International Journal of Medical Laboratory 2019;6(3):219-225.

International Journal of Medical Laboratory
Shahid Sadoughi University of Medical Sciences

Original Article

Effects of Aqueous and Ethanolic Extracts of *Myrtus Communis* Leaves on Trophozoites and Cysts of *Acanthamoeba*: An *In Vitro* Study

Tooran Nayeri Chegeni[1,2] Ph.D., Fatemeh Ghaffarifar[3*] Ph.D.
Fariba Khoshzaban[4] Ph.D., Abdolhosein Dalimi Asl[3] Ph.D.
Hoda Mirzaian[3] M.Sc., Farnoosh Jameie[5] Ph.D.

[1]Department of Parasitology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
[2]Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran.
[3]Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
[4]Department of Parasitology, Shahed University of Medical Sciences, Tehran, Iran.
[5]Department of Parasitology, Razi Vaccine and Serum Reaserch Institute, Agricultural Reaserch, Edjucation and Extension Organization (AREEO), Karaj, Alborz, Iran.

ABSTRACT

Article history
Received 13 Dec 2018
Accepted 2 Jul 2019
Available online 31 Aug 2019

Key words
Acanthamoeba
Keratitis
In vitro
Myrtus communis

Background and Aims: *Acanthamoeba* is a ubiquitous amphizoic organism which can cause lethal diseases such as granulomatous amoebic encephalitis and unfortunately, the infection has now increased in the world. The aim here was to evaluate *in vitro* anti-Acanthamoeba properties of crude aqueous and ethanolic extracts of *Myrtus communis*.

Materials and Methods: In this experimental research, a clinical isolate of *Acanthamoeba* was cultured and genotyped. The aqueous and ethanolic extracts of *Myrtus communis* were prepared. Then, various concentrations of *Myrtus communis* extracts (1.25, 2.5, 5, and 10 mg/ml) were tested at three different times (24, 48 and 72 hr) on trophozoites and cysts of *Acanthamoeba in vitro*. The viability of trophozoites or cysts was tested by trypan blue method. Unstained (viable) and stained (nonviable) parasites were evaluated by counting with a neobar lam.

Results: The percentage of viablity of trophozoites and cysts after adding ethanolic extract of *Myrtus communis* was 0% and 8.62%, respectively. Moreover, at 10 mg/ml concentration of aqueous extract of *Myrtus communis*, 0% trophozoites and 31.10% cysts lived after 72 h.

Conclusions: This extract can be used as a safe anti-Acanthamoeba agent against trophozoites and cysts of *Acanthamoeba* and further investigations are recommended to show the effects of this plant as an antiparasitic drug in animal models and volunteer infected people.

[*]**Corresponding Author:** Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. **Tel/Fax:** +98 21 82884553, **Email:** ghafarif@modares.ac.ir

Introduction

Acanthamoeba are ubiquitous amphizoic organisms are found in variety of environments including water, sewage, air, soil, food items, swimming pool, dust, dialysis units, humidifiers, and healthy individuals [1-3]. *Acanthamoeba* keratitis (AK) is a painful sight-threatening ocular disease that occurs generally among immunocompetent individuals. AK symptoms include redness, irritation, tearing, photophobia, ocular pain, lid edema, corneal ring, perineural infiltrates, loose corneal epithelium, and blurred vision [2]. About 85-88% of AK cases are connected with contact lens wearers [4]. The treatment of ocular acanthamoebiasis is extremely difficult and protracted. The combination of cationic antiseptics and aromatic diamidines inhibit membrane functions and DNA synthesis [5, 6]. One of medicinal plants called *Myrtus communis* is a perennial shrub having a maximum height of 5 meters (m) that belongs to the Myrtaceae family. The leaves of this plant are mostly used in medicine [7]. *Myrtus communis* extract are useful against different pathogenic organisms and there are good results in terms of antibacterial [8], antiviral [9], bioactivity and antioxidant activity of this plant as well as its antiparasitic [10-12] and antifungal [13] properties. Anti-*Acanthamoeba* properties of *Myrtus communis* are not identified; thus, we focused to explain Anti-*Acanthamoeba* activity of this extract against trophozoites and cysts of *Acanthamoeba*.

