In vitro effect of *Acanthospermum australe* (Asteraceae) extracts on *Acanthamoeba polyphaga* trophozoites

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ABSTRACT: *Acanthamoeba* is a free-living protozoan widely distributed in the environment, occurring in vegetative trophozoite and resistance cyst stages during its life cycle. It constitutes an etiological agent of *Acanthamoeba* keratitis, a disease that may cause severe ocular inflammation and blindness. New drugs can be developed from molecules found in plants and thus help in its difficult treatment. *Acanthospermum australe* (Asteraceae), a plant used in folk medicine, had its effect tested on *Acanthamoeba polyphaga*. Aqueous and ethanolic extracts of *A. austral* were obtained from aerial parts for infusion and static maceration, respectively. Concentrations of 10, 5, 2.5, 1.25 and 0.625 mg/ml of the extract were tested against *Acanthamoeba polyphaga* trophozoites. The cytotoxic effect of the extracts was tested in mammalian cells using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RESULTS: The 10 mg/ml concentration of ethanolic extract was lethal to 100% of the *A. polyphaga* trophozoites in 24 h and both extracts presented cytotoxic effect against mammalian cells. These findings suggest that the *A. austral* ethanolic extract may have compounds with relevance to the development of new amoebicidal drugs.

Keywords: *Acanthamoeba polyphaga*, amoebicidal activity, *Acanthospermum australe.*

RESUMO: Efeito *in vitro* de extratos de *Acanthospermum australe* (Asteraceae) sobre trofozoitos de *Acanthamoeba polyphaga. Acanthamoeba* é um protozoário de vida livre amplamente distribuído no ambiente, ocorrendo sob a forma trofozoítica (metabolicamente ativa) e cística (de resistência), durante seu ciclo de vida. O protozoário constitui um agente etiológico da Ceratite Amebiana, uma doença que pode causar inflamação ocular severa e cegueira. Novos fármacos podem ser desenvolvidos a partir de moléculas encontradas em plantas e assim ajudar em seu difícil tratamento. Aqui, *Acanthospermum australe* (Asteraceae), uma planta utilizada na medicina popular, teve seu efeito sobre trofozoítos de *Acanthamoeba polyphaga* testado. O extrato aquoso e etanólico de *A. australe* foram obtidos das partes aéreas por infusão e maceração estática, respectivamente. As concentrações 10, 5, 2,5, 1,25 e 0,625 mg/ml dos extratos foram testadas contra trofozoítos do protozoário. O efeito citotóxico dos extratos foi testado em células de mamífero utilizando o ensaio de brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio (MTT). A concentração de 10 mg/ml do extrato etanólico foi letal a 100% dos trofozoítos de *A. polyphaga* em 24 h e ambos os extratos apresentaram efeito citotóxico contra as células de mamífero. Estes resultados sugerem que o extrato etanólico de *A. australe* pode ter componentes com relevância para o desenvolvimento de novos fármacos amebicidas.

Palavras Chave: *Acanthamoeba polyphaga*, atividade amebicida, *Acanthospermum australe.*

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INTRODUCTION

The free-living amoebae (FLA) are a group of protozoa widely dispersed in nature, being found in soil, water and air. Some species can live as facultative parasites in humans and domestic animals. *Acanthamoeba*, a FLA genus, can occur in trophozoite (metabolically active) and resistant cyst forms during its life cycle. Some species of *Acanthamoeba* are opportunistic pathogens that can cause *Acanthamoeba* Granulomatous Encephalitis (AGE) and *Acanthamoeba* keratitis, but may also be associated with cutaneous lesions and sinusitis in immunocompromised patients (Khan, 2006).

