Efficacy of flaxseed oil and when loaded on ZIF-8 nanoparticles in experimental cryptosporidiosis

Original Article

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ABSTRACT

Background: Nitazoxanide (NTZ) is not effective in treating cryptosporidiosis in immunocompromised individuals with moderate effectiveness in immunocompetent individuals.
Objective: To evaluate the efficacy of flaxseed oil alone or loaded on ZIF nanoparticles (NPs) in *Cryptosporidium*-infected mice.
Material and Methods: This case-control experimental study was conducted on 60 immunocompromised mice equally divided into 6 groups; group 1: non infected non treated, group 2: infected non treated, group 3: infected and NTZ treated (100 mg/kg/day), group 4: infected and flaxseed oil treated (300 mg/kg/day), group 5: infected and treated with a combination of NTZ and flaxseed oil, group 6: infected and treated with flaxseed oil loaded on ZIF-8 NPs. To achieve our objective, oocyst shedding, superoxide dismutase antioxidant enzyme (SOD), oxidative stress marker malondialdehyde (MDA), and intestinal histopathology were investigated.
Results: The best oocyst reduction rate was observed in mice treated with combination of flaxseed oil and NTZ (84.5%), followed by those treated with NTZ (80.4%), treated with flaxseed oil loaded on ZIF-8 NPs (79.0%), and treated with flaxseed oil alone (74.7%). The highest value of SOD and lowest value of MDA were observed in mice treated with combination of flaxseed oil and NTZ, followed by those treated with flaxseed oil loaded on ZIF-8 NPs, and treated with flaxseed oil alone. Histopathologically, all treated groups showed improvement, but the mice treated with combination of flaxseed oil and NTZ showed the best improvement.
Conclusion: Adding flaxseed oil to NTZ improves the outcome of cryptosporidiosis. Moreover, ZIF-8 proved to be a successful drug delivery method.

Keywords: *Cryptosporidium*; drug delivery; flaxseed oil; ZIF-8 nanoparticles; NTZ; oxidative stress markers.

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INTRODUCTION

Cryptosporidiosis is a prevalent cause of diarrhea outbreaks around the world[1] and is ranked second to Rotavirus, as a primary cause of food-borne and water-borne diarrhea in human outbreaks, particularly in immunocompromised patients and children[2]. Although immunocompetent individuals may not exhibit any symptoms, immunocompromised individuals are more prone to persistent and severe cryptosporidiosis[3]. Experimental and epidemiological research generally supports the idea that cryptosporidiosis and cancer are related[4]. Notably, NTZ is now the best treatment for cryptosporidiosis and has demonstrated potential effectiveness against a variety of protozoa and helminths[5]. It is currently utilized to treat adults and immunocompetent individuals with cryptosporidiosis; however, it is ineffective in children and immunocompromised patients[6].

Flaxseed comes from the seed of the flax plant (*Linum usitatissimum*) which belongs to the family Linaceae and genus *Linum*. It is categorized as a natural superfood with many bioactive components and several health benefits[7]. Flaxseed has high protein content accounting for 23% of the entire seed weight, which rises from 35 to 40% following oil extraction[8]. Because of its distinct chemical composition, the health benefits of flaxseed oil are well established. It is an abundant source of polyunsaturated Omega-3 fatty acids of plant origin (39.35–60.11% α-linolenic acid, 15.89–26.21% oleic acid, 12.09–17.45% linoleic acid, 6.19–11.64% palmitic acid, and 4.67–7.68% stearic acid)[9]. As an anti-inflammatory agent, α-linolenic acid is widely utilized because it reduces the synthesis of inflammatory cytokines, lipoproteins and lipids[7]. Additionally, it exhibits antimicrobial effects through the suppression of bacterial enoyl-acyl carrier protein reductase[10]. Flaxseed oil has several positive health impacts, such as lowering cholesterol and lowering

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the risk of cardiovascular and cancer disorders[8]. Its anticancer properties were established by detecting the direct inhibition on cancer cell growing *in vitro*[11]. Flaxseed oil also has cyclic peptides known as cyclolinopeptides which exhibit antimalarial and antifungal properties[12]. It also exhibits an antioxidant action as it is richly endowed with many antioxidants like polyphenols, tocopherols, phytosterols, flavonoids, and beta-carotene[13].

