

## THE EVALUATION OF THE AMEBICIDAL ACTIVITY OF *ERYNGIUM PLANUM* EXTRACTS

MONIKA DERDA<sup>1\*</sup>, BARBARA THIEM<sup>2</sup>, JAROMIR BUDZIANOWSKI<sup>2</sup>, EDWARD HADAŚ<sup>1</sup>,  
WALDEMAR JERZY WOJT<sup>1</sup> and AGNIESZKA WOJTKOWIAK-GIERA<sup>1</sup>

<sup>1</sup>Department of Biology and Medical Parasitology, Poznan University of Medical Sciences,  
10 Fredry St., 61-701 Poznań, Poland

<sup>2</sup>Department of Pharmaceutical Botany, Poznan University of Medical Sciences,  
14 Św. Marii Magdaleny St., 61-861 Poznań, Poland

**Abstract:** Selected fractions of ethanolic extracts obtained from leaves and roots of *Eryngium planum* (Apiaceae) were evaluated *in vitro* for amebicidal activity against *Acanthamoeba castellanii*. This free-living amoeba is the cause of *Acanthamoeba* keratitis, which is a painful, vision-impairing disease of the eyes, and chronic granulomatous amoebic encephalitis. Treatment is very difficult and not always effective because of encystation, which makes the amoebae highly resistant to anti-amoebic drugs. The search for novel natural amebicidal agents is still of current interest. Fractions of *E. planum* ethanolic extract from basal leaves: flavonoid fraction (Lf), flavonoid-saponin fraction (Lf-s), saponin fraction (Ls) and phenolic acids fraction (La) and from roots: saponin fraction (Rs) and phenolic acids fraction (Ra) were assayed for anti-amoebic activity. In the presence of the saponin fractions and phenolic acid fractions (ranging from 1-5 mg/mL), the number of the trophozoites of *Acanthamoeba castellanii* viable strain 309 decreased during the experimental period (0-72 h). On the other hand, the flavonoid fraction from leaves showed a stimulating activity on the amoebae. Almost all fractions (except the flavonoid fraction) showed a time- and dose-dependent amoebistatic activity on the trophozoites. Of the fractions tested, the phenolic acid fraction from roots at the concentration of 5 mg/L showed the amebicidal activity on the trophozoites.

**Keywords:** *Acanthamoeba* sp., acanthamebiasis, treatment, *Eryngium planum*, amebicidal activity

Free-living amoebae belonging to the genera *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Learamoeba*, *Naegleria*, *Mastigina*, *Tetramintus*, *Valhikampfia*, *Vannella*, *Vexillifera* and *Willaertia* are small free-living organisms, which feed on bacteria, fungi, and other particulate matter and are highly adaptable to their environment (1). While many free-living amoebae are ubiquitous and harmless to humans, several genera are pathogenic.

Amoebae of the genus *Acanthamoeba* are a group of protozoa that are opportunistic pathogens in humans (2, 3). They constitute an etiological factor of chronic granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), amoebic pneumonitis (AP), as well as changes occurring in other human and animal organs. In immunocompromised individuals they may also cause cutaneous and sinus diseases (4).

*Acanthamoeba* keratitis is usually associated with trauma and exposure to contaminated water or

soil, often in agricultural workers. Additional potential risk factors include the use of contaminated tank-fed water in the home, warm weather and poor socioeconomic conditions. AK has also been reported after surgical trauma (including penetrating keratoplasty and radical keratotomies) and in contact lens users (5). An increased incidence of AK is observed during the summer, which may be connected with increased recreational activity and increased presence of *Acanthamoeba* in the environment. The development of AK in contact lens users is much more strongly related to poor lens hygiene and contaminated water.

In order to understand the diagnostic and treatment strategies knowledge of some aspects of the biology of *Acanthamoeba* and of the epidemiology and pathogenesis of *Acanthamoebiosis* is required. The life cycle consists of the infective trophozoite and resilient cyst stages. The trophozoite has an amoeboid shape with pseudopodia and it feeds on

\* Corresponding author: e-mail: mderda@ump.edu.pl

bacteria, other protozoans and small algae. Trophozoite encystment allows the organism to survive in an adverse environment, and is the form of the organism responsible for persistent disease.

