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ORIGINAL ARTICLE

AMOEBICIDAL AND CITOTOXIC ACTIVITY OF GREEN PROPOLIS AQUEOUS EXTRACT AGAINST *Acanthamoeba castellanii* TROPHOZOITES AND CYSTS

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ABSTRACT

The genus *Acanthamoeba* belongs to the group of free-living amoebae and is widely distributed in the environment. These protists are known to cause severe diseases, including Granulomatous Amebic Encephalitis in immunocompromised patients and amebic keratitis, especially in immunocompetent contact lens wearers. Green Propolis is a resinous and balsamic substance, known in alternative medicine because of its several biological activities. In this study we evaluated the amoebicidal activity of an aqueous extract of green propolis against trophozoites and cysts of *Acanthamoeba castellanii.* At concentrations of 10 and 20 mg/mL, the extract was able to inactivate 100% of trophozoites in 24 and 48 hours, while at the concentration of 5 mg/mL, 100% of trophozoites were inactivated in 72 hours. *Acanthamoeba* cysts were inactivated following 24 hours exposure to the extract at 40 mg/mL. When evaluated on human corneal epithelial (HCE) cells using a MTT-based viability assay, the extract had no significant cytotoxic effect at concentrations of 0.312, 0.625, 1.25 and 2.5 mg/mL. The adhesion test performed showed that the propolis extract reduced the attachment of *Acanthamoeba* to HCE cells, in a dose-dependent manner. Thus, this study demonstrated the antiparasitic activity of propolis against both forms of *Acanthamoeba* proving it to be a promising substance especially for the formulation of solutions for disinfection of surfaces, since it showed no toxicity to the HCE cells. However, more studies are needed to understand its mechanism of action.

KEY WORDS: *Acanthamoeba castellanii*; propolis; amoebicidal activity.

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RESUMO

Atividade amebicida e citotóxica do extrato aquoso de própolis verde contra trofozoítos e cistos de *Acanthamoeba castellanii*

O gênero *Acanthamoeba* pertencente ao grupo das amebas de vida livre e é amplamente distribuído no ambiente. Estes protistas são conhecidos por causarem doenças graves, como a Encefalite Amebiana Granulomatosa em pacientes imunocomprometidos e ceratite amebiana, especialmente em usuários de lentes de contato imunocompetentes. Própolis verde é uma substância resinosa e balsâmica, conhecida na medicina alternativa por exibir várias atividades biológicas. Neste estudo avaliou-se a atividade amebicida de um extrato aquoso de própolis verde contra trofozoítos e cistos de *A. castellanii.* Nas concentrações de 10 e 20 mg/mL, o extrato foi capaz de inativar 100% de trofozoítos no prazo de 24 horas e 48 horas, enquanto a uma concentração de 5 mg/mL 100% dos trofozoítos foram inativados em 72 horas. Os cistos foram inativados após 24 horas de exposição ao extrato à concentração de 40 mg/mL. O efeito do extrato foi avaliado sobre células HCE (epiteliais de córnea humana), empregando-se ensaio de viabilidade baseado na redução do sal de tetrazólio MTT. O extrato não apresentou efeito citotóxico significativo sobre as células HCE, nas concentrações de 0,312, 0,625, 1,25 e 2,5 mg/mL. O teste de adesão realizado mostrou que a fixação de *Acanthamoeba* a células HCE apresenta comportamento dose- dependente em relação ao extrato de própolis. Assim, este estudo demonstrou a eficácia da própolis verde contra trofozoítos e cistos de *Acanthamoeba* e provou ser uma substância promissora especialmente para a formulação de soluções para desinfecção de superfícies. No entanto, mais estudos são necessários para entender seu mecanismo de ação.

DESCRITORES: *Acanthamoeba castellanii*; própolis; atividade amebicida.

