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RESEARCH ARTICLE

CHEMICAL COMPOSITION AND SCREENING OF ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OIL OF PISTACIA KHINJUK AGAINST BACILLUS SUBTILIS (ATCC NO. 21332)

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ABSTRACT

Medicinal plants are considered modern resources for producing agents that could act as alternatives to antibiotics in demeanor of antibiotic-resistant bacteria. The aim of the study was to evaluate the chemical composition and antibacterial activities of the essential oil of *Pistacia khinjuk* (Combined with the dominance γ-terpinene) against *Bacillus subtilis*. The chemical composition of the *P. khinjuk* was identified using gas chromatography coupled with mass spectrometer detector (GC-MS). As a screen test to detect antibacterial properties of the *P. khinjuk*, agar well diffusion and agar disk diffusion methods were employed. Macrobroth tube test was performed to determinate MIC. According to results of GC-MS analysis, γ-terpinene (81.14%) (w/w), β-Pinene (3.93%) (w/w), α-Terpinolene (2.38%) (w/w) were the abundant components of the *P. khinjuk*. The MIC and MBC values were 0.031 g/ml for *P. khinjuk* in case of the bacterium. We believe that the article provide support to the antibacterial properties of the *P. khinjuk*. In fact, the results indicate that the essential oil of *P. khinjuk* can be useful as medicinal or preservative composition. Fractionation and characterization of active molecules will be the future work to investigate.

INTRODUCTION

Infections due to bacterial species also stay a serious clinical difficulty. Emerging resistance of bacterial species is seriously reducing the number of efficient antimicrobials. Because of increasing pressure of consumers and legal authorities, the food industry has tended to decrease the use of chemical preservatives in their products to either entirely nil or to adopt more indigenous alternatives for the maintenance or extension of product shelf life[1].

Essential oils are made from a very intricate mixture of volatile molecules that are produced by the secondary metabolism of aromatic and medicinal plants and can be obtained by various methods, including the use of low or high pressure distillation of various parts of plants or the employment of liquid carbon dioxide or microwaves. The span of the essential oils action versus bacteria may achieve values that only prevent the bacterial growth (bacteriostatic) or may be used at either high concentrations or are inherently more aggressive and their action results in a reduction in the number of bacterial cells (bactericide)[2-5]. The bacteriostatic action has a reversible character since, after frustration of the agent, the microbial cells will meliorate their reproductive capacity. In contrast, the bactericidal effect has a constant effect; as even after the neutralization of the agent, the microbial cells are not capable of growth and reproduction[6]. The genus Pistacia (family Anacardiaceae), is widely distributed in the Mediterranean and Middle East areas[7]. Among the 15 known species of pistachios, only 3 species flourish in Iran, including *Pistacia vera*, *Pistacia khinjuk* and *Pistacia atlantica*[8]. These are shrubs and small trees growing to 5-15 m tall. The leaves are alternate, innately compounds and can be either evergreen or deciduous depending on species. *P. khinjuk* is circumfuse in places where the attitude is 700 - 2000 meter above sea level[9]. *P. khinjuk* is an indigenous plant in Iran, which the plant has been used as an indigestion, toothache and astringent in Bakhtiari folk medicine. The plant is known as Khenjuk or Kelkhong in Persian[10]. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos and Galenos have recommended use of mastic gum obtained from genus Pistacia for gastrointestinal derangements like gastralgia, dyspepsia and peptic ulcer[11,12] . Some species of Pistacia have been used

in folk medicine in eczema treatment ,throat infections, renal stones, asthma and stomach ache, and as a astringent, anti-inflammatory ,antipyretic, antibacterial, antiviral, pectoral and stimulant[13-15]. Essential oils of some *Pistacia* 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 have the same molecular formula and carbon fuselage, but they vary 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 activities in several assay systems. And have been isolated from a versatility of plant sources, such like coriander oil, lemon oil, cumin oil and fragrant celery oil. Also present in the grapes, celery, cinnamon, cloves, cumin seeds, ginger, pepper and tea[16, 17].

