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Biosynthesized Zinc Oxide Nanoparticles Regulating Immune Response and Liver Enzymes in Reducing Inflammation from Cryptosporidiosis-induced Infection

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ABSTRACT

Background: *Cryptosporidium*, a global intestinal protozoan parasite, induces mild to severe diarrhea in several vertebrate hosts, potentially resulting in life-threatening sickness. This study aimed to assess the efficacy of biosynthesized nano-zinc oxide using cellulose nanocrystals and *Zingiber officinale* extract (Bio-ZnNPs) in the treatment of experimentally infected mice with cryptosporidiosis.

Methods: A total of 20 male mice were allocated into four experimental groups (G1-G4), with triplicates. G1 (Control); non-infected, non-treated, G2 (Inf); infected with *Cryptosporidium parvum*, G3 (Bio-ZnNPs); infected- treated with Bio-ZnNPs at 200 mg/kg B.W. and G4 (NTZ) infected- treated with nitazoxanide (NTZ) at 100 mg/kg B.W. Mice were inoculated with 3000 oocysts of *C. parvum*. All treatments commenced on the initial day of oocyst shedding and persisted for five continuous days for all groups, excluding the negative control one. Oocysts count was assessed daily from the 1st to the 5th day post-treatment. Hematological analyses, blood biochemistry, immunoglobulin level assessments and histopathological evaluations were performed on day 5 post-treatment.

Result: The Bio-ZnNPs treated group exhibited the highest percentage reduction in *C. parvum* oocyst excretion. Furthermore, Bio-ZnNPs treatment resulted in notable enhancements in hematological parameters, liver enzymes and immunoglobulin levels, including immunoglobulin G and immunoglobulin M using Bio-ZnNPs. Additionally, a significant improvement in intestinal lesions with no intraluminal oocysts was noted in the Bio-ZnNPs treated group. Taken all together, this work demonstrated the promising performance of nanocomposites composed of cellulose nanocrystals and zinc oxide nanoparticles as an effective drug delivery strategy to enhance the efficacy of *Zingiber officinale* extract against experimental infected mice with *C. parvum*.

Key words: Cellulose nanocrystals, *Cryptosporidium*, Nanocomposite, Zinc oxide, *Zingiber officinale* extract.

INTRODUCTION

Cryptosporidiosis is a significant cause of diarrhea in both immunocompetent and immunocompromised individuals that is caused by *Cryptosporidium* species. It is self-resolving in immunocompetent persons. But in immunocompromised individuals, it can lead to chronic fluid, vitamin and electrolyte malabsorption, which causes wasting and sometimes fatal diarrhea (Moawad *et al.,* 2021; Ipek, 2023). Cryptosporidium is one of the few protozoans that are resistant to chemotherapy (Mead, 2002). Lack of anticryptosporidial medications may be due to its unique properties, such as cellular location and autoinfection potential, distinguishing it from other protozoans (Plutzer and Karanis, 2009).

Aromatic plants, especially their essential oils or components, have been known for centuries to fight bacteria, viruses, fungi, insects (Basile *et al.,* 2006; Moon *et al.,* 2006) and protozoan parasites like cryptosporidiosis. Notable examples include cinnamon and onion (Abu El Ezz *et al.,* 2011), pomegranate (Al-Mathal and Alsalem, 2012) as well as garlic (Abdel Megeed *et al.,* 2015) are often more affordable, easier to produce and process, more readily available and generally more effective than prescription drugs, with fewer adverse effects (Calixto, 2019).

Ginger (*Zingiber officinale* Roscoe) is a popular natural nutraceutical (Santini and Novellino, 2018; Daliu *et al.,* 2018). Ginger's medical and culinary properties made it a

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popular spice and herb globally. Ali *et al.* (2008) found that it is beneficial for arthritis, fever, vomiting and ulcer. The rhizome of this plant is a widely utilized medicinal herb due to its numerous pharmacological properties, including antioxidant, potent antibacterial (Mahady *et al.,* 2003), strong antifungal (Ficker *et al.,* 2003), anti-helminthic (Iqbal *et al.,* 2001), anti-inflammatory and antiallergic activities (Joshi *et al.,* 2017). Moreover, its anti-inflammatory characteristics, attributed to immune response regulation during the cellular phase, have been documented (Ballester *et al.,* 2022).

