Original Article

Nano copper-organic framework as a delivery system to improve the efficiency of commercially available drugs for cryptosporidiosis: *In vivo* experimental study

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ABSTRACT

Background: Considering low efficacy, and high toxicity of the commonly used drugs in cryptosporidiosis, in addition to the development of drug resistance, trial for a novel approach is required.
Objective: To evaluate the efficacy of Nifuroxazide (Antinal), Trimethoprim-Sulfamethoxazole (Septrin), Nitazoxanide (NTZ), loaded-on copper-benzene tricarboxylate (Cu-BTC) metal organic frameworks (MOH) for treatment of *Cryptosporidium*-experimentally infected mice.
Material and Methods: Ninety male Swiss albino mice were divided into nine groups. Except for the 1st group (control negative), all mice were infected with 10^3 *Cryptosporidium* oocysts. Except for 2nd group (infected non treated), the remaining groups of infected mice were administered the tested drugs 2nd day post infection (PI) for 7 days. Daily stool examination for oocyst shedding from day 3 to 18 PI was conducted to evaluate drugs' efficacy. Furthermore, to confirm *Cryptosporidium* complete eradication, real time PCR (RT-PCR) was performed on stool samples collected after 3 weeks PI. Survival rate was also calculated for 3 weeks PI (at 7th, 14th and 21st d) to detect drugs' efficacy. Besides, physical characterization and toxicity of Cu-BTC were performed before mice treatment.
Results: Electron micrographs revealed 300-500 nm pyramidal crystals of Cu-BTC. Non-loaded Antinal, Septrin, and NTZ showed 67.2%, 62.2% and 76.6% reduction in oocyst shedding, while loaded drugs showed 88.4%, 66.7%, and 98.3% reduction, respectively. Additionally, RT-PCR revealed that the best treatment for cryptosporidiosis was NTZ@Cu-BTC, followed by antinal@Cu-BTC. Interestingly, using Cu-BTC alone had a relative positive therapeutic effect. Concerning the survival rate, Cu-BTC alone had 90% total survival that increased to100% when Cu-BTC was incorporated with studied drugs in all treated groups.
Conclusion: The results indicated that Cu-BTC is an efficient delivery system that improved the efficacy of the commonly used drugs.

Keywords: antinal; cryptosporidiosis; Cu-BTC; delivery system; nitazoxanide; septrin; therapeutic efficacy.

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INTRODUCTION

Cryptosporidium spp. are universal obligatory intracellular protozoa that infect most mammalians, targeting the brush border of epithelial cells of the digestive and respiratory tracts of a broad spectrum of animals[1,2]. Zoonotic transmission from animal to human was reported under poor hygienic conditions[3]. Intestinal cryptosporidiosis causes self-limited diarrhea in immunocompetent individuals and severe illness in immunocompromised patients[4]. In Africa and Asia, it is the second cause of severe diarrhea in young children[5]. Due to the extensive host range, low infective threshold, high excretion of resistant oocysts from infected patients, and water-borne transmission, *Cryptosporidium* spp. were prevalent in developing countries[6]. Nitazoxanide was initially prescribed as an oral antibiotic to treat traveler's diarrhea or colitis[7]. Because of the poor bioavailability of the commonly used drugs, a vehicle or carrier is required to facilitate their dispersion, selectivity, and activity[8]. Nitazoxanide has good efficacy as demonstrated by fast patients' recovery, good tolerance due to its unique pharmacokinetics, absence of systemic side effects, and great patients' satisfaction. It can be recommended as the first-choice empirical treatment in adult patients with acute diarrheal syndrome[9].

