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Efficacy of albendazole in combination with thymol against *Echinococcus multilocularis* protoscoleces and metacestodes

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ABSTRACT

The larval (metacestode) stage of the tapeworm *Echinococcus multilocularis* causes alveolar echinococcosis (AE), a mainly hepatic disease characterized by continuous asexual proliferation of metacestodes by exogenous budding, resulting in the tumor-like, infiltrative growth of the parasite lesion. Current chemotherapeutical treatment of AE relies on the use of benzimidazoles, albendazole (ABZ) and mebendazole, but these drugs act parasitostatic rather than parasitocidal, and due to their low success rate they imply a lifelong application causing severe side effects. Thymol is one of the major components of the essential oils of Thymus and is a widely known anti-microbial agent. The aim of the present work was to compare the efficacy of albendazole (ABZ) and thymol separately or combined on *E. multilocularis* protoscoleces and metacestodes. For this purpose, microscopical examinations at different time points were carried out. Moreover the tegumentary enzyme gamma glutamyl transferase (GGT) was measured to quantify the damage in metacestodes. Even though treatments of in vitro cultured *E. multilocularis* protoscoleces or metacestodes with ABZ or/and thymol showed that the drugs have an adverse effect on parasite viability, the combination of the two compounds at the concentration of 10 μg/ml showed the maximum anti-parasitic effect. Three days postincubation the first effects of the treatment were detected on protoscoleces and a marked reduction in viability (33%) was registered at day 18. Incubation of *E. multilocularis* metacestodes in the presence of ABZ 10 μg/ml + thymol 10 μg/ml during 10 days resulted in dramatic alterations such as strongly irregular and fissured surface and markedly disrupted vesicles. Scanning electron microscopy showed that protoscoleces as well as the germinal layer of *E. multilocularis* metacestodes were dramatically damaged following ABZ or/and thymol treatment. Also an important increase of tegumentary enzyme GGT was registered after 72 h postincubation with both drugs. The data reported in this article demonstrate a clear in vitro effect of ABZ + thymol against *E. multilocularis* protoscoleces and metacestodes.

1. Introduction

Alveolar echinococcosis (AE) is a parasitic disease caused by the larval stage of *Echinococcus multilocularis* with tumor-like growth primarily affecting the liver. Traditionally, treatment of echinococcosis relies on surgery and/or chemotherapy, depending on different factors such as metacestode size and location, viability status, the interaction between the expanding parasite and the adjacent host tissue, bacterial and fungal infection, and potential complications related to cyst rupture and spillage of protoscoleces (Kern, 2006).

The only drugs available for the treatment of human AE are benzimidazole carbamate derivatives, namely mebendazole (MBZ) and albendazole (ABZ). However, these drugs are parasitostatic rather than parasitocidal for *E. multilocularis* (Reuter et al., 2004) with an overall success-rate of between 55 and 97% (Reuter et al., 2000). This fact implies lifelong application of benzimidazoles causing severe side effects to the patients.

On the other hand, the in vitro parasitocidal effect of other drugs, such as nitazoxanide (a broad spectrum drug against a wide variety of intestinal parasites and enteric bacteria) (Stettler et al., 2003), 2-methoxyestradiol (documented to possess an anti-angiogenic and broad spectrum anti-tumor activity) (Spicher et al., 2008), genistein (an isoflavonoid that inhibits growth and metastasis of a number

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of cancer cell lines) (Naguleswaran et al., 2006), triclabendazole (a liposoluble benzimidazole derivative successfully applied against *Fasciola* spp.) (Richter et al., 2013), DB1127 (a diguanidino compound) (Stadelmann et al., 2011), mefloquine (a synthetic analogue of quinine commonly used in malaria prophylaxis) (Küster et al., 2011) and several anti-tumor drugs (Hübner et al., 2010; Hemer and Brehm, 2012) have been reported against *E. multilocularis*.

However, no reliable chemotherapeutic alternative has yet been developed, stimulating the research of new treatment strategies such as the use of medicinal plants. 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). In previous works, several encouraging findings have been reported using thymol on *E. granulosus* protoscoleces, microcyst and metacestodes (Elissondo et al., 2008, 2013). Also the in vitro and in vivo activity of thymol against leishmania (Robledo et al., 2005) and anisakis larvae (Hierro et al., 2004) was proven.

