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In vitro scolicidal effect of *Satureja khuzistanica* (Jamzad) essential oil

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Objective: To investigate the scolicidal effect of the *Satureja khuzistanica (S. khuzistanica)* essential oil from aerial parts of this herbal plant. **Methods:** The essential oil was obtained by hydrodistillation method. Gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) were employed to determine the chemical composition of the essential oil. Protoscolices were collected aseptically from sheep livers containing hydatid cyst. Protoscolices were exposed to various concentrations of the oil (3, 5 and 10 mg/mL) for 10, 20, 30, and 60 min. Viability of protoscolices was confirmed by 0.1% eosin staining. **Results**: A total of 19 compounds representing 97.6% of the total oil, were identified. Carvacrol (94.9%) was found to be the major essential oil constituent. Scolicidal activity of *S. khuzistanica* essential oil at concentration of 3 mg/mL was 28.58, 32.71, 37.20 and 42.02%, respectively. This essential oil at concentration of 5 mg/mL killed 51.33, 66.68, 81.12, and 100% of protoscolices after 10, 20, 30 and 60 min, respectively. One hundred scolicidal effect was observed with *S. khuzistanica* essential oil at the concentration of 10 mg/mL after 10 min (comparing with 7.19% for control group). **Conclusions:** The essential oil of *S. khuzistanica* is rich in carvacrol and may be used as a natural scolicidal agent.

1. Introduction

Human cystic echinococcosis (hydatid disease) continues to be a substantial cause of morbidity and mortality in many parts of the world[1]. Although scientists and clinicians have accumulated much experience in the diagnosis and treatment of human echinococcosis, there are still many questions and problems. Among the most pressing of these are early and accurate diagnosis by imaging, immunological techniques or needle biopsy, development of reliable follow-up methods and a need for rapidly acting, effective and safe protoscolicides, development of PAIR techniques under ultrasound guidance, improvement in surgical procedures and chemotherapeutic approaches for the increase of clinical cure, prevention of secondary infection and reduction of other complications[2].

In general, radical resection of the parasitic mass, if possible, represents the preferred treatment strategy. Surgery is usually complemented by pre-, and/or post-surgical chemotherapy, and in inoperable cases, chemotherapy is the only option. For this, benzimidazole carbamate derivatives such as albendazole and mebendazole are currently the drugs of choice[3].

With regard to benzimidazole chemotherapy for human hydatid disease, all studies show similar rates of success (10%-30%), improvement (40%-60%) and no change (10%-30%) [2]. In human patients, benzimidazoles have to be applied in high doses for extended periods of time, and adverse side effects are frequently observed[4].

Scolicidal solutions remain indispensable in the treatment of hydatid cyst disease and surgeons need less harmful but more effective drugs in hydatid disease[5]. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as plants, animals and microorganisms. Recently, herbal medicines have increasingly been used to treat many diseases including several infections[6]. Recently, antibacterial[7], antiviral[8], and antifungal[9] effects of *Satureja* species have been reported from different parts of the world.

Satureja khuzistanica (*S. khuzistanica*) Jamzad belonging to the Lamiaceae family is an endemic plant that widely distributed in the southern parts of Iran. It is a subshrub, branched stem ± 30 cm high, densely leafy, broadly ovate-orbicular and covered with white hairs. Base of the leaves is attenuate and petioliform[10]. It is famous for its medical uses as an analgesic and antiseptic in folk medicine[11]. *S. khuzistanica* has been reported to be antispasmodic, antidiarrhea[12], vasodilator[13], anti-inflammatory[14],

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antihyperlipidemic[11] and antioxidant[11,14]. *S. khuzistanica* has been also known to possess antifungal[15], antiviral[8] and antimicrobial[16], properties. Furthermore, it is effective in improvement in rat fertility[17] and is useful in the treatment of recurrent aphthous stomatitis (RAS)[18]. It also protects rats from hemorrhagic cystitis induced by cyclophosphamide (a widely used antineoplastic drug) by reduction of free radical-induced toxic stress[19].

As far as the authors are aware, there are no published reports regarding the protoscolicidal effect of essential oils. Therefore, the aim of the present work was to determine the *in vitro* protoscolicidal effect of the essential oil from *S. khuzistanica* at various concentrations and at different exposure times.

2. Materials and methods

2.1. Experimental design

Hydatid cyst protoscolices were exposed to the essential oil of *S. khuzistanica* for 10, 20, 30, and 60 min. Three concentrations (3, 5 and 10 mg/mL) of the essential oil were used in this study. The experiments were performed at 37 ℃. To determine the scolicidal activity of *S. khuzistanica* essential oil, the treated protoscolices were stained with 0.1% eosin for 15 min and the mortality rate of protoscolices was monitored by a light microscope.

