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Research Article

Ethnomedicinal Plants: Study on Antifungal Activity of Essential Oil of *Pistacia khinjuk* (Combined with the Dominance γ-Terpinene) Against *Candida albicans*.

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ABSTRACT

Medicinal plants are considered modern resources for producing agents that could act as alternatives to antifungal drugs in demeanor of antifungal drug-resistance fungi. *Pistacia khinjuk* is a native plant in Iran, which this 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 antifungal activity against common pathogens (*Candida albicans*) 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 antifungal activity of *P. khinjuk* essential oil was evaluated by micro-dilution method in Sabouraud-Dextrose broth medium and agar well and disk diffusion assay. 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 essential oil. The results revealed that the essential oil exhibited strong levels of antifungal activity against this tested microorganism. Regarding the MIC and MFC values *C. albicans* was very high sensitivity to the essential oil. Our findings indicated that *P. khinjuk* essential oil had a potential to be applied as antifungal agent.

Keywords: *Pistacia khinjuk*, Essential Oil, Chemical Composition, Antifungal Ativity.

INTRODUTION

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. 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 microorganisms[1,2]. In recent years, interest in essential oils has been increased for pharmacological studies which claim that the essential oil has beneficial efficacy for the control and inhibition of human microorganism's growth[3-5]. 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. The genus *Pistacia* (family Anacardiaceae), is widely distributed in the Mediterranean and Middle East areas[6]. Among the 15 known species of pistachios, only 3 species grow in Iran, including *Pistacia vera*, *Pistacia khinjuk* and *Pistacia atlantica*[7]. These are shrubs and small trees growing to 5-15 ml 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[8]. P. khinjuk is a native plant in Iran, which resin this plant has been used as an indigestion, tonic, toothache and astringent in Bakhtiari folk medicine. In addition, fruits of *P. khinjuk* used edible wild fruits. The plant is known as Khenjuk or Kelkhong in Persian[9]. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos and Galenos have recommended use of mastic gum obtained from genus *Pistacia* for gastrointestinal disorders like gastralgia, dyspepsia and peptic ulcer[10,11]. Some species of *Pistacia* have been used in folk medicine as antiflammatory, antipyretic, antibacterial, antifugal, antiviral, in treatment diarrhea and throat infection[12-14]. 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 each have the same molecular formula and carbon framework, but they differ in the position of carbon-carbon

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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[15,16]. Based on knowledge of author, in comparison to many other pharmaceutical-industrial plants, there is a very little data about chemical composition and antifungal 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 antifungal activity of the essential oil against common pathogens (*C. albicans*) with broth macro-dilution and agar disk diffusion methods.

MATERIAL AND METHODS

Plant samples collection

In this empirical-experimental study, medicine plants collected from Kermanshah. The samples were 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 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 oil were done using dimethyl sulfoxide (DMSO).

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 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 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.

Source of microorganisms

C. albicans (ATCC No. 2091) was procured from Iranian Research Organization for Science and Technology as lyophilized. Fungus strain was activated on Sabouraud-Dextrose broth, constant at 37°C for 18 h. Then 100 μl of the broth was transferred to Sabouraud-Dextrose agar and incubated at 37°C for another 24 h; cell concentration was then adjusted to obtain final concentration of 10^6 CFU/ml using Sabouraud-Dextrose broth.

Culture media

Sabouraud-Dextrose Agar 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, Sabouraud-Dextrose broth containing different concentrations of the essential oil and of the final fungus inoculums (1×10^6 CFU/ml) were added in to each well.

Evaluation of antimicrobial activities

Agar disk diffusion was used as screen test to evaluate antifungal 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. 100 μl of each dilution was poured on each disk in order[17]. 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 Amphotericin B was used as positive control. Minimum inhibitory concentration (MIC) means the lowest concentration of the probable antimicrobial agent which prevents growing of fungi (regardless of killing the fungi or stopping the growth of them). The lowest dilution which no gross microbial growth has been seen indicates MIC. Minimum fungicidal concentration (MFC) means the lowest concentration of the agent which causes death to test fungi. The last can be revealed by pouring 100 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 MFC. For determination of MIC value, macrobroth dilution method was applied. Interpretation of the results was done due too national accepted letter[17].

Statistical Analysis

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

RESULTS

Chemical composition

The chemical constituents identified by GC and GC/MS. 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%.

Agar good diffusion test

In regard to *P. khinjuk* essential oil, the widest zones were seen in 1 g/ml (64 mm). It was no growth inhibition in negative control and less for *C. albicans*. The data are

Table 1: The diameters of growth inhibition zones in different dilutions of essential oil of *P. khinjuk*.

