

# The Effects of Commercially Available *Syzygium aromaticum*, *Anethum graveolens*, *Lactobacillus acidophilus* LB, and Zinc as Alternatives Therapy in Experimental Mice Challenged with *Cryptosporidium parvum*

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**Background:** Waterborne cryptosporidiosis is the second cause of diarrhea in young children and immunocompromised hosts after rotavirus. Except for nitazoxanide (NTZ), there is no accredited cryptosporidiosis treatment to date. Therefore, there is an urgent need to find an effective and safe treatment for cryptosporidiosis. This study aimed to investigate the possible anti-protozoal effects of *Syzygium aromaticum* (clove) oil, *Anethum graveolens* (dill) seeds oil, *Lactobacillus acidophilus* LB, and zinc against *Cryptosporidium parvum* in comparison to NTZ.

**Methods:** Besides the negative control, mice from six experimental groups (T1–T6) were infected with *Cryptosporidium parvum* oocysts. On the seventh day post-infection (PID), mice from five groups were treated for 8 consecutive days with NTZ, clove oil, dill seed oil, *Lactobacillus acidophilus* LB, and zinc commercial forms (T2–T5). Oocysts shedding rate, differences of mice body weight, serum IL10, and TNF- $\alpha$ , cryptosporidial antigen, and cd3 at the intestinal mucosa were evaluated at the end of the experiment.

**Results:** The mean of the *C. parvum* oocysts' shedding rate was significantly lower in all treated groups than in the non-treated group. The oocysts reduction rate was the highest in zinc-treated mice (98.3%), *Lactobacillus acidophilus* LB and dill-treated groups (95.77%), and the NTZ-treated group (91.55%). Clove oil was the least effective, with a 74.65% reduction rate. Excluding the clove oil-treated group, immunohistochemical analysis revealed the clearance of the *Cryptosporidium* antigen in the intestinal tissue in all treated groups.

**Conclusion:** The study has provided a rational basis for using these safe, cheap, and commercially available alternatives in treating cryptosporidiosis combined with NTZ.

**Keywords:** *Cryptosporidium parvum*, *Syzygium aromaticum*, *Anethum graveolens* seeds oil, *Lactobacillus acidophilus* LB, zinc, nitazoxanide

## Plain Language Summary

Cryptosporidiosis is one of the most worldwide distributed neglected tropical diseases and is difficult to be treated in immunocompromised hosts. The current study tried to find out effective, safe, available, cheap, and natural therapeutic alternatives. Clove oil, dill seed oil, *Lactobacillus acidophilus* LB probiotic, and zinc commercial forms were tested in cryptosporidiosis infected animals. All tested alternatives greatly reduced the *Cryptosporidium*

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oocysts shedding rate. The oocysts reduction rate was the highest in zinc-treated mice, while it was the lowest in the clove oil-treated group. Dill, *Lactobacillus* spp., zinc, and NTZ cause 100% clearance cryptosporidial infection from intestinal mucosal tissue. The study provides a rational foundation to use the four alternatives in treating cryptosporidiosis together with NTZ.

## Introduction

*Cryptosporidium*, an apicomplexan parasite, is a major human parasite in the last and early part of the current century. Water is an important environmental transmission route for *Cryptosporidium* spp.<sup>1</sup> Treating infected waters and patients costs developed countries a large amount of money annually. Infected water consumption was responsible for most of the cryptosporidiosis outbreaks recorded worldwide.<sup>2</sup> There is little information on *Cryptosporidium* in Egyptian drinking water. However, available data show variable prevalence rates of between 3% and 100%.<sup>3–7</sup> The results may vary depending on different diagnostic techniques, sample sizes, and geographic locations.

Watery diarrhea and malabsorption are the most common clinical forms of the parasite. The parasite targets epithelial cells in the small intestines of humans and animals. The severity of the clinical manifestation increases in young children and immunocompromised individuals, leading to long-lasting diarrhea, considerable deformation of the intestinal epithelium, and malnutrition.<sup>8</sup> In contrast, a good immune system in immunocompetent hosts ends up with self-limiting signs and symptoms.

Histological changes caused by intestinal cryptosporidiosis are non-specific, but they include dampening of villi, hyperplasia of intestinal crypt cells, and inflammatory cells infiltration into the luminal propria. All previous changes are associated with the intensity of *C. parvum* infection. However, the pathophysiological mechanisms underlying intestinal *C. parvum* infection are not well understood. Studies discussing parasite pathogenesis are limited, and all are sourced from experimental animal studies.<sup>9</sup>

To date, there is no accredited efficient treatment for cryptosporidiosis. Infected hosts eradicate the parasite through innate and acquired immunity.<sup>10</sup> Therefore, the hypothesis that cryptosporidiosis might be controlled by the administration of natural commercially available products came to the surface recently. Modulation of innate and acquired immunity at mucosal and systemic levels is

the supposed target mechanism by which they overcome cryptosporidial infection.<sup>11,12</sup>

