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In vitro and *in vivo* efficacy of carvacrol against *Echinococcus granulosus*

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ABSTRACT

Currently, benzimidazoles are used as chemotherapeutic agents and as a complement to surgery and PAIR in the treatment of cystic echinococcosis (CE). They are generally applied at high doses causing side effects and, 50% of cases do not respond favorably to such chemotherapy. The use of essential oils obtained by distillation from aromatic plants would be an effective alternative or complementary to the synthetic compounds, because would not bring the appearance of side effects. Carvacrol and his isomer thymol are the main phenolic components from essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme). The aim of the present work was to evaluate the *in vitro* and *in vivo* efficacy of carvacrol against *Echinococcus granulosus* metacestodes. For the *in vitro* assay, protoscoleces and cysts of *E. granulosus* were incubated with carvacrol at the following final concentrations: 10, 5 and 1 μg/ml of carvacrol. The maximum protoscolicidal effect was found with 10 μg/ml of carvacrol. Results of viability tests were consistent with the structural and ultrastructural damage observed in protoscoleces. Ultrastructural studies revealed that the germinal layer of cysts treated with carvacrol lost the multicellular structure feature. In the clinical efficacy study, a reduction in cyst weight was observed after the administration of 40 mg/kg of carvacrol during 20 days in mice with cysts developed during 4 months, compared to that of those collected from control mice. Given that the *in vivo* effect of carvacrol was comparable with the treatment of reference with ABZ and the fact that is a safe compound, we postulated that carvacrol may be an alternative option for treatment of human CE.

1. Introduction

The larval stage (metacestode) of *Echinococcus granulosus* causes cystic echinococcosis (CE) in humans and livestock. This cestode parasite has a worldwide distribution, particularly affecting pastoral and poor rural communities where people raise livestock in close contact with dogs (Alvarez Rojas et al., 2014). CE is characterized by cystic lesions, most commonly in the liver and lungs (Budke et al., 2013).

The WHO-Informal Working Group on Echinococcosis (WHO-IWGE) proposes four different treatment modalities for CE: surgery, percutaneous treatment (PAIR: puncture, aspiration, injection, re-aspiration), drug treatment and observation (watch and wait). Treatment indications are based on cyst characteristics, available medical/surgical expertise and equipment, and presence/absence of complications (Brunetti et al., 2010). Currently, chemotherapy is used as an adjuvant to surgery either preoperatively or postoperatively or both. This produces the decrease in the viability of cysts and reduces its tension, making surgery easier. It is also used complementarily with PAIR treatment and, in both cases; it prevents the recurrence of the disease in the form of secondary hydatidosis (Arif et al., 2008).

In human patients, benzimidazoles have to be applied at high doses for extended periods of time and adverse side effects are frequently observed, such as abnormalities in liver function, leucopenia and alopecia (Walker et al., 2004). Mebendazole, albendazole (ABZ) and its major metabolite albendazole sulfoxide, are the only anthelmintics approved by the FDA (Food and Drug Administration, USA), but its effectiveness is about 50% (Pawlowski et al., 2001).

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Because of the serious difficulties in achieving treatment success and toxicity of the compounds used in chemotherapy, the proposal of an effective drug against hydatid disease would be a great alternative treatment to surgery. Many efforts have been made to discover new compounds from various kinds of sources such as plants, animals and microorganisms. Currently, herbal medicines are being increasingly used to treat many diseases including several infections (Khan et al., 2010). For this reason, the use of essential oils obtained by distillation from plants would be an effective alternative or complementary to the synthetic compounds, because they would not bring the appearance of side effects (Pessoa et al., 2002; Carson and Riley, 2003).

Essential oils are very complex natural mixtures, synthesized by all organs of the aromatic plants as secondary metabolites. They contain 20–60 components at very different concentrations, but they are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. These major components determine the biological properties of the essential oils (Bakkali et al., 2008). Because of its hydrophobic character, the essential oils and their components present a great potential for pharmacological applications as antimicrobial agents (Edris, 2007; Santoro et al., 2007; Tavares et al., 2008; Arana-Sánchez et al., 2010; Machado et al., 2010).

Some essential oils have demonstrated activity against *E. granulosus* as *Rosmarinus officinalis* (rosemary), *Pistacia khinjuk* (pistachio), *Mentha* spp, *Trachyspermum ammi* (ajowan), *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) (Albanese et al., 2009; Taran et al., 2009; Maggiore et al., 2012; Moazeni et al., 2012; Pensel et al., 2014).

Carvacrol and its isomer thymol are the main phenolic components from essential oils of thyme and oregano (Santoro et al., 2007). The *in vitro* effect of thymol was shown against protoscoleces, microcysts and cyst of *E. granulosus*, as well also its *in vivo* effect in a murine model (Elissondo et al., 2008, 2013; Maggiore et al., 2015). Nevertheless, the effect of carvacrol against *E. granulosus* it has not been studied yet. The aim of the present work was to determine the *in vitro* and *in vivo* efficacy of carvacrol against *E. granulosus*.