Treatment of an *Acanthamoeba* infection is always very difficult and not always effective. In addition to antibiotics, which prevent further tissue damage, compounds that are amebicidal or amebistatic, such as biguanides and diamidines, are also used. These substances are frequently strong irritants and toxic for humans (6, 7).

The most important factors affecting prognosis include the severity of the disease at presentation and interval between the onset of symptoms and the start of effective therapy. More than 3 weeks is associated with a worse prognosis. The effective therapy is dependent on the elimination of viable cysts (5, 8, 9).

Many natural compounds have demonstrated antiparasitic effects (10, 11), yet the search for novel natural amebicidal agents is still of current interest (12, 13).

*Eryngium planum* L. (Flat Sea Holly), a species that belongs to the family Apiaceae and to the subfamily Saniculoideae, has been reported as a medicinal plant and used in traditional medicine in Europe (*E. plani herba* and *E. plani radix*). Infusions from aerial parts and roots of *E. planum* have been used in European folk remedies as an antitussive, diuretic, appetizer, stimulant and aphrodisiac (14). *E. planum* is a perennial erect herb with silvery-blue stems, widespread in Central and Southeast Europe. The presence of secondary metabolites such as phenolic acids (rosmarinic, chlorogenic and caffeic acids), triterpenoid saponins (barrigenol derivatives), flavonoids (kaempferol and quercetin derivatives),

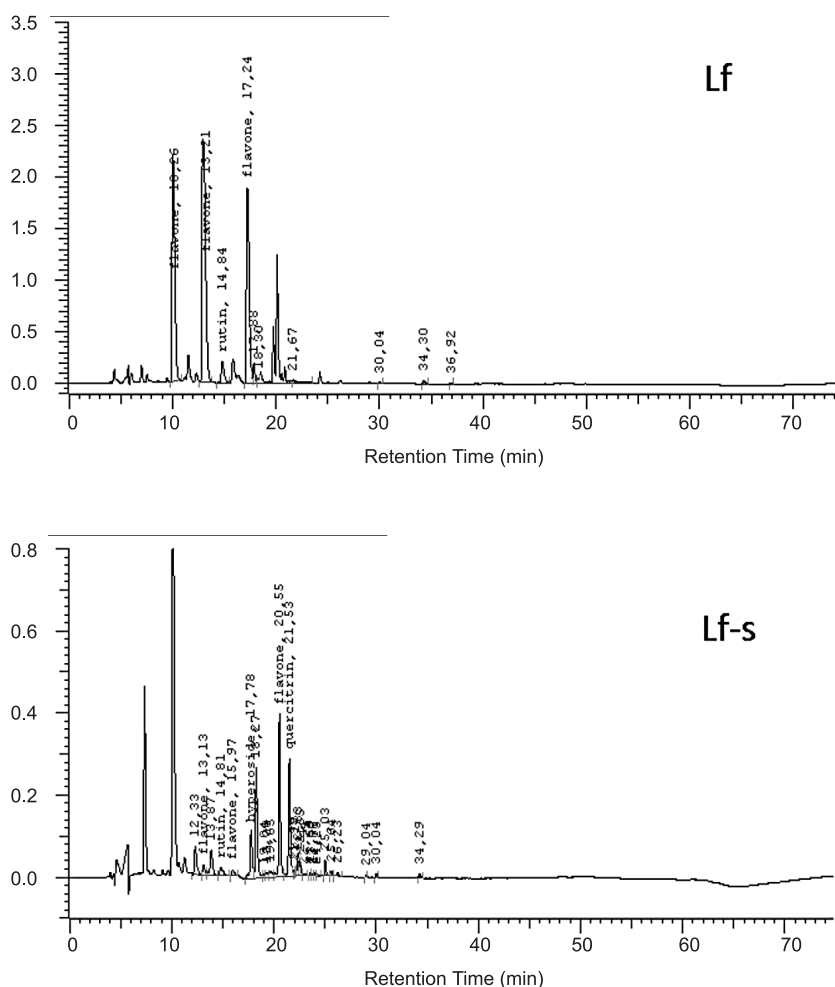


Figure 1. HPLC chromatograms of the flavonoid (Lf) and flavonoid-saponin (Lf-s) fractions from leaves of *E. planum*.

essential oils and coumarins is considered to determine their multidirectional pharmacological activities: diuretic, antidiabetic, expectorant, spasmolytic, anti-inflammatory, antinociceptive, hemolytic and antimycotic (14-16). Rosmarinic acid (RA) and chlorogenic acid (CGA), which are known for their antioxidant activity, have been described for many *Eryngium* species (17). Recently, essential oils and their identified components have been found in *E. planum* organs (18).