In recent decades, medicinal plants have gained greater recognition due to the awareness that these plants have lesser side effects and improved efficacy than their synthetic counterparts.<sup>13</sup> Several studies documented variable degrees of antibacterial, antiviral, anticarcinogenic, antifungal, and antiparasitic activities of different herbs, including cinnamon, oregano, clove, thyme, dill, garlic, black seed, and mint.<sup>14,15</sup> However, *Syzygium aromaticum*, popular as clove and *Anethum graveolens* known as dill, gained much attention among other spices due to its strong antimicrobial, including antiparasitic and antioxidant properties.<sup>16,17</sup> Both herbs are cultivated widely worldwide. They have a very long history of use, going back to Ancient Egypt. *Syzygium aromaticum* and *Anethum graveolens* seeds essential oil and extracts exerted anti-inflammatory activity against various microorganisms, including *Entamoeba histolytica* and *Helicobacter pylori*.<sup>13,18</sup>

Nitazoxanide (NTZ) is used to treat cryptosporidiosis. It has a broad-spectrum parasitocidal activity approved by the US FDA for treating diarrhea in both children and adults.<sup>19</sup>

Several probiotics, mainly *Lactobacillus* strains, have been investigated for cryptosporidiosis treatment and prevention. According to Travers et al, the possible mechanisms of action of *Lactobacillus* include modulating the intestinal environment by competing with pathogens (nutrients, adhesion site, etc), improving barrier function, and modulating peristalsis and mucus secretion, which favor beneficial microflora. In addition, probiotics can also modulate host immunity by stimulating CD4<sup>+</sup> differentiation, stimulating Th1/Th2 response; cytokines induction (IL10, IL12, IFN $\gamma$ ); stimulation of IgA producing cells and IgA secretion.<sup>20</sup>

Although zinc supplementation proved beneficial in managing diarrhea, its effects on intestinal cryptosporidial infection remain unknown. Therefore, whether the benefits of zinc supplementation in diarrhea are similar to cryptosporidial infection remains unclear. The expected possible effects of zinc in *Cryptosporidium* spp. infection arises from the famous role of zinc in priming the immune system and tissue repair.<sup>21</sup> Epithelial cell and tissue integrity are maintained partially by trace nutrients as zinc promotes cell growth and suppressing apoptosis; thus, it may protect against *Cryptosporidium* infection.<sup>22,23</sup>

The study aimed to investigate the effects of four naturally commercially available products, including *Syzygium aromaticum*, *Anethum graveolens* seeds oil, *Lactobacillus acidophilus* LB, and zinc to treat cryptosporidiosis in *C. parvum* challenged mice compared to NTZ-treated mice.

## Materials and Methods

### Ethics Concerns

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University (approval no. 17300385), following the internationally accepted principles for laboratory animal use and care, under the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS).<sup>24</sup>

### The Experimental Animal Models

Forty-two pathogen-free Swiss albino male mice, 8 weeks old with 32 grams mean weight, were used in the study.

### The Parasite

*Cryptosporidium parvum* oocysts were isolated from positive water samples collected from the local community drinking water supply according to the previous recommendation of Hassan et al using a specially designed filter system.<sup>25</sup> Discontinuous sucrose gradient flotation was used to isolate and purify the parasite.<sup>26</sup> Water samples were subjected to molecular characterization using nested polymerase chain technique (nPCR).<sup>25</sup> Briefly, DNA extraction was performed by FavorPrep DNA Isolation Mini Kit (Favorgen Biotech Co., Taiwan) following the manufacturer's instructions then amplified, using two sets of primers as shown in Table 1. *Cryptosporidium* oocyst wall protein (COWP) fragments digestion was determined by electrophoresis in 3.2% agarose gels comprising ethidium bromide, thereafter, fragments visualization was done under UV light to explore the *Cryptosporidium* genotype.

### The Treatment Forms

#### Nitaxanide Preparation

Mice received Cryptonaz (Copad Pharma, Egypt) commercially available as 100 mg/5 mL in a 60-mL syrup bottle.

#### *Syzygium aromaticum* Oil

Clove plant oil in a 30-mL commercial container was available as Zongle USDA Certified Organic Clove Essential Oil (Zongle Therapeutics, Georgia, USA).

#### *Anethum graveolens* Oil

The commercial form of pure dill seed oil was supplied in a 30-mL container by Hemani Award (Dubai, United Arab Emirates).

#### *Lactobacillus acidophilus* LB

A commercial form of *Lactobacillus acidophilus* LB from human origin was provided as Lactéol Fort 340-mg sachets each containing 10 billion *L. acidophilus* LB (Axcan Pharma, Egypt).

#### Zinc Supplements

It was supplied as zinc sulfate preparation in the commercial form as zinc Origin 10 mg/5 mL (Origin International Pharma, Egypt).