INTRODUCTION

The genus *Acanthamoeba* comprises the protozoa that along with *Naegleria, Balamuthia* and *Sappinia* constitute the group of free-living amoebae (FLA), which are potentially pathogenic or opportunistic (19). *Acanthamoeba* spp. are widely spread throughout nature, and have been isolated from soil, air, and water (1, 5, 6, 32). This organism has a life cycle with two stages: a vegetative trophozoite and a resistant cyst stage (10, 39). These amoebae, when they invade immunosupressed hosts, can cause some diseases, including granulomatous amoebic encephalitis, skin infections, lung diseases as well as disseminated diseases. In addition, when in contact with the injured cornea of healthy individuals this protist may behave as a potential pathogen and cause an infection known as Amebic Keratitis (AK). Patients with AK may experience pain with photophobia, ring-like stromal infiltrate, epithelial defects and lid oedema. If AK is not treated adequately and aggressively, it can lead to loss of vision. The ability of *Acanthamoeba* to produce infection requires specific adhesins, the production of toxins, and its ability to resist immune/environmental factors and chemotherapeutic agents (3, 16). The treatment of such diseases involves the utilization of a set of cationic antiseptic drugs, aminoglycosides besides aromatic diamines, amphotericin

B, and metronidazole (8, 11). However, these drugs may promote the manifestation of side effects and the development of disabilities, such as loss of hearing and vision (15). One of the difficulties for the successful treatment of amoebic keratitis is the resistance of the cystic form of *Acanthamoeba* to various concentrations of antibiotics, and the tolerance by the ocular surface (12). Considering the increasing records of clinical cases of *Acanthamoeba* infections, the resistance of these microorganisms to drugs and the toxic effects of many of them on human cells, the development of natural medicines to promote more effective treatment is important.

Propolis, a complex and heterogeneous mixture, composed of resinous and balsamic material, with a strong and characteristic aroma, is collected by honeybees from different plants (17, 33). Several studies have reported important activities of propolis, such as anti-inflammatory, antioxidant, antitumor (30), antiparasitic (40), antibacterial, antifungal, and antiviral (14). Propolis is found in southeastern Brazil and its botanical origin is *Baccharis dracunculifolia*, a plant of the family Asteraceae popularly known as "Rosemary's field" or "broom" (25). Due to the presence of various substances in propolis and mainly because of the variation of its polarity, many solvents are used to extract their chemical compounds (4, 29). Moura et al. (24) found by HPLC/ESI/MS that the major chemical compounds in the aqueous extract of propolis were caffeoylquinic acids and cinnamic acid derivatives such as artepelin C and dupranin. Several biological activities have been attributed to these compounds, such as antimicrobial (20), antioxidant (31), antitumor (27) and apoptosis-inducing factor (22). Studies have been performed showing the amoebicidal activity of natural products against *Acanthamoeba* (34). Thus, the aim of this study was to evaluate the amoebicidal activity of the crude green propolis aqueous extract (GPAE) against trophozoites and cysts of *Acanthamoeba castellanni* in order to develop an alternative treatment, and use it in the composition of lens or surface cleaning solutions. We also studied the cytotoxicity of the extract to human corneal epithelium cells and the adherence of the trophozoites on these cells treated with the extract.

MATERIALS AND METHODS

Propolis samples and preparation of extract

Propolis samples were acquired from the Natucentro Apiary Industry company and Centro Oeste Ltda. (Bambuí, MG), lot 41/11 in November 2011. The extract preparation was performed according to Moura et al. (24) with modifications. Briefly, Brazilian green propolis was crushed and 200 g were added to 500 mL of sterile distilled water and the mixture remained undisturbed for 24 hours in the absence of light. After, the solution was heated to 70 °C in

a water bath with constant stirring for 2 hours, filtered, frozen at -80 °C and lyophilized. In total, 20 g of dry extract was obtained, which was solubilized in sterile PBS (1x) and filtered through a 0.22 µm membrane to obtain a stock solution of 100 mg/mL.

Microorganisms, culture media, and growth conditions

Acanthamoeba castellanii (ATCC 50492) belonging to the T4 genotype was used in all experiments. Trophozoites were grown at 30 °C in tissue culture flasks in PYG medium (2% proteose peptone, 0.2% yeast extract and 1.8% glucose) without agitation (36). For the experiments, 1 mL of culture was centrifuged (5 min at 300 x g), the supernatant was discarded and the pellet was twice washed with phosphate buffered saline (PBS). The trophozoites were diluted in PYG at a final density of $3x10^4$ organisms/mL (34). For cyst production, 1 mL of amoebae cultured in PYG was taken and washed twice in PBS. The supernatant was discarded and the pellet was resuspended in 5 mL of Neff´s encystment saline (0.1M KCl, 0.008 M $MgSO_4$, 0.0004 M $CaCl_2$, 0.01 M $NaHCO_3$- pH 9.0) and maintained at 30 °C for 7 days (13, 26). Monoxenic culture of the amoebae was also performed after incubation of trophozoites and cysts in test solutions. For this, 10 µL of each culture sample (treated with the different concentrations of the extract) were inoculated in 1.5% non-nutrient agar covered with heat-killed (63 °C – 2 hours) *Escherichia coli*. Plates were incubated at 30 °C for 7 days and monitored under an optical microscope (36).