The aim of the present study was to investigate the *in vitro* amebicidal or amebistatic effect of the fractions of ethanolic extracts obtained from leaves and roots of *E. planum* on the growth and development of free-living amebae.

## MATERIALS AND METHODS

### Plant material

Plants of *Eryngium planum* L. – (Flat Sea Holly, Eryngo) were collected from its natural habitats in Poland, Kujawy region, in September 2009. Thanks to the Department of Pharmaceutical Botany and Plant Biotechnology, K. Marcinkowski University of Medical Sciences in Poznań, the voucher specimens were deposited in the Herbarium

of Medicinal Plant Garden of the Institute of Natural Fibers and Medicinal Plants. For our investigations, roots and basal leaves of the plants in the first year of vegetation were collected.

### Preparation of plant extracts and fractions

Dried and powdered basal leaves of *E. planum* (82.8 g) were extracted with boiling 70% ethanol (4 × 600 mL). The extracts were combined and concentrated under reduced pressure to give a dry extract (32.4 g), a portion of which (27.7 g) was separated by column chromatography (CC) on polyamide MN-6 (Macherey-Nagel; grain size 0.05-0.16 mm) using water, 20, 40, 60, 80 and 100% methanol and methanol with 0.1% ammonia for elution. Column fractions were combined according to the results of TLC examination (see section Phytochemical screening) to give sugar, coumarin, flavonoid (Lf), flavonoid-saponin (Lf-s), saponin (Ls) and phenolic acids (La) fractions. Dried and powdered roots of *E. planum* (351.0 g) were extracted with boiling 70% ethanol (4 × 3 L). The combined extracts were evaporated to give a dry extract (145.5 g), a portion of which (136.0 g) was separated by CC on polyamide using water, 100% methanol and methanol with 0.1% ammonia for elu-

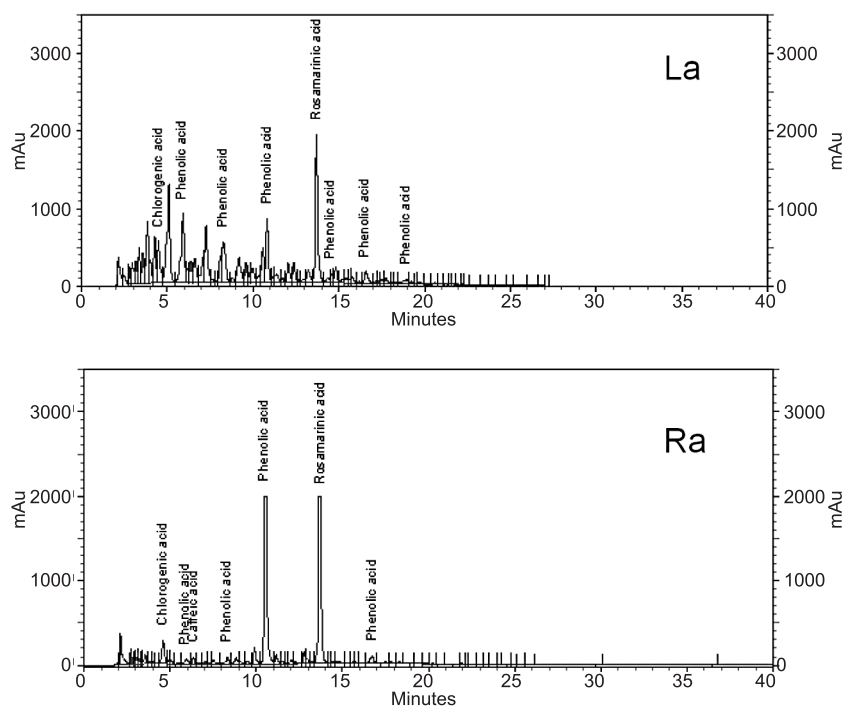


Figure 2. HPLC chromatograms of phenolic acids fractions from leaves (La) and roots (Ra) of *E. planum*.

