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Giardia lamblia: The effects of extracts and fractions from *Mentha x piperita* Lin. (Lamiaceae) on trophozoites

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Abstract

Giardia lamblia is a parasite that causes giardiasis in humans and other mammals. The common treatment includes different classes of drugs, which were described to produce unpleasant side effects. *Mentha x piperita*, popularly known as peppermint, is a plant that is frequently used in the popular medicine to treat gastrointestinal symptoms. We examined the effects of crude extracts and fractions from peppermint against *G. lamblia* (ATCC 30888) on the basis of trophozoite growth, morphology and adherence studies. The methanolic, dichloromethane and hexanic extracts presented IC_{50} values of 0.8, 2.5 and 9.0 μg/ml after 48 h of incubation, respectively. The aqueous extract showed no effect against the trophozoites with an $IC_{50} > 100$ μg/ml. The aqueous fraction presented a moderate activity with an IC_{50} of 45.5 μg/ml. The dichloromethane fraction showed the best antigiardial activity, with an IC_{50} of 0.75 μg/ml after 48 h of incubation. The morphological and adhesion assays showed that this fraction caused several alterations on plasma membrane surface of the parasite and inhibited the adhesion of *G. lamblia* trophozoites. Cytotoxic assays showed that *Mentha x piperita* presented no toxic effects on the intestinal cell line IEC-6. Our results demonstrated antigiardial activity of *Mentha x piperita*, indicating its potential value as therapeutic agent against *G. lamblia* infections.
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Index Descriptors: Protozoan; *Giardia lamblia*; *Mentha x piperita*; Giardiasis; Treatment

1. Introduction

Giardia lamblia is a parasite that colonizes the duodenum and upper jejunum of humans causing a disease known as giardiasis. The common treatment for this illness includes different drugs such as metronidazole, furazolidone and the class of benzimidazoles. In spite of their large use, these drugs can produce many side effects on patients including headache, vertigo, nausea, gastrointestinal disturbance, anorexia and dizziness (Gardner and Hill, 2001). Strains of *G. lamblia* have also developed resistance to some of these remedies (Kollaritsch et al., 1993; Liu et al., 2000).

Due to the factors presented above, the search for new agents to treat giardiasis has increased. A great number of papers is attempting to prove the efficacy of plant extracts against *G. lamblia* trophozoites (Cowan, 1999; Harris et al., 2000; Khan et al., 2000; Arrieta et al., 2001; McAllister et al., 2001; Ankli et al., 2002; Calzada et al., 2003, 2005; Gadelha et al., 2005; Sawangjaroen et al., 2005).

Mentha x piperita Lin. (Lamiaceae), commonly called peppermint, is a well-known herbal remedy used for a variety of symptoms and diseases (Guedón and Pasquier, 1994). In the popular medicine, it is used to treat nausea, flatulence, vomiting, indigestion, stomach cramps, menstrual cramps and parasitosis (Fonseka-Kruel and Fernandes, 2003). It is also recognized for its carminative, stimulant, antispasmodic, antiseptic, anti-inflammatory, antibacterial and antifungal activities (Guedón and Pasquier, 1994; Gershenzon et al., 2000; Inoue et al., 2002; Samarth and Kumar, 2003; Ruiz del Castillo et al., 2004; Duarte et al., 2005). As usual for a member of the plant group, *Mentha x*

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piperita produces a variety of metabolites such as terpenes, tannins, flavonoids and phenolic acids (Guedón and Pasquier, 1994). Among the identified compounds some had already been reported as having antimicrobial activity, including 1,8-cineole, limonene, linalool and menthol (Mazzanti et al., 1998; Iscan et al., 2002).

The aim of the present work is to analyze the activity of extracts and fractions from *Mentha x piperita* against trophozoites of *G. lamblia*. Using video-microscopy and transmission electron microscopy, we analyzed the effects on the morphology of the parasite. The IC_{50} value of different extracts and fractions derived from *Mentha x piperita* was also obtained. Adhesion assays were done to evaluate the activity of the plant on the adhesion capacity of *G. lamblia*. Cytotoxic assays were performed with the intestinal epithelial cell line IEC-6 using different concentrations of *Mentha x piperita*.

