A broad spectrum antiparasitic effect of nitazoxanide: an important advancement

Amani A. Soliman[a], Sayeda M. Aufy[a], Hanaa M. Ezzat Moussa[a], Maha M. Saber[b], Mayssa M. Zaki[a], Dina M.H. El Akkad[a]

Departments of [a]Parasitology, [b]Pharmacology, Faculty of Medicine, Cairo University, Giza, Egypt

Correspondence to Mayssa Mohamed Zaki, MD, Department of Parasitology, Faculty of Medicine, Cairo University, Giza, 11369, Egypt
Tel: 22687726; Fax: 22900816;
e-mails: mayssaabcd@gmail.com and mmzaki@kasralainy.edu.eg

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Background
Parasitic infection has grown to be one of the most significant causes of morbidity and mortality worldwide. Nitazoxanide, a new antiparasitic drug, is believed to have a remarkable effect. This work aimed to study the in-vitro effects of nitazoxanide on *Echinococcus granulosus*, as an example of helminthic infection, and *Blastocystis* spp., as an example of protozoal infection, compared with the standard drugs in use.
Materials and methods
Hydatid protoscoleces and *Blastocystis* spp. were isolated and cultivated each on its specific medium. Nitazoxanide and standard drugs (albendazole for hydatid or metronidazole for *Blastocystis* spp.), or a combination of nitazoxanide and the standard drug for each were added to separate cultures, and parasite motility and viability were assessed. Furthermore, electron microscopic study for both parasites was carried out.
Results
The results showed a directly proportional relationship between number of dead protoscoleces and the tested nitazoxanide concentration; 50% of protoscoleces were killed (LD50) at 6 h incubation with 15 μg/ml of nitazoxanide, compared with LD50 of albendazole that was 30 μg/ml at 6 h. For *Blastocystis* spp., LD50 was 2 μg/ml after 48 h, whereas that for metronidazole was 10 μg/ml at the same culture duration. On combining nitazoxanide and the standard drugs, it showed variable effect, especially against *Blastocystis* spp.
Conclusion
These advantageous results toward nitazoxanide may allow trying the drug for both helminthic and protozoal infections.

Keywords:
Blastocystis spp, culture, electron microscope, hydatid, nitazoxanide

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Introduction

Parasitic infections rank among the most significant causes of morbidity and mortality worldwide, especially in the poor communities [1].

Despite the development of cases refractory to treatment and the withdrawal of several antiparasitic drugs from the market due to their adverse effects, there has been very little effort to develop new agents to treat such human parasitic infections [2]. Mainly, economic factors are to be accused for lack of innovation of new drugs [1].

In this context, the development of nitazoxanide is quite remarkable [3]. It affects electron transfer reaction through parasite cell membrane, which is important for anaerobic glucose energy metabolism, resulting in cell swelling and membrane damage [4]. Many scientists suggested this drug to be effective in the treatment of a range of helminthic and protozoal infections [1,5,6]. Cystic echinococcosis and blastocystosis were selected in the present study to investigate the in-vitro effect of nitazoxanide on both infections.

The present work aimed to study the effect of nitazoxanide *per se* or in combination with the drug of choice for each of the mentioned parasitic infections.

Materials and methods

This in-vitro study was carried out in four places: Medical Parasitology Department, Kasr Al-Ainy

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Faculty of Medicine; Parasitology Department, Faculty of Veterinary Medicine; Electron Microscopy Department, Faculty of Agriculture, Cairo University; and Electron Microscopy Department, Faculty of Science, Ain Shams University. The procedures followed were in accordance with the ethical standards of the experimentation.

Preparation of samples

Protoscoleces were aseptically collected from fertile hydatid cysts obtained from infected camel livers at a slaughterhouse in Cairo, Egypt. Hydatid cysts (2–5 cm in diameter) were cut, opened aseptically, and the vesicle fluid (containing protoscoleces) was separated, washed, and assessed for viability by observing peristaltic motility under the microscope, and then placed into 10 ml culture tubes.

