

Research Paper:**Toxicity and Antiparasitic Efficacy of Essential Oils: Analyses of the Biochemical Compositions and Potencies****Emad Soleimani¹ , Hossein Sarmadian² , Mohammad Arjomand Zadegan³ , Reza Ghasemikhah^{4*} , Hasan Taher Ahmadi⁵** *1. Student Research Committee, Arak University of Medical Sciences, Arak, Iran.**2. Department of Infectious Disease, School of Medicine, Arak University of Medical Sciences, Arak, Iran.**3. Department of Microbiology, Infectious Diseases Research Center (IDRC), School of Medicine, Arak University of Medical Sciences, Arak, Iran.**4. Department of Pediatrics, School of Medicine, Arak University of Medical Sciences, Arak, Iran.**5. Department of Parasitology and Mycology, School of Medicine, Arak University of Medical Sciences, Arak, Iran.*

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Background: Hydatidosis is a common disease of both humans and animals, resulting from infection with the larvae of *Echinococcus granulosus* tapeworm. The aim of this study was to investigate the antiparasitic (protoscolicidal) activities of three essential oils in vitro.

Methods: This study was designed to evaluate the biochemical composition and in vitro antiparasitic effects of *Zataria multiflora*, *Origanum vulgare* and *Mentha pulegium* essential oils. Gas chromatography was performed to identify the main components of the herbal oils. To determine the antiparasitic properties of the essential oils, live protoscoleces from hydatid cysts were exposed to three concentrations of the herbal oils and were incubated at 37°C for 5, 10, 15, 20 or 25 minutes.

Results: The biochemical analysis of these oils indicated that carvacrol and thymol were the major compounds of the *Zataria* oil. Further, carvacrol and thymol in *Origanum* essential oil and pulegone and piperitone in *Mentha* oil were the major compounds. The quickest and slowest antiparasitic effect was achieved from *Zataria* and *Origanum* (10%) or from *Zataria* (0.6%), respectively. The statistical analysis showed a significant difference between the mortality rate of protoscoleces exposed to 0.6% and 1% concentrations, respectively, of *Zataria* and *Origanum* at the predetermined exposure times ($P<0.05$). The three concentrations of *Mentha* had the same significant statistical differences ($P<0.05$).

Conclusion: Essential oils, *Zataria multiflora*, *Origanum vulgare* and *Mentha pulegium* had significant protoscolicidal activities that were dependent on the concentration of the oils and the exposure times.

Keywords: Protoscoleces, Essential oils, *Zataria multiflora*, *Origanum vulgare*, *Mentha pulegium*

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School of Medicine, Arak University
of Medical Sciences, Arak, Iran.**E-mail: ghasemikhah@yahoo.com***Introduction**

Hydatid disease (hydatidosis) is caused by infection with *Echinococcus* larvae. The disease is severe and the treatment is complicated. The current treatment choice is

surgery, which has serious side effects and is costly. The most complications of hydatid surgery are rupture of the cyst and recurrence of disease due to formation of secondary cysts. So far, various mechanical methods and chemical agents have been tested in order to prevent the formation of secondary hydatidosis. The antiparasitic ef-

fects of direct electric current [1], magnetic field [2], x-ray [3], UV light [4], gamma irradiation [5], ultrasound [6, 7], nanoparticles [8], hypotonic saline [9], silver nitrate [10], vinegar [11] and various herbal extracts or essential oils have been evaluated on protoscoleces to certain extents [12-14].

Zataria multiflora, Origanum vulgare and Mentha pulegium have been widely used in traditional medicine, from which numerous therapeutic properties have been suggested. The most effective components of the Zataria multiflora, Origanum vulgare are thymal and caracrol, which are confirmed antibacterial, antifungal, and anti-parasitic agents in vitro [15, 16]. The essential oil from Mentha pulegium has been traditionally used as an anti-septic and antibacterial agent [13].

The current study aimed at evaluating the efficacy of micro and macro-emulsions of Zataria multiflora, Origanum vulgare and Mentha pulegium on protoscoleces of hydatid cyst origins.

Materials and Methods

Preparation of protoscoleces: This in vitro study was conducted on protoscoleces derived from hydatid cysts. Sheep hydatid cysts were collected from a slaughter house in Arak, Iran, and were transferred to the Parasitology Laboratories at Arak University of Medical Sciences. The contents of the cysts were completely evacuated by sterile syringes and washed 3 times with normal saline. The concentration of protoscoleces was set by normal saline so that the number of protoscoleces per mL of the suspension was 6000 at more than 90% viability. The suspension of live protoscoleces was transferred to a dark container and stored at 4°C for the subsequent experiments.