Based on knowledge of authors, in comparison to many other pharmaceutical-industrial plants, there is a very little data about chemical composition and antibacterial activities of the essential oil collected from Kermanshah province, west of Iran, and there is no study on antibacterial effects of essential oil of *P. khinjuk* (Combined with the Dominance γ-Terpinene) in all over the world. Hence, the aim of the current study was (1): Determination of chemical composition of its hydro-distilled essential oil obtained from Kermanshah city, west of Iran by GC–MS, (2): Evaluation of antibacterial activities of the *P. khinjuk* against common pathogen (*B. subtilis*) with broth macro-dilution and agar well and disk diffusion methods.

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. 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 conjuncted 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 was liberated from the plant material and vaporized 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 compress the steam. The steam was applied for 3h. After settling the recovered mixture, essential oil was withdrawn. The supernatant essential oil was purged through anhydrous Na_2SO_4 to dry the yielded essential oil. Then, the essential oil was collected in tightened vials and stored in a refrigerator. For the antimicrobial activities test, several dilutions of the oil were done using dimethyl sulfoxide (DMSO).

Gas chromatography mass spectrometry (GC/MS)

Essential oil of *P. khinjuk* 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 essential oil 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

Bacterial specie namely *B. subtilis* (ATCC No. 21332) were procured from Iranian Research Organization for Science and Technology as lyophilized. 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 distributed at 20 ml per plate in 12 x 12 cm 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 bacterium inoculums (1×10^8 CFU/ml) were added in to each well.

Evaluation of antimicrobial activities

Agar well diffusion and agar disk diffusion were used as screen tests to evaluate antibacterial properties of *P. khinjuk* based on standard protocol. The solution of the compound 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 cephalothin was used as positive control in case of *B. subtilis*. 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[18].

Statistical Analysis

Antibacterial effects was determined by One way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p\leq0.01$.

RESULTS

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 the *P. khinjuk*. Composition of the plant using Gas chromatography mass spectrometry method can be perceived in table 1[19].

Table 1 Identified main composition of the essential oil of *P. khinjuk* using Gas chromatography mass spectrometry method[19].

No	compound	Area (%)
1	Tricyclene	0.35
2	γ-Terpinene	81.14
3	Camphene	1.6
4	Sabinene	1.09
5	β-Pinene	3.93
6	β-Myrcene	1.1
7	α-Terpinene	0.18
8	1-Phellandrene	0.11
9	DELTA.3-Carene	0.72
10	m-Cymene	0.39
11	dl-Limonene	1.45
12	Terpan	0.43
13	α-TERPINOLENE	2.38
14	α-Pinene epoxide	0.3
15	Linalol	0.2
16	α-Campholenal	0.4
17	trans-Pinocarveol	0.33
18	3-Cyclohexene-1-carboxaldehyde	1.25
19	p-Cymen-8-ol	0.2
20	α-TERPINEOL	0.34
21	Myrtenol	0.16
22	l-Bornyl acetate	0.81
	Total	98.86

Agar well diffusion test

In regard to, the widest zone was seen in 0.062 g/ml (12 mm). It was no growth inhibition in negative control. The data are discoverable in table 2.

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

Dilution(g.ml^{-1})	Inhibition zone in well diffusion (mm)
Microorganism	***B. subtilis***
1/16 (0.062)	12
1/32 (0.031)	10
1/64 (0.015)	9
1/128 (0.007)	8
1/256 (0.003)	0
1/512 (0.002)	0
Negative control (DMSO)	0

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

Dilution(g.ml^{-1})	Inhibition zone in disk diffusion (mm)
Microorganism	*B. subtilis*
Positive control	32
1/16 (0.062)	18
1/32 (0.031)	12
1/64 (0.015)	10
1/128 (0.007)	8
1/256 (0.003)	0
1/512 (0.002)	0
Negative control (DMSO)	0

Agar disk diffusion test

The widest zone was formed due to positive control (32 mm) and after it, the widest zone was formed due to 0.062 g/ml of the *P. khinjuk* (18 mm) and it was no halo in negative control. The data are discoverable in table 3.