Blastocystis spp. were recovered from stool samples of patients attending the outpatient clinic at Kasr Al-Ainy Hospital.

Media and culture

For hydatid, Roswell Park Memorial Institute medium was used, and penicillin, streptomycin, and amphotericin B were added. Washed protoscoleces were assessed for viability by observing peristaltic motility under the microscope and then placed into 10 ml culture tubes. The tubes were kept in an upright position in an incubator at 37°C and 5% CO_2 [7,8].

For *Blastocystis* spp., a culture described by Jones [9] was used to which horse serum is added. Samples were placed in 5 ml volume test tubes of prepared Jones medium. The tubes were kept in an upright position in an incubator at 37°C and subcultures were prepared every 2 days [10].

Chemotherapeutics

Nitazode oral suspension (each 5 ml suspension contains 100 mg nitazoxanide; Sigma, United States Pharmacopeia (USP)) as antiparasitic synthetic agent was used against hydatid protoscoleces and *Blastocystis* spp.

Flagyl oral suspension (40 mg/ml metronidazole benzoate; Sanofi Aventis) was used against *Blastocystis* spp.

Alzental oral suspension (20 mg/ml albendazole; Egyptian International Pharmaceutical Industries Company) was used against hydatid.

Stocks of the tested drugs were prepared with different concentrations after being dissolved in dimethylsulfoxide and were added to hydatid and *Blastocystis* spp. culture tubes.

Bioassay of antiparasitic effect

Viability test

Hydatid culture tubes were supplemented with either nitazoxanide at concentrations of 1, 10, 15, 20, 25, and 30 μg/ml or albendazole [11] at concentrations of 10, 20, 30, 40, and 50 μg/ml or combinations of both drugs (10 μg/ml nitazoxanide+30 μg/ml albendazole and 15 μg/ml nitazoxanide+20 μg/ml albendazole). Control culture tubes (equal amount of Dimethyle sulfoxide (DMS)) were supplemented with this drug [12].

The vitality/viability of protoscoleces was observed for each drug concentration together with the control culture at 3, 6, 24, 48, and 72 h. The number of viable protoscoleces was assessed both by observing their morphological criteria and peristaltic motility under the inverted microscope and using the trypan blue exclusion test, in which live cells with intact cell membranes did not take the dye [8,12]. A small sample of each culture was processed for scanning electron microscopy (SEM) in the Parasitology Department, Faculty of Agriculture, Cairo University.

The viability of noncystic forms of *Blastocystis* spp. was observed for each drug concentration together with the control culture at 24 and 48 h [10]. Quantitative assessment of *Blastocystis* spp. cultures was carried out using neutral red. This stain is capable of penetrating intact cellular membranes [13]. Cells were considered viable when they appeared with a thin rim of cytoplasm and peripheral nuclei and were stained red [10].

Subsequently, cells were washed and processed for transmission electron microscopy (TEM) in the Electron Microscopy Department, Faculty of Science, Ain Shams University.

Each concentration was determined in five cultures and the mean was calculated.

The viability in the exposed samples was determined.

For hydatid, the number of viable protoscoleces in 100 viable organisms was counted. Five readings were taken and the average was obtained. For *Blastocystis* spp., the number of viable *Blastocystis* spp. per 25 organisms was counted. Five readings were taken and the average was obtained. Median lethal dose that kills 50% (LD50) and 90% (LD90) of the exposed samples was used to determine the efficacy of each drug [10].

Electron microscopic study

Hydatid protoscoleces were processed according to Hemphill and Croft and inspected on a JEOL 840 SEM (SOI, Massachusetts, USA) operating at 25 kV to compare both viable drug-treated and control samples.

Likewise, *Blastocystis* spp. controls and drug-treated samples were processed and cut using an ultramicrotome, to be examined by means of TEM [14].