The development of new molecules for parasite targets is a process that requires a high investment in time and money (Hennessy, 1997). However it is extremely necessary to optimize the use of existing drugs and also search for tools to improve the behaviour of these drugs and their pharmacologic effect. Moreover, the simultaneous or sequential application of different drugs is an appealing approach for potentially enhancing effectiveness, shortening long-term use of these substances and therefore decreasing the toxicity.

The work reported here aimed to compare the efficacy of albendazole (ABZ) and thymol separately or combined on *E. multilocularis* protoscoleces and metacestodes.

2. Materials and methods

2.1. Parasite material

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 U.S. National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. All experiments were carried out using parasite isolate 8065 (kindly provided by Klaus Brehm, Institute for Hygiene and Microbiology, University of Würzburg) and that was propagated in the peritoneum of CF1 mice. For metacestodes retrieval isolated cyst with a diameter of 4 to 8 mm were recovered from the cystic mass dissected from experimentally infected female CF1 mice after 3 month post-infection. They were washed several times with an antibiotic solution (60 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin in phosphate-buffered saline [PBS]) and maintained in the same solution overnight before the experiments start. For protoscoleces isolation, metacestodes were pressed through a metal tea strainer and the suspension obtained was washed first with the antibiotic solution and then several times with PBS. The freshly isolated parasitic material was diluted with PBS to obtain a 1:4 suspension und vigorously shaken for approximately 10 min to release the protoscoleces from the metacestode material. Afterward, the suspension was firstly sieved through a polyester gauze (pore size 150 μm). Then, the flow through was decanted onto a second polyester gauze (pore size 30 μm). On the top of the gauze, the retained material contained the protoscoleces, which were collected by resuspension in PBS. Viable and free protoscoleces (1500 per Leighton tube) or metacestodes (10 per tube) were cultured in medium 199 (Gibco), supplemented with 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).

2.2. Drug treatment

Thymol (Sigma) was dissolved in dimethyl sulphoxide (DMSO) at a drug concentration of 10 mg/ml. Albendazole (Sigma-Aldrich) solution was prepared by dissolution of 10 mg of pure standard drug in 1 ml of DMSO. The two drugs were added to the protoscoleces medium either separately or in combination at the following final concentrations: 10, 5, 1 μg/ml ABZ; 10, 5, 1 μg/ml thymol; 10 μg/ml ABZ + 10 μg/ml thymol; 5 μg/ml ABZ + 5 μg/ml thymol and 1 μg/ml ABZ + 1 μg/ml thymol.

Protoscoleces incubated in culture medium containing 10 μl DMSO served as controls. Each experiment was perform in triplicates and repeated three times. During the experiments, culture tubes were followed microscopically everyday to determine the appearance of morphological alterations. Samples of protoscoleces (approximately 90 to 100 PCS 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. Percentages were plotted. Each point represents the mean percentage of vital protoscoleces from three different experiments.

Metacestodes were treated with 10 μg/ml ABZ, 10 μg/ml thymol or 10 μg/ml ABZ + 10 μg/ml thymol. After 1, 3 and 6 days postincubation 300 μl of the culture supernatant was collected, centrifuged at 10,000 × *g* for 30 min at 4 °C, and the supernatant was recovered for subsequent measurement of enzymatic activity. Metacestodes incubated in culture medium containing 10 μl DMSO served as controls.

2.3. Enzymatic assay

Enzyme activities were determined from culture metacestodes supernatants following the procedure described below. In all cases, the activities were measured at 37 °C in a recording Shimadzu model UV–vis spectrophotometer, the volume of the reaction mixture was 1 ml with 100 μl of enzyme samples and measurements made after 60 min of incubation. Gamma-glutamyl-transferase (GGT) activity was determined as Cumino et al. (2012). The rate of increase in absorbance is due to release of *p*-nitroanaline with a molar extinction coefficient of 9.9. The absorbance was read at 405 nm against appropriate blanks. The substrate solution is an aqueous buffered solution containing 2.9 mM L-gamma-glutamylp-nitroanilide, 100 mM glycylglycine and 5 mM MgCl2. All data were shown as arithmetic means ± SD.

2.4. Morphological and ultrastructural studies

To visualize the ultrastructural alterations caused by albendazole and thymol treatment separately or combined, protoscoleces and metacestodes were processed for scanning electron microscopy (SEM) at different time points as described by Elissondo et al. (2006, 2007).

