

Could thymol have effectiveness on scolices and germinal layer of hydatid cysts?

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ABSTRACT

Scolicidal solutions remain indispensable in the treatment of hydatid cyst disease. Properties of an ideal solution would be inexpensiveness and the promotion of a rapid and complete scolical effect with an absence of local and systemic side effects. From this point of view, no ideal solution and agents have been described yet. The aim of the present work was to determine the in vitro effect of high concentrations of thymol against protoscoleces, microcyst and cyst of *Echinococcus granulosus* and to evaluate its possible role as a scolical agent during surgery or PAIR. After short exposure times, a rapid effect was observed depending on the parasitic material. After 2 min of exposure to thymol, viability of protoscoleces was approximately 1.3% at a concentration of 250 µg/ml. The protoscolical effect is dose and time dependent. The results of the in vitro treatment with thymol were similar in both microcysts and secondary murine cysts. The employment of SEM and TEM allowed us to examine, at an ultrastructural level, the effects induced by thymol on *E. granulosus* protoscoleces, microcysts and murine cysts. In conclusion, the data obtained clearly demonstrated that thymol caused severe damages to the parasite even after short incubation times. This fact and the lack of toxicity at the evaluated concentrations, allow us to propose it as a possible scolical agent during hydatid cysts surgery and/or PAIR.

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1. Introduction

Human cystic echinococcosis, caused by the larval stage of the cestode *Echinococcus granulosus*, is a chronic, complex, and still neglected parasitic infection disease (Brunetti et al., 2011). WHO-IWGE classification provides the basis for choosing basically four treatment and management options for cystic echinococcosis: surgery, percutaneous sterilization, drug treatment, and observation (watch and wait) (Brunetti et al., 2010). The appropriate treatment depends on cyst characteristics (for hepatic cysts, size and stage are the most important criteria), the therapeutic resources available, and the physician's preference. The level of evidence supporting one therapeutic modality over the other is low because only few prospective, randomized studies comparing different treatments are available (Brunetti and White, 2012).

Surgery remains the therapy of choice in complicated cysts (i.e., rupture, biliary fistula, compression of vital structures, superinfection, hemorrhage), or large cysts with many daughter vesicles that are not suitable for percutaneous treatments. Traditional

approaches have included radical resectioning including pericystectomy or more conservative techniques. Laparoscopic surgery has emerged as an alternative to open procedures in some cases (Brunetti et al., 2010). Instillation of a scolical agent into hepatic hydatid cysts to reduce the risk of spillage of viable protoscoleces is an integral part of the surgical technique for many surgeons (Adas et al., 2009).

Percutaneous techniques aim either to destroy the germinal layer with scolical agents or to evacuate the entire endocyst. The most popular method in the first group is PAIR (puncture, aspiration, injection of a scolical agent, and reaspiration) (Brunetti and White, 2012). PAIR confirms the diagnosis and removes parasitic material. It is minimally invasive, less risky and usually less expensive than surgery.

Protoscolicides used during surgery and PAIR are mainly 20% NaCl and 95% ethanol (Brunetti et al., 2010). Moreover, other substances were proposed as scolical agents such as povidone iodine, silver nitrate, albendazole sulfoxide, cetrimide, octenidine hydrochloride, octenidine dihydrochloride and chlorhexidine gluconate (Adas et al., 2009; Altindis et al., 2004; Ciftci et al., 2007; Puryan et al., 2005).

For sterilization of the cyst, several parasiticidal substances have been used. Scolical solutions remain indispensable in the treatment of hydatid cyst disease. Properties of an ideal solution would be inexpensiveness and the promotion of a rapid and complete

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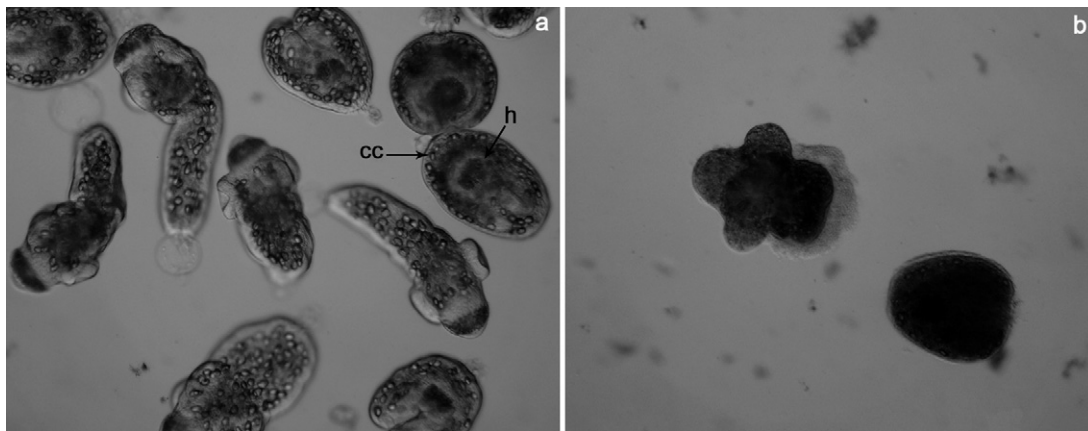