Recently, nanoparticles (NPs) have garnered attention for drug delivery and remediation (De Jong and Borm, 2008). Smart systems using NPs enable regulated drug delivery to specific areas. Rizvi and Saleh (2018) and Ezike *et al.* (2023) discovered that targeted administration of medication minimizes side effects and improves patient compliance by reducing dose frequency. Nanostructured drug delivery methods enhance the therapeutic properties, efficacy and safety of plant extracts due to their reduced particle size, increased surface area and accelerated dissolution rate (Priotti *et al.,* 2016; Khaleil *et al.,* 2023). Biopolymers like cellulose nanocrystals are safe for medical applications including drug delivery (Mehany, 2022). The trace element zinc is crucial to human enzymatic activity. It plays a critical role in phagocytosis, gene replication, transcription, translation and cytokine synthesis, as well as immunoglobulins levels (Begum *et al.,* 2022; Faisal, 2021; 2022). Zinc oxide nanoparticles (ZnO NPs) are one of five zinc compounds presently considered safe by the US Drug and Food Administration (US FDA) (Najoom *et al.,* 2021). ZnO NPs, due to their diminutive particle size, are more readily absorbed by the body. ZnO NPs are cheaper and less toxic than other metal oxide NPs, making them ideal for biomedical applications like drug administration, anti-inflammatory, anti-diabetic, antibacterial and anticancer (Jiang *et al.,* 2018).

Our previous research (Khaleil *et al.,* 2024) proved that the self-assembly of cellulose nanocrystals with *Zingiber officinale* extract provided with zinc oxide nanocomposites had an *in vitro* anti-cryptosporidial effect against its oocysts. Therefore, the present study was designed to study the *in vivo* anti-cryptosporidial effect of nanocomposites from cellulose nanocrystals-*Zingiber officinale* extract supported by zinc oxide nanoparticles against experimental infected mice with *C. parvum*.

MATERIALS AND METHODS

Preparation of *zingiber officinale* (ginger) extract (ZOE)

Fresh *Zingiber officinale* roots were peeled and thinly sliced. The *Zingiber officinale* slices were submerged in 70% ethyl alcohol to be extracted according to Abouelsoued *et al.* (2020).

Preparation of nanomaterials

Cellulose nanocrystals (CNCs) were synthesized and obtained as described before by Khaleil *et al.* (2024). Then, an equal quantity (0.06 g) of CNCs and ZOE were mixed to form CNCs-ZOE nanocomposite (CNCs-ZOE NCP). Zinc oxide nanoparticles (ZnO-NPs) was synthesized by dissolving 0.2 M of zinc acetate dihydrate in double distilled water, then 1 M KOH was added dropwise until a white precipitate formed, washed and finally dried. The formation of CNCs-ZOE NCP that supported by ZnO-NPs (Bio-ZnNPs) was synthesized by adding about 0.02 gram of the formed ZnO-NPs to CNCs-ZOE mixture that was mixed, filtered and dried at 40^{o}C. The characterization of CNCs-ZOE NCP and CNCs-ZOE/ZnO was previously documented in Khaleil *et al.* (2024), indicating that ZOE exhibits characteristic regular crystals with significant dispersion on the CNCs surface, whereas in CNCs-ZOE/ZnO, noticeable spherical ZnO nanoparticles appeared with extensive surface dispersion.

Experimental animals

This study was carried out between March 2024 and August 2024 at the Zoology Department, Faculty of Science, Kafrelsheikh University, Kafr Elsheikh, Egypt, while formal analysis was done in Saudi Arabia. The study involved 20 male Swiss albino mice (*Mus musculus*), 20-25 g (4-6 weeks old). The animals were kept in a housing at 22±2^{o}C on a standard food and with sufficient water. Mice feces was examined for 3 consecutive days to confirm the absence of intestinal parasite infection using zinc sulfate ($ZnSO_4$) floatation concentration technique (Current and Reese, 1986) and modified Ziehl-Neelsen staining method (Henriksen and Pohlenz, 1981). Mice were allowed to be adapted in the lab for a week. Following one-week adaptation, mice were randomly distributed into 4 equal groups (G1-G4) with triplicates. G1 (Control) was the negative control (non-infected, non-treated) and G2 (Inf) was the positive control (infected, non-treated) with 3000 *C. parvum* oocysts in 100 µl saline. The remaining two groups were infected with 3000 *C. parvum* oocysts and treated with Bio-ZnNPs at 200 mg/kg B.W. (G3) (Yanamala *et al.,* 2014) and nitazoxanide (NTZ) at 100 mg/kg B.W. (G4) as drug of choice for cryptosporidium and used for comparison of the result (El-Wakil *et al.,* 2021). G3 and G4 received treatments on the first day of oocyst shedding for 5 continuous days.