### Design of the Experiment

Seven main different experimental conditions were evaluated. Each contained six mice.

Group C: Negative control group: None infected non-treated group.

Group T: Includes six sub-groups. Each group contained six mice. All mice in these groups were infected orally with *C. parvum* oocysts on the first PID. Following the recommendation of Sayed et al, the infectious dose was adjusted to contain approximately 600 oocysts.<sup>7</sup>

T1 group contained six mice; each received the *C. parvum* infectious dose on the first PID. No treatments were given.

T2 group: Infected and treated with NTZ in a dose of 200 mg/kg/day orally on the seventh PID and for 8 consecutive days twice daily.<sup>27</sup>

T3 group: Infected and treated with clove oil, 33 mg/kg was given on the seventh PID and for 8 consecutive days, once daily.<sup>28</sup>

T4 group: Infected and treated with dill seed oil at a dose of 20 µL twice a day on the seventh PID and for 8 consecutive days, once daily.<sup>29</sup>

T5 group: Infected on the first PID and treated with  $5 \times 10^8$  *Lactobacillus acidophilus* LB dissolved in 200 µL distilled water on the seventh PID and for 8 consecutive days, once daily.<sup>8</sup>

T6 group: Infected and on the seventh PID mice were treated with 5 mg of zinc in 500 µL syrup for eight

**Table 1** Primers Sets Used for nPCR Targeting COWP Gene

Primers	Sequences	Expected Product Size (bp)	Annealing Temperature (°C)
1st PCR (E-COWP = Extended COWP) <sup>73</sup>			
BCOWPF	5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3'	769	65
BCOWPR	5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'		
2nd PCR (N-COWP = Nested COWP) <sup>74</sup>			
Cry-15	5'-GTA GAT AAT GGA AGA GAT TGT G-3'	553	54
Cry-9	5'-GGA CTG AAA TAC AGG CAT TAT CTT G-3'		

consecutive days, till the end of the experiment, once daily. The zinc dose was adjusted according to the annual report of the US Environmental Protection Agency, which demonstrates the safe daily intake of zinc.<sup>30</sup> Table 2 illustrates a summary of treatment type and dose given to the mice of T subgroups.

## Evaluation of Experimental Conditions

- Infection monitoring: After oocysts inoculation, all mice were observed daily for general health status, mortalities, fecal pellets color, and consistency. The bodyweight of mice in all groups was measured in grams and documented daily throughout the 14 days of the experiment. Pooled fecal samples from each group were collected daily from the second day and onward.<sup>7</sup> Fecal smears were performed daily along the experiment period using Modified Ziehl–Neelsen stain.<sup>31</sup> The efficacy of different treatments used and NTZ was assessed by daily measuring oocysts shedding count in feces until the experimental end by haemocytometer.<sup>26</sup> The number of oocysts was

calculated as (Total no. of oocysts counted × dilution factor)/(tested stool volume in gm).<sup>7</sup>

- Treatments' efficacy: The efficacy of each product was done by calculating the oocysts discharge reduction rate<sup>32</sup> as follows:

$$\text{Efficacy} = \frac{\text{Oocysts shedding of the control group} - \text{oocysts shedding by the treated group}}{\text{Oocysts shedding of the control group}} \times 100$$

- IL10 and TNF-α serum level among groups: concentrations of TNF-α and IL10 were determined in the serum samples of the animals at the end of the experiment. An enzyme-linked immunosorbent assay (ELISA) technique was performed according to the manufacturer protocol guideline, using antibody pairs from Bio Legend, Inc. (San Diego, USA).
- Detection of whole *Cryptosporidium* oocysts antigen and CD3 by immunohistochemistry: Terminal ileum were collected from sacrificed animals, tissue sections (4 mm thick) were placed in 10 mmol/l citrate buffer solution at pH of 6.0 and 80 °C for 30 min and

**Table 2** Treatment Type and Dose Were Given to the Mice of T Subgroups

Groups	Treatment	Treatment Dose	Time of Treatment in Relation to Infection	Duration of Treatment
T1	No	–	–	–
T2	NTZ	200 mg/kg/day, twice daily	7th PID	Eight consecutive days
T3	Clove oil	33 mg/kg, once daily		
T4	Dill seed oil	20 µL, once daily		
T5	<i>Lactobacillus acidophilus</i> LB	5×10 <sup>8</sup> dissolved in 200 µL distilled water, once daily		
T6	Zinc	5 mg in 500 µL syrup, once daily		

washed in water then application of 3.0% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min to block endogenous peroxidase activity. All slides were incubated with normal goat serum at room temperature for 20 min to avoid non-specific staining. Mouse monoclonal anti-*Cryptosporidium* antibody (1:50 Novus Biologicals, USA, Catalog No: NB100 65674UV) and rabbit polyclonal anti- CD3 (1:100) were then added for 1 h at room temperature. The previous steps were repeated with negative control. Next, the biotinylated secondary antibody, streptavidin horseradish peroxidase-conjugated tertiary antibody, and diaminobenzidine were applied according to the manufacturer's instructions. Sections were then counterstained with Mayer's Haematoxylin.

