Tropical Biomedicine 34(1): 127–136 (2017)

The amoebicidal activity of *Ziziphus vulgaris* extract and its fractions on pathogenic *Acanthamoeba* trophozoites and cysts

Dodangeh, S.[1,2], Niyyati, M.[1*], Kamalinejad, M.[3], Lorenzo-Morales, J.[4], Haghighi, A.[2] and Azargashb, E.[5]
[1]Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]School of Pharmacology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Avda. Astrofísico Fco. Sánchez, S/N, 38203 La Laguna, Tenerife, Canary Islands, Spain
[5]Department of Family Medicine, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[*]Corresponding author e-mail: maryamniyati@yahoo.com
Received 23 April 2016; received in revised form 11 June 2016; accepted 15 June 2016

Abstract. *Acanthamoeba* genus includes pathogenic species which are causal agents of a severe sight-threatening infection of the eye known as *Acanthamoeba* keratitis (AK). Furthermore, the number of AK is worryingly increasing worldwide, mostly in contact lens users. Until present, there is a general failure to reach a fully effective treatment against AK which is mainly due to the amoebic double-walled cyst stage which forms a protective barrier against drugs. Therefore, drug discovery research towards AK treatment is a must. In this study, *Ziziphus vulgaris*, a native plant of Asian countries, was checked for its activity against *Acanthamoeba*. For this purpose and in order to determine the *in vitro* amoebicidal effects of *Ziziphus vulgaris* aqueous extract and its fractions (chloroformic, remaining aqueous and primary alcoholic) against *Acanthmoeba* trophozoites and cysts, activity and sensitivity assays were performed. Moreover, the toxic effect of the extract and its fractions was also tested on murine peritoneal macrophages using a colorimetric tetrazolium salt (MTT) test. The obtained results showed that the chloroformic fraction presented a higher anti-*Acanthamoeba* activity when compared to the other fractions (Trophozoites/cysts were eliminated, when incubated in a concentration of 50 mg/ml of the fraction, after 24 hours). The calculated active concentrations against *Acanthamoeba* of these extracts did not shown any high cytotoxicity levels.This study suggests that the *Ziziphus vulgaris* chloroformic fraction, may present compounds with relevance for the development of novel anti-*Acanthamoeba* drugs.

INTRODUCTION

Free living amoebae (FLA) belonging to the genus *Acanthamoeba* spp. are the causative agents of a severe sight-threatening corneal infection called *Acanthamoeba* Keratitis (AK). To date, 20 genotypes (T1-T20) of *Acanthamoeba* have been established based on the diagnostic fragment (DF3) sequence of 18S rRNA gene (Corsaro *et al*., 2015). Among all the reported genotypes, T4 have been related to the higher number of AK worldwide and in Iran (Khan 2009; Niyyati and Rezaeian, 2015). Other genotypes reported in AK cases include T2, T3, T4, T5, T6, T9 and T11 have been isolated in Iran and worldwide (Hajialilo *et al*., 2015; Lorenzo-Morales *et al*., 2015). It is also important to mention that more than 80% of AK cases reported worldwide are related to lack of hygiene in the use of contact lenses (Rezeaian *et al*., 2007; Niyyati *et al*., 2009;

Hajialilo *et al*., 2015). It is also important to note that in 2007, the number of AK cases was estimated to be more than 3000 worldwide (Govinda *et al*., 2007). Among the clinical symptoms of AK, severe pain, eye-redness, photophobia, edema, perforation, epithelial loss, and vision-loss are the most commonly reported. Moreover, the characteristic clinical symptom of *Acanthamoeba* keratitis, is still the presence of a ring like stromal infiltrate (Kaur *et al*., 2011).

A faster clinical response could be achieved, with early and efficient diagnosis tests. Unfortunately, most of the AK cases show frequent and serious recurrences, due to the encystment of *Acanthamoeba* within the corneal stroma (Khan 2009; Shoaib *et al*., 2013). Treatment of AK is usually a problematic issue and not always effective. This is mainly due to the presence of *Acanthamoeba* rigid double-layer wall of the cysts. Most of the available drugs demonstrated treatment failure and recurrence of infection does occur (Khan 2009). In addition, monotherapy is not an effective way for elimination of the cysts and thus poor response to treatment is a usual phenomenon. On the other hand, combination therapy with two or more chemical drugs may lead to corneal toxicity (Khan 2009; Malatyali *et al*., 2012). In addition, *Acanthamoeba* trophozoites exhibit a higher level of resistance to the usual anti-amoebic compounds and also reaching enough concentration of the drug in the cornea remains an issue (Khan 2009; Lorenzo-Morales *et al*., 2015).

