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ORIGINAL PAPER

Amoebicidal activity and chemical composition of *Pterocaulon polystachyum* (Asteraceae) essential oil

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Abstract *Acanthamoeba* species are free-living amoebae that constitute an etiological agent of *Acanthamoeba* keratitis, an illness that may cause severe ocular inflammation and blindness and has a very difficult treatment. These molecules that are found in plants may be an alternative for the development of new drugs. Plants of the genus *Pterocaulon* (Asteraceae) are used in folk medicine as an antiseptic and antifungal agent. In this work, we analyzed *Pterocaulon polystachyum* essential oil and assessed its amoebicidal activity against *Acanthamoeba polyphaga*. The leaves of the fresh plant submitted to hydrodistillation yielded 0.15% (*w*/*v*) of essential oil that was analyzed by gas chromatography–mass spectrometry being *E*-sesquilavandulyl acetate as the major component, representing 43.8% of the oil. For the assessment of the amoebicidal activity, concentrations of 20, 10, 5, 2.5, and 1.25 mg/mL of essential oil were tested, being lethal to 100% of the *A. polyphaga* trophozoites at the concentrations of 10 and 20 mg/mL in 24 and 48 h. The cytotoxic effect of essential oil was also tested in mammalian cells using MTT assay. Amoebicidal activity results are in accordance with previous work in which the lipophilic compounds from this plant were active against *Acanthamoeba castellanii*. However, further studies with the major component of the essential oil will be carried out.

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Introduction

Acanthamoeba is a free-living protozoan widely distributed in the environment, occurring in vegetative trophozoite, and resistance cyst stages during its life cycle. *Acanthamoeba* can cause two well-recognized diseases: *Acanthamoeba* keratitis and *Acanthamoeba* granulomatous encephalitis. *Acanthamoeba* keratitis has been recognized as a significant ocular microbial infection, being an acute inflammation of the cornea that can result in blindness when not properly treated in the initial stage (Schuster and Visvesvara 2004; Khan 2006). The incidence of the illness has enhanced due to the increasing number of contact lens wearers. Contact lenses exposed to contaminated water and inappropriately cleaned are among the main risk factors of the infection. If there is a trauma or hypoxia in the corneal epithelium, the invasion of the parasite into the stroma is facilitated, and it adheres to the host tissue initiating a cytopathic effect (Clarke and Niederkorn 2006; Kliescikova et al. 2011).

Early diagnosis followed by adequate treatment is indispensable to patients presenting *Acanthamoeba* keratitis. The infection is difficult to cure because the treatment must be maintained for a long period. The recommended

treatment includes a biguanide (polyhexamethylene biguanide or chlorhexidine digluconate) together with a diamidine (propamidine isethionate or hexamidine) (Khan 2006). Inadequate treatment can cause reinfection once the trophozoite can encyst under adverse conditions, which makes the recommended correct utilization of the drugs during the whole treatment. Therefore, more effective drugs against *Acanthamoeba* must be developed and medicinal plants can be useful in this search.

Pterocaulon (Asteraceae) has been used in traditional medicine as antiseptic, antifungal, and antiparasitic agent (Avancini 2002). A study of Stein and colleagues (2005) and Daboit et al. (2010) showed that *Pterocaulum alopecuroides*, *Pterocaulum balansae*, and *Pterocaulum polystachyum* extracts were active against a range of pathogenic fungi. The hexane extract of the latter plant showed amoebicidal activity against a pathogenic strain of *Acanthamoeba* (Ródio et al. 2008). So, the aim of present study is to analyze the essential oil obtained from the aerial parts of *P. polystachyum*, to evaluate its in vitro amoebicidal activity against *Acanthamoeba polyphaga* and its cytotoxic effect in mammalian cells.

Materials and methods

Plant material

The aerial parts of *P. polystachyum* were collected in the city of Butiá, Rio Grande do Sul, Brazil, in December, 2009. A voucher specimen was deposited at the Herbarium of the Department of Botany of the Federal University of Rio Grande do Sul (ICN 136584).

Essential oil

The essential oil was obtained from the fresh aerial parts of the plants by hydrodistillation using a Clevenger-type apparatus for 4 h. The essential oil was collected, dried over sodium sulfate, and stored in amber-colored vials at 4°C until analysis.

GC and GC-MS analysis

The oil was analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS), using a chromatograph (Shimadzu GC-17A) equipped with a fused silica capillary column (30 m, 0.25 mm, 0.25 μm, coated with DB-5). The temperature was programmed from 60°C to 300°C at 3°C/min. Injector and detector temperatures were set at 220°C and 250°C, respectively. The GC apparatus was equipped with a flame ionization detector, while the GC/MS analysis had a quadrupole MS system (QP 5000) operating at 70 eV and mass range 40–400 amu. The relative composition of the oil was obtained from electronic integration, without taking into account relative response factors. The identification of compounds was based on a comparison of retention indices (determined relatively to the retention times of *n*-alkanes homologous series), mass spectra with those of authentic samples, and data from Nist GS–MS library with the literature (Adams 2007).

