Ethnomedicinal Plants: In vitro antibacterial effect of essential oil of Pistacia khinjuk

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Abstract: Pistacia khinjuk is a native plant in Iran, which the plant has been used as an indigestion, tonic, toothache, antiflammatory, antipyretic and astringent in Iran. The aim of the current study was to determine chemical composition of P. khinjuk essential oil and evaluate its antibacterial activity against common pathogens (Escherichia coli O157:H7 and Staphylococcus aureus) with broth macro-dilution and agar well and disk diffusion methods. The chemical composition of the essential oil was identified using gas chromatography coupled with mass spectrometer detector (GC-MS). The antibacterial activity of P. khinjuk essential oil was evaluated by macro-dilution method in Mueller-Hinton broth medium and agar well and disk diffusion methods. According to results of GC-MS analysis, γ-terpinene (81.14%) (w/w) was the abundant component of the essential oil. The results revealed that the essential oil exhibited strong levels of antibacterial activity against E. coli and S. aureus. Regarding the MIC and MBC values E. coli were more sensitivity to the essential oil than S. aureus. Our findings indicated that P. khinjuk essential oil had a potential to be applied as antimicrobial agent.

Keywords: Pistacia khinjuk, Essential oil, Chemical composition, Antibacterial effect.

1 Introdution

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. Essential oils are also known as volatile oils, ethereal oils, aetherolea, or simply as the oil of the plant from which they were extracted, such as oil of clove. Essential oils could be extracted from different parts like leaves, stems, flowers, roots including bushes and trees through distillation. They are effective on a wide range of Gram-negative and positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Listeria monocytogenes, Salmonella typhimurium* and *Escherichia coli O157:H7* [1], [2]. In recent years, interest in essential oils has

been increased for pharmacological studies which claim that the essential oil have beneficial efficacy for the control and inhibition of human and food-borne pathogens and food spoilage microorganism's growth [3], [4], [5]. In addition, essential oils are being used in perfumes, cosmetics and for flavoring of foods including meat and meat products and also milk and dairy products [6]. The use of plant compounds to treat infections is an old practice in a large part of the world, especially in developing countries where there is dependence on traditional medicine for a variety of diseases. Interest in plants with antimicrobial properties has revived as a result of current problems associated with the use of antibiotics [7]. The genus *Pistacia* (family Anacardiaceae), is widely distributed in the Mediterranean and Middle East areas [8]. Among the 15 known species of pistachios, only 3 species grow in Iran, including *Pistacia vera*, *Pistacia khinjuk* and *Pistacia atlantica* [9]. These are shrubs and small trees growing to 5-15 m tall. The leaves are alternate, pinnately compounds and can be either evergreen or deciduous depending on species. They are the most important species of pistachio in Iran is known as the origin of pistachios. *P. khinjuk* is spread in places where the attitude is 700 - 2000 meter above sea level [10]. In addition, fruits of *P. khinjuk* used edible wild fruits. The plant is known as Khenjuk or Kelkhong in Persian. *P. khinjuk* is a native plant in Iran, which the plant has been used as an indigestion, tonic, toothache and astringent in Bakhtiari folk medicine [11]. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos and Galenos have recommended use of mastic gum obtained from genus *P. khinjuk* for gastrointestinal disorders like gastralgia, dyspepsia and peptic ulcer [12], [13]. Some species of *P. khinjuk* have been used in folk medicine as antiflammatory, antipyretic, antibacterial, antiviral, in treatment diarrhea and throat infection[14], [15], [16]. Essential oils of some *P. khinjuk* species consist of components such as γ-terpinene, cymene, linalool, β-caryophyllene, α-thujene, fenchene, sabinene, α-phellandrene, cineol, α-fenchone, borneol and α-terpineil. The terpinenes are a group of isomeric hydrocarbons that are classified as terpenes. They each have the same molecular formula and carbon framework, but they differ in the position of carbon-carbon double bonds. γ- terpinene is a monoterpene and a major component of essential oils made from plants fruit and shows strong antioxidant activity in various assay systems[17], [18].