Acanthamoeba keratitis is a chronic inflammation of the cornea caused by infection with several *Acanthamoeba* species. Keratitis primarily affects users of contact lenses, which in recent years have greatly increased around the world. The available treatments include aromatic diamidines (hexamidine, pentamidine, or propamidine isothionate), cationic antiseptics, aminoglycosides, imidazoles and polyenes (amphotericin B) (Auran *et al.* 1987; Chomicz *et al.*, 2005; Obeid *et al.,* 2003). Because these drugs do not have great efficacy against the cystic form of this organism, the treatment is long and complex. Thus, the search for new drugs is crucial to develop dynamic therapies and facilitate treatment (Obeid *et al.*, 2003).

Members of the Asteraceae have been used in traditional medicine as antiseptic, antifungal and antiparasitic agents (Portillo *et al.*, 2001; Ródio *et al.*, 2008). The asteracean *Acanthospermum australe* is used in popular medicine to treat different diseases, but scientific studies demonstrating its efficiency and safety are not available. *Acanthospermum hispidum* has been widely studied and shows antiparasitic (Sanon *et al.*, 2003; Bero *et al.*, 2011; Ganfon *et al.*, 2012) and antibacterial (Arena *et al.*, 2011; Alva *et al.*, 2012) activity. Here, we tested the effect of *Acanthospermum australe* extracts on *Acanthamoeba polyphaga* trophozoites, and also evaluated the cytotoxicity of these extracts to mammalian cells.

MATERIALS AND METHODS

Plant material

The aerial parts of *A. australe* (Loefl.) O. Kuntze were collected at the city of Lajeado, Rio Grande do Sul, Brazil, in April, 2009. The plant material was identified by botanical Dra. Elisete Maria de Freitas. Voucher specimen (HVAT 2346) was deposited at the Herbarium of the Centro Universitário UNIVATES.

Ethanolic Extract (EE)

The EE of *A. australe* was obtained by static maceration, using 100 g of powdered dried leaves in 1.5 L of 90% ethanol for 7 days. The extract was filtered and the solvent was completely removed at 40 °C, under reduced pressure in a rotary evaporator. The yield of the EE was 12.8% (w / w).

Aqueous Extract (AE)

The AE of *A. australe* was obtained by infusion, using 100 g of powdered dried leaves in 1.5 L of boiling distilled water for 30 minutes. The extract was filtered and the solvent was completely removed at 40 °C, at reduced pressure on rota-evaporator. The yield of AE was 17.1% w/w. The dried extracts were reconstituted in methanol to obtain final concentrations of 100 µg/ml and 200 µg/ml.

Amoeba cultures

The clinic strain of *A. polyphaga* (ATCC 30461) was obtained from the American Type Culture Collection. The axenic cultures were kept in PYG medium (2% proteose peptone, 0.2% yeast extract and 1.8% glucose) at a constant temperature of 30°C. For the experiment, one ml (1ml) of the culture was centrifuged for 5 min at 670 x *g*, the supernatant discarded, and the precipitate washed twice with phosphate-buffered saline buffer (PBS). The precipitate of amoebae was diluted in PYG medium to obtain a final concentration of 2 $\times 10^4$ trophozoites per milliliter.

Assessment of amoebicidal activity

Amoebicidal activity was performed according to Sauter *et al.* (2011). Briefly, the extracts were solubilized with 1% Tween 20 and water to a final concentration of 20 mg/ml and were tested at final concentrations of 10, 5, 2.5, 1.25 and 0.625 mg/ml. For the assessment of amoebicidal activity, 100 ìL of *A. polyphaga* culture and 100 ìL of each test solution were inoculated into each well of a 96-well plate and incubated at 30°C. *Acanthamoeba* were counted in a Fuchs-Rosenthal counting chamber after 24 hours. Viability was assessed using trypan blue dye exclusion method. The control used was sterile water containing 1% Tween 20. All experiments were performed in triplicate with at least three repetitions.