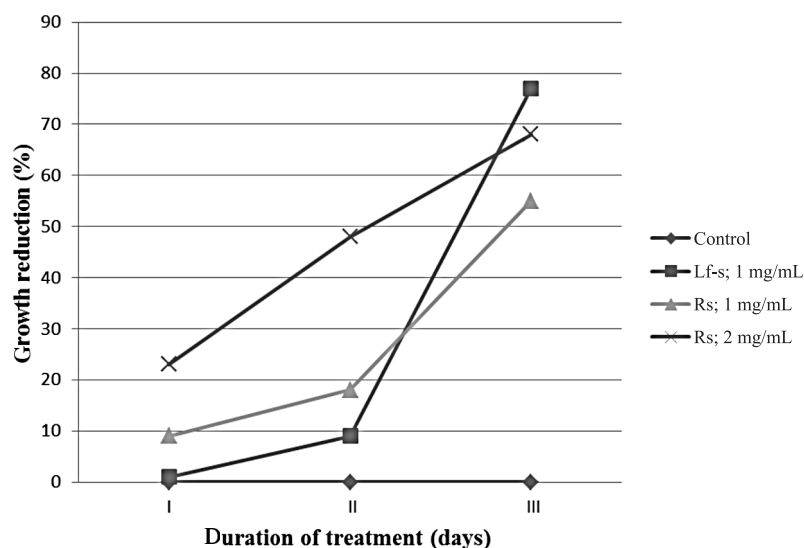


Figure 3. Percentage of growth reduction of *A. castellanii* trophozoites in culture medium after exposure to different concentrations of flavonoid – saponin fraction from basal leaves (Lf-s) and saponin fraction from roots (Rs)

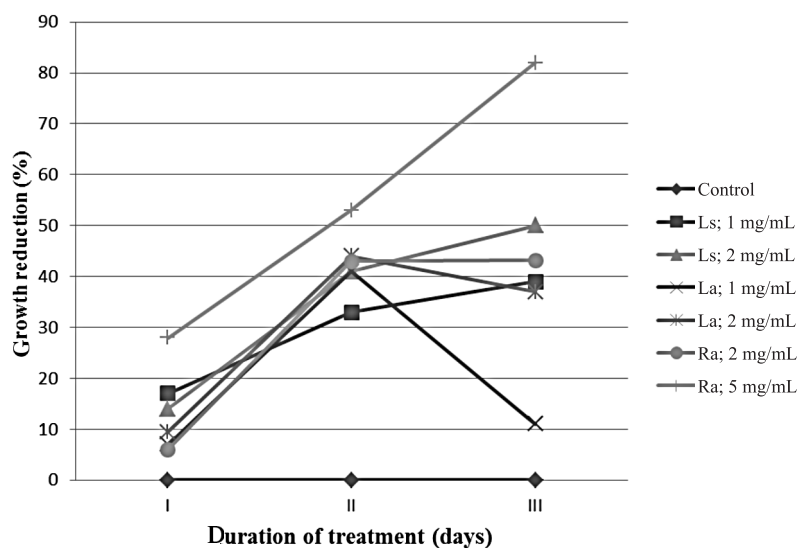


Figure 4. Percentage of growth reduction of *A. castellanii* trophozoites in culture medium after exposure to different concentrations of saponin fraction from basal leaves (Ls), phenolic acids fraction from basal leaves (La) and phenolic acids fraction from roots (Ra)

tion. Column fractions were combined according to the results of TLC examination (see section Phytochemical screening) to give sugar, saponin (Rs) and phenolic acids (Ra) fractions.

#### Phytochemical screening

Phytochemical analysis of the extracts and fractions was performed using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Equal portions of each

extract or fraction (0.1 g) were dissolved in 1 mL of 70% ethanol. An analysis of phenolic acids and flavonoids was performed on cellulose or silica gel HPTLC plates (Merck) developed with ethyl acetate-acetic acid-water (8 : 1 : 1, v/v/v). The plates were viewed under UV<sub>254</sub> and UV<sub>366</sub> nm light before and after spraying with 0.1% NA (2-aminoethanol diphenylborate) in ethanol. Brown bands changing color to yellow or orange fluorescent were considered as those of flavonoids, while