3. Results

The survival of *E. multilocularis* protoscoleces after exposure to different concentrations of ABZ + thymol combination as compared with the same concentrations of ABZ and thymol alone is shown in Fig. 1. Control protoscoleces incubated in medium 199 remained 97% viable after 18 days of incubation, and no changes in structure and ultrastructure were observed throughout the experimental period (Figs. 2A and 3A).

Although all treatments had a protoscolicidal effect, the combination of albendazole 10 μg/ml with thymol 10 μg/ml showed

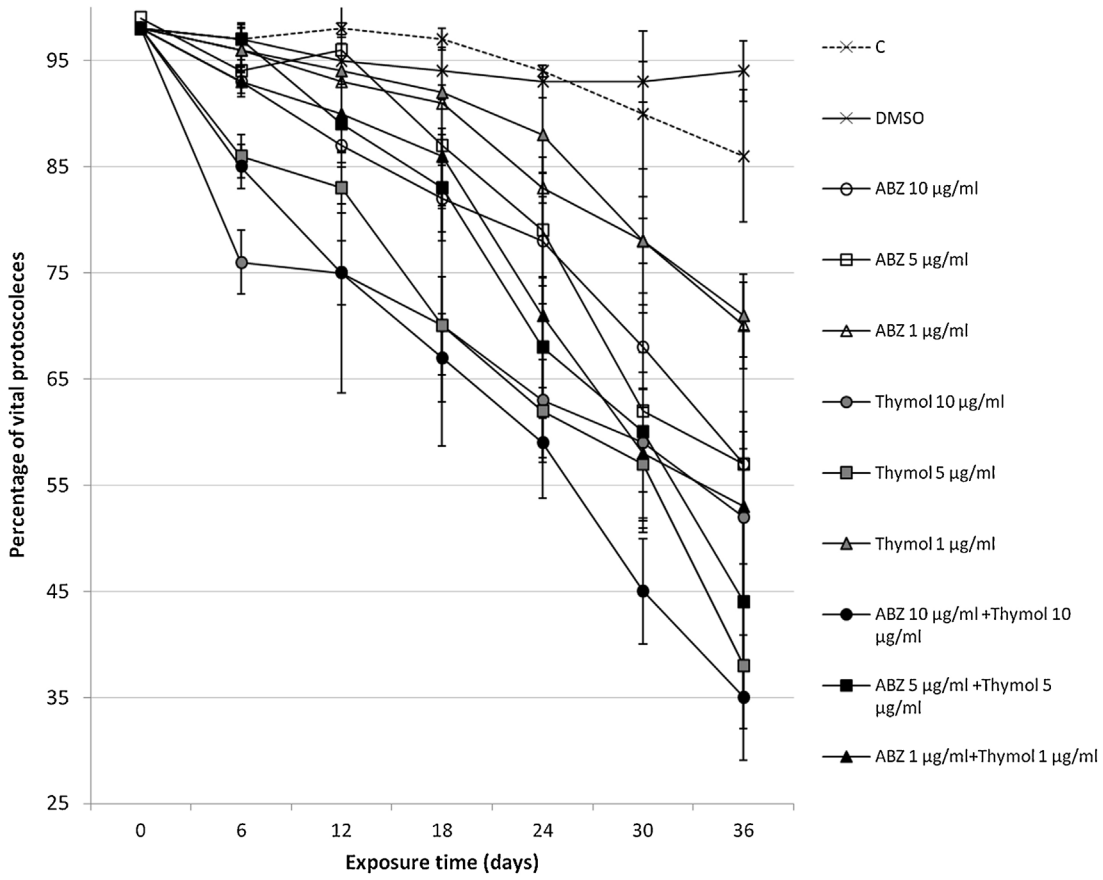

Fig. 1. Survival of *Echinococcus multilocularis* protoscoleces after exposure to albendazole (ABZ), thymol and the combination of both drugs. Each point represents the mean percentage of vital protoscoleces from three different experiments ± SD.

the maximum scolicidal effect. After 3 days postincubation (p.i.) the first effects of the treatment were detected. They consisted in contraction of the soma region, formation of blebs on the tegument and rostellar disorganization (Figs. 2D and 3D). Moreover, a marked reduction in protoscoleces viability (33%) was detected at day 18, accompanied by an increase in structural and ultrastructural damage (Figs. 2F and 3F).

On the other hand, both drugs separately or combined at 1 μg/ml provoked a later effect. At day 6 p.i. the first signs of damage appeared (Figs. 2C and 3B and C), which became more severe at day 18 (Figs. 2B and E and 3E).