Statistical analysis

The obtained data were statistically analyzed using computer software package SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA). For quantitative variables, mean (as a measure of central tendency) and SEM (as a measure of variability) were calculated.

Results

On studying the in-vitro effect of nitazoxanide on both *Echinococcus granulosus* and *Blastocystis* spp. as compared with standard drugs the following were observed.

Echinococcus granulosus

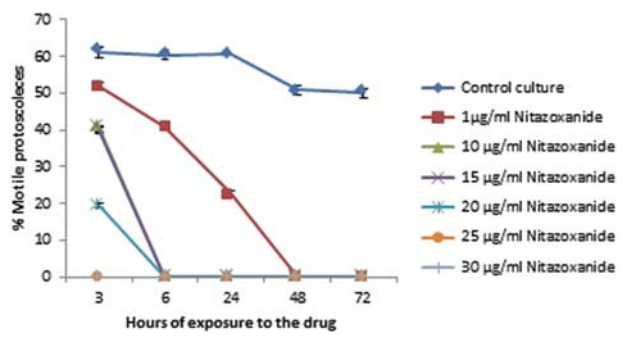
Microscopic examination showed viable protoscoleces to have intact contour and brilliant color with sharply demarcated suckers. They did not take the bluish coloration of trypan blue. In contrast, dead ones were small in size, dark in color, and nonmotile. They had irregular shape and unclearly demarcated suckers or rostellum. They took the bluish stain of trypan blue.

There was a directly proportional relationship between loss of motility and tested nitazoxanide concentration and exposure time (Fig. 1). At the least dose of 1 μg/ml of the drug, there was a slight decrease in the motility of protoscoleces that was completely lost after 48 h. At a dose of 10 μg/ml of nitazoxanide, the decrease in the motility of the protoscoleces became evident after 6 h, whereas at concentrations of 25 and 30 μg/ml there was loss of movement at 3 h. As regards viability, 50% of protoscoleces were killed at 6 h of incubation with 15 μg/ml of nitazoxanide (LD50 of nitazoxanide was 15 μg/ml at 6 h). At the same dose of nitazoxanide, no viable protoscoleces could be seen in the field after 24 h – that is, LD90 was 15 μg/ml at 24 h.

As regards albendazole, the drug directly affected the peristaltic movement of protoscoleces and complete cessation of movement was reached at 6 h at a dose of 50 μg/ml of albendazole (Fig. 2). For viability, LD50 of albendazole was 30 μg/ml at 6 h, whereas LD90 was 30 μg/ml at 48 h.

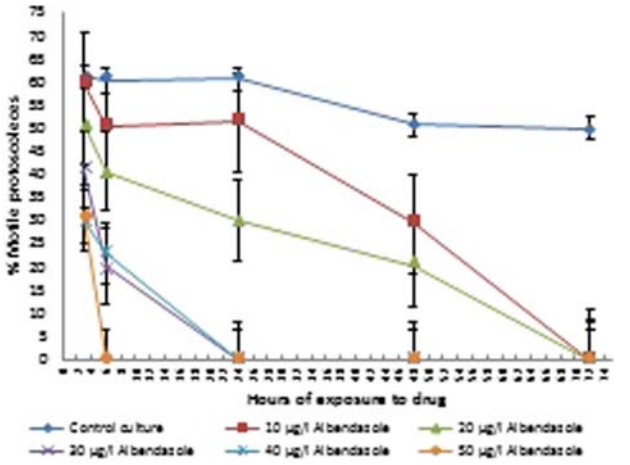
The two doses of combined nitazoxanide and albendazole showed complete loss of protoscoleces motility at 6 h (Fig. 3). Loss of movement was evident at same time as previously noticed using nitazoxanide alone with same dose.

