screening and Antioxidant Studies in the Pulp Extracts of Cucurbita maxima. *South Asian Journal of Engineering and Technology*. 2016; 2(24):131-140.
4. Patil SB, Lende MY, Thakur VS, Naikwade NS, Magdum CS, Chavan GM. Protective effect from UV rays by Medicinal flowers. Asian J. Res. Pharm. 2012; 2(1): 24-25.
5. Samydurai P, M. Saradha. Effects of Various Solvent on the Extraction of Antimicrobial, Antioxidant Phenolics from the Stem Bark of Decalepis hamiltonii Wight and Arn. Asian J. Res. Pharm. 2016; 6(2): 129-134.
6. Shokri A, Hatami T, Khamforoushb M: Mathematical and artificial neural network modeling, *Journal of Supercritical Fluids*, 2011; 58 (1): 49-57
7. Al-Bayati FA. Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts, *Journal of Ethnopharmacology*, 2008; 116(3): 403-6.
8. Tunc I, Berger BM, Erler F, Dagli F. Ovicidal activity of essential oils from five plants against two stored-products insects, *Journal of Stored Products Research*, 2000; 36: 161-168
9. 9 Clinical and laboratory standards institute (CLSI), M7-A7, 26(2), 2006.
10. Charde RM, Charde MS, Fulzele SV, Satturwar PM, Kasture AV, Joshi SB. Evaluation of Ethanolic Extract of Moringa Oleifera for Wound Healing, Anti-inflammatory and Antioxidant Activities on Rats. *Research Journal of Pharmacy and Technology*. 2011; 2(4): 254-258.
11. Abhijeet P, Jui V, Jagtap, Polshettiwar SA, Kuchekar BS. Formulation and Evaluation of Antibacterial and Antifungal Activity of Herbal Gel Containing Aloe vera, Azadirachta indica and Lycopersicon esculentum Seed Extract. *Research Journal of Pharmacy and Technology*. 2011; 4(4): 552-554.
12. Suresh M, Rath PK, Panneerselvam A, Dhanasekaran D, Thajuddin N. Anti-Mycobacterial Effect of Leaf Extract of Centella asiatica (Mackinlayaceae). *Research Journal of Pharmacy and Technology*. 2010; 3(3): 872-876.
13. Venkatachalam T, Kishor Kumar V, Satheesh Kumar P, Kalaiselvi P, Chitra M, Senthil Kumar N. In-vitro Anti Oxidant and Antimicrobial Activities of Ethyl Acetate Extract of Evodia lunu-Ankenda (Gaertn) Merr. Bark. Research J. Pharmacognosy and Phytochemistry. 2009; 1(3): 201-203.
14. Jafferi SAH, Sandhya S, Vinod KR, Narender Prasad D, Venkataramana K. Evaluation of Anti Bacterial and Anti Platelet Activity of Ethanolic Leaf Extract of Physalis angulata. Research J. Pharmacognosy and Phytochemistry. 2010; 2(1): 67-69.
15. Ladda PL, Naikwade NS, Magdum CS. Antimycobacterial and Antimicrobial Activity of Leaf Extracts of Vitex negundo Linn. Research J. Pharmacognosy and Phytochemistry 2010; 2(2): 166-168.
16. Sarac N, Ugur A. The in vitro antimicrobial activities of the essential oils of some Lamiaceae species from Turkey. *J Med Food*. 2009; 12: 902-7.

17. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymer. 2011; 3: 1377-1397.
18. De M, De AK, Sen P, Banerjee AB. Antimicrobial properties of star anise (Illicium verum Hook f). *Phytother Res*. 2002; 16 (1): 94–5.
19. Kubo I, Fujita K. Naturally occurring anti-Salmonella agents. *J. Agric. Food Chem.* 2001; 49 (12): 5750–4.
20. Weissinger WR, McWatters KH, Beuchat LR. Evaluation of volatile chemical treatments for lethality to Salmonella on alfalfa seeds and sprouts. *J. Food Prot.* 2001; 64 (4): 442–50.
21. Fujita K, Fujita T, Kubo I. Anethole, a potential antimicrobial synergist, converts a fungistatic dodecanol to a fungicidal agent. *Phytother Res.* 2007; 21 (1): 47–51.
22. A. Akhtar AA, Deshmukh AV, Bhonsle. "In vitro Antibacterial activity of *Pimpinella anisum* fruit extracts against some pathogenic bacteria," *VeterinaryWorld*, 2008; 1(9): 272–274.
23. Gulcin I, Oktay M, Kirecci E, Kufrevioglu OI. "Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) Seed extracts," *Food Chemistry*, 2003; 83(3): 371–382.