## Statistical Analysis

SPSS program version 16 was used to analyze data using the chi-square test to compare qualitative variables, while the independent test was used to compare quantitative variables. Qualitative data were presented in frequencies and proportions, while the mean and standard deviation were used to express quantitative data. Statistically, significance was considered when *P*-value less than 0.05 and 95% confidence intervals did not overlap.

## Results

### Rate of Infection

The isolated *C. parvum* oocysts were significantly able to infect mice in all tested groups. The number of +ve samples stained with Modified Ziehl–Neelsen showed a significant decrease at the end of the experiment in all treated groups than the non-treated group except for clove oil-treated one [Table 3](#).

### Oocyst Shedding Count

In positive control (infected untreated group), the mean of *C. parvum* oocysts shedding was significantly higher than all treated groups. At the same time, maximum shedding of *C. parvum* oocysts in that group was observed on the 14th PID, with a mean of 4970 oocysts/gm stool (*P* = 0.011). In all treated groups, maximum oocyst shedding was observed on the 7th and 8th PID and minimum on the 14th PID.

The oocyst reduction rate was affected variably among different tested treatments; it was the highest in zinc-treated mice (T6) (98.3%). Both *Lactobacillus acidophilus* LB

**Table 3** Rate of Infection Throughout the Experiment

Group	No. Samples	–ve Samples	+ve Samples	Percentage of +ve Samples
Control	46	46	0	0
T1	47	12	35	74.46
T2	39	24	15	38.5**
T3	49	15	34	69.38
T4	38	19	19	50**
T5	42	20	22	52.4**
T6	40	22	18	45**
Total	301	158	143	47.5**

**Note:** Values in the last column followed by \*\*are highly significant (*P*-value ≤0.001).

and dill seed oil-treated groups (T4 and T5) are equally reduced oocysts shedding (95.77%). The oocysts reduction rate recorded in NTZ-treated group (T2) was 91.55%. Clove oil (T3) was the least effective, with a 74.65% reduction rate.

However, the difference in oocysts shedding found between the NTZ, dill seed oil, *Lactobacillus acidophilus* LB, and zinc was not a significant treatment, unlike the oocysts shedding reduction effect in the clove oil-treated group, which was statistically lower than other treated groups (*P* < 0.0001) [Table 4](#).

### Bodyweight (BW)

At the beginning of the study, there was no significant difference in the mice's body weights of all groups compared to the negative control. By the end of the study, the

**Table 4** Comparison of Means Oocysts/gm Stool Detected in All Groups at the End of the Experiment and Reduction Rate of Each Treatment

Groups	Mean of Oocysts/gm Stool	Std. Deviation	Reduction Rate
T1	4970 <sup>a</sup>	±135.29	— <sup>a</sup>
T2	420	±4.38	91.55 <sup>b</sup>
T3	1260	±10.57	74.65 <sup>bc</sup>
T4	210	±1.96	95.77 <sup>b</sup>
T5	210.00	±1.80	95.77 <sup>b</sup>
T6	98.00	±1.76	98.03 <sup>b</sup>

**Note:** Oocysts discharge reduction rates among different groups differ significantly (*P* <0.05) when they are not sharing any common superscript letter (a–c).



**Table 5** Body Weights in All Groups Throughout the Experiment Compared to the Control Group

Groups	Mean	Std. Deviation	P-value
C	30.2457	±0.21547	–
T1	27.8214	±0.24862	**
T2	29.1000	±0.15191	**
T3	27.9286	±0.22678	**
T4	28.5571	±0.08516	*
T5	29.0643	±0.17305	**
T6	30.8071	±0.12157	*

**Note:** Values in each column followed by \* means P-value is significant (P-value ≤ 0.05) and that followed by \*\* are highly significant (P-value ≤ 0.001).

following data were observed (Table 5): In the infected untreated group and clove oil-treated group (T1 and T3), there was a highly significant reduction in BW compared to the control group and other treated groups (P-value ≤ 0.0001). But this reduction was nearly equal between the two groups.

In the groups treated with NTZ and *Lactobacillus acidophilus* LB (T2 and T5), the body weight was decreased compared to the control group, which improved 5 days after NTZ and probiotic administration. The weight gain was more in the NTZ-treated group (P < 0.001).

In both dill seed oil- and zinc-treated groups (T4 and T6), BW decreased compared to the control group, which improved 6 days after dill and Zn administration. The weight gain was more in the zinc-treated group (P < 0.05).