Based on knowledge of author, in comparison to many other pharmaceutical-industrial plants, there is a very little data about chemical composition and antibacterial activity of *P. khinjuk* essential oil collected from Kermanshah province, west of Iran. Hence, the aim of the current study was (i): determination of chemical composition of its hydro-distilled essential oil obtained from Kermanshah city, west of Iran by GC–MS, (ii): evaluation of antibacterial activity of the essential oil against common pathogens (*E. coli* and *S. aureus*) with broth macro-dilution and agar well and disk diffusion methods.

Material and Methods

2.1 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.

2.2 Essential oil extraction

Essential oil from fresh, clean, weighed aerial part *P. khinjuk* 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 was done using dimethyl sulfoxide (DMSO).

2.3 Gas chromatography mass spectrometry (GC/MS)

Essential oil of *P. khinjuk* was analysed 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 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, or by retention indices (RI) and mass spectra.

2.4 Source of microorganisms

Two bacterial species namely *Escherichia coli O157:H7* (ATCC No. 25922) and *Staphylococcus aureus* (ATCC No. 25923) 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.

2.5 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. Then, Mueller-Hinton broth containing different concentrations of the essential oil and of the final bacteria inoculums (1×10^8 CFU/ml) were added in to each well.

2.6 Evaluation of antimicrobial activities

Agar disk diffusion was used as screen test to evaluate antibacterial property of essential oil of *P. khinjuk* based on standard protocol. The solution of this compound was yielded in 1g/ml from which two 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. DMSO was used as negative control whereas gentamicin and cephalothin 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 three 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 [19].

2.7 Statistical Analysis

Antibacterial effect was determined by One way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p\leq0.05$.

3 Results

3.1 Chemical composition

The most substance found in essential oil of *P. khinjuk* was γ-terpinene. In contrast, *1-Phellandrene* was the least constituents discovered in this essential oil. Composition of this plant using Gas chromatography mass spectrometry method can be perceived in table 1.

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

No	compound	RT(min)	Area (%)
1	Tricyclene	6.454	0.35
2	γ-terpinene	7.302	81.14
3	Camphene	7.538	1.6

4	Sabinene	8.349	1.09
5	β-pinene	8.493	3.93
6	β-myrcene	9.022	1.1
7	α-terpinene	-	0.18
8	1-phellandrene	9.505	0.11
9	Delta.3-carene	9.746	0.72
10	M-cymene	10.347	0.39
11	Dl-limonene	10.537	1.45
12	Terpan	10.619	0.43
13	α-terpinolene	13.161	2.38
14	α-pinene epoxide	13.541	0.3
15	Linalol	13.695	0.2
16	α-campholenal	14.82	0.4
17	Trans-pinocarveol	15.369	0.33
18	3-Cyclohexene-1-carboxaldehyde	15.729	1.25
19	P-cymen-8-ol	17.557	0.2
20	α-Terpineol	17.798	0.34
21	Myrtenol	18.06	0.16
22	l-Bornyl acetate	22.636	0.81
	Total		98.86

3.2 Agar well diffusion test

In regard to *P. khinjuk* essential oil, the widest zone was seen in 0.062 g/ml, due to *E. coli* (19 mm). It was no growth inhibition in negative control and less for both bacteria. The data are discoverable in table 2.

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

Dilution(g/ml)	**Inhibition zone in well diffusion (mm)**	
Microorganism	***E. coli***	***S. aureus***
1/16 (0.062)	19	15
1/32 (0.031)	14	13
1/64 (0.015)	13	13
1/128 (0.007)	10	11
1/256 (0.003)	9	9
1/512 (0.002)	8	8
Negative control (DMSO)	0	0

3.3 Agar disk diffusion test

The widest zone was formed due to positive controls (38 mm for gentamicin and 21 mm for cephalothin) and after it, the widest zone was formed due to 0.062 g/ml of the essential oil in *E. coli* culture (20 mm) and it was no halo in negative control and less for both bacteria. The data are discoverable in table 3.

Table 3. The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of essential oil of *P. khinjuk*.