Cytotoxicity assay

Cytotoxic effect of the *A. australe* extracts were evaluated by 3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann 1983). The tests were performed according to Sauter *et al.* (2011). Briefly, Vero cells (African Green Monkey

Kidney, ATCC CCL-81) received Eagle's minimal essential medium supplemented with 10% fetal bovine serum (E-MEM/FBS); (GIBCO) with extract at different concentrations (10, 5, 2.5, 1.25 and 0.625 mg/ml). Medium without extract was used how control. The cells were incubated at 37 ºC in a humidified 5% CO_2 atmosphere. After 48h, 50µL of MTT (Sigma Chemical Co., Saint Louis, MO, USA) solution (2 mg/ml) was added to each well and incubated for further 4 h. The plates were centrifuged (1400 × *g* for 5min) and the untransformed MTT was removed. Ethanol (100 µL) was added to each well to solubilize formazan crystals and the optical density (OD) measured in an ELISA reader (Anthos 2020) at 550 nm with a 620 nm reference filter. The amount of formazan produced was directly proportional to the number of living cells in culture. Results were expressed as the percentual OD of viable cells in comparison to the OD of untreated control cells.

Statistical analysis

The means were submitted to the ANOVA test with Tukey Post Hoc (p<0.01) in the SPSS Statistical Software 17.0.

RESULTS

The amoebicidal activity of *A. australe* (Loefl.) O. Kuntze extract was tested against *A. polyphaga* trophozoites, which were chosen as the standard of clinical origin (isolated from an *Acanthamoeba* keratitis lesion). Through the tests in the study, we determined the amoebicidal activity of *A. australe* extracts after 24 hours of treatment. *A. australe* AE showed no activity at the concentrations tested (Figure 1), compared to the control group. There was no statistical difference among them, but the concentration of 0.625 mg/mL increased the proliferation of trophozoites, suggesting that the compounds present in the AE did not show amoebicidal activity under the conditions used in this *in vitro* assay.

The EE was tested, using the same *in vitro* assay. *A. polyphaga* trophozoites underwent the same test with different doses of *A. australe* EE. The dose of 10 mg/mL showed activity against the amoeba, eliminating 100% of viable trophozoites (Figure 2). The concentrations of 5, 2.5 and 1.25 mg/mL had different degrees of amoebicidal activity, allowing 15, 65 and 89% of trophozoites to remain viable, respectively, compared to the control group. The concentration 0.625 mg/mL allowed an increase in the trophozoite growth compared to the control: the number of trophozoites increased 11% after 24 h. All concentrations were statistically different (p <0.01) by the Tukey test.

The results for *in vitro* amoebicidal activity showed that the EE of *A. australe* was active in a dose-dependent manner, as assessed by linear regression (Figure 3), i.e., the activity was directly proportional to the increase in the dose. Thus, the minimum inhibitory concentration of EE against *A. polyphaga* was 8.77 mg/mL. The ethanolic extract from *A. australe* showed high activity against the trophozoites. However, at all concentrations tested, some trophozoites encysted, unlike the control group, which showed no encystment. This is very important because the ability of trophozoites to encyst during therapy is the major problem leading to reinfection (Schuster and Visvesvara, 2004).

Cytotoxic effect of EE and AE on mammalian cells

The MTT assay was used to test the cytotoxic effect, and showed that both the aqueous and hydroethanolic extracts were toxic against