Amoeba cultures

The pathogenic strain of *A. polyphaga* (ATCC 30461) was obtained from the American Type Culture Collection. The axenic cultures were kept in 2% proteose peptone, 0.2% yeast extract, and 1.8% glucose (PYG) medium at a constant temperature of 30°C. For the experiment, 1 mL of the culture was centrifuged for 5 min at 2,000 rpm, the supernatant was discarded, and the precipitate was washed twice with phosphate-buffered saline buffer. The precipitate of amebae was diluted in PYG medium to obtain a final concentration of 1.6×10^4 trophozoites per milliliter.

Assessment of amoebicidal activity

The essential oil was solubilized with 1% Tween and water to a concentration of 40 mg/mL and was tested at final concentrations of 20, 10, 5, 2.5, and 1.25 mg/mL. For the assessment of amoebicidal activity, 100 μL of culture of *A. polyphaga* and 100 μL of each test solution were inoculated into each well of a 96-well plate. The plate was sealed and incubated at 30°C, monitored by means of an inverted microscope, and counted in a Fuchs–Rosenthal counting chamber after 24 and 48 h. Viability was assessed using methylene blue. The controls used were sterile water and sterile water containing 1% Tween 20. The experiments were performed in triplicate and repeated in three different days.

Cytotoxicity assay

Essential oil cytotoxic effect was evaluated by 3-(4,5-dimethyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). Briefly, Vero cells (African Green Monkey Kidney, ATCC CCL-81) were cultured in Eagle's minimal essential medium (E-MEM) supplemented with 10% fetal bovine serum [(E-MEM/FBS); (GIBCO)] and antibiotics (penicillin 100 UI/mL; streptomycin 100 μg/mL). Cells were seeded at a concentration of 4.0×10^4 cells per well on 96-well microplates and maintained at 37°C under a 5% $C0_2$ atmosphere. After 24 h, the medium was removed and 100 μL of E-MEM/FBS with essential oil at different concentrations (10, 5 and 2.5 mg/mL) were added

Table 1 Percentage composition of *P. polystachyum* essential oil

Component	RI[a]	%
α-Cubebene	1,348	1.7
α-Copaene	1,378	5.4
β-Bourbonene	1,388	1.4
β-Caryophyllene	1,401	10.0
α-Humulene	1,435	0.5
Germacrene D	1,463	3.4
Bicyclogermacrene	1,478	1.2
α-Muurolene	1,483	0.5
δ-Cadinene	1,506	2.2
Elemol	1,537	0.5
E-nerolidol	1,554	1.4
Caryophyllene oxide	1,572	0.4
E-sesquilavandulyl	1,627	17.3
Tau-cadinol	1,636	1.7
α-Eudesmol	1,645	0.6
α-Cadinol	1,650	2.7
E-sesquilavandulyl acetate	1,736	43.8

[a] *RI* retention indices on DB-5 column

to each wells in triplicate. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 48 h, 50 μL of MTT (Sigma Chemical Co., Saint Louis, MO, USA) solution (2 mg/mL) was added to each well and incubated for a further 4 h. The plates were centrifuged (1400×*g* for 5 min) and the untransformed MTT was removed. Ethanol (100 μL) was added to each well for solubilizing formazan crystals and the optical density (OD) was 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 results are expressed as percentage and analyzed by analysis of variance and comparison of averages with the Tukey's test. Statistical significance was defined as $p<0.05$.

Results

Essential oil analysis

The essential oil yield, based on fresh weight (*w*/*v*), was 0.15%. Essential oil analysis showed 17 volatile compounds, representing 94.5% of the total oil. Its chemical composition is presented in Table 1 and summarized in Fig. 1. *E*-sesquilavandulyl acetate was the major component, representing 43.8% of the oil, followed by *E*-sesquilavandulyl (17.3%) and b-caryophyllene (10.0%).

Amoebicidal activity

P. polystachyum essential oil has never been investigated neither for the chemical composition nor the biological activities. Here, the amoebicidal activity of *P. polystachyum* essential oil was tested against *A. polyphaga* trophozoites. The essential oil showed activity in all tested concentrations. When tested the amoebicidal activity in 24 h, the essential oil killed all trophozoites in the concentrations of 10 and 20 mg/mL (Fig. 2). With 1.25, 2.5, and 5 mg/mL, the oil was able to kill 60%, 71.6%, and 81.1% of the trophozoites, respectively (vs control).

