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Investigation of In Vitro Amoebicidal Activities of *Trachystemon orientalis* on *Acanthamoeba castellanii* Cysts and Trophozoites

Trachystemon orientalis'in *Acanthamoeba castellanii* Kistleri ve Trofozoitleri Üzerine In Vitro Amoebisidal Aktivitelerinin Araştırılması

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ABSTRACT

Objective: Acanthamoeba species cause important diseases such as Acanthamoeba keratitis, granulomatous amoebic encephalitis and cutaneous acanthamoebiasis. In this study, we investigated the amoebicidal action of methanol and ringer extracts of *Trachystemon orientalis* plant on cyst and trophozoite forms of *Acanthamoeba castellanii* by evaluating cell viability percentage and IC_{50} values.

Materials and Methods: The in vitro amoebicidal effects of *T. orientalis* methanol and ringer extracts prepared at different concentrations on *A. castellanii* trophozoites and cysts were investigated.

Results: The IC_{50} value of *A. castellanii* trophozoite form at 72^{nd}, 48^{th}, 24^{th}, 8^{th}, 6^{th}, 3^{rd} and 1^{st} hours were 4, 7.2, 8.7, 11.1, 14.1, 21.4, and 23.8 mg/mL with methanol extract of T. orientalis, respectively, and 8.5, 11.1, 14.4, 15.9, 20.9, 23.9 and 25.8 mg/mL, with ringer extract of T. orientalis respectively. T. orientalis 80 mg/mL methanol extract showed lethal effect for the all trophozoites at 72^{nd} hour. The viability (%) of the ringer extract of T. orientalis at 72^{nd} hour was 1.6 ± 0.3.

Conclusion: Methanolic extract of T. orientalis was found to be more effective than ringer extract on Acanthamoeba trophozoites. *A. castellanii* cysts showed similar sensitivity to methanolic and ringer extracts of T. orientalis. Both extracts showed greater amoebicidal activity on trophozoites when compared to cysts. Whether the concentrations explored in the existing study are cytotoxic for mammalian cells, or have toxic effects on experimental animals should be examined with future in vivo studies. Furthermore, the mechanism of action for the active substances responsible for biological activity should be investigated in future studies.

Key Words: *Acanthamoeba castellanii*, Amoebicidal effect, Trachystemon orientalis

ÖZET

Amaç: Acanthamoeba türleri, Acanthamoeba keratiti, granülomatöz amibik ensefalit, kutanöz acanthamoebiasis gibi önemli hastalıkların etkenidir. Bu çalışmada, *Trachystemon orientalis*'den elde edilen ringer ve metanol özütlerinin *Acanthamoeba castellanii* kist ve trofozoitleri üzerindeki yüzde (%) canlılık etkisi ve IC_{50} değeri araştırılmıştır.

Materyal ve Yöntemler: Farklı konsantrasyonlarda hazırlanan *T. orientalis*'in metanol ve ringer özütlerinin *A. castellanii* trofozoitleri ve kistleri üzerinde in vitro amoebisidal etkisi incelenmiştir.

Bulgular: *A. castellanii* trofozoit formu üzerindeki IC_{50} değeri sırasıyla 1., 3., 6., 8., 24., 48., ve 72. saatlerde T.orientalis'in metanol özütünde, 23.8, 21.4, 14.1, 11.1, 8.7, 7.2 ve 4 mg/mL, T. orientalis ringer özütünde ise, 25.8, 23.9, 20.9, 15.9, 14.4, 11.1 ve 8.5 mg/mL olarak saptanmıştır. *T.orientalis*'in 80 mg/mL'deki metanol özütü 72. saatte tüm trofozoitleri öldürmüştür. T.orientalis'in ringer özütünde ise 72. saatte % canlılık oranı 1.6 ± 0.3 olarak bulunmuştur.

Sonuç: *T. orientalis*'in metanol özütü ile ringer özütünün A. castellanii trofozoitleri üzerine amoebisidal etkileri karşılaştırıldığında, T. orientalis'in metanol özütünün, ringer özütünden daha etkili olduğu bulunmuştur. *A. castellanii* kistlerinin her iki özüte karşı duyarlılığı benzer olup Acanthamoeba trofozoitleriyle karşılaştırıldıklarında, trofozoitlerin özütlere olan duyarlılığının kistlerden daha fazla olduğu görülmüştür. Araştırmada etkili bulunan konsantrasyonların memeli hücresi ve deney hayvanları için toksik olup olmadığının tespit edilmesi amacıyla in vivo çalışmaların yapılması ve bu özütlerin biyolojik aktivitesini sağlayan etken maddelerin etki mekanizmalarının araştırılması gerektiği önerileri sunulmuştur.

