Parasitology

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Anthelmintic activity of *Stevia multiaristata* extract against *Echinococcus granulosus sensu stricto*

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Research Article

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Abstract

Cystic echinococcosis is a zoonotic disease caused by the larval stage of the parasite *Echinococcus granulosus sensu lato*. The available anti-parasitic treatment is mostly limited to a continuous administration of albendazole. However, due to its numerous side-effects and efficacy of around 50%, there is a need to find new drugs to improve the treatment for this disease. In the current study, the *in vitro* and *in vivo* efficacy of a *Stevia multiaristata* extract against *E. granulosus sensu stricto* (*s.s.*) was demonstrated. *Stevia multiaristata* extract (100 and 50 $\mu g\,mL^{-1}$) caused a quick viability decrease on protoscoleces which was consistent with the observed tegumental alterations. Loss of turgidity was detected in 95 ± 3.4% of cysts incubated with *S. multiaristata* extract during 2 days (100 $\mu g\,mL^{-1}$) and the collapse of the germinal layer was observed in 60 ± 9.3% of cysts treated with 100 $\mu g\,mL^{-1}$ of the *S. multiaristata* extract during 4 days. The half maximal effective concentration value was 69.6 $\mu g\,mL^{-1}$ and the selectivity index for *E. granulosus s.s.* cysts was 1.9. In this clinical efficacy study, the treatment of infected mice with the *S. multiaristata* extract (50 $mg\,kg^{-1}$) caused a significant decrease in the weight of the cysts compared with the control group. These results coincided with the tissue damage observed in the cysts at the ultrastructural level. In conclusion, we observed high protoscolicidal and cysticidal effects, and significant reduction in the weight of the cysts in experimentally infected mice following treatment with the *S. multiaristata* extract.

Introduction

Cystic echinococcosis (CE) is a worldwide zoonotic disease caused by the larval stage of the parasite *Echinococcus granulosus sensu lato* (*s.l.*) which provokes long-term infections in humans and animals, being a serious public health problem (Pavletic *et al.*, 2017). The life cycle of this parasite includes an intermediate host (domestic and wild ungulates and occasionally humans) and a definitive host (dogs and other canids) which have the adult worm in the intestine and release eggs with their feces. The eggs can be ingested accidentally by an intermediate host that can develop a hydatid cyst (Moro and Schantz, 2009).

Based on the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) classification of cyst stages by imaging-based techniques, four treatment options have been suggested: percutaneous–aspiration–injection and re-aspiration (PAIR), surgery, anti-parasitic therapy with benzimidazoles (BZM) and a watch-and-wait approach for inactive cysts (Siles-Lucas *et al.*, 2018). For PAIR and surgery options, pre- and post-treatment and peri-interventional treatment with anti-parasitic drugs are also used (Brunetti *et al.*, 2010).

Available anti-parasitic treatment against CE is mostly limited to the administration of BZM, mainly albendazole (ABZ) and mebendazole, which are the only anti-infective clinically efficient drugs to interrupt the larval growth of *Echinococcus* spp. More specifically, ABZ is the preferred option due to its high bioavailability and easy administration to patients (Wen *et al.*, 2019). ABZ oral administration is recommended at a dosage of 10–15 $mg\,kg^{-1}\,day^{-1}$ divided

in two doses and a continuous therapy is suggested. However, ABZ has undesirable side-effects such as abnormal liver function, leucopoenia, thrombocytopoenia and alopecia in 3–5% of patients and their effectiveness is about 50% (Pawlowski *et al.*, 2001). A third of patients generally have been cured, 30–50% have developed a partial response while 20–40% of cases do not respond to treatment (Moro and Schantz, 2009). Based on the problems described, new treatment alternatives are urgently needed, including the identification, development and assessment of novel compound classes and drug targets (Siles-Lucas *et al.*, 2018).

In the last few decades, there has been an increased interest in studying the anthelmintic activity of medicinal plants focusing on the research of clinically important parasites, including several species of trematodes, nematodes and cestodes (Tagboto and Townson, 2001; Tandon *et al.*, 2011). Plant extracts or their components can be used as new options or in combination with actual anti-parasitic treatments.

In this context, many plant extracts have shown promising *in vitro* results on the viability of *E. granulosus s.l.* protoscoleces (Al-Abodi *et al.*, 2019; Tabatabaei *et al.*, 2019; Ali *et al.*, 2020; Cheraghipour *et al.*, 2021). However, only a few plant extracts have been tested on the *E. granulosus s.l.* murine model, showing a reduction in cyst development and, in some cases, a marked damage in the germinal layer of the hydatid cysts (Lv *et al.*, 2013; Moazeni *et al.*, 2014; Haji Mohammadi *et al.*, 2018, 2019; Luo *et al.*, 2018; Al-Abodi *et al.*, 2019; Labsi *et al.*, 2019).

Plants from the *Stevia* genus (Asteraceae) are a potential source of anti-protozoal and anti-microbial compounds (Ruiz-Ruiz *et al.*, 2017; Borgo *et al.*, 2021). Recently, it has been shown that dichloromethane extracts of *Stevia* species presented a significant activity on *Trypanosoma cruzi* epimastigotes (Beer *et al.*, 2016). On the other hand, several derivatives isolated from *S. alpina* showed antiparasitic activity against *T. cruzi* and *Leishmania braziliensis* making these promising candidates for the development of effective compounds for the treatment of Chagas disease and leishmaniasis (Sülsen *et al.*, 2019; Elso *et al.*, 2020).

In the current study, we demonstrated the *in vitro* efficacy of a *Stevia multiaristata* extract against protoscoleces and murine cyst of *E. granulosus sensu stricto* (*s.s.*). Moreover, we investigated the clinical efficacy of this extract in a murine model of CE.

Materials and methods

Plant material

The aerial parts of *S. multiaristata* Spreng. (Asteraceae) were collected in the province of Buenos Aires, Argentina, in February 2012. A voucher specimen was deposited at the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires under the number BAF 742.

Preparation of S. multiaristata crude extract

For the preparation of the crude extract of *S. multiaristata*, the dried aerial parts were extracted by maceration twice with dichloromethane (10% w/v). The extract was filtered through filter paper (Schleicher & Schuell – Whatman, grade 0859, medium, smooth, 90 mm) and taken to dryness in a rotary evaporator.