Dilution(g/ml)	Inhibition zone in disk diffusion (mm)
Microorganism	*C. albicans*
1 (1)	64
1/2 (0.5)	54
1/4 (0.25)	32
1/8 (0.125)	18
1/16 (0.062)	14
1/32 (0.031)	12
1/64 (0.015)	11
1/128 (0.007)	9
1/256 (0.003)	8
1/512 (0.002)	8
1/1024 (0.001)	8
Negative control (DMSO)	0

Table 2: The diameters of growth inhibition zones in different dilutions of essential oil of *P. khinjuk*.

Dilution(g/ml)	Inhibition zone in disk diffusion (mm)
Microorganism	*C. albicans*
Positive control	16
1 (1)	50
1/2 (0.5)	36
1/4 (0.25)	22
1/8 (0.125)	13
1/16 (0.062)	13
1/32 (0.031)	12
1/64 (0.015)	11
1/128 (0.007)	10
1/256 (0.003)	9
1/512 (0.002)	8
1/1024 (0.001)	8
Negative control (DMSO)	0

discoverable in table 1.

Agar disk diffusion test

The widest zone was formed due to 1 g/ml (50mm) of the essential oil and it was no halo in negative control and less for the fungus. The data are discoverable in table 2.

Minimum inhibitory concentration (MIC) determination

In this essential oil, MIC was 0.003 g/ml for *C. albicans* (Table 3).

Minimum fungicidal concentration (MFC) ascertaining

In this essential oil, MFC was 0.003 g/ml for the fungus (Table 3).

As the table showed, essential oil of *P. khinjuk* have excluded the growth of *C. albicans*. Also, by increasing the concentration of this essential oil, the inhibition zone augmented. The results defined that in tested fungus, there was a considerable discrepancy in terms of sensitivity to *P. khinjuk* essential oil.

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. Because of their safety and low cost as well as their impact on a large number of microbes[18], medicinal plants may have the ability to treat fungus resistance to many types of antifungal drugs. The antimicrobial effects of aromatic oils extracted from a large number of plants have been evaluated and reviewed[19], and the mechanisms that enable the natural ingredients of herbs and spices to resist microbes have been discussed[20]. The results show that these mechanisms vary greatly depending on the components of the essential oil[21,22]. P. khinjuk is an endemic and resistance species in dry and sub-dry forests in mountainous regions of Western Iran. These plants have played important roles in folk medicine and are used in eczema treatment, throat infections, renal stones, asthma and stomach ache, and as an astringent, anti-inflammatory, antipyretic, antibacterial, antiviral, pectoral and stimulant[23]. Yield and analysis of essential oil of P. khinjuk. The chemical constituents identified by GC and GC/MS. 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. In a previous study[24], 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). 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[25]. Results of a recent study[26] 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. It is possible that our result on the composition of this essential oil related to method of essential oil extraction.

Antifungal activity

The antifungal results showed that the essential oil of *P. khinjuk* inhibited the fungus and the activities were considerably dependent upon concentration. In fact, the results indicated that *P. khinjuk* essential oil with 0.003 g/ml concentration has prevented from the growth and has destroyed *C. albicans*, actually MIC and MFC are equal for the fungus. Thus, the research represents the antifungal effects of the medical herb on *C. albicans*. Concerning the method of essential oil, extraction 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. Its bioactive components may be γ-terpinene and other components that we do not know. Our results agree with the previous

Table 3: Minimum inhibitory concentrations (MIC) and Minimum fungicidal concentration (MFC) for the essential oil of *P. khinjuk*.

Microorganism	*C. albicans*
MIC(g/ml)	1/256(0.003)
MFC(g/ml)	1/256(0.003)

antifungal studies related to this specie[14,23,26]. In this essential oil, the main constituent was found to be γ-terpinene. γ-terpinene was assessed for its ability to induce cellular protein leakage in Gram negatives and Gram positives bacteria and fungi. Both the Gram negative and Gram positive test bacteria and fungi 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 assessed for its ability to induce cellular lipid leakage in Gram negatives and Gram positives bacteria and fungi[27]. The effect of γ-terpinene might be the result of its phenolic structure which interferes with the lipid bilayer of the outer membranes[28]. 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[29]. Flavonoids are hydroxylated phenolic substances and they have been found in vitro to be effective antimicrobial substances against a wide array of micro-organisms[30]. 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 antimicrobial properties[31,32]. Additionally, terpinolen has been identified as antioxidant agent[33].

From this study it can be concluded that the essential oil of *Pistacia khinjuk* (Combined with the Dominance γ-terpinene) possess antifungal effect, and the antifungal activity of the essential oil was due to the presence of various active compounds. Hence, the phytochemical compounds responsible for the antifungal effects of *C. albicans* can be subjected to isolation of the therapeutic antimicrobials. Our results defend the use of the plant in traditional medicine and offer that some of the plant oil possess compounds with good antifungal properties. They can be used as antifungal supplements in the developing countries towards the development of new remedial agents. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of the plant as one antifungal 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 antifungal 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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