2. Materials and methods

2.1. Parasites culture

Giardia lamblia trophozoites, Portland-1 strain (ATCC 30888), were axenically cultivated in TYI-S-33 medium, enriched with 0.1% bovine bile and 10% fetal bovine serum, at 37 °C, for 48–72 h (Keister, 1983). Subcultures were done twice a week. Tubes containing cells at log phase were used in the experiments.

2.2. Intestinal epithelial cell line culture

Monolayers of IEC-6 (ATCC CRL-1592) were cultured at 37 °C in 25 cm^2 flasks and grown in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., MO, USA) supplemented with 10% of fetal bovine serum and 100 U/ml of human regular insulin in an atmosphere of 5% CO_2 and 95% air (McCabe et al., 1991).

2.3. Plant material

The dry leaves of *Mentha x piperita* (Lin.) were obtained on a store specialized in medicinal plants in Niterói City, in the state of Rio de Janeiro, Brazil, in December of 2003. A voucher specimen (RB 412917) was deposited at the herbarium of Botanic Garden of Rio de Janeiro, Rio de Janeiro, Brazil.

2.4. Extraction

Dry leaves (5 g) were extracted with 100 ml of different solvents: methanol (Tedia, USA, HPLC grade), dichloromethane (EM-Science, USA, HPLC grade) and *n*-hexane (Tedia, USA, HPLC grade) for 15 days, at room temperature. The infusion was obtained by adding 100 ml of boiled water on 5 g of dry leaves of *Mentha x piperita*.

The extracts were filtered, then concentrated under vacuum conditions, and stored at 4 °C until further use. The infusion was lyophilized to obtain a dry extract.

2.5. Fractionation

The methanolic extract was dissolved in 100 ml of a solution of ethanol/water (3:7), and further fractionated by liquid–liquid extraction using 50 ml dichloromethane. The fractions were filtered and the solvents were removed under reduced pressure. Two fractions were obtained: (1) dichloromethane fraction (DCM) and (2) residual fraction. On the day of the experiments, the dry extracts and fractions were dissolved in DMSO (Merck, Germany) on a final concentration of 10 mg/ml.

For the sake of simplicity, the methanolic extract and the dichloromethane fraction will be designated, from now on, as follows: methanolic extract, MeOH extract; dichloromethane fraction, DCM fraction.

2.6. Growth inhibition assays

Trophozoites (5×10^4 cells/ml) were grown in 1.5 ml tubes in the presence of the extracts/fractions (Campanati et al., 2001). The concentrations of extracts and fractions employed were 1, 10, 50 and 100 μg/ml in culture medium, leading to a final concentration of DMSO (solvent used in stock solution) less than or equal to 1%. In our previous work, it was established that the above concentration range of DMSO did not induce alterations of the cell morphology or of any other cell parameter tested (Campanati et al., 2001). Trophozoites were exposed to the extracts and fractions for 2, 4, 6, 24 and 48 h. The total number of cells was obtained using a hemocytometer (Neubauer chamber). Dose–response graphics were elaborated. In addition, the concentration that causes 50% reduction on the number of thophozoites (IC_{50}) was calculated for each compound (Katiyar et al., 1994). The IC_{50} values of metronidazole and furazolidone were calculated in our previous work using the same methodology described above (Campanati and Monteiro-Leal, 2002).

These samples were also used to study the trophozoite morphology by video-microscopy and transmission electron microscopy.

2.7. Video-microscopy

An aliquot of cells was removed and mounted between slide and coverslip. Video-microscopy images were obtained using the Zeiss Axiophot microscope (Zeiss, Germany) equipped with a digital camera SIS CC-12 with the help of AnalySIS 3.1 (SIS-Soft Imaging System, Munster, Germany). The images were optimized by the use of the Paint Shop Pro program (JASC software, Inc., USA).