Fig. 1. Light microscopy of *E. granulosus* protoscoleces incubated in vitro with thymol. (a) Control protoscoleces (cc, calcareous corpuscles; h, hooks; 240 \times) and (b) altered protoscoleces (200 μ g/ml, 2 min p.i., 320 \times).

scolicidal effect with an absence of local and systemic side effects. From this point of view, no ideal solution and agents have been described yet (Adas et al., 2009).

Many drugs originate from herbal sources: a century ago, most of the effective drugs were plant based. The development of drugs from plants continues, with drug companies engaged in pharmacological screening of herbs (Jazani et al., 2009).

The pharmaceutical properties of aromatic plants are partially attributed to essential oils. To date, essential oils are presented as valuable therapeutic options against a number of diseases (Edris, 2006). Purified compounds derived from essential oils such as carvacrol, eugenol, linalool and thymol inhibit a variety of microorganisms, such as bacteria and fungi (Hulin et al., 1998). Moreover, several essential oils and their constituents have been found to possess antiparasitic activity (Garg, 1997; Hammond et al., 1997).

To date, there are few works that study the role of essential oils specifically against parasitic helminths (Anthony et al., 2005; Hammond et al., 1997; Pessoa et al., 2002). The in vitro effect of the essential oils of *Rosmarinus officinalis* (rosemary), *Mentha* sp., *Pistacia khinjuk* (pistachio) and *Trachyspermum ammi* (ajowan) was demonstrated against protoscoleces of *E. granulosus* (Albanese et al., 2009; Maggiore et al., 2012; Moazeni et al., 2012; Taran et al., 2009). Moreover, the in vitro effect of low concentrations of thymol (principal component of essential oils extracted from *Origanum vulgare* and *Thymus vulgare*) was shown against the causative agent of cystic echinococcosis. The viability reduced to 53.5% after 12 days of incubation (Elissondo et al., 2008).

The aim of the present work was to determine the in vitro effect of high concentrations of thymol against protoscoleces, microcyst and cyst of *E. granulosus*.

2. Materials and methods

2.1. In vitro culture of *E. granulosus* protoscoleces and drug treatment

Protoscoleces of *E. granulosus* were isolated under aseptic conditions from liver and lung hydatid cysts of infected cattle slaughtered in an abattoir located in the southeast of the Buenos Aires province, Argentina. Viability was assessed as previously described (Elissondo et al., 2006). Viable and free protoscoleces ($n = 1500$) were cultured under aseptic conditions in 3 ml/well of medium 199 (Gibco) supplemented with antibiotics (penicillin, streptomycin and gentamicin 100 μ g/ml) and glucose (4 mg/ml) in sterile tissue culture plates (Nunc, 12 well).

Thymol was dissolved in 0.5 ml of dimethyl sulphoxide (DMSO) at a drug concentration of 100 mg/ml. Then different volumes of this final solution (7.5, 6, 4.5 and 3 μ l) were added to the medium resulting in final concentrations of 250, 200, 150 and 100 μ g/ml, respectively. So the bigger volume was 7.5 μ l. For this reason protoscoleces incubated in culture medium containing 7.5 μ g/ml DMSO served as controls. Each experiment was repeated three times. During the experiments, culture plates were followed microscopically continuously to determine the appearance of morphological alterations. Samples of protoscoleces (approximately 100 protoscoleces in 150 μ l of incubation medium) from each of the dosing groups and the controls were taken for viability assessment using the methylene blue exclusion test. Additionally, ultrastructure studies with scanning and transmission electron microscope (SEM and TEM, respectively) were performed.