2. Materials and methods

2.1. Chemicals

Carvacrol and ABZ were purchased from Sigma-Aldrich. For *in vitro* studies, carvacrol was dissolved in dimethyl sulphoxide (DMSO) at a drug concentration of 10 mg/ml and added to the medium resulting in final concentrations of 10, 5 and 1 μg/ml. For *in vivo* treatments, ABZ suspension (2.5 mg/ml) was prepared by dissolution of ABZ pure standard in deionized water (pH 7.0) by shaking on a mechanical shaker (12 h). Carvacrol was dissolved in olive oil at a drug concentration of 4 mg/ml. ABZ suspension and carvacrol were vigorously shaken before their intragastric administration to mice.

2.2. Protoscoleces collection

Protoscoleces of *E. granulosus* were collected aseptically from liver and lung hydatid cysts of naturally infected cattle slaughtered in an abattoir located in the southeast of Buenos Aires province, Argentina. The area where the cattle came from is known to include only the G1 strain of *E. granulosus* (Andresiuk et al., 2013). Viability was assessed by the methylene blue exclusion test as previously described (Elissondo et al., 2006).

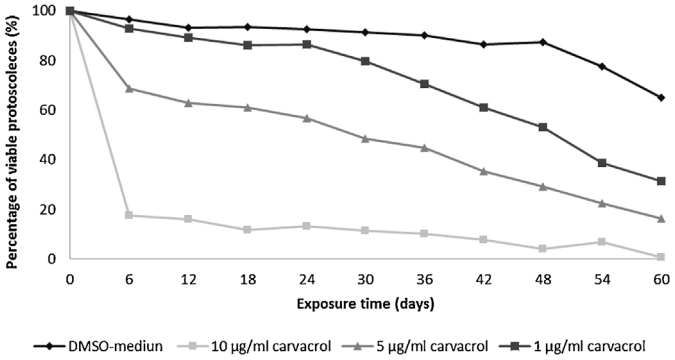

Fig. 1. Survival of *E. granulosus* protoscoleces after *in vitro* exposure to carvacrol. Each point represents the mean percentage of viable protoscoleces from three different experiments (DMSO: dimethyl sulphoxide).

2.3. Experimental animals and infection

Animal procedures and management protocols were approved by the Institutional Animal Care and Use Committee (RD 148/15) of the Faculty of Exact and Natural Sciences, National University of Mar del Plata, Mar del Plata, Argentina and carried out in accordance with the revised form of The Guide for the Care and Use of Laboratory Animals (National Research Council US, 2011). Unnecessary animal suffering was avoided throughout the study. Female CF-1 mice (bodyweight 25 g ± 5) were infected by intraperitoneal inoculation with 1500 *E. granulosus* protoscoleces/animal, suspended in 0.5 ml of medium 199 (Lab. Microvet SA, Argentina). Animals were housed in a temperature-controlled (22 ± 1 °C) and light-cycled (12 h light/dark cycle) room. Food and water were given *ad libitum*.

2.4. Experimental design

2.4.1. In vitro *experiments*

2.4.1.1. In vitro *drug treatment of protoscoleces with carvacrol.* Viable and free protoscoleces (2000 per Leighton tube) were cultured in medium 199 containing 60 μg/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 4 mg/ml glucose. Cultures were performed in 10 ml of incubation medium at 37 °C without changes of medium (Elissondo et al., 2006).

Carvacrol was added to the medium resulting in final concentrations of 10, 5 and 1 μg/ml. Protoscoleces incubated in culture medium containing 10 μl DMSO served as controls (Elissondo et al., 2006). Each experiment was repeated three times. During the experiments, culture tubes were followed microscopically everyday to determine the appearance of morphological alterations. Samples of protoscoleces (approximately 90–100 protoscoleces in 180 μl of incubation medium) from each of the dosing groups and the controls were taken every 6 days for viability assessment using the methylene blue exclusion test.

2.4.1.2. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). The detection mechanism of apoptosis on *E. granulosus* protoscoleces by the action of carvacrol was performed using the TUNEL method. A commercially available kit (Apop Tag Plus *In Situ* Apoptosis Detection Kit S7101; Chemicon International a Serologicals Company; USA and Canada) was used to detect the 3′-OH ends of the DNA strands according to manufacturer's instruction. Protoscoleces incubated 24 h with 10 μg/ml of carvacrol were fixed in Karnovskyís solution (paraformaldehyde 2% (w/v), glutaraldehyde 2.5% (v/v), 0.05 M cacodylate pH 7.2) at 4 °C for 48 h, and then embedded in paraffin. Tissue sections (5 μm) were deparaffinized and pretreated with Proteinase-K solution (20 μg/ml) at room temperature for 15 min. The endoge-