Cell culture

The human hepatic cell line Huh7 was cultured in DMEM culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin (DMEM-C) at 37°C under a 5% CO_2 atmosphere. Cell viability at the beginning of each experiment was assessed by the trypan blue exclusion test.

Chemicals

For *in vitro* studies involving *E. granulosus*, the *S. multiaristata* extract was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 35 mg mL^{-1} and added to the culture medium resulting in final concentrations of 100, 50, 10 and 5 μg mL^{-1}. The maximum amount of DMSO added in the culture medium never exceeded 3 μL mL^{-1}. For *in vitro* studies involving Huh7 cells, the *S. multiaristata* extract (10 mg) was sequentially resuspended in 20 μL of DMSO, 40 μL of ethanol 96% and finally, 40 μL of deionized water, giving a solution of 100 mg mL^{-1}. Dilutions were carried out in complete DMEM-10% FBS. For *in vivo* treatments, ABZ (Pharmaceutical grade, Parafarm, Argentina) suspension (5.25 mg mL^{-1}) was prepared by dispersion of pure ABZ in distilled and deionized water (pH = 7.0) with carboxymethylcellulose (CMC, Todo Droga, Córdoba, Argentina) (0.1% w/v, pH = 6.0). *Stevia multiaristata* extract was dissolved in olive oil with DMSO (0.5% v/v) at a drug concentration of 6.67 mg mL^{-1}. Both suspensions were shaken for 5 h and sonicated for 1 h.

Parasite material, protoscoleces collection and cyst obtention

Liver and lung hydatid cysts were obtained from cattle slaughtered in an abattoir located in the Buenos Aires province, Argentina. Protoscoleces were removed aseptically from cysts as previously described (Elissondo *et al.*, 2006). Parasitic material was genotyped by sequencing a fragment of the gene coding for mitochondrial cytochrome *c* oxidase subunit 1 (CO1), as previously described (Cucher *et al.*, 2011). Based on sequencing analysis, the G1 genotype was identified. To obtain murine cysts female CF-1 mice (body weight 25 ± 5 g) were infected by intraperitoneal inoculation with 1500 *E. granulosus s.s.* (G1 genotype) protoscoleces/animal, suspended in 0.5 mL of medium 199 (Mediatech, USA). At 6 months post inoculation, 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).

In vitro assays

Protoscolicidal activity

Viable and free protoscoleces (2000 per Leighton tube) were cultured in 6 mL of culture medium 199 at 37°C without changes in medium during the entire experiment as previously described (Elissondo *et al.*, 2006). *Stevia multiaristata* extract was added and the final achieved concentrations were 100, 50, 10 and 5 μg mL^{-1}. Control protoscoleces were incubated in culture medium with DMSO (3 μL mL^{-1}). Cultures were performed in triplicate and the experiment was repeated three times. To determine the appearance of morphological alterations, culture tubes were followed microscopically every day. Viability assessment using the methylene blue exclusion test was performed every day until day 6 and then, every 3 days. Samples of protoscoleces from each of the treatment groups and the control were periodically taken for ultrastructural studies by scanning electron microscopy (SEM).

Cysticidal activity

Groups of 10 cysts were placed in Leighton tubes containing 6 mL of medium 199. *Stevia multiaristata* extract was added to the medium resulting in final concentrations of 100, 50, 10 and

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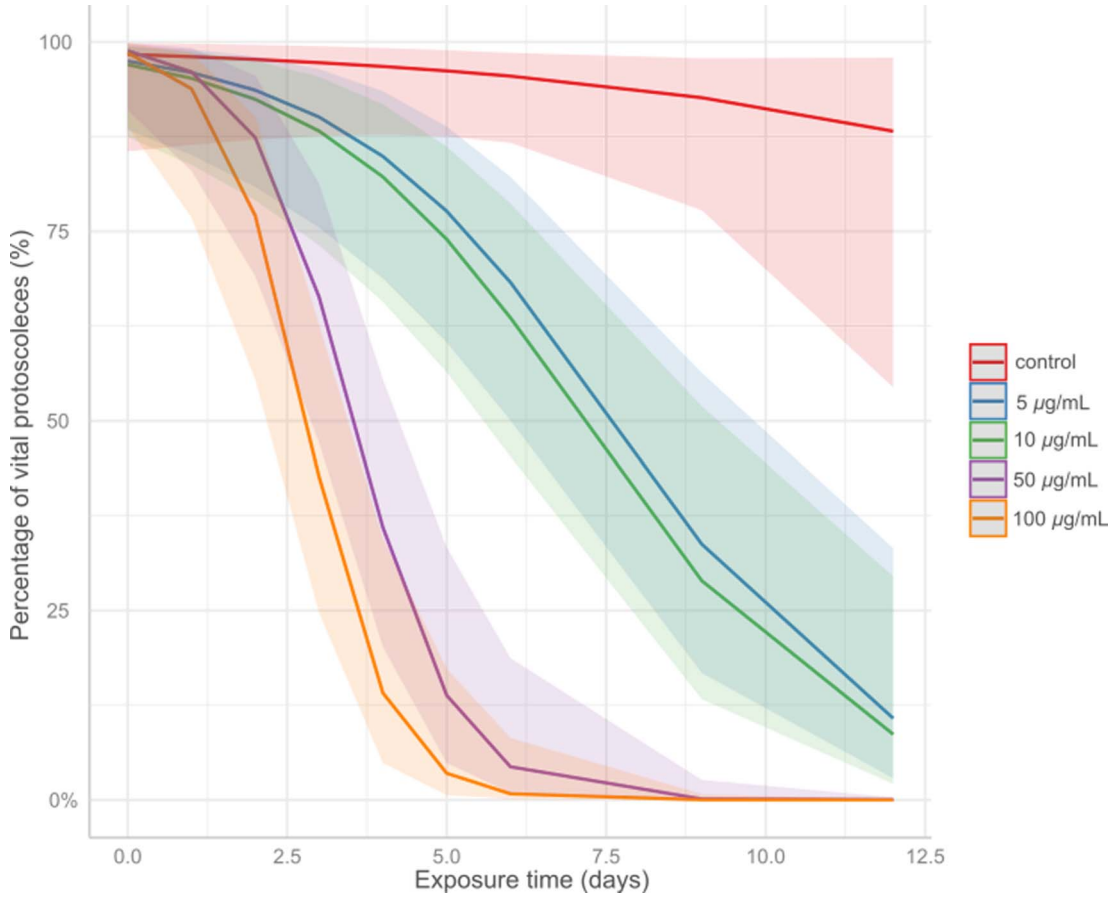

Fig. 1. Estimates of *Echinococcus granulosus s.s.* protoscoleces survival after *in vitro* exposure to the *Stevia multiaristata* extract. The lines and ribbons indicate the predicted fits and 95% confidence intervals from a generalized linear mixed-effects model.