Dilution(g/ml)	**Inhibition zone in disk diffusion (mm)**	
Microorganism	***E. coli***	***S. aureus***
Positive control	38	21
1/16 (0.062)	20	16
1/32 (0.031)	19	13
1/64 (0.015)	15	12
1/128 (0.007)	11	11
1/256 (0.003)	9	9

1/512 (0.002)	8	8
Negative control (DMSO)	0	0

3.4 MIC determination

In the essential oil, MIC was 0.015 g/ml for *E. coli*, Whereas MIC was 0.031 g/ml for *S. aureus* (Table 4).

3.5 MBC ascertaining

In the essential oil, MBC was 0.015 g/ml for *E. coli*, Whereas MBC was 0.031 g/ml for *S. aureus* (Table 4).

Table 4. MIC and MBC for the essential oil of *P. khinjuk*.

Microorganism	***E. coli***	***S. aureus***
MIC(g/ml)	1/64(0.015)	1/32 (0.031)
MBC(g/ml)	1/64 (0.015)	1/32 (0.031)

As the table showed, essential oil of *P. khinjuk* have excluded the growth of *E. coli* and *S. aureus*. Also, by increasing the concentration of the essential oil, the inhibition zone augmented. The results defined that in tested bacteria, there was a considerable discrepancy in terms of sensitivity to *P. khinjuk* essential oil. The most sensitivity was apperceived in *E. coli*.

Discussion

The type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture 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 affect 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 large number of microbes [20], medicinal plants may have the ability to treat bacterial resistance to many types of antibiotics. The antimicrobial effects of aromatic oils extracted from a large number of plants have been evaluated and reviewed [21], [22], and the mechanisms that enable the natural ingredients of herbs and spices to resist microbes have been discussed [23]. The results show that these mechanisms vary greatly depending on the components of the essential oil [24], [25]. Since the antibacterial effectiveness of medicinal plants varies dramatically 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 same plant, but from two different regions [26]. *P. khinjuk* is an endemic and resistance species in dry and sub-dry forests in mountainous regions of Western Iran. The plant have played important roles in folk medicine and are used in eczema treatment, throat infections, renal stones, asthma and stomach ache, and as a astringent, anti-inflammatory ,antipyretic, antibacterial, antiviral, pectoral and stimulant [21].

4.1 Yield and analysis of essential oil of *P. khinjuk*

The chemical constituents identified by GC and GC/MS, the results concerning the qualitative and quantitative analysis of the essential oil are presented in the Table 1. In the essential oil of *P. khinjuk*, 22 compounds were identified. The main constituents were found to be γ-terpinene (81.14%) (w/w), β-Pinene (3.93%) (w/w), α-Terpinolene (2.38%) (w/w), Camphene (1.6%) (w/w), dl-Limonene (1.45%) (w/w), 3-Cyclohexene-1-carboxaldehyde (1.25%) (w/w), β-Myrcene (1.1%) (w/w), and sabinene (1.09%) (w/w). Other components (14 compounds) were present in amounts less than 1%. Studies in related species have identified triterpenes in the galls of *P. terebinthus* L. and *P. Lentiscus* and in the bled resin of *P. uera* L. were reported to contain α-pinene, P-pinene, limonene and myrtenol (or pinocarveol) [27], [28], [29], [30]. In a previous study, the main components of the green external skin of fruits of *P. khinjuk* were reported to be 1, 8 – Cineole (11.09%) (w/w), 1, 5 - Heptadien -4-one, 3, 3, 6 trimethyl (35.76%) (w/w), Camphor (26.34%) (w/w) and β-Selinene (10.15%) (w/w) [31]. De Pooter et al., reported that the essential oils of leaves of *P. khinjuk* Stocks, *P. chinensis* Bunge and *P. lentiscus* L, prepared by hydrodistillation, and studied by GC and GC-MS, showed qualitative and quantitative differences. All three were found to be rich in monoterpene hydrocarbons. In *P. lentiscus* 4 % sesquiterpene alcohols were found, and no monoterpene alcohols, whereas in *P. khinjuk* and *P. chinensis* 16% and 8% monoterpene alcohols respectively were detected, and no sesquiterpene alcohols. Some major constituents of essential oil from the aerial parts of *P. khinjuk* are α-pinene, β- pinene, Myrcene, beta-caryophyllene, Germacrene B and Spathulenol [32]. Results of a recent study showed that some of the major constituents of essential oil from the aerial parts of *P. khinjuk* (Kermanshah, western part of Iran) are α-pinene, β-pinene, myrcene, beta-caryophyllene, germacrene B and spathulenol [33]. It is possible that our result on the composition of this essential oil related to method of essential oil extraction.