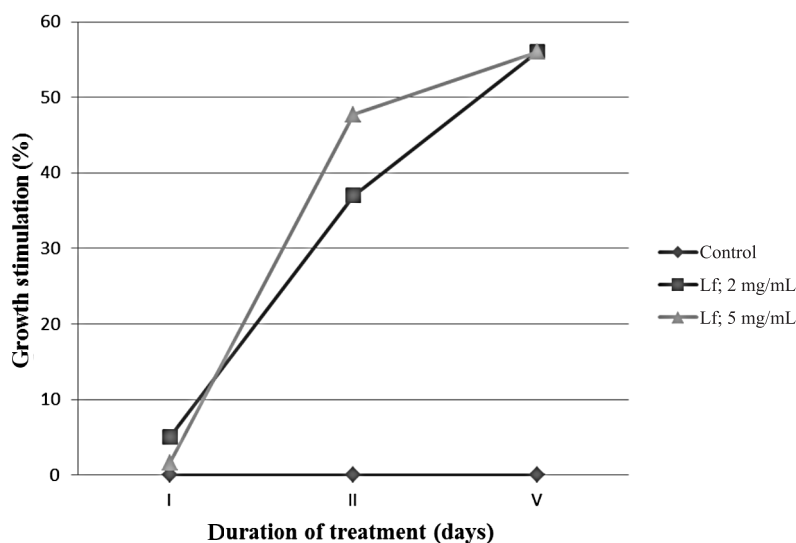


Figure 5. Percentage of growth stimulation of *A. castellanii* trophozoites in culture medium after exposure to different concentrations of flavonoid fraction from basal leaves (Lf)

blue bands changing to strong blue or blue-yellow fluorescent were considered as those corresponding to phenolic acids. Coumarins were recognized by strong blue fluorescence without staining. A respective analysis for saponins was performed by TLC on silica gel F plates (Merck) eluted with 1-butanol-acetic acid-water (4 : 1 : 5, v/v/v) (organic phase) and the detection was carried out by spraying with vanillin-sulfuric acid or Libermann-Burchard reagent followed by heating for 5 min at 100°C. Saponins appeared as violet-pink bands in daylight. HPLC-DAD was applied for an analysis of phenolic compounds (flavonoids and phenolic acids), while UHPLC-MS/MS was used for an analysis of saponins.

#### Culture of *Acanthamoeba* strain

The influence of the therapeutic fractions obtained from plant ethanolic extracts was tested *in vitro* on *Acanthamoeba castellanii* strain 309 (pathogenic for mice, isolated from the environment) (19).

The amoebae strains were axenically cultured on a liquid medium containing 2% Bacto-Casitone and 10% horse serum (20, 21). The plant extracts were dissolved in normal saline with a small quantity of DMSO. Plant extracts were added to the axenic culture of amoebae containing  $5 \times 10^4$  cells/mL at the following concentrations: 1, 2 and 5 mg/mL. The increase or decrease in the number of amoebae was checked at 24 h intervals during three days in a Thoma hemocytometer chamber. The control group

was a culture of amoebae without extracts. All measurements were repeated 18 times.

#### Statistical analysis

The average number of amoebae and standard deviation were calculated in each measurement group. The statistical analysis was determined using the Mann-Whitney and ANOVA tests. Statistical significance was defined as  $p < 0.05$ .

#### RESULTS

In the present study, *Eryngium planum*, a member of the Apiaceae family, was evaluated for its antiamebic activity using fractionated extracts prepared from the leaves and roots. Extracts prepared from those organs with 70% ethanol were separated by column chromatography to give flavonoid fraction (Lf), flavonoid-saponin fraction (Lf-s), saponin fraction (Ls) and phenolic acids fraction (La) from basal leaves and saponin fraction (Rs) and phenolic acids fraction (Ra) from roots. The fractions were further assayed for antiamebic activity. The preliminary analysis of those fractions by TLC was supported with HPLC-DAD and showed the presence of four flavonoids in the Lf fraction and eight phenolic acids including rosmarinic, chlorogenic and caffeic acids as the main compounds in the La and Ra fractions (Figs. 1, 2). On the HPLC chromatograms some unidentified peaks were labelled as phenolic acids on the basis of their DAD recorded UV spectra, which were very similar to those of the

Table 1. The effect of the flavonoid-saponin fraction from basal leaves (Lf-s) and saponin fraction from roots (Rs) on the proliferation of *A. castellanii* trophozoites.