The current treatments for AK include a combination of chemotherapeutic agents, such as biguanides (poly-hexamethylene biguanides, and/ or chlorhexidine which inhibit the membrane functions), and diamidines such as propamidine isethionate which inhibit DNA synthesis. These drugs, have been reported to successfully heal some patients suffering from AK (Roongruangchai *et al*., 2008; El Sayed *et al*., 2012). However, the drugs mentioned above, are highly toxic to the human corneal cells. Moreover, it should also be noted that propamidine is poorly cysticidal and resistance in some *Acanthamoeba* strains has been previously reported (Uemura *et al*., 2010). The MIC of propamidine isethionate for clinical strain of *Acanthamoeba* trophozoites was calculated as 10 µg/ml and for cysts was 125 µg/ml at 48 h (Khan 2009). This level of drug is well over the recommended dose. Moreover, propamidine isethionate revealed a high levels of host cell toxicity (Khan 2009). The other treatment regimen includes biguanides such as chlorohexidine (0.02%) which lead to permeability changes in the cell wall. The concentration of chlorohexidine needs to be higher than 100 µg/ml which could lead to shrinkage of cyst wall, however this concentration is highly toxic to the cornea (Perrine *et al*., 1995). In a previous study regarding the amoebicidal efficiencies of various diamidines such as propamidine against two strains of pathogenic *Acanthamoeba* spp. it was shown that diamidines including hexamidine, hepta-midine, octamidine and nonamidine presented a faster amoebicidal action than propamidine (Perrine *et al*., 1995; Khan 2009).

Taking in to account the poor prognosis of AK and common treatment failure, there is an imperative priority to discover new compounds for the treatment of this infection. To date, it has been reported that natural agents, are a source of new active compounds for different diseases (Tipu *et al*., 2006). The herb *Ziziphus vulgaris*, belongs to the Rhamnaceae family. This plant is cultivated in many parts of India, Iran and Burma (Goli-malekabadi *et al*., 2014).

Phytochemical screening of *Ziziphus vulgaris*, has shown the presence of bioactive compounds such as cyclopeptide alkaloids, among others. According to various studies, the anti-plasmodial and anti-mycobacterial properties of *Ziziphus*, is due to the presence of cyclopeptide alkaloids. Moreover, other compounds such as saponin glycosides, alkaloids, steroids, polysaccharides and terpenoids which could be amoebicidal have been also reported in the plant (Panseeta *et al*., 2011).

In this study, the activity of the aqueous total extract and fractions of *Ziziphus vulgaris* was evaluated against *Acan-*

thamoeba trophozoites and cysts. Moreover, the toxic effects of these extracts was tested using a murine macrophage cell line.

MATERIALS AND METHODS

Procedure of the aqueous extract and fractions

Ziziphus vulgaris specimens were collected from the Birjand area in Iran. The plant was carefully identified by Mr. Mohammad Kamali Nejad of the Pharmacognosy Department, at the Shahid Beheshti University of Medical Science (Tehran, Iran). This plant was chosen, based on its ethnobotanical data, and local information. The aqueous extract was prepared, by mixing 100 g of the dried plant, with 2 L of distilled boiling water. Briefly, the pan was quickly removed from the heat, covered with a foil, and after 4 h, the contents of the pan were squeezed and filtered, using a 0.22 µm filter (Whatman paper). The prepared extract, was dried for 24 h in a water bath, to evaporate the solvent (Mehrabani Natanzi *et al*., 2012). To obtain the fractions, the successive liquid-liquid partitioning was used. Five grams of the aqueous extract were dissolved in 50 ml of ethanol, which was evaporated *in vacuo*, to give a semi-dried extract denoted as a primary alcohol extract. The remaining aqueous phase, was successively extracted with distilled water and $CHCl_3$. Each organic phase was evaporated *in vacuo*, to give the corresponding remaining aqueous and chloroformic respectively (Vyas *et al*., 2008).

Acanthamoeba strain

A clinical isolate of *Acanthamoeba* was tested in this study, and was obtained from a corneal scrape collected from a soft contact lens wearer suffering AK in Iran. The diagnosis of AK, was based on both culture test and PCR sequencing. Briefly, the sample was cultured onto the surface of 1.5% Non-Nutrient Agar (NNA) plates, enriched with heated *Escherichia coli* and incubated at room temperature for up to 72 hours. Following the DNA extraction, PCR was used to confirm the microscopic diagnosis. The PCR analysis, sequencing, and BLAST search of the isolate, revealed the presence of T4 genotype in the sample. Cloning of the amoeba was done, in order to eliminate any contaminations of bacterial and fungal (Khan, 2009; Init *et al*., 2010). The isolated *Acanthamoeba* strain was then kept in NNA plates, until performance of further experiments.

Trophozoites and cysts preparation

Acanthamoeba was grown at 26ºC in an axenic medium, which was described in a previous study (Rahdar *et al*., 2012). One hundred microliters of the axenic medium was inoculated directly onto the surface of 1.5% non-nutrient agar (NNA) plates, and incubated at 26ºC. The Trophozo-ites at the stage of exponential growth (72 h to 96 h), and cysts after 3 weeks, were gently identified by the use of an inverted microscope (Perrine *et al*., 1995). Amoebic forms were concentrated separately, by centrifugation at 1500 g for 5 min. The supernatant was removed, and the sediment was washed twice in Phosphate-Buffered Saline (PBS). The trophozoites/cysts in the obtained suspension were counted with a hemocytometer, and the suspension was adjusted to 25×10^4 amoebae/ml, for the amoebicidal activity assays (El-Sayed *et al*., 2012; Malatyali *et al*., 2012).