Oocysts excretion

Feces samples were collected daily from the 1st to 5th day post treatment (DPT) for estimating *C. parvum* oocysts per gram (OPG) according to Benamrouz *et al.* (2012).

Hematology and blood biochemistry

On day 5 post-treatment (DPT), blood was collected from the jugular vein of mice using vacutainer EDTA tubes for the measurement of red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), total leukocyte count (TLC), lymphocytes and eosinophils during cervical dislocation. Additional blood was drawn using non-anticoagulated weatherman tubes to separate serum and store it at -20°C for further analysis. Comprehensive liver function kits (MxP® Quant 500) were used for kinetic measurement of liver enzymes like alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).

Immunoglobulins level

Serum levels of immunoglobulin G and M (IgG and IgM) were measured on day 5 post-treatment following the instructions provided with the BD Pharmingen™ mouse immunoglobulin isotyping ELISA kit (B 1500).

Histopathological studies

Upon completion of the experiment on day 5, Ileal sections were fixed in 10% neutral buffered formalin, routinely managed and stained using hematoxylin and eosin (HandE) (Jeena *et al.,* 2013). The stained slides were inspected with a LEICA DM650 light microscope (Germany) to identify any histopathological alterations.

Statistical analysis

The data were evaluated using an Analysis of Variance (ANOVA) test conducted with SPSS 25 software. The Tukey post hoc test was employed. Probability levels (P values) less than 0.05 were deemed statistically significant.

RESULTS AND DISCUSSION

Cryptosporidiosis is among the most prevalent infectious diarrheal diseases in immunocompromised patients globally. In underdeveloped nations, *Cryptosporidium* spp. ranks as the second predominant cause of infectious diarrheal illnesses among children under five years old (Gebretsadik *et al.,* 2018). Targeting and designing new nanoparticle dependent drug carriers achieves enhancement of treatment efficacy (Abd El Wahab *et al.,* 2021). In this study, the therapeutic effect of nanocomposites from cellulose nanocrystal-*Zingiber officinale* extract supported by zinc oxide nanoparticles (Bio-ZnNPs) were evaluated against infected mice with *C. parvum* indicated by oocysts excretion, hematology, blood biochemical analysis, serum immunoglobulins levels and histopathological studies.

In this study, using the modified Ziehl Neelsen stain revealed that the stained *C. parvum* oocysts had a spherical to oval morphology, characterized by a pink hue on a blue-green backdrop (Fig 1). In the current study, the therapeutic use of Bio-ZnNPs against cryptosporidiosis revealed a lower significant (P<0.05) OPG count excretion from the first to the last day of treatment (1-5 DPT) in comparison to the Inf group. Bio-ZnNPs group had 47.2±0.25 $\times 10^3$ OPG, while NTZ group had 92.00±2.59$\times 10^3$ OPG (Fig 2). Abouel-Nour *et al.* (2015) and Abouelsoued *et al.* (2020) reported that oocyst shedding in ginger-treated mice gradually declined until it completely stopped. Also, Ginger and its phytochemicals were tested for its anti-cryptosporidiosis action (Batiha *et al.,* 2020). In addition, Dkhil *et al.* (2015) discovered that ZnO-NPs decreased oocyst excretion and jejunal inflammatory damage in mice infected with *Eimeria papillata*. This the same of Mohi-Eldin *et al.* (2018), who said that *Allium sativum* loaded ZnO-NPs decreased the oocyst in feces of rabbits infected with *Eimeria stiedae*. Ginger medication may directly affect parasite growth in the intestines, sexual stage production and oocyst generation, reducing and eliminating fecal oocyst shedding (Abdelgelil *et al.,* 2023). The antibacterial efficacy of zinc oxide particles is believed to stem from the production of reactive oxygen species (Sabir *et al.,* 2014). Joe *et al.* (2017) shown that ZnO nanoparticles can compromise the oocyst wall glycoproteins, enabling penetration and hence elevating the release of detrimental Zn^{2+} ions.