2.2. Microcysts obtainment and drug treatment

Protoscoleces of *E. granulosus* were collected aseptically as described above. The culture protocols were carried out as described previously (Elissondo et al., 2004) using medium 199 (Gibco) supplemented with antibiotics (penicillin, streptomycin and gentamicin 100 μ g/ml), glucose (4 mg/ml) and 20% (v/v) fetal calf serum. Cultures were maintained at 37 $^{\circ}$ C, and the medium was changed every 3–4 days. After 28–30 days, microcysts developed under in vitro conditions were recovered and transferred to tissue culture plate (Nunc, 12 well) for incubation with thymol in final concentrations of 250, 200, 150 and 100 μ g/ml. Microcysts incubated in culture medium containing 7 μ l DMSO served as controls. Culture plates were followed microscopically continuously to determine the appearance of morphological alterations. The criteria for microcysts vitality assessment included detachment and complete destruction of germinal layer.

2.3. Mouse infection and procedures for in vitro incubation of cysts

Animal procedures and management protocols were carried out in accordance with the 2011 revised form of The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. Female CF1 mice (body weight 25 ± 5 g) were infected by intraperitoneal infection of 1500 protoscoleces in 0.5 ml of medium 199 to produce experimental secondary hydatid disease. Animals were housed in a temperature-controlled (22 ± 1 $^{\circ}$ C),