Evaluation of amoebicidal activity

The Aqueous total extract, were prepared at the following concentrations: 25mg/ml, 50mg/ml, 100mg/ml, 200mg/m, 400mg/ml, and 500 mg/ml. In addition, the fractions concentrations were obtained at: 12/5 mg/ml, 25 mg/ml, and 50 mg/m. 2 hundred microliters of the calibrated cyst/trophozoite suspension (25×10^4/ml), and the same volume of each extract/fraction concentration, were mixed thoroughly in the microcentrifuge tubes. Incubation was done at 26ºC for different incubation periods (24 hours, 48 hours, and 72 hours). In addition, the negative control con-taining only the parasite plus PBS (without extracts) and the positive control containing the parasite plus 0.02% chlorhexidine glu-conate (prepared from a solution 20% in water CHX, C-9394; Sigma),

were used. Three tubes were used for the evaluation of each concentration and measurements were repeated 5 times (Polat *et al*., 2008).

Efficacy of aqueous extract and its fractions against cultured trophozoites and cysts

Following the incubation periods, twenty five µl from each suspensions of test or control well, was mixed with the same volume of 0.1% Eosine. The viable amoebae (unstained parasites), were counted using a microscope. Approximately, 100 *Acanthamoeba* trophozoites were examined at each time. Additionally, the cultures containing no viable cysts, were transferred onto an NNA agar enriched with *E. coli* and incubated at 26ºC for 3 more days, to confirm the observed results (Polat *et al*., 2008).

Cytotoxic assay of extract/fractions on murine peritoneal macrophages

Elicited peritoneal macrophages were obtained from a 5 week-old mouse. Briefly, the injection of 1 ml of Brewer Thioglycollate Broth (4.05 g/100 ml), to the peritoneal and lavage of peritoneal of the mouse were performed. The elicited cells were washed twice and added to RPMI-1640 containing 10% FBS, and 2% antibiotic (penicillin and streptomycin) (Klimetzek and Remold, 1980).

The macrophages were kept at 37ºC, in a 5% CO2 air incubator and passaged every day. Ten microliters of the total extract concentrations (25mg/ml-6000 mg/ml) and the plant fractions (12.5mg/ml-500 mg/ml), were added to all wells except the controls, and the plates were mixed well. The cells were then maintained at 37°C in a 5% CO2 air incubator, for 24 h. Exposed cells in a 96-well plate, were incubated in the MTT solution for 4 h. Then a formazan dye was quantified using an ELISA reader with 540 nm wavelength, after 15 min (viability (%) = O.D. TESE/O.D. CONTROL X 100) (Wagner *et al*., 1999; Hussai *et al*., 1993). The Growth inhibition was compared with that of the controls, to find the extract/fractions concentration that inhibited growth by 50% (IC_{50}).

Statistical analysis

The statistical analysis of data was performed using SPSS software version 15.0. P values < 0.001, were considered statistically as highly significant. The IC_{50} calculation was done using Graph Pad Prism 6.

RESULTS

***Acanthamoeba* growth inhibition**

This study revealed the amoebicidal activity of *Ziziphus vulgaris* extract/fractions. The anti-amoebicidal activities of the aqueous extract at concentrations of (25mg/ml, 50mg/ml, 100mg/ml, 200mg/ml, 400mg/ml, and 500 mg/mL), and various fractions of the plant at concentrations of (12.5mg/ml, 25mg/ml, and 50 mg/ml), are shown in Fig. 1 and 2. Comparatively, the chloroform fraction of *Ziziphus vulgaris* was found to be more active than other fractions and the total extract of this plant (Fig. 3). As expected, cysts demonstrated more resistance to the extraction/fractions, than trophozoites. The lowest concentration of 12.5 mg/ml chloroform fraction failed to cause the total death of the trophozoites, though, it showed growth reduction by 79.1–91.8% in the trophozoites, and 18–87% in the cysts in all incubation periods. In the 50 mg/ml concentration, all the trophozoites/cysts were eliminated during the duration of the experiments.

Cytotoxicity Assay

The effect of *Ziziphus vulgaris* total aqueous extract and its fractions on murine peritoneal macrophages, were examined by the MTT assay. Dose response curves which were constructed within the range of 25mg/ml-6000 mg/ml for the total aqueous extract, and 12.5mg/ml-500 mg/ml for the fractions respectively, showed a decreasing number of viable cells, with an increasing concentration of extract/fractions (Fig. 4, 5). The calculation of IC_{50} value was done using the Graph Pad Prism Software (Ver. 6.0). The susceptibility of the cells to the extract/fractions, was characterized by the IC_{50}.