2.2. Preparation of plant material

Aerial parts including flowers and leaves of *S. khuzistanica* (Mazhin, Lorestan, Iran), were collected from the wild growing plants at the full flowering stage in September to October 2009 (Altitude 490 m, Coordinate N 33^0 00′ E 47^0 40′). Voucher specimen of the species (MPH-1582) was deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran.

2.3. Essential oil isolation

The leaves and flowers of *S. khuzistanica* were dried under shade, ground mechanically using a commercial electric blender. One hundred grams of the resulting powder was subjected to hydrodistillation for 3 h in an all glass Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia[20]. The extracted oil samples were dried over anhydrous sodium sulphate and stored in sealed vials at 4 ℃ for gas chromatography (GC), GC/mass spectrometry (MS) analysis and scolicidal assessments.

2.4. GC analysis

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was carried out on fused silica capillary DB-5 column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The injector and detector temperatures were kept at 250 ℃ and 300 ℃, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.1 mL/min, oven temperature program was 60-250 ℃ at the rate of 4 ℃/min and finally held isothermally for 10 min. Split ratio was 1:50.

2.5. GC/MS analysis

GC-MS analysis was carried out by use of Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (60 m × 0.25 mm i.d.; film thickness 0.25 μm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 200 ℃ and 250 ℃, respectively. Mass range was from 35 to 456 amu. Oven temperature program was the same given above for the GC.

2.6. Identification and quantification of the oil components

The constituents of essential oils were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C_6-C_{24}) and the oil on a DB-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Adams and Wiley 7.0) or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature[21,22]. For quantification purposes, relative area percentages obtained by FID were used without the use of correction factors.

2.7. Collection of protoscolices

Hydatid cysts from livers of naturally infected sheep were obtained from Shiraz abattoir in southern Iran. Protoscolices were removed from cysts under aseptic conditions and washed several times with normal saline. Viability was assessed by muscular movements and 0.1% eosin staining test. The live protoscolices were finally transferred into a dark container containing normal saline solution and stored at 4 ℃ for further use.

2.8. Scolicidal assay

In this study, four concentrations of *S. khuzistanica* essential oil (1, 3, 5, and 10 mg/mL) were used for 10, 20, 30 and 60 min. To prepare the above concentrations, 10, 30, 50 and 100 μL of essential oil was dissolved in 9.7 mL of normal saline, in a test tube respectively. To enhance the dispersion of the essential oil in normal saline, 0.3 mL of tween 80 (Sigma,Germany) was added to the test tube. The resulting solution was mixed properly using a magnetic stirrer. In each experiment, 2.5 mL of the solution was placed in a test tube, to which a drop of protoscolex-rich sediment was added. The contents of the tube was gently mixed. The tube was then incubated at 37 ℃. At the end of each incubation time (10, 20, 30 and 60 min) the upper phase was carefully

removed so as not to disturb the protoscolices. One milliliter of 0.1% eosin stain was then added to the remaining settled protoscolices and mixed gently. The upper portion of the solution was discarded after 15 min of incubation. The remaining pellet of protoscolices was then smeared on a manually scaled glass slide, covered with a cover glass (24 mm × 50 mm), and examined under a light microscope. The percentages of dead protoscolices were determined by counting a minimum of 700 (usually more than 1 000) protoscolices. Protoscolices in the control group were treated only with normal salin containing tween 80. The experiments were performed in triplicate[23].

2.9. Viability test

In the present study, eosin stain with the concentration of 0.1% (1 g of eosin powder in 1 000 mL distilled water) was used to check the viability of the protoscolices[23]. Fifteen minutes after exposure to the stain the protoscolices with no absorbed dye were considered potentially viable (Figure 1); otherwise, they were recorded as dead (Figure 2).

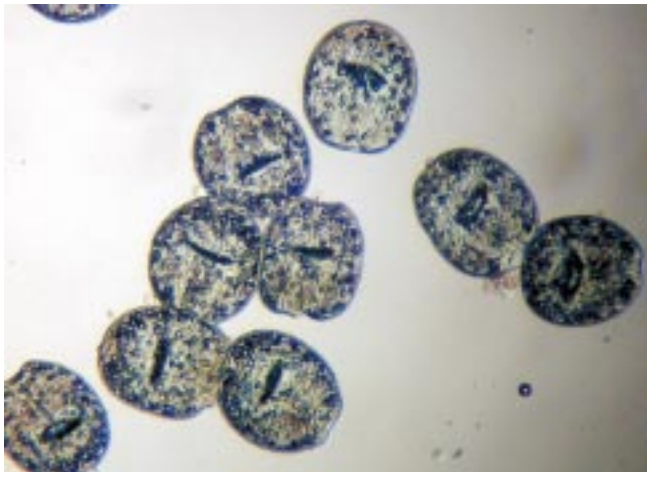

Figure 1. Live protoscolices after staining with 0.1% eosin.