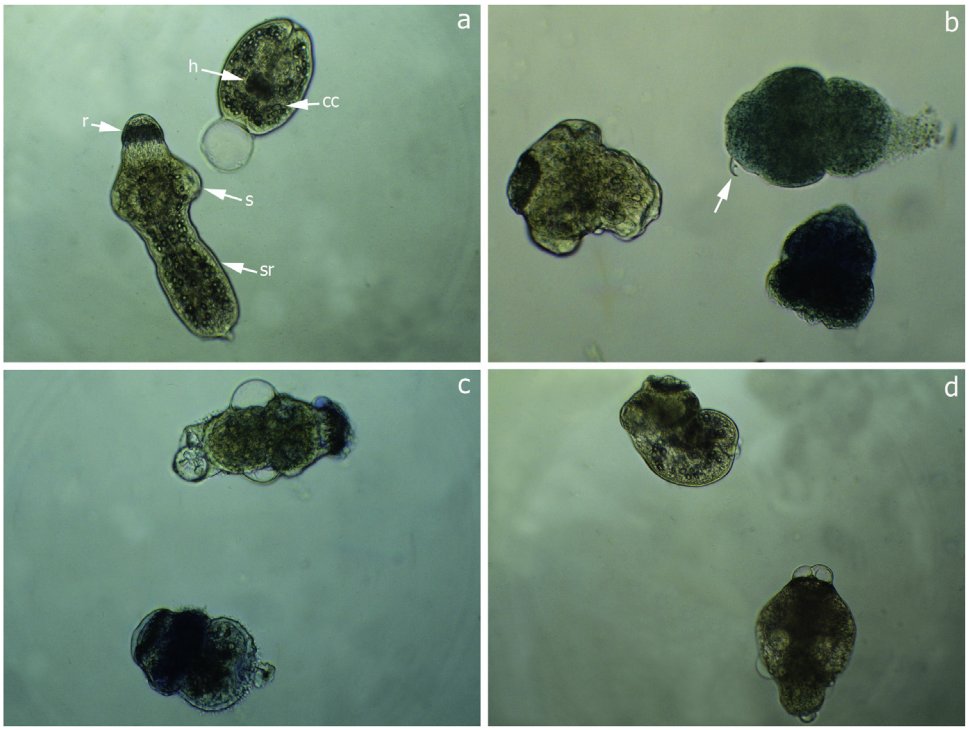

Fig. 2. Light microscopy of *E. granulosus* protoscoleces incubated *in vitro* with carvacrol and stained with methylene blue (1:10000). Blue protoscoleces are not viable. (a) Control protoscoleces after 6 days of incubation (cc, calcareus corpuscles; h, hooks; r rostellar region, s sucker, sr soma region, 360×); (b) Altered protoscoleces with contracted soma, 1 day p.i. with 10 μg/ml of carvacrol (490×). Note the presence of blebs in the tegument and loss of hooks (arrow); (c) Protoscoleces with contracted soma and blebs in the tegument incubated with carvacrol (3 days p.i., 5 μg/ml, 360×). (d) Protoscoleces with the presence of blebs in the tegument (3 days p.i., 1 μg/ml of carvacrol, 390×).

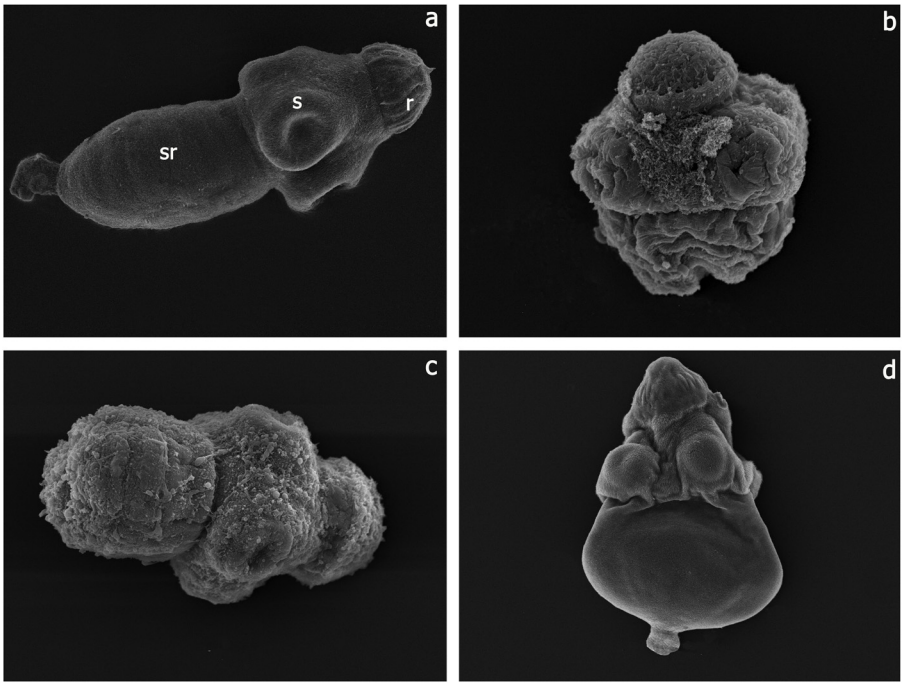

Fig. 3. Scanning electron microscopy of *E. granulosus* protoscoleces incubated *in vitro* with carvacrol during 6 days. (a) Evaginated control protoscolex, (r rostellar region, s sucker, sr soma region, 600×); (b) Protoscolex incubated with 10 μg/ml of carvacrol. The tegument is markedly altered with loss of microtriches and formation of vesicles. Note loss of hooks and contracted soma (750×); (c) Evaginated protoscolex (5 μg/ml of carvacrol). Note the altered tegument with vesicles and loss of microtriches (800×); and (d) Protoscolex incubated with 1 μg/ml of carvacrol. Note the contraction of soma region (430×).

