Evaluation of the therapeutic effect of *Zingiber Officinale* loaded on nanoparticles for cryptosporidiosis in experimentally immunosuppressed mice

Original Article

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ABSTRACT

Background: Cryptosporidiosis causes retractable diarrhea with drastic dehydration, especially in immunocompromised patients. Search for a novel therapeutic approach is urgently required.
Objective: To assess the potential effect of *Zingiber Officinale* (ginger) loaded on chitosan nanoparticles (ginger CSNPs) versus Nanazoxid® (NTZ) in treatment of cryptosporidiosis.
Material and Methods: Seventy mice were immunosuppressed and divided into seven groups (10 mice each), of which two groups were negative and positive control groups. Except for the negative control group, cryptosporidiosis was experimentally induced, and different drug regimens (NTZ, ginger, CSNPs, ginger CSNPs, and NTZ combined with ginger CSNPs) were given for seven consecutive days. Stool samples were collected daily for oocysts count, followed by sacrifice of mice one week after drug administration. Histopathological examination was conducted for small intestine, liver, and lung tissues.
Results: Treatment with ginger CSNPs recorded the highest significant reduction in oocysts shedding, followed by treatment with NTZ/ginger CSNPs combined therapy. Histopathological examination of small intestine showed the best amelioration results in mice treated with ginger CSNPs signified by remarkable improvement in the form of intact intestinal mucosa, with complete healing of intestinal surface epithelium, and with preserved brush border and absence of mucosal ulceration. Histopathologic effect in liver showed various degrees of expansion of portal tracts with moderate inflammation among the treated groups. The lung showed congestion with various degrees of emphysematous changes and inflammatory infiltration among the treated groups.
Conclusion: Ginger CSNPs possessed anti-cryptosporidial activity, diminished the oocysts shedding, and protected the intestinal epithelial from deleterious effects of cryptosporidiosis.

Keywords: chitosan; cryptosporidiosis; ginger; histopathology; immunosuppressed; *in vivo*; nanoparticles.

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INTRODUCTION

Cryptosporidium spp. are coccidian intracellular protozoa causing an important waterborne disease in developing countries. Pathology of cryptosporidiosis is induced by invasion of the apical tip of ileum by sporozoites and merozoites. The disease causes severe life threating acute diarrhea in immunocompromised patients. Autoinfection is commonly reported rendering *Cryptosporidium* spp. unique among other coccidians[1]. Unfortunately, there is no efficient treatment against cryptosporidiosis in immunocompromised patients. The only approved treatment, NTZ, inhibits the parasite anaerobic energy metabolism and the result depends on its efficacy on the host immune status. Thus, it proved to have poor efficacy in patients at high risk of disease, i.e., immunocompromised patients[2]. Moreover, drug development against cryptosporidiosis is challenging owing to the parasite's limited genetic controllability, absence of conventional drug targets, unique intracellular location within the host, and the scarcity of culture cell lines for maintenance and propagation[3]. Nanazoxid® is the only FDA-approved treatment for cryptosporidiosis; however, it has limited efficacy in immunosuppressed patients, and malnourished children[4].

Over the last few years, natural products were used for treatment of several pathogenic infections all over the world. These serve as a very important source of bioactive molecules, including antibiotic, antiviral, and antifungal molecules. Such compounds are often obtained or synthesized from either plants and marine sources, or microbiota[5]. The rhizome of *Zingiber Officinale* (Zingiberaceae), commonly known as ginger is one of the world's most widely used aromatic herbal medicinal treatments. Its variable

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pharmacological activity includes antiprotozoal[6], antioxidant, antineoplastic[7], anthelmintic[8], anti-inflammatory[9], and antimicrobial effects[10]. Additionally, ginger contains diverse bioactive compounds, such as gingerols, shogaols, and paradols with antioxidant and anti-inflammatory properties. Its potential use in treatment of neurodegenerative diseases was attributed to its capability to reduce inflammation and oxidative stress[11]. Generally, the main disadvantage of herbal medicine is its poor bioavailability, and bio-solubility. Therefore, a nano particle (NP) delivery system is required to promote the therapeutic effect[12]. It is worth mentioning that NPs, produced with a wide variety of polymers and nanotechnologies, received considerable attention as potential drug delivery vehicles that can significantly eliminate drug side effects[12]. Additionally, NPs became of significant interest as a drug delivery system due to their small size and large surface area. The small sizes of NPs increase efficacy for accurate intracellular uptake of the drug in the desired cellular targets[13]. Notably, chitosan (CS) is a polysaccharide known to be a favorable pharmaceutical material because of its biocompatibility, biodegradability, low toxicity, and it became an ideal hydrophilic carrier system[14]. The aim of our study is to evaluate ginger CSNPs effects in treatment of cryptosporidiosis in immunosuppressed mice by assessment of parasitological and histopathological parameters.

