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ORIGINAL ARTICLE

Scolicidal effectiveness of essential oil from *Zataria multiflora* and *Ferula assafoetida*: disparity between phenolic monoterpenes and disulphide compounds

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Abstract Hydatidosis in humans and animals is a substantial cause of deaths in many parts of the world. Surgery and the chemotherapeutic agents are the main treatments for hydatid disease; however, most of them are accompanied by adverse side effects. In this study, the scolicidal effect of essential oils from *Zataria multiflora* and *Ferula assafoetida* against *Echinococcus granulosus* protoscolices was investigated. Essential oils from *Z. multiflora* and *F. assafoetida* were prepared by hydrodistillation and analyzed by gas chromatography–mass spectrometry. Carvacrol (29.2 %), thymol (25.4 %), *p*-cymene (11.2 %), linalool (9.6 %), and γ-terpinene (8 %) were detected as the main components of the *Z. multiflora*, while the main components of *F. assafoetida* were E-1-propenyl-sec-butyl disulfide (62.7 %), β-ocimene (21.7 %) and β-pinene (5 %). Scolicidal activities of the essential oils against protoscolices for *Z. multiflora* and *F. assafoetida* were obtained at concentrations more than 17 and 60 μg/mL, respectively. Accordingly, it was concluded that both essential oils can selectively reduce protoscolices viability, and *Z. multiflora*, which contains phenolic monoterpenes, is more cytotoxic rather than *F. assafoetida* which contains disulfide compounds. Therefore, *Z. multiflora* and *F. assafoetida* could be recommended as a treatment against hydatid cyst.

Keywords *Zataria multiflora* · *Ferula assafoetida* · Essential oil · *Echinococcus granulosus* · Scolicidal

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Introduction

Hydatidosis in humans and animals is a substantial cause of deaths in many parts of the world. Surgery and the administration of chemotherapeutic agents are the main treatments for hydatid disease (Craig et al. 2007; Kahriman et al. 2011). Various chemotherapeutic agents such as hypertonic saline (Albi et al. 2002), warm water (Moazeni and Alipour-Chaharmahali 2010), acidic and alkaline solutions (Moazeni and Larki 2010), nitric oxide metabolites (Zeghir-Bouteldja et al. 2009), silver nitrate (Caglar et al. 2008), cetrimide (Besim et al. 1998), ethyl alcohol (Erzurumlu et al. 1998), povidone iodine (Landa-Garcia et al. 1997), albendazole (Adas et al. 2009), and chlorhexidine gluconate (Topcu et al. 2009) have been experienced as scolicidal agents. However, many of these scolicidal agents may cause undesirable complications that limit their usage. Therefore, comprehensive scolicidal agents with no local or systemic side effects are needed. In the recent decade, herbal therapy is widely employed for treatment of human and animal diseases. For example, essential oils or the natural product derived from the traditional herbs have been experienced for bioactive components (Bakkali et al. 2008), antioxidant (Sze et al. 2010), antibacterial (Solorzano-Santos and Miranda-Novales 2011), antiparasitic (Anthony et al. 2005), antifungal (Reichling et al. 2010), antidiabetes (Hui et al. 2009), anticancer (Olaku and White 2011), and anti-inflammation (Harikumar and Aggarwal 2008) activities.

Zataria multiflora Boiss, with the Persian name Avishan Shirazi, grows only in warm parts of Iran, Afghanistan, and Pakistan. This aromatic plant belongs to the Lamiaceae family. In traditional medicine in the Middle East, *Zataria* is used in flavoring and preserving food and drinks for its