Nanotechnology has effective valuable applications in various fields including diagnosis, drug delivery, gene therapy, and treatment[14]. Combining several components in a single nanostructure is one of the most amazing features of nanotherapeutics. Metal-organic frameworks (organic inorganic hybrid porous materials) have been widely investigated as perfect nanocarriers for loading many drugs because of their highly porous structure, very large surface area, easy chemical modification and low toxicity. Zeolite imidazolate frameworks (ZIF-8) are a subclass of metal-organic frameworks which have been widely studied[15]. They are made up of the pharmacologically active imidazole linker and the bioactive zinc metal ion (Zn^{2+}). Besides, ZIF-8' structure is flexible and stable with a wide surface area[16]. The aim of the present study is to assess the efficacy of flaxseed oil either alone or loaded on ZIF-8 NPs in *Cryptosporidium*-infected mice.

MATERIAL AND METHODS

This case-control experimental study was carried out between March 2024 and October 2024 at Parasitology Department, Faculty of Medicine, Benha University, and Zoonotic Diseases Department, National Research Institute, Giza, Egypt.

Study design: Swiss albino mice were infected with *Cryptosporidium* oocysts after seven days of immunosuppression. Seven days post infection (dpi), mice were treated orally for one week by NTZ, flaxseed oil, combination of NTZ plus flaxseed oil, and flaxseed oil loaded on ZIF-8 NPs. The mice were sacrificed 24 h after the last dose of treatment. Oocyst shedding, antioxidant enzyme SOD, oxidative stress marker MDA, and intestinal histopathology were investigated.

Parasites: *Cryptosporidium* spp. oocysts were obtained from a strain maintained in mice at the National Research Center's Department of Zoonotic Diseases in Giza, Egypt. A modified Ziehl-Neelsen stain was utilized to identify the oocysts, which were extracted from naturally infected calf stool samples using concentrated Sheather's sugar solution. At 4°C, the collected oocysts were preserved in a 2.5% potassium dichromate solution. Prior to inoculation, the infecting dose of oocysts in phosphate buffer saline (PBS) solution was adjusted to $3x10^3$ oocysts/ml using a hemocytometer[17].

Experimental animals: Sixty male Swiss albino mice (20–25 g and two weeks of age) were utilized in this study. National Research Centre in Dokki, Egypt provided us with the animals which were maintained under control with water and pellet food. Their bedding was changed every day while their cages had perforated lids and were ventilated. The globally recognized guidelines were followed for conducting the animal experiment.

Mice immunosuppression: Before inoculation with *Cryptosporidium* oocysts and until the end of the experiment, the mice were inoculated with corticosteroids for immunosuppression (0.5 mg Dexazone tablets, Al Kahira Pharmaceutical and Chemical Industries Company, Egypt) at a dose of 0.25 µg/g body weight/day for seven days[18].

Mice infection: All mice, with the exception of group (1), received gastric gavage of 0.1 ml of the oocyst inoculum ($3x10^3$ oocysts/ml) using a plastic tube at the tip of a 25-gauge needle[17].

Drug administration: Beginning on the 7th dpi, the treated mice groups received the treatment orally through an oesophageal tube for seven days. Flaxseed oil was acquired from the Agricultural Research Center in Giza, Egypt, and administered as 300 mg/kg/day[19]. Nitazoxanide was provided as Nanazoxid 100 mg/5 ml powder for oral solution following reconstitution (Utopia Pharmaceuticals). Administration dose was 100 mg/kg/day[20].

Preparation of ZIF-8: All of the chemicals were analytical grade and purchased from Sigma (Ronkonkoma, NY 11779, USA), including methyl alcohol, zinc nitrate [Zn $(NO_3)^2$], methylimidazole (Hmim), sodium hydroxide (NaOH), and hydrochloric acid (HCl). Methanol (100 ml) was used to dissolve 2-methylimidazole (Hmim, 4.10 g, 50 mmole). This solution was then added to a lignin-containing methanol solution of Zn $(NO_3)^2 \cdot 6H_2O$ (2.97 g, 10 mmole). The mix solution was left to stand for 24 h at room temperature with continuous stirring followed by centrifugation to separate the final solids. These were then cleaned with methanol and deionized water and then dried under reduced pressure for 24 h at 60°C[21].