nous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide in phosphate buffered saline (PBS) at room temperature. Sections were incubated in a mixture of terminal deoxynucleotidyl transferase and digoxigenin-labeled dideoxynucleotide in a humidified chamber at 37 °C for 1 h. After reacting with a stop buffer, the sections were incubated with an anti-digoxigenin peroxidase conjugate for 30 min. Peroxidase activity was detected by exposing the sections to a solution containing 3.3′-diaminobenzidine

tetrahydrochloride (DAB). Sections were then counter-stained with hematoxylin. Negative controls were treated with distilled water in place of the terminal deoxynucleotidyl transferase enzyme. After color development, slides were observed under light microscope. Nuclei of apoptotic cells were stained brown with TUNEL reagents; normal nuclei had no brown staining but showed a blue color with hematoxylin.

2.4.1.3. Collection of murine cysts and in vitro *incubation with carvacrol.* Female CF-1 mice were infected with *E. granulosus* protoscoleces as described above (see Section 2.3). At 6 months p.i., mice with experimental secondary CE were euthanized and necropsy was carried out immediately thereafter. At necropsy, the peritoneal cavity was opened and the hydatid cysts were carefully removed (Elissondo et al., 2009).

Groups of 5 cysts were placed in Leighton tubes containing 10 ml of supplemented medium 199. Carvacrol was added to the medium resulting in final concentrations of 10, 5 and 1 μg/ml. Cysts incubated with the culture medium containing 10 μl DMSO were used as controls. Cultures were maintained at 37 °C without changes of medium during the entire drug incubation period (Elissondo et al., 2007). Each experiment was repeated three times. Culture tubes were followed macro and microscopically every day. Samples of cysts from each of the dosing groups and the controls were taken and then fixed for electron microscopy. The criteria for cysts viability assessment included the loss of turgidity, the collapse of cysts and the ultrastructural observation of the germinal layer as described by Elissondo et al. (2007).

2.4.2. In vivo *experiments*

At 4 months post-infection (as described in Section 2.3), forty mice were allocated into the following experimental groups (10 animals/group) and treated as follows: a) unmedicated water control group, animals receiving distilled water as a placebo; b) unmedicated oil control group, animals receiving olive oil as a placebo; c) ABZ group, animals treated with ABZ suspension (25 mg/kg) every 24 h for 30 days; d) carvacrol group, animals treated with carvacrol (40 mg/kg) every 24 h for 20 days. Treatments were performed by intragastric administration (0.3 ml/animal). At the end of the treatment period, all mice remained alive and, they were euthanized. Necropsy was carried out immediately thereafter.

At necropsy, the peritoneal cavity was opened and the hydatid cysts were carefully removed. The weight of the cysts collected from each individual animal was recorded using an analytical balance. The efficacy of treatments (based on the weight of cysts from infected mice), was calculated by use of the following formula: [(mean weight from untreated control group − mean cysts weight from treated group)/mean cysts weight of untreated group] × 100. Samples of cysts from each group were taken and then fixed for electron microscopy.

2.5. Statistical analysis

Log-rank test was used to assess the survival differences of protoscoleces after *in vitro* exposure to different concentrations of carvacrol. Statistical analysis was performed with the software BioEstat 5.0 software (Ayres et al., 2007). Cysts weights (reported as mean ± SD) recovered for *in vivo* assay were compared statistically by nonparametric method (Kruskal Wallis test followed by Dunnís multiple comparison test). The statistical analysis were performed using the Instat 3.0 software program (GraphPad Software, San Diego, CA, USA). For all statistical comparison, P values less than 0.05 ($P < 0.05$) were considered to be statistically significant.