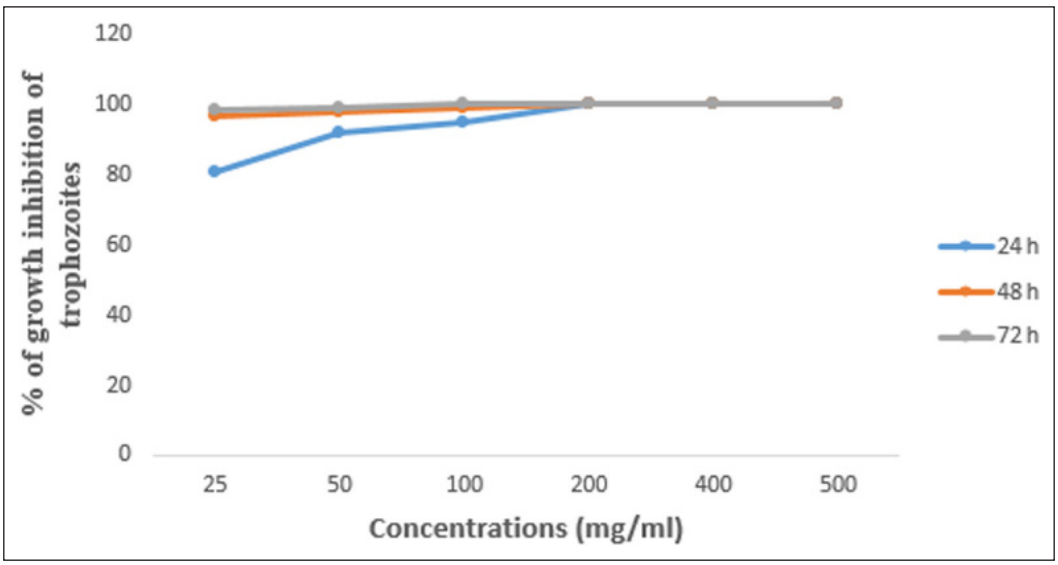

Figure 1. Effect of the total extract on the growth inhibition of *Acanthamoeba* trophozoites at different concentrations (mg/ml) after 24, 48, 72 h.

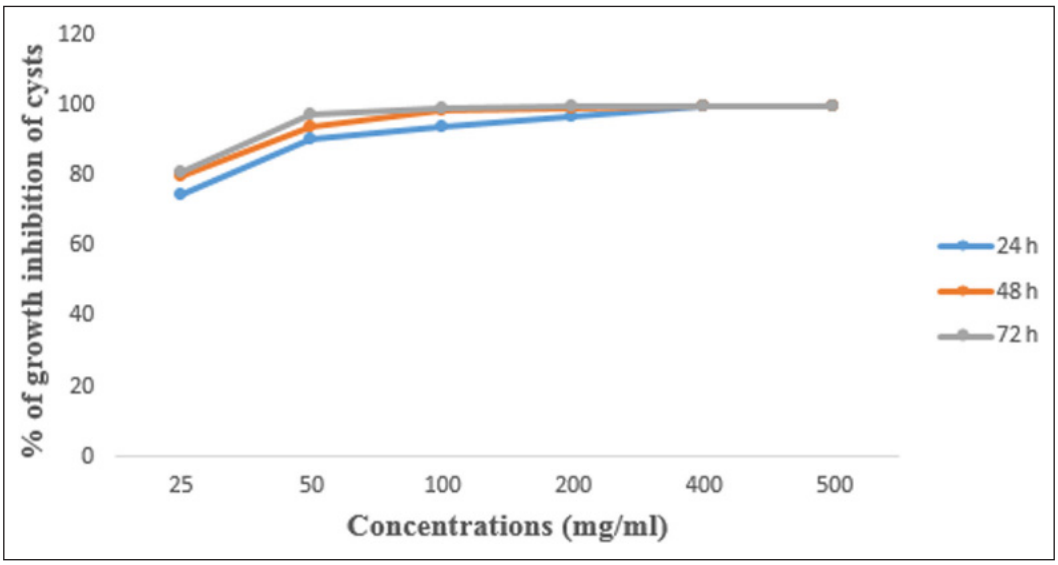

Figure 2. Effect of the total extract on the growth inhibition of *Acanthamoeba* cysts at different concentrations (mg/ml) after 24, 48, 72 h.

Results indicate that *Ziziphus vulgaris* is completely non-cytotoxic in used concentrations of anti-amoebicidal, since the $IC_{50,}$ total extract, chloroformic, remaining aqueous and primary alcoholic fractions of the plant were evaluated in the order of 904.3mg/ml, 397.8mg/ml, 530.6mg/ml, and 257.6 mg/ml.

DISCUSSION

Acanthamoeba keratitis is a potentially devastating and sight-threatening disease that constantly presents difficulties in treatment. The reported treatment drugs in the literature, have numerous effects