Dosage of treatment	I duration of treatment		II duration of treatment		III duration of treatment	
	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation
Control	4.28 $\pm$ 0.98	0	4.06 $\pm$ 0.97	0	13.00 $\pm$ 1.22	0
Lf-s - 1 mg/mL	4.39 $\pm$ 1.05	2.6 (+)	3.72 $\pm$ 0.88	8.8 (-)	3.11 $\pm$ 0.85*	76.1 (-)
Rs - 1 mg/mL	4.67 $\pm$ 1.11	9.1 (+)	4.83 $\pm$ 0.91	19.0 (+)	5.78 $\pm$ 0.99*	55.5 (-)
Rs - 2 mg/mL	5.28 $\pm$ 0.98	23.4 (+)	6.00 $\pm$ 1.15	47.8 (+)	4.33 $\pm$ 0.89*	66.7 (-)

(+) – stimulation; (-) – inhibition; \*p < 0.05 statistically significant difference in comparison with the control during the same time interval.

Table 2. The effect of the saponin fraction from basal leaves (Ls), phenolic acids fraction from basal leaves (La) and phenolic acids fraction from roots (Ra) on the proliferation of *A. castellanii* trophozoites.

Dosage of treatment	I duration of treatment		II duration of treatment		III duration of treatment	
	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation
Control	18.83 $\pm$ 3.33	0	21.00 $\pm$ 2.88	0	30.78 $\pm$ 4.45	0
Ls - 1 mg/mL	22.06 $\pm$ 4.01*	17.2 (+)	14.06 $\pm$ 2.15*	33.1 (-)	19.05 $\pm$ 3.85*	39.1 (-)
Ls - 2 mg/mL	21.44 $\pm$ 3.99*	13.9 (+)	12.39 $\pm$ 2.00*	41.0 (-)	15.11 $\pm$ 2.22*	50.1 (-)
La - 1 mg/mL	20.17 $\pm$ 3.05*	7.1 (+)	12.44 $\pm$ 1.75*	40.8 (-)	27.39 $\pm$ 3.15	11.1 (-)
La - 2 mg/mL	17.06 $\pm$ 2.69	9.4 (-)	11.78 $\pm$ 1.99*	43.9 (-)	19.44 $\pm$ 3.18*	36.8 (-)
Ra - 2 mg/mL	19.94 $\pm$ 2.55	5.9 (+)	12.06 $\pm$ 2.04*	42.6 (-)	17.50 $\pm$ 2.99*	43.1 (-)
Ra - 5 mg/mL	13.5 $\pm$ 1.88	28.3 (-)	9.89 $\pm$ 0.89*	53.0 (-)	5.56 $\pm$ 0.72*	81.9 (-)

(+) – stimulation; (-) – inhibition; \*p < 0.05 statistically significant difference in comparison with the control during the same time interval.

Table 3. The effect of the flavonoid fraction from basal leaves (Lf) on the proliferation of *A. castellanii* trophozoites.

Dosage of treatment	I day duration of treatment		II day duration of treatment		III day duration of treatment	
	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation	Average number of ameba $\pm$ SD	% of growth inhibition or stimulation
Control	9.72 $\pm$ 0.99	0	10.72 $\pm$ 1.33	0	31.67 $\pm$ 4.98	0
Lf - 2 mg/mL	9.22 $\pm$ 1.11	5.1 (-)	14.72 $\pm$ 1.56	37,3 (+)	49.33 $\pm$ 5.68*	55.8 (+)
Lf - 5 mg/mL	9.56 $\pm$ 0.86*	1.6 (-)	15.83 $\pm$ 1.13	47,7 (+)	49.42 $\pm$ 5.01*	56.1 (+)

(+) – stimulation; (-) – inhibition; \*p < 0.05 statistically significant difference in comparison with the control during the same time interval.

reference phenolic acids, like rosmarinic, caffeic and chlorogenic acids. The results of TLC and UHPLC-MS/MS indicated the presence of a complex of triterpenoid saponins (10-11 compounds) in the Ls, Lf-s and Rs fractions (data not shown). The Lf-s (flavonoid-saponin) fraction contained five

flavonoids and about ten saponins and shared only one flavonoid with the Lf fraction and probably two saponins with the Ls fraction.