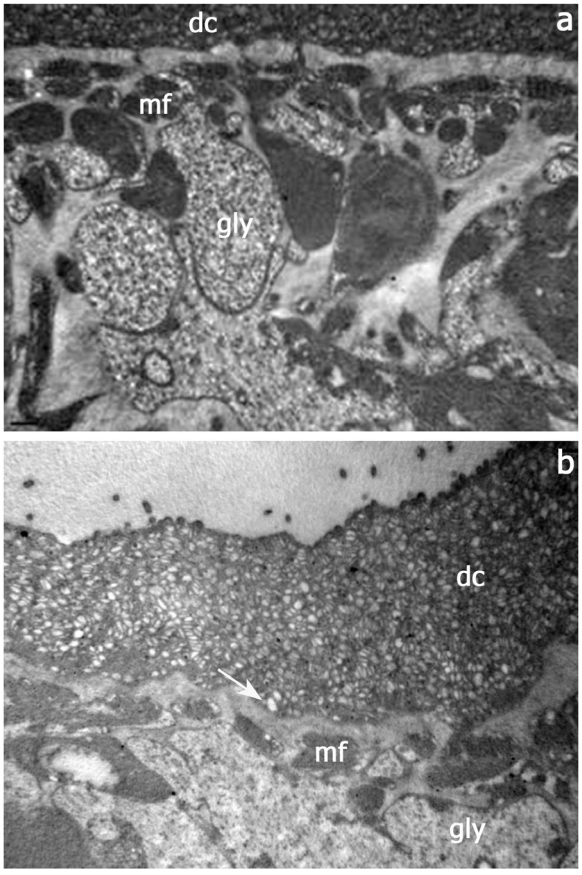

Fig. 4. Transmission electron microscopy of *E. granulosus* protoscoleces incubated *in vitro* with carvacrol during 12 days (15,000×). (a) Control protoscolex. Note glycogen storage cells (gly, glycogen) densely packed (dc, distal cytoplasm; mf muscle fibers); and (b) Treated protoscolex (10 μg/ml of carvacrol). Note decrease in glycogen reserves (gly) and appearance of small vacuoles in the distal cytoplasm (dc, arrow; mf, muscle fibers).

2.6. Electron microscopy

Samples of protoscoleces and cysts cultured *in vitro* as well as cysts recovered from mice involved in the efficacy study were processed for transmission and/or scanning electron microscopy (TEM and/or SEM) as described by Elissondo et al. (2006, 2007).

3. Results

3.1. Evaluation of in vitro efficacy of carvacrol against *E. granulosus* protoscoleces and cysts

The survival of *E. granulosus* protoscoleces after exposure to different concentrations of carvacrol is shown in Fig. 1. Control protoscoleces viability was 87.45 ± 4% after 48 days post-incubation (p.i.). The maximum protoscolicidal effect was found with 10 μg/ml of carvacrol ($P < 0.0001$). Viability was reduced to 17.62 ± 13.93% after 6 days p.i. and reached 0% after 60 days. Carvacrol at concentrations of 5 and 1 μg/ml provoked a later protoscolicidal effect, reducing viability of protoscoleces to 16.17 ± 1.26% and 31.24 ± 10.52% respectively, after 60 days p.i. (Fig. 1).

The results of viability tests coincide with the structural and ultrastructural alterations observed by light microscopy, SEM and TEM. Control protoscoleces remained viable throughout the experimental period and no changes in structure and ultrastructure were observed (Figs. 2 a, 3 a and 4 a). However, after the first day p.i., formation of blebs on the tegument, soma contraction and rostellar

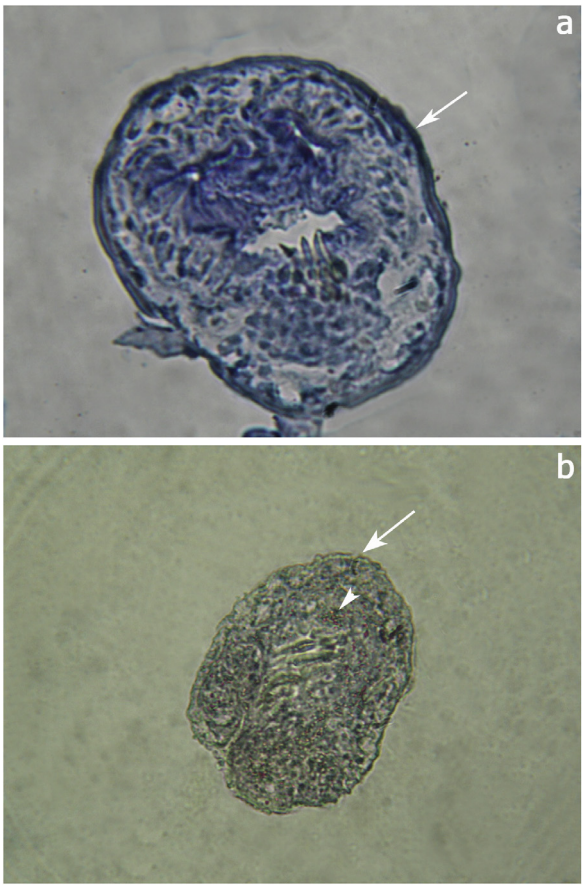

Fig. 5. Light micrographs of TUNEL staining of *E. granulosus* protoscoleces incubated *in vitro* during 24 h with 10 μg/ml of carvacrol. (a) Invaginated control protoscolex (1720×). Note the integrity of the tegument (arrow); (b) Protoscolex treated with carvacrol. Note the presence of apoptotic area (arrowhead) and loss of the integrity of the tegument (arrow) (1440×).

disorganization were observed in protoscoleces incubated with the highest concentration of carvacrol (Fig. 2b). With the other concentrations, the same alterations could be observed later, at 3 days p.i. (Figs. 2c and d). At ultrastructural level, SEM demonstrated alterations on protoscoleces incubated *in vitro* with 10 and 5 μg/ml of carvacrol. After 6 days p.i., loss of microtriches and hooks of the scolex and the presence of blebs on the tegument were detected (Fig. 3b and c). After 6 days p.i. with the lower concentration, the damage was lower but equally remarkable in relation to the control group. The contraction of soma region was observed (Fig. 3d). TEM revealed the presence of ultrastructural alterations on the internal tissue of protoscoleces incubated during 12 days with 10 μg/ml of carvacrol. The presence of vacuoles in the distal cytoplasm and a marked decrease in glycogen reserves were observed (Fig. 4b).