**Table 6** IL10 Difference Among Groups Compared to Control

Group	No. of Samples	Mean (pg/mL)	Std. Deviation	P value
C	6	1749.9	23.53	–
T1	6	1170.4	45.98	00.028
T2	6	3029.4	33.59	00.037
T3	6	1575.8	62.51	00.538
T4	6	2176.9	33.89	00.030
T5	6	2013.3	17.81	00.053
T6	6	1947.3	82.12	00.081

**Table 7** TNF-α Difference Among Groups Compared to Control

Group	No. of Samples	Mean (pg/mL)	Std. Deviation	P value
C	6	5917	2.26	
T1	6	896.30	169.45	0.000
T2	6	247.03	17.33	0.006
T3	6	749.84	31.85	0.002
T4	6	16.4700	8.55	0.001
T5	6	530.67	53.51	0.000
T6	6	20.0117	2.49	0.0071

## IL10 Concentration Levels (Table 6)

Variable concentrations of IL10 serum level were recorded in different groups: levels were decreased in both the infected non-treated and the clove oil-treated groups compared to healthy controls; however, the reduction was significant only in the infected non-treated animals. The IL10 serum level was higher than the control in the NTZ, dill seed oil, *Lactobacillus acidophilus* LB, and zinc-treated groups; the increase was significant in both the NTZ and dill seed oil-treated groups.

## TNF-α Concentration Levels (Table 7)

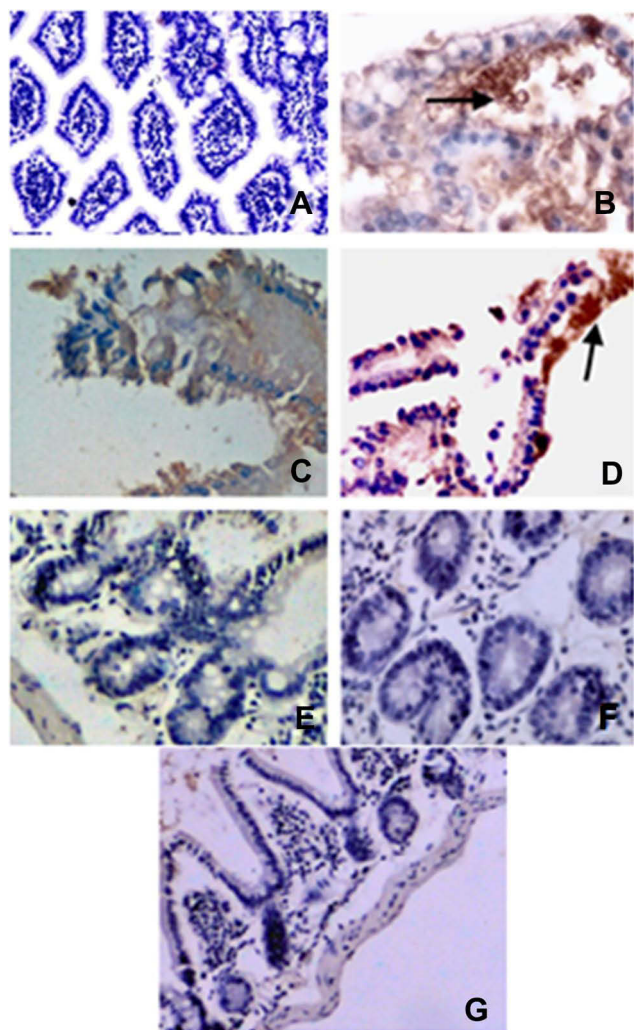
There was a significant reduction of TNF-α serum level in all tested groups compared to the healthy control. The reduction was remarkable in both zinc and dill seed oil-treated groups.

## Immunohistological Evaluation

Intestinal tissue revealed the presence of the *Cryptosporidium* antigen in intestinal tissue only in the positive control group and the clove oil-treated group (Figure 1B and D), compared to their absence in the healthy control, NTZ, dill seed oil, *Lactobacillus acidophilus* LB, and zinc-treated groups (Figure 1A, C, E, F, and G).

## The Expression of CD3 Antigens in Intestinal Tissue of Tested Mice

Immunohistological evaluation of the formalin-fixed paraffin revealed overexpression of the CD3 protein in lymphocytes that infiltrate the intestinal tissue of all tested groups versus healthy control (Figure 2A–G).