5 $\mu g\,mL^{-1}$. Cysts incubated with the culture medium containing DMSO (3 $\mu L\,mL^{-1}$) were used as control. Culture tubes were maintained at 37°C without changes in medium during the entire experiment (Elissondo *et al.*, 2007). Cultures were performed in triplicate and the experiment was repeated two times. Culture tubes were followed macro and microscopically every day. The criteria for cyst viability assessment included the loss of turgidity and the collapse of the germinal layer (Fabbri *et al.*, 2016).

In vitro *cytotoxicity*

Huh7 cells were harvested with trypsin 0.25% EDTA. Cell pellets were resuspended after centrifugation in dye mix [100 $\mu g\,mL^{-1}$ acridine orange (AO), 100 $\mu g\,mL^{-1}$ ethidium bromide (EB) in phosphate-buffered saline (PBS)] and visualized by fluorescence microscopy (Olympus BX51, America Inc.). Huh7 cells were cultured in the presence of the *S. multiaristata* extract (5–250 $\mu g\,mL^{-1}$) or DMEM-C or solvent as controls, during 48 and 96 h. At least 200 cells were counted for each dose and cells were discriminated according to its stain pattern. Cells with homogeneous AO staining without EB stain were considered alive and cells with homogeneous EB staining were considered death (Cavaliere *et al.*, 2014). All experiments were performed in triplicate.

Selectivity index

Stevia multiaristata extract selectivity index (SI) was calculated at 96 h as the relation between CC_{50} (50% cytotoxicity concentration) on Huh7 cells and the EC_{50} (half maximal effective concentration) value determined by germinal layer collapse of *E. granulosus s.s.* cysts.

In vivo *assay*

Experimental animals and infection

Female CF-1 mice (body weight 25 ± 5 g) were intraperitoneally infected with 1500 *E. granulosus s.s.* protoscoleces/animal, suspended in 0.5 mL of medium 199.

Clinical efficacy study

At 6 months post-infection, female CF-1 mice ($n = 30$) were allocated into the following experimental groups (10 animals/group): (1) control group, (2) ABZ group, animals treated with ABZ suspension (25 $mg\,kg^{-1}$) every 24 h for 30 days and (3) *S. multiaristata* group, animals treated with the extract (50 $mg\,kg^{-1}$) every 24 h for 20 days. Treatments were performed by intragastric administration. At the end of the treatment period, all mice remained alive and they were euthanized. Necropsy was carried out immediately thereafter, the peritoneal cavity was opened and the hydatid cysts were carefully removed. The weight of the cysts collected from each animal was recorded using an analytical balance. Samples of cysts from each group were taken and fixed for histopathological analysis and SEM.

Blood biochemical assays

At the end of the clinical efficacy study, animals were anaesthetized with a mixture of ketamine (100 $mg\,kg^{-1}$) and xylazine (10 $mg\,kg^{-1}$), and blood samples were collected by cardiac puncture. Plasma was separated by centrifugation at 2000 ***g*** for 15 min and stored at −20°C until further analysis. To evaluate liver function, plasma levels of the enzymes alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), and glutamate-pyruvate transaminase (GPT) were measured using commercial kits from

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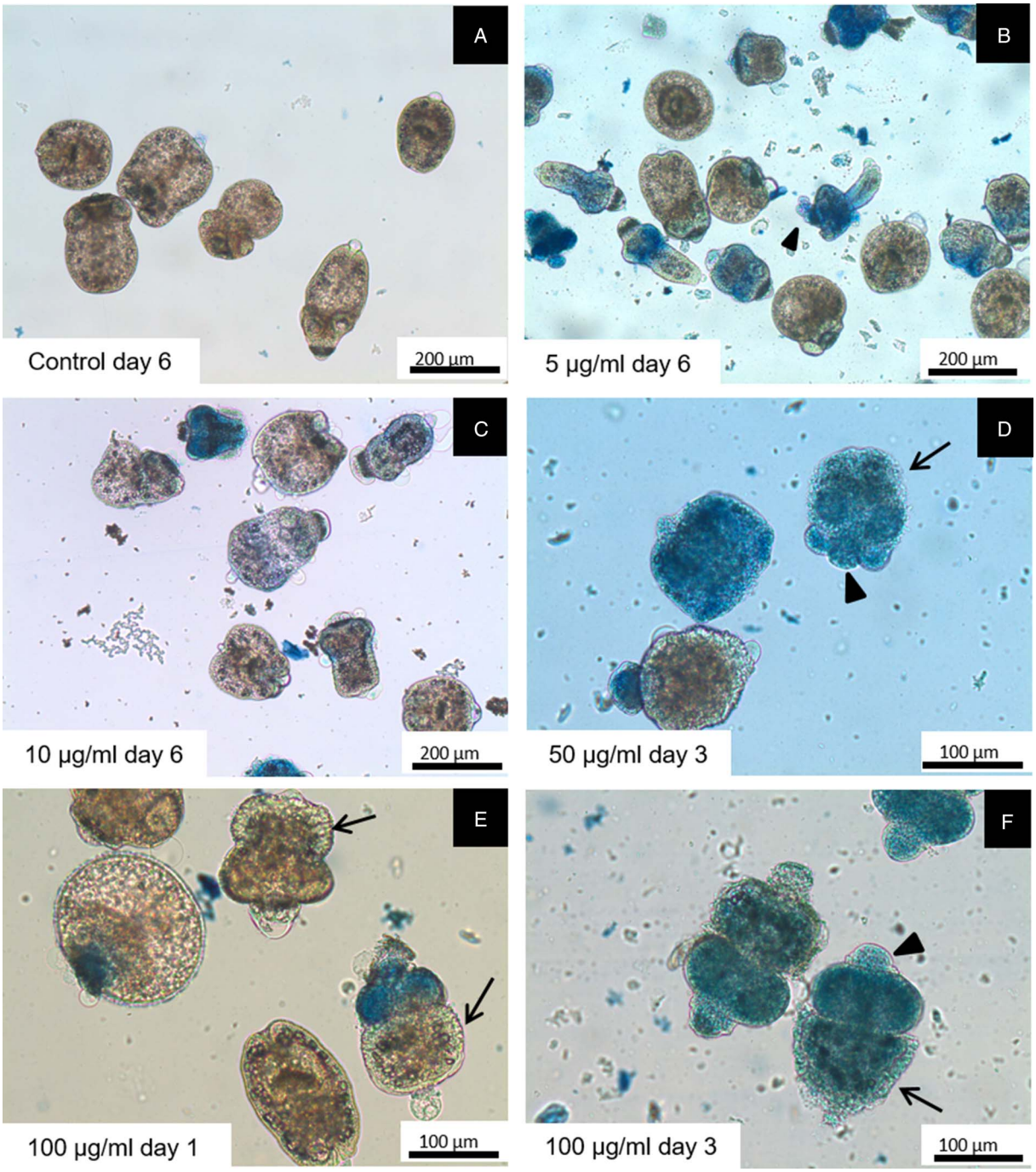