Viability test: The viability of protoscoleces was tested by 0.1% eosin staining. For this purpose 0.1 gram of eosin was dissolved in 100mL of distilled water. Equal volumes of protoscoleces suspension and 0.1% eosin solution were mixed. After three minutes, the protoscoleces were examined under light microscopy. The stained protoscoleces were considered dead and the unstained ones were recorded as being alive [17].

Preparation of medicinal plants and essential oils: Zataria multiflora, Origanum vulgare and Mentha pulegium were obtained from the Agricultural Research Center in Isfahan province and were transferred to the laboratory of Infectious Diseases Research Center at the School of Medicine, Arak University of Medical Sci-

ences. The young leaves form each plant were separated, dried in the dark at low humidity and used for the extraction of the essential oils. The oils from Zataria multiflora, Origanum vulgare and Mentha pulegium were prepared by steam distillation in a Clevenger apparatus. Briefly, a 30 g sample of the dried leaves was added to a 1000mL distillation balloon to which 300 mL distilled water was added. The Clevenger's refrigerant and rotator of cold water were placed on the distillation balloon then the heating unit was turned on.

Micro emulsion preparation: The essential oils dissolved in Tween 80 (Polysorbate 80) were separated from the water on a magnet stirrer for approximately five minutes. Using this method, a milky appearing macro-emulsion was prepared for each plant at 10% concentration. The bright appearing micro-emulsion was prepared at 0.6% or 1% dilution.

Gas chromatography & mass spectrometry

Analysis of essential oils: The analysis of the oil samples was carried out, using an Agilent 7890 Ampere with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 50°C for 3 min and increased progressively to 300°C at a rate of 5°C per min, and kept constant at 300°C for 5 min. The injector and interface temperatures were set at 270°C and 300°C, respectively. The atomic mass unit ranged between 50°C and 300°C. The flow rate of helium as the carrier gas was 2 mL/min. The oven was set at the same temperature as mentioned above for the gas chromatography. See Tables 1, 2 and 3.

Identification of components: The oils' components were identified and confirmed in comparison with their mass spectra and those of a computer library or with the authentic compounds.

Experiments: The experiments were conducted in eleven groups. Each group included six test tubes, each of which was used for a single incubation time. All of the test tubes contained 300 µL protoscoleces suspension (~1800 protoscoleces) plus 300 µL of the herbal materials. The tests were performed as follows:

1: 0.6, 1 or 10% concentration of Zataria multiflora was added to the first, second or third groups of test tubes, respectively.

2: 0.6, 1 or 10% concentration of Origanum vulgare was added to the fourth, fifth or sixth groups of test tubes, respectively.

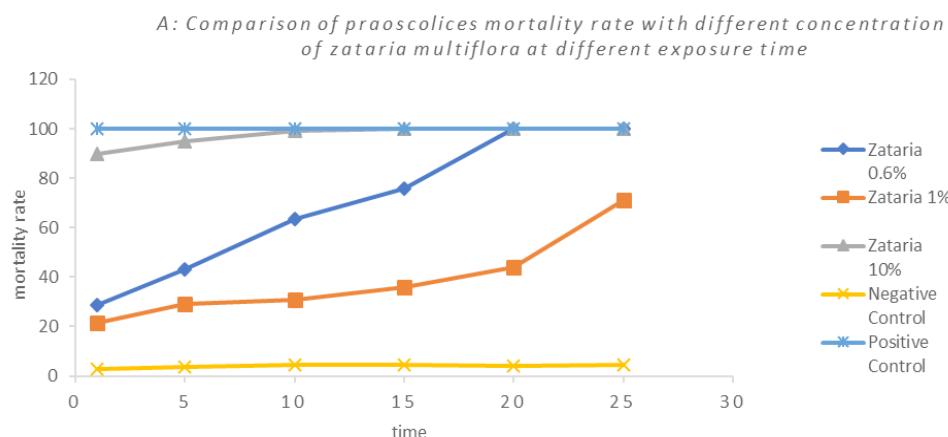


Figure 1. Comparison of protoscoleces mortality rates at varying concentrations of *Zataria multiflora* versus exposure times.

3: 0.6, 1 or 10% concentration of *Mentha pulegium* was added to the seventh, eighth or ninth groups of test tubes, respectively.

An aliquot of normal saline (negative control) or 20% concentration of hypertonic saline (positive control) was added to the tenth or eleventh group, respectively. The test tubes' contents were vortexed then incubated at 37° C for 1, 5, 10, 15, 20 or 25 min. Each experiment was repeated three times. At the end of each incubation time, the tubes contents were mixed with 0.1% eosin. The protoscoleces' mortality rate was examined and the numbers recorded under light microscopy.

