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The Effect of Aqueous Extract of *Artemisia aucheri* Seed on *Acanthamoeba In vitro*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Acanthamoeba* cause dangerous diseases in humans such as encephalitis and keratitis as an opportunistic pathogen. Due to the antioxidant, antimicrobial, antifungal, anti-*Acanthamoeba* and anti-leishmania activities of *Artemisia,* the aim of this study is investigate the effect of aqueous extract of *Artemisia aucheri* seed on *Acanthamoeba* trophozoites and cysts *in vitro*.
Materials and Methods: *Acanthamoeba* trophozoites and cysts were propagated in appropriate culture medium. Aqueous extract of *Artemisia aucheri* were prepared at concentrations of 2000, 1000, 500, 250, 125 and 62.5 µg/ml and were added to both protozoa forms (trophozoites and cysts). Then, three techniques including trypan blue, MTT and flowcytometry were used to investigate the effect of this extract on *Acanthamoeba* trophozoites and cysts.
Results: It was found that increasing the time and concentration of aqueous extract of *Artemisia aucheri* seed significantly reduced the number of live *Acanthamoeba* trophozoites and cysts ($P \leq 0.05$). At the concentration of 2000 µg/ml the number of live trophozoites was 0% and at the concentration of 62.5 µg/ml the number of live trophozoites was 57.7%.

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Conclusion: The results of this study showed that the aqueous extract of *Artemisia aucheri* has anti-acanthamoeba activity and seems to have beneficial pharmacological effects on some diseases and complications caused by *Acanthamoeba*. Further research is needed to determine this issue.

Keywords: Aqueous extract; Artemisia aucheri; Acanthamoeba; amoebicidal activity; cytotoxic potential.

1. INTRODUCTION

Acanthamoeba is a free-living protozoan widely distributed in nature. This amoeba is capable of growing, dividing and feeding in the trophozoite phase. The parasite forms resistant cysts in adverse environmental conditions. Such cysts can enter the body tissues and cause pathogenesis along with water, soil and dust from outside the body or from a primary point on the lungs, nose, and even skin ulcers. The parasite provides two types of infection: 1) the cause of granulomatosis disease is Granulomatous Amoebic Encephalitis (GAE) among immunocompromised patients and those suffering from AIDS, and 2) another type of infection is amoebic keratitis that may occur among healthy individuals [1,2]. The latter is a painful disease that is accompanied by such symptoms as infiltrated corneal ring, superficial corneal epithelial destruction and severe eye pain (1). Typically, only one eye is affected, but both eyes may be infected simultaneously. Above 80% of amoebic keratitis cases have been reported among those using contact lens [3]. It has been seen among both men and women in the range of 23 and 67 years old [4].

The immunological, biochemical, physiological and genetic criteria are nowadays used to identify and classify *Acanthamoeba* species [3]. Current advances in taxonomy of *Acanthamoeba* have led to identify 17 different genotypes based on 18S rRNA gene sequencing using molecular techniques. The T4 is the most common type of *Acanthamoeba* genotype that causes human disease and is globally the main genotype associated with *Acanthamoeba* keratitis [5].

In recent years, studies in Iran for the diagnosis of amoebic keratitis have shown an increasing rate for incidence of this disease in such a territory [3]. The important point regarding *Acanthamoeba* keratitis is to recognize the right medication or combination to treat the disease while being effective on different *Acanthamoeba* species and not toxic to eye tissue. Herbal remedies compose a group of medicines with worldwide popularity in recent years because of their favorable benefits and high efficacy, and according to the report of World Health Organization (WHO), herbal remedies account for 20% of the total pharmaceutical market. Many of the commonly used medications today have herbal origin [6].

Presence of such features as lack of harmful and side effects of chemical medications on one hand and the environmental pollution that threatens the planet on the other hand, as well as the much lower cost of the herbal products compared to chemical pharmaceutical industry have increased the rate of taking herbal medications.

Besides, *Artemisia* is one of the herbs used today in the treatment of many diseases. These plants belong to the family Asteraceae and have different species, most of which have bitter and fragrant leaves with medicinal properties. Artemisia in Iran has 34 annual and multi-annual herbaceous species scattered throughout Iran. Artemisia has antioxidant, antimicrobial, antifungal, anti-acanthamoeba, anti-malarial and anti-leishmania activities [7-12].