Materials and Methods

Preparation of the plant

The dried leaves of *Myrtus communis* were purchased from the Kerman area in the southeast Iran. The plant was identified and approved by pharmacognosist (Faculty of Pharmacognosy, Shahid Beheshti University, Tehran, Iran).

Extraction of the ethanolic extract

The ethanolic extract of *Myrtus communis* was obtained by incubating 50 grams (g) of powdered dried leaves in 500 milliliters (ml) of 85-87% ethanol for 3 days. In all cases, the extracts were centrifuged (5000 rpm) for 30 minutes (min) and the supernatants were harvested and filtered using Whatman paper No. 1. The extract was then filtered.

Afterwards, a rotary vacuum evaporator at 40°C was used in order to remove the solvent.

Extraction of the aqueous extract

Fifty grams of powdered dried leaves was kept in 200 mL of distilled water for half an hour. Then, the solvent from the extract was removed by rotary evaporator. The residues of extract were collected and kept in the freezer (-20°C) for the experiment.

Acanthamoeba strain

The sample used in this study were obtained from a patient with keratitis. AK diagnosis was based on culture, sequencing analysis and Basic Local Alignment Search Tool (BLAST) search. The sequencing results showed the existence of T4 genotype (accession number KU877552). The specimen was grown on non-nutrient agar (NNA) plates [10] coated with

Escherichia coli at 26˚C. The *Acanthamoeba* strain was kept in non-nutrient agar culture for further use.

Trophozoites

Acanthamoeba was cultivated in NNA medium at 26°C. After 72 to 96 h, trophozoites were washed twice with sterile page's saline and concentrated at 1500 rpm for 5 min [14]. The number of viable trophozoites were calculated by a neobar lam [15]. The final amount was adjusted to 15×10^4 trophozoites per ml. Initial cultures were used for testing after 14-21 days and centrifuged at 2000 rpm for 5 min. Cysts were counted and the final amount was adjusted to 15×10^4 cysts per ml.

Evaluation of activity

In this study, 100 microliters (µl) of the calibrated cyst/ trophozoite suspension (15×10^4/ml) was inoculated in each micro-centrifuge tube and 100µl of each extract concentration (1.25, 2.5, 5, 10 mg/ml) was added to the tubes. Then, the tubes were kept at 26°C for 24, 48, and 72 hr. In this test, the control tubes included only trophozoites or cysts suspension and sterile distilled water. Three tubes were used for the evaluation of each concentration and measurements were repeated 3 times [16].

Amoebicidial activity of the *Myrtus communis* on trophozoites and cysts

The samples were incubated with different concentrations of the extract for 24, 48, and 72 h. Then, 25 µl from each test and control well was added into 25 µl from 0.4% trypan blue and the number of live and dead cysts was counted with a neobar lam. This study was approved by the ethical committee of Tarbiat Modares University, Tehran, Iran.

Statistical analysis

The statistical analysis of the data was made by one-way ANOVA and repeated measures tests (compare three or more dependent groups) using SPSS version 18.0. The number of trophozoites and cysts was expressed as mean±SD and percent survival. The significance level was considered as $p<0.05$.