Statistical analyses: Statistical analyses were performed on SPSS V. 16 for Windows. The data were presented as the mean values of three separate experiments and expressed as means±standard deviations for **Table 1**. The essential oil composition of *Mentha pulegium*

each group. The differences among the groups and the control were statistically analyzed by one-way ANOVA test. The statistical significance was defined as P<0.05.

Results

Analyses of essential oils: The major compounds in the essential oils derived from the three plants were as follows: Carvacrol and thymol were found in *Zataria* and *oregano* while pulegone and piperitone were identified in *Mentha*.

Antiparasitic effects: The most potent antiparasitic effect was recorded in both *Zataria* and *Origanum* at 10% concentration, which killed all protoscoleces after one minute exposure. The slowest antiparasitic effect was detected for *Zataria* at 0.6% concentration, which caused the death of all of the protoscoleces after 20 minutes of

Peak No.	Retention Time (Min.)	Compound	Combined (%)
1	4.199	Octane	1.479
3	11.48	1,8-Cineole	5.937
5	15.578	Borneol L	1.547
6	16.244	Alpha Terpineol	1.437
7	17.601	Pulegone	6.842
8	20.577	Piperitenone	30.120
9	21.234	Piperitenone Oxide	18.307
10	22.155	Caryophyllene	1.720
11	25.714	Caryophyllene Oxide	2.597

Table 2. Essential oil composition of *Origanum vulgare*

Peak No.	Retention Time (Min.)	Compound	Combine (%)
1	4.193	Octane	2.458
3	8.207	Alpha-Pinene	1.420
4	10.087	Beta-Myrcene	1.789
5	10.961	Alpha-Terpinene	2.907
6	11.284	O-Cymene	11.074
7	12.475	Gamma-Terpinene	17.950
8	15.838	4-Terpineol	1.286
9	19.529	Carvacrol	49.427
10	20.895	Carvacryl Acetate	1.851

Table 3. Essential oil composition of *Zataria multiflora*

Peak No.	Retention Time (Min.)	Compound	Combine (%)
1	4.188	Octane	3.141
2	8.22	Alpha-pinene	4.699
3	10.066	2-ethyl-Butanal	1.063
4	10.373	Decane	1.500
5	10.94	(+)-2-Carene	1.883
6	11.273	Cymol	14.372
7	12.317	Gamma-terpinene	6.006
8	13.529	Linalool	1.116
9	16.341	Dodecane	1.506
10	17.284	Thymyl methyl ether	2.438
11	19.227	Thymol	33.894
12	19.465	Carvacrol	7.759
13	20.387	AcetateThymol	2.024
14	22.092	Trans (beta)-Caryophyllene	3.620
15	22.515	Aromadendrene	1.854
16	23.723	Ledene	1.142
17	25.555	(+) Spathulenol	1.924
18	25.666	Caryophyllene oxide	1.242

B: Comparison of praoscolices mortality rate with different concentration of organum vulgare at different exposure time

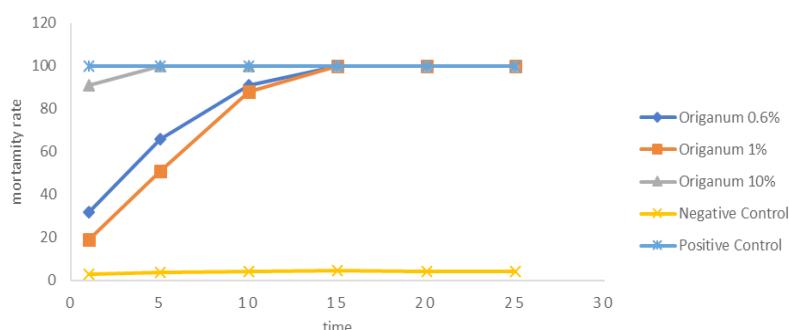


Figure 2. Comparison of protoscoleces mortality rates at varying concentrations of *Organum vulgare* versus exposure times

exposure (Table 1). The survival rates of the protoscoleces from each herbal material are shown in Figure 1.

The statistical analysis revealed a significant difference between the mortality rate of protoscoleces exposed to *Zataria* and *Origanum* at 0.6% and 1% concentrations, respectively, versus the exposure times ($P<0.05$). However, the three *Mentha* oils concentrations revealed similar statistical differences ($P<0.05$). See details in Figures 1, 2 and 3.

