In vitro and *ex vivo* activity of *Melaleuca alternifolia* against protoscoleces of *Echinococcus ortleppi*

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SUMMARY

Cystic echinococcosis is a zoonotic disease of difficult diagnosis and treatment. The use of protoscolicidal agents in procedures is of utmost importance for treatment success. This study was aimed at analysing the *in vitro* and *ex vivo* activity of *Melaleuca alternifolia* oil (tea tree oil – TTO), its nanoemulsion formulation (NE-TTO) and its major component (terpinen-4-ol) against *Echinococcus ortleppi* protoscoleces obtained from cattle. Concentrations of 2·5, 5 and 10 mg mL^{-1} of TTO, 10 mg mL^{-1} of NE-TTO and 1, 1·5 and 2 mg mL^{-1} of terpinen-4-ol were evaluated *in vitro* against protoscoleces at 5, 10, 15 and 30 min. TTO was also injected directly into hydatid cysts (*ex vivo* analysis, $n = 20$) and the viability of protoscoleces was evaluated at 5, 15 and 30 min. The results indicated protoscolicidal effect at all tested formulations and concentrations. Terpinen-4-ol (2 mg mL^{-1}) activity was superior when compared with the highest concentration of TTO. NE-TTO reached a gradual protoscolicidal effect. TTO at 20 mg mL^{-1} showed 90% protoscolicidal action in hydatid cysts at 5 min. The results showed that TTO affects the viability of *E. ortleppi* protoscoleces, suggesting a new protoscolicidal option to the treatment of cystic equinococcosis.

Key words: *Echinococcus ortleppi*, tea tree oil, protoscolicidal, nanoemulsions.

INTRODUCTION

Cystic echinococcosis (CE) is an important zoonosis with worldwide distribution. The disease is caused by *Echinococcus granulosus sensu lato* and has a considerable impact on economic and public health (Nakao *et al*. 2013). The larval stage of the parasite, the hydatid cyst, is usually found in liver and lungs of intermediate hosts, as well as in humans (Eckert *et al*. 2001). In Rio Grande do Sul state, southern Brazil, the predominant species are: *E. granulosus sensu stricto*, reported in humans, cattle, sheep, pigs and dogs, *Echinococcus canadensis* (G7), reported in cattle and pigs and *Echinococcus ortleppi*, reported in cattle, humans and dogs (de la Rue *et al*. 2006, 2011; Balbinotti *et al*. 2012; Monteiro *et al*. 2014). Human infections caused by *E. ortleppi* were reported in several countries such as Argentina, Mexico, Brazil, India and France (Guarnera *et al*. 2004; Maravilla *et al*. 2004; de la Rue *et al*. 2011; Sharma *et al*. 2013; Grenouillet *et al*. 2014).

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CE treatment depends on the parasitized organ and on the larval stage of the hydatid cyst. The most common approaches include surgical procedure, drug and percutaneous treatment (PAIR – puncture, aspiration, injection and reaspiration) or only long-term monitoring (Brunetti *et al*. 2010). The PAIR method consists of an ultrasound-guided puncture of the cyst followed by aspiration of the content and injection of protoscolicidal agents to inactivate protoscoleces inside the cyst. The procedure is finished with the reaspiration of all liquid present inside the cyst. This protocol reduces disease recurrence, since the leakage of viable protoscoleces in the body can cause secondary echinococcosis (Silva *et al*. 2001; Adas *et al*. 2009; Moazeni and Roozitalab, 2012). The protoscolicidal agent recommended by the literature is composed of 20% ethanol or hypertonic saline and, although efficient, may cause a strong osmotic gradient across the cuticle membrane, causing an increase in the hydatid cyst days after the procedure (Peláez *et al*. 2000; Silva *et al*. 2001). In the search for more efficient protoscolicidal agents against *Echinococcus* spp. protoscoleces, studies have emphasized the use of medicinal plants and essential oils as potential antiparasitic substances (Hailong *et al*. 2013; Pensel *et al*. 2014).