Figure 1



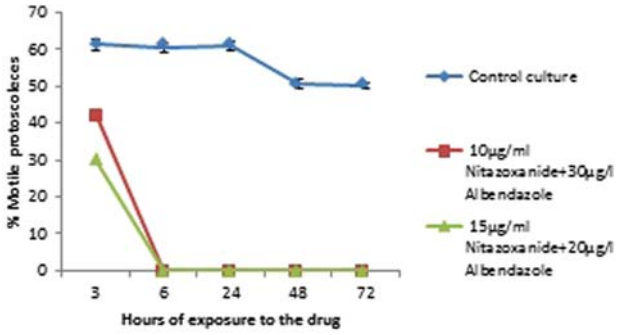
Effect of nitazoxanide on *Echinococcus granulosus* motility.

Figure 2



Effect of albendazole on *Echinococcus granulosus* motility.

Figure 3



Effect of nitazoxanide and albendazole combination on *Echinococcus granulosus* motility.

For viability, different drug concentrations were tried; only the 10 µg/ml nitazoxanide and 30 µg/ml albendazole combination showed a higher mortality rate than that produced by nitazoxanide alone. Other concentrations of drug combination showed a lower mortality rate than that produced by nitazoxanide alone.

Compared with control, 80% of the parasites were still viable after 5 days of culture.

***Blastocystis* spp.**

Viable *Blastocystis* spp. had intact cell membrane and took the neutral red stain, whereas dead cells appeared with corrupted morphology, loss of cell membrane continuity, and were not stained with neutral red.

Nitazoxanide 2 µg/ml was shown to induce 50% reduction of viable noncystic forms of *Blastocystis* spp. after 48 h in culture (LD50), whereas LD90 was 10 µg/ml at 48 h (Fig. 4). No deaths were detected in the non-drug-treated cultures.

For metronidazole, LD50 was 10 µg/ml at 48 h. However, a high concentration of the drug up to 200 µg/ml did not cause complete death of *Blastocystis* spp. in all cultures (Fig. 5).

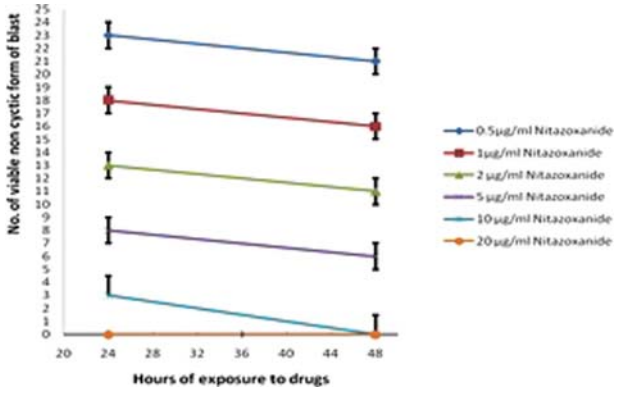
Combined treatment (Fig. 6) showed better effect than that produced by metronidazole alone in one culture tube and was less effective in other culture tubes. In contrast, the combination of 2 µg/ml nitazoxanide +10 µg/ml metronidazole almost had the same effect of 2 µg/ml nitazoxanide alone.

Electron microscopic study

SEM for hydatid verified that the drug-induced morphological and structural damage upon drug-treated protoscoleces in different drug-treated cultures without clear difference in the degree of damage of protoscoleces with different drugs used (Fig. 7).

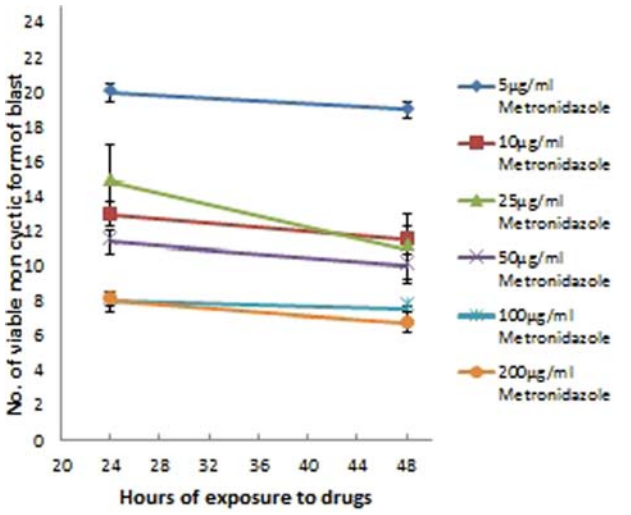
Similarly, TEM for *Blastocystis* spp. showed drug-induced cytoplasmic vacuoles with necrotic cells together with disruption of the normal morphology of *Blastocystis* spp. in different drug-treated cultures without clear difference in the degree of damage of *Blastocystis* spp. with different drugs used (Fig. 8).