MATERIAL AND METHODS

This case-control study was conducted at the biological unit of Theodor Bilharz Research Institute (TBRI) during the period from March to May 2022.

Study design: Laboratory mice were immunosuppressed for 14 d before induction of experimental cryptosporidiosis. Different drug regimens were administered after infection, and the survived mice were sacrificed 7 d after drug administration. The effects of drug regimens were evaluated by parasitological and histopathological parameters.

Animal source and handling: Parasite-free laboratory bred female, white Albino mice of CDI strain, ~4–6 weeks old and 20–25 g weight, were obtained from Theodor Bilharz Animal house. Animal experiments were performed in well-ventilated plastic cages with clean wood-chip bedding in conditioned rooms (27°± 2°C) and away from direct sunlight, with good sanitary conditions.

Animal groups: Immunosuppressed mice were divided into 7 groups; 10 mice each. While mice of group 1 were non-infected (negative control), those of group 2 were infected and non-treated (positive control). Mice of groups 3-7 were infected and treated with NTZ, ginger, CSNPs, ginger CSNPs, and NTZ/ginger NPs combination, respectively.

Drugs and doses: Dexazone® tablets (0.5 mg) were provided by Kahira Pharmaceuticals and Chemical Industries Company, Egypt. Ginger tablets (400 mg) were provided by Arab Company for Pharmaceutical and Medical Plants MEPACO-MEDIFOOD, Sharkeya, Egypt. Nanazoxid® suspension (100 mg) was provided by Medizen Pharmaceutical Industries for Utopia Pharmaceuticals, Egypt. The CSNPs were provided by Nanogate Company, Nasr city, 11765, Egypt. They were prepared according to the ionotropic gelation process[15]. Blank NPs were obtained upon the addition of a 2 ml tripolyphosphate (TPP) aqueous solution to a 5 ml CS solution, followed by magnetic stirring at 1000 rpm for one h at room temperature. The CSNPs were separated from aqueous suspension by centrifugation at speed of 20,000 g and temperature of 14°C for 30 min and stored at 4°C.

Oral drugs administration started on the 7th d post infection for seven consecutive days. While ginger was administered in a dose of 100 mg/mouse/day, NTZ was administered in a dose of 65 mg/mouse/day. Drug doses were adjusted according to Paget and Barnes[16].

Preparation of ginger encapsulated CSNPs: Ginger was dissolved in CSNPs prepared solution (10 ml). To prepare a final concentration of 10%; TPP was slowly added into 10 ml solution under magnetic stirring for 20 min[17].

Immunosuppression: All mice were daily administered Dexazone® (0.25 µg/g/day) orally *via* tuberculin tube for two successive weeks before oral inoculation with *Cryptosporidium* oocysts[18] and was maintained weekly till the end of the study[19].

Mice infection: Stool samples were collected from diarrheic calves and were examined by direct and concentration techniques to exclude the presence of any other parasitic infections other than *Cryptosporidium* spp. Oocysts were identified by modified Ziehl Neelsen (MZN) technique[20], preserved in 2.5% potassium dichromate ($K2Cr_2O_7$), and stored at 4°C[21]. Just before use, the oocysts were washed by phosphate buffer saline (PBS), repeatedly concentrated by sedimentation and centrifugation to obtain clear oocysts sediment[22]. Using a pilot study, a dose of 3000 *Cryptosporidium* oocysts established the infection but a further increase to 5000 oocysts caused death of mice, and the lower dose of 2000 oocysts did not cause infection. So, the infection dose for groups 2-7 was 3000 oocysts that were dissolved in 200 ul PBS. Mice were orally infected with *Cryptosporidium* oocysts using tuberculin tube. Stool samples were collected from mice and examined by MZN stain for oocyst shedding one week following infection to ensure infection of all mice[23]. Based on the results of the pilot study, the infection dose was

estimated by calculating the average of three counts of oocysts stained by MZN stain in one ml of stool sediment, i.e., average of cyst counts in 3 different fields in 100 µl stool X 10[23].