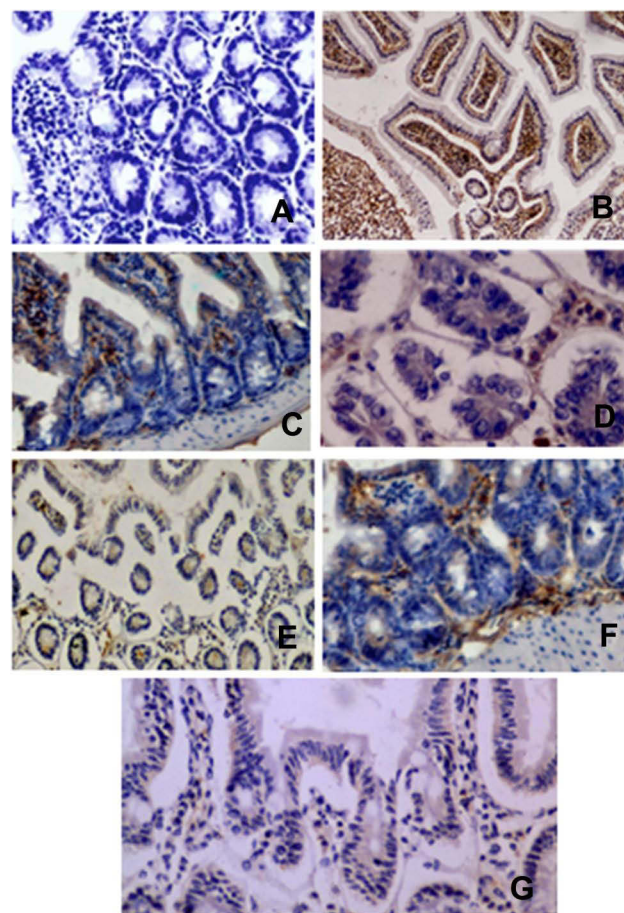


**Figure 1** Immunohistochemical analysis of *Cryptosporidium* antigen in intestinal mice tissues of different groups: the control group and T1, T2, T3, T4, T5, and T6 were respectively represented in (A–G).

## Discussion

*Syzygium aromaticum* oil, *Anethum graveolens* seeds oil, *Lactobacillus acidophilus* LB, zinc, and NTZ commercial forms were supplied as treatment of *Cryptosporidium* infection. As HSE (2008) reported that the incubation period for cryptosporidiosis is 1–12 days (average, 7 days), they were supplied on the seventh PID, after oocysts discharge established and not for prophylaxis.<sup>33</sup>

Although all the tested products failed to eradicate *C. parvum* infection, they modulated infection outcomes upon their administration in the experimental animal models. Oocysts shedding, body weight, local mucosal immunohistochemical changes, and systemic cytokines were affected by the investigated products with variable degrees.



**Figure 2** Immunohistochemical analysis of CD3 expression in intestinal mice tissues of different groups: the control group and T1, T2, T3, T4, T5, and T6 were represented in (A–G), respectively.

NTZ, clove oil, dill seed oil, *Lactobacillus acidophilus*, and zinc had affected the multiplication of *C. parvum*, as they lowered the oocysts discharge rate. Oocysts discharge was probably decreased by hindering their multiplication nature in the animal models and regaining intestinal integrity. This decrease is supported by a marked difference in oocysts discharge between the untreated and treated groups, which reflects the good efficacy of all types of supplements in decreasing the in vivo ability of the parasite oocysts to multiply. This finding is consistent with numerous in vivo human and animal studies that clearly illustrated the preventative or therapeutic potential of one or more of these alternatives.<sup>13,18,34</sup> Logically recovery of intestinal integrity and decreased oocysts multiplication correlate with the ability of the parasite to cause the disease manifestations.

BW was improved after all treatments except for the clove oil-treated group; this could be postulated by simultaneous improvement of host immune status, oocysts shedding

rate reduction. This correlated with previous preliminary researches showing the possibility of NTZ, dill, probiotics, and zinc to treat various forms of gastroenteritis; therefore, cryptosporidiosis manifestations improved by reducing both stool duration and frequency.<sup>35–38</sup> The current results oppose previous studies discussing the gastroprotective role of clove oil in different animal models.<sup>39</sup>

According to Qin, *Lactobacillus* caused marked improvement of intestinal epithelia-infected animal intestines.<sup>40</sup> The intestinal integrity was completely restored after *Lactobacilli* treatment, probably through competing with the parasite for nutrient hindering or preventing the parasite colonization along the intestinal tract and improving the mucosal immunity to fight back the infection.<sup>20</sup> These could explain both negative *C. parvum* antigen and CD3 overexpression at the local mucosal level in *Lactobacillus* treated group.