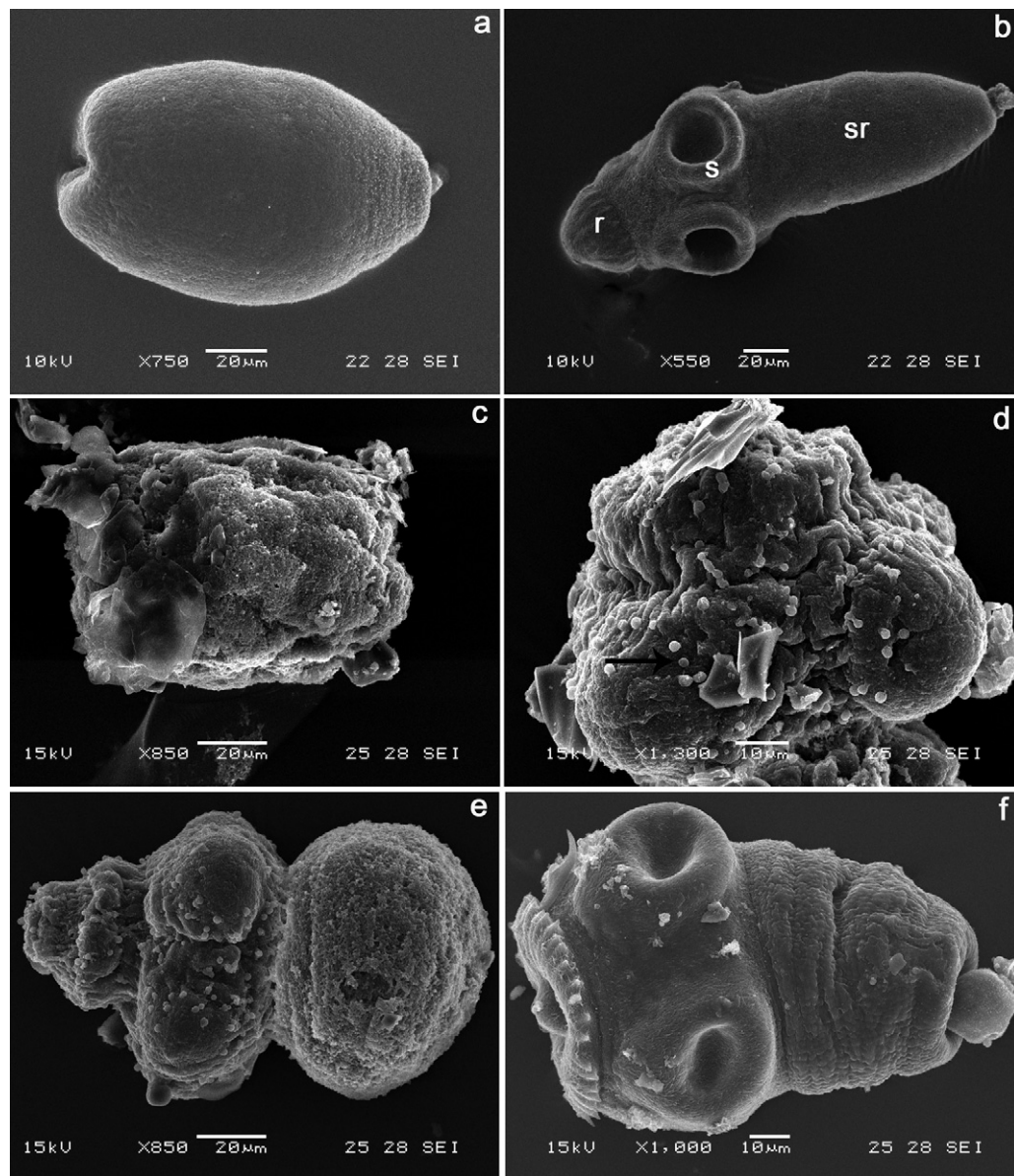


Fig. 2. Scanning electron microscopy of *E. granulosus* protoscoleces incubated in vitro with thymol. (a) Invaginated control protoscolex (750 \times); (b) evaginated control protoscolex (r, rostellar region; s, sucker; sr, soma region; 550 \times); (c) note the complete loss of morphology (10 min p.i., 250 μ g/ml, 850 \times); (d) scolex region of an evaginated protoscolex (10 min p.i., 200 μ g/ml, 1 \times , 300 \times). Note the loss of microtriches and the blebs (black arrow); (e) evaginated protoscolex (10 min p.i., 150 μ g/ml, 850 \times). The altered tegument of soma region and loss of microtrichias of scolex region can be observed. Note the loss of hooks; and (f) evaginated treated protoscolex (20 min, 100 μ g/ml, 1000 \times). The soma region is contracted and altered.

light-cycled (12-h light/dark cycle) room. Food and water were provided ad libitum.

After 6 months post-infection, mice were euthanized, and necropsy was carried out immediately thereafter. At necropsy, the peritoneal cavity was opened, and the hydatid cysts were carefully removed.

Groups of 10 peritoneal cysts were placed in Leighton tubes containing 10 ml of culture medium. Thymol was added to the medium resulting in final concentrations of the 250, 200, 150 and 100 μ g/ml. Cysts incubated with culture medium containing DMSO were used as controls. Each experiment was repeated three times.

Culture tubes with cysts were continuously followed macro and microscopically. Samples of cysts from each of the dosing groups and the control were taken and then fixed for electron microscopy. The criteria for cysts vitality assessment included the loss of turgidity, the collapse of cysts, and the ultrastructural observation of the germinal layer (Elissondo et al., 2007).

2.4. Electron microscopy

Samples of protoscoleces and cysts cultured in vitro were processed for SEM and TEM as described by Elissondo et al. (2004, 2006, 2007).

3. Results

3.1. In vitro protoscoleces incubation

Control protoscoleces remained viable and no changes in structure and ultrastructure were observed throughout the experimental period (Figs. 1a, 2a and b, and 3a). After 30 s post-incubation (p.i.) the first effects of the treatment with thymol were detected. Contraction of the soma region, formation of blebs on the tegument and rostellar disorganization became evident in some protoescoleces.