Anahtar Kelimeler: *Acanthamoeba castellanii*, Amoebisidal etki, Trachystemon orientalis

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Introduction

As in other countries, plants of medicinal significance have long been used in Turkey in the form of tea or spices to treat various diseases. They are also widely used in various paints, fragrance and taste industries, food additives, perfumes, cleaning products and cosmetics industry, and their new benefits are discovered over time (1,2).

Many microorganisms causing infectious diseases and hospital acquired infections have developed resistance against many antibiotics that have been used for their treatment. Additionally, use of some antifungal, anttiparasitic and antiviral drugs has been restricted because of their high toxicities (3). Since parasites have eukaryotic structures as human cells, it is difficult to design drugs that will show selective toxicity (4,5).

Acanthamoeba species, which are among the protozoa commonly found in the environment, show a wide distribution in the world. Dust, soil, air, mud, hot springs, seawater, swimming pools, sewers, air purifying units, tap and bottled water, hospitals, dialysis units, dental treatment units, contact lenses, lens storage containers and lens cleaning solutions, bacterial and fungal cultures and mammalian cell cultures are among the habitats of Acanthamoeba species (6,7). Acanthamoeba keratitis (AK) which appear especially in contact lens wearers, cutaneous acanthamoebiasis and granulomatous amoebic encephalitis (GAE), which are mostly lethal, are important diseases caused by Acanthamoeba species. Besides, Acanthamoeba species are clinically important because they are involved as vectors in many viral and bacterial diseases (8,9,10).

Many of the drugs used against Acanthamoeba infections may have toxic effects, or microorganisms can develop resistance against these drugs. Therefore, new natural resources are required to develop new, effective and more reliable drugs. Examination of natural plants for his purpose is the most effective way for discovering new active compounds. In our review of literature, we did not encounter any study examining amoebicidal, antiviral or bactericidal actions of the plant *T. orientalis*. Nevertheless, *T.orientalis* contains volatile fats, tanin, nitrate salts, saponin, mucilage and resin. Diuretic and antipyretic actions have been reported. It has been reported that it may also have antideppressant effect (11). It is known as blood cleaner by local people (12). With the assumption of possible antiparasitic effects of the seondary metabolites present in the plant, it was aimed to investigate the percent viability and IC_{50} values of methanolic and Ringer extracts of T.orinetalis against Acanthamoeba castellanii cyst and trophozoites in the present study.

Material and Methods

Preparation of Plant Extract: *T. orientalis* plant, which is known as 'kaldirik' by local people, was taken from a local bazaar in the Ordu province. The flowers, leaves and stem parts of the plant was washed with sterile distilled water in laboratory, and then dried with drying paper. Sixty grams samples from the leaf, flower and stem parts of the plant were weighed in an analytical scale, and grounded in 250 mL of methanol. The obtained mixture was mixed for 72 hours in a shaker microclimate device. At the end of this time, the mixture was filtered through a filter paper to provide coarse filtration. Later on, the mixture was passed through a vacuum filter containing a bacterial filter (pore size: 0.22 μm) for elimination of bacteria. Two hundred mL of the filtrate was taken into a sterile volumetric flask, and the methanol was evaporated via evaporator. The final concentration of the T. orientalis extract was calculated as 80 mg/mL.