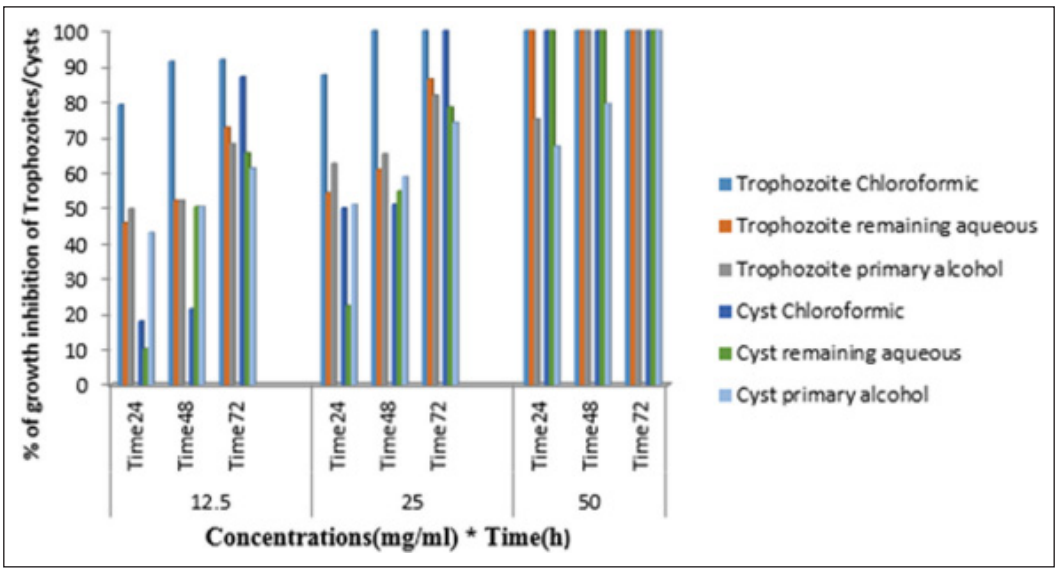

Figure 3. Effect of different fractions of *Ziziphus vulgaris* on the growth inhibition of *Acanthamoeba* trophozoites/cysts at different concentrations (mg/ml) after 24, 48, 72 h.

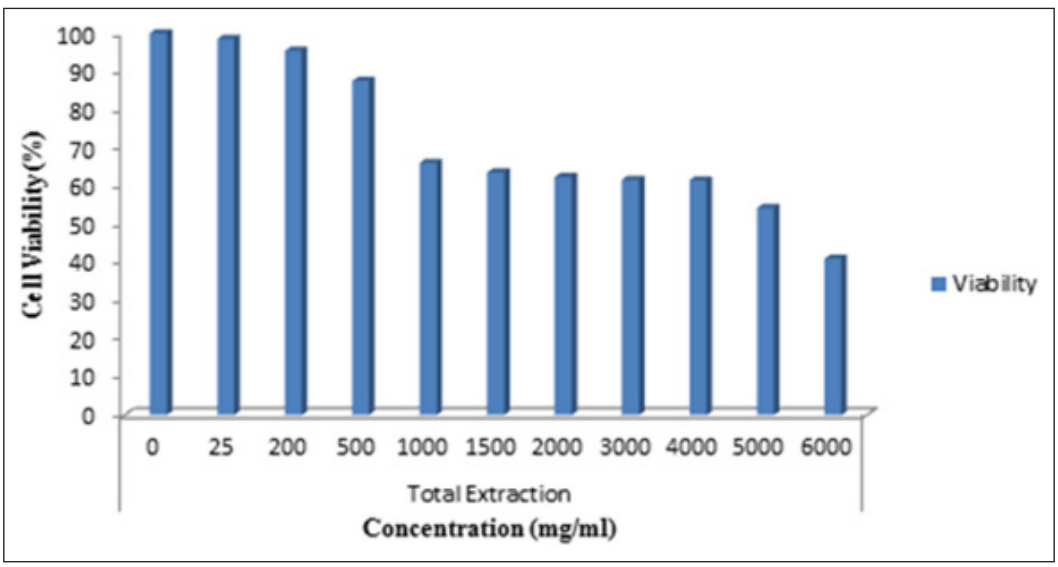

Figure 4. Test of cell viability (MTT). Action of total extract of *Ziziphus vulgaris* on murine peritoneal macrophages, presented as percentage of cell viability. The columns represent the average number of viable cells compared to the control ($p<0.001$ vs. control).

widely depending on the level of affectation of the disease, the health condition of the patients cornea, and the experience of the ophthalmologist (Ficker *et al.*, 1990). For example, in the *Acanthamoeba* keratitis case reported by Naginton *et al.*, various topical antimicrobial drugs were used combined with steroids, but in the end, both required grafting. Polyhexamethylene biguanide (PHMB, 0.02%) and chlorhexidine (0.02%) have been reported mostly effective for the treatment of the infection and 0.1% propamidine isethionate and 0.15% dibromopropamidine, have reported success only in the early stages of the disease (Lorenzo-Morales *et al.*, 2013). Unfortunately,

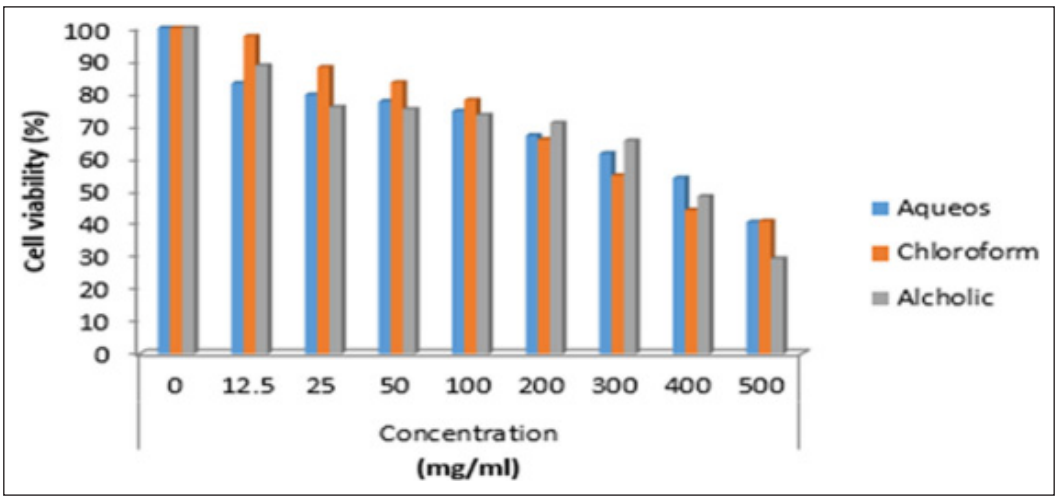

Figure 5. Test of cell viability (MTT). Action of different fractions of *Ziziphus vulgaris* on murine peritoneal macrophages, presented as percentage of cell viability. The columns represent the average number of viable cells compared to the control ($p<0.001$ vs. control).