Laboratory tests were also carried out to determine the essential oil amoebicidal activity from *P. polystachyum* in 48 h (Fig. 2). The results were similar to those obtained in 24 h. All trophozoites were killed with the concentrations of 10 and 20 mg/mL, while with 1.25, 2.5, and 5 mg/mL, the oil killed 46%, 46.3%, and 80.7% of the trophozoites, respectively (vs control). Diminished effect observed in the concentrations of 1.25 and 2.5 mg/mL at the 48 h was probably due to grow's retake of trophozoites. Essential oil was active in a dose-dependent manner assessed by linear regression (Fig. 3), showing that the activity was directly proportional to the increase of the dose. The essential oil, at all concentrations used, was able to prevent the encystment of the trophozoites, once that there was not cyst formation.

In order to apply *P. polystachyum* essential oil as a topical agent, the oil must not induce the cytotoxic effects on human cornea. Therefore, we examined the essential oil's cytotoxic effect on a mammalian cell linage. *P. polystachyum* essential oil showed toxic effect against Vero

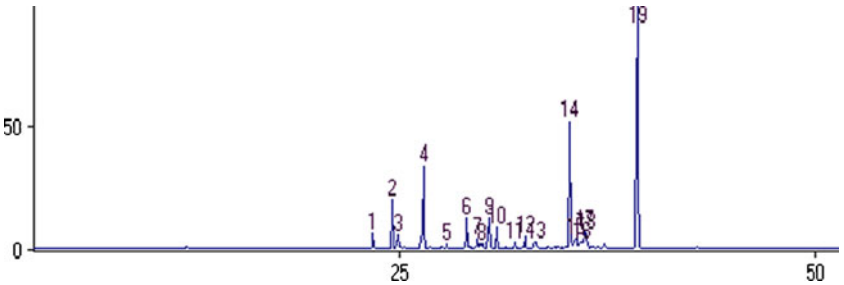

Fig. 1 Chromatogram of the essential oil from *P. polystachyum* by GC-MS

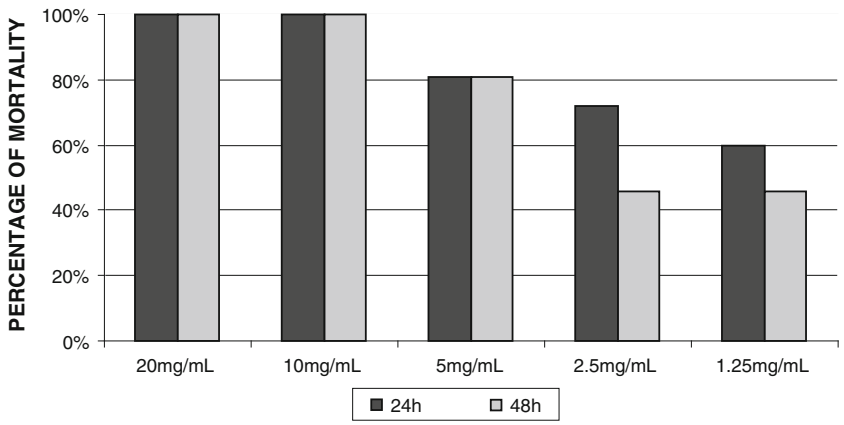

Fig. 2 Amoebicidal activity of essential oil of *P. polystachyum* presented as percentage of mortality of *A. polyphaga* trophozoites ($p < 0.05$ vs control)

cells (Fig. 4). The doses were selected from the amebicial activity test and had a cytotoxic activity of 13% (vs control).

Discussion

Plants and their extracts have been used for many centuries as treatments for ailments from headaches to parasite infections (Jones 1996). Plants of the genus *Pterocaulon* have shown activities against different fungi, bacteria, and protozoa (Alarcón et al. 2008; Ródio et al. 2008; Daboit 2010). This study has demonstrated that *P. polystachyum* essential oil can reduce the *A. polyphaga* trophozoites viability. Essential oil analysis showed that sesquiterpenes are the major compounds in the oil. *E*-sesquilavandulyl acetate is not commonly found in essential oils but it was identified in the essential oil obtained from aerial parts of *Pluchea quitoc* (syn. *Pluchea sagittalis*), a native plant of Southern Brazil. From *P. quitoc* essential oil, 42 compounds were identified being sesquilavandulyl acetate, sesquilavandulol, and α-gurjunene as the main components (Simionatto et al. 2007). It is interesting to comment that both *Pterocaulon* and *Plucheae* are closely related genera, belonging to the same tribe—Plucheeae (Bremer 1994).