Although some of the medicinal effects of *Artemisia aucheri* (e.g. antioxidant, anti-parasitic and anti-leishmania activities) are well known [13] and due to their anti-malarial activity, it has received worldwide attention and studies, its effect on *Acanthamoeba* has not been investigate in Iran. Therefore, the aim of the present study was to evaluate the effect of *Artemisia aucheri* seed on *Acanthamoeba* trophozoites and cysts *in vitro*.

2. MATERIALS AND METHODS

This study was approved by the Ethics Committee for Research at Semnan University of Medical Sciences with the ID MUMS.REC.1396.263.

2.1 Preparation of *Artemisia aucheri* and Protozoa *Acanthamoeba*

Artemisia aucheri is a native plant of Semnan province. In autumn, this plant was collected

from its growing medium near Semnan and was approved by the herbarium expert (Database of dried and preserved plants) and Semnan Agricultural Jihad Research Center.

The *Acanthamoeba* species used in this study are those isolated from soil of Varamin Parks. Specimens were cultured on the culture medium of 1.5% non-nutrient agar along with Escherichia coli as a nutrition and then they were incubated at a temperature of 26ºC.

2.2 Preparation of Dry Extract of *Artemisia aucheri*

After transferring to laboratory, the *Artemisia aucheri* was dried under ideal and optimum conditions (i.e. shade, room temperature and appropriate humidity). In order to prepare the extract, 50 g of seed was completely crushed and milled, then 500 ml of sterile distilled water was added and incubated at room temperature for 72 hours. Afterwards, it was inserted above the Chauffe Ballon Heating Mantle for 2 hours at a moderate temperature in order to be boiled. Then the solution was entirely filtered off by sterile gas and filter paper.

The filtered liquid was poured into 50 ml Falcone tubes which were then placed in a freezer with a temperature of -70ºC. The tubes were then inserted in the lyophilizing apparatus for 24 hours as long as preparation of dried extract. Until use, the dried extract was stored in the freezer with a temperature of -20ºC.

2.3 Preparation of Trophozoites and Cysts of *Acanthamoeba*

The protozoa *Acanthamoeba* was cultured on NNA plates at a temperature of 26ºC along with Escherichia coli. After 72-96 hours, the trophozoites were washed twice by sterile saline solution and centrifuged twice with a speed of 1500 rpm for 5 minutes. To prepare the cysts, *Acanthamoeba* was cultured in the same conditions and after three weeks, the cysts were washed twice in plates by sterile saline solution and centrifuged twice with a speed of 2000 rpm for 5 minutes.

The number of trophozoites and also cysts were determined by Trypan Blue staining and counting them directly on the Neubauer chamber. The final concentration of 15×10^4 was found to be trophozoites or cysts per ml and these protozoan forms were rapidly used in the experiment.

2.4 Preparation of Different Concentrations of *Artemisia aucheri*

First, 20 mg of dried *Artemisia aucheri* extract was weighed, poured into a sterile micro-tube and finally 1 ml of distilled water was added to the extract. Concentrations of 2000, 1000, 500, 250, 125 and 62.5 µg/ml were prepared through this aqueous extract.

2.5 Cellular Life Evaluation

Evaluation of the efficacy of aqueous extract of *Artemisia aucheri* seed on coloration of cysts and trophozoites of *Acanthamoeba* was determined using Trypan blue staining, MTT Assay as well as Flow-Cytometry.

2.5.1 Trypan blue test

In this method, alive cells will remain colorless due to their intact and robust membrane and thus resistive against color import, however, the dead or dying cells which have lost their membrane resistance will turn blue (their nucleus and cytoplasm are stained), indicating the efficacy of this medication on cysts and trophozoites of *Acanthamoeba*. After sufficient growth, the cultured cells were first treated by *Artemisia auchery* aqueous extract with the concentrations of 2000, 1000, 500, 250, 125 and 62.5 µg/ml. After two or more days, it was incubated by trypan blue with a concentration of 0.4% for 10 minutes and then using Neubauer chamber and optical microscope, the number of dead and living cells was counted and reported. In each experiment, a negative control (including parasite and distilled water) and a positive control (including parasite and 0.01% polyhexanide drop) were used, as well.