Discussion

Hydatidosis is a chronic disease that may occur in various parts of the world. The application of benzimidazole and the Punctuation, Aspiration, Injection and Re-aspiration (PAIR) approach to the management of this disease dates back to many decades ago, even though a definitive medical cure is out of reach [18, 19]. Except for surgery, that is the popular approach, there is no consensus on the best conservative treatment for hydatidosis within the medical community [18, 20, 21]. The most common risk

of surgery is the rupture of cysts, which may lead to the spread of protoscoleces and formation of secondary cysts in the patient at a minimum [11, 22]. The World Health Organization has recommended using herbal medicines and natural alternatives against parasitic diseases [23].

For this reason, plant-derived agents have attracted the attention of many researchers and clinicians, because most herbal compounds are safe and some of them are consumed as foods [15, 17, 24].

In previous studies, the effect of several herbal extracts and their essential oils were tested on protoscoleces from hydatid cysts [16, 17, 25]. In the present study, the antiparasitic effects of three medicinal herbs at varying concentrations were tested versus their exposure times (Figures 1, 2 and 3). Comparing the lethal dosages on the hydatid protoscoleces revealed that the effect of the macro-emulsion was more potent than that of the micro-emulsion. However, the micro-emulsion at low concentration provided desirable effects.

C: Comparison of praoscolices mortality rate with different concentration of mentha pulegium at different exposure time

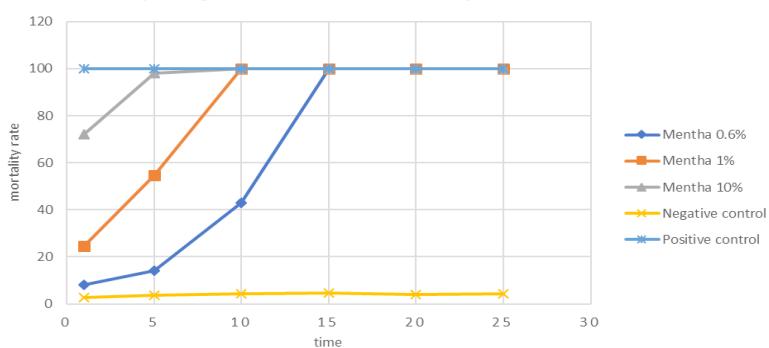


Figure 3. Comparison of protoscoleces mortality rates at varying concentrations of *Mentha pulegium* versus exposure times

Studies have investigated the effects of various plants' essential oils on protoscoleces. For examples, the essential oils from *Satureja khuzistanica* and *Nigella sativa* have destroyed 100% of protoscoleces in 10 minutes. Also, *Rhus coriaria* has been shown to kill 100% of protoscoleces over a 30-min exposure [17, 26]. In this context, the essential oils we prepared were more potent than those used in previous studies. For instance, the essential oil from *Zataria multiflora* and *Origanum vulgare* killed 100% of protoscoleces in one minute while the oil extracted from *Mentha pulegium* achieved the same effect in 10 minutes. The differences in the antiparasitic effects of the essential oils prepared in the current study might be due to differences in their solvents.

In previous studies, DMSO was used as the solvent of essential oils; however, the solubility in water is not comparable to that of DMSO [27], which may induce agglomeration. Of note, the essential oils prepared in this study in macro- and micro-emulsion forms were entrapped in nano-scale vesicles, which made them more potent than other forms of essential oils.

The antiparasitic effects of *Zataria multiflora*, *Origanum vulgare* and *Mentha pulegium* depended on the concentrations of their essential oil and exposure time. In this respect, the *Origanum*'s essential oil at 0.6% concentration demonstrated the best antiparasitic effect compared to those extracted from *Zataria* and *Mentha* plants.

Conclusions

Based on the findings of this study, the extraction, purification and application of natural compounds, such as essential oils, from medicinal plants are promising steps to developing effective treatment for hydatid cysts in humans. We discovered that the essential oil derived from *Origanum* at 0.6% concentration provided the best antiparasitic effect compared to those from *Zataria* and *Mentha* plants. The conservative approach suggested by this study is likely to provide an equally effective treatment for parasitic cysts while offering a safer approach than surgery. Limitations of the Study: Obtaining non-infectious hydatid cysts with 90% viable protoscoleces was one of the limitations of this study.

Recommendations for Future Research: For better effects of the essential oils on hydatid cysts protoscoleces, we recommend that the optimal oil concentrations be determined on protoscoleces grown in a cell culture medium, such as RPMI.

Ethical Considerations

Compliance with ethical guidelines

All instructions and ethical considerations were followed in this project as set by the Research Council of Arak University of Medical Science. The Ethics Committee ethically approved this study (Code: IR.ARAKMU.REC.1394.310).

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Author's contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interests.

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