Polyhexamethylene biguanide has a highly cytotoxic effect on the human keratocytes, even at the lowest cysticidal concentrations (Uemura *et al*., 2010). The length of treatment is also very high, and it could even last up to six months (Reinhard and Sundmacher, 2000). To reduce inflammation and pain, corticosteroids are also prescribed sometimes, but by reducing the ocular immune response, they can simultaneously increase the pathogenicity of the *Acanthamoeba*. The main drawback of the listed drugs is their toxicity and poor cysticidal effect (McClellan *et al*., 2001; Visvesvara, 2010).

Drug resistance is mainly due to the double-walled cyst, which forms a protective barrier against drugs. In the last few years, there has been a rising trend to shift resources from the current chemical drugs to plant drugs (Shinwari, 2010). Indeed, finding a new natural product compound with amoebicidal and cysticidal effect, and that is non-toxic for human cells, is of utmost importance in the control of AK cases. In the present study we have used the *Z. vulgaris* and its fractions against a pathogenic *Acathamoeba* strain belonging to T4 genotype, the sample was taken from AK patient, however more researches regarding the efficacy of the studied extract and its fractions on other *Acanthamoeba* genotypes such as T2 and T3 and also standard isolates (ATCC) are of utmost importance. Previous study conducted by El- Sayed *et al.* (2012) revealed that ethanol extracts of *Arachis hypogaea* L., *Curcuma longa* L. and *Pancratium maritimum* L. on *Acanthamoeba castellanii* cysts have significant cysticidal activity in a time and dose dependent way. Interestingly, all extracts seemed to have more efficiency in comparison to chlorhexidine alone. It is worthy to mention that *Curcuma longa* L. (turmeric) contains curcumin which exhibits a great variety of activities including anti-protozoal properties such as *anti-Plasmodium* and anti-*Leishmania* activity.

Although the exact anti-microbial process and underlying molecular mechanism of *Ziziphus* will be a focus for further studies, but *Z. vulgaris* could produce the free radicals especially reactive oxygen species which lead to several disorders by damaging biomolecules like Deoxyribonucleic Acid (DNA), proteins, and membrane lipids (Asgarpanah and Haghighat, 2012). These properties may affect the cyst wall of amoebae. On the other hand, the common drugs such as propamidine isethionate affect DNA and chlorhexidine destroy membrane permeability. It seems that *Z. vulgaris* is able to affect both organelles (Khan 2009; Asgarpanah and Haghighat, 2012). However

more studies are needed to clarify this issue. Moreover, the presence of the high amount of the flavonoids, saponin, tannin and alkaloids may contribute to the observed activity against *Acanthamoeba* spp. It is also seems that *Z. vulgaris* contain important antibacterial compounds such as tannin which lead to antimicrobial properties including *anti-mycobacterial* and antiparasitic effect. It is worthy to mention that *Ziziphus spina Christi* (another species of *Ziziphus*) contains main phytochemicals as *Z. vulgaris* such as flavonoids, alkaloids and saponins. Interestingly, this plant has been used in alternative medicine for cure of eye disease (Asgharpanah and Haghighat, 2012).

There are no previous studies regarding the amoebicidal activity of *Zizphus vulgaris* against *Acanthamoeba*, and thus, our research is the first report of the amoebicidal and cysticidal effect of this plant. The presence of tannin creates the ability of *Ziziphus*, to play a major role as an antidiarrhoeal and antihemorrhagic agent (Asquith and Butler, 1986), and has various physiological effects like antimicrobial such as *mycobacterial* and antiparasitic, such as malaria (Panseeta *et al*., 2011). Also, tannin inhibits the growth of many fungi, yeasts, and bacteria (Chung *et al*., 1998). Studies have shown that saponin is an active antifungal (Sadipo *et al*., 1991).

In addition, the *Ziziphus* is a well-known medicinal plant used in the treatment of some diseases such as obesity, weakness, digestive disorders, diarrhea, liver complaints, urinary disorders, diabetes, skin infections, fever and insomnia (Steiner, 1986; Abdel-Zaher *et al*., 2005; Scartezzini and Speroni, 2000). The present research clearly indicated that *in vitro*, *Zizphus vulgaris* extract/fractions were able to eliminate the trophozoites and cysts of *Acanthamoeba* in the tested concentrations. It should be mentioned that in comparison to other researches regarding other medicinal plants, we tested higher concentrations for the cytotoxic evaluation on macrophage cells. The results confirm that the *Zizphus vulgaris* plant, has no cytotoxic effect on the culture of macrophage cells with the dose of anti-*Acanthamoeba*. However, further studies are needed to evaluate the cytotoxicity of *Z. vulgaris* extracts and their fractions using human corneal cell line and *in vivo* models.