Parasitological examination: Before sacrifice, stool samples were collected from mice of each group, pooled, and examined after MZN stain[20] to count oocysts/100 ul in three high power fields. Mean ± SD of oocyst count was calculated and multiplied by 10 to express mean ± SD/gm stool for each group[23].

Histopathological examination: The upper parts of small intestine, liver and lung of surviving mice were excised. One segment (one cm length) from each organ was fixed in 10% formaldehyde. Then, the tissues were processed for paraffin embedding. Histopathological sections of 4 µm thickness were stained with hematoxylin and eosin (H&E)[24] and then submitted to examination microscopically under oil immersion lens (X1000) to assess the pathological changes and cure rates after drug administration.

Statistical analysis: Data were analyzed using IBM SPSS statistics version 22 (IBM corp. Armink, NY). Continuous numerical data were presented as mean ± standard deviation (SD). Between-group differences were compared using one-way analysis of variance (ANOVA) with application of the Tukey-Kramer post hoc test for pairwise comparison. Generalized linear model with gamma and log link was used to examine therapeutic efficacy. Statistical significance was considered at *P* value <0.05.

Ethical consideration: This study was approved by Scientific Research Ethics Committee of Beni-Suef University, Faculty of Medicine on 10/12/2019. All the experiments were carried out according to the Animal Internationally Ethics Procedures and Guidelines.

RESULTS

Parasitological examination for the stools of different study groups: On comparing *Cryptosporidium* oocyst excretion among the studied groups, group treated with ginger CSNPs showed the highest reduction of the mean oocyst count, followed by group treated with NTZ/ginger CSNPs combination, group treated with CSNPs, group treated with ginger then group treated with NTZ, and lastly positive control group. Each group had statistically significant lower oocyst count compared with the positive control group (Figure 1).

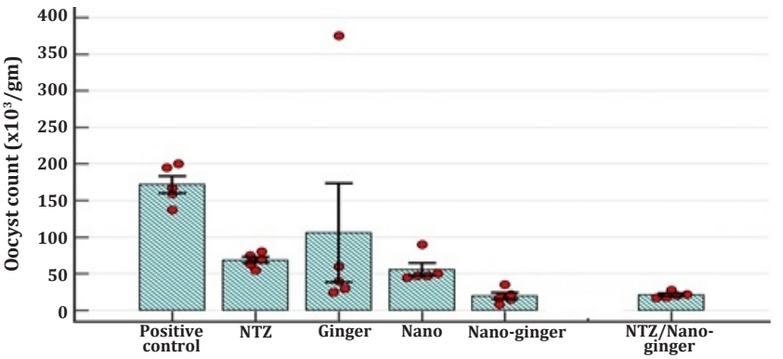


Fig. 1. Mean oocyst count in the studied groups. Error bars represent the standard deviation (SD). **NTZ:** Nanazoxid; **Nano:** chitosan nanoparticles (CSNPs).

Histopathological examination

Intestine: Figure (2) shows the histopathological pictures of small intestinal sections of all groups. Negative control group showed normal villous architecture with average width and length of villi with healthy well-formed brush border and average number of goblet cells (Fig. 2 a, b). Small intestinal sections of the positive control group showed marked mucosal histopathological changes including variable grades of villous architectural changes from shortened broad villi with decreased ratio of villous height to crypt length, up to villous atrophy. Goblet cell depletion, mucosal ulceration, and inflammatory infiltrate of lamina propria were observed. In addition, the section showed inflammatory cells mainly lymphocytes, plasma cells and macrophages with lymphoid follicles hyperplasia (Fig. 2 c, d). *Cryptosporidium* oocysts were detected in the intestinal lumen along the brush border as purple-stained tiny structures (4-6 µm) (Fig. 2d). Groups treated with NTZ, ginger, and CSNPs alone showed partial improvement in the histopathological changes of small intestine with detection of small number of *Cryptosporidium* oocysts in the lumen, and along the brush border (Fig. 2 e, f, and g, respectively). Mice that were treated with ginger CSNPs showed a significant improvement of the histopathological changes in the form of intact intestinal mucosa with complete healing of epithelium and with no *Cryptosporidium* oocysts (Fig. 2h). Mice infected and treated by combined NTZ and ginger CSNPs showed partial healing of intestinal mucosa with partial villous blunting (Fig. 2i).