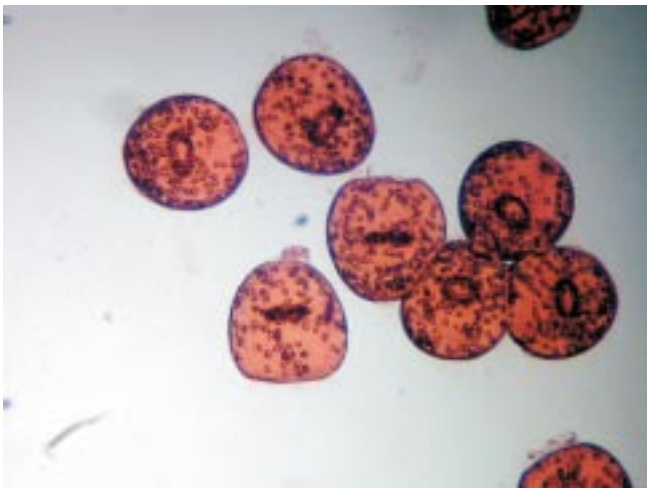

Figure 2. Dead protoscolices after exposure to *S. khuzistanica* essential oil and staining with 0.1% eosin.

2.10. Statistical analysis

Differences between the test and control groups were analyzed with *Chi*-square test. Statistical analysis was performed with GraphPad InStat software. *P* values less than 0.05 were considered to be significant.

3. Results

The chemical composition of *S. khuzistanica* essential oil is shown in Table 1. A total of 19 compounds, representing of 97.6 % of total oil, were identified. The major constituent of *S. khuzistanica* essential oil was carvacrol (94.9%). p-Cymene and γ-terpinene, as precursors of carvacrol, were present in low concentrations (Table 1). The mortality rates of *E. granulosus* protoscolices after exposure to different concentrations of *S. khuzistanica* essential oil at various

Table 1
Essential oil composition (%) of *S. khuzistanica*.

No	RI	Percentage (%)	Compound	Identification method
1	925	t	α-Thujene	RI, MS
2	933	t	α-Pinene	RI, MS, CoI
3	981	t	Myrcene	RI, MS
4	1013	0.26±0.02	α-Terpinene	RI, MS, CoI
5	1017	0.55±0.28	p-Cymene	RI, MS, CoI
6	1026	0.32±0.54	Limonene	RI, MS, CoI
7	1036	0.36±0.17	Z-β-Ocimene	RI, MS
8	1053	0.49±0.07	γ-Terpinene	RI, MS, CoI
9	1081	0.11±0.04	trans-Sabinene hydrate	RI, MS
10	1163	t	Terpin-4-ol	RI, MS
11	1175	t	α-Terpinole	RI, MS
12	1266	t	Thymol	RI, MS, CoI
13	1282	94.97±0.68	Carvacrol	RI, MS, CoI
14	1329	t	Thymyl acetate	RI, MS
16	1425	0.13±0.01	β-Caryophyllene	RI, MS, CoI
17	1427	t	α-Humulene	RI, MS
18	1501	0.36±0.17	β-Bisabolene	RI, MS
19	1522	t	trans-β-Bisabolene	RI, MS

RI: Retention indices relative to C6-C24 n-alkanes on the DB-5 column; MS: Mass spectroscopy; CoI: Co-injection; t: Trace <0.05%.

exposure times are shown in Tables 2-4. The scolicidal activity of *S. khuzistanica* essential oil at the concentration of 3 mg/mL was 28.58, 30.12, 37.20, and 42.02% after 10, 20, 30 and 60 min of application, respectively (comparing with 7.19% for control group). The difference between the scolicidal effect of *S. khuzistanica* essential oil at this concentration was statistically highly significant comparing to the control group ($P<0.001$).The scolicidal power of *S. khuzistanica* essential oil at the concentration of 5 mg/mL was 51.33, 66.68, 81.12, and 100%, respectively. One hundred scolicidal effect was observed with *S. khuzistanica* essential oil at the concentration of 10 mg/mL after 10 min of application. All experiments of our work, exhibited dose-dependent and also time-dependent scolicidal effect of *S. khuzistanica* essential oil on the protoscolices of hydatid cyst. The results of the present study indicated that the essential oil of *S. khuzistanica* has high scolicidal activity and might be used as a scolicidal agent.

Table 2
Scolicidal effect of *S. khuzistanica* essential oil at concentration of 3 mg/mL following various exposure times (mean±SD).