2.8. Transmission electron microscopy (TEM)

The cells were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in PHEM buffer (Schliwa and van Blerkom, 1981), overnight at 4 °C. The samples were

washed twice in phosphate-buffered saline (PBS), pH 7.2, and post-fixed in 1% osmium tetroxide (OsO_4) supplemented with 0.8% potassium ferricyanide (FeCNK) for 40 min. Afterwards they were dehydrated in crescent concentrations of acetone, embedded in Epon (Ted Pella, Redding, CA, USA) and sectioned with a Reichert ultramicrotome (Vienna, Austria). The sections were collected on copper grids and contrasted in 5% aqueous uranyl acetate and lead citrate (Gadelha et al., 2005). The preparations were evaluated with a Zeiss EM 906 transmission electron microscope (Oberkochen, Germany). The images were digitized directly from the microscope by an F-View XS (SIS-Germany) digital camera attached to it with the use of AnalySIS 3.1.

2.9. Adherence inhibition assays

Trophozoites (5×10^4 cells/ml) were grown on 12 mm glass coverslips in 24-well tissue culture plates, at 37 °C, under microaerophilic conditions. The culture was made in TYI-S-33 medium with 10% of fetal bovine serum in presence or not (control conditions) of the DCM fractions at concentrations of 1, 10, 50 and 100 μg/ml for 6, 24 and 48 h.

After the incubation, the culture medium, with unattached cells, was removed and the remaining attached parasites fixed and stained with the Panotico Kit (Laborclin, Paraná, Brazil). The number of cells on control and treated coverslips was determined by counting cells using a computer module, a computer routine, prepared by our group. This module runs in the Zeiss image-processing system KS-400 (Zeiss-Vision, Oberkochen, Germany) (Gadelha et al., 2006).

2.10. Cytotoxic assays

For cytotoxic assays, IEC-6 cells were trypsinized, and then an aliquot of 2×10^5 cells/ml, each, was incubated on 12 mm glass coverslips in 24-well tissue culture plates under microaerophilic condition. When the monolayers reached confluence (3–4 days), the medium was removed and the cells were then incubated with fresh medium plus different concentrations of DCM fraction, diluted in phosphate-buffered saline (PBS) pH 7.2, for 48 h (Gadelha et al., 2005). After this period of incubation, the cells were fixed and stained with the Panotico Kit. The number of cells in control and treated coverslips was determined by counting cells using the same macro mentioned in Section 2.9 (Gadelha et al., 2006).

2.11. Statistical analysis

Values were expressed as means ± standard error of the mean (SEM), and compared by repeated measures analysis of variance (ANOVA), followed by Dunnet's multiple comparison test when appropriate. p values less than 0.05 were considered significant.

3. Results

3.1. Growth assays

All extracts from *Mentha x piperita*, except for the infusion, reduced the in vitro growth of *G. lamblia* trophozoites.

Table 1 shows the IC_{50} of extracts and fractions after 48 h of incubation. The IC_{50} values of metronidazole and furazolidone were determined in our previous work and are also shown as positive control (Campanati and Monteiro-Leal, 2002). The MeOH extract presented the highest inhibitory activity against the multiplication of trophozoites with an IC_{50} of 0.8 μg/ml.

The residual fraction of MeOH extract presented a moderate activity with an IC_{50} value of 45.5 μg/ml and the DCM fraction showed a strong inhibition activity with an IC_{50} of 0.75 μg/ml, as displayed in Table 1 and Fig. 1.

Since the DCM fraction presented the best performance among all other compounds, in the assays described above, all subsequent experiments were performed using only this preparation.

3.2. Video-microscopy

The morphology of untreated trophozoites (control cells) of *G. lamblia* is shown in Fig. 2A. The micrograph shows the characteristic pear-shaped body and other

Table 1
Antigiardial activity of pure extracts and fractions from *Mentha x piperita* Lin.

Compounds	IC_{50} (μg/ml)
Methanolic extract	0.8[b]
Dichloromethane fraction	0.75[b]
Residual fraction	45.5[b]
Dichloromethane extract	2.5[b]
Hexane extract	9.0[b]
Infusion	>100[b]
Metronidazole[a]	0.8[c]
Furazolidone[a]	0.65[c]

[a] Positive control.
[b] Values after 48 h of incubation.
[c] Values after 24 h of incubation.