Studies have shown that many plants present activity against different parasites being this effect related to some components of the essential oil. A study demonstrated that low concentrations of two Lavandula essential oil can completely eliminate *Trichomonas vaginalis*, *Giardia duodenalis*, and *Hexamita inflata* in vitro (Moon et al. 2006). *Baccharis dracunculifolia* (Asteraceae) essential oil showed activity against promastigote forms of *Leishmania donovani*. Its essential oil displayed high activity in the schistosomicidal assay, since all pairs of *Schistosoma mansoni* adult worms were dead after incubation with the essential oil. *B. dracunculifolia* essential oil was neither cytotoxic against Vero cells nor active in the antimicrobial and antiplasmodial assays (Parreira et al. 2010).

Nevertheless, other protozoa parasites were susceptible to this group of natural compound. Essential oil antiparasitic activity was demonstrated for Monzote et al. (2007), which showed that the *Chenopodium ambrosioides* essential oil has a synergic activity after incubation in conjunction with pentamidine against promastigotes of *Leishmania amazonensis*. In other study, the chemical composition and biological activities of 19 *Lippia* essential oils and seven of their major components were tested against free and intracellular forms of *Leishmania chagasi* and *Trypanosoma cruzi* parasites. The essential oil of *Lippia alba* exhibited the highest activity against *T. cruzi* epimastigotes

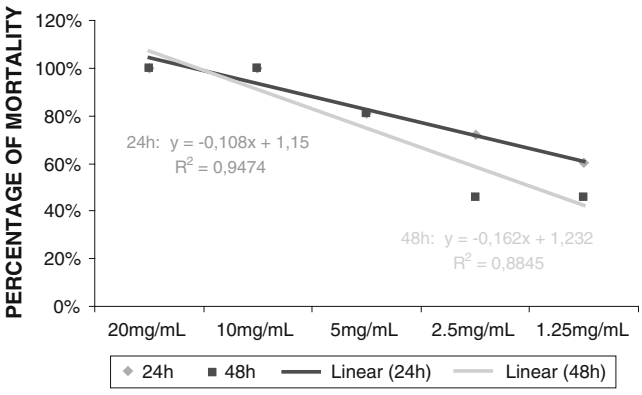

Fig. 3 Linear regression from the concentrations of *P. polystachyum* essential oil front of the percentage mortality of *A. polyphaga* trophozoites

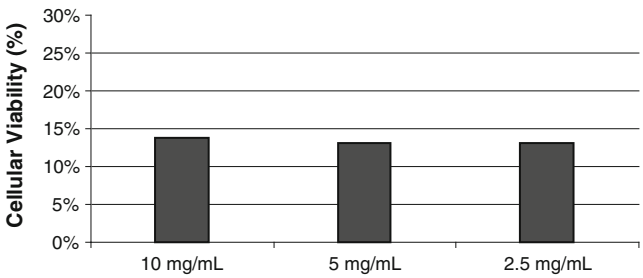

Fig. 4 Cellular viability of Vero cells front different concentrations of *P. polystachyum* essential oil. ($p < 0.05$ vs control)

and intracellular amastigotes, while *Lippia origanoides* essential oil was active against *L. chagasi* promastigotes and exhibited no toxicity in mammalian cells (Escobar et al. 2010).

Our data corroborate the study of Ródio et al. (2008), where the hexane extract of *P. polystachyum* showed antiamoebic activity against *Acanthamoeba castellanii* trophozoites. However, amoebicial activity of the essential oil was not previously investigated. The amoebicidal activity found in the *P. polystachyum* essential oil could be occurring by the action of specific compounds within the oil or the synergistic action of several molecules. A study of Martín-Navarro et al. (2010) showed that the α-cyperotundone, a natural sesquiterpene isolated from the root bark of *Maytenus retusa*, was able to inhibit the in vitro growth of the amoebae at relatively low concentrations. However, the identification of the molecular targets of this sesquiterpene and its effects on *Acanthamoeba* has yet to be discovered.

Here, *P. polystachyum* essential oil was able to prevent cyst formation. This essential oil property is very important because the ability of trophozoites to turn in cyst form during the therapy is the major problem for reinfection (Schuster and Visvesvara 2004). The doses used in the tests were high and cytotoxic to Vero cells; however, these results preclude the direct use of oil on mammalian cells, but do not rule out their use, once its activity against the trophozoites would allow the oil to be incorporated into contact lenses cleaning solutions. Importantly, the oil not only destroyed the trophozoites but also prevented cysts formation. Thus, the use of essential oil of *P. polystachyum* in contact lenses cleaning solutions would be a great alternative.

In conclusion, *P. polystachyum* essential oil showed an important amoebicidal activity against trophozoites of *A. polyphaga*. However, further studies are necessary to evaluate the possible active compound from *P. polystachyum* essential oil, as well as identify the molecular targets of these products and thus determine its possible therapeutic use.

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