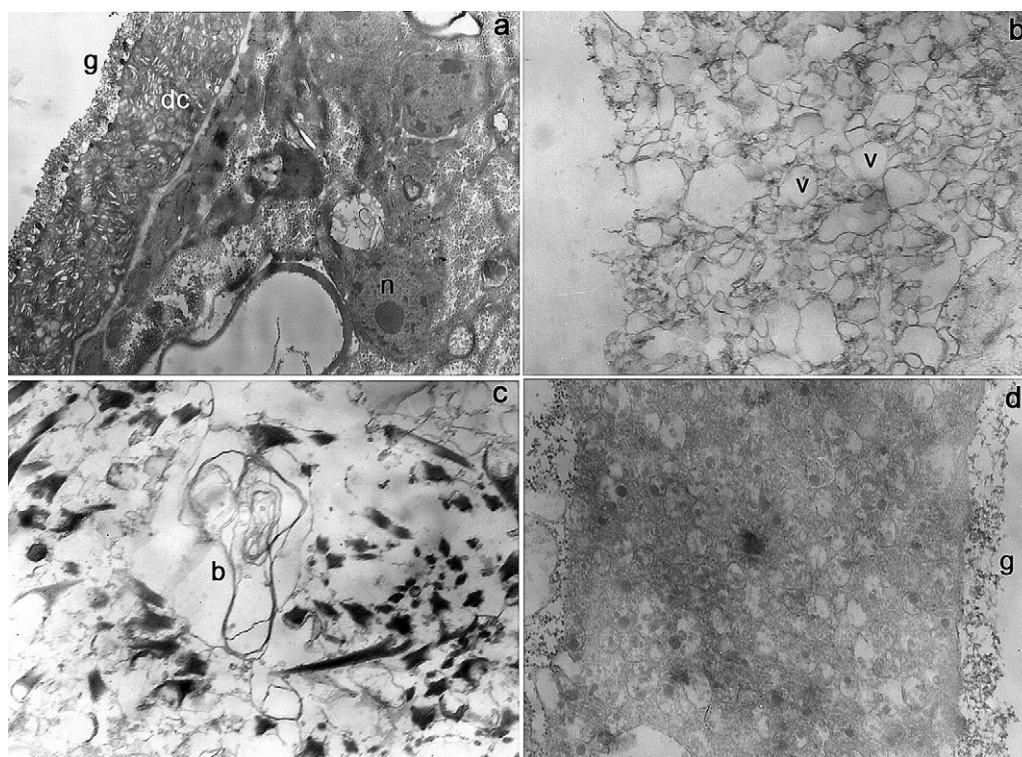


Fig. 3. Transmission electron microscopy of *E. granulosus* protoscoleces incubated in vitro with thymol. (a) Soma region of a control protoscolex (g, glycocalyx; dc, distal cytoplasm; n, nucleus; 6000 \times); (b) treated protoscolex (10 min p.i., 200 μ g/ml thymol, 12,000 \times). Note the presence of large vacuoles (v) in the distal cytoplasm; (c) note the presence of residual lamellar bodies (b) (10 min p.i., 200 μ g/ml, 12,000 \times); and (d) note the vacuolation of the distal cytoplasm (10 min p.i., 100 μ g/ml, 13,000 \times).

The survival of *E. granulosus* protoscoleces after exposure to different concentrations of thymol is shown in Table 1. Dose and time-dependent effects were observed. After 2 min p.i. with 250 and 200 μ g/ml, viability decreased rapidly reaching 1.3% and 14.6%, respectively. Thymol at 150 and 100 μ g/ml provoked a later effect.

The results of viability test coincide with the tegumental alterations observed by inverted microscope and with the tissue damage determined at the ultrastructural level (Figs. 1b, 2c and d, and 3b–d).

SEM demonstrated the drug-induced ultrastructural damage imposed upon thymol-treated protoscoleces (Fig. 2). Between 2 and 10 min, alterations in the tegument of the soma and scolex region were observed. The damage was most evident in protoscoleces treated with the higher concentrations (Fig. 2c–e) where the loss of hooks and microtriches of the scolex region and the presence of numerous blebs were detected after a short incubation time. At the lowest concentration tested, the damage was lower but equally remarkable in relation to the control group (Fig. 2f).

TEM revealed the presence of severe ultrastructural alterations on the internal tissue of the protoscoleces with the presence of numerous vacuoles and residual lamellar bodies (Fig. 3b–d).

Table 1

Survival of *E. granulosus* protoscoleces after exposure to thymol. Each value represents the mean percentage of vital protoscoleces from three different experiments.

Thymol concentrations (μ g/ml)	Percentage of viable protoscoleces (%)			
	Time (min)			
	2	5	10	20
0 (control)	100	100	100	100
250	1.3	0.1	0	0
200	14.6	0.3	0	0
150	32.4	15.9	5.8	2.2
100	41.5	23.9	13.6	12.8

3.2. In vitro microcysts incubation

Control microcysts remain unaltered during the entire experiment (Fig. 4a). On the other hand, thymol-treated microcysts were markedly affected by thymol after very short incubation times. Dose and time-dependent effects were observed (Table 2). Light microscopy observations demonstrated the drug-induced damage. Detachment of the germinal layer occurred as a first sign of alteration (Fig. 4b). This effect was more rapidly detected in microcysts than in cysts obtained from mice (see below). After a few seconds, the complete destruction of the germinal layer was observed in the microcysts incubated at the higher concentrations of thymol (Fig. 4c and d).

3.3. In vitro cysts incubation

All control cysts appeared turgid with no observable collapse of the germinal layer over the course of the in vitro experiment. Moreover, control cultures exhibit no ultrastructural alterations in parasite tissue during the whole incubation period (Figs. 5a and 6a).

At macroscopic observations, a loss of turgidity was detected in all thymol-treated cysts between 5 and 30 min p.i. (Table 3).

Table 2

Time of appearance (seconds post-incubation) of different indicators of tissue damage under light microscopy on *E. granulosus* microcysts, after its incubation with thymol, under in vitro conditions (gl, germinal layer).