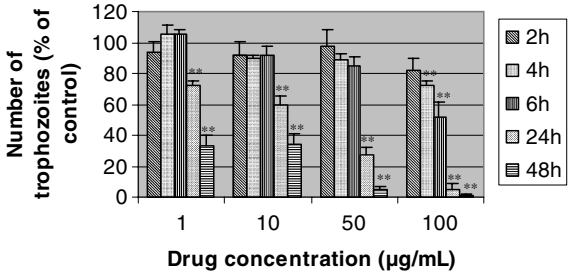

Fig. 1. Inhibition of *Giardia lamblia* growth by the dichloromethane fraction (DCM) from *Mentha x piperita*. The concentrations used were 1, 10, 50 and 100 μg/ml, for 2, 4, 6, 24 and 48 h of exposure. Values are expressed as means and SEM ($n = 6$). $^{**}p < 0.01$.

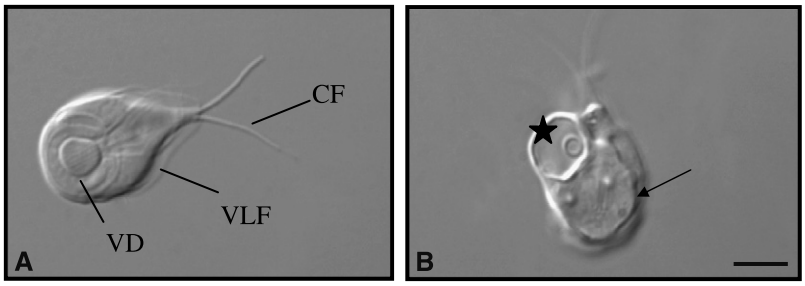

Fig. 2. (A) Control trophozoites of *Giardia lamblia* (untreated parasites) as seen by video-microscopy. VLF, ventro-lateral flange; CF, caudal flagella; VD, ventral disk. (B) Cells treated with 1 μg/ml of dichloromethane fraction (DCM) over 48 h. It is possible to see some protrusions (arrow) and holes on the surface membrane (star). Bar = 5 μm.

structures such as the flagella, the adhesive disk and the ventro-lateral flange.

The addition of the DCM fraction to the culture medium containing trophozoites induced, in these cells, a large spectrum of morphological changes. The modifications started mainly after 6 h of incubation and became more frequent with increasing exposure times and with crescent concentrations of the fraction (data not shown). Within 48 h after the initial addition of the DCM fraction to the culture medium, alterations on the surface membrane of the majority of the trophozoites, such as holes and protrusions, were clearly visible (Fig. 2B).

3.3. Transmission electron microscopy (TEM)

An electron micrograph of control *G. lamblia* is shown in Fig. 3A. The characteristic two nuclei of *Giardia* were evident, as well as its adhesive disk, axonemes of the flagella and peripheral vesicles. Compared to control cells, trophozoites incubated with the DCM fraction presented abnormal morphology, altered plasmatic membrane, increased peripheral vesicles, lamellar body-like structures and abnormal nuclei (Fig. 3B–D). As visible by means of light-microscopy, the dorsal surface of treated trophozoites was affected. In Fig. 3B it is possible to see plasma membrane blebbing and increased peripheral vesicles.

Lamellar body-like structures were seen close to the cellular membrane, apparently inducing the membrane disruption (Fig. 3C). Trophozoites incubated with 100 μg/ml of the DCM fraction, the lamellar structures were observed in close association to the nuclei (Fig. 3D).

3.4. Adherence inhibition assay

A dose–response graphic showing the percentage of parasites adhered to the coverslip, after incubation with the DCM fraction for 6, 24 and 48 h, is displayed in Fig. 4.

The adherence of the parasite was affected with increasing exposure times and fraction concentrations. Only after 48 h of incubation, and for all concentration tested, the decline in the percentage of adhere trophozoites become significant ($p < 0.01$).

3.5. Cytotoxic assay

The DCM fraction did not caused a significant alteration on the final number of treated IEC-6 cells when compared to control cells, and did not induced alterations on the morphology of the cells when they were observed by light-microscopy (not shown).

4. Discussion

The present study describes the antigiardial activity of extracts and fractions from *Mentha x piperita*. The effects were observed affecting the multiplication, adhesion and morphology of trophozoites of *G. lamblia*. Besides that, cytotoxic assays were made with an intestinal cell line and no toxicity was observed.