*Lactobacilli* also prevent parasite colonization along the intestinal tract.<sup>8</sup> The other suggested mechanism is the effect on intestinal microflora and lowering intestinal pH, regulating intestinal motility, and mucus production.<sup>41</sup> According to Desouza et al, the non-pathogenic probiotic bacteria interact with the gut epithelial cells and immune cells to start the immune signals in the form of modulating immunoglobulin production. This interaction increases the number of IgA-producing cells at the level of mucosal immunity and increases certain cytokines profiles, including (TNF- $\alpha$  IFN- $\gamma$ , IL-10, IL-12) to up or downregulate the immune responses and maintain intestinal homeostasis.<sup>42</sup> Furthermore, *Lactobacillus acidophilus* reduces the duration and number of *C. parvum* oocysts shed in feces of experimentally infected mice.<sup>43,44</sup>

Guitard et al had investigated the effects of *Lactobacillus casei* on *C. parvum*-infected rats, among many parameters including weight gain, parasite burden, mucosal histology, and mucosal cytokines' production (IFN $\gamma$ , IL10, and TNF $\alpha$ ).<sup>8</sup> The authors' findings agreed with the current study, rapid clearance of the parasite was noted in rats treated with probiotics, but it was unable to eradicate it. In contrast to the present study results, they could not find any significant effect of probiotic administration in weight gain or kinetics of mucosal cytokines during infection. Also, in a clinical trial performed in Peru, Salazar-Lindo et al found that the administration of a milk formula containing *Lactobacillus* is not useful in infants with acute diarrhea due to various microbial agents, including *C. parvum*. However, this low activity of probiotic treatment is surprising in light of previously

published data on the beneficial effect of some probiotics on infectious diarrhea.<sup>12,45–47</sup> The first case of successful resolution of prolonged cryptosporidiosis with probiotic treatment was a 12-year-old girl of previous good health supplied with a 4-week course of *Lactobacilli*; her clinical symptoms had completely resolved within 10 days of treatment inception.<sup>48</sup> Probiotics might excrete harmful substances to one of the developmental stages of the parasite leading to infection inhibition, offering new therapeutic agents for cryptosporidiosis treatment. Foster et al evaluated the in vivo effect of *Lactobacillus acidophilus* from human intestines on the oocyst stage of *C. parvum* using a flow cytometric viability assay. They found that *Lactobacillus* significantly reduced oocyst viability up to 81%. These results suggest antimicrobial substance(s) against the oocyst stage of *C. parvum* in *Lactobacillus acidophilus* cultures.<sup>49</sup>

While Zn probably improves the nutritional state of the infected hosts, leading to improvement of immunity, making them able to win the infection battle.<sup>22</sup> This finding explains the marked improvement of BW reduction of oocysts discharge rate, negative *C. parvum* antigen, and CD3 overexpression at the local mucosal level at the end of the experiment.

Although the benefits of zinc supplementation in the management of diarrhea have been established, the mechanisms by which zinc beneficially affects the pathological changes remain unknown in the current study. However, several postulations have been established for its specific effects in intestinal cryptosporidial infection, such as priming the immune system and tissue repair, as supported by Mathers et al. Zinc also plays a part in maintaining epithelial and tissue integrity through promoting cell growth and differentiation, suppressing apoptosis, improving immune function, and intestinal transport of water and electrolytes<sup>22</sup>; thus, it may protect against *Cryptosporidium* infection. Zinc also has an antioxidant and an anti-inflammatory agent.<sup>50</sup> Zinc deficiency is associated with an increased risk of gastrointestinal infections, with its adverse effects on the structure and function of the gastrointestinal tract and impaired immune function.<sup>22,51,52</sup> According to Yones et al, a coincident decrease in serum Zn level was more prominent among *Cryptosporidium* sp. infected malnourished children.<sup>23</sup> The positive association between *Cryptosporidium* infection and zinc deficiency could be explained by the changes in the absorptive villous architecture that is often severely disrupted, inflamed, or destroyed by *Cryptosporidium* sp., which is observed in



the present study and regained by probiotic and zinc treatment. There is currently an extensive clinical interest in Zn as a protective agent against various *Cryptosporidium* spp. infection aspects.

NTZ is reportedly effective against a wide spectrum of human microorganisms.<sup>53</sup> Over the years, through experimental and clinical trials, NTZ range of action extended to include enteric pathogens including protozoa (*Cryptosporidium parvum*, *Giardia lamblia*, *Entamoeba*, *Blastocystis hominis*, *Cyclospora*, *Isospora*), helminths (*Taenia saginata*, *Hymenolepis nana*), and bacteria (*Clostridium*, *Bacteroides*, *Helicobacter*).<sup>54,55</sup> It reduces symptom severity and duration of gastroenteritis, mainly diarrhea.<sup>38</sup> Yet, its efficacy remains contested among immunocompromised patients. Moreover, Amadi et al found no significant benefit of using NTZ in HIV children infected with cryptosporidiosis despite high dose and longer treatment duration through a randomized, double-blind, placebo-controlled trial performed in Lusaka, Zambia.<sup>56</sup> The current study revealed the positive therapeutic effects of NTZ at the levels of oocysts shedding rate and local mucosal clearance, yet this effect did not reach a complete cure. This agrees with several previous studies targeting the same issue recorded variable degree of efficacy. NTZ reduced the oocysts shedding rate by 85% and 76.1% in drug-treated calf and mice in two studies performed by Ollivett et al and Sadek et al, respectively.<sup>14,57</sup> Thus many authors recommended using NTZ with other drugs or herbal products to achieve more efficacy.<sup>14,58</sup>

Contrary to the current result, Theodos et al stated that NTZ did not show the expected positive effect on *Cryptosporidium* spp. burden in a gnotobiotic piglet diarrhea model.<sup>59</sup> Different experimental designs may explain these disagreements between studies, drug doses and diverse animal models used, and various diagnostic techniques used to evaluate the experimental outcomes.

