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BRIEF COMMUNICATION

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In vitro effects of *Blepharocalyx salicifolius* (H.B.K.) O. Berg on the viability of *Echinococcus ortleppi* protoscoleces

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ABSTRACT

Scolicidal agents are important in the treatment of cystic echinococcosis. This study evaluated the scolicidal activity of the plant *Blepharocalyx salicifolius* (H.B.K.) Berg against *Echinococcus ortleppi* protoscoleces. The parasite species was identified by amplifying a fragment of the gene cytochrome *c* oxidase subunit 1 (COX 1). *B. salicifolius* crude extract at concentrations of 100, 200, 300 and 400 mg/mL was analyzed at different times (5, 10, 15, 30, 45 and 60 min). N-butanol and ethyl acetate fractions (100 and 200 mg/ mL) were also analyzed at 5, 10, 15 and 30 min. Both fractions showed 100% scolicidal activity at the concentration of 200 mg/mL at 5 min. Gallic acid, identified as the major compound of the ethyl acetate fraction- was responsible for the observed scolicidal activity. The results showed that crude extract and fractions of *B. salicifolius* have scolicidal effect against *E. ortleppi* protoscoleces.

KEYWORDS: *Echinococcus ortleppi*. Protoscolicidal. Scolicidal agent. Gallic acid.

INTRODUCTION

Echinococcus ortleppi is one of the species of *Echinococcus* spp. found in Southern Brazil[1] and in some regions of Europe, Africa, South Asia and the Americas[2]. There are also reports of *E. ortleppi* causing cystic echinococcosis (CE) in humans[3,4,5].

Until the 80's, surgery was the only option for treatment of CE. Subsequently, chemotherapy with benzimidazole compounds and, later, treatment by PAIR (puncture, aspiration, injection and reaspiration) was introduced[6]. One of the major surgical complications of CE is recurrence, after the primary hydatid disease treatment. Dissemination of protoscolex-rich fluid during surgery is a major cause of recurrence and multiple secondary CE[7]. The use of an effective scolicidal solution during the surgical procedure is an important tool in CE treatment, and this can significantly reduce the rate of disease recurrence[8].

Among the countless plant species of medicinal interest, *Blepharocalyx salicifolius* (H.B.K.) O. Berg (Myrtaceae) has been used in the treatment of leukorrhea, diarrhea, digestive problems and to treat cystitis and urethritis[9]. In folk medicine, *B. salicifolius* is known as "*murta*" and is distributed throughout South America, from Ecuador to Uruguay[10]. *In vitro* antiparasitic activity of *B. salicifolius* extract has been reported against *Leishmania amazonensis*[11]. Therefore, due to the fact that scolicidal agents are limited and may cause side effects that can be harmful to human health, the aims of this study were to evaluate the scolicidal

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action of *B. salicifolius* on protoscoleces of *E. ortleppi* and to determine the compounds present in the extract that are likely to be responsible for such action.

MATERIAL AND METHODS

Preparation of plant material

Crude extract

B. salicifolius leaves were collected in the city of Nova Prata, Rio Grande do Sul State, Brazil, in November 2011. A fertile branch was identified and deposited at the Herbarium of Universidade Federal de Santa Maria (catalog number SMDB 13.515). The leaves were dried in a laboratory oven, with air circulation, at 40 °C for 72 h, and then ground in a Wiley knife mill. Plant material was submitted to an aqueous extraction for 30 min at 70 °C. Thereafter, the extract was stored in Petri dishes and transferred into an oven with air circulation, at 42 °C for 48 h. The crude extract consisted of plate scrapings[12].

Fractions

For obtaining the component fractions, a crude extract portion was dissolved in 50 mL of distilled water. Initially, ethyl acetate (EtOAc) fraction was extracted (3 x 20 mL) in a separatory funnel, and the solvent was dried through evaporation until the total dryness of the EtOAc fraction. The same procedure was carried out to obtain the n-butanol (BuOH) fraction. The remaining aqueous extract was lyophilized yielding the aqueous fraction.

HPLC-DAD quantitative analysis of rutin and gallic acid

Reverse phase chromatography analyses were carried out under gradient conditions using a Techno Sciences C-18 column (4.6 mm x 250 mm). The flow rate was 0.6 mL/min, the injection volume was 40 µL and the gradient elution was conducted according to Boligon *et al.*[13], with minor modifications. Mobile phase consists of water containing 2% acetic acid (solvent A) and acetonitrile (solvent B).

In an attempt to identify the compounds present in the plant under study, tests were performed with different commercial standards, where the presence of rutin and gallic acid was detected. The ultraviolet (UV) absorption spectra of the standards rutin (Sigma-Aldrich, Brazil) and gallic acid (*Vetec Química*, Brazil) and of the samples were recorded in the range of 230-400 nm. Samples, standard solutions and the mobile phase were degassed and filtered through a 0.45 µm membrane filter (Merck-Millipore, Billerica, MA, USA). Chromatographic operations were carried out at room temperature and in triplicate. Identification of the compounds was done by comparison of their retention times and UV absorption spectrum with the respective standards. The substance contents were obtained for the calibration curves (gallic acid, y = 71293x – 76386, r = 0.9958; rutin, y = 75045x – 18282, r = 0.9929).