Four different extracts from dry leaves of peppermint were screened, in attempt to obtain the most effective one. All extracts, except for the infusion, were able to inhibit the multiplication of the trophozoites and, among all, the MeOH extract presented the lowest IC_{50} of 0.8 μg/ml. The further fractionation of the MeOH extract resulted in two other fractions. Of these, the DCM fraction displayed the strongest inhibitory activity, with an IC_{50} of 0.75 μg/ml, after 48 h of exposure.

Other works have also demonstrated an antigiardial activity of a variety of plant species (Harris et al., 2000; Khan et al., 2000; Arrieta et al., 2001; McAllister et al., 2001; Ankli et al., 2002; Calzada et al., 2003, 2005; Sawangjaroen et al., 2005). These papers showed the effects of different plant extracts, fractions or purified compounds, on the multiplication, adhesion and, in some of them, on the morphology of *G. lamblia* trophozoites. In our previous work (Gadelha et al., 2005) we found that the DCM fraction of *Hovenia dulcis* caused a great inhibition effect on the multiplication of *G. lamblia*. The IC_{50} value was 12 μg/ml after 48 h of exposure. Comparing the IC_{50} from the extracts and fractions of *Mentha x piperita* with the IC_{50} from some of the above-cited papers, including ours, one can notice that peppermint presented a better efficacy indicated by its lower IC_{50}.

In attempt to observe the effects of DCM fraction on trophozoite morphology, we performed assays using light-