Nanomedicine is the use of nanotechnology in treatment, monitoring, prevention, control, and vaccination of biological diseases. In order to use

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nanomedicine, it is necessary to pinpoint the precise targets (cells and/or receptors) associated with the clinical disease and choose the most effective nanoparticles for the delivery system to reduce the negative effects of the original medication. Macrophages, endothelial, dendritic, and tumor cells are examples of these exact targets[10]. Nanotechnology has demonstrated its potential by creating a liposome of amphotericin B for treating leishmaniasis[11]. To improve immunity against a variety of infections, including *M. tuberculosis* and *T. gondii*, nanoparticles are employed as an adjuvant or as an immunostimulant adjuvant[12]. It is worth mentioning that Cu-BTC possesses good biocompatibility, high drug loading capacity and excellent surface-enhanced Raman scattering effects[13]. Besides, Cu-BTC has the potential to be a promising treatment for chronic toxoplasmosis, especially when combined with herbal extracts[14].

Therefore, we designed a new trial by loading NTZ, Antinal and Septrin onto a nano copper-based MOH. The present study aimed to explore Cu-BTC efficacy as delivery system to improve the bioavailability of the commercially used drugs for cryptosporidiosis. In addition, we insured that all drugs do not affect Cu-BTC framework for its crystallinity using X-ray diffraction (XRD). It is a versatile non-destructive analytical technique used to examine the physical characteristics of Cu-BTC regarding phase composition, crystal structure and orientation[15].

MATERIAL AND METHODS

This case-control study was conducted at Zoonotic Diseases Department, National Research Centre, Cairo, Egypt, during the period from September 2022 to December 2022.

Study design: Groups of experimentally infected mice with *Cryptosporidium* oocysts were respectively treated with Antinal, Septrin, NTZ, and Cu-BTC metallic organic framework (MOF) loaded drugs. Except for the 1st group (control negative) and 2nd group (control positive), all infected were treated on the 2nd day post infection (PI) for 7 d. Parameters used to evaluate drugs' efficacy included 1) daily stool examination for oocyst shedding from day 3 to 18 PI, 2) RT-PCR on stool samples, and duodenal tissues collected after 3 weeks PI, and survival rate for 3 weeks. Physical characterization and toxicity of Cu-BTC were also performed.

Chemicals: Table (1) shows chemicals and reagents used in the study.

Table 1. Compounds used in the study according to their prescribed method.

Methods	Chemicals	Company
Preparation of Cu-BTC	• Dimethylformamide (DMF) • Copper (II) nitrate trihydrate • Benzene-1,3,5-tricarboxylic acid	Sigma-Aldrich (Oakville, ON, Canada)
Preparation of oocysts	• 2.5% potassium dichromate solution	
Cu-BTC toxicity	• Normal human dermal fibroblast (BJ1 cell line) • Methylthiazol tetrazolium (MTT) • Dulbecco's modified eagle medium • Antibiotic-antimycotic mixture • 96-well microtiter plastic plates • 10% Sodium dodecyl sulphate (SDS) • Doxycycline (DOX)	Sheldon (TC2323, OR, USA)
DNA extraction for RT-PCR	• MagMAXTM CORE nucleic acid kit • Primers • 2 HERA qPCR kit (Cat. No. WF10302001)	Willowfort (UK)

Drugs: Antinal (nifuroxazide 220 mg/5 ml), Septrin (each 5 ml contains 40 mg Trimethoprim and 200 mg Sulfamethoxazole) and NTZ (100 mg/5 ml powder for oral solution after reconstitution), were obtained from local Egyptian companies, Amon, GlaxoSmithKline, and Utopia Pharmaceuticals, respectively.

Parasites: Oocysts of *C. parvum* were kindly provided from a strain maintained in mice at the Department of Zoonotic Diseases, National Research Centre, Cairo, Egypt.

Animals: The study utilized 90 male Swiss albino mice weighing 20–25 g, two weeks of age. The animals were obtained from the National Research Centre in Dokki, Egypt, and kept with normal pellet food and water. They were housed in ventilated cages with perforated lids, and their bedding was changed daily.