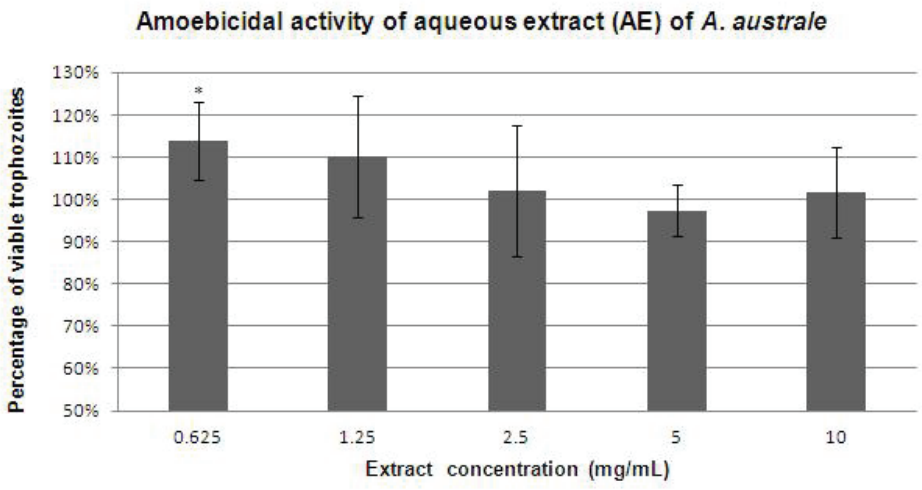

FIGURE 1. Amoebicidal activity of aqueous extract (AE) of *A. australe* presented as percentage of viability of *A. polyphaga* trophozoites.

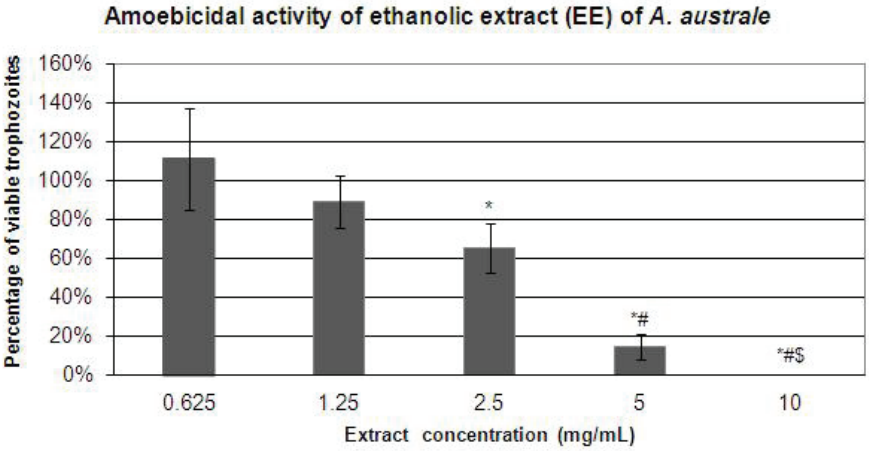

FIGURE 2. Amoebicidal activity of ethanolic extract (EE) of *A. australe* presented as percentage of viability of *A. polyphaga* trophozoites.

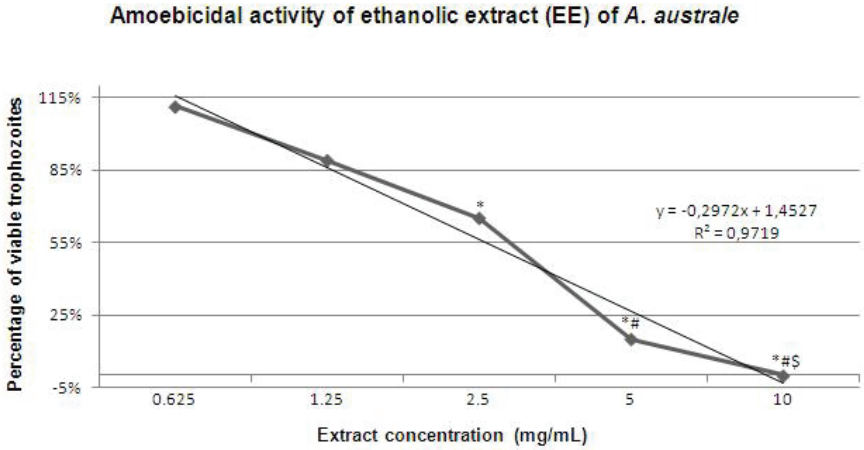

FIGURE 3. Linear regression from the concentrations of *A. australe* ethanolic extract (EE) front of the percentage viability of *A. polyphaga* trofozoites.