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antiseptic, analgesic, and carminative properties (Zargari 1993). The composition, antioxidant, antibacterial, antifungal, and anti-inflammation activities of the essential oil from *Z. multiflora* have been previously studied (Amirghofran et al. 2011; Zomorodian et al. 2011; Saei-Dehkordi et al. 2010). *Ferula assafoetida*, with the Persian name Angozeh, is a flowering plant in the family Apiaceae that grows broadly in warm parts of Iran, Afghanistan, and Pakistan. It is herbaceous and perennial and grows up to 2 m in length. The plant has several medicinal properties including antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, analgesic, and antiseptic characteristics (Iranshahy and Iranshahi 2011; Nazari and Iranshahi 2011). Even though the composition, antioxidant, antibacterial, and antifungal activities of the essential oils from these plants have been previously studied, however, there is still no any information about the scolicidal activity of their essential oils. Thus, in the present study, the composition and effective scolicidal activities of essential oils from *Z. multiflora* and *F. assafoetida* against *Echinococcus granulosus* protoscolices were tested. Our results for the first time indicated that essential oil from *Z. multiflora* and *F. assafoetida* exhibited high scolicidal properties even at low concentrations and scolicidal activity of Z. *multiflora* is higher than that of *F. assafoetida*.

Materials and methods

Plant materials from *Z. multiflora* and *F. assafoetida*

The aerial parts (leaves) of *Z. multiflora* were obtained from wild plants in the mountains of Arsenjan (Fars, Iran). The plant was taxonomically identified by a senior plant taxonomist, Prof. Ahmad Reza Khosravi at the Department of Biology, Shiraz University, Shiraz, Fars Province, Iran. The leaves of the plant (5 years old) were separated from their stem and dried for 72 h in the shade. *F. assafoetida* from the mountains of Darab (Fars, Iran) was taxonomically identified by a senior plant taxonomist, Prof. Ahmad Reza Khosravi at the Department of Biology, Shiraz University, Shiraz, Fars Province, Iran. The latex of the plant was collected by incision method. The air-dried latex of the plant (50 g) was solved in 1,000 mL distilled water.

Essential oil preparation

The air-dried leaves (100 g) and latex of the plant (50 g) were hydrodistilled for 3 h using an all-glass Clevenger-type apparatus according to the method outlined by the British Pharmacopeia (1988). The essential oil thus obtained was dried over anhydrous sodium sulfate (Sigma-Aldrich) and stored at 4 °C before the gas chromatography–mass spectrometry (GC-MS) analysis and further experiments.

Identification of the oil components

The GC analysis was carried out using an Agilent Technology chromatograph with HP-5 column (30 m×0.32 mm i.d.×0.25 μm). Oven temperature was performed as follows: 60 to 210 °C at 3 °C/min; 210 to 240 °C at 20 °C/min and hold for 8.5 min, injector temperature 280 °C; detector temperature, 290 °C; carrier gas, N_2 (1 mL/min), and split ratio of 1:50. The GC-MS analysis was carried out using an Agilent 7890, operating at 70 eV ionization energy, and equipped with a HP-5 MS capillary column (phenyl methyl siloxane, 30 m×0.25 mm i.d×25 μm.) with He as the carrier gas and split ratio 1:50. Retention indices were determined using retention times of *n*-alkanes that were injected after the essential oil under the same chromatographic conditions. The retention indices for all components were determined according to the method using *n*-alkanes as standard. The compounds were identified by the comparison of retention indices (RRI, HP-5) with those reported in the literature and by comparison of their mass spectra with the Wiley GC/MS Library, Adams Library, Mass Finder 2.1 Library data published mass spectra data (Adams 2007; McLafferty 2009).

Scolicidal assay

The protoscolices of *E. granulosus* were obtained from the infected livers of sheep killed at Shiraz abattoir in southern Iran. The hydatid fluid was transferred into a glass cylinder. The protoscolices settled at the bottom of the cylinder after 30 min. The settled proscolices were washed three times with normal saline. The viability of the protoscolices was confirmed by their motility under a light microscope (Nikon Eclipse E200, Japan). The protoscolices were transferred into a dark container containing normal saline and stored at 4 °C. In this study, we used different concentrations of essential oils from *Z. multiflora* and *F. assafoetida* (1–100 μg/mL in Tween 80). The essential oils (900 μL) and protoscolices in normal saline (100 μL) were placed in separate test tubes. The contents of the tubes were mixed gently. The tubes were then incubated at 37 °C for 10 min. Thereafter, the upper phase was carefully removed, and 2 mL of 0.1 % eosin stain (Sigma-Aldrich) was then added to the remaining settled protoscolices and mixed gently. After 15 min incubation, the upper portion of the solution was discarded. The remaining pellet of protoscolices was then smeared on a manually scaled glass slide and examined under a light microscope. The percentage of the dead protoscolices was determined by counting a minimum of 1,350 protoscolices. The effective concentrations which totally killed protoscolices, i.e., by 100 % (EC_{100}), were calculated.