Drug loading: For loading the drug and ZIF-8 NPs, ethanol (100 ml) was mixed with varying amounts of flaxseed oil and NTZ (100–1000 ppm). Drug solutions were mixed with 1 g of ZIF-8 NPs for 90 min at room temperature using a magnetic stirrer set to 600 rpm. The solution was then allowed to stand for the entire night. After that, the suspension was centrifuged at 5,000 rpm for five minutes to separate the precipitate and supernatant. The amount of loaded NTZ and flaxseed oil was determined by comparing their concentration in the solution before and after loading. The NTZ and flaxseed oil loading percentage was

calculated according to the equation: Drug loading percentage = [(A−B)/A]×100; where A and B are the initial and final NTZ and flaxseed oil concentration of the solution[22].

Characterization of ZIF-8: The phase purity and crystallinity of the materials were determined using X-ray diffraction (XRD) patterns (X'Pert MPD Philips diffractometer; the used monochromated was Cu Kα). The materials' nanostructure morphology was verified by scanning electron microscope (SEM: Hitachi SU-70, JP)[23].

Study groups: Six groups of immunocompromised mice were included; group 1: non infected non treated (control negative), group 2: infected non treated (control positive), group 3: infected and treated with NTZ (100 mg/kg/day) (drug control), group 4: infected and treated with flaxseed oil (300 mg/kg/day), group 5: infected and treated with combination of 100 mg/ kg NTZ and 300 mg/kg flaxseed oil, and group 6: infected and treated with flaxseed oil loaded on ZIF-8 NPs. Mice were sacrificed at the end of the experiment, 24 h after the final dose of the drugs.

Parasitological evaluation: Stool samples were collected on 1st and 7th day of treatment to count oocysts floated in Sheather's sugar solution. They were identified by modified Ziehl-Neelsen staining. The amount of oocyst in each group was expressed as mean±SD. Reduction rate was calculated as follows: R% is equal to 100 × (mean number of oocysts in the infected control group minus mean number in the treated group)/mean number of oocysts in the infected control group[17].

Oxidative stress and antioxidant markers evaluation: A colorimetric method[24,25] was used to detect the levels of the antioxidant enzyme SOD, and the serum oxidative stress marker MDA. Both kits were obtained from Biodiagnostic, Dokki- Giza, Egypt.

Histopathological evaluation: At the end of experiment, samples from various small intestinal sections of mice in each group were excised, and immediately preserved in 10% formalin. Dehydration, clearing, paraffin embedding, sectioning at 4 μm, and hematoxylin and eosin (H&E) staining were all performed on the fixed samples[26].

Statistical analysis: The SPSS software version 19 was used to analyze the data. The significance between all groups was determined by one way ANOVA test, but independent t-test was used between every two groups. *P* values less than 0.05 were statistically significant.

Ethical consideration: The study was ethically accepted by Research Ethics Committee, Faculty of Medicine, Benha University, Egypt (Code number: RC1122024).

RESULTS

Characterization of flaxseed oil@ ZIF-8: The SEM image of flaxseed oil@ ZIF-8 showed cubic-like morphology and size ranges of approximately 80–150 nm (Fig. 1). X-ray diffraction (XRD) measurements were used to analyze the phase purity and crystallographic structure of flaxseed oil@ZIF-8. The XRD spectra of flaxseed oil@ZIF-8 showed peak positions with different intensities of diffractions (Fig. 2). These intense diffraction peaks indicated good crystallinity.

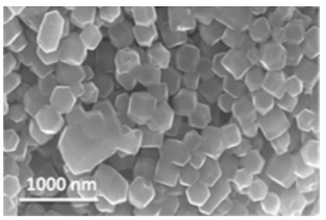

Fig. 1. SEM micrograph of flaxseed oil@ZIF-8.

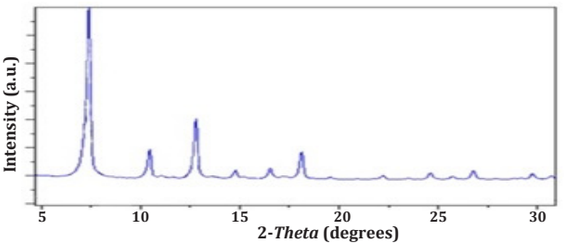

Fig. 2. The PXRD pattern of flaxseed oil@ ZIF-8.