2.5.2 MTT assay

The MTT assay is a standard colorimetric test to evaluate cellular metabolic activity and to assess the toxicity of the materials versus the cell life. MTT powder (Dimethylthiazol Diphenyltetrazolium Bromide) is resuscitated by mitochondrial succinate dehydrogenase enzyme in intact cells and forms purple Formazan precipitate, which is absorbed at the wavelength of 570 nm.

The solution containing trophozoites or cysts was poured into plate wells and added to wells of aqueous extract of *Artemisia aucheri* seed with concentrations of 200, 100, 50 and 25 µg/ml.

After 24 hours of the specimen treatment, MTT material was added to the wells. Afterwards, the plate was inserted in darkness and an incubator having a temperature of 37ºC for 3 hours. Then, the optical absorption of the wells was read by a micro-plate reader at 570 nm, which is directly related to the number of living cells. In this experiment, by examining the results, the appropriate medicinal dose with the most efficacy can be determined.

2.5.3 Evaluation of cellular death using flow-cytometry

In flow-cytometry, by examining the diffraction of the laser beam emitted into a cellular set, the cell's state (e.g. size, shape, and structure) can be largely ascertained. The intensity of light refraction was directly correlated with cell size, whereas the amount of refraction at right angle against the reflected laser light was correlated with cell density, indicating the presence of intracellular organelles.

Annexin-V staining method was used to differentiate necrotic and apoptotic cells of *Acanthamoeba* cysts exposed to different doses of the plant extract. Propidium Iodide (PI) and annexin staining can also be used individually to perform the test. In this study, the precise kit (Roche, Germany) was used for more accuracy.

2.6 Statistical Analysis

Analysis of the data resulted from effect of the plant extract on the protozoa was performed using three or more dependent groups (repeated measures) and one-way analysis of variance (ANOVA) to determine the significant percentage. Also, the software SPSS Ver.18 was used for data analysis and the level of significance was set at 0.05.

3. RESULTS

In vivo staining of trophozoites with Trypan Blue: Using in vivo trypan blue staining, the number of living *Acanthamoeba* trophozoites were counted after 24 and 48 hours compared to the control group. Data analysis was performed using comparative test of the few dependent groups to determine the significant percentage. It was found that increased time and concentration of *Artemisia aucheri* extract significantly reduced the number of living *Acanthamoeba* trophozoites (P≤0.05), such that the number of living trophozoites was reported to be 0.0% and 57.7%, respectively, at concentrations of 2000 µg/ml and 62.5 µg/ml (Table 1 and Chart 1).

In vivo staining of Cysts with Trypan Blue: Using in vivo trypan blue staining, the number of living *Acanthamoeba* cysts were counted after 24, 48 and 72 hours compared to the control group. Data analysis was performed using comparative test of the few dependent groups to determine the significant percentage. It was found that increased time and concentration of *Artemisia aucheri* extract significantly reduced the number of living *Acanthamoeba* Cysts (P≤0.05), such that in the highest concentration (i.e. 2000 µg/ml), the number of living cysts was reported to be 3.4%, while it was reached 0.0% after 48 and 72 hours (Table 2 and Chart 2).

3.1 Evaluation of Macrophages' Survival by MTT Assay

Macrophages'survival rate after effect of aqueous extract of *Artemisia aucheri* seed (with

Table 1. Survival rate of *Acanthamoeba* trophozoites after effect of different concentrations of aqueous extract of *Artemisia aucheri* seed. Data is derived from mean value of at least three independent replicates

Concentration (µg/ml)	Survival rate (Mean±SD) 24 hours	Survival rate (Mean±SD) 48 hours
Parasite without medication (negative control)	100±0	100±0
2000	0000±0	0000±0
1000	7.7±.7	4±.7
500	11.5±.7	7.2±.7
250	26.9±.7	20±.7
125	42.3±.7	28±.7
62.5	57.7±.7	44±.7
0.01% Polyhexanide (Positive Control)	42.3±.7	36±.7