Untreated protoscolices were considered as the control group in each experiment. The essential oil was dissolved in Tween 80 and then diluted in normal saline at appropriate concentrations. The final Tween 80 concentration was less than 1 % in all tests, and at this concentration, it had not any effects on the cell viability (Moazeni and Nazer 2010).

Statistical analysis

All data are expressed as the means plus standard deviations of at least three independent experiments. The significant differences between treatments were analyzed by one-way analysis of variance test at $P<0.05$ using statistical package for the social sciences (Abaus Concepts, Berkeley, CA) software.

Results

The main components of *Z. multiflora* essential oil as well as their physicochemical properties are showed in Table 1. The main components were carvacrol (29.2 %), thymol (25.4 %), *p*-cymene (11.2 %), linalool (9.6 %), and γ-terpinene (8 %). The essential oil yield from the leaves was 2.5 % (*w*/*w*). Therefore, the essential oil from *Z. multiflora* supplemented with high levels of phenolic monoterpenes and monoterpenes. The main components essential oil from the latex of *F. assafoetida* as well as their physicochemical properties is showed in Table 2. The main components were E-1-propenyl-sec-butyl disulfide (62.7 %), β-ocimene (21.7 %), and β-pinene (5 %). The essential oil yield from latex was 7 % (*w*/*w*). Therefore, the essential oil from *F. assafoetida* supplemented with high levels of disulfide compounds and monoterpenes.

Different concentrations of the essential oils from *Z. multiflora* and *F. assafoetida* were used for 10 min to investigate scolicidal activity. Viability of protoscolices was confirmed by 0.1 % eosin staining. The scolicidal activity of *Z. multiflora* essential oil is summarized in Table 3. Our results indicated that all protoscolices were killed after 10 min of exposure to concentrations more than 17 μg/mL of essential oil from *Z. multiflora*. The scolicidal activity of *F. assafoetida* essential oil is summarized in Table 4. Our results indicated that all protoscolices were killed after 10 min of exposure to concentrations more than 60 μg/mL of essential oil from *F. assafoetida*. Therefore, in comparison with essential oil from *F. assafoetida*, *Z. multiflora* essential oil displayed more scolicidal potential. Hence, essential oil bearing phenolic monoterpenes exhibited more scolicidal activity rather than the essential oil bearing disulfide compounds. In the present study, eosin staining was used as to check the viability of the protoscolices. Fifteen minutes after exposure to the stain, the protoscolices with no absorbed dye were considered potentially viable (Fig. 1). Protoscolices with absorbed color dye were recorded as dead (Fig. 2). Accordingly, it was concluded that both essential oils can selectively reduce protoscolices viability, and also, *Z. multiflora* is more cytotoxic rather than *F. assafoetida* that might be related to phenolic monoterpenes.

Discussion

In this research, the essential oils from Z. *multiflora* and *F. assafoetida* were used to investigate scolicidal activity. The main components of the essential oil from *Z. multiflora* were carvacrol, thymol, *p*-cymene, linalool, and γ-terpinene. Our results indicated that the essential oil from *Z. multiflora* exhibited a potent scolicidal activity even at low concentrations and at concentrations more than 17 μg/mL, where it killed all protoscolices after 10 min application. In addition, the essential oil from latex of *F. assafoetida* was also used to investigate its scolicidal activity. The main components of

Table 1 Physicochemical properties of carvacrol, thymol, *p*-cymene, γ-terpinene, and linalool from *Z. multiflora* essential oil