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Tea tree oil (TTO) is an essential oil extracted from the leaves of an Australian native plant known as *Melaleuca alternifolia*. The main components of this oil are: terpinen-4-ol (majority component), 1,8-cineol, α-terpinene, γ-terpinene, α-pinene, β-pinene, α-terpineol, p-cymene and sesquiterpene alcohols (Carson *et al*. 2006). TTO is widely used especially due to its antibacterial (Cox *et al*. 2000), antifungal (Hammer *et al*. 2006), anti-inflammatory (Hart *et al*. 2000), antiviral (Carson *et al*. 2008) and antiparasitic properties (Walton *et al*. 2004; Baldissera *et al*. 2014). Nanotechnology has been used to improve the efficiency of many compounds by altering their biodistribution and thereby improving their delivery to the action site (Schaffazick *et al*. 2003; Anton *et al*. 2008; Monalisa *et al*. 2013). Promising results of selenium nanoparticles were previously reported by Mahmoudvand *et al*. (2014) against *Echinococcus* spp. protoscoleces.

Due to the need for CE alternative therapies, the aim of this study was to analyse the *in vitro* and *ex vivo* scolicidal activity of TTO, its nanoemulsion formulation (NE-TTO) and its major compound (terpinen-4-ol) against *E. ortleppi* protoscoleces in a short period of time.

MATERIALS AND METHODS

Sample collection and viability

Hydatid cysts ($n = 25$) were obtained from naturally infected bovine lungs collected from a slaughterhouse in the central region of Rio Grande do Sul state, Brazil. The hydatid cysts were aseptically punctured with an 18-gauge hypodermic needle and syringe. Protoscoleces viability in hydatid liquid was evaluated using 0·1% eosin. Motile and non-stained protoscoleces were considered viable. Only samples with 100% viable protoscoleces were used for the *in vitro* and *ex vivo* studies.

Molecular analysis

An aliquot of protoscoleces obtained from a hydatid cyst was used for DNA extraction, according to the protocol established by Petrigh and Fugassa (2013). Polymerase chain reaction (PCR) was performed using the cytochrome *c* oxidase subunit I (COX I) gene following the protocol described by Bowles *et al*. (1992). PCR products were submitted to DNA sequencing, and the sequences obtained were compared with those in the GenBank database. The analyses were performed using the BLAST program (http://www.ncbi.nlm.nih.gov).

TTO and terpinen-4-ol dilution

TTO was obtained from Laszlo Aromatherapy (Belo Horizonte, Brazil) and terpinen-4-ol was obtained from Sigma-Aldrich (São Paulo, Brazil). TTO and terpinen-4-ol were diluted in sterile water until the concentrations of 2·5, 5, 10 and 20 mg mL^{-1} and 1, 1·5, 2 and 2·5 mg mL^{-1}, respectively.

NE-TTO preparation

NE-TTO was prepared ($n = 3$) according the conditions described by Flores *et al*. (2011). An organic phase comprising TTO (0·5 g), sorbitan monostearate (0·383 g) and acetone (25 mL) was added to an aqueous solution (50 mL) containing polysorbate 80 (0·383 g) and kept under moderate magnetic stirring for 10 min. The formulation was concentrated and the organic solvent was removed on a rotary evaporator (Fisatom, São Paulo, Brazil) at 60 rpm and at a temperature of 30–35 °C. The final volume of the formulations was of 50 mL to give an oil concentration of 10 mg mL^{-1}. Particles sizes and polydispersity indices ($n = 3$) were measured by photon correlation spectroscopy after appropriate dilution of an aliquot of the samples in purified water (Zetasizer® Malvern Instruments, Worcestershire, UK). Zeta potential values were measured using the same instrument at 25 °C after dilution of the samples in 10 mM NaCl. NE-TTO pH values were measured directly on the sample using a calibrated potentiometer (Mettler, São Paulo, Brazil), kept at room temperature. In order to compare the oil effectiveness, formulations without essential oil were also prepared (data not shown).