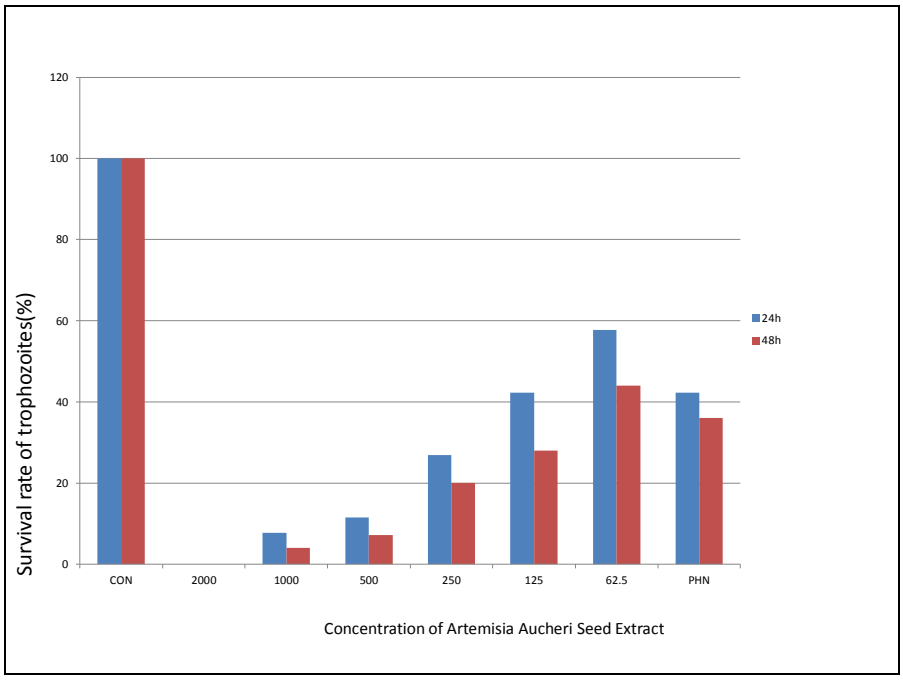

Chart 1. Column chart of survival rate of *Acanthamoeba* trophozoites at times of 24 and 48 hours after effect of different concentrations of aqueous extract of *Artemisia aucheri* seed. Data is derived from mean value of at least three independent replicates. Vertical lines indicate standard deviation

Table 2. Survival rate of *Acanthamoeba* cysts after effect of different concentrations of aqueous extract of *Artemisia aucheri* Seed. Data is derived from mean value of at least three independent replicates

Concentration (µg/ml)	Survival rate (Mean±SD) 24 hours	Survival rate (Mean±SD) 48 hours	Survival rate Mean±SD) 72 hours
Parasite without medication (negative control)	100±0	100±0	100±0
2000	3.4±.7	0000±0	0000±0
1000	20.6±.7	17.3±.7	3.4±.7
500	31±.7	27.6±.7	17±.7
250	34.5±0	31±.7	24±.7
125	51.7±.7	38±.7	31±.7
62.5	65.5±.7	52±.7	45±.7
0.01% Polyhexanide (Positive Control)	58.6±.7	31±.7	20±0

concentrations of 200, 100, 50 and 25 µg/ml) was determined after 24 hours using MTT assay (Table 3 and Chart 3). According to our results, macrophages'survival rate reduced with increasing concentration and at concentration 25, the highest macrophages'survival rate was observed which was/was not statistically significant.

The amount of IC50 content of aqueous extract of *Artemisia aucheri* seed on trophozoites, cysts and macrophages after 24 hours; the results indicated that in the concentrations of 93.75, 125 and 150 µg/ml of the extract, half of the trophozoites, cysts, and macrophages, respectively, is killed. Thus, at a lower concentration of the extract, half of the protozoa is killed.

3.2 Results of Flow-cytometry

In the control group, the rate of living cells and apoptosis after 24 hours was 98.6% and 1.5%, respectively. In the specimens treated with

aqueous extract of *Artemisia aucheri* seed, after 24 hours, living cells, apoptotic, secondary apoptotic and necrosis were seen to be 92%, 1.5%, 4.9% and 1.5%, respectively (Fig. 1).