Hepatic histopathology (Fig. 3 a-g): Normal control group showed preserved architecture (a). Positive control group revealed slightly disturbed architecture with scattered foci of spotty necrosis and interface

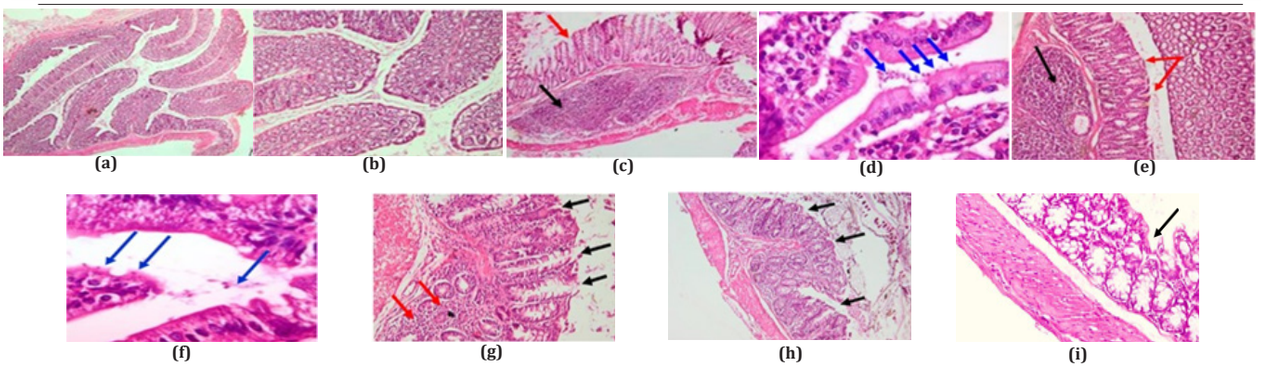


Fig. 2. Small intestinal histopathology (Fig. 2 a-h): Normal control group shows normal villous architecture with normal brush border (H&E stain x 40 and x100) **(a, b)**. Positive control group revealing marked villous blunting with focal ulceration (red arrow), marked mononuclear inflammatory infiltrate, and lymphoid follicle hyperplasia (black arrow) (H& E stain x 100) **(c)**; *Cryptosporidium* oocysts (tiny purple stained structures) in the intestinal lumen and on the mucosal brush border (blue arrows) (H&E stain x 1000 "oil immersion") **(d)**. Section of small intestine in group infected treated with NTZ revealing partial healing of the intestinal villi with villous blunting and some ulcerated areas (red arrows) with moderate inflammatory infiltrate (black arrow) (H&E stain x 100) **(e)**. Few *Cryptosporidium* oocysts detected in the intestinal lumen (blue arrows) (H&E stain x 1000) **(f)**. Mice infected and treated with ginger showing partial healing of intestinal mucosa with partial villous blunting, mild goblet cell depletion and focally ulcerated mucosa (black arrows) and patchy inflammatory cellular infiltration of lamina propria (red arrows) (H&E stain x200) **(g)**. Mice infected and treated by ginger CSNPs showing partial healing of intestinal mucosa with focally ulcerated mucosa, mild goblet cell depletion and mild decrease in the ratio between villous height to crypt length and with partial villous blunting (black arrows) (H&E stain x100) **(h)**. Mice infected and treated by combined NTZ and ginger CSNPs showing partial healing of intestinal mucosa with partial villous blunting (black arrow) (H&E stain x100) **(i)**.

activity. Expanded fibrotic portal tracts with moderate lymphocytic infiltration are detected (b). Infected group treated with NTZ revealing expanded portal tracts with mild inflammation (c). Infected group treated with ginger revealing slightly expanded portal tracts with moderate inflammation (d). Infected group treated by ginger CSNPs revealing slightly expanded portal tracts with moderate inflammation (H&E stain x200) (e-f). Group (7) treated with combined NTZ and ginger CSNPs showed no changes (g).