Figure 4



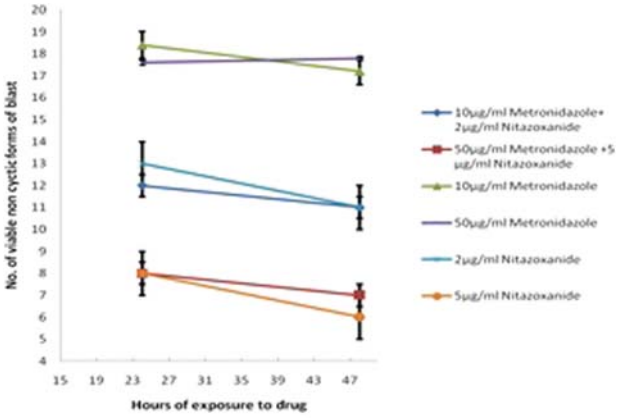
Effect of nitazoxanide on noncystic forms of *Blastocystis* spp.

Figure 5



Effect of metronidazole on noncystic forms of *Blastocystis* spp.

Figure 6

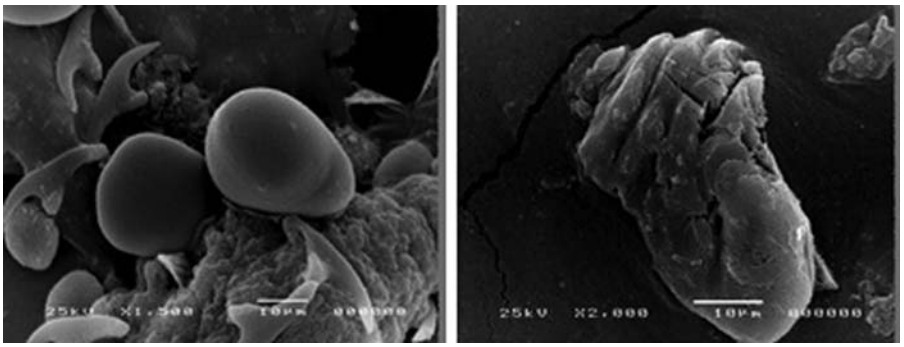


Effect of nitazoxanide and metronidazole combination on *Blastocystis* spp.

Discussion

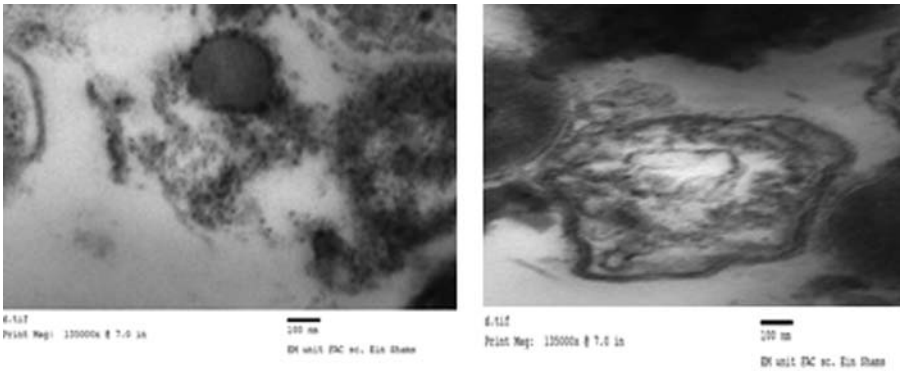
For hydatid cysts, surgery or puncture, aspiration, injection, reaspiration remains the mainstay of treatment. However, it is usually combined with