Trophozoites: The suspension of *Escherichia coli* (ATCC 25922) was cultured in ringer agar growth mediums. Then, *A. castellanii* (ATCC 30010) trophozoite strain was inoculated to the same mediums. The growth mediums were incubated at 26 ± 1 °C for 3 days. At the end of this time, the growth mediums were washed with sterile ringer solution for three times in a biosafety cabinet while taking caution not to damage the trophozoites. The surface was then kindly swept with a sterile loop, and collected in 15 mL sterile falcon tube, and the suspension was centrifuged at 10°C and 3000 rpm for 5 minutes. After centrifugation, the supernatant portion was discarded, and the sediment was taken into Eppendorf tubes to be used in the experiments. The sediment was then taken to a Thoma cell counter, and the trophozoites were counted under a light microscope. The number of trophozoites per mL was calculated. The final concentration was set to $2x10^6$ trophozoites/mL before starting the experiment. In order to test the vitality of the trophozoite, 0.4% trypan blue was added to the sediment, the mixture was vortexed and allowed to stand at room temperature for 3 minutes. The mixture was put onto a slide and covered with a coverglass, and examined with 40x magnification of light microscope. The trophozoites that took up the dye and appeared blue were evaluated as dead. The experiment was initiated with 98% viability.

Cysts: After inoculating the trophozoites into the ringer agar medium, a part of the growth mediums

were incubated at 30 ± 1 °C for 2 weeks to permit transformation to cyst forms. At the end of this time, the same procedures that were applied to the trophozoite forms were also performed to the cyst forms. The final concentration was set to $2x10^6$ cysts/mL prior to the experiment. The experiment was initiated with 100% viability.

Experimental Design: 1 mL of the plant extract (T. orientalis 80 mg/mL) was taken and put into sterile Eppendorf tubes. Seven serial dilutions were prepared as indicated to obtain different concentrations of the plant extract. The dilutions were prepared using (1%) methanol and ringer solution. The resulting concentrations were 1.25, 2.5, 5, 10, 20, 40, 80 mg/mL.

Testing Amoebicidal Activity of Plant Extracts on *A. castellanii* Trophozoites and Cysts: 100 µl of *A. castellanii* trophozoite/cyst suspension was added to each sterile Eppendorf tube. Subsequently, 100 µl from each of the plant extract methanol (1%) /ringer solution dilutions was added. The tubes were vortexed, and later incubated at 26 ± 1 °C. The Eppendorf tubes were taken out from the incubator at 1st, 3rd, 6th, 8th, 24th, 48th and 72nd hour, respectively for the performing the counts. Before counting, cell viability was evaluated by treating with 0.4% trypan blue. 20 µL of 0.4% trypan blue dye solution was added to each of the seven sterile eppendorf tubes. Later on, 20 µL of each of the 80 mg/mL, 40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, and 1.25 mg/mL dilutions series, and control solution (contains only methanol (1%)/ringer solution and *A.castellanii* (trophozoite/cyst) suspension but no plant extract) was added to the tubes, and vortexed. After standing for 3 minutes at room temperature, the number of viable cells in each tube was examined using a Thoma cell counter, starting from the tube with highest concentration. Trophozoite and cyst counts were evaluated respectively for both ringer and methanol dilution series. All experiments were performed with three repetitions and statistical analyses were performed to determine the cell viability percentage.

Statistical Analysis: The data concerning the changes observed at 1st, 3rd, 6th, 8th, 24th, 48th and 72nd hour in control and plant extracts were entered to Microsoft Excel and SPSS programs. SPSS 18 package software was used to make illustrative data analysis, and to generate the graphics. Data are expressed as mean ± standard error (SE). Comparative analyses were performed within 95% confidence interval. $P<0.05$ was accepted as statistically significant. Cell viability % outcomes indicating the amoebicidal activity of the extracts were analysed with multiple comparison test (Post-hoc) in SPSS 18 software. Dual comparison of the groups was made with Tukey test. In order to calculate IC_{50} (plant extract concentration showing lethal impact on half of the trophozoites; 50% inhibitory concentration), logarithmic regression analysis was used. The graph plotted in the logarithmic regression analysis was used to define the IC_{50} value. The amoebicidal activity observed with distinct concentrations of plant extracts at different intervals were compared against the control cells, and expressed as percent inhibition (% cell death). IC_{50} values of plant extracts in distinct concentrations were determined after experiments made in three repeats.

Results

The amoebicidal activities of ringer and methanolic (1%) extracts obtained from *T. orientalis* plant on A. castellanii cysts and trophozoites were studied by calculating cell viability percentage (%) and IC_{50} values. The values observed at the 72nd hour were compared against the values observed at other timepoints, and presence of statistically significant difference ($p<0.05$) was represented in Table 1 and 3.