Parameters of the study	Seconds post-incubation Thymol concentrations (μ g/ml)				
	0	250	200	150	100
Detachment of the gl	–	30	40	60	90
Complete destruction of gl	–	60	80	110	150

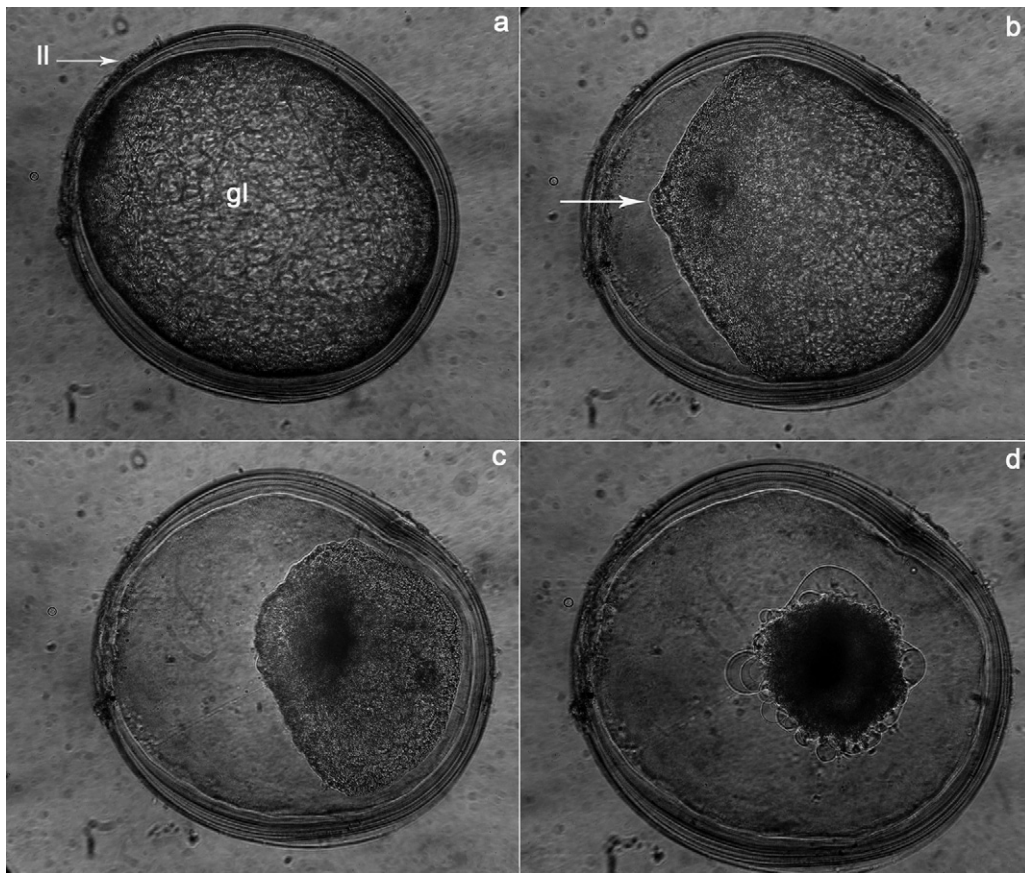


Fig. 4. Light microscopy of *E. granulosus* microcysts incubated in vitro with 250 $\mu\text{g/ml}$ of thymol. (a) Control microcyst (gl, germinal layer; ll, laminar layer; 20 \times); (b) treated microcyst. Note the detachment of the germinal layer (arrow; 30 s p.i., 20 \times); (c) microcyst incubated with thymol (40 s p.i., 20 \times); and (d) Complete destruction of the germinal layer (60 s p.i., 20 \times).

Table 3

Time of appearance (minutes post-incubation) of different macroscopic indicators of tissue damage on *E. granulosus* murine cysts, after its incubation with thymol, under in vitro conditions.

Parameters of the study	Minutes post-incubation Thymol concentrations ($\mu\text{g/ml}$)				
	0	250	200	150	100
Loss of cyst turgidity	–	5	5	10	30
Appearance of collapsed cysts	–	10	15	30	60

There was a correlation between the intensity of damage and the concentration assayed. As was mentioned for protoscoleces, the concentrations of 250 and 200 $\mu\text{g/ml}$ produced faster effects than the lower concentrations analyzed. Between 10 and 15 min p.i., the collapse of the germinal layer was observed. Nevertheless, the ultrastructural alterations observed by SEM and TEM were similar in all the evaluated concentrations. Studies by SEM revealed that the germinal layer of treated cysts lost the feature multicellular structure (Fig. 5b). TEM showed the presence of severe changes on