Assessment of amoebicidal activity

To assess the amoebicidal activity, propolis aqueous extract was serially diluted and tested against trophozoites (final concentrations of 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 mg/mL) and cysts (30, 20 and 10 mg/mL). For this, 100 µL of *A. castellanii* culture ($3x10^4$ trophozoites or cysts/mL) and 100 µL of each test solution were added into the wells of a 96-well plate. The plate was sealed and incubated at 30 °C, and monitored in an inverted microscope. Cell numbers were determined in a Fuchs-Rosenthal counting chamber after 24, 48 and 72 hours. Viability was assessed using trypan blue. The negative control consisted of 100 µL of amoebae in PYG and 100 µL of PBS. For a positive control, 100 µL of amoebae in PYG and 100 µL of 0.02% chlorhexidine were used. The experiments were performed in triplicate and repeated on three different days (42).

Human corneal epithelial cell cultures

The human corneal epithelial cells (HCE) were obtained from the Riken Cell Bio Resource Center, Japan. HCE cells were cultured in DMEM/

F12 (GIBCO) supplemented with fetal bovine serum (15%), insulin (5 µg/mL), human epidermal growth factor (10 ng/mL), 0.5% DMSO and antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL). Under these conditions, the HCE cells exhibited their characteristic morphology. For the experiments the cells were maintained in culture bottles and incubated at 37 °C with 5% CO_2 until the tests (2).

Cytotoxicity assays

The cytotoxic effect of propolis extract was evaluated by 3-(4,5-dimethyl)-2,5- diphenyltetrazolium bromide (MTT) assay (23) modified by the authors. Briefly, 200 µL of HCE cell suspensions ($4x10^5$ /mL) were added into the wells of 96-well plates and incubated at 37 °C with 5% CO_2 atmosphere. After the formation of confluent monolayers, the medium was changed and HCE cells were treated with the propolis aqueous extract at 10.0, 5.0, 2.5, 1.25, 0.625 and 0.312 mg/mL. After 24 hours, 40 µL of MTT reagent (Sigma Chemical Co., Saint Louis, MO, USA) solution (5 mg/mL) were added to each well and incubated for a further 3 hours. The plates were centrifuged (1400 x g for 5 min) and the untransformed MTT was removed. Then, ethanol (100 µL), instead of acid-isopropanol (as originally), was added to each well for solubilizing formazan crystals and the optical density (OD) measured in an ELISA reader (Anthos 2020) at 550 nm with a 620 nm reference filter. Results were expressed as percentages of the OD in relation to that of untreated control cells.

Adhesion Assay

Adhesion assays were performed as previously described (38). Briefly, HCEC were grown to confluency in 24-well plates as described above. To determine the effects of propolis extract, 0.5 mL of a suspension of $6x10^4$ trophozoites of *Acanthamoeba* per well, previously treated with 20, 10, 5, 2.5, 1.25, and 0.625 mg/mL of propolis extract for 45 min at 30 °C, was incubated with HCEC monolayers. The plates were incubated at 37 °C in a 5% CO_2 incubator for 60 min. The numbers of unbound amoebae in the supernatants were determined by hemocytometer counting. The percentage of bound amoebae was calculated as follows: number of unbound amoebae/total number of amoebae × 100 = % unbound amoebae. The number of bound amoebae was deducted as follows: 100 − % unbound amoebae = % bound amoebae. The negative control was a suspension of amoebae without contact with propolis extract in PBS.

Statistical Analysis

To detect differences between treatments, analysis of variance (ANOVA) followed by Tukey test was performed, using the program BioEstat 5.0. The level of significance was defined as $p < 0.05$.