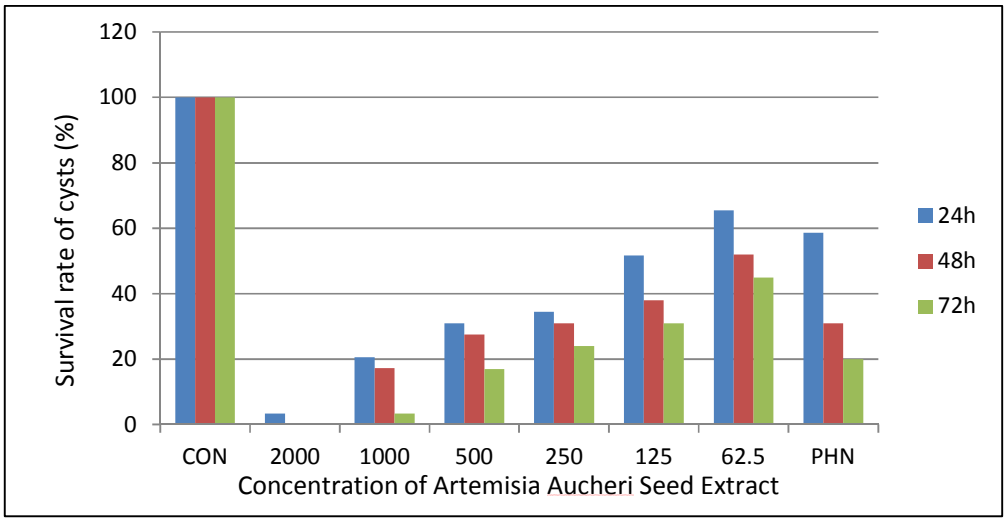

Chart 2. Column chart of survival rate of *Acanthamoeba* cysts at times of 24, 48 and 72 hours after effect of different concentrations of aqueous extract of *Artemisia aucheri* seed. Data is derived from mean value of at least three independent replicates. Vertical lines indicate standard deviation

Table 3. Macrophages' survival rate

Concentration (µg/ml)	Macrophages' survival rate
200	42.7
100	62.3
50	71.6
25	76.99