Lung histopathology (Fig. 4 a-g): Normal control group showing slight congestion, otherwise unremarkable(a). Positive control group showing markedly congested lung with emphysematous change and marked inflammatory infiltrate with foci of suppuration (b). Infected group treated with NTZ revealing slightly congested lung with emphysematous change and significant inflammatory infiltrate (c). Infected group treated with ginger revealing slightly congested lung with emphysematous change and mild inflammatory infiltrate (d). Infected group treated by ginger CSNPs revealing slightly congested lung with emphysematous change (red arrow) (H&E stain x200) (e-f). Infected group treated with combined NTZ and ginger CSNPs showed normal lung pathology (g).

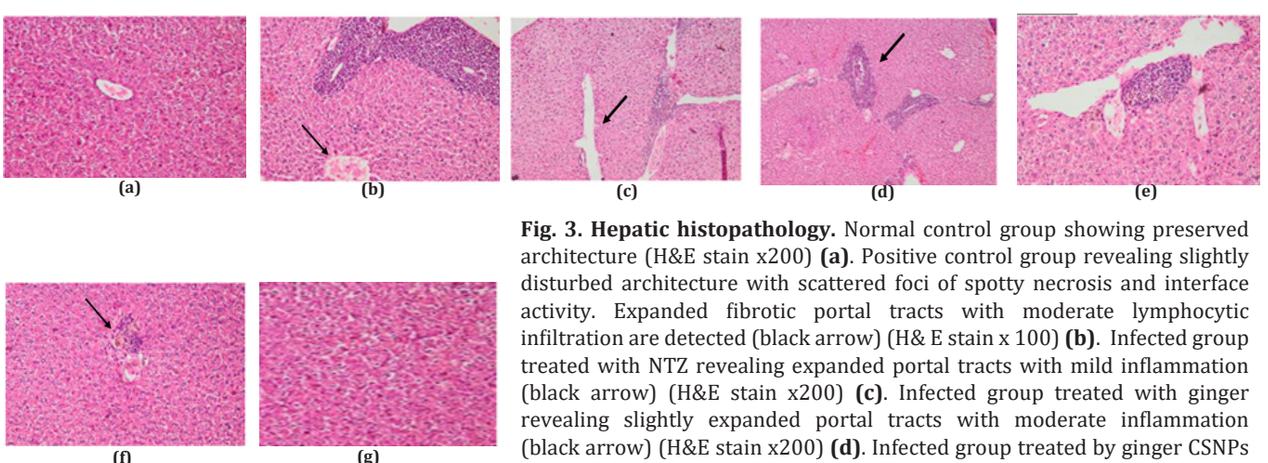
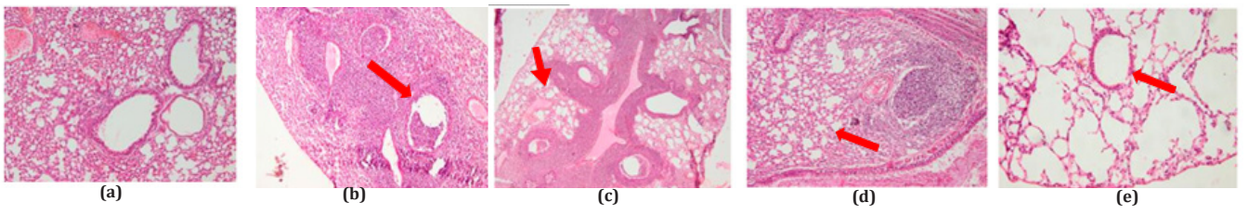


Fig. 3. Hepatic histopathology. Normal control group showing preserved architecture (H&E stain x200) **(a)**. Positive control group revealing slightly disturbed architecture with scattered foci of spotty necrosis and interface activity. Expanded fibrotic portal tracts with moderate lymphocytic infiltration are detected (black arrow) (H& E stain x 100) **(b)**. Infected group treated with NTZ revealing expanded portal tracts with mild inflammation (black arrow) (H&E stain x200) **(c)**. Infected group treated with ginger revealing slightly expanded portal tracts with moderate inflammation (black arrow) (H&E stain x200) **(d)**. Infected group treated by ginger CSNPs revealing slightly expanded portal tracts with moderate inflammation (H&E stain x200) **(e-f)**. Group (7) treated with combined NTZ and ginger CSNPs showed no changes **(g)**.