Fig. 2. Light microscopy of *E. granulosus s.s.* protoscoleces incubated *in vitro* with different concentrations of *S. multiaristata* extract and stained with methylene blue. Blue protoscoleces are not viable. Observe the contracted soma (arrows) and rostellar disorganization (arrowheads).

Wiener Lab (Rosario, Argentina). The activity of ALP was determined using the protocol described by Bessey *et al.* (1946). Plasma GGT activity was measured using the modified Szasz method (IFCC) (Szasz, 1969; Shaw *et al.*, 1983). For the estimation of GPT activity, the optimized UV method (IFCC) was followed (Bergmeyer *et al.*, 1976). The enzymatic activities were expressed in units/litre (U/L).

Histopathological analysis

Echinococcus granulosus cysts were washed twice with PBS and fixed in 10% neutral-buffered formalin at 4°C for 72 h. Then, the fixed samples were dehydrated in an ascending series of ethanol, embedded in paraffin, cut into 5 μm sections and stained with haematoxylin and eosin for microscopic examination.

Electron microscopy

Samples of protoscoleces taken from the *in vitro* studies and samples of metacestodes taken from *in vivo* studies were processed for SEM following the protocol described by Elissondo *et al.* (2007).

Statistical analysis

All statistical analyses were conducted within the R environment (R Core Team, 2021). For all tests *P* values less than 0.05 were considered statistically significant. For the *in vitro* incubation of protoscoleces with the *S. multiaristata* extract, a generalized linear model with a quasi-binomial distribution was fitted with the proportion of viability as a response variable and treatments and time in days as explanatory variables. To determine whether time–

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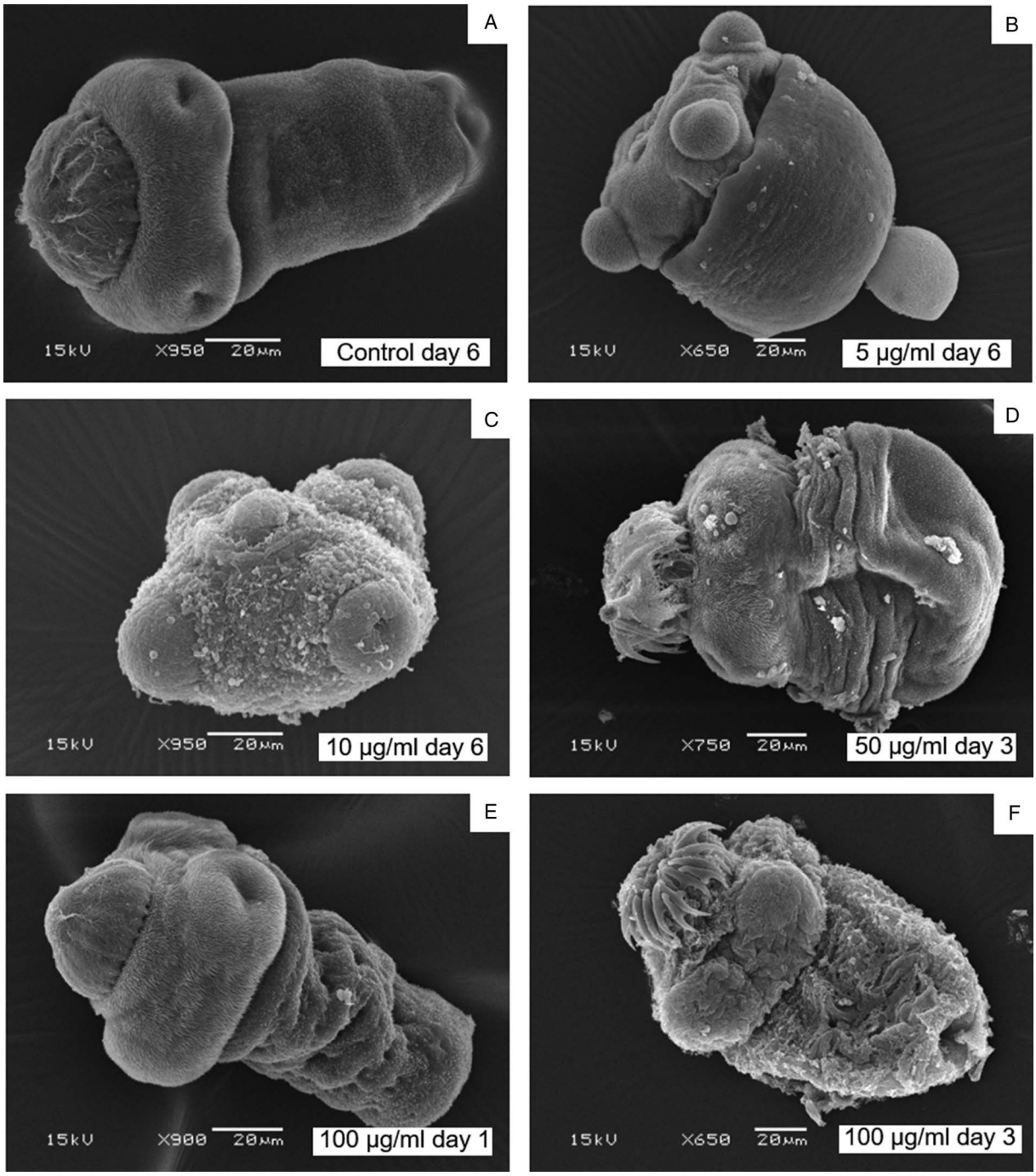

Fig. 3. SEM of *E. granulosus s.s.* protoscoleces incubated *in vitro* with different concentrations of the *S. multiaristata* extract.

treatment interactions had to be included in the model, we used the 'ANOVA' command from the 'car' package (Fox and Weisberg, 2019). Differences among the *S. multiaristata* extract concentrations and control were assessed by pairwise contrasts of the interaction means using the 'emmeans' package (Lenth, 2021).