4.2 Antibacterial activity

The antibacterial results showed that the essential oil of *P. khinjuk* inhibited the two bacteria and the activities were considerably dependent upon concentration. In fact the results indicated that *P. khinjuk* essential oil with 0.015/0.031 g/ml concentration has prevented from the growth *E. coli* and *S. aureus*, respectively, also in 0.015/0.031 g/ml concentration has destroyed *E. coli* and *S. aureus*, respectively. Thus, the research represents the antibacterial effects of the essential oil of *P. khinjuk* on *E. coli* and *S. aureus*. Concerning the method of essential oil, extraction and preventing from using high temperature to decrease the rate of destruction of effective herbal compound. Its bioactive components may be γ-terpinene and other components that we do not know. There is a partial difference between these results and the similar studies. Our results agree with the previous antibacterial studies related to these species [21], [34], [35]. The result indicated that in essential oil of *P. khinjuk*, the main constituent was found to be γ-terpinene. γ-terpinene was assessed for its ability to induce cellular protein leakage in *P.vulgaris* and *E. coli*

(Gram negatives) as well as *L. monocytogenes* and *S. pyogenes* (Gram positives). Both the Gram negative and Gram positive test bacteria showed a similar trend of protein leakage when treated with γ-terpinene. Protein leakage could be used as an indicator of the membrane damage caused by chemical and physical agents. It has been suggested that the cytoplasmic membrane is also a target for γ-terpinene action and the results evidencing the protein leakage corroborated this hypothesis. γ-terpinene was determined for its capability to persuade cellular lipid leakage in *P. vulgaris* and *E. coli* as well as *L. monocytogenes* and *S. pyogenes* [36]. The effect of γ-terpinene might be the result of its phenolic structure which interferes with the lipid bilayer of the outer membranes [37]. The essential oil of *P. khinjuk* content flavonoids and flavonoid glycosides. Also, several members of the genus *Pistacia* have been chemically investigated. They are characterized mainly by the occurrence of flavonoids and flavonoid glycosides [38]. Flavonoids are hydroxylated phenolic substances and they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls [39]. *P. khinjuk* have also been reported to contain phenolic compounds and triterpenoids [40], [41]. Terpenenes or terpenoids are active against bacteria [39]. It should be noted that the two major volatile constituents, α-pinene (0.3% in this essential oil) and terpinolene (2.38% in this essential oil), are compounds with interesting antibacterial properties [42], [43]. Additionally, terpinolen has been identified as antioxidant agent [44].

5 Conclusion

From the study it can be concluded that the essential oil of *P. khinjuk* extract possess antibacterial activity. In fact essential oil of *P. khinjuk* have prevented the growth of *E. coli* and *S. aureus*. Also, by increasing the concentration of the essential oil, the inhibition zone increased. The results determined that in tested bacteria, there was a significant difference in terms of sensitivity to *P. khinjuk* essential oil. In other words, the most sensitivity was observed in *E. coli*. Our results support the use of the plant in traditional medicine and suggest that essential oil *P. khinjuk* possess compounds with good antibacterial properties. They can be used as antibacterial supplements 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 the plant as an antibacterial agent in topical or oral applications.

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Authors' Contribution

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 Mohammad Mahdi Zangeneh and Akram Zangeneh. Essential oil extraction provided by Reza Tahvilian, Rohallah Moradi, Hossein Zhale, Hossein Yazdani and Majid Hajialiani.

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