This study suggests that the *Ziziphus vulgaris* chloroformic fraction, may have compounds with relevance to the development of new anti-*Acanthamoeba* drugs.

Acknowledgments. Thanks are due to University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Tenerife, Canary Islands, Spain; JLM was funded by RYC-2011-08863 grant. The authors thanks for the grant from Traditional Medicine and Materia Medica Research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant No: 92/149).

REFERENCES

Abdel-Zaher, A., Salim, S., Assaf, M. and Abdel-Hady, R. (2005). Antidiabetic activity and toxicity of Zizyphus spina-christi leaves. *Journal of Ethnopharmacology*, **101(1)**: 129-138.

Asgarpanah, J. and Haghighat, E. (2012). Phytochemistry and pharmacologic properties of Ziziphus spina christi (L.) Willd. *African Journal of Pharmacy and Pharmacology*, **6(31)**: 2332-2339.

Asquith, T.N. and 10.1016/S0031-9422(00)81214-5 Butler, L.G. (1986). Interaction of condensed Tannins with selected proteins. *Phytochemistry*, **25(7)**: 1591-1593.

Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W. and Lin, Y. (1998). Tannins and human healt: a review. *Critical Reviews in Food Science and Nutrition*, **38(6)**: 421-464.

Corsaro, D., Walochnik, J., Kohsler, M. and Rott, M.B. (2015). *Acanthamoeba* misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for *Acanthamoeba micheli* sp. nov. (genotype T19). *Parasitology Research*, **114(7)**: 2481-90. 10.1016/S0031-9422(00)81214-5.

El-Sayed, N.M., Ismail, K.A., Ahmed, S.A. and Hetta, M.H. (2012). *In vitro* amoebicidal activity of ethanol extracts

of *Arachishypogaea L.*, *Curcuma longa L.* and *Pancratiummaritimum L.* on *Acanthamoeba castellanii* cysts. *Parasitology Research*, **110(5)**: 1985-1992.

Ficker, L., Seal, D., Warhurst, D. and Wright, P. (1990). *Acanthamoeba* keratitis resistance to medical therapy. *Eye*, **4(6)**: 835-838.

Goli-malekabadi, N., Asgary, S., Rashidi, B., Rafieian-Kopaei, M., Ghannadian, M., Hajian, S. and Sahebkar, A. (2014). The protective effects of *Ziziphus vulgaris* L. fruits on biochemical and histological abnormalities induced by diabetes in rats. *Journal of Complementary and Integrative Medicine*, **11(3)**: 171-177.

Govind, S., Visvesvara, G.S., Moura, H. and Schuster, F.L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp, *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *Immunology & Medical Microbiology*, **50(1)**: 1-26.

Hajialilo, E., Niyyati, M., Solaymani, M. and Rezaean, M. (2015). Pathogenic Free-Living Amoebae Isolated From Contact Lenses of Keratitis Patients. *Iranian Journal of Parasitology*, **10(4)**: 541-546.

Hussai, R.F., Nouri, A.M. and Oliver, R.T. (1993). New approach for Measurment of Cytotoxicity using Colorimetric assay. *Journal of Immunological Methods*, **160(1)**: 89-96.

Init, I., Lau, Y.L., ArinFadzlun, A., Foead, A.I., Neilson, R.S. and Nissapatorn, V. (2010). Detection of free living amoebae, *Acanthamoeba* and *Naegleria*, in swimming pools, Malaysia. *Tropical Biomedicine*, **27(3)**: 566-577.

Kaur, J., Singh, H. and Khan, M.U. (2011). Multifarious therapeutic potential of fenugreek: A comprehensive review. *International Journal of Research in Pharmaceutical and Biomedical*, **2(3)**: 863-871.

Khan, N.A. (2009). *Acanthamoeba* Biology and pathogenesis. *Caister Academic Press*.

Klimetzek, V. and Remold, H.G. (1980). The murine bone marrow macrophage, a sensitive indicator cell for murine migration inhibitory factor and a new method for their harvest. *Cellular Immunology*, **53(2)**: 257-266.

Lorenzo-Morales, J., Martín-Navarro, C.M., López-Arencibia, A., Arnalich-Montiel, F., Piñero, J.E. and Valladares, B. (2013). *Acanthamoeba* keratitis: an emerging disease gathering importance worldwide? *Trends in Parasitology*, **29(4)**: 181-187.

Lorenzo-Morales, J., Khan, N. and Walochnik, J. (2015). An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite*, **22(10)**.

Malatyali, E., Tepe, B., Degerli, S. and Berk, S. (2012). *In vitro* amoebicidal activities of *Saturejacuneifolia* and *Melissa officinalis* on *Acanthamoeba castellanii* cysts and trophozoites. *Parasitology Research*, **110(2)**: 2175-2180.

Mehrabani Natanzi, M., Pasalar, P., Kamalinejad, M. and Gerayesh-Nejad, S. (2012). Effect of aqueous extract of Elaeagnusangustifolia fruit on experimental cutaneous wound healing in rats. *Acta Medica Iranica*, **50(9)**: 589-596.