For the *in vitro* incubation of cysts and hepatic cells with *S. multiaristata* extract, differences between treatments and exposure times were tested by fitting an analysis of variance model with extract concentrations and exposure time as explanatory variables, and the percentage of cysts with loss of turgidity and germinal layer collapse, and cell death as response variables for each case. Then a Tukey's HSD test was applied for pairwise contrasts. Additionally, the EC_{50} of the extract was calculated using the 'ec50estimator' package in R program (Alves, 2020).

For the *in vivo* experiments, differences between treated groups in the weight of the cysts and the plasma levels of ALP, GGT and GPT enzymes were all assessed by using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. The weights of the cysts for each treatment are reported as median and interquartile range (IQR).

Ethic statement and experimental animals

Animal procedures and management protocols were approved by the Institutional Animal Care and Use Committee (RD No. 211/2018) of the Faculty of Exact and Natural Sciences, National University of 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).

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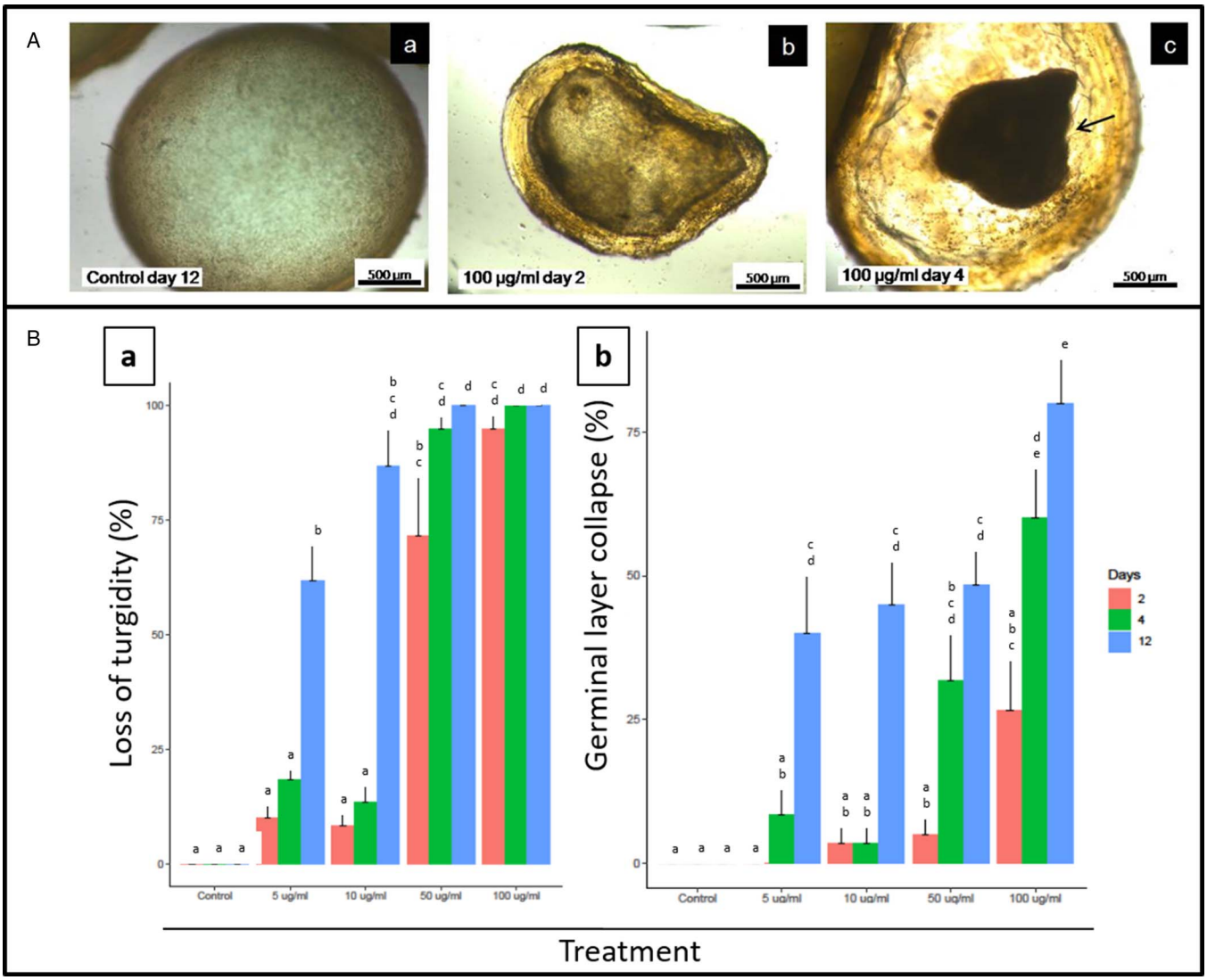

Fig. 4. (A) Light microscopy of *E. granulosus s.s.* cysts incubated *in vitro* with 100 μg mL^{-1} of the *S. multiaristata* extract. Observe the loss of turgidity (arrowhead) and collapse of the germinal layer (arrow). (B) Effect of *S. multiaristata* extract on *E. granulosus s.s.* cysts after 2, 4 and 12 days of *in vitro* exposure to different concentrations. The criteria used to evaluate the cysticidal effect were: (a) loss of turgidity and (b) germinal layer collapse. Each bar represents the mean percentage ± S.D. (standard deviation). Similar letters indicate no differences ($P < 0.05$).

Unnecessary animal suffering was avoided throughout the study. Animals were housed in a temperature-controlled (22 ± 1°C), light-cycled (12 h light/dark cycle) room. Food and water were given *ad libitum*.