The amebicidal and amebistatic activity of the aforementioned fractions was investigated. The study was conducted on pathogenic trophozoites of

*Acanthamoeba castellanii* strain 309 under *in vitro* conditions. It demonstrated that the saponin fraction (Ls) and the phenolic acids fraction from both leaves (La) and roots (Ra) and the flavonoid-saponin fraction from leaves (Lf-s) (at the concentration of 1.0–2.0 mg/L) exhibited an amebistatic activity (Tables 1, 2 and Figs. 3, 4). The flavonoid-saponin fraction from leaves (Lf-s) showed the strongest amebistatic effect, i.e., a 76% inhibition of the amebae growth. Other fractions inhibited the growth of amebae in the range from 36 to 55%. The phenolic acid fraction obtained from roots (Ra) exhibited amebicidal activity at a concentration of 5.0 mg/L. The percentage decrease of the number of trophozoites was approximately 82% compared to the control. On the other hand, the flavonoid fraction from leaves (Lf) stimulated growth of the amebae. That fraction at concentrations of 2.0 and 5.0 mg/L contributed to an over 56% stimulation of the amebae growth compared to the control (Table 3 and Fig. 5).

## DISCUSSION

Some secondary metabolites that are synthesized in plants have the ability to protect the plants from pathogens, insects and other herbivores (22). Extracts of plants with medicinal properties are being examined with increasing frequency for antibacterial, antifungal, antiviral and antiprotozoal activity due to their natural bioactive ingredients. Recently, some reports have been published on several medicinal species that exhibit amebicidal or amebistatic effects against *Acanthamoeba* trophozoites and cysts *in vitro* (12, 23–28).

In the present study, *E. planum*, a member of the Apiaceae family was evaluated for its amebicidal activity. Earlier reports on extracts of some plants belonging to that family, such as *Peucedanum* spp. and *Pastinaca*, have demonstrated their properties against *Acanthamoeba* (23–25). Previous studies have demonstrated that ethanolic extracts from *E. planum* that were active in the present study show antibacterial and antifungal activity, too (16).

The amebicidal effect of the fractions of phenolic acids from leaves and roots obtained in this study may be attributed to the phenolic acids which are the major phenolic compound with antioxidant properties in *Eryngium* species (17). Phenolic acids, caffeic acid derivatives, and rosmarinic acid (a depside composed of two caffeic acid molecules) which is the major compound in *E. planum*, possess antioxidant properties. In Roongruangchai's opinion (13), phenolic compounds may cause damage to the plasma membrane, which results in a leakage of intra-

cellular constituents from the cell. Phenolic compounds act as oxidizing agents causing cell membrane damage by reacting with cellular proteins, lipids, nucleic acids and carbohydrates. Plant phenolics have been regarded as important defence compounds against pathogens and insects (22).

Another important group of active constituents present in the two fractions of the *E. planum* extract were triterpenoid saponins. These compounds exhibit various biological activities (29, 30). The amebistatic effect of the saponin fractions from leaves and roots seems to be resulting from the complexity of those compounds. The maximum activity, that of the flavonoid-saponin fraction from leaves, may be due to the synergies between those two groups of compounds. In their review on the biological action of saponins in animals, Francis and co-workers (29) have reported that saponins have high toxicity against fungi. Some saponins and sapogenins, for example a mixture of purified saponins from *Maesa lanceolata*, have been shown to have virucidal activity. Moreover, saponins show an effect on protozoa. The toxicity of those compounds to protozoans seems to be widespread and non-specific and it obviously is a result of their detergent effect on the cell membrane. The main mechanism suggested for the antifungal activity of saponins (that are also defence compounds) is their interaction with membrane sterols (29, 31).

The amebicidal effect of some fractions of *E. planum* may be associated with some specific interactions of the active phytochemicals with the cell wall of the *Acanthamoeba* or with a more effective mechanism of penetration into the parasites through membrane channels. These mechanisms have also been proposed by Malatyali and co-workers (26).

Plants are a potential source of new bioactive natural compounds for pharmaceutical purposes. Natural plant products may provide a new series of chemotherapeutic agents and may be used for treatment of amebic keratitis. Some substances of plant origin have already been used as natural agents to treat other parasitic diseases (11).

Further studies using purified compounds of plant extracts or fractions are necessary to identify the active components and to evaluate the mechanism of action of the effective substances both *in vitro* and *in vivo*. To our knowledge, this is the first report on the amebicidal activity of *E. planum* extracts against *A. castellanii*.

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