Hematological studies revealed a substantial reduction (P<0.05) in RBCs count, Hb level and PCV percentage in mice infected with *C. parvum* oocysts against the control group on 5 DPT (Table 1). The Bio-ZnNPs treated group exhibited significant rise on 5 DPT in their levels in relation to the Inf group (Table 1). Kabu (2023) demonstrated that hemoglobin levels were elevated during therapy, potentially linked to significant fluid loss. Despite the observation of alterations in hemoglobin, red blood cell counts and packed cell volume in calves suffering from diarrhea caused by Cryptosporidiosis, all hematological parameters remained within the established reference ranges (Aiello, 2016). Besides, in this study, TLC and differential leukocytic count, comprising lymphocytes (%) and eosinophils (%), in the Inf group compared to the control group (Table 2). However,

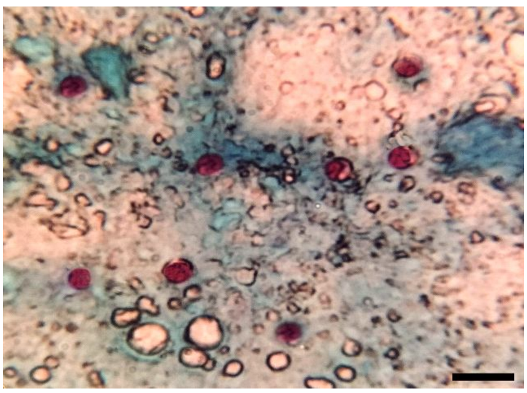


Fig 1: Stained ziehl neelsen fecal smear showing *C. parvum* oocysts. Scale bar= 10 µm.

significant reductions were noted in the Bio-ZnNPs treated group, related to the Inf group on 5 DPT (Table 2). Nanoparticles initially engage with blood and its constituents, potentially inducing various immunogenic responses, inflammation and alterations in hematological parameters, including leukocytes (Kim *et al.,* 2009). The alterations in white and red blood cells documented herein may be attributable to an augmented immunogenic response (Kawata *et al.,* 2009) or disruptions in signaling pathways and cellular maturation (Gopinath *et al.,* 2010), which can influence red blood cells alongside the proliferation and differentiation of other cells.

Cryptosporidiosis in mice may result in a chronic disease state affecting the gastrointestinal system and/or extraintestinal locations, potentially causing hepatic impairment (Mead *et al.,* 1994). This study indicated that the Inf group had substantial elevations ($p<0.05$) in liver enzymes such as ALT, AST and ALP compared to the control group. In contrast, the Bio-ZnNPs group showed a significant ($P < 0.05$) drop in the three enzyme levels relative to the Inf and NTZ groups on 5 DPT (Fig 3). These findings corroborated that cryptosporidiosis may have extraintestinal consequences (Chalmers and Davies, 2010). Malekizadeh *et al.* (2012) discovered that a 3% supplementation of ginger powder reduced serum ALT and AST activity in laying hens. Similar benefits of ginger were noted in broilers (Sahoo *et al.,* 2019) and laying quails (Herve *et al.,* 2019). The enhanced hepatic function may be ascribed to the antioxidant chemicals, including 6-gingerol, present in ZOE. Prior research shown the preventive properties of 6-gingerol against oxidative stress-induced liver damage both in vitro and in animal (Joshi *et al.,* 2017; Vipin *et al.,* 2017). The radical-scavenging activity of antioxidant compounds in ZOE may elucidate this phenomenon, as they inhibit lipid peroxidation (Si *et al.,* 2018) and enhance organ function, as indicated by reduced ALT and AST serum activities, thereby promoting the synthesis of antioxidant enzymes (Li *et al.,* 2019). Mahmoud *et al.* (2021) observed no adverse effects on liver histology, blood physiology, immunological function, or DNA integrity in broilers following prolonged low-dose ZnO NP administration. Zinc treatment restored AST and ALT activity, demonstrating its hepatoprotective impact by controlling protein synthesis. Zinc's antioxidant capabilities can also protect hepatocytes from adverse effects and reduce enzyme leakage into the bloodstream (Kouadria *et al.,* 2020).