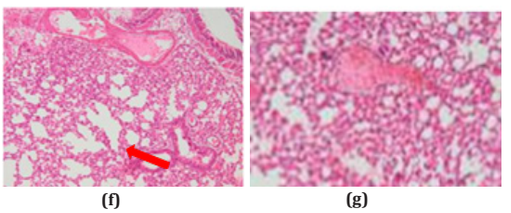


Fig. 4. Lung histopathology. Normal control group showing slight congestion, otherwise unremarkable (H&E stain x 200) **(a)**. Positive control group showing markedly congested lung with emphysematous change and marked inflammatory infiltrate with foci of suppuration (red arrow) (H&E stain x100) **(b)**. Infected group treated with NTZ revealing slightly congested lung with emphysematous change and significant inflammatory infiltrate (red arrow) (H&E stain x100) **(c)**. Infected group treated with ginger revealing slightly congested lung with emphysematous change and mild inflammatory infiltrate (red arrow) (H&E stain x100) **(d)**. Infected group treated by ginger CSNPs revealing slightly congested lung with emphysematous change (red arrow) (H&E stain x200) **(e-f)**. Infected group treated with combined NTZ and ginger CSNPs showed normal lung pathology **(g)**.

DISCUSSION

In the current study, our results were supported by Abouelsoued *et al*.[25] who reported that significant dose-dependent reduction of fecal oocyst count was detected with ginger treatment. High and low doses of ginger extract diminished cryptosporidiosis oocysts count significantly in experimentally infected mice. Similar results about anti-protozoal effects of ginger extracts were recorded against *G. lamblia* trophozoites, *T. gondii* both *in vitro* and *in vivo*, *Trypanosoma* spp. and *Blastocystis* spp.[7]. Our results were also supported by Kara *et al*.[26] regarding *in vitro* use of thyme extract in treatment of cryptosporidiosis. Apparently ginger has the ability to increase digestive fluids and can absorb and neutralize toxins and stomach acid. Moreover, its extract causes inactivation of apoptotic proteins in infected host cells through direct inhibition of the parasite and inhibition of inflammatory cytokine secretion *in vivo*[27]. The present study showed that use of CSNPs with ginger and NTZ was associated with statistically significant lower *Cryptosporidium* oocyst excretion. The nano-technique was also successfully applied[28] using silver (AgNPs) and copper (CuONPs) nanoparticles, resulting in the inactivity of *E. histolytica* cysts and *Cryptosporidiium* oocysts after 180 min of exposure time to both NPs. The authors advocated that AgNps, gold and oxidized metals nanoparticles showed growth inhibitory or cytotoxic effects on various other parasites, including *G. lamblia, Leishmania* spp., *Plasmodium* spp., *T. gondii* and insect larva[28].

Smith[29] studied the AgNPs effect on cryptosporidiosis using a murine model and di-electrophoresis, and found that ionic silver released from the NPs had a modest but measurable effect on cryptosporidiosis infectivity. On the other hand, the use of metal oxide NPs exhibited antimicrobial activity offering the possibility of efficient removal of pathogens from water. It was concluded that NPs may not have pronounced antimicrobial activity when compared to the bulk formulations of the metal oxide or solutions of metal salts. Nevertheless, the characteristic stability and slow release of metal ions from NPs are advantageous[30]. Our histopathological results disagree with El-Shewehy *et al*.[31] who showed that ginger treatment in *Cryptosporidium* infected mice promoted desquamation of the gastric mucosa, and degenerative changes within the parietal cells associated with the presence of oocysts within the epithelial cell and lumen of the gastric glands.

In conclusion, ginger CSNPs NPs possessed anti-cryptosporidial activity, diminishing oocysts shedding and protecting the intestinal epithelium from deleterious effects of cryptosporidiosis. The current study provides another novel, effective, safe, and inexpensive drug therapy alternative. The use of CSNPs apparently increased the efficiency of the standard NTZ chemotherapeutic agents currently used in treatment of *Cryptosporidium* spp. infection especially in immunocompromised individuals.

Author contribution: Zalat RS, Abdelmaksoud HF, Nahas E, Mousa MA contributed in parasitological examination, and data attainment. Elgohary SA performed the histopathological examination. All authors collaborated in writing the original draft, reviewing, editing, and approving the final manuscript.
Conflict of interest: The authors declare that they have no conflict of interest.
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