According to Table 1, % viability rate of cysts at the end of 72nd hour in 80 mg/mL concentration methanolic extract of T.orientalis was 84.6 ± 0.3. This can be interpreted as highly potent amoebicidal effect of 80 mg/mL methanolic (1%) extract of T.orientalis. As seen in Figures 1 and 2, increasing concentrations resulted in a time-dependent decrease in the % viability of the parasite. IC_{50} value of the methanolic extract of the plant at 72nd hour was 4 mg/mL (Table 2). Table 3 shows that 80 mg/mL and 40 mg/mL concentrations of Ringer extract of T.orientalis yielded a rapid decrease in the viable trophozoite count at the end of 1st hour, while viability rates of trophozites at the end of 72nd hour were 1.6 ± 0.3 and 6.3 ± 0.3, respectively. This can be explained by high amoebicidal action of the 80 mg/mL concentration Ringer extract of *T.orientalis* (Table 3, Figures 3 and 4). 72nd hour IC_{50} value of the Ringer extract of the plant was 8.5 mg/mL (Table 4). This result can be interpreted as that even very low concentrations of the plant can show amoebicidal effect.

Based on these results, the methanolic extract of T. orientalis plant was found to have higher amoebicidal effect on Acanthamoeba trophozoites when compared to the ringer extract of the plant. A. castellanii trophozites were found to be more responsive than the cyst forms to both methanolic and ringer extracts of *T. orientalis* plant. Both extracts showed similar activity on the cysts (Tables 1 and 3).

Table 1. Percent (%) viability effect of different concentrations of *T. orientalis* methanol extract on A. castellanii trophozoite and cyst forms

Concentration	Morphological form	Experimental period						
		1st hour	3rd hour	6th hour	8th hour	24th hour	48th hour	72nd hour
80 mg/mL	Trophozoite	5.6±0.3a	4.3±0.3b	3.3±0.3c	2.6±0.3d	2.0±0.0	1.0±0.0	0.0±0.0
	Cyst	93.6±0.3a	93.3±0.3b	91.6±0.6c	89.6±0.6d	87.6±0.3e	86.6±0.3f	84.6±0.3
40 mg/mL	Trophozoite	9.6±0.3a	7.3±0.3b	5.3±0.3c	4.3±0.3d	3.3±0.3	2.6±0.3	1.6±0.3
	Cyst	95.3±0.3a	94.0±0.0b	92.6±0.3c	91.3±0.3d	89.6±0.3e	88.0±0.0f	86.0±0.0
20 mg/mL	Trophozoite	66.0±0.5a	56.3±0.8b	34.0±0.5c	28.3±0.8d	23.3±0.3e	18.3±0.3f	12.3±0.3
	Cyst	96.3±0.3a	94.6±0.3b	93.6±0.3c	92.3±0.3d	90.0±0.5e	88.6±0.3	87.6±0.3
10 mg/mL	Trophozoite	85.0±0.5a	78.3±0.3b	69.0±0.5c	57.0±0.0d	45.0±0.5e	34.6±0.3f	29.0±0.5
	Cyst	96.6±0.3a	95.3±0.3b	94.3±0.3c	92.6±0.3d	90.6±0.3e	89.6±0.3	88.6±0.3
5 mg/mL	Trophozoite	96.3±0.3a	89.3±0.3b	85.3±0.3c	83.3±0.3d	80.3±0.3e	71.3±0.3f	46.0±0.5
	Cyst	97.6±0.3a	96.6±0.3b	95.3±0.3c	93.6±0.3d	92.6±0.3e	91.3±0.3	89.6±0.3
2.5 mg/mL	Trophozoite	98.0±0.0a	96.0±0.0b	94.0±0.0c	93.0±0.0d	84.0±0.0e	75.3±0.3f	64.0±0.0
	Cyst	98.6±0.3a	97.3±0.3b	96.0±0.0c	94.6±0.3d	93.6±0.3e	92.3±0.3f	90.3±0.3
1.25 mg/mL	Trophozoite	99.0±0.0a	97.0±0.0b	95.0±0.0c	94.0±0.0d	90.6±0.3e	85.3±0.3f	76.3±0.3
	Cyst	99.6±0.3a	98.3±0.3b	96.6±0.3c	95.3±0.3d	94.3±0.3e	92.3±0.3	91.3±0.3
Control	Trophozoite	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	99.0±0.5	98.0±0.5	97.3±0.3
	Cyst	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	99.3±0.3	99.3±0.0	99.0±0.3