Figure 7



Scanning electron microscopy of in-vitro-cultivated *Echinococcus granulosus* protoscoleces. Nondrug treated to the left: showing the normal smooth outer surface of suckers and the scattered hooks; drug treated to the right: showing damaged rough outer surface of the protoscoleces and the damaged hooks.

Figure 8



Transmission electron micrograph of cultured *Blastocystis* spp. Nondrug treated to the left: showing apparently vacuolar form with a central vacuole and thin rim of cytoplasm peripheral nuclei; right: drug treated *Blastocystis* spp. culture revealed necrotic cell with cytoplasmic vacuolations.

chemotherapy and may be the only solution if other lines of treatment are contraindicated. Nevertheless, treatment of nonresected cysts with benzimidazoles (albendazole or mebendazole) results in cyst disappearance in 30% of cases with variable effects on the remaining 70% of the treated patients [15]. Being a broad spectrum, both antiprotozoal and antihelminthic agent, nitazoxanide does offer a new ray of hope for the treatment of intestinal parasitic infections [16].

The current study revealed that there was a directly proportional relationship between loss of motility of protoscoleces and the tested nitazoxanide concentration together with the exposure time. The decrease in the vitality of the protoscoleces became evident after 6 h following the addition of 10 μg/ml nitazoxanide.

These findings were previously observed by Walker *et al.* [12], who reported that following isolation of protoscoleces from hydatid cysts, about 60% showed distinct movements. However, a significant decrease in the motility of the protoscoleces was noticed 3–4 h after incubation with nitazoxanide.

In this study, using 10 μg/ml of albendazole resulted in complete loss of motility after 72 h. By increasing the dose to 30 μg/ml, movement was lost only at 24 h.

However, in the research by Parashar and Arya [17], they found that the viability of protoscoleces was 82.5% with 10 μg/ml of albendazole 72 h after incubation. However, on adding ivermectin, maximum protoscolicidal effect was detected with absence of cyst formation following their inoculation into mice.

On studying either nitazoxanide alone or the combination of nitazoxanide and albendazole, complete loss of protoscoleces motility was noticed at 6 h. This proves that there is no synergistic effect of both drugs together, with nitazoxanide taking the upper hand.

However, in a research conducted on different *Echinococcus* spp. (*Echinococcus multilocularis*), Casado *et al.* [11] found that there was profound improvement in the antiparasitic effect of combined drugs compared with using each separately.

The present study illustrated loss of protoscolex viability in nitazoxanide-treated cultures, which became clearer after 24 h of incubation, with LD50 being 10 μg/ml. In contrast, LD90 of nitazoxanide was 15 μg/ml at the same exposure time. On increasing the dose to 30 μg/ml, no viable protoscoleces could be seen in the field at 3 h after incubation. This indicated the rapid protoscolicidal effect of nitazoxanide.

These results are in agreement with those of Walker *et al.* [12], who compared the in-vitro effect of benzimidazole carbamate derivatives, such as mebendazole and albendazole, currently used for chemotherapeutic treatment of cystic echinociccosis, and that of nitazoxanide. They reported that the former drugs have to be applied at high doses for extended periods of time to have the same protoscolicidal effect produced by 5 and 10 μg/ml nitazoxanide. In addition, they tried resuspension of nitazoxanide-treated protoscoleces in fresh medium for a period of several weeks without the drug; however, this resulted in no changes, indicating that none of the parasites had survived the treatment.

The current study showed that combined nitazoxanide and albendazole showed complete loss of viable protoscoleces after 48 h and that 10 μg/ml nitazoxanide and 30 μg/ml albendazole was the most effective concentration with higher mortality rate than that produced by each drug alone.