Results

The results of this research are presented as survival percentage of trophozoites and cysts in Tables 1 and 2. The concentrations of 1.25, 2.5, 5 and 10 mg/ml of aqueous and ethanolic extracts of the *Myrtus communis* were used against trophozoites and cysts at three different times (24, 48 and 72 hr). The mortality of trophozoites and cysts exposed to the different extracts arised with increase in time of exposure and extract concentration (Fig. 1). The ethanolic extract of *Myrtus communis* is more effective than the aqueous extract. After adding 10 mg/ml ethanolic extract of *Myrtus communis* to the medium culture, 0% trophozoites and 8.62% cysts were viable after 48 and 72 hr, respectively. In the case using 5 mg/ml ethanolic extract of *Myrtus communis*, 28.88% trophozoites and 35.80 % of the cysts were detected in 72 hr. By adding 2.5 mg/ml of extract and after 72 hr percentages of trophozoites and cysts viabilitywere 43.78% and 56.06%, respectively. Upon administration of extract at concentration of 1.25 mg/ml, 56.98% trophozoites and 75.59% cysts were

alive. The difference between these results was statistically significant ($p<0.05$).

Anti-amoebic activity of extract from *Myrtus communis* at 10 mg/ml on trophozoites and cysts of *Acanthamoeba* caused removal of all trophozoites, and 31.10% cysts were viable in 72 h. In the case of 5 mg/ml ethanolic extract of *Myrtus communis*, 42.22% trophozoites and 63.77 % of the cysts were detected in 72 h. At another concentration, 2.5 mg/ml, 57.33% trophozoites and 69.58% cysts survived. Anti-amoebic activity at 1.25 mg/ml of the extract showed survival of 69.96% of trophozoites and 85.34% of cysts. The difference between the results of the effect of extract on parasite was statistically significant ($p<0.05$). Finally, among the extracts evaluated, ethanolic extract of *Myrtus communis* revealed the strongest anti-*Acanthamoeba* activity on the trophozoites and cysts.

Table 1. Effect of *Myrtus communis* ethanolic extract on the survival and growth of *Acanthamoeba*

Concentrations (mg/ml)	**Effect on**	**%Viability of *Acanthamoeba***		
		24th hour	48th hour	72th hour
10.0 (mg/ml)	Trophozoites	19.99 ±5.44	00.00	00.00
	Cysts	27.68±4.25	20.38 ±2.71	8.62±0.65
5.0 (mg/ml)	Trophozoites	37.77±3.14	33.33±00	28.88±3.14
	Cysts	40.00±00	37.68±4.25	35.80±5.43
2.5 (mg/ml)	Trophozoites	48.66±8.37	46.66±00	43.78±8.80
	Cysts	71.10±7.70	66.50±3.52	56.06±3.86
1.25 (mg/ml)	Trophozoites	64.44±3.13	60.31±9.01	56.98±6.32
	Cysts	88.32±2.35	76.92 ±00	75.59±2.59
Control	Trophozoites	100±00	100±00	100±00
	Cysts	100±00	100±00	100±00

Data are expressed as mean±SD

Table 2. Effect of *Myrtus communis* aqueous extract on the survival and growth of *Acanthamoeba*

Concentrations (mg/ml)	**Effect on**	**%Viability of *Acanthamoeba***		
		24th hour	48th hour	72th hour
10.0 (mg/ml)	Trophozoites	36.01±9.09	33.33±00	00.00
	Cysts	52.22±1.56	46.66 ±6.66	31.10±3.14
5.0 (mg/ml)	Trophozoites	54.33±7.93	48.66±2.01	42.22±3.13
	Cysts	66.50 ±3.52	64.09± 3.62	63.77±7.38
2.5 (mg/ml)	Trophozoites	69.58±2.75	65.13±8.55	57.33±3.35
	Cysts	80.16±4.59	71.10±7.70	69.58±0.09
1.25 (mg/ml)	Trophozoites	74.52 ±1.69	71.10±7.70	69.96±1.03
	Cysts	92.85±7.14	86.66±00	85.34±0.52
Control	Trophozoites	100±00	100±00	100±00
	Cysts	100±00	100±00	100±00