Exposure time (min)	Protoscoleces	Dead protoscoleces	Mortality rate
10	808.00±33.15	231.00±15.39	28.58
20	901.66±46.73	295.00±25.23	32.71
30	898.66±55.82	334.33±36.85	37.20
60	1 140.66±95.13	479.33±35.92	42.02
Control	1014.00	73.00	7.19

Table 3
Scolicidal effect of *S. khuzistanica* essential oil at concentration of 5 mg/mL following various exposure times (mean±SD).

Exposure time (min)	Protoscoleces	Dead protoscoleces	Mortality rate
10	951.33±88.55	488.30±30.03	51.33
20	1 194.66±28.50	796.66±15.56	66.68
30	1 054.33±29.00	855.33±52.99	81.12
60	938.33±53.59	938.33±53.59	100.00
Control	1 014.00	73.00	7.19

Table 4
Scolicidal effect of *S. khuzistanica* essential oil at concentration of 10 mg/mL following various exposure times(mean ± SD).

Exposure time (min)	Protoscoleces	Dead protoscoleces	Mortality rate
10	875.66±160.00	875.66±160.00	100.00
Control	1 014.00	73.00	7.19

4. Discussion

The control of helminthosis and, generally of all parasitic diseases is usually made with synthetic anthelmintics. The appearance of resistance to synthetic anthelmintics stimulated the research of alternatives, such as medicinal plants active substances[24]. According to circumstances and depending on their efficacy, naturally produced plant anthelmintics offer an alternative that can overcome some of these problems and is both sustainable and environmentally acceptable[25]. essential oils which are accumulated in aromatic plants, are chiefly used as flavors or fragrances, but currently a renewal interest in natural substances has focused attention on plants rich in bioactive compounds. Among these components, essential oils well known for their antimicrobial properties[26]. In the present study, the essential oil of *S. khuzistanica* represented high scolicidal activity. The scolicidal power of this oil at the concentration of 5 mg/mL was 51.33, 66.68, 81.12, and 100% after 10, 20, 30 and 60 min, respectively. One hundred scolicidal effect was observed with *S. khuzistanica* essential oil at the concentration of 10 mg/mL after 10 min of application(comparing with 7.19% for control group).

Up to date, many scolicidal agents have been used for inactivation of the hydatid cyst protoscolices. Many of these scolicidal agents may cause undesirable complications that limit their use. For example adverse side effects has been reported for 20% hypertonic saline, 20% silver nitrate, 0.5% -1% cetrimide, ethyl alcohol, and 20 mg/mL albendazole sulfoxide[23].

According to the results of this work, the scolicidal activity of *S. khuzistanica* essential oil at the concentration of 5 mg/mL (60 min) or 10 mg/mL (10 min) was comparable with scolicidal power of 20% hypertonic saline (15 min), 20% silver (20 min), 0.5%-1% cetrimide (10 min), and 95% ethyl alcohol (15 min).

In the present study, the results of essential oil analysis showed that carvacrol was the major oil component of *S. Khuzistanica* (94.9%). Carvacrol is a monoterpenoid phenol, biosynthesized via aromatization of γ-terpinene to *p*-cymene and the subsequent hydroxylation of *p*-cymene. This phenol along with its precursors (γ-terpinene and *p*-cymene) appears as the major components in numerous phenolic essential oils (thyme, oregano and savory) of Lamiaceae family. It is also one of the most important components of many species including those belong to *Satureja* genus. This component has been reported as one of the strongest antimicrobial agents[27]. This phenolic compound has shown antiseptic, antibacterial, antifungal as well as anti-nocceptive and anti-inflammatory properties[28]. It has been shown that the antimicrobial activity of several essential oils has been attributed to the presence of phenolic compounds such as carvcrol[26].

Ultee *et al*[29] hypothesized that the hydroxyl group and the presence of a system of delocalized electrons are important for the antimicrobial activity of phenolic compounds, such as carvacrol and thymol[29]. Such a particular structure would allow compounds to act as proton exchanger, thereby reducing the gradient across the cytoplasmic membrane. The resulting collapse of the proton motrice force and depletion of the ATP pool lead eventually to cell death[26].

As far as we know, this is the first report on the scolicidal activity of S. *khuzistanica* essential oil. The results of this study allowed us to suggest that *S. khuzistanica* is a rich source of carvacrol that could be used as an effective scolicidal agent. *S. khuzistanica* is an edible plant therefore it is safe for human. Oral administration of *S. khuzistanica* essential oil to rats induced a marked antioxidant, antidiabetic, antihyperlipidemic, and reproduction

stimulatory effects without occurrence of any toxic or adverse effects[30].The results of present study open the possibility of more investigations of *in vivo* scolicidal effect of this traditional medicine.

Conflict of interest statement

We declare that we have no conflict of interest.

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