Parasitological evaluation: There was significant reduction in oocyst count after seven days of treatment in every treated group in comparison with infected non treated group (P<0.001). The group that received flaxseed oil plus NTZ combination showed the lowest oocyst count (47.0±27.30) with the best reduction rate (84.5%). Reduction in oocyst count was recorded in group 3 that received NTZ (59.5±23.97), followed by group 6 receiving flaxseed oil loaded on ZIF-8 NPs (63.5±21.09), then group 4 receiving flaxseed oil alone (76.5±20.96) (Table 1, Fig. 3).

Oxidative stress and antioxidant markers evaluation: There was increase in the antioxidant enzyme SOD in each treated group. The group receiving combination of (flaxseed oil plus NTZ) showed the highest value of SOD (103.59±10.13), followed by the group receiving flaxseed oil loaded on ZIF-8 NPs (97.77±7.21), then the group treated with flaxseed oil alone (91.00±3.51). The lowest value of SOD was observed in the group treated with NTZ alone (87.34 ±5.57) (Table 2). There was decrease in MDA level in all treated groups. The best result was observed in mice group treated with flaxseed oil plus NTZ combination

where the percent of reduction was 76.3%, followed by mice group treated with flaxseed oil loaded on ZIF-8 NPs with percent of reduction 37.0%, then mice group treated with flaxseed oil alone with percent of reduction 30.8%. The minimum reduction was obtained in the group treated with NTZ alone where the percent of reduction was 20% (Table 3).

Histopathological evaluation: Histopathological examination of intestinal tissue sections showed small intestinal villi with normal architecture and average width and length in negative control group; while the positive control group showed hyperplasia in epithelium of intestinal villi with villous architectural loss, villous atrophy, and villous shortening and widening. While the treated groups showed improvement in the histopathological alterations in the form of decrease in the parasite load at the brush borders and in the inflammatory cells' infiltration. The group treated with flaxseed oil plus NTZ combination showed the best improvement in the form of restoring the normal villus pattern, and no parasites at the brush borders with minimal inflammatory cells (Fig. 4).

Table 1. Assessment of oocyst shedding and reduction% (R%) on 7th and 14th dpi among the study groups.

Groups	7th dpi		14th dpi		Statistical analysis 7th versus 14th
	Oocyst shedding Mean±SD	R%	Oocyst shedding Mean±SD	R%	
G1	-	-	-	-	-
G2	285.5±39.75	-	302.9±31.79	-	0.321
G3	270.0±21.60	5.4	59.5±23.97	80.4	< 0.001*
G4	280.0±27.18	1.9	76.5±20.96	74.7	< 0.001*
G5	258.0±19.32	9.6	47.0±27.30	84.5	< 0.001*
G6	267.0±24.06	6.5	63.5±21.09	79.0	< 0.001*
Statistical analysis Versus G2	*P* = 0.849 (G3), 1.00 (G4), 0.211 (G5), 0.698 (G6)		*P* < 0.001* (G3), < 0.001* (G4), < 0.001* (G5), < 0.001* (G6)		

G1: Non infected non treated (control negative); **G2:** Infected non treated (control positive); **G3:** Infected and treated with NTZ (drug control); **G4:** Infected and treated with flaxseed oil; **G5:** Infected and treated with NTZ and flaxseed oil; **G6:** Infected and treated with flaxseed oil loaded on ZIF-8; ***:** Significant (*P*≤0.05).

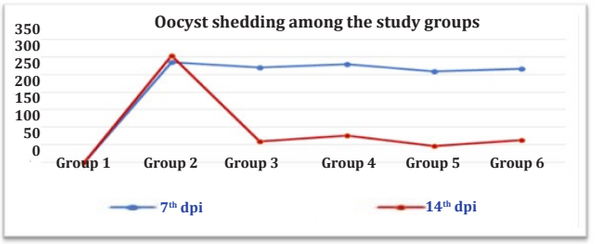

Fig. 3. Assessment of oocyst shedding on 7th and 14th dpi among the study groups.

Table 2. Assessment of SOD among the study groups.