Data are expressed as mean±SD

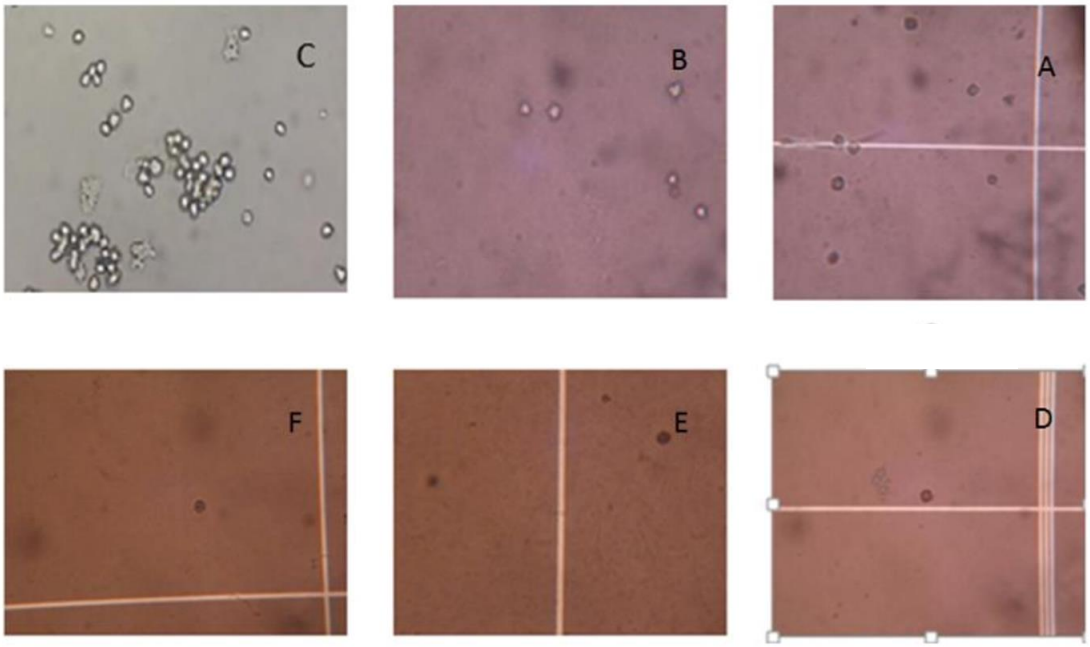

Fig. 1. The effect of *Myrtus* extract on *Acanthamoeba* (40×). (A and B) Live cysts, (C) Live trophozoites, and (D, E, and F) Dead cysts.

Discussion

AK is an infrequent serious complication in contact lens users that can lead to severe visual loss [17] and promotes with corneal abrasions, poor contact lens hygiene, and home-made saline solutions [2]. The disease is efficintly treated by using antibiotics, propamidine, chlorhexidine, and administering eye drops [18, 19]. Despite combination therapy, only half of the patients have been reported to improve after treatment with the therapeutic regimens when the disease is not early diagnosed [20]. Failure to treat *Acanthamoba* infections is due to the following: 1) failure to achieve the optimum dose for the treatment of infections, 2) phenotypic switching (transformation of trophozoite to double-walled cyst), 3) unwanted side effects due to non-selective drugs [21]. *Myrtus communis* with the common name "myrtle" is a medicinal herb the leaves, branches, berries, and fruits of which have been used widely as a traditional medicine for the treatment of various diseases [22]. The leaves of *Myrtus communis* are useful in cerebral, stomach and liver diseases, pulmonary disorders, deep sinuses, hair fall, inflammation, haemorrhage, and diarrhea [23]. In this context, anti-Acanthamoeba effect of aqueous and ethanolic extracts of *Myrtus communis* against T4 genotype of *Acanthamoeba* was evaluated. The results of this study showed that aqueous and ethanolic extracts of *Myrtus communis* on trophozoites are more effective than cysts. Rigid double-layered wall of cyst causes a difference in the sensitivity to drug in the trophozoites and cysts [24].