Preparation of Cu-BTC: Fifty ml Dimethylformamide (DMF) were used to dissolve 2.077 g of copper (II) nitrate trihydrate and 1.0 g of benzene-1,3,5-tricarboxylic acid. The mixture was heated in open

air at 160°C until the extra DMF had evaporated. After cooling at room temperature, the solid Cu-BTC was separated by centrifugation and five times washing with ethanol to eliminate unreacted components. The Cu-BTC product was then dried in a 105°C oven overnight until needed[13].

Drug loading: Dried Cu-BTC (200 mg) was dissolved in 20 ml water/methanol (1:1), followed by 0.1 mmol Cu-BTC. The NTZ, Antinal, and Septrin were added separately at room temperature and stirred for one hour. The reaction mixture was centrifuged, and the deposited solids were allowed to soak in methanol five times for 3 h before being removed. In an oven, the solids were dried at 105°C for six h and stored at room temperature in dry conditions until use[16].

Physical characterization of the studied drugs loaded with Cu-BTC: The X-ray diffraction (XRD) patterns were studied on the X'Pert MPD Philips diffractometer (UK) coupled with Cu.Kα monochromated, as a detector. Prepared compounds were measured using scanning electron microscope (SEM) (Hitachi SU-70-JP, Japan)[17].

Investigating Cu-BTC toxicity: A biosafety assay was done to determine the used Cu-BTC dose. The mitochondrial dependent reduction of yellow methylthiazol tetrazolium (MTT) to purple formazan was used to measure cell vitality. The operations were performed in a biosafety class II level laminar flow cabinet in a sterile environment (Baker, SG403INT, Sanford, ME, USA). At 37°C with 5% CO_2, cells were suspended in Dulbecco's modified eagle medium (DMEM-F12) with 1% antibiotic-antimycotic mixture (10,000 U/ml potassium Penicillin, 10,000 g/ml Streptomycin sulfate and 25 g/ml Amphotericin B). Using a water jacketed carbon dioxide incubator, cells were batch grown for 10 d before being seeded at a concentration of 10^4 cells per well in new complete growth media in 96-well microtiter plastic plates. Cells were incubated either alone (negative control) or with various sample dilutions to obtain a final concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ug/ml. To terminate the reaction and dissolve formed crystals, 200 µl of 10% sodium dodecyl sulphate (SDS) in deionized water were added to each well and incubated overnight at 37°C. Under the same circumstances, doxycycline was utilized as a positive control[18]. In our study, the total extract was screened in the same manner and the 100 µg concentration was found most suitable for application[19].

Mice infection: Oocysts from experimentally infected mice were collected and isolated using concentrated Sheather's sugar solution and confirmed microscopically by staining with the modified Ziehl-Neelsen method[20]. Hemocytometer was used to count number of oocysts in a phosphate buffer saline (PBS) solution before inoculation, and 3000 oocysts in 100 µL PBS were used to infect each mouse[3]. Except for group (1), all mice were infected with 1x103 *Cryptosporidium* oocysts/mouse[21] *via* gastric gavage, utilizing a 25-gauge needle with a plastic tube at the tip[22].

Study groups: Ten mice were allocated in each of the 9 groups. Group 1 was retained as the adverse control (neither infected nor-treated). All other groups were infected with *C. parvum* oocysts. Group 2 included infected non-treated mice (control positive). While group 3 received Cu-BTC treatment alone; groups 4-9 received NTZ, NTZ@Cu-BTC, Antinal, Antinal@Cu-BTC, Septrin, and Septrin@Cu-BTC, respectively.

Drugs and Cu-BTC dosage administration: Except for group (2), all infected mice were administered 100 mg/kg Cu-BTC, NTZ[23], NTZ@Cu-BTC, Antinal[24], Antinal@Cu-BTC, Septrin[25], and Septrin@Cu-BTC on the 2nd day PI for seven days. Doses were given daily through stomach tubes one hour before meals.

Treatment effect on oocyst shedding: From day 3 to 18 days PI, stool samples were collected, and microscopically examined to count *C. parvum* oocysts shedding from each mouse in 50 fields[26] (oil immersion), and the mean number for each group was calculated.