In the *C. parvum* infection group, sera levels of IgG and IgM increased with a significant ($p<0.05$) difference in comparison to the control group. On the other hand, the Bio-ZnNPs group showed the lowest blood titres of IgG and IgM than NTZ group, compared to the Inf group (Fig 4). Khan *et al.* (2004) indicated that diarrhea was associated with lower IgA and IgM levels. Ginger therapy modulated immunological responses, according to Abdelgelil *et al.* (2023). Piglets' blood IgM and IgG levels improved with ZnO and nano ZnO supplementation (Sun *et al.,* 2019).

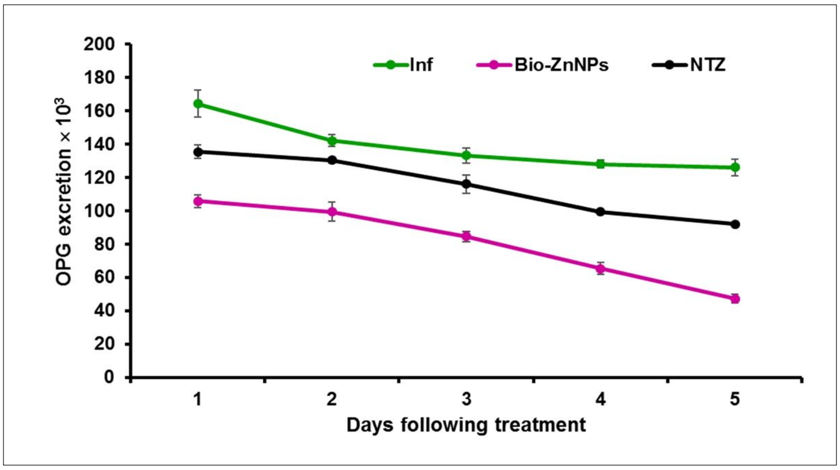


Fig 2: *Cryptosporidium parvum* oocysts excretion from experimentally *C. parvum* infected mice that treated with Bio-ZnNPs. Results are means± SD.

Table 1: RBCs counts, Hb and PCV percentage from experimentally *C. parvum* infected mice that treated with Bio-ZnNPs.

Groups	RBCs ($\times 10^6$/UL)	Hb (gm/dl)	PCV (%)
Control	10.3±0.25	14.6±0.53	45.9±5.28
Inf	9.10±0.3*	12.1±0.27*	38.1±3.62*
Bio-ZnNPs	11.6±0.61*#	14.5±0.18*#	45.6±2.95#
NTZ	9.20±0.18#	13.0±0.2*#	41.6±5.0#

Results are means± SD. *, # Statistically significant when P<0.05 against negative and positive groups, respectively.

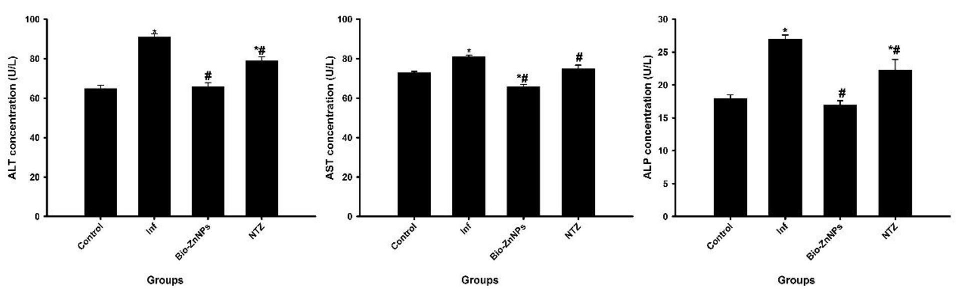
Zinc strongly affects humoral and innate immune activity (Knoell and Liu, 2010). Serum IgG and IgM can protect the extravascular compartment against pathogenic viruses and bacteria (Li *et al.,* 2007). Nanoparticles has the ability to treat experimental cryptosporidiosis through disruption of the normal physiological bioactivities of the parasite, such as food absorption, reproduction and mobility (Hamdy *et al.* 2023). Immune system enhancement by stimulating the natural killer cells' activity and phagocytosis may be another mechanism (Gaafar, 2012).