Incubation of *E. multilocularis* metacestodes at 37 °C in the presence of ABZ 10 μg/ml, thymol 10 μg/ml or ABZ 10 μg/ml + thymol 10 μg/ml during 10 days resulted in dramatic alterations. Fig. 4A

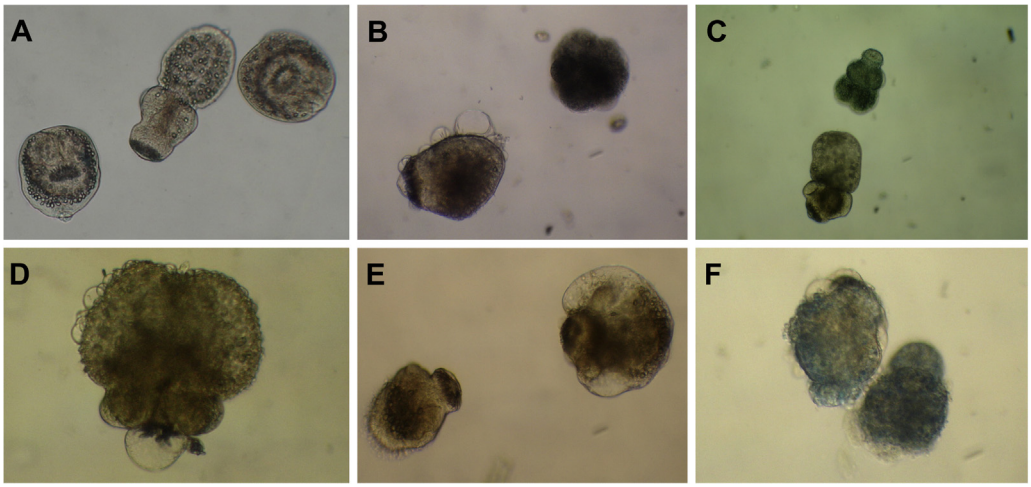

Fig. 2. Light microscopy of *Echinococcus multilocularis* protoscoleces incubated in vitro with albendazole (ABZ), thymol, and the combination of both drugs. (A) Control protoscoleces after 18 days post-incubation (p.i.). (B) Protoscoleces incubated with ABZ (18 days p.i., 1 μg/ml). (C) Protoscoleces incubated with thymol (6 days p.i., 1 μg/ml). (D) Altered protoscolex after 3 days p.i. with ABZ 10 μg/ml + thymol 10 μg/ml. Note the presence of numerous blebs in the tegument and on the scolex region. (E) Protoscoleces incubated with ABZ + thymol (18 days p.i., 1 μg/ml). (F) Protoscoleces incubated with ABZ + thymol (18 days p.i., 10 μg/ml). (600× magnification).