Groups	SOD (u/ml)		Statistical analysis Versus G2
	Mean±SD	Change from group II (%)	
G1	115.87 ± 11.07	--	<0.001*
G2	83.83 ± 6.81	--	--
G3	87.34 ± 5.57	-4.2	0.985
G4	91.00 ± 3.51	-8.6	0.597
G5	103.59 ± 10.13	-23.6	<0.001*
G6	97.77 ± 7.21	-16.6	0.014*

G1: Non infected non treated (control negative); **G2:** Infected non treated (control positive); **G3:** Infected and treated with NTZ (drug control); **G4:** Infected and treated with flaxseed oil; **G5:** Infected and treated with NTZ and flaxseed oil; **G6:** Infected and treated with flaxseed oil loaded on ZIF-8; ***:** Significant (*P*≤0.05).

Table 3. Assessment of SOD among the study groups.

Groups	MDA (mmol/l)		Statistical analysis Versus G2
	Mean±SD	Change from group II (%)	
G1	1.70 ± 0.41	--	<0.001 *
G2	17.97 ± 7.34	--	--
G3	14.37 ± 6.43	20.0	0.661
G4	12.43 ± 5.56	37.0	0.145
G5	4.25 ± 0.94	30.8	<0.001*
G6	11.33 ± 3.52	76.3	0.039*

G1: Non infected non treated (control negative); **G2:** Infected non treated (control positive); **G3:** Infected and treated with NTZ (drug control); **G4:** Infected and treated with flaxseed oil; **G5:** Infected and treated with NTZ and flaxseed oil; **G6:** Infected and treated with flaxseed oil loaded on ZIF-8; ***:** Significant (*P*≤0.05).

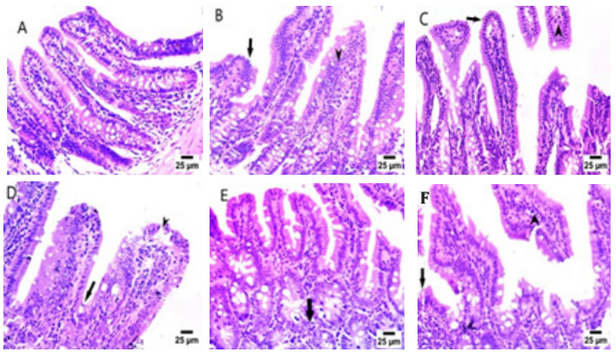

Fig. 4: Histopathology of small intestinal sections (H&E stain): **(A)** Normal villus pattern with no pathological lesions (negative control group). **(B)** Hyperplasia in epithelium of intestinal villi (arrow head) with degenerative changes in epithelium of some villi (arrow) (positive control group). **(C)** *Cryptosporidium* developmental stages at the villi's brush border (arrow), and sloughing of some villi's upper tips (arrow head) (NTZ treated group). **(D)** *Cryptosporidium* developmental stages at the villi's brush border (arrow), presence of degenerative changes in epithelium of some villi (arrow head) (flaxseed oil treated group). **(E)** Infiltration of intestinal mucosa by mononuclear inflammatory cells (arrow) (flaxseed oil and NTZ treated group). **(F)** *Cryptosporidium* developmental stages at the villi's brush border (arrow) with sloughing of some villi's upper tips (arrow head) (flaxseed oil@ZIF-8 treated group).

DISCUSSION

Although NTZ is the most common therapeutic drug in treatment of cryptosporidiosis, it exhibited dose-dependent toxicity in addition to its poor efficacy in children and immunocompromised patients[27]. Therefore, the aim of this study is the assessment of the efficacy of flaxseed oil either alone or loaded on ZIF-8 NPs in *Cryptosporidium*-infected mice. To our best knowledge, our study is the first to investigate the influence of flaxseed oil on experimental cryptosporidiosis.

In our research, adding flaxseed oil to NTZ resulted in the least number of oocysts count with reduction percentage 84.5%. Guillaume *et al*.[28] claimed that the *in vitro* toxic effect of phospholipases A2 against *P. falciparum* resulted from generating arachidonic acid and other polyunsaturated fatty acids (PUFAs) that were harmful to the parasite. Zhang *et al*.[29] recommended fatty acid supplements in immunocompromised patients to inhibit toxoplasmosis as the investigators found that giving fish oil to experimentally infected mice led to impairment of fatty acid metabolism in the parasites. Choi *et al*.[30] suggested that omega-3 polyunsaturated fatty acids (ω3PUFAs) were essential in reducing toxoplasmosis both *in vitro* and *in vivo* through triggering autophagy. El-Beshbishi *et al*.[31] reported that adding ω-3PUFAs to artemether significantly reduced liver and spleen indices, worm count, egg burdens, granulomas count and diameter, hepatic nitric oxide and serum interleukin-4 level. Górniak *et al*.[32] noticed that adding flaxseed oil to horses' food infected with Strongylidae significantly reduced the worm burden in fecal samples.