Furthermore, in the present study, histopathological examination results showed that the control group exhibited normal ileal tissue architecture, characterized by normal villi and goblet cells (Fig 5A). additionally, the Inf group with *C. parvum* displayed pronounced villous atrophy and sloughing epithelium, characterized by a considerable presence of goblet cells, alongside severe intraluminal infection by *Cryptosporidium* oocysts and significant infiltration of inflammatory cells (Fig 5B). Similar results were reported by Bhagat *et al.* (2017) and Sood *et al.* (2019). Conversely, the Bio-ZnNPs treated group displayed almost normal elongated ileal villi, well-organized intestinal crypts and a reduced quantity of goblet cells, with an absence of intraluminal *Cryptosporidium* oocysts (Fig 5C). On the other hand, the NTZ treated group exhibited regenerative moderate crypts generating new villi, with the presence of goblet cells (Fig 5D). Various plant extracts, including garlic, ginger, curcumin and black seeds, were evaluated on

Table 2: TLC counts, lymphocytes and eosinophils percentages from experimentally *C. parvum* infected mice that treated with Bio-ZnNPs.

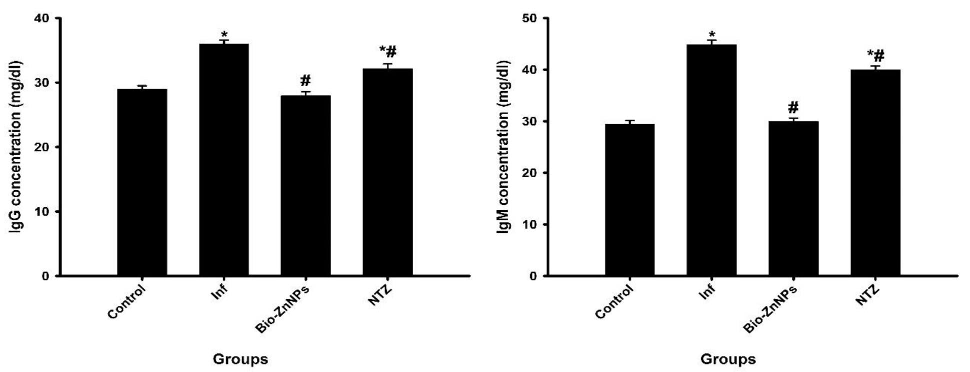
Groups	TLC (×103/UL)	Lymphocytes (%)	Eosinophils (%)
Control	7.5±2.25	59.0±5.95	1.3±0.2
Inf	11.0±0.49*	67.2±5.9*	2.3±2.1*
Bio-ZnNPs	8.4±0.75*#	58.1±0.7*#	1.3±0.3#
NTZ	9.10±0.82#	63.0±3.0#	1.9±0.2#

Results are means± SD. *, #Statistically significant when P<0.05 against negative and positive groups, respectively.



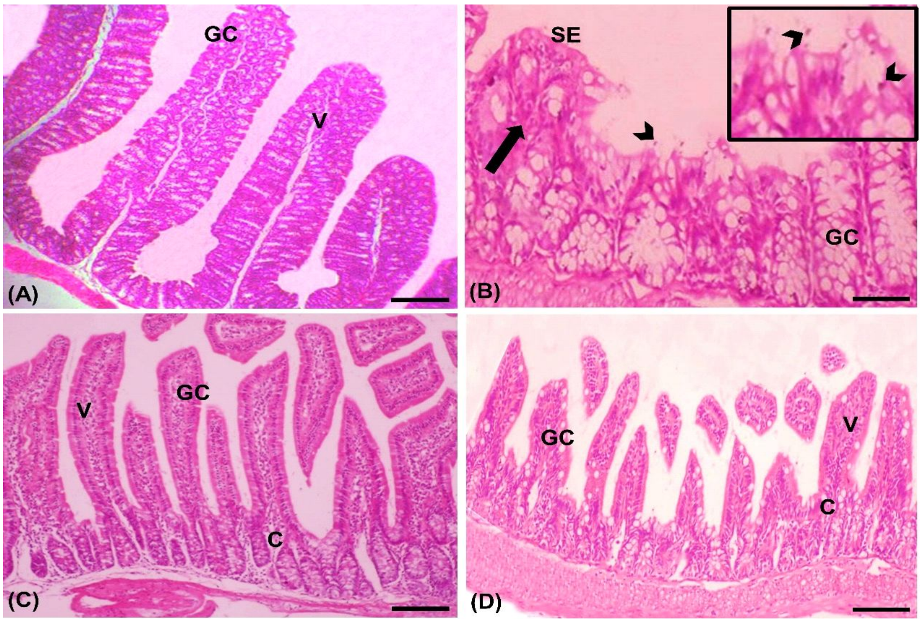
Results are means± SD. *, # Statistically significant when P < 0.05 against negative and positive groups, respectively.