RESULTS

Amoebicidal activity

The GPAE showed 100% of activity in 24 hours in the concentrations of 10 and 20 mg/mL (Figure 1). With 1.25, 2.5, and 5 mg/mL, the extract was able to kill 89.6, 58, and 34.7% of the trophozoites, respectively (vs control). Lower doses showed no statistically significant activity. Also, the amoebicidal activity increased over time (48 hours and 72 hours), indicating that the contact time is a variable to be considered when the biological activity of propolis is being evaluated. No encystment was observed throughout the experiment. Between 24 and 48 hours of treatment, the concentrations of 1.25, 0.625, and 0.312 mg/mL showed significant differences ($p<0.05$), showing greater activity in 48 hours. The concentration of 5 mg/mL showed the same activity over all the 72 hour period. There was no significant difference ($p<0.05$) between trials of 48 and 72 hours. Also, the GPAE showed amoebicidal activity at 24 hours from 1.25 mg/mL and at all concentrations at 48 and 72 hours (Figure 1). At 40 mg/mL the GPAE was able to kill 100% of the cysts after 24 hours of exposure (Figure 2). There were no statistical differences between treatments of 24 and 48 hours at each dose.

Cytotoxic Assay

The GPAE showed toxic effects against HCE at the concentrations of 5 and 10 mg/mL killing 47.6 and 55.4% of the cells, respectively. Lower doses (0.312, 0.625 and 1.25 mg/mL) showed no toxicity to the HCE cells, not differing from the control ($p>0.05$). Although the concentration of 2.5 mg/mL had not shown significant differences when compared with control, further studies are necessary to determine their possible topical use on the cells of the cornea (Figure 3).

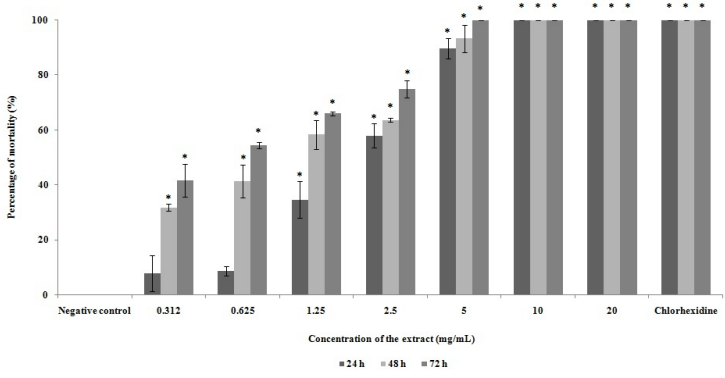

Figure 1. Amoebicidal activity of GPAE concentrations (0.312 to 20 mg/ml) against trophozoites of *A. castellanii* in 24, 48 and 72 h expressed as percentage of mortality. Cell numbers were determined in a Fuchs–Rosenthal counting chamber. Viability was assessed using trypan blue. Negative control was amoebae in PYG and PBS. Positive control was amoebae in PYG and 0.02% chlorhexidine. The experiments were performed in triplicate and repeated in three different days. * Significant difference compared to the negative control ($p < 0.05$). Bars represent standard deviation

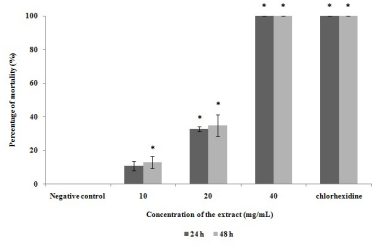

Figure 2. Amoebicidal activity of GPAE concentrations (10, 20 and 40 mg/mL) against cysts of *A .castellanii* in 24 and 48 h expressed as percentage of mortality. Cell numbers were determined in a Fuchs–Rosenthal counting chamber. Viability was assessed using trypan blue. Negative control was amoebae in PYG and PBS. Positive control was amoebae in PYG and 0.02% chlorhexidine. The experiments were performed in triplicate and repeated in three different days. * Significant difference compared to the negative control ($p < 0.05$). Bars represent standard deviation

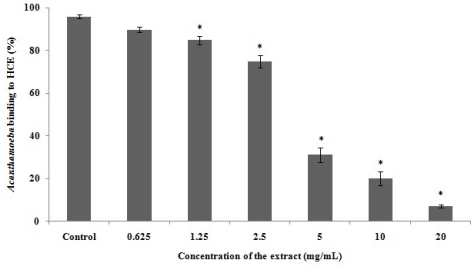

Figure 3. Viability of HCE cells treated with the GPAE concentrations (0.625 to 10 mg/mL) after 24 h expressed as percentage of cellular viability. The cytotoxic effect of the propolis extract was evaluated by 3-(4,5-dimethyl)-2,5- diphenyltetrazolium bromide (MTT) assay. * Significant difference compared to negative control ($p < 0.05$). Bars represent standard deviation

Adhesion Assay

The trophozoites of *Acanthamoeba* treated (1 hour at 37 °C) with GPAE in the concentrations of 20, 10 and 5 mg/mL showed that 7.25, 20.15 and 31.15% respectively, remained adherent to HCE cells compared to 95.9% of the non treated control. Organisms treated with lower concentrations of GPAE were more adherent at the same conditions (Figure 4).