TTO characterization

Oil composition and yield were analysed by gas chromatography-mass spectrometry in a 7890N GC system coupled to a 5977A mass-selective detector (Agilent Technologies, USA). The separation was achieved on a DB-5MS 30 m × 0·25 mm × 0·25 μm capillary column and the injector temperature was set to 250 °C. The injection volume was 1 μL and helium of 99·999% purity at a flow rate of 1·3 mL min^{-1} was used as the carrier gas. The column oven temperature was programmed as follows: 50 °C held for 0·5 min, then programmed at 6·5 °C min^{-1} to 200 °C and further increased at 50 °C min^{-1} to 280 °C, which was held for 4·83 min. The transfer line temperature was 290 °C. The mass spectrometer was operated in electron impact ionization mode operating at 70 eV. The quadrupole temperature was set to 150 °C. The source of the mass instrument was operated at 300 °C. Identification of components was performed comparing with the mass spectra library search (NIST and Wiley). The relative amounts of individual components were calculated based on the resultant peak areas.

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In vitro *analysis*

Protoscoleces used for *in vitro* analysis were pre-washed with PBS (phosphate buffered saline) and stored at 4 °C. For each analysis, 500 μL of the solution to be tested (TTO, NE-TTO or terpinen-4-ol) were added to test tubes containing 5 μL of sediment rich in protoscoleces (~800 larvae). Tubes were incubated at 5, 10, 15 and 30 min at room temperature. Subsequently, the excess of liquid was removed and added to 500 μL of 0·1% eosin for 15 min. All viable and non-viable protoscoleces present in the tube were counted in an optical microscope. The tests were performed in triplicate. Water for injection (500 μL) was used as positive control to viability.

Ex vivo *analysis*

Fertile hydatid cysts ($n = 20$) in lungs obtained from naturally infected cattle were used for *ex vivo* analysis. Initially, 80% of the hydatid liquid was aspirated to confirm protoscoleces viability using 0·1% eosin and to perform the molecular identification of the species. This initial analysis was used as a positive control sample. Ten hydatid cysts were used for each concentration of TTO (10 and 20 mg mL^{-1}). TTO was injected to fill the complete interior of the cyst. An aliquot was removed from the liquid with protoscoleces inside the cyst at 5, 15 and 30 min and 0·1% eosin was added to the precipitate. After 15 min, viable and non-viable protoscoleces were counted at the microscope. Each new puncture of the liquid inside the cyst was stirred for a greater uniformity of action of the product being tested.

Statistical analysis

Data were plotted using Kaplan–Meier analysis and differences in protoscoleces viability were analysed 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

Molecular analysis

PCR analysis of all samples obtained in this study amplified a fragment of approximately 366 bp, corresponding to the mitochondrial gene COX I. DNA sequences generated were compared with sequences in GenBank, showing high similarity to *E. ortleppi*.

TTO characterization

The characterization of TTO identified four major compounds, in decreasing order: terpinen-4-ol (35·4%), α-terpinene (11%), γ-terpinene (20·4%) and 1,8-cineole (3·4%).

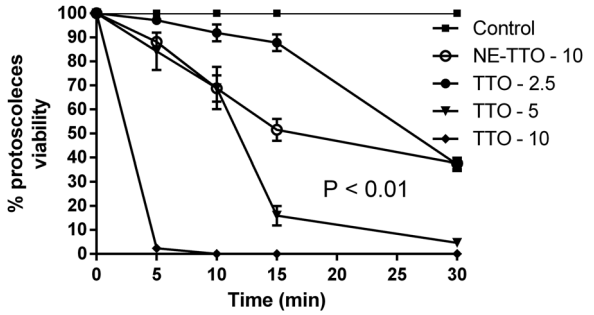

Fig. 1. Per cent viability of *Echinococcus ortleppi* protoscoleces exposed *in vitro* to different concentrations of tea tree oil (TTO) and to a nanoemulsion formulation of TTO (NE-TTO). The log-rank test was used to compare curves with the control (water). Data are expressed as mean ± S.D. ($n = 3$).

NE-TTO

NE-TTO at 10 mg mL^{-1} showed 267 nm of diameter and polydispersity index lower than 0·25, indicating adequate homogeneity of these systems. The formulations showed an acid pH (4·86) and ζ (zeta) potential of −11·5 ± 0·78 mV.