Treatment effect on *Cryptosporidium* eradication

Sample collection: After sacrifice, stool samples were collected from each mouse and transferred in ice boxes to the laboratory for three sterile PBS buffer washes (PH 7.4).

***Cryptosporidium* DNA extraction:** For extraction of DNA from oocysts the QIAamp DNA Mini Kit (Qiagen, Cat. No. 51304) was used according to the magnetic bead-based separation technique. It is a quick, simple, and efficient tool to separate the particles after the nucleic binding or elution step and a far less rigorous method than traditional techniques, e.g., centrifugation that generates shear forces leading to nucleic acids degradation. Therefore, DNA is completely separated from cell lysate components that can inhibit the enzyme (Taq polymerase) used in the PCR reaction[27].

Performance of RT-PCR: Primers that specifically target the *Cryptosporidium* species-specific small subunit (SSU) ribosomal RNA gene were used. The primers were P1 5-CAA TTGGAG GCC AAG TCT GGT GCC AGC-3, and P2 5-CCT TCC TAT GTC TGG ACCTCG TGA GT-3[27]. The reaction mixture was prepared using positive and negative controls that contained 12.5 µl of the 2 HERA qPCR Kit (Cat. No. WF10302001, Willowfort, UK), 1 µl (100 nmol) of each primer, and 2 µl of crude template DNA, and was completed to 25 µl using deionized double distilled water. The manufacturer's standard technique for amplification was followed (2

min at 95°C, followed by 40 cycles of 95°C for 20 s, 68°C for 20 s and 72°C for 30 s). Using an MX30005P Agilent RT-PCR System (Germany), amplification, data collection, and analysis were carried out. Cycle threshold values were exported to Microsoft Excel for additional examination. Melting curve analyses were used to verify each amplified PCR product's positive sample specificity. With each PCR run, both positive and negative controls were employed. The cycle threshold (Ct), which identified the cycle when a given sample's fluorescence considerably outperformed the baseline signal, was used to express the results. Higher Ct value indicated lesser *Cryptosporidium* spp. load, whereas lower Ct value indicated higher *Cryptosporidium* spp. load (DNA). Negative Ct results indicated the parasite was completely eradicated. The SYBR Green method of RT-PCR was used.

Survival rate: For each group, mice survival rate was recorded daily for 3 weeks PI (at 7th, 14th and 21st day).

Statistical analysis: Means and standard deviations of the oocyst shedding count data were calculated. Using SPSS version 15 software, t-test, Duncan, and analysis of variance (ANOVA) were used to determine the statistical significance between the study groups. Significant difference is considered when $P<0.05$.

Ethical consideration: The study was ethically approved by research ethics committee of Benha Faculty of Medicine. Experimental animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines. The study is reported in accordance with ARRIVE guidelines.

RESULTS

Physical characterization of Cu-BTC and loaded drugs: Figure (1) shows X-ray diffraction (XRD) of Cu-BTC and Cu-BTC after immobilization with drugs (Antinal, Septrin and NTZ). The obtained data were matched with the XRD of commercial Cu-BTC and the results clearly showed that Cu-BTC MOF retained its crystalline structure. Before addition of drugs electron micrography illustrated Cu-BTC crystals of pyramidal shape and dimensions in the range of 300-500 nm (Fig. 2a). Figures (2 b-d) show similar well diffraction patterns of NTZ@Cu-BTC, Antinal@Cu-BTC, and Septrin@Cu-BTC that confirmed drugs encapsulation inside Cu-BTC pores without destroying its framework.