Results

In vitro *incubation of protoscoleces with* S. multiaristata *extract*

Protoscoleces survival after incubation with *S. multiaristata* extract is shown in Fig. 1. Viability in the control group was 90.9 ± 3.6 after 12 days of incubation. Viability decreased quickly with 100 and 50 μg mL^{-1} of *S. multiaristata* extract, reaching 0% on days 7 and 8, respectively. The concentrations of 10 and 5 μg mL^{-1} reduced the viability to approximately 50% between days 7 and 8; at the end of the experiment, approximately above 10% of the protoscoleces treated with these concentrations remained viable.

Figures 2 and 3 show representative images of protoscoleces treated with different concentrations of *S. multiaristata* extract. Tegumental alterations were consistent with the results of the viability test. Control protoscoleces remained unaltered throughout the experimental period and no changes in structure and ultrastructure were observed (Figs 2A and 3A). After 6 days post incubation, protoscoleces treated with the lowest concentrations (5 and 10 μg mL^{-1}) showed rostellar disorganization and alterations in the tegument (Fig. 2B and C). At day 3 post incubation, protoscoleces treated with 50 or 100 μg mL^{-1} presented total loss of hooks and blebs in the tegument (Fig. 2D and F). Soma contraction could be detected in the first day of incubation with 100 μg mL^{-1} (Figs 2E and 3E). Studies by SEM revealed that the ultrastructure of protoscoleces treated during 3 days with 50 or 100 μg mL^{-1} were altered with tegumental alterations, loss of microtriches of the scolex region, rostellar disorganization and soma contraction (Fig. 3D and F). Protoscoleces incubated during 6 days with the lowest concentrations showed milder ultrastructural alterations (Fig. 3B and C).

In vitro *incubation of cysts with* S. multiaristata *extract*

Control cysts appeared macroscopically turgid with no observable collapse of the germinal layer throughout the *in vitro* experiment (Fig. 4Aa). In contrast, loss of turgidity was detected in 95 ± 3.4% of cysts incubated during 2 days with 100 μg mL^{-1} of *S. multiaristata* extract (Fig. 4Ab and Ba). After 4 days post incubation, the collapse of the germinal layer was observed in 32 ± 8.7% and 60 ±

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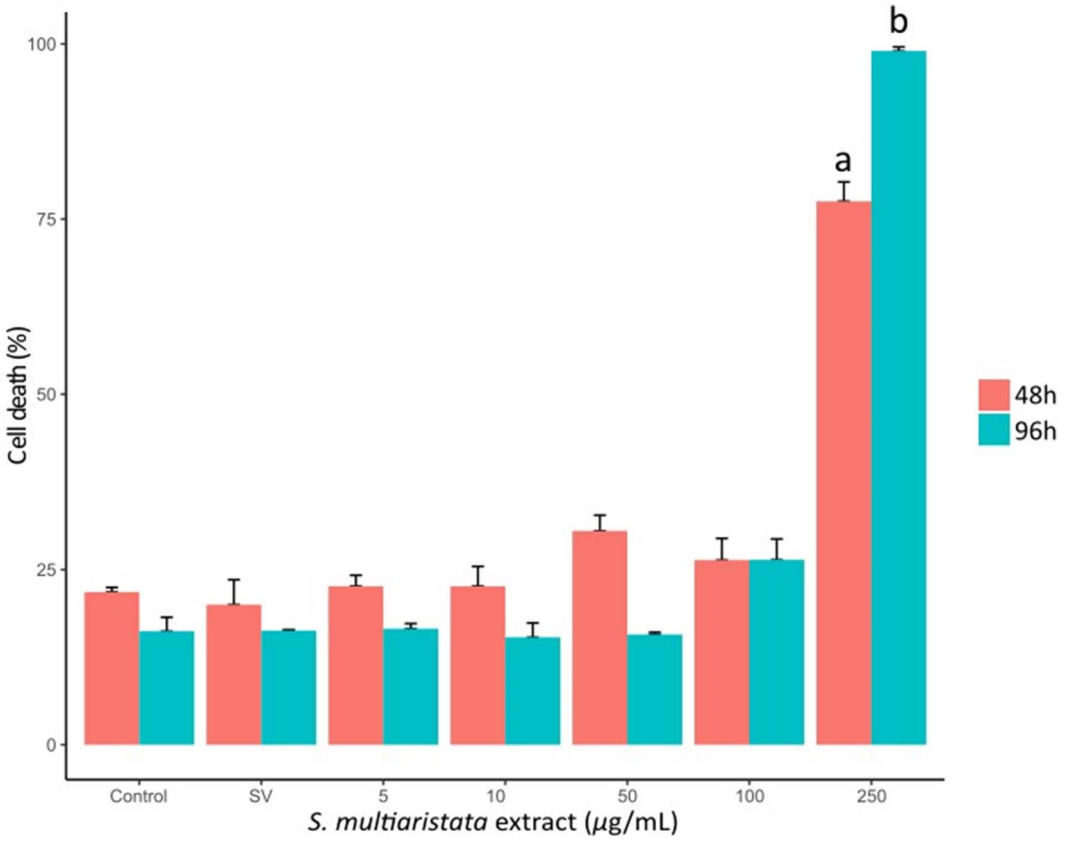

Fig. 5. Effect of different concentrations of *S. multiaristata* extract on Huh7 cells after 48 and 96 h of *in vitro* exposure. The graph shows the percentage of cell death determined as % cells PI+/total cell. 'Control' corresponds to cells cultured with 10% DMEM, 'SV' corresponds to cells culture in the presence of the solvent used to resuspend the *S. multiaristata* extract. Each bar represents the mean percentage ± S.D. Three independent experiments were performed. Different letters indicate significant differences ($P < 0.0001$).

Table 1. Clinical efficacy study

Group	Median weight of cysts (g)	IQR
Control	5.47	5.385
ABZ	3.75	1.705
Stevia multiaristata	2.265*	0.965

Median weight (g) and IQR of the *Echinococcus granulosus* cyst recovered from experimentally infected mice from the unmedicated and treated groups.
*Statistically significant differences with the control group ($P < 0.05$).

9.3% of cysts treated with 50 and 100 $\mu g\,mL^{-1}$ (Fig. 4Ac and Bb) of the *S. multiaristata* extract, respectively. *Stevia multiaristata* extract showed a dose and time dependent effect against cysts (Fig. 4B, Fig. S1).

The EC_{50} value of the *S. multiaristata* extract against *E. granulosus s.s.* cysts was 69.6 $\mu g\,mL^{-1}$.