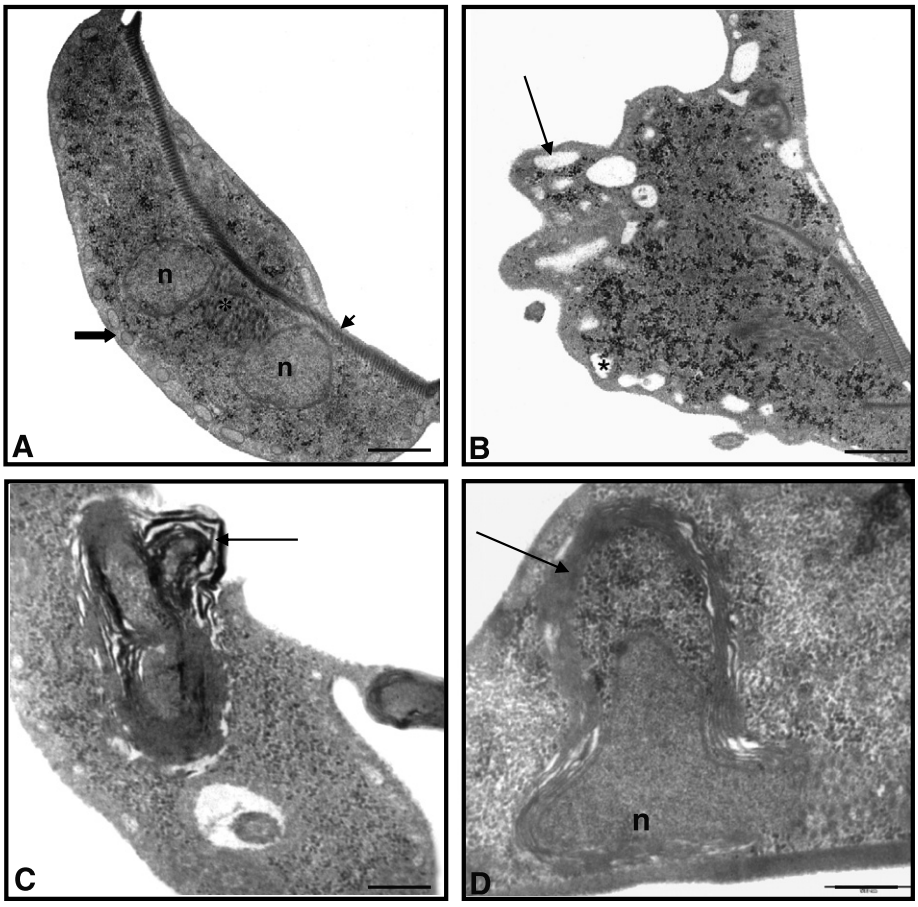

Fig. 3. (A) The general aspect of *G. lamblia* as seen by transmission electron microscopy. The electron micrograph shows, in cross section, the two nuclei (n), three pairs of axonemes from the flagella (asterisk), the adhesive disk (arrowhead), the peripheral vesicles (arrow) and the typical aspect of the cytoplasm. (B and C) Cells grown in the presence of 50 μg/ml of dichloromethane fraction (DCM) for 24 h. (B) The dorsal side of the cell presented an uncommon undulated profile (arrow). Peripheral vesicles were bigger than normal (asterisk). (C) Lamellar body-like structure apparently causing the rupture of the cell membrane (arrow). (D) Trophozoite exhibiting an altered nucleus (n) morphology with a lamellar structure surrounding it (arrow), after incubation with 100 μg/ml of dichloromethane fraction (DCM) for 24 h. Bar in (A–C) = 0.5 μm. Bar in (D) = 0.2 μm.

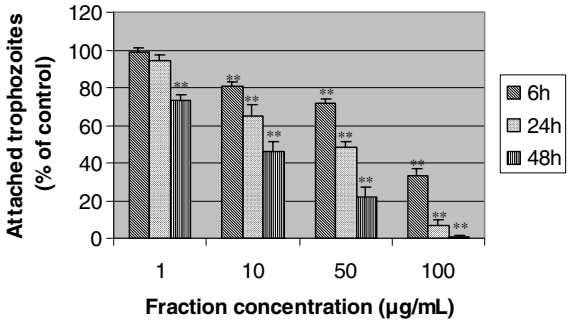

Fig. 4. Inhibition of *G. lamblia* trophozoite adherence over 6, 24 and 48 h by dichloromethane fraction (DCM) from *Mentha x piperita*. Values are expressed as means and SEM ($n = 6$). $^{**}p < 0.01$.

microscopy and transmission electron microscopy. The light-microscopy images showed that the general aspect of the cell was deeply changed, especially in what concerns the cell surface membrane (Fig. 2B). Abnormal structures, such as, protrusions and holes, were commonly observed on the dorsal side of the trophozoite. The possible target of the effective components present in this fraction could be the plasmatic membrane. The exact way in which these compounds were affecting the membrane still needs further investigation.

The ultrastructural modifications of DCM-treated trophozoites included changes of the general aspect, alterations of the surface membrane, increased volume of the peripheral vesicles and the presence of lamellar bodies on the cytoplasm near the plasma membrane and the nucleus (Fig. 3). In some cases, a disruption of the cell membrane was observed, in association with the presence of the lamellar bodies. Comparable lamellar structures were observed in our previous studies, when trophozoites were treated with commercial drugs and with the DCM fraction from *Hovenia dulcis* (Campanati et al., 2001; Campanati and Monteiro-Leal, 2002; Gadelha et al., 2005).

Despite of these large morphological alterations, there were no visible modifications on the adhesive disk or of the axonemes. The stable microtubular-based cytoskeleton of the cell remained intact. However, as derived from the adhesion assays (Fig. 4), concentrations above 10 μg/ml inhibited, in more than 50%, the attachment of trophozoites to glass surfaces, suggesting that other metabolic or

morphological phenomena than those accessible to microscopic analysis could have been affecting the trophozoite's adhesion capabilities.

Mentha x piperita, as other plants, synthesizes numerous classes of natural products such as terpenes, tannins, flavonoids and phenolic acids. Among these compounds, terpenoids have attracted special interest due to the great variety of biological properties displayed by most of them (Ruiz del Castillo et al., 2004). Some examples are the toxic activities over a wide range of gram-positive and gram-negative species of bacteria (Deans and Ritchie, 1987; Panizzi et al., 1993; Sivropoulou et al., 1995; Veličković et al., 2002; Mimica-Dukić et al., 2003; Duarte et al., 2005) and fungi (Panizzi et al., 1993; Mimica-Dukić et al., 2003; Cardenas-Ortega et al., 2005; Duarte et al., 2005). Further investigations are in course in our laboratory, in order to identify the active compound(s) of *Mentha x piperita*, being responsible for the toxic effects on *G. lamblia* trophozoites.

In conclusion, the investigation performed by light and electron microscopy, growth and adherence assays, demonstrated the antigiardial activity of the herb *Menta x piperita*, indicating its potential value for treatment of giardiasis.

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