McClellan, K., Howard, K., Niederkorn, J.Y. and Alizadeh, H. (2001). Effect of steroids on *Acanthamoeba* cysts and trophozoites. *Investigative Ophthalmology & Visual Science*, **42(12)**: 2885-2893.

Niyyati, M., Lorenzo-Morales, J., Rahimi, F., Motevalli-Haghi, A., Martín-Navarro, C.M., Farnia, S., Valladares, B. and Rezaeian, M. (2009). Isolationand genotyping of potentially pathogenic *Acanthamoeba* strains from dust sources in Iran. *Tropical Medicine*, **103(4)**: 425-427.

Niyyati, M.A. and Rezaeian, M. (2015). Current Status of *Acanthamoeba* in Iran: A Narrative Review Article. *Iranian Journal of Parasitology*, **10(2)**: 157-163.

Panseeta, P., Lomchoey, K., Prabpai, S., Kongsaeree, P., Suksamrarn, A., Ruchirawat, S. and Suksamrarn, S. (2011). Antiplasmodial and anti-mycobacterial cyclopeptide alkaloids from the root of *Ziziphus mauritiana*. *Phytochemistry*, **72(9)**: 909-915.

Perrine, D., Chenu, J.P., Georges, P., Lancelot, J.C., Saturnino, C. and Robba, M. (1995). Amoebicidal efficiencies of various Diamidines against two strains of *Acanthamoeba polyphaga*. *Antimicrobial Agents and Chemotherapy*, **39(2)**: 339-342.

Polat, Z.A., Vural, A., Ozan, F., Tepe, B., Özcelik, S. and Cetin, A. (2008). *In vitro* evaluation of the amoebicidal activity of garlic (*Allium sativum*) extract on *Acanthamoeba castellanii* its cytotoxic potential on corneal cells. *Journal of Ocular Pharmacology and Therapeutics*, **24(1)**: 8-14.

Rahdar, M., Niyyati, M., Salehi, M. and Farnia, S. (2012). Isolation and genotyping of *Acanthamoeba* strains from environmental sources in ahvaz city, khuzestan province, southern iran. *Iranian Journal of Parasitology*, **7(4)**: 22-26.

Reinhard, T. and Sundmacher, R. (2000). Clinical aspects and therapy of *Acanthamoeba* keratitis. *Ophthalmology*, **97(6)**: 446-459.

Rezeaian, M., Farnia, Sh., Niyyati, M. and Rahimi, F. (2007). Amoebic keratitis in Iran (1997-2007). *Iranian Journal of Parasitology*, **2(3)**: 1-6.

Roongruangcha, K., Roongruangchai, J., Kaewmanee, S. and Manoot, D. (2008). Comparing lethal dose of povidone-iodine and Virkon to the *Acanthamoeba* cyst *in vitro*study. *Siriraj Medical Journal*, **60(4)**: 187-189.

Sadipo, O.A., Akanji, M.A., Kolawole, F.B. and Odutuga, A.A. (1991). Saponin is the active antifungal principle in Garcinia Kola, heckle seed. *Bioscience Biotechnology Research Communications*, **3**: 171.

Scartezzini, P. and Speroni, E. (2000). Review on some plants of Indian traditional medicine with antioxidant activity. *Journal of Ethnopharmacology*, **1(1)**: 123-143.

Shinwari, Z.K. (2010). Medicinal plants research in Pakistan. *Journal of Medicinal Plants Research*, **4(3)**: 161-176.

Shoaib, H.M., Muazza, A., Mir, A.G., Jung, S. and Matin, A. (2013). Evaluation of inhibitory potential of some selective methanolic plants extracts on biological characteristics of *Acanthamoeba castellanii*using hu-man corneal epithelial cells *in vitro*. *Parasitology Research*, **112(3)**: 1179-1188.

Steiner, R. (1986). Folk medicine: the art and the science. *Journal of the American Chemical Society*, **7(2)**: 223.

Tipu, M.A., Akhtar, M.S., Anjum, M.I. and Raja, M.L. (2006). New dimension of medicinal plants as animal feed. *The Pakistan Veterinary Journal*, **26(3)**: 144-148.

Uemura, T., Hirai, S., Mizoguchi, N., Goto, T., Lee, J.Y., Taketani, K., Nakano, Y., Shono, J., Hoshino, S., Tsuge, N., Narukami, T., Takahashi, N. and Kawada, T. (2010). Diosgenin present in fenugreek improves glucose metabolism by promoting adipocyte differentiation and inhibiting inflammation in adipose tissues. *Molecular Nutrition & Food*, **54(11)**: 1596-1608.

Visvesvara, G.S. (2010). Amebic meningo-encephalitides and keratitis: challenges in diagnosis and treatment. *Current Opinion in Infectious Diseases*, **23(6)**: 590-594.

Vyas, S., Agrawal, R.P., Solanki, P. and Trivedi, P. (2008). Analgesic and anti-inflammatory activities of *Trigonella foenum-graecum* (seed) extract. *Acta poloniae pharmaceutica*, **65(4)**: 473-476.

Wagner, U., Burkhardt, E. and Failling, K. (1999). Evaluation of Canine Lymphocyte Proliferation: Comparison of the different Colorimetric Methods With the H- thymidine Incorporation assay. *Veterinary Immunology*, **70(3)**: 151-159.