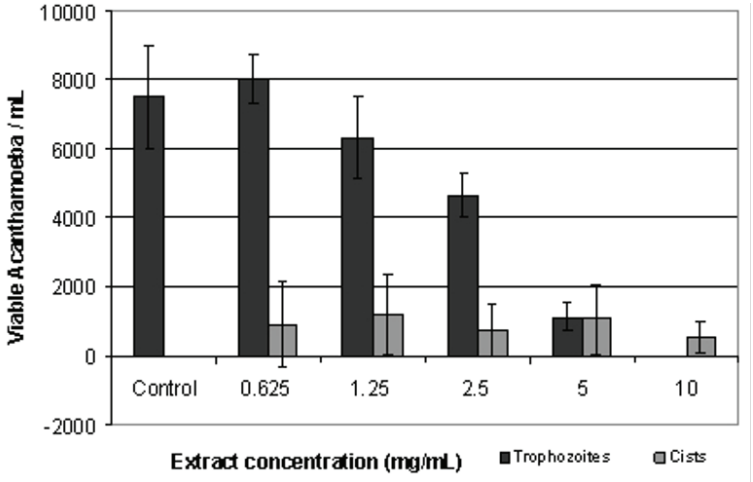

FIGURE 4. Amoebicidal activity of ethanolic extract (EE) of *A. australe* presented as viable *Acanthamoeba* per milliliter.

mammalian cells (data not shown). All concentrations killed 100% (vs control) of the Vero cells. This level of cytotoxicity shows that the use of these extracts on the cornea for treatment of keratitis is not feasible.

DISCUSSION

The investigation of plants used in traditional medicine is a widely used strategy for finding alternative antibiotics (Brantner and Grein, 1994). Recently, several substances obtained from plants have been studied for amoebicidal activity, and many of these compounds have proven to be more effective than the currently used therapy (Di Stasi, 1995; Polat *et al.*, 2008).

The family Asteraceae has been of interest to researchers due to the presence of many compounds that are active against a range of microorganisms. *A. australe* (Loefl.) O. Kuntze, a member of this family, has been studied in different areas of application. Studies showed that *A. australe* exerts effects on myelopoiesis that may be implicated in antitumor immune responses (Mirandola *et al.*, 2002). Rocha Martins *et al.* (2011) reported for the first time the antiviral activity of extracts and fractions from *A. australe* aerial parts. The antifungal activity of aqueous, dichloromethane and methanol extracts from *A. australe* was assayed *in vitro* against 11 fungal strains including several ûlamentous fungi and yeasts (*Aspergillus fumigatus, Aspergillus níger, Candida albicans, Cladosporium cladosporioides, Cryptococcus neoformans, Microsporum gypseum, Penicillium purpurogenum, Saccharomyces cerevisiae, Trichophyton mentagrophytes, Fusarium oxysporum and Neurospora crassa*), showing activity as well (Portillo *et al.*, 2001).

Amoebicidal activity has been reported in recent years, for several different plants. Extracts and essential oil of plants from the family Asteraceae have been found in many studies to show high levels of activity against *Acanthamoeba*. Ródio *et al.* (2008) showed that a hexane extract of *Pterocaulon polystachyum* (Asteraceae) has amoebicidal activity against a clinical strain of *Acanthamoeba*. Essential oil of *P. polystachyum* also showed high activity in the same conditions (Sauter *et al.*, 2011).

Medicinal plants can produce highly complex molecules, some of which might serve as a basis for new products to be used against *Acanthamoeba*. In this study, we were able to show that the *A. australe* EE has activity against trophozoites of *A. polyphaga*. However, the cytotoxic assay showed that EE from aerial parts of *A. australe* is inappropriate for direct use in keratitis therapy. Therefore, additional bioprospecting studies are needed to identify and isolate compounds present in *A. australe.* Further biological tests should be conducted to identify molecular targets of these products, aiding in the development of new drugs against *Acanthamoeba* keratitis.

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