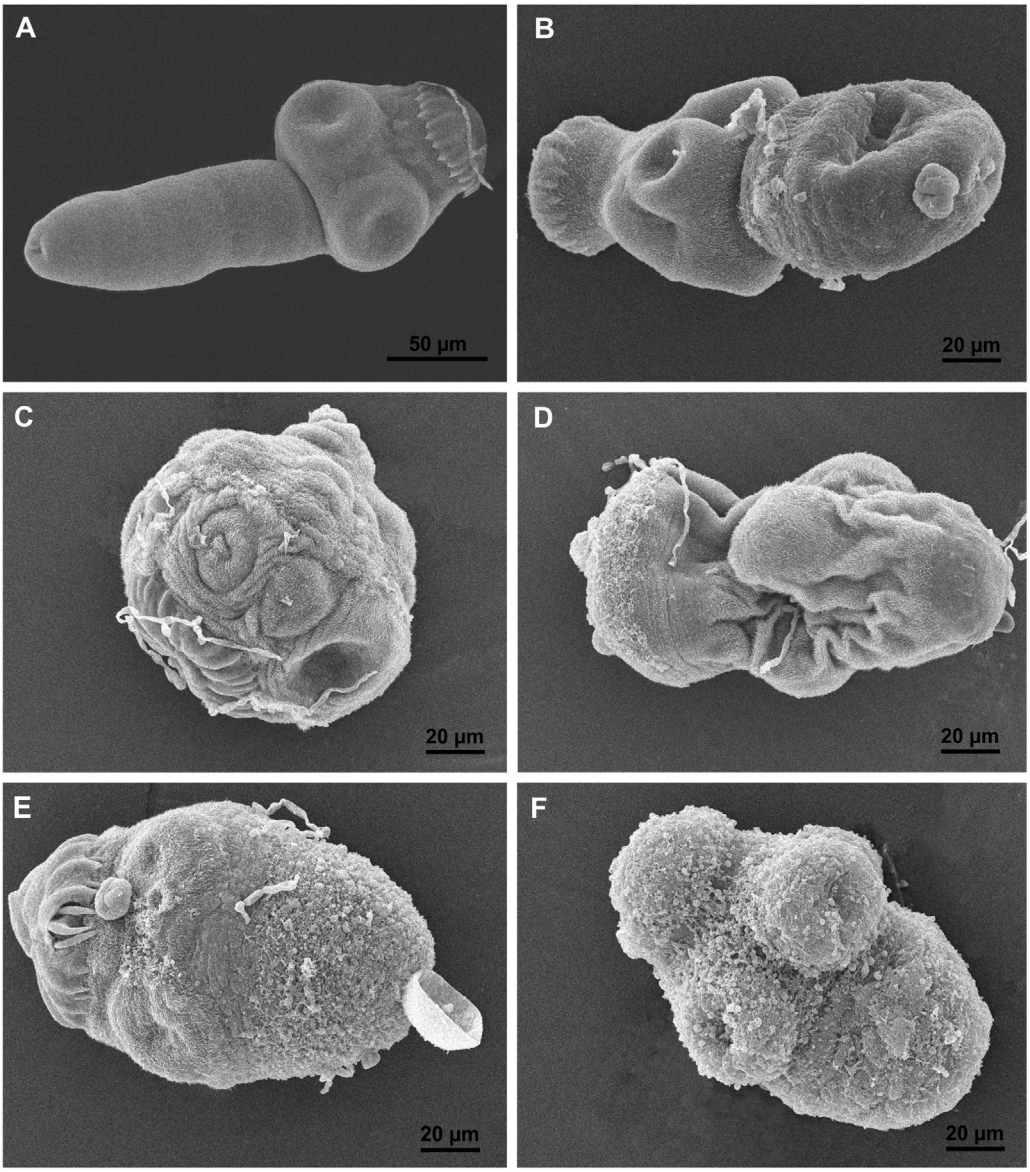

Fig. 3. Scanning electron microscopy of *Echinococcus multilocularis* protoscoleces incubated in vitro with albendazole (ABZ), thymol, and the combination of both drugs. (A) Evaginated control protoscolex at day 18 post-incubation (p.i.) (600× magnification) (B) Protoscolex incubated with ABZ 1 μg/ml (6 days p.i.). (600× magnification) (C) Altered protoscolex after 6 days p.i. with thymol 1 μg/ml. (450× magnification) (D) Protoscolex incubated with ABZ 10 μg/ml + thymol 10 μg/ml during 3 days. Note the tegumental alterations of the soma region. (750× magnification) (E) Protoscolex incubated with ABZ 1 μg/ml + thymol 1 μg/ml (18 days p.i.). (600× magnification) (F) Loss of morphology, rostellar disorganization, loss of hooks, shedding of microtriches, and formation of tegumental vesicles 18 days p.i. (ABZ 10 μg/ml + thymol 10 μg/ml). (600× magnification).

shows an untreated vesicle, turgid, with a smooth surface and intact germinal and laminated layers. Treatment with ABZ 10 μg/ml or thymol 10 μg/ml showed vesicles with reduced turgidity, thin membranes and strongly irregular and fissured surface as well as completely disrupted vesicles (Fig. 4B). The best results were obtained with the combination ABZ 10 μg/ml + thymol 10 μg/ml where a complete disruption of the vesicles occurred. In all the vesicles, the parasite tissue detached from the interior lining of the laminated layer and formed a densely packed aggregate inside them (Fig. 4C).

These results were confirmed on the ultrastructural level by SEM (Fig. 5). After 10 days post-incubation, control cultures exhibited no ultrastructural alterations in parasite tissue during the whole incubation period (Fig. 5A). SEM revealed that treated metacestodes suffered a devastating impact, with a major portion of the germinal layer being largely distorted by the drugs, and only tissue residues were present (Fig. 5B–D). Only a few cells could be observed, and these were collapsed, deformed and surrounded by blebs (Fig. 5E).