Many plant extracts have been reported as potent inhibitors of parasites. In the case of *Acanthamoeba*, different medicinal plants and herbal extracts have been studied as sources of amoebicidal agents. Nayeri Chegeni et al. in 2016 [25] demonstrated that in the presence of 10 mg/ml alcoholic extract in medium culture after 72 hr, 30.51% trophozoites and 91.40% cysts of *Acanthamoeba* were viable. However, in the presence of 10 mg/ml aqueous extract of *Artemisia annua*, 58.25% trophozoites and 81.53% cysts were alive in the medium culture after 72 hr. Furthermore, anti-amoebic effects of *Peganum harmala* ethanolic extract were tested against *Acanthamoeba in vitro* which ultimately with the effect of 10 mg/ml of extract, 0% trophozoites and 21.10% cysts were identified as alive after 72 hr [26]. Dodangeh et al. revealed that in the presence of 10 mg/ml Chloroformic extract of *Trigonella Foenum Graecum*, all trophozoites and cysts were removed after 48 and 72 hr, respectively [27]. Polat et al. reported that methanolic extract of *Thymus sipyleus subsp* was effective on *Acanthamoeba castellanii*. Finally, 32 mg/ml of the methanolic extract eliminated all trophozoites in 3 h. At the same concentration, no viable cysts were recognized in 12 hr [28]. There are many investigations on effectiveness of plant extracts on parasites that show the importance of natural products for treatment of parasitic disease.

Conclusion

Our findings demonstrated that *Myrtus communis* inhibits the growth rate of trophozoites and cysts of *Acanthamoeba* with a dose of 10 mg/ml. It is recommended that cell culture be performed and the extract be used for the animal models in order to determine its exact efficacy and side effects on the human eye.

Conflict of Interest

There is no conflict to declare.

Acknowledgments

We would like to thank the staff of the department of parasitology in Tarbiat Modares University for their help. Also special thanks goes to Dr. Bita Bakhshi, the associate professor of medical bacteriology and Dr. Majid Pirestani assistant professor of medical parasitology in Tarbiat Modares University for their sincere cooperation in this project.

References

[1]. Khan NA, Tareen NK. Genotypic, phenotypic, biochemical, physiological and pathogenicity-based categorisation of Acanthamoeba strains. Folia Parasitol. 2003; 50(2): 97-104.

[2]. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev. 2003; 16(2): 273-307.

[3]. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol. 2004; 34(9): 1001-1027.

[4]. Dart JK, Saw VP, Kilvington S. Acanthamoeba keratitis: diagnosis and treatment update 2009. Am J Ophthalmol. 2009; 148(4): 487-99.

[5]. Khunkitti W, Lloyd D, Furr JR, Russell A. Aspects of the mechanisms of action of biguanides on trophozoites and cysts of Acanthamoeba castellanii. J Appl Microbiol. 1997; 82(1): 107-14.

[6]. Hargrave SL, McCulley JP, Husseini Z, Group BS. Results of a trial of combined propamidine isethionate and neomycin therapy for