In vitro *analysis*

In vitro results indicated protoscolicidal effect in all formulations and concentrations tested in this study. NE-TTO at 10 mg mL^{-1} obtained its maximum action at 30 min, with 62·4% of mortality of protoscoleces (Fig. 1), whereas TTO obtained its best action at the concentration of 10 mg mL^{-1} at 10 min. A superior effect was observed for terpinen-4-ol, which showed 100% protoscolicidal effect at the concentration of 2 mg mL^{-1} at 5 min (Fig. 2). A great deposition of eosin was observed in the outer cuticular membrane of non-viable protoscoleces after contact with TTO at the concentrations tested (Supplementary Fig. 1, available from http://journals.cambridge.org/PAR).

Ex vivo *analysis*

Ex vivo analysis evaluated the protoscolicidal action of TTO at the concentrations of 10 and 20 mg mL^{-1} against *E. ortleppi* protoscoleces directly in the hydatid cyst. The concentration of 10 mg mL^{-1}, even though effective *in vitro*, did not demonstrate the same action in the *ex vivo* analysis, requiring a longer time to ensure a strong protoscolicidal effect. Notwithstanding, at 20 mg mL^{-1} and exposure time of 5 min, approximately 90% of protoscoleces were non-viable within the cyst (Fig. 3).

DISCUSSION

Echinococcus ortleppi is the species among the genus *Echinococcus* with greater predilection to infect

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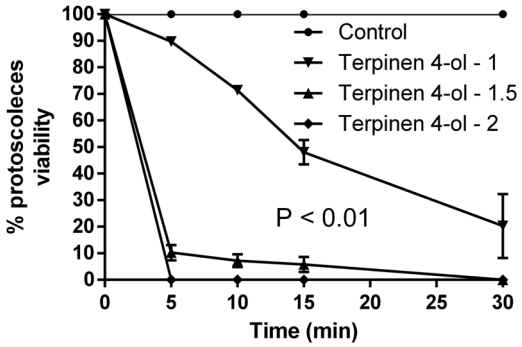

Fig. 2. Per cent viability of *Echinococcus ortleppi* protoscoleces exposed *in vitro* to different concentrations of terpinen-4-ol (mg mg^{-1}). The log-rank test was used to compare curves with the control (water). Data are expressed as mean ± S.D. ($n = 3$).

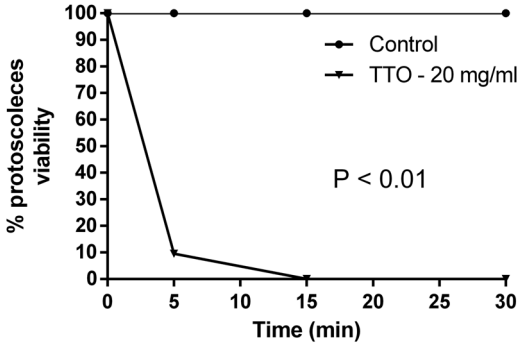

Fig. 3. Per cent viability of *Echinococcus ortleppi* protoscoleces exposed *ex vivo* to TTO. The log-rank test was used to compare the treated group with the control (water). Data are expressed as mean ± S.D. ($n = 3$).

cattle (Romig *et al.* 2015). This information was confirmed in our study, where we identified the presence of this species in cattle in Rio Grande do Sul state. Indeed, all hydatid cyst samples analysed in this study were infected by *E. ortleppi*.