In order to quantify the damage caused by the treatment of metacestodes with ABZ and thymol alone or combined, the releasing of membrane enzyme GGT to the culture media was measured (Fig. 6). Control treatment did not lead to increase of total GGT activity. However, on culture supernatants of treated metacestodes GGT was detectable at day 3 and continued increasing after 6 days of incubation. Interestingly, we found that the activity of GGT was always significantly higher in the supernatants of metacestodes treated with ABZ and thymol combined.

4. Discussion

AE is one of the last non-curable helminthic infections of humans. The urgency of developing new therapy options is much

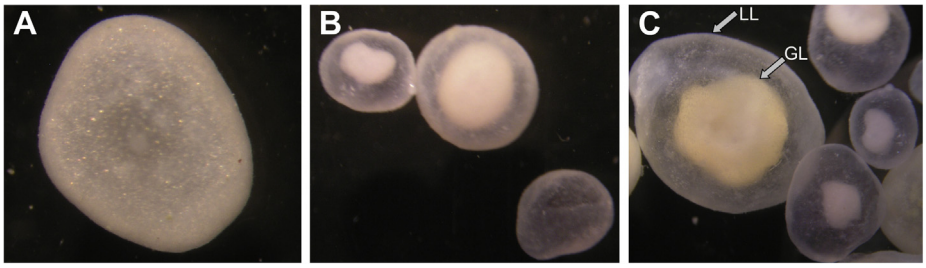

Fig. 4. Morphological effects of albendazole (ABZ) and thymol treatment on *Echinococcus multilocularis* metacestodes. (A) Metacestodes kept in normal cultivation medium during 10 days. (B) Metacestodes incubated with thymol 10 μg/ml. (C). Dramatic morphological changes after 10 days of incubation with ABZ 10 μg/ml + thymol 10 μg/ml. The germinal layer (GL) detaches rapidly from the laminated layer (LL). (40× magnification).

needed considering that this widespread disease usually leads to death if left untreated (Brunetti et al., 2010). The current strategies for treating human AE are surgical resection of the parasite mass complemented by chemotherapy with benzimidazoles such as mebendazole or albendazole, and for inoperable cases chemotherapy alone is applied. Albendazole treatment has been proven to inhibit parasite proliferation but is rarely curative, resulting in a long duration of treatment, high costs and an elevated risk of adverse effects (Torgerson et al., 2008; Hemphill et al., 2010).

Looking for new chemotherapeutic tools, phytomedicine offers a valuable option against a number of diseases (Edris, 2006). Innovatively, promising results on *E. granulosus* protoscoleces and metacestodes have been obtained with a wide variety of essential oils (Albanese et al., 2009; Maggiore et al., 2012; Moazeni et al., 2012). It has been reported that thymol affects the surface electrostatics of the cell membrane and membrane integrity (Lambert et al., 2001; Sánchez et al., 2004). This mechanism was proposed to explain the severe damages caused by thymol on *E. granulosus* protoscoleces and metacestodes (Elissondo et al., 2008, 2013) and on *Haemonchus contortus* eggs and adult worms (Boubaker Elandalousi et al., 2013). On the other hand, Lei et al. (2010) demonstrated the nematicidal activity of thymol suggesting the interaction of this compound with the nematode tyramine receptor (TyrR) as possible mode action. However, no previous publications were found about the antiparasitic effect of thymol on *E. multilocularis* protoscoleces or metacestodes.

Essential oil compounds such as found in thyme extract are frequently used for the therapy of chronic and acute bronchitis.