Fig 3: Liver function tests from experimentally *C. parvum* infected mice that treated with Bio-ZnNPs.



Results are means± SD. *, # Statistically significant when P < 0.05 against negative and positive groups, respectively.

Fig 4: Levels of serum immunoglobulins from experimentally *C. parvum* infected mice that treated with Bio-ZnNPs.



(A) Control group showing normal ilea tisuue architecture with intact villi (V) and goblet cells (GC). **(B)** Inf group with *C. parvum* showing noticeable villous atrophy and sloughing epithelium (SE) with massive number of goblet cells (GC). Intraluminal heavy infection with Cryptosporidium oocysts (arrowheads) and considerable inflammatory cells infiltration (arrow). **(C)** Bio-ZnNPs treated group showing almost normal long ileal villi (V), well organized intestinal crypts (C) and few goblet cells (GC) with absence of intraluminal Cryptosporidium oocysts. **(D)** NTZ treated group showing regenerative moderate crypts (C) forming new villi (V) with observed goblet cells (GC). Scale Bar = 50 µm.

Fig 5: Histopathological HandE stained ileal sections.

Cryptosporidium-infected experimental mice and shown an anti-Cryptosporidial effect by safeguarding the intestinal epithelial tissue (Abouel-Nour *et al.,* 2015; Asadpour *et al.,* 2018; Sadek *et al.,* 2020). Plant extracts may inhibit or compete for receptor sites on the gut surface, hence reducing *C. parvum* colonization (Harp *et al.,* 1996). The pronounced positive and rectified impact of ginger in the current investigation may be due to its anti-inflammatory activities, notable antioxidant and immunomodulatory capabilities (Adewusi *et al.,* 1996). Ali *et al.* (2008) elucidated the immunological effects of ginger, ascribed to its active constituents, including zingerone, paradol, gingerols and shogaols. The most promising biopolymers in nanotechnology are natural substances like CNCs. This nanomembrane material is optimal owing to its superior mechanical strength and toughness (Li *et al.,* 2021). CNCs are biopolymers that ensure safety for medical applications such as medication delivery systems (El-Shafai *et al.,* 2022; Mehany *et al.,* 2022). Sharaf-El-Deen *et al.* (2023) indicated that ZnONPs inhibited the attachment of *C. parvum* oocysts to the intestinal tissue of mice. Hamdy *et al.* (2023) noted that animals treated with *A. sativum*-loaded ZnO-NPs demonstrated the most pronounced enhancement in histological intestinal lesions with no *Cryptosporidium* oocysts detected in the intestinal villi.

CONCLUSION

In conclusion, the results demonstrated that biosynthesized nano-zinc oxide using cellulose nanocrystals and *Zingiber officinale* extract could function synergistically resulting in significant anti-cryptosporidial, anti-inflammatory and immunomodulatory effects. It offers a cost-effective and environmentally sustainable method for innovative delivery synthesis in the treatment of parasite illnesses. Nonetheless, it is imperative to investigate the molecular processes that account for the observed effects. Subsequent research should focus on clarifying the specific routes by which this nanocomposite demonstrated their anti-cryptosporidial properties.

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Disclaimers

The views and conclusions expressed in this article are for the authors solely and do not represent the views of the affiliated institutions.

Informed consent

All animals were handled according to the Committee of Experimental Animal care and handling techniques of the Institutional Animal Care and Use Committee of Kafrelsheikh University (KFS-IACUC) under ethical license of KFS-IACUC/201/2024.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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