In vitro *cytotoxicity on a hepatic cell line*

The percentage of viable cells after treatment with *S. multiaristata* extract at concentrations between 5 and 100 $\mu g\,mL^{-1}$ was higher than 69.5%, at both times evaluated. Only at a dose of 250 $\mu g\,mL^{-1}$ the extract induced a significant percentage of cell death in relation to the control ($P < 0.0001$), with values of 77.5 ± 4.8% and 99.0 ± 0.6% cell death after 48 and 96 h, respectively (Fig. 5). Moreover, there is no time-dependence in the response of Huh7 cells in the presence of the *S. multiaristata* extract. The CC_{50} values for 48 and 96 h were 207.7 and 132.2 $\mu g\,mL^{-1}$, respectively. The SI for *E. granulosus s.s.* cysts was 1.9 (96 h).

Clinical efficacy study

The behaviour and appearance of the animals were normal throughout the entire experimental period. Furthermore, no statistical differences were found in ALP, GGT and GPT activities between control and treated mice ($P > 0.05$).

Hydatid cysts developed in all the infected animals involved in the clinical efficacy study. Table 1 summarizes the cyst weights (median and IQR) recorded after treatments of the different experimental groups involved in the study. Although the median weight of cysts recovered from ABZ-treated mice was lower than that observed in the control group, no significant differences were found ($P > 0.05$). In contrast, *S. multiaristata* treatment caused a significant decrease in the weight of the cysts compared with the control group ($P < 0.05$).

An intact consecutive germinal layer and laminated layer were observed in the control group (Fig. 6A). Histopathological analysis showed no differences between control and treated mice (Fig. 6B and C).

Studies by SEM of metacestodes from the control group revealed the typical features of *E. granulosus* cysts, with an intact germinal layer composed of a multitude of different cell types (Fig. 7A). Cysts recovered after treatment with ABZ 25 $mg\,kg^{-1}$ showed a germinal layer with reduction in the number of cells and damaged cells (Fig. 7B). On the other hand, cysts recovered after treatment with the *S. multiaristata* extract 50 $mg\,kg^{-1}$ displayed a severe impairment of the germinal layer and a significant decrease in the number of cells (Fig. 7C).

Discussion

Phytotherapy has a high potential in the fight against neglected diseases for which prevention and cure are not sufficiently

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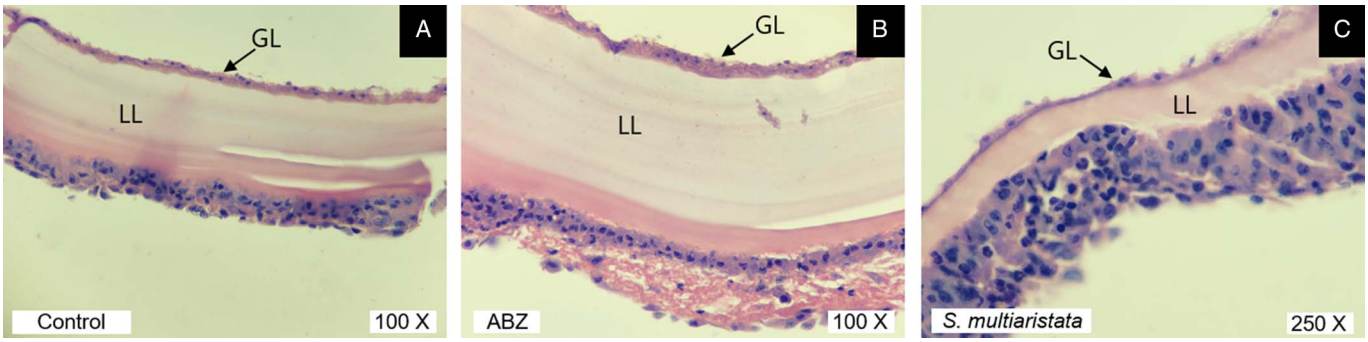

Fig. 6. Histopathological images of hydatid cysts recovered from infected mice. Cuts were stained with haematoxylin–eosin: (A) control; (B) ABZ (25 mg kg^{-1}) and (C) *S. multiaristata* extract (50 mg kg^{-1}). LL, laminated layer; GL, germinal layer.

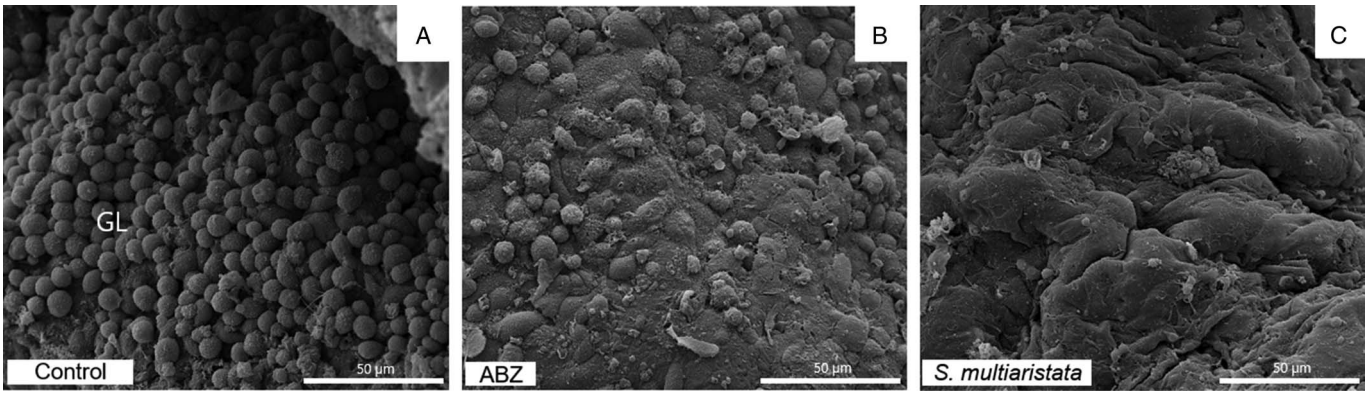

Fig. 7. SEM of *E. granulosus* metacestodes recovered from infected mice treated with ABZ or *S. multiaristata* extract during the clinical efficacy study. Treatments were administered orally at the doses of 25 mg kg^{-1} of ABZ and 50 mg kg^{-1} of *S. multiaristata* extract every 24 h. (A) Metacestode from the control group. Observe the germinal layer (GL) with different types of cells. (B) Germinal layer of metacestode from mice treated with ABZ suspension showing loss of cells. (C) Altered metacestode recovered from *S. multiaristata* extract treated mice. Note the extensive areas without cells and the presence of cellular debris.

available. As stated by the World Health Organization, up to 80% of the population in developing countries depends on the use of traditional medicine and medicinal herbs for primary health care (WHO, 2013).