Collection of protoscoleces

Hydatid cysts from lungs of naturally infected bovines were collected in a slaughterhouse in the central region of Rio Grande do Sul State. The hydatid cyst fluid was aseptically transferred to glass cylinders. After 30 min, protoscoleces were deposited on the bottom. The supernatant was then removed and the protoscoleces were washed three times in 0.9% saline for later use. Protoscoleces viability was assessed using 0.1% eosin[14]. The percentage considered suitable for the development of our experimental test was of at least 98% of viability.

Molecular analysis

DNA extraction was performed using an aliquot of the liquid containing hydatid protoscoleces, using a commercial kit (QIAamp tissue, QIAGEN Inc., Chatsworth, CA), according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a pair of primers (5'TTTTTTGGGCATCCTGAGGTTTAT3' and 5'TAAAGAAAGAACATAATGAAA ATG 3') to amplify a fragment of COX-I gene[15], with modifications. The reactions were carried out in a thermocycler model PTC-100™ (MJ Research, Inc.). To detect the pattern of bands, electrophoresis was performed on 1% ethidium bromide-stained agarose gel and visualized under UV light. The similarity of DNA samples sequenced with the COX-I gene was carried out by using the BLAST program (http://www.ncbi.nlm.nih.gov).

Scolicidal assay

For analysis of the scolicidal effect, 100 µL of hydatid fluid containing protoscoleces were added to 100 µL of each of the agents tested (crude extract, fractions and standards), along with 200 µL of 0.1% eosin, yielding a final volume of 400 µL for each analysis. Distilled water was used as a dilution vehicle for the agents. The negative control group received 100 µL of hydatid fluid, 100 µL of distilled water and 200 µL of 0.1% eosin.

Crude extract and fractions of *B. salicifolius*; gallic acid and rutin standards

A crude extract was tested at concentrations of 100, 200, 300 and 400 mg/mL at 5, 10, 15, 30, 45 and 60 min, at room temperature. BuOH and EtOAc fractions were analyzed at 100 and 200 mg/mL at 5, 10, 15 and 30 min. Gallic acid and rutin standards were evaluated in order

to find the lowest concentration and the lowest time that showed full scolicidal action. The negative control group was also assessed in the same conditions.

Viability test

In the present study, 0.1% eosin was used to check the viability of the protoscoleces. The viability was evaluated by the same optical microscope, at different times and concentrations described, observing motility and eosin staining, counting 100 protoscoleces at each evaluation. The protoscoleces were considered viable when they remained unstained[14,16].

Statistical analysis

Data were plotted using the Kaplan-Meier analysis, and differences in protoscoleces viability were analyzed by the log-rank test using GraphPad software (version 6.1., La Jolla, CA). Each experiment was performed in triplicate. A -p-value of < 0.05 was considered statistically significant.

RESULTS

The identification and quantification of the compounds present in EtOAc and BuOH fractions of *B. salicifolius* are shown in Table 1. Gallic acid and rutin were not found in the crude extract.

Table 1 - HPLC/DAD determination of gallic acid and rutin in fractions obtained from *B. salicifolius* leaves

Fractions	Compounds (mg/g of dry fraction)	
	Gallic acid	Rutin
Crude extract	-	-
EtOAc	91.3 ± 0.7	95.2 ± 0.88
BuOH	66.22 ± 1.09	65.26 ± 0.93

Not detected (-). Values are expressed as the mean ± SD (n=3).

The protoscoleces obtained in this study were molecularly identified as the *E. ortleppi* species. Figure 1 shows the *in vitro* effects of *B. salicifolius* on the viability of *E. ortleppi* protoscoleces exposed to different concentrations of the plant leaf crude extract. It is noteworthy that *B. salicifolius* extract showed dose-dependent and time-dependent effects against the protoscoleces. The negative control group did not present changes throughout the experimental time, maintaining a viability of 98%.