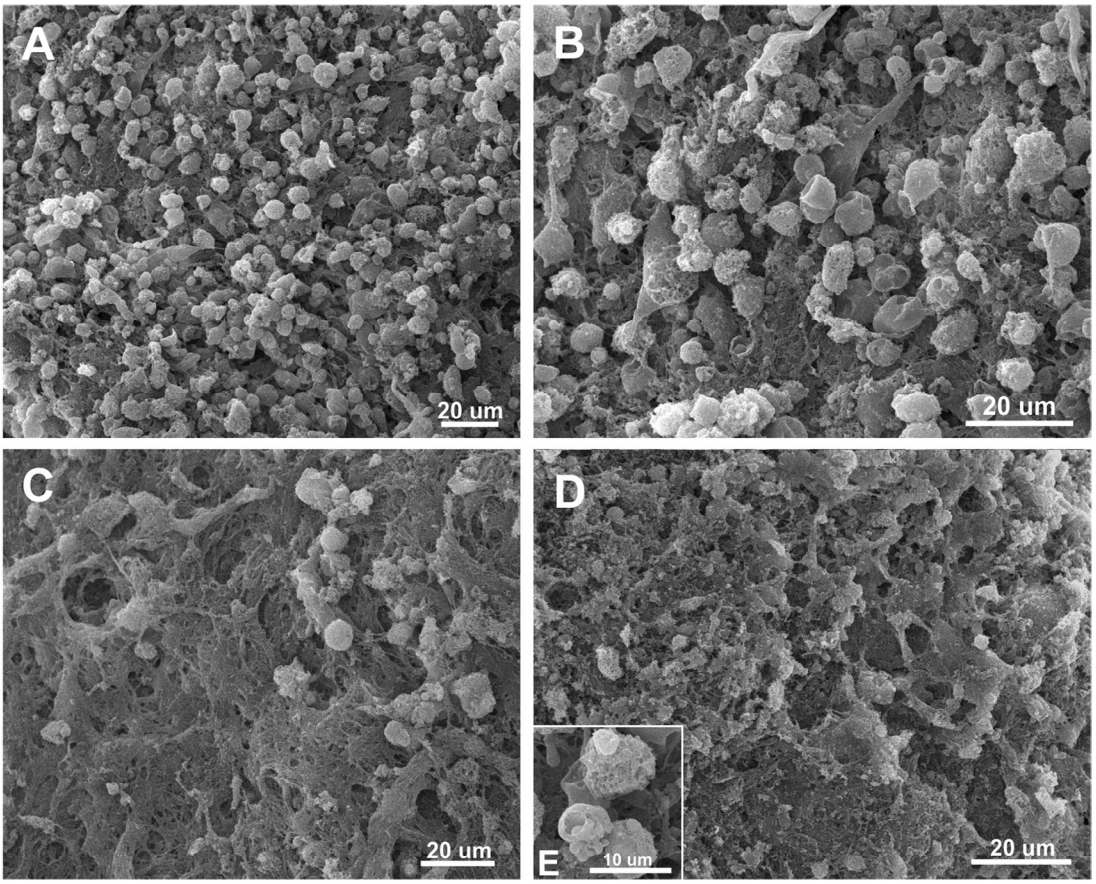

Fig. 5. Ultrastructural effects of albendazole (ABZ) and thymol on *Echinococcus multilocularis* metacestodes visualized by scanning electron microscopy after 10 days of incubation. (A) Untreated metacestode with an intact germinal layer. (B) Metacestode incubated with thymol 10 μg/ml. Note presence of damaged and collapsed cells (C). Metacestode incubated with ABZ 10 μg/ml. (D) Metacestode incubated with ABZ 10 μg/ml + thymol 10 μg/ml. Note the extensive damage of the germinal layer. Only cellular debris could be observed. (E) High magnification image showing the cellular morphology after treatment with ABZ 10 μg/ml + thymol 10 μg/ml.