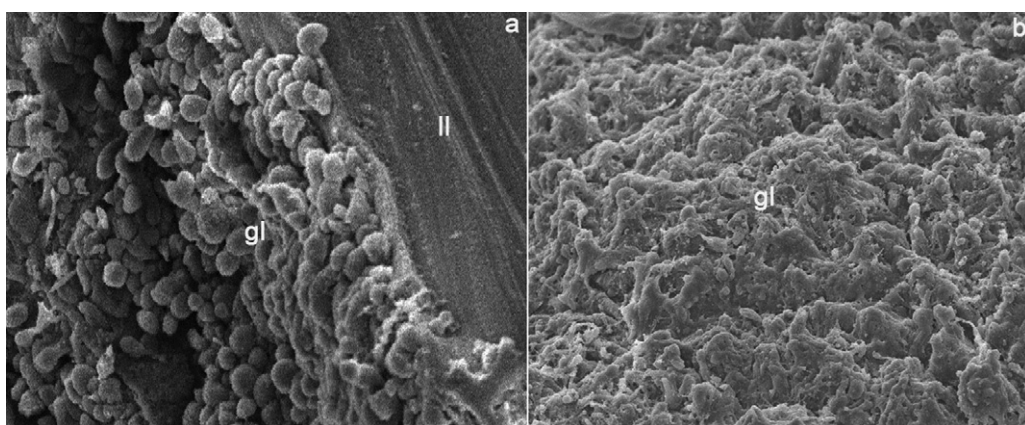


Fig. 5. Scanning electron microscopy of *E. granulosus* murine cysts incubated in vitro with thymol. (a) Control cyst with an intact germinal layer (gl, germinal layer; ll, laminar layer, 600 \times); and (b) murine cyst incubated with thymol (150 $\mu\text{g/ml}$, 10 min p.i., 700 \times). Note the extensive damage of the germinal layer. Only cellular debris could be observed.

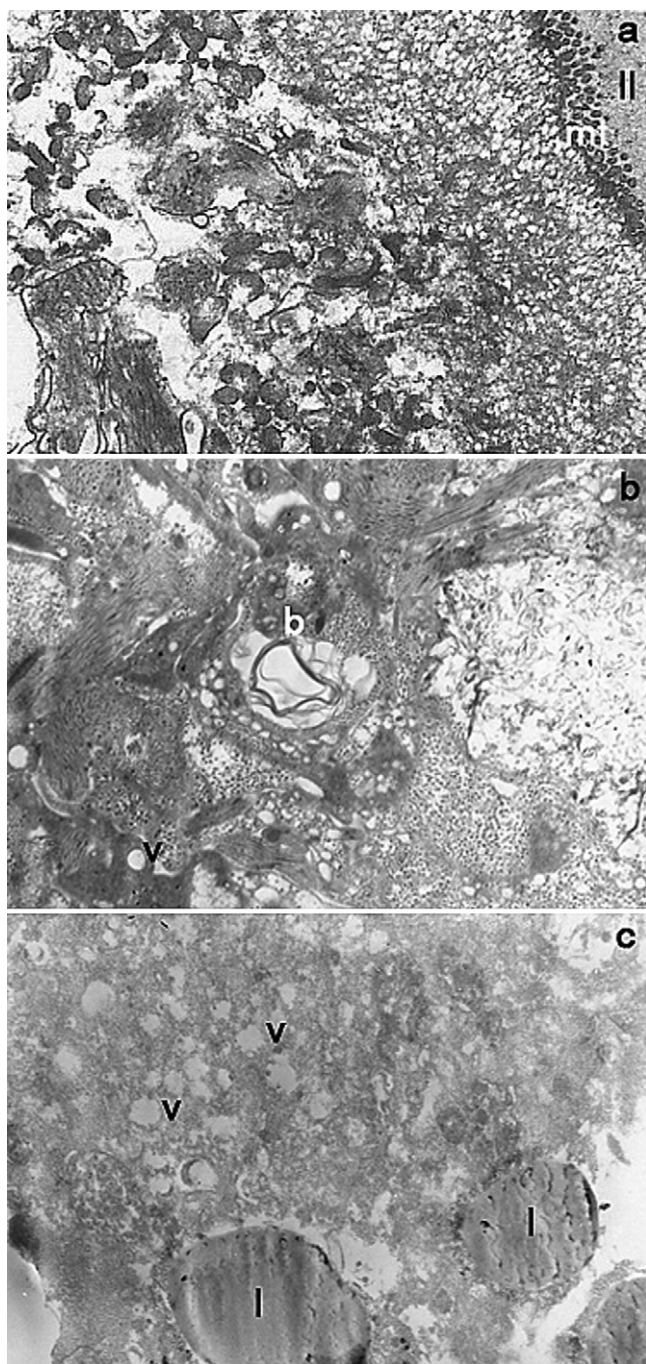


Fig. 6. Transmission electron microscopy of *E. granulosus* murine cysts incubated in vitro with thymol. (a) Control cyst (ll, laminar layer; mt, microtriches; 12,000×); (b) treated murine cyst (10 min p.i., 250 µg/ml thymol, 6000×). The internal tissue is altered with the presence of vacuoles (v) and residual lamellar bodies (b); and (c) murine cyst incubated during 15 min with 200 µg/ml of thymol (12,000×). Note the presence of lipid droplets (l) and vacuoles (v).

the internal tissue, including the presence of numerous vacuoles, lipid droplets and residual lamellar bodies (Fig. 6b and c).