Properties	Carvacrol	Thymol	*p*-Cymene	Linalool	γ-Terpinene
Percentage in oil	29.2	25.4	11.2	9.6	8
Physical occurrence	Liquid	Crystalline	Liquid	Liquid	Liquid
Formula	$C_{10}H_{14}O$	$C_{10}H_{14}O$	$C_{10}H_{14}$	$C_{10}H_{18}O$	$C_{10}H_{16}$
Molar mass (D)	150.22	150.22	134.24	154.25	136.24
Density (g/mL)	0.977	0.960	0.857	0.860	0.853
Melting point (°C)	<−20	51	<−20	<−20	<−20
Boiling point (°C)	233	232	177	198	183
Water solubility (g/L)	830	846	6	754	1
Octanol solubility	Soluble	Soluble	Soluble	Soluble	Soluble
P(o/w)	3.6	3.3	4.1	3.8	4.5
Phenol content	Yes	Yes	No	No	No
H-band capacity	Yes	Yes	No	Yes	No

Table 2 Physicochemical properties of E-1-propenyl-sec-butyl disulfide, β-ocimene, and β-pinene from *F. assafoetida* essential oil

Properties	E-1-propenyl-sec-butyl disulfide	β-Ocimene	β-pinene
Percentage in oil	62.7	21.7	5
Physical occurrence	Liquid	Liquid	Liquid
Formula	$C_7H_{14}S_2$	$C_{10}H_{16}$	$C_{10}H_{16}$
Molar mass (D)	162.32	136.24	136.24
Density (g/mL)	0.812	0.818	0.872
Melting point (°C)	<−20	<−20	<−20
Boiling point (°C)	215	177	165
Water solubility (g/L)	Insoluble	Insoluble	Insoluble
Octanol solubility	Soluble	Soluble	Soluble
P(o/w)	4.07	4.27	4.37
Phenol content	No	No	No
H-band capacity	No	No	No

the essential oil were: E-1-propenyl-sec-butyl disulfide, β-ocimene, and β-pinene. Our results also indicated that essential oil from the *F. assafoetida* exhibited a potent scolicidal activity even at low concentrations and at concentrations more than 60 μg/mL, where it killed all protoscolices after 10 min application. Accordingly, scolicidal activity of essential oil from *Z. multiflora*, which contains high levels of phenolic monoterpenes, is higher than the essential oil from *F. assafoetida* which contains high levels of disulfide compounds.

To our knowledge, this is the first report on the scolicidal activity of the essential oils from *Z. multiflora* and *F. assafoetida*. However, scolicidal effects of several natural products derived from medicinal plants were tested by various research groups. For example, methanolic extract from *Z. multiflora* at 25 mg/mL (Moazeni and Roozitalab 2010), methanolic extract from *Allium sativum* at 50 mg/mL (Moazeni and Nazer 2010), and essential oil from *Satureja khozestanica* at 10 mg/mL (Moazeni et al. 2012) exhibited scolicidal potential. Accordingly, the essential oils from *Z. multiflora* and *F. assafoetida* display strong scolicidal activity at very lower concentrations, as compared with previous studies. This activity could be reflected to the main constitute of the essential oil.

Generally, the main components of *Zataria* oil are phenolic monoterpenes, carvacrol, and thymol. They are biosynthesized from γ-terpinene through *p*-cymene. Therefore, these two compounds are always present in oils containing carvacrol and thymol. All of these compounds are monoterpenic nature containing methyl and isopropyl function groups in para position to each other. The main difference between γ-terpinene or *p*-cymene and carvacrol or thymol is the substitution of hydroxyl group on the phenol ring in the thymol and carvacrol, and the only difference between carvacrol and thymol is the position of hydroxyl group (Baser and Demirci 2007).