In the view of the mentioned data, nitazoxanide showed a remarkable rapid effect over albendazole and the two drugs combined, giving a new hope to use it in the future *in vivo* with better rapid results.

As regards *Blastocystis* spp., metronidazole treatment is considered the first-line therapy. Nevertheless, variation in treatment response suggests the presence of metronidazole-resistant subtypes of the *Blastocystis* spp. [18].

The present work focused on isolation of *Blastocystis* spp. and studying the efficacy of nitazoxanide as compared with the standard drug.

Results obtained in the current study showed that LD50 of nitazoxanide was 2 μg/ml at 48 h and LD90 of the drug was 10 μg/ml at 48 h. No deaths were recorded in non-drug-treated cultures.

This is in agreement with the findings of Stensvold *et al.* [19], who proved the in-vivo effect of nitazoxanide in persistent diarrhea and enteritis associated with *Blastocystis hominis*, as it caused complete remission of the treated patients.

Similar studies were performed by Rossignol and colleagues [20–21]. They tested the in-vitro effect of nitazoxanide against other protozoa such as *Cryptosporidium* spp., *Giardia* spp., *Trichomonas* spp., and *Balantidium coli.* They concluded that all tested protozoa were markedly affected after adding the drug.

The present study showed that LD50 of metronidazole was 10 μg/ml after 48 h. Nevertheless, metronidazole did not produce complete death of viable *Blastocystis* spp. in all cultures, used up to a concentration of 200 μg/ml. This may be explained by the presence of metronidazole-resistant subtypes.

This is in the agreement with the findings of Diaz *et al.* [22], who studied the in-vitro susceptibility of clinical isolates of *B. hominis* to different concentrations of metronidazole and reported that the resistance of *B. hominis* to metronidazole at 10 μg/ml was 40%.

As regards metronidazole and nitazoxanide combination, variable effects were noticed in different *Blastocystis* spp. cultures, which may be explained by the presence of different subtypes of *Blastocystis* spp. with different drug susceptibilities.

This is in accordance with the findings of Yakoob *et al.* [23], who evaluated the effect of metronidazole and nitazoxanide against *Blastocystis* spp. subtypes 4 and 7, which had been suggested to represent pathogenic zoonotic subtypes. They also documented that nitazoxanide was effective against both subtypes.

Thus, the present work showed that nitazoxanide was effective against *Blastocystis* spp., whereas some degree of resistance to the standard drug metronidazole was shown by *Blastocystis* spp., which suggested the presence of resistant subtypes among isolated samples. A variable effect of the combination drug could be observed.

SEM of drug-treated cultures of hydatid demonstrated morphological and structural damage, without clear difference in the degree of damage of protoscoleces with different drugs used.

These findings are similar to those of Yakoob *et al.* [23], who tested the in-vitro effects of nitazoxanide on *E. granulosus* protoscoleces and metacestodes. They noted the extensive damage caused by nitazoxanide at the ultrastructural level using SEM.

In drug-treated cultures of *Blastocystis* spp., TEM illustrated drug-induced cytoplasmic vacuoles with necrotic cells, together with disruption of the normal morphology of *Blastocystis* spp. and loss of the continuity of the outer surface coat without clear difference in the degree of damage of *Blastocystis* spp. with different drugs used. The same ultrastructural changes were described by Mirza *et al.* [24], who concluded that metronidazole induced programmed cell death in *B. hominis*.

Conclusion

Nitazoxanide was shown to be an antiparasitic synthetic agent with remarkable effects against both *E. granulosus* and *Blastocystis* spp. Combined drug effects were variable against hydatid, which may be due to drug interactions, whereas in *Blastocystis* spp. the variable effect may be attributed to the presence of different subtypes of *Blastocystis* spp. with different drug susceptibilities. Study of nitazoxanide effect on a wider scale of parasites is recommended to pave the way for generalizing the drug for human parasitic infections.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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