Fig. 5 shows representative TUNEL images of control and treated protoscoleces with 10 μg/ml of carvacrol during 24 h. Cells from control sections (Fig. 5a) showed no apoptotic nuclei. TUNEL assay clearly reveals the presence of *in situ* DNA fragmentation in the nuclei of the treated protoscoleces (Figs. 5b).

Macroscopically, all control cysts appeared turgid with no observable collapse of the germinal layer throughout the *in vitro* experiment (Table 1). Ultrastructurally, the germinal layer was observed without alterations (Fig. 6a). In contrast, loss of turgidity was detected in cysts incubated during 3 days at the highest concentrations. After 3 days p.i., the collapse of the germinal layer was observed in cysts treated with 10 μg/ml of carvacrol (Table 1). Studies by SEM revealed that the highest concentration of carvacrol caused a major effect against cysts. The germinal layer of

Table 1
Time of appearance (days p.i.) of different indicators of tissue damage on *E. granulosus* murine cysts, after their incubation with carvacrol, under *in vitro* conditions.

Parameters of the study	Days p.i.			
	Carvacrol (μg/ml)			
	10	5	1	Control
Loss of cyst turgidity	3	3	–	–
Appearance of collapsed cysts	3	–	–	–

Table 2
Clinical efficacy study. Mean (±SD) weights (g) of the hydatid cysts recovered from artificially infected mice from the unmedicated control and treated groups. Treatments were performed after 4 months of inoculation, by intragastric administration (0.3 ml/animal) every 24 h, at the doses rate of 25 mg/kg of ABZ (30 days) and 40 mg/kg of carvacrol (20 days).

	Clinical efficacy study	
	Wet weigh (g) of cysts Mean ± SD	% of efficacy
Unmedicated control water group	5.26 ± 3.14^{a}	–
Unmedicated control oil group	6.50 ± 3.18^{a}	–
ABZ suspension	1.41 ± 1.72^{b}	73.20
Carvacrol	1.80 ± 1.78^{b}	72.31

Different letters (a, b) indicate statistically significant differences ($P < 0.05$) between groups.

cysts incubated during 3 days, lost the feature multicellular structure (Fig. 6b). On the other hand, cysts incubated with 5 μg/ml of carvacrol showed a lower reduction in the number of cells (Fig. 6c). The germinal layer of cysts incubated with 1 μg/ml of carvacrol was unaltered after 6 days p.i. (Fig. 6d).

3.2. Clinical efficacy study of carvacrol

Hydatid cysts developed in all the infected animals involved in the efficacy study. Carvacrol treatment resulted in a statistically significant reduction on the weight of the cysts compared to those obtained for unmedicated mice ($P < 0.05$) (Table 2). On the other hand, no significant difference between ABZ (1.41 ± 1.72 g) and carvacrol groups (1.80 ± 1.78 g) was detected ($P > 0.05$). The efficacy of the treatment during 20 days with carvacrol (72.31%) was similar to the efficacy observed with a 30 days ABZ-treatment (73.20%).

All cysts in the samples removed from control mice appeared turgid, showing no observable collapse of the germinal layer. At ultrastructural level, the cysts showed the typical features of *E. granulosus* metacestodes, with a germinal layer comprised of different cell types (Fig. 7a). In contrast, the ultrastructural study of cysts developed in mice treated with ABZ suspension and carvacrol revealed the lost of the characteristic multicellular structure (Fig. 7b and c).

4. Discussion

Essential oils are among the classes of vegetable substances reported to possess anthelmintic activity and can be used as an alternative to current therapies (Anthony et al., 2005; Ribeiro et al., 2013). They contain large amounts of terpenes, which are secondary metabolites that interfere with biochemical and physiological functions of parasites (Kaplan et al., 2014; Nordi et al., 2014). Carvacrol and its isomer thymol are phenolic monoterpenes that are found as major components in essential oils of plants belonging to the genera *Origanum* and *Thymus* (Guimaraes et al., 2015).