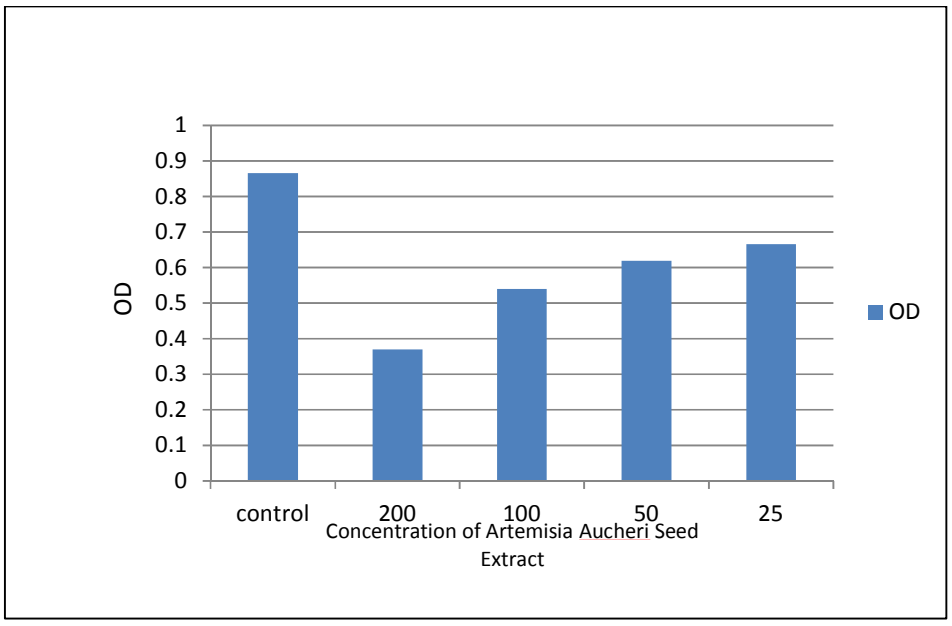

Chart 3. Column chart for Macrophages' survival rate

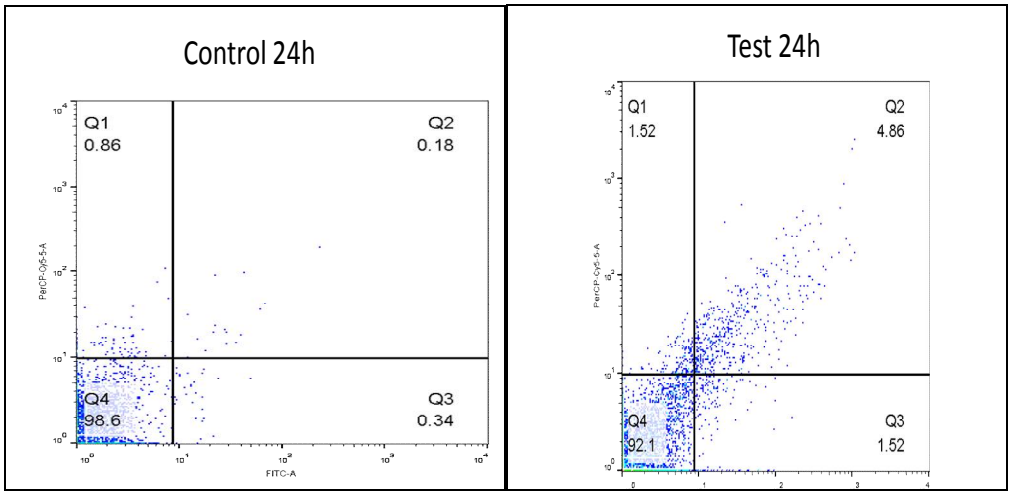

Fig. 1. Flow-cytometric analysis of *Acanthamoeba* cysts on specimens as well as negative control after effect of aqueous extract of *Artemisia aucheri* seed

4. DISCUSSION

Artemisia, which has a characteristic odor and taste, is known as aromatic plant due to its monoterpene and sesquiterpene. For this reason it is used in folk remedies and its phytotoxic activity makes it a natural herbicide as a candidate [14]. *Acanthamoeba* is an amoeba with a very wide distribution in the nature and different environments of human life. Given the wide and inclusive spread of this amoeba, its frequent exposure is inevitable, as anti-*Acanthamoeba* antibodies is often found among healthy individuals, indicating frequent exposure to this amoeba [15]. *Acanthamoeba* is known to cause two major diseases that lead to major health problems: 1) *Acanthamoeba* keratitis is a painful disease that is mainly seen among healthy individuals (in terms of immunity) and can lead to vision loss [16]; 2) another disease is granulomatous amoebic encephalitis, or GAE, which is a rare and chronic illness most commonly seen among immunocompromised individuals and usually results in death. Diagnosis and treatment of *Acanthamoeba* encephalitis is highly controversial. The rate of improvement in GAE is rare due to delay in diagnosis. Therefore, early molecular testing by valid diagnostic centers is recommended for early detection of the disease [17].

In present study, the effect of different concentrations of aqueous extract of *Artemisia aucheri* seed on *in vitro* conditions was investigated. Based on the results of different concentrations of aqueous extract on trophozoites and *Acanthamoeba* cysts *in vitro*, with increased extract concentration at a given time,the mean lethal percentage on trophozoites increased; also with increasedproximity duration of aqueous extract at each concentration,the lethal percentage and the effectiveness of the extract on killing the trophozoites and *Acanthamoeba* cysts significantly increased, especially at the concentration of 2000 µg/ml. In this study, the survival rate of *Acanthamoeba* trophozoites followed by effect of extract's different concentrations after 24 reached 0% at the highest concentration of the extract, i.e. 2000 µg/ml; it reached 0% after 48 hours, as well. Also, the survival rate of *Acanthamoeba* cysts followed by effect of a concentration of 2000 µg/ml of the aqueous extract reached 3.4% and 0% after 48 and 72 hours, respectively.

Unfortunately, there are currently only a few medicationsin the market to treat a large number of parasitic diseases. Therefore, using the biochemical and biological properties of the parasitic species should increase the effectiveness of the medications; in addition, in case of long-term use, many medications lead to variable and toxic effects [18]. According to the researchers, a medication is of clinical value that destroys both the trophozoite and cystic *Acanthamoeba* stages. The results of all pharmacological tests on the parasite show that the trophozoite form of the parasite is much more sensitive than its cystic form [19].

Despite the harmful effects of some Artemisia species, it is widely used in various fields,

including pharmaceuticals, landscape architecture and agricultural areas. Some species of this genus are selected as notable classical drugs for the treatment of malaria, hepatitis, cancer, inflammation and infection by fungi, bacteria, and viruses In previous studies, *Artemisia* species was reported to have anti-malarial, anti-cancer, anti-diabetic, anti-hepatitis, anti-inflammatory, anti-hypertensive, anti-bacterial, anti-oxidant, anti-melanogenic and anti-viral effects [20,21].