Clove oil significantly reduced the oocysts discharge rate compared to the infected non-treated group in the present study. However, it showed the least reduction rate between all tested alternatives, including NTZ. This could be explained by the non-clearance of *C. parvum* infection from the intestinal mucosa, by finding the parasite antigen in the intestinal mucosa.

Among the limited studies published, addressed the effects of clove oil and or its major compounds on parasitic infection, Tanghort et al stated that clove essential oil resulted in a significant decrease in the number of *C. baileyi* and *C. galli* oocysts by about 85% up to 98% in

time and dose-dependent manners.<sup>60</sup> In another study conducted by Fang et al, clove oil has shown 100% acaricidal efficacy by killing all *Sarcoptes scabiei* mites subjected to it within 20.<sup>61</sup> Eugenol, one of the main clove oil components, has the potential to precipitate in the treatments of many parasites as *Giardia lamblia*, *Leishmania infantum*, *Trypanosoma cruzi*, and *Plasmodium falciparum* at high doses as stated in many in-vitro studies.<sup>62–65</sup> Eugenol also proved to be effective against *Schistosoma mansoni*. It reduced the tested worm burden by 19.2%, but it did not affect egg development.<sup>66</sup>

Based on previous studies investigating the possible effects of clove oil on different bacteria and protozoa other than *Cryptosporidium* sp., the mechanism of action hypothesized the ability of essential oil to disturb the cell function and morphology. This can be explained by the lipophilic properties of the plant elements, so they cross the cell membrane and disrupt the phospholipid layer structure. It also has the cytotoxic ability, leading to membrane damage, leakage of macromolecules, depolarization of the action of the mitochondrial membrane in the cytoplasm and nucleus, and finally, lysis.<sup>67</sup>

As far as we know, no previous study addressed the potential antiparasitic effects of dill seed oil against *Cryptosporidium* sp., even though its anti-inflammatory proprieties are widely discussed in different studies. Essential oil of dill has been reported to have a good inhibitory action on a broad spectrum of microorganisms, especially gram-positive bacteria were generally more liable to be affected by oils.<sup>68</sup> Two studies examined the effects of dill seed extracts on the parasitic population. First, the study investigated the effect of aqueous dill extract in children infected with giardiasis. The study revealed that 5 days' consumption of the extract and the regular dose of metronidazole improved symptoms earlier than treatment with metronidazole alone.<sup>69</sup> The second study evaluated the protective effects of dill extracts on amoebic liver abscess through in vivo and in vitro studies at multiple concentrations. The study revealed very promising anti-protozoal effects in comparison to metronidazole effects.<sup>13</sup>

The thorough mechanism by which dill seed oil exhibited antiparasitic effects is undetermined and needs further studies. However, it could be the dill chemical structure and its major components, such as dillapiole and anethole, that form hydrogen bonds with the active site of the target enzymes.<sup>70</sup> Dill is also popular for being mucosal

protective, antioxidant, and anti-secretory action at the intestinal mucosa.<sup>71</sup>

The precise cause explains the discharge of oocysts in mouse feces at end of the experiment while *Cryptosporidium* antigen was not visualized on ileal sections at the same period is still unknown and need further investigations, perhaps it may be attributed to the single antigen of *Cryptosporidium* oocysts that the Immunohistological analysis targeted in the current study, as there are other endogenous developmental stages: meront and gamonts beside oocysts. They reproduce within the intestinal epithelial cells.<sup>72</sup>

## Conclusion

Our work provided pieces of evidence for the eminent antiprotozoal activity of *Anethum graveolens* seeds oil, zinc, and *Lactobacillus acidophilus* LB commercial forms against *Cryptosporidium* infection by clearance of the parasite from intestinal mucosa and significant reduction of oocysts shedding rate. Collectively, our results provide a rational basis for using these safe, cheap, and commercially available products to treat cryptosporidiosis. We recommend more investigatory studies on a larger scale in both experimental animals and calves, as well as clinical trials to set a determined valid accredited therapy strategy for cryptosporidiosis including single and combined alternatives.

## Author Contributions

Gaber and Galal contributed to the study concept and design, acquisition of data, analysis and interpretation of data, drafting and revision of the manuscript. Farrag, Badary, Alkhalil and Elossily contributed to the acquisition of data, analysis and interpretation of data, and revision of the article. All authors gave final approval of the version to be published, agreed on the journal to which the article has been submitted and agreed to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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