4. Discussion

In a previous study (Elissondo et al., 2008), the in vitro protoscolicidal effect of low concentrations of thymol on cultured *E. granulosus* was established. Long incubation times were needed to completely kill the parasites. This work describes the effect of high concentrations of thymol on protoscoleces, microcysts and murine

Table 4

Comparison between the present work and previous researches on the evaluation of scolical agents against protoscoleces.

Author	Agents/time of exposure (min)	Mortality (%)
Own data	250 µg/ml thymol/5	99.9
	250 µg/ml thymol/2	98.7
Adas et al. (2009)	20% NaCl/5	98.2
	3% H ₂ O ₂ /5	90.3
	Cetrimide/5	86.9
	Albendazole sulfoxide/5	98.4
Puryan et al. (2005)	0.04% chlorhexidine gluconate/5	100
Altindis et al. (2004)	0.1% octenidine hydrochloride/5	90
Ciftci et al. (2007)	0.1% octenidine dihydrochloride/15	100

cysts. After short exposure times (minutes or even seconds), a rapid effect was observed depending on the parasitic material.

After 2 min of exposure to thymol, viability of protoscoleces was approximately 1.3% at a concentration of 250 µg/ml. The protoscolicidal effect is dose and time dependent. Table 4 shows a comparison between previous studies related to the evaluation of scolical agents and the results obtained in the present study working with thymol.

In conjunction with prevention of cystic fluid spillage, total evacuation and prevention of any contact of the germinal layer with the peritoneal surface are essential because the germinal layer can contain viable protoscoleces despite proper cyst fluid inactivation (Adas et al., 2009). Moreover, embryonic or stem cells present on this layer have the potentiality to develop new protoscoleces or brood capsules (Menezes da Silva, 2011). Regarding this, no previous publications were found about the effect of cysticidal agents that could kill germinal cells and could be applied during surgery or PAIR.

The results of the in vitro treatment with thymol were similar in both microcysts and secondary murine cysts. The higher concentrations affected more rapidly the cysts. However, the treatment with thymol produces the collapse or detachment of germinal layer in the cultured cysts in less time than in murine cysts. This could be explained by the fact that the cultured cysts are smaller and the laminated layer is thinner than in murine cysts (Casado et al., 1996). Moreover, murine cysts have a fibrous layer being another obstacle for drug diffusion.

The employment of SEM and TEM allowed us to examine, at an ultrastructural level, the effects induced by thymol on *E. granulosus* protoscoleces, microcysts and murine cysts. Germinal layer of cysts appeared markedly affected. We demonstrated that identical ultrastructural changes were induced in each of the incubation conditions. However, at the lower concentrations the alterations were detected later.

The in vitro and in vivo cytotoxicity activity of thymol was evaluated by Robledo et al. (2005). The cytotoxic activity (50% lethal concentration) in U-937 human promonocytic cells was 400 ± 0 µg/ml. By the other hand, at an orally dosage of 40 mg/kg of body weight/day, thymol was not toxic to Golden hamsters based on corporal weight, behavior and serum levels of bilirubin, uric acid and glucose. The concentrations assayed in the present study were considerably lower than the described LC50.

Moreover, the in vivo effect of thymol on the *E. granulosus* murine model was observed by us (manuscript on preparation). The chemoprophylactic activity and the effects on secondary hydatid disease of thymol were demonstrated working with a dose of 40 mg/kg of body weight/day. No toxic effects were detected on CF1 mice.

In conclusion, the data obtained clearly demonstrated that thymol caused severe damages to the parasite even after short incubation times. This fact and the lack of toxicity at the evaluated concentrations, allow us to propose it as a possible scolical agent

during hydatid cysts surgery and/or PAIR. However, during these medical interventions the potential communication between the hydatid cyst and the biliary tree substantially increases the safety requirements for using protoscolicides, which can cause chemical cholangitis leading to frequently fatal subsequent sclerosing cholangitis, as it occurs when formalin is used (Pawłowski et al., 2001). For this reason, more exhaustive evaluation of the toxicity on the liver and biliary tract should be carried out before testing it in humans.

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