The data are expressed as mean ± standard error
a, b, c, d, e, f: different statistical values from 72. hours, p (0.05)
a: 1. – 72.hours; b: 3. – 72.hours; c: 6.-72. hours; d: 8.-72.; e: 24.– 72.hours; f: 48th - 72nd hours

Table 2. IC_{50} value of different concentrations of T. orientalis methanol extract on A.castellanii trophozoite form

A. castellanii	Experimental period	50 % inhibitor concentration (IC50)
Trophozoite	72	4 mg/mL
	48	7.2 mg/mL
	24	8.7 mg/mL
	8	11.1 mg/mL
	6	14.1 mg/mL
	3	21.4 mg/mL
	1	23.8 mg/mL

Dıscussıon

Increasing resistance of microorganisms against antimicrobial agents used for treatment, and the high cost of producing new generation drugs drive the pharmaceutical industry to explore alternative antimicrobial agents and to conduct further research (3,13).

Antiparasitic drugs have recently been ineffective in the treatment of infections related to Acanthamoeba species since they fail to show the desired level of action in certain anatomical regions such as CNS and eye. Acanthamoeba keratitis is currently treated with cationic antiseptics such as chlorhexidine and polyhexamethylene biguanide, either alone or in combination (14,15).

Some of the antiamoebic drugs show only amoebastatic effect and the molecules used in the treatment may show cytotoxic effects for the host as well. In addition, treatment with these drugs cannot be tolerated sufficiently by the patient when they are applied for a long time, and the microorganisms may develop resistance to these drugs over time (14).

There is a need for alternative treatment methods to eliminate the problems such as the long term treatment duration and related side effects and the

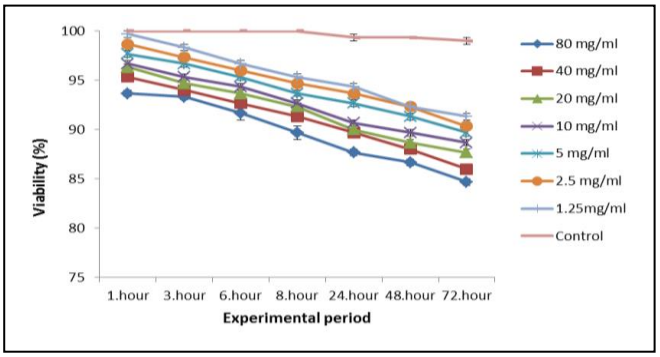

Fig. 1. Diagram of amoebicidal activity of different concentrations of methanolic extract of *T. orientalis* on *A. castellanii* trophozoites at different times

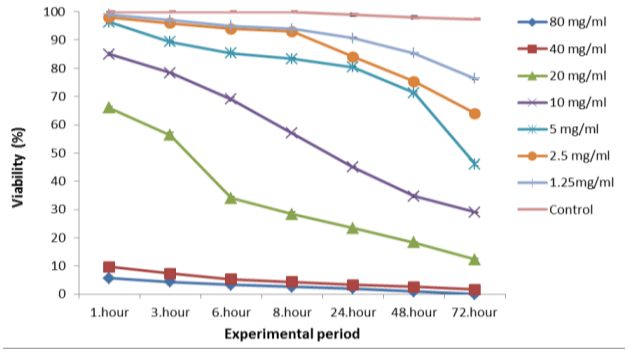

Fig. 2. Diagram of amoebicidal activity of different concentrations of methanolic extract of T. orientalis on A. castellanii cysts at different times

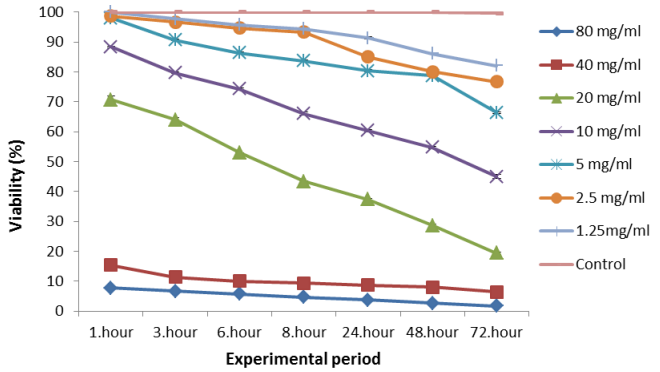

Fig. 3. Diagram of amoebicidal activity of different concentrations of ringer extract of T. orientalis on A. castellanii trophozoites at different times