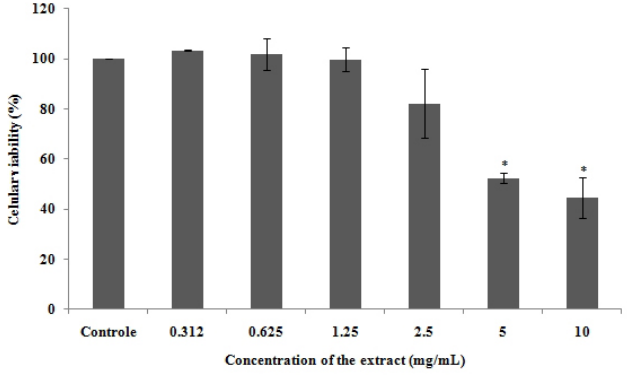

Figure 4. Inhibitory effect of GPAE on adhesion of *A. castellanii* trophozoites to HCE cells. The results are expressed as percentage of binding trophozoites. * Significant difference compared to negative control ($p < 0.05$). Bars represent standard deviation

DISCUSSION

In the present study, GPAE showed amoebicidal activity at the concentration of 10 mg/mL killing 100% of trophozoites in 24 hours. To our knowledge this is the first evaluation of the amoebicidal activity of GPAE. A study conducted at Turkey using an ethanolic extract of propolis showed activity against *A. castellanii* trophozoites in 24 hours using the concentration of 8 mg/mL and against cysts in 48 hours using the concentration of 15.6 mg/mL (41). Such activity could be associated with some phytochemical compound interacting with the cell membrane of *Acanthamoeba*, or penetration of the compound inside the membrane channels of the parasite (18). In relation to the cysticidal activity, the result obtained by Topalkara et al. (41) corroborates our work, showing that higher doses are required to inactivate cysts of *A. castellanii*. This could be explained by the presence of a double layer composed of cellulose and protein conferring greater resistance to the cystic form (10, 37). Martin and colleagues (21) evaluated the activity of an aqueous extract of a sample of Brazilian propolis on corneal cells previously injured and it did not show any toxic effect at a concentration of 10 mg/mL. The authors also observed the effect on wound healing, and they concluded that the extract accelerated the healing process and reduced inflammation. The effects shown by the extract are similar to the results of other authors, confirming that Brazilian green propolis has the ability to reduce necrosis and apoptosis of retinal ganglion cells (9). However, the aqueous extract of propolis evaluated in our study showed cytotoxic effects from the concentration of 5 mg/mL. In the same way, Vural et al. (2007) studying the effect of propolis on experimental *Acanthamoeba* keratitis found that concentrations of propolis higher than 7.8 mg/mL caused damage to corneal epithelial cells. However, the keratitis grade on day 5 of the treatment was significantly lower using a propolis solution of 8 mg/mL (43).

Since the adhesion process of several microorganisms on the cell surfaces is an initial step to establish an infection, the ability of GPAE to inhibit adhesion of *Acanthamoeba* on HCE cells was evaluated in this study. Research evaluating the ability of adhesion of bacterial cells on buccal cells showed that when bacteria were exposed to a hydro-alcoholic extract of propolis, the adhesion of *Haemophilus influenzae* decreased significantly (7). The mechanisms by which propolis affects the adhesion of bacteria and protozoa on cell surfaces are not well known (35). It is believed that propolis may act on the cytoplasmic membrane and interfere with the emission of pseudopodia of this protist, which are used for movement and adhesion (28). In our study the ability of GPAE to inhibit the adherence of an *A. castellanii* strain belonging to the T4 genotype was shown. This finding is relevant because T4 is the genotype more related with diseases. This highlights the importance of further studies related to the development of natural drugs. Furthermore these substances in higher

concentrations could be used for the disinfection of surfaces, thus preventing the spread of this organism, which is capable of carrying other pathogenic microorganisms.

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