Acanthamoeba keratitis. Ophthalmology 1999; 106(5): 952-57.
[7]. Sobel JD. Bacterial vaginosis. Annu Rev Med. 2000; 51(1): 349-56.
[8]. Alem G, Mekonnen Y, Tiruneh M, Mulu A. In vitro antibacterial activity of crude preparation of myrtle (Myrtus communis) on common human pathogens. Ethiop Med J. 2008; 46(1): 63-9.
[9]. Moradi MT, Karimi A, Rafieian M, Kheiri S, Saedi M. The inhibitory effects of myrtle (Myrtus communis) extract on herpes simplex virus-1 replication in baby hamster kidney cells. J Shahrekord Univ Med Sci. 2011; 12(4): 54-61.
[10]. Gortzi O, Lalas S, Chinou I, Tsaknis J. Reevaluation of bioactivity and antioxidant activity of Myrtus communis extract before and after encapsulation in liposomes. Eur Food Res Technol. 2008; 226(3): 583-90.
[11]. Mahmoudvand H, Ezzatkhah F, Sharififar F, Sharifi I, Dezaki ES. Antileishmanial and cytotoxic effects of essential oil and methanolic extract of Myrtus communis L. Korean J Parasitol. 2015; 53(1): 21-30.
[12]. Abdollahy F, Ziaei H, Shabankhani B, Azadbakht M. Effect of essential oil and methanolic extract of Myrtus communis on Trichomonas vaginalis. Iran J Pharm Res. 2004; 3(2): 35-41.
[13]. Azad ED, Emami M, Adimi P, Amin G. Survey on antifungal effect of Myrtus communis leave extract on saprophytes and dermatophytes fungi. J Microbiol Knowledge 2010; 2(5): 27-31.
[14]. Garcia LS, Bruckner DA, Brewer TC, Shimizu RY: Techniques for the recovery and identification of Cryptosporidium oocysts from stool specimens. J Clin Microbiol. 1983; 18 (1): 185-190.
[15]. Khan NA. Acanthamoeba: biology and pathogenesis. BioMed Central Parasite Vectors 2009; 2(1):1-2.
[16]. Malatyali E, Tepe B, Degerli S, Berk S, Akpulat HA. In vitro amoebicidal activity of four Peucedanum species on Acanthamoeba castellanii cysts and trophozoites. Parasitol Res. 2012; 110(1): 167-74.
[17]. Mahgoub AM. Acanthamoeba keratitis. Parasitol United J. 2010; 3(1): 9-18.
[18]. Seal D, Hay J, Kirkness C. Chlorhexidine or polyhexamethylene biguanide for Acanthamoeba keratitis. The Lancet. 1995; 345(8942): 136.
[19]. Seal D. Acanthamoeba keratitis update-incidence, molecular epidemiology and new drugs for treatment. Eye. 2003; 17(8): 893-900.
[20]. Ficker L, Seal D, Warhurst D, Wright P. Acanthamoeba keratitis-resistance to medical therapy. Eye (Lond) 1990; 4(1): 835-38.
[21]. Khan NA. Acanthamoeba: biology and increasing importance in human health. FEMS Microbiol Rev. 2006; 30(4): 564-95.
[22]. Alipour G, Dashti S, Hosseinzadeh H. Review of pharmacological effects of Myrtus communis L. and its active constituents. Phytother Res. 2014; 28(8): 1125-136.
[23]. Sumbul S, Ahmad MA, Asif M, Akhtar M. Myrtus communis Linn.-A review. Indian J Nat Product Resourc. 2011; 2(4): 395-402.
[24]. Leitsch D, Köhsler M, Marchetti-Deschmann M, Deutsch A, Allmaier G, Duchêne M, et al. Major role for cysteine proteases during the early phase of Acanthamoeba castellanii encystment. Eukaryotic cell. 2010; 9(4): 611-18.
[25]. Nayeri Chegeni T, Ghaffarifar F, Khoshzaban F, Dalimi Asl A. The effects of artemisinin and aqueous and alcoholic extracts of Artemisia annua on Acanthamoeba genotype T4 in vitro. Modares J Med Sci Pathol. 2016; 19(2): 75-87.
[26]. Nayeri Chegeni T, Ghaffarifar F, Khoshzaban F, Dalimi Asl A. Evaluation of anti-amoebic activity of Peganum harmala ethanolic extract on Acanthamoeba in vitro. AMUJ. 2018; 20(129): 74-82.
[27]. Dodangeh S, Niyyati M, Kamalinejad M. Anti-Acanthamoeba activities of chloroformic fractions of Trigonella Foenum graecum (seed) and their cytotoxity on mice macrophage cell. NBM. 2015; 3(4): 182-88.
[28]. Polat ZA, Tepe B, Vural A. In vitro effectiveness of thymus sipyleus subsp. sipyleus var. sipyleus on Acanthamoeba castellanii and its cytotoxic potential on corneal cells. Parasitol Res. 2007; 101(6):1551-555.