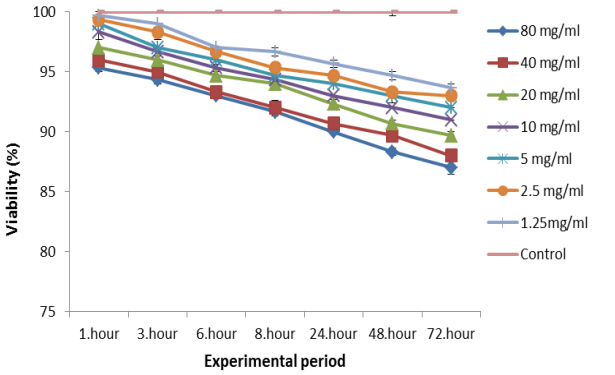

Fig. 4. Diagram of amoebicidal activity of different concentrations of ringer extract of T. orientalis on A. castellanii cysts at different times

cyst forms observed in chronic infections that are resistant to drugs. In this context, a trend towards herbal chemicals is observed. In the pharmaceutical industry and in modern medicine, there is a growing trend towards the use of chemicals of herbal origin (16). The search for effective, well tolerated and non-cytotoxic drugs on trophozoites and cysts of Acanthamoeba species is still ongoing (15,17).

There are many studies that examined the in vitro amoebicidal effects of methanolic extracts of various plants on A. castellanii trophozoites and cysts. Polat et al. (18) studied the in vitro amoebicidal activities of the methanolic extracts of four plant species from Turkey (Allium sivasicum, Allium dictyoprosum, Allium scrodoprosum subsp. rotundum, and Allium atroviolaceum) against A. castellanii, and examined the cytotoxicity in corneal cells. As a result of their studies, they emphasized that Allium scrodoprosum subsp Rotundum has the strongest amoebicidal effect. Malatyalı et al. (19) evaluated the in vitro amoebicidal activity of methanolic extracts of Satureja cuneifolia and Melissa officinalis on A. castellanii trophozoites and cysts. They stated that S. cuneifolia showed a stronger amoebicidal effect. Tepe et al. (20) evaluated the in vitro amoebicidal efficacy of methanol extracts of Teucrium polium and Teucrium chamaedrys on A. castellanii trophozoites and cysts. T. chamaedrys showed the strongest amoebisidal effect.

There are also other studies that used extracts obtained with solvents other than methanol. For example, Rodio et al. (21) tested extracts of Pterocaulon polystachium obtained with hexane, dichloromethane and methanol against A. castellanii. They stated that the most effective amoebicidal activity against A. castellanii was shown by the extract prepared with hexane. Değerli et al. (22) evaluated the in vitro amoebicidal efficacy of the extracts of Pastinaca armenea and Inula oculus-christi prepared using deionized water against A. castellanii. They emphasized that Inula oculus-christi had the strongest amoebicidal effect on trophozoites and cysts. Derda et al. (23) tested the deionized water, alcohol and chloroform extracts of the Artemisia annua L plant in vivo in combination with antibiotics in the treatment of acanthamoebiasis. They found that extracts prepared with chloroform were more effective.

Table 3. Percent (%) viability effect of different concentrations of T. orientalis ringer extract on A. castellanii trophozoite and cyst form