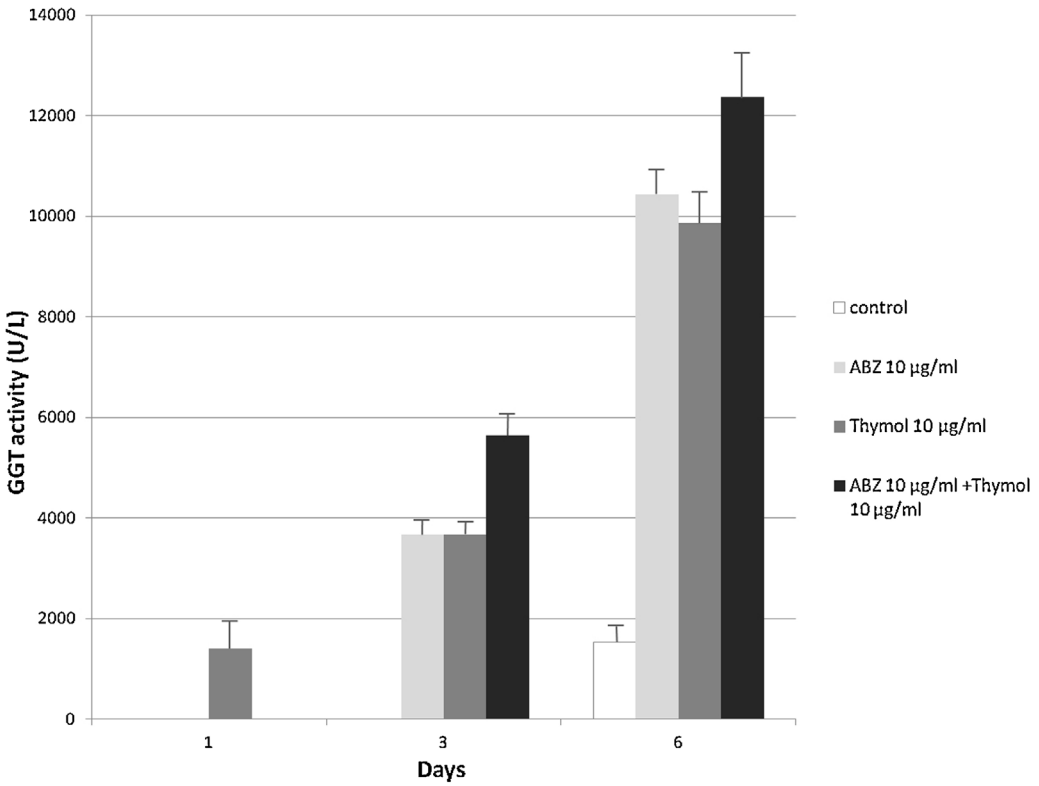

Fig. 6. Determination of the tegumental enzyme gamma glutamyl tranferase (GGT) activity. *E. multilocularis* metacestodes were treated with 10 μg/ml ABZ, 10 μg/ml thymol or 10 μg/ml ABZ + 10 μg/ml thymol and GGT activity was measured in culture supernatants each 3 days.

For these indications, several clinical trials have been carried out (Ernst et al., 1997; Matthys et al., 2000). Systemic availability and the pharmacokinetics after oral administration of 1.08 mg thymol in humans was determined (Kohlert et al., 2002). The metabolites thymol sulfate and thymol glucuronide were found in urine, however only thymol sulfate was detectable in plasma with a maximum plasma concentration of 93.1 ng/ml reached after 1.97 h. It should be stated that the single dose in that study was very low: 1.08 mg per human volunteer. Furthermore, a maximum plasma concentration of approximately 3 μg/ml was detected in piglets after an oral administration of 13.2 mg/kg of thymol (Michiels et al., 2008).

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, behaviour 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.

Combination treatment is an interesting approach for the utilization of synergistic effects of two drugs and for the reduction of toxicity (Reuter et al., 2010). Here, we described the combined effect of ABZ and thymol on *E. multilocularis*, with the aim of a more potent antiparasitic activity and a reduction of toxicity.

Protoscoleces cultured with ABZ + thymol were killed considerably faster than protoscoleces cultured with ABZ or thymol alone. After 25 days of exposure to ABZ 10 μg/ml + thymol 10 μg/ml, viability was approximately 50%. However, 50% of viability with thymol 10 μg/ml or ABZ 10 μg/ml was reached at day 36 and 45, respectively (data not shown).

Even though both drugs produced morphological and ultrastructural changes, alterations appear earlier with the ABZ 10 μg/ml + thymol 10 μg/ml combination. The alterations included contraction of the soma region, formation of blebs on the tegument, rostellar disorganization, loss of hooks and complete shedding of microtriches.

Metacestodes incubated with ABZ + thymol underwent more dramatic changes than when the drugs were used separately. As was mentioned for protoscoleces, the higher concentration (10 μg/ml) was more effective causing total collapse of germinal layer after 10 days post-incubation and loss of normal ultrastructure. The observed changes were consistent with those reported by other authors employing triclabendazole sulfoxide (Richter et al., 2013), mefloquine (Küster et al., 2011) or 2-methoxyestradiol (Spicher et al., 2008).

Tegumentary enzyme GGT was used to quantify the damage induced by the drugs ABZ and thymol on metacestodes. Interestingly we observed the release of enzyme occurred before we detect structural damages, suggesting it would be a sensitive viability marker against *E. multilocularis* metacestodes. In accordance with reports by Cumino et al. (2012) where they treated *E. granulosus* metacestodes with flubendazole.

The availability of new drugs with activity against *E. multilocularis* offers hope against this deadly disease. In vitro testing of drug combinations is mandatory in order to exclude inhibitory interactions, as shown in the present experiments.

The results obtained demonstrated the favorable effect of the combination of ABZ and thymol against *E. multilocularis* protoscoleces and metacestodes. Moreover, in vivo studies to evaluate the potential of the combination as treatment option against human AE are currently in progress.

Acknowledgments

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