The use of a protoscolicidal agent is essential to increase the efficiency of CE treatment, surgically or and by the PAIR method, because it reduces the risk of dissemination of viable protoscoleces in the body preventing disease recurrence (Silva *et al.* 2001; Puryan *et al.* 2005). Smego and Sebanego (2005) recommend 20–30 min of exposure to a protoscolicidal agent using the PAIR method. This time is considered too long for patients submitted to moderately invasive procedure. In our study, the *in vitro* efficacy of protoscolicidal agents was analysed in a shorter period of time, as observed in the treatment with TTO at 10 mg mL^{-1}. Thus, we considered the lowest time of TTO treatment that was able to kill *E. ortleppi* protoscoleces in order to significantly decrease the procedure time of CE treatment. Baldissera *et al.* (2014) reported the antiparasitic action of TTO at 0·9 mg mL^{-1} against *Trypanosoma evansi*, *in vitro* and *in vivo*, successfully controlling the infection. Carson *et al.* (2006) addressed the antibacterial mechanism of action of TTO and terpinen-4-ol, which consists in compromising the integrity of the cell membrane, leading to intracellular material leakage and loss of microbial capacity to maintain homeostasis. In line with this finding, we reported a high dye concentration (0·1% eosin) in the outer cuticular membrane of protoscoleces exposed to TTO treatment, an indicative of membrane damage. The protoscolicidal action of the TTO major compound, terpinen-4-ol, was very promising since non-viable *E. ortleppi* protoscoleces were observed at the concentration of 2 mg mL^{-1} within 5 min of exposure. Therefore, it can be inferred that terpinen-4-ol has more effective protoscolicidal action when compared with TTO. Bakkali *et al.* (2008) reported that the different pharmacological activities between essential oils and their isolated compounds may have antagonizing effects among compounds, which could alter the bioavailability of active components. Baldissera *et al.* (2016) reported the antiprotozoal action *in vitro* and *in vivo* of terpinen 4-ol, α-terpinene and γ-terpinene against *T. evansi*, highlighting that α-terpinene showed greater *in vitro* trypanocidal activity when compared with other compounds. Thus, it is observed that the antiparasitic activity of the compounds present in TTO differs according to the parasite evaluated.

In this study, we reproduced the PAIR method, with some modifications, using hydatid cysts removed from naturally infected cattle lungs. It was observed that the procedure demanded expertise in handling the hydatid cyst. Protoscolicidal should fill the entire cyst cavity, seeking contact with the protoscoleces, which sometimes are deposited in the cyst wall. It was observed decreased activity of TTO at the concentration of 10 mg mL^{-1} in *ex vivo* analysis. Therefore, in order to ensure the protoscolicidal effect, we used a concentration of 20 mg mL^{-1}, obtaining a quick and lasting effect in *ex vivo* analysis.

NE-TTO at the concentration of 10 mg mL^{-1} was used to minimize volatilization of the compound and to increase the protoscolicidal effect at the action site, i.e. in a shorter time. It was observed a constant effect on the protoscoleces viability; thus, it is believed that this effect can be enhanced in studies *in vivo*, thereby generating an extended action within the hydatid cyst. Flores *et al.* (2013) evaluated the antifungal activity of polymeric nanoparticles of TTO against *Trichophyton rubrum* isolates, reporting that the formulation was effective in reducing fungal growth. Sagave *et al.* (2015) studied the antimicrobial activity of TTO against a *Rhodococcus equi* isolate, and reported that the activity of the oil was enhanced when used as nanoformulation. Nanotechnology is an important feature of the pharmaceutical industry, especially in the development of more efficient

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formulations that can remain for long periods at the action site (Putheti *et al*. 2008). Ahmadnia *et al*. (2013) used albendazole sulfoxide nanoparticles in animals experimentally infected with CE and observed ultrastructural changes in hydatid cysts. Gradual decrease in the viability of protoscoleces is expected when using nanostructured systems because of the slow and gradual release of active formulations.

To the authors' knowledge, this is the first report demonstrating the protoscolicidal effect of *M. alternifolia* against *E. ortleppi* protoscoleces *in vitro* and *ex vivo*. The results showed that both the formulations TTO and NE-TTO were active against the protoscoleces. This activity was related to the presence of the major compound of the oil, terpinen-4-ol, which demonstrated strong protoscolicidal activity when evaluated alone. The potential use of *M. Alternifolia* oil and terpinen-4-ol in the treatment of CE should be further evaluated *in vivo*.

SUPPLEMENTARY MATERIAL

The supplementary material for this paper can be found at https://doi.org/10.1017/S0031182016001621.

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