The *in vitro* efficacy of thymol and *T. vulgaris* and *O. vulgare* essential oils against protoscoleces and cysts of *E. granulosus* and, the *in vivo* activity of thymol and aromatic water of *Zataria multiflora* on hydatid cysts were previously demonstrated (Elissondo

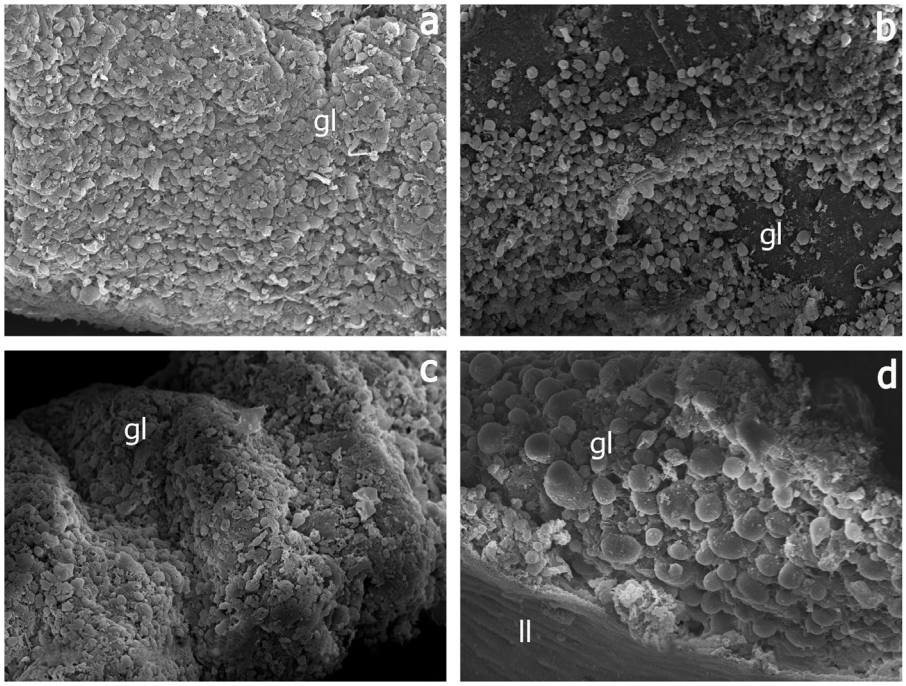

Fig. 6. Scanning electron microscopy of *E. granulosus* murine cyst incubated *in vitro* with carvacrol. (a) Control cyst with an intact germinal layer (gl, germinal layer, 3 days p.i.) (400×); (b) Murine cyst incubated with 10 μg/ml of carvacrol (3 days p.i.). Note the reduction in the number of cells of germinal layer (gl, 400×); (c) Cyst (5 μg/ml of carvacrol, 3 days p.i.). The germinal layer (gl) is altered (400×); and (d) Murine cyst incubated with 1 μg/ml of carvacrol after 6 days (ll, laminar layer) (800×).

et al., 2008, 2013; Pensel et al., 2014; Moazeni et al., 2014; Maggiore et al., 2015). There are very few studies that evaluate the anthelmintic and antibacterial action of carvacrol. This work is the first report of the *in vitro* and *in vivo* effect of carvacrol against the *E. granulosus* larval stage.

The *in vitro* efficacy of carvacrol against *E. granulosus* protoscoleces and murine cysts was demonstrated. In both cases, carvacrol showed a time and dose dependent effect. A correlation between the intensity of damage and the assayed concentration was observed, as was demonstrated at the ultrastructural studies by SEM. The alterations in protoscoleces included contraction of soma region, bleb formation in tegument, rostellar disorganization and loss of hooks and microtriches. In carvacrol-treated cysts, the germinal layer lost the characteristic multicellular structure. The ultrastructural alterations were similar to those observed in *E. granulosus* protoscoleces and cysts incubated *in vitro* with thymol and the essential oils of oregano and thyme (Elissondo et al., 2008; Pensel et al., 2014).

When the *in vitro* effects of carvacrol on protoscoleces of *E. granulosus* was compared with those produced by thymol (Elissondo et al., 2008) and with the essential oils of *T. vulgaris* and *O. vulgare* (Pensel et al., 2014), the observed effectiveness was: carvacrol > thymol » essential oils of *T. vulgaris* and *O. vulgare*.

The extraction product of essential oils can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage (Masotti et al., 2003; Angioni et al., 2006). If an essential oil obtained from a particular plant demonstrates be effective for treatment from any disease, it would be very difficult to obtain a product of a constant composition. For this reason, and considering the possibility of antagonic effect of the minor components of essential oils, it may be more feasible to determine which specific components in which ratios are responsible for the antimicrobial activity of an essential oil and to combine these purified or synthetically derived compounds in the precise ratio desired (Burt, 2007).

The similarity in chemical structure of carvacrol and thymol suggests a similar mechanism of antimicrobial activity (Ultee et al., 2002). Due to their hydrophobic nature, both compounds interact with the lipid bilayer of the cytoplasmic membrane (Helander et al., 1998; Ultee et al., 1999; Lambert et al., 2001; Trombetta et al., 2005). Moreover, due to the presence of a free hydroxyl group, these compounds can act as proton translocators (Ben Arfa et al., 2006), inserted into the cytoplasmic membrane, which generates change in their physical and chemical properties, affecting the order of lipid and the stability of the bilayer, resulting in increased passive flow of protons through the membrane (Xu et al., 2008). The collapse of the proton motive force and ATP depletion can lead to cell death (Ultee et al., 2002).