Study on the antimicrobial activity of monoterpenes indicated that the extent of antibacterial activity induced by monoterpenes can be related to intrinsic hydrophobicity. The hydrophobicity index can be determined experimentally by its partition coefficient in octanol/water ($P_{o/w}$). Carvacrol, thymol, *p*-cymene, and γ-terpinene have a log $P_{o/w}$ of 3.3, 3.64, 4.1, and 4.7, respectively; however, thymol and carvacrol could successfully diffuse through the cell membrane and

Table 3 Scolicidal activity of essential oil from *Z. multiflora* against *E. granulosus*

Essential oil (μg/mL)	Total cells	Live cells	Killed cells	Mortality (%)
Tween 80	1,456	1,425	31	2.12
1	1,341	1,189	152	11.33
10	1,526	732	794	52.03
15	1,452	465	987	68
17.5	1,385	0	1,385	100
20	1,457	0	1,457	100
25	1,416	0	1,416	100
50	1,398	0	1,398	100
75	1,407	0	1,407	100
100	1,370	0	1,370	100

Table 4 Scolicidal activity of essential oil from *F. assafoetida* against *E. granulosus*

Essential oil (μg/mL)	Total cells	Live cells	Killed cells	Mortality (%)
Tween 80	1,365	1,332	33	2.41
1	1,524	1,360	164	10.76
10	1,632	507	1,125	69
25	1,235	395	840	68
50	1,487	208	1,279	86
60	1,543	0	1,543	100
70	1,642	0	1,642	100
80	1,491	0	1,491	100

disrupt cells. Thus, in addition to carbon skeleton, the hydroxyl group is required for biological activity (Dorman and Deans 2000; Griffin et al. 1999). Accordingly, the hydrophobicity index cannot be the main determinant of cell disruption. Hence, a more specific approach is required for cytotoxic activity of essential oils.

Although the scolicidal mechanisms of phenolic monoterpenes did not currently recognized, however, the cytotoxic activities of phenolic monoterpenes against other eukaryote cells to some extent are known. Phenolic monoterpenes exert its cytotoxic activity through cytoplasmic and mitochondrial membrane and through apoptosis induction. Collectively, in eukaryotic cells, the cell membrane and mitochondrial membrane are the major sites of essential oil and phenolic monoterpenes actions. Essential oil passes through cytoplasm membranes, disrupts the structure of lipid bilayer, and changes membrane permeability. The permeabilization of membranes enhances the leakage of ions from cell and reduces membrane electric potential. Reduction of membrane electric potential enhances the leakage of ions, ATP, amino acids, and proteins from cell membrane. The leakage of these ions, specially potassium and calcium, are a clear indication of membrane damages and cell death (Arunasree 2010; Deb et al. 2011; Diaz et al. 2008; Peres et al. 2009; Yang et al. 2010). As shown in Fig. 2, eosin stain could diffuse to protoscolices. It is expected that the essential oil might be exerted its scolicidal activity by change membrane permeabilization. In the mitochondria, essential oils induce membrane depolarization and reduce membrane potential. The reduction of membrane potential affects ionic channel and reduces pH gradient. The reduction of pH gradients affects the proton pomp and the ATP pool. In addition, the essential oil changes the fluidity of mitochondrial membrane, which results in the leakage of radicals, cytochrome c, calcium, and proteins. The leakage of calcium and cytochrome c from mitochondrial membrane leads to the cell death by apoptosis (Chang et al. 2011; Hsu et al. 2011; Kumar et al. 2008; Yin et al. 2011). Accordingly, although more expert studies are required to identify the precise mechanisms of scolicidal activity of phenolic monoterpenes, this scolicidal effect might be applied by apoptosis induction.

Ferula are known for their complex content in bioactive secondary metabolites such as coumarins, phenylpropanoids, sesquiterpenes, and disulphide-bearing compounds. To our knowledge, there is no any information on the