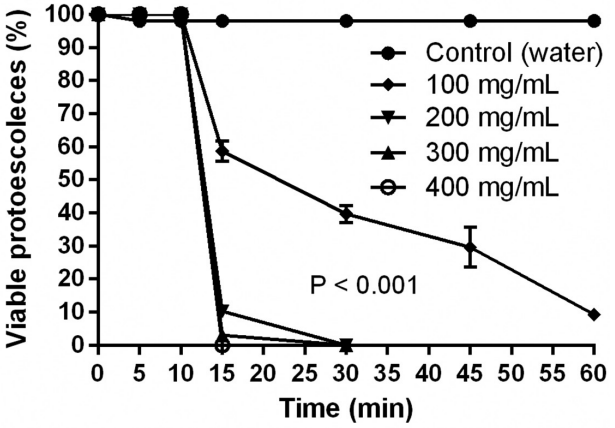

Figure 1 - Percentage viability of *E. ortleppi* protoscoleces exposed to different concentrations of *B. salicifolius* leaf crude extract. Data are means ± SD of triplicate samples. P < 0.001 in comparison to the control group (water)

The viability assays for BuOH and EtOAc fractions are shown in Figure 2. The HPLC/DAD analysis revealed two distinct patterns in the EtOAc and BuOH fractions of *B. salicifolius*: gallic acid and rutin. The assay performed with gallic acid standard showed 100% scolicidal activity at 5 min. at the concentration of 25 mg/mL. Conversely, rutin standard did not present any scolicidal action in this study.

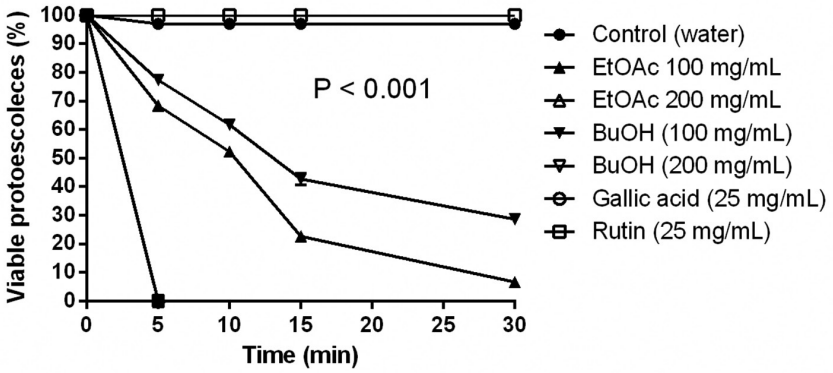

Figure 2 - Percentage viability of *E. ortleppi* protoscoleces exposed to different concentrations of ethyl acetate (EtOAc) and n-butanol (BuOH) fractions, gallic acid and rutin. Data are means ± SD of triplicate samples. Values of p- < 0.001 in comparison to the control group (water), except for rutin (p> 0.05). EtOAc (200 mg/ mL), BuOH (200 mg/ mL) and gallic acid curves are overlapped

DISCUSSION

The identification of *E. ortleppi* in this study corroborates previous studies that have reported the presence of this *Echinococcus* species in cattle raised in Rio Grande do Sul State[1,17]. According to Nakao *et al*.[18], this parasite is highly fertile and prolific in cattle. Importantly, a human case of CE caused by *E. ortleppi* has been previously reported in the studied area[5]. In this study, the tests with *B. salicifolius* showed highly significant scolicidal effects. According to the results obtained in the tests, 100% scolicidal activity using the crude extract was obtained at the concentration of 400 mg/mL at 15 min., which is comparable to the scolicidal action of hypertonic saline (at 20% during 15 min) and with the action of ethyl alcohol (at 95% during 15 min)[19]. In line with these results, BuOH and EtOAc fractions reached 100% scolicidal effect at 200 mg/mL at 5 min, presenting the same results regarding the action and time-exposure reported in another study using 20% sodium chloride[20]. These results were able to validate the methodology used in this study and, most importantly, confirmed the scolicidal action of the plant evaluated in our experiment.

The analysis of the compounds present in *B. salicifolius* fractions identified gallic acid, which was found in higher proportion in EtOAc when compared to BuOH fraction. The greater scolicidal action of EtOAc when compared to BuOH fraction confirmed the gallic acid as the scolicidal agent of the extract. Indeed, the result obtained with the gallic acid standard, which showed 100% scolicidal activity at the concentration of 25 mg/mL at 5 min, was considered ideal, demonstrating effectiveness against *E. ortleppi*. Studies with gallic acid and some of its derivatives have already been reported as effective against *Trypanosoma cruzi*[21] and *T. brucei brucei*[22] *in vitro*. The mechanisms determining the action of gallic acid that leads to death of these parasites have not been completely elucidated, but it is reported that the presence and the size of the alkyl chain in gallic acid can be related to lipid solubility characteristics and, consequently, it can provide different degrees of cell permeability. These findings may explain a likely mechanism of action of gallic acid that leads to protoscoleces death. Importantly, *B. salicifolius* raw extract might have other compounds with scolicidal action that have not been determined yet.

Considering the need for new perspectives for the treatment of CE, this study identified satisfactory scolicidal actions of *B. salicifolius* leaf crude extract, of EtOAc and BuOH fractions and of gallic acid, at all concentrations tested, against *E. ortleppi* protoscoleces. Further studies are necessary to investigate the mechanism of action and the potential scolicidal effects of *B. salicifolius in vivo*.

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