Concentration	Morphological form	Experimental period						
		1st hour	3rd hour	6th hour	8th hour	24th hour	48th hour	72nd hour
80 mg/mL	Trophozoite	7.6±0.3a	6.6±0.3b	5.6±0.3c	4.6±0.3d	3.6±0.3	2.6±0.3	1.6±0.3
	Cyst	95.3±0.3a	94.3±0.3b	93.0±0.0c	91.6±0.3d	90.0±0.0e	88.3±0.3	87.0±0.5
40 mg/mL	Trophozoite	15.3±0.3a	11.3±0.3b	10.0±0.5c	9.3±0.3d	8.6±0.3e	8.0±0.5	6.3±0.3
	Cyst	96.0±0.0a	95.0±0.0b	93.3±0.3c	92.0±0.5d	90.6±0.3e	89.6±0.3	88.0±0.0
20 mg/mL	Trophozoite	70.6±1.2a	64.0±0.5b	53.0±0.0c	43.3±0.3d	37.3±0.3e	28.6±0.3f	19.3±0.3
	Cyst	97.0±0.0a	96.0±0.0b	94.6±0.3c	94.0±0.0d	92.3±0.3e	90.6±0.3	89.6±0.3
10 mg/mL	Trophozoite	88.3±0.3a	79.6±0.3b	74.3±0.3c	66.0±0.5d	66.3±0.3e	54.6±0.3f	45.0±0.5
	Cyst	98.3±0.6a	96.6±0.3b	95.3±0.3c	94.3±0.3d	93.0±0.0e	92.0±0.0	91.0±0.0
5 mg/mL	Trophozoite	98.0±0.0a	90.6±0.3b	86.3±0.3c	83.6±0.3d	80.3±0.3e	78.6±0.3f	66.3±0.3
	Cyst	99.0±0.0a	97.0±0.0b	96.0±0.0c	94.6±0.3d	94.0±0.0e	93.0±0.5	92.0±0.0
2.5 mg/mL	Trophozoite	98.6±0.3a	96.6±0.3b	94.6±0.3c	93.3±0.3d	85.0±0.0e	80.0±0.0f	76.6±0.6
	Cyst	99.3±0.3a	98.3±0.6b	96.6±0.3c	95.3±0.3d	94.6±0.3d	93.3±0.3	93.0±0.0
1.25 mg/mL	Trophozoite	100.0±0.0a	97.6±0.3b	95.6±0.3c	94.3±0.3d	91.3±0.3e	86.0±0.0f	82.0±0.0
	Cyst	99.6±0.3a	99.0±0.0b	97.0±0.0c	96.6±0.3d	95.6±0.3e	94.6±0.3	93.6±0.3
Control	Trophozoite	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	99.6±0.3
	Cyst	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0

The data are expressed as mean ± standard error

a, b, c, d, e, f: different statistical values from 72. hours, p (0.05)

a: 1. – 72.hours; b: 3. – 72.hours; c: 6.-72. hours; d: 8.-72.; e: 24.– 72.hours; f: 48th - 72nd hours

Table 4. IC_{50} value of different concentrations of *T. orientalis* ringer extract on *A.castellanii* trophozoite form

A. castellanii	Experimental period	50 % inhibitor concentration (IC50)
Trophozoite	72	8.5 mg/mL
	48	11.1 mg/mL
	24	14.4 mg/mL
	8	15.9 mg/mL
	6	20.9 mg/mL
	3	23.9 mg/mL
	1	25.8 mg/mL

Dodangeh et al., (24) investigated the in vitro amoebicidal effects of the aqueous extracts of Ziziphus vulgaris (chloroform, water and alcohol) on Acanthamoeba trophozoites and cysts. In addition, they tested their cytotoxicity on mouse peritoneal macrophages. They showed that the chloroform extract had a higher amoebicidal activity than the other extracts and emphasized that the extracts did not show any cytotoxicity.

In related literaure, T.orientalis has been reported to contain volalite fats, tannins, nitrate salts, saponin, mucilage and resin. Diuretic, antipyretic and antidepressant effects have been reported (11). The plant is known to have blood cleansing effect by the local people. We did not encounter any previous study examining its antiparasitic, bactericidal, antifungal or antiviral effects (12). Assuming that the secondary metabolites present within the plant may have antiparasitic effect, we examined its antiamoebicidal action in the present study. Our results showed that methanolic extract of the plant had more potent amoebicidal action against A.castellanii trophozoites and cysts compared to the Ringer extract of the plant.

Extracts obtained from T. orientalis under controlled conditions can be used as an alternative treatment choice or in combination with other treatments. T. orientalis plant extracts showed amoebicidal effect against both trophozoite and cyst forms of A. castellanii. Whether the concentrations explored in the existing study are cytotoxic for mammalian cells, or have toxic effects on experimental animals should be examined with future in vivo studies. Furthermore, the mechanism of action for the active substances responsible for biological activity should be investigated in future studies.

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