In traditional medicine whole plants or mixtures of plants are used rather than isolated compounds. There is evidence that crude plant extracts often have greater *in vitro* and/or *in vivo* anti-parasitic activity than isolated constituents at an equivalent dose (Rasoanaivo *et al.*, 2011). One hypothesis that could explain this is the synergistic interaction or multi-factorial effects between compounds present in herbal extracts (Duke and Bogenschutz-Godwin, 1999; Gilbert and Alves, 2003).

Natural compounds have been used as medicines against infectious diseases caused by fungi, bacteria, viruses and parasites (Cos *et al.*, 2006). In the last few decades, a large number of plant extracts and compounds with anthelmintic activity against *E. granulosus s.l.* were reported. However, in most of these studies, only *in vitro* experiments were carried out (Ali *et al.*, 2020).

In Argentina, the genus *Stevia* is represented by more than 30 species and several varieties growing in the northern and central areas of the country (Zuloaga *et al.*, 2008); however, the majority have not been investigated so far. *Stevia multiaristata* Spreng. is a native species which has shown antiprotozoal activity against *T. cruzi* (Beer *et al.*, 2016).

The *in vitro* efficacy of *S. multiaristata* extract against *E. granulosus s.s.* protoscoleces and murine cysts was demonstrated. In both cases, the effect was time and dose dependent. *Stevia multiaristata* extract caused a marked reduction on protoscoleces viability which was consistent with the alterations observed such as contraction of soma region, the presence of blebs in the tegument, rostellar disorganization and loss of hooks and microtriches. Cysts treated with the *S. multiaristata* extract showed a rapid loss of turgidity and germinal layer collapse. The ultrastructural alterations were similar to those observed in *E. granulosus s.s.* protoscoleces and cysts incubated *in vitro* with other drugs and natural products as thymol, carvacrol and essential oils of oregano and thyme (Elissondo *et al.*, 2012; Pensel *et al.*, 2014; Fabbri *et al.*, 2016).

Even though the *S. multiaristata* extract showed a low SI, neither of the experimental concentrations (5–100 μg mL^{-1}) caused a cytotoxic effect, indicating that this compound has the capacity to inhibit the parasite growth without displaying a significant toxicity on the host's cells at the assayed concentrations.

In the current study, the effect of *S. multiaristata* extract was also evaluated in an *in vivo* murine model of CE. To the present, there are only a few plant extracts that were investigated for preventive or therapeutic activities against *E. granulosus s.l.* in the search for new alternative treatment for CE (Ali *et al.*, 2020). Some of these studies include the use of the methanolic extract of *Allium sativum*, the aqueous extract of Huaier, the aqueous extract of *Punica granatum* peel, the aqueous extract of *Sophora moorcroftiana* seeds and the methanolic extract of *Zataria multiflora*. All the extracts caused a decrease in the weight of the cysts recovered from treated mice compared with the control groups (Lv *et al.*, 2013; Moazeni *et al.*, 2014; Labsi *et al.*, 2016; Haji Mohammadi *et al.*, 2018; Luo *et al.*, 2018). Moreover, and in accordance with our results, the presence of ultrastructural alterations in the germinal layer was reported (Moazeni *et al.*, 2014; Luo *et al.*, 2018).

The median lethal dose (LD_{50}) of *S. multiaristata* extract has not been described yet. On the other hand, Kujur *et al.* (2010)

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reported a nontoxic effect of the leaves of *S. rebaudiana* in mice and suggested that the LD_{50} would be greater than 5 g kg^{-1} body weight in mice. This value is much greater than the dose used in the current study. No adverse side-effects on mice were observed during the entire treatment period. Moreover, we found no differences in the enzymes ALP, GGT and GPT activities between control and treated mice suggesting no hepatotoxic effect.

Oral administration of 50 mg kg^{-1} of *S. multiaristata* extract during 20 days in infected mice has a therapeutic effect on the hydatid cyst causing a significant reduction of the parasite weight. We found that the *S. multiaristata* extract caused both an *in vitro* effect on protoscoleces and cysts and also an *in vivo* therapeutic effect.

Stevia genus is characterized by the presence of sesquiterpene lactones, diterpenoids and flavonoids, among other phytochemical groups (Borgo *et al.*, 2021). Anti-parasitic activity has been described for many compounds belonging to these groups (Sanchez Alberti *et al.*, 2018; Boniface and Ferreira, 2019; Sessa *et al.*, 2020; Sülsen, 2021). Sesquiterpene lactones are one of the major phytochemical groups of compounds present in the genus *Stevia*. In relation to their mechanism of action it has been reported that they exert their biological activities throughout the inhibition of proliferation by the induction of apoptosis when they were applied on cancer cells (Rasul *et al.*, 2013; Moujir *et al.*, 2020). Regarding diterpenes with anti-parasitic activity, it has been shown that they inhibit nitric oxide production and inducible nitric oxide synthase (iNOS) expression by blocking the activation of STAT1, IRF3 and NF-κB on macrophages (Byoung, 2012). Flavonoids constitute another relevant phytochemical group found in the genus *Stevia* and the mode of action of these compounds may be multifaceted. It has been suggested that they can counter drug resistance, inhibit important enzymes or proteins or induce apoptosis of the parasites, among others (Mead and McNair, 2006). The results of the *in vitro* and *in vivo* anti-*Echinococcus* activity of *S. multiaristata* could indicate the presence of bioactive compounds of these types in the organic extract. Phytochemical analysis and fractionation of the extract will be undertaken in order to isolate the active molecules.

In conclusion, we demonstrated high protoscolicidal and cysticidal effects, and significant reduction in the weight of the cysts in experimentally infected mice following treatment with the *S. multiaristata* extract.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021002109

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Author contributions. AC and CE conceived and designed the study; EO, PD, GD, GB, BM and VS produced and provided plant extract; PA provided parasite material; AC, JF, PP and LF carried out the experiments and analysed and interpreted the data; NF performed statistical analyses; ZP carried out histopathological analyses; EN and GG performed biochemical analyses; AC, CE, JF, PP and VS wrote the article.

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Conflict of interest. None.

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