Additionally, Al-Khalaifah *et al*.[33] reported that PUFAs can improve macrophages' phagocytic capacity. Cucchi *et al*.[34] reported that PUFAs are immunomodulatory as they are able to stimulate endogenous cytokines production in response to bacterial, viral, fungal, and parasitic infections. In addition to fatty acids, a study showed that cyclolinopeptides, a class of hydrophobic peptides found in flaxseed oil, are responsible for the oil's antimicrobial properties[35].

In our study, loading flaxseed oil on ZIF-8 NPs increased its efficacy. This agrees with the documented increased potential efficacy of different drugs when loaded on ZIF-8 NPs. Jermy *et al*.[36] reported significant reduction of *Blastocystis* cysts after using ZIF-8/curcumin and ZIF-8/resveratrol. ZIF-8 has promising physicochemical characters including its pore size, allowing for maximum drug loading and effective controlled release[37]. It also has biocompatible properties and thermal and hydrothermal stability[38]. Ali *et al*.[39] reported that ZIF-8 NPs improved the effect of NTZ and *E. purpurea* in experimental cryptosporidiosis. Jiang *et al*.[40] recorded improvement of antigiardial effect of dihydroartemisinin when loaded on ZIF-8 NPs.

Cryptosporidiosis causes damage of intestinal mucosa stimulating leukocytes and activating processes leading to reactive oxygen species (ROS) production as hydrogen peroxide, nitric oxide, and superoxide radicals[41]. Moreover, ROS cause cellular injury and decrease the availability of antioxidants such as vitamins (A, C, and E) or glutathione which can increase free radicals' production and peroxidation of lipids. Oxidative stress, which results from ROS formation and antioxidant system suppression, causes oxidative changes of cell components, primarily lipids that produce MDA[42].

According to our results, a significant increase in the SOD antioxidant enzyme and a significant decrease in MDA oxidative stress marker were observed in mice receiving flaxseed oil. This is in harmony with the report of Huang *et al*.[43] who claimed that flaxseed polyphenol extracts preserved cell vitality and morphology by significantly decreasing ROS, GSSG (oxidized glutathione) and MDA levels, while raising CAT (catalase enzyme) and SOD activity. Such antioxidant activity of flaxseed oil depends on its chemical constituents. Tocopherols, polyphenols, phytosterols, beta-carotene, and flavonoids are among the many antioxidants found in flaxseed oil. Alpha-tocopherol is the best peroxyl radicals scavenger in phospholipid bilayers, protecting cells from ROS induced lipoperoxidative damage[13]. The best result was observed in mice group receiving combination of flaxseed oil and NTZ. This is probably

due to NTZ augmented antioxidant activity of flaxseed oil causing eradication of more parasites and thus decreasing oxidative stress due to infection.

Regarding histopathological evaluation, intestinal sections from the infected mice typically revealed villous widening and shortening, moderate to severe inflammation, and decrease of goblet cells. These results agree with Dragomirova[44] who reported villous atrophy and intestinal inflammatory infiltration as a result of cryptosporidiosis. Nevertheless, all treated mice showed improvement in the intestinal pathological alterations. According to our current findings, the group that received NTZ plus flaxseed oil combination presented the best improvement in the intestinal pathological alterations among all the treated groups. This finding may be explained by the abundance of omega-3 PUA (α-linolenic acid) in flaxseed oil. A study with animal models and humans complaining of colitis and inflammatory bowel disease, illustrated the effects of α-linolenic acid on INF-γ, IL-6, TNF-α, NF-κB and intestinal permeability[45]. Another possibility may be that the above-mentioned improvement in intestinal inflammatory alterations is due to the combined action of NTZ and flaxseed oil on oocyst clearance. In conclusion, flaxseed oil has anti-*Cryptosporidium* and anti-oxidant activities. Furthermore, ZIF-8 proved to be a successful drug delivery system.

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