MIC determination

In the essential oil MIC was 0.031 g/ml concentration.

MBC ascertaining

MBC of *P. khinjuk* was 0.031 g/ml for *B. subtilis*.

As the tables showed, *P. khinjuk* have excluded the growth of *B. subtilis*. Also, by increasing the concentration of the essential oil, the inhibition zone in many of samples augmented. The results defined that in tested bacterium, there was a considerable discrepancy in terms of sensitivity to essential oil of *P. khinjuk*.

DISCUSSION

The use of plant compounds to remedy 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 versatility of diseases. Interest in plants with antimicrobial properties has revived as a result of new obstacles associated with the use of antibiotics[20-25]. *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 as antiflammatory, antipyretic, antibacterial, antiviral, in treatment diarrhea and throat infection[26].

Yield and resolution of essential oil of *P.khinjuk*

The chemical constituents recognized by GC and GC/MS, the results concerning the qualitative and quantitative analysis of the *P. khinjuk* are presented in the table 1. In the *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%[19]. 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 apinene, P-pinene, limonene and myrtenol (or pinocarveol)[27-30]. 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]. In the other study 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 found, 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 main 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.

Antibacterial activities

The antibacterial results showed that the *P. khinjuk* inhibited the bacterium and the activities were considerably dependent upon concentration. In fact the results indicated that *P. khinjuk* with 0.031 g/ml concentration has prevented from the growth *B. subtilis*, also in 0.031 g/ml concentration has destroyed *B. subtilis*, actually MIC and MBC are equal for the bacterium. Thus, the research represents the antibacterial effects of the medical plant on *B. subtilis*. Concerning the method of essential oil, extraction and preventing from using high temperature to decrease the rate of destruction of impressive herbal compound. Its bioactive components may be γ-terpinene and other components that we do not know. Our results agree with the past antibacterial studies related to these species[26,34,35]. In the *P. khinjuk*, the main constituent was found to be γ-terpinene. γ-terpinene was assessed for its ability to induce cellular protein leakage in *Proteus vulgaris* and *Escherichia coli* (Gram negatives) as well as *Listeria monocytogenes* and *Streptococcus pyogenes* (Gram positives). Both the Gram negative and Gram positive test bacteria showed a similar trend of protein permeation when treated with γ-terpinene. Protein permeation could be used as an index of the membrane detriment caused by chemical and physical agents. It has been offered 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 assessed for its capability to induce cellular lipid permeation in *P. vulgaris* and *E. coli* as well as *L. monocytogenes* and *S. pyogenes*[36]. The effects of γ-terpinene might be the result of its phenolic structure which interferes with the lipid bilayer of the outer membranes[37]. *P. khinjuk* content flavonoids and flavonoid. 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 efficient antimicrobial substances against an extensive array of microorganisms. Their activities is probably due to their ability to complex with extracellular and solvable proteins and to complex with bacterial cell walls[39]. These plants (such as *P. khinjuk*) have also been reported to contain phenolic compounds and triterpenoids[40,41]. Terpenenes or terpenoids are active against bacteria [29]. It should be noted that the two main volatile constituents, α-pinene (0.3% in the *P. khinjuk*) and terpinolene (2.38% in the *P. khinjuk*), are compounds with interesting antibacterial properties[42,43]. Additionally, terpinolen has been identified as antioxidant agent[44].

From this study it can be concluded that the essential oil of *P. khinjuk* (Combined with the Dominance γ-terpinene) possess antibacterial effects, and the antibacterial activities of the *P. khinjuk* was due to the presence of various active compounds. Hence, the phytochemical compounds responsible for the antibacterial effects of bacterium can be subjected to isolation of the therapeutic antimicrobials. Our results defend the use of the plant in traditional medicine and offer that essential oil of *P. khinjuk* possess compounds with good antibacterial properties. They 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.

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