The induction of apoptosis was demonstrated for several essential oils (Cha et al., 2010) and, drug induced apoptosis in *E. granulosus* protoscoleces has previously been reported (De et al., 2012). Moreover, thymol and essential oils of *T. vulgaris* and *O. vulgare* induce apoptosis in the cells of protoscoleces (Pensel et al., 2014). On the other hand, the induction of apoptosis by carvacrol on different cancer cells was observed (He et al., 1997; Karkabounas et al., 2006; Arunasree, 2010; Yin et al., 2012). In this study, the TUNEL assay indicated that carvacrol appear to induce apoptosis in the cells of protoscoleces after short incubation times.

In the present work, the effect of carvacrol was also evaluated in an *in vivo* murine model of CE. Carvacrol has the status of generally recognized as safe food additive (GRAS), which is endorsed by the Flavor and Extract Manufacturers' Association (FEMA) and the Food and Drug Administration (FDA) of the U.S.A. (Furia and Bellanca, 1975). Andre et al. (2016) determined that the LD50 by oral route in mice was 919 (693.3–1,251.9) mg/kg.

The ultrastructural alterations in germinal layer induced *in vivo* with carvacrol were similar to those described.

Oral administration of 40 mg/kg of carvacrol during 20 days in infected mice has a therapeutic effect on the hydatid cyst. Moreover, no toxic effects were detected on CF1 mice during the entire experiment. Carvacrol led to a significantly reduced parasite weight

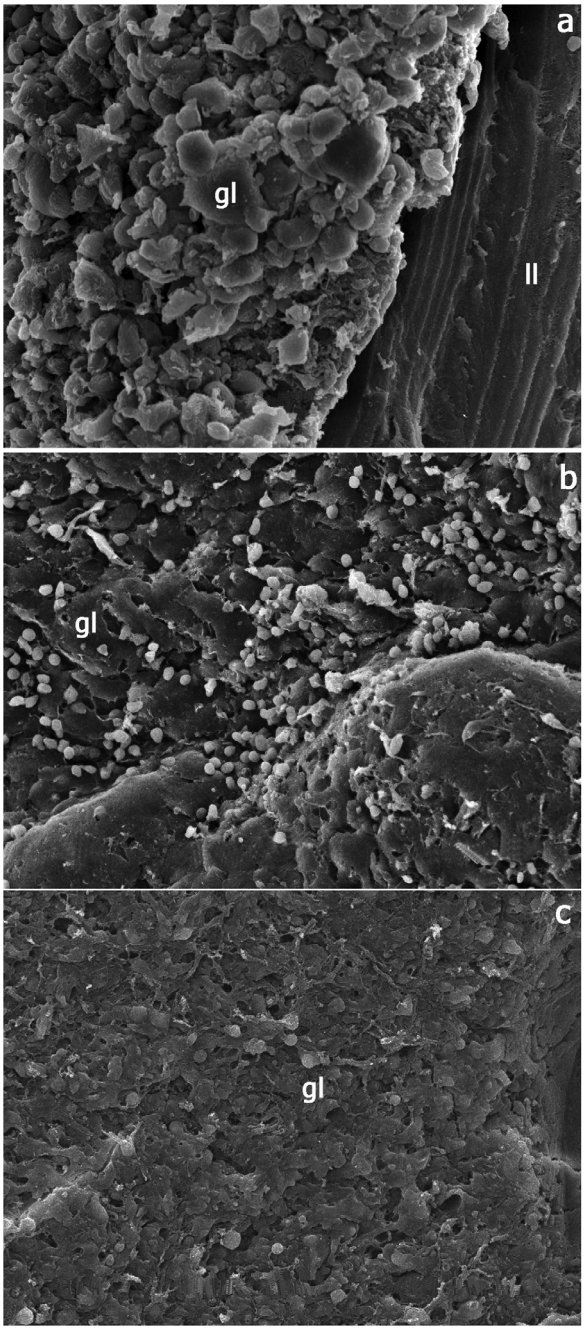

Fig. 7. Scanning electron microscopy of *E. granulosus* murine cyst recovered from infected mice treated with albendazole (25 mg kg^{-1} day $^{-1}$, 30 days) or carvacrol (40 mg kg^{-1} day $^{-1}$, 20 days) (800×). (a) Control cyst with an intact germinal layer (gl germinal layer, ll laminal layer); (b) Murine cyst recovered from mice treated with albendazole. Note the lost of the characteristic multicellular structure of germinal layer; (c) Cyst recovered from mice treated with carvacrol. Alterations in germinal layer and only debris of cells can be observed.

comparable with ABZ suspension (25 mg/kg during 30 days). The ultrastructural changes induced *in vivo* by carvacrol were similar to those observed for cysts incubated *in vitro*. These similarities could be indicating the entrance of the drug into the cysts in treated mice. Moreover, the alterations coincide with the changes reported for cysts recovered from mice treated with thymol (Maggiore et al., 2015).

In conclusion, our data suggest that carvacrol has *in vitro* and *in vivo* activity against the larval stage of *E. granulosus*. A short period of treatment was sufficient to achieve a pharmacological effect. Given that the *in vivo* effect of carvacrol was comparable with the treatment of reference with ABZ and the fact that is a safe compound, we postulated that carvacrol may be an alternative option for the treatment of CE.

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