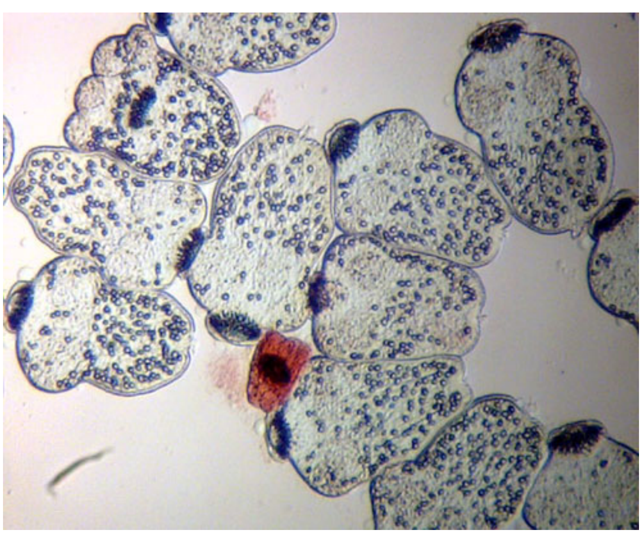

Fig. 1 Live protoscolices after staining with 0.1 % eosin

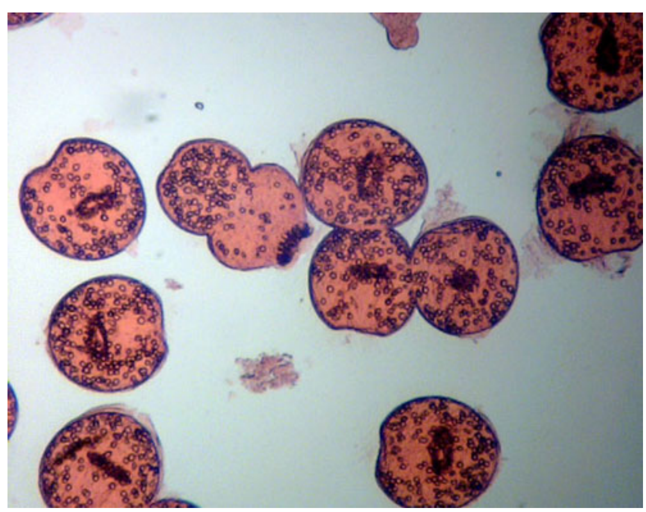

Fig. 2 Dead protoscolices after exposure to essential oils and staining with 0.1 % eosin

scolicidal activity of essential oils from *F. assafoetida* or essential oils bearing disulphide compounds. Although the scolicidal mechanisms of *F. assafoetida* or essential oils bearing disulphide compounds did not currently recognized, however, the cytotoxic activities of essential oils from *F. assafoetida* against other eukaryote cells to some extents are known. The essential oils of different *Ferula* species exhibited cytotoxic effect against human tumor cell lines with lethal concentration (LC_{50}) values in the range of 6–321 μg/mL (Bagheri et al. 2010; Kuete et al. 2011; Mollazadeh et al. 2010; Mazzio and Soliman 2011). The cytotoxic activities of stylosin (a monoterpene extracted from *Ferula ovina*) and mogoltacin (a sesquiterpene-coumarin from *Ferula badrakema*) indicated that they might have cytotoxic properties against human tumor cell lines via quickly induced DNA lesions and increased the number of apoptotic cells (Hanafi-Bojd et al. 2011; Rassouli et al. 2011a, b). In Addition, galbanic acid decreased androgen receptor abundance and signaling and induces G1 arrest in prostate cancer cells. Galbanic acid preferentially suppressed prostate cancer cell growth. Galbanic acid induced a caspase-mediated apoptosis that was attenuated by a general caspase inhibitor (Dall Acqua et al. 2011; Kim et al. 2011; Zhang et al. 2012). Therefore, although more expert studies are required to identify the precise mechanisms of scolicidal activity of disulfide compounds, this scolicidal effect might be exerted by apoptosis induction.

Accordingly, these results indicated that the essential oils from *Z. multiflora* and *F. assafoetida* have scolicidal activities and could kill *E. granulosus*. The scolicidal activity of essential oil from *Z. multiflora* and *F. assafoetida* is very high even at low concentration. Consequently, the benefits of this herbal therapy with essential oil is its potency at lower concentrations, proper performance in a shorter period of exposure, lower toxicity, low viscosity, low cost, and ability to be prepared rapidly. However, further study is also necessary to confirm scolicidal activities of these essential oils in vivo conditions. Thus, the present study recommends the use of essential oil from *Z. multiflora* and *F. assafoetida* for the treatment of hydatid cyst.

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