Kolören, et al. (2016) first reported the genetic variety of *Artemisia* species in Ordu province in Turkey by confirming the previously reported species from this region with molecular techniques [20].

Acanthamoeba must be in the trophozoite stage to bind to human corneal epithelial cells [15]. Recent studies have shown that cysts are ineffective. When the *Acanthamoeba* trophozoite form binds to the corneal epithelium, it produces various proteases which facilitate invasion to the cornea and cause corneal cytolysis. The infection leads to destroy the corneal epithelium and stroma, to penetrate the inflammatory cells and eventually thins the cornea and creates a hole. Resistance of *Acanthamoeba* cysts to antibiotics is the major problem in the treatment of *Acanthamoeba* keratitis. In fact, *Acanthamoeba* may remain in the cyst form for months and may reactivate after discontinuation of treatment [22].

Occurrence of *Acanthamoeba* keratitis in Iran has been reported to be in the range 25-34% [23, 24]. If keratitis is treated appropriately in the early stages, treatment outcomes will be favorable. The best treatment outcome can be achieved by early diagnosis, adequate treatment, and a high level of patient's compliance [25].

Previous studies on the effects of Artemis extract on *Acanthamoeba* have been performed. Chegini, et al. (2017) reported that the alcoholic extract of aerial organs of *Artemisia annua* had a stronger effect on trophozoites compared to the aqueous extract; and aqueous extract of *Artemisia annua* showed better effect on *Acanthamoeba* cysts. Also, the survival rate of Acanthamoebater fozoites followed by effect of different concentrations of the extract after 24, 48 and 72 hours at the highest concentration of extract (i.e. 10,000 µg/ml) reached to 72.69%, 67.06% and 58.25%, respectively. The survival rate of *Acanthamoeba* cysts followed byeffect of a concentration of 10,000 µg/ml of aqueous extract reached 86.02%, 84.97% and 81.53% after 24, 48 and 72 hours, respectively [9].

Comparison of our results with those of Chegini, et al. indicate that aqueous extract of *Artemisia aucheri* seed at lower concentration (2000 µg/ml) was more effective than aqueous extract of *Artemisia annua*at higher concentration (10,000 µg/ml).

Koloren, et al. in a study to investigate amoebicidal and amoebistatic effects of Artemisia argyi methanolic extracts on *Acanthamoeba* castellanii Trophozoites and Cysts, indicated that Artemisia argyi leaf extract has cytotoxic and anti-amoebic activities. In their study, the growth of trophozoites stopped in Artemisia argyi extract with 50% inhibitory concentrations (IC50)/8 h for 37.4 mg/ ml and 74.8 mg/ml extract solution and had stronger amoebicidal activity on the cysts with IC50/72 h. Also, Artemisia showed stronger inhibitory effects on bronchial epithelial cells at the concentrations of 9.4, 18.7, 37.4 and 74.8 mg/ml [26].

Cecilia, et al. (2016) revealed that use of 200 µg/ml of Artemisin in content for 24 hours reduced the number of trophozoites; and reduction of this content to 100 µg/ml was less effective compared to a content of 200 µg/ml [27].

Baldemir, et al. [28] reported effectiveness of *Artemisia asteraceae* on *Acanthamoeba*. In these studies, the results of the anti-amoebic activity of Artemisia was dose and time dependent, which is in line with conclusion of the present study [28].

In general, the results of this study were consistent with previous studies and aqueous extract of *Artemisia aucheri* seed can be effective in removing *Acanthamoeba* trophozoites and cysts *in vitro*.

5. CONCLUSION

The results of this study showed that the aqueous extract of *Artemisia aucheri* has anti-*Acanthamoeba* activity and also seems to have beneficial pharmacological effects on some diseases and complications of *Acanthamoeba* (especially *Acanthamoeba* keratitis). Obviously, for approval of this issue and use of this extract as a therapeutic agent, more research needs to be conducted.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the Ethics Committee for Research at Semnan University of Medical Sciences with the ID MUMS.REC.1396.263.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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