Toxicity of Cu-BTC: The sample was tested against cultured normal human dermal fibroblast cell line (BJ1) in a concentration range between 100 to 0.78 µg/ml using MTT assays. It was demonstrated that up to 12.5 ppm concentration, Cu-BTC showed zero TC50 while TC90 (toxicity concentration that causes the death of 50%, and 90% of cells in 48 h, respectively) was zero% TC50 100%. These results were compared with DMSO

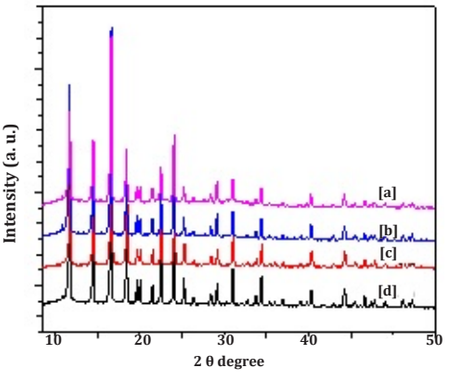

Fig. 1. The XRD of Cu-BTC **[a]**, NTZ@Cu-BTC **[b]**, Antinal@Cu-BTC **[c]**, and Septrin @Cu-BTC **[d]**.

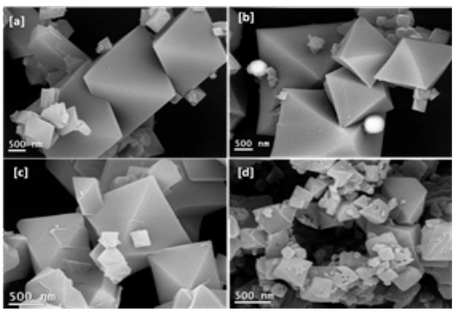

Fig. 2. SEM of Cu-BTC **[a]**, NTZ@Cu-BTC **[b]**, Antinal@Cu-BTC **[c]**, and Septrin @Cu-BTC **[d]**.

at 100 ppm that showed 1%, and 100% for its TC50 and TC90, respectively.

Oocyst shedding: The *C. parvum* oocyst counts from all infected and treated groups (2nd to 9th group) revealed that there was a gradual reduction in oocyst shedding in the infected non-treated group and continued till a few oocysts were found at day 18 PI. There was a statistically significant reduction in oocyst shedding in antinal@ Cu-BTC and NTZ@Cu-BTC groups till no oocysts were found at days 17 and 18 PI, respectively. Oocyst shedding in Cu-BTC and Antinal, NTZ administrated groups diminished gradually till reaching negligible numbers of oocysts at day 15-18 PI. Treatment with NTZ@Cu-BTC, and antinal@Cu-BTC diminished *C. parvum* oocysts count significantly in experimentally infected mice than other treatments on almost all days PI. Examination of stools of both mentioned groups at days 17 and 18 PI were negative especially group NTZ@ Cu-BTC, while antinal@Cu-BTC was nearly negative for *C. parvum* oocysts. Although Septrin treated group showed highest oocysts shedding than other groups, the Septrin @Cu-BTC revealed fewer excreted oocysts than the control non treated group (Table 2, and Fig. 3).

Results of RT-PCR: The amplification plot revealed that the Ct value came later when compared with the untreated control (A1); such a result indicated the anti-*Cryptosporidium* effect of the used drugs in the current

study (Figs. 4 and 5). The dissociation curve of all the tested samples showed the melting temperature (Tm) of the products around 78°C. These results indicated the amplification of the target sequence (no primer dimmer noticed). All tested samples gave positive results except for sample G1 (negative control), and the lowest Ct was for sample G1 (positive control), while the highest Ct samples (with lowest parasite load) were as follows: G9, G7, G6, G5, G4, G3 and finally G2 (Table 3). This means that the best treatments for *Cryptosporidium* spp. were NTZ@Cu-BTC followed by antinal@Cu-BTC. Interestingly, using Cu-BTC alone had a relative positive therapeutic effect.

Survival rate: Table (4) showed that treatment with Cu-BTC (G3) resulted in 90% total survival; this percentage changed when Cu-BTC was incorporated with the other studied drugs. The survival rate in the infected untreated group (G2) was reduced by the 3rd week PI to 80%. In the case of Septrin alone (G8) the survival rate was 80%. There was no statistically significant increase in the survival rate as compared to infected untreated control. On the other hand, G4-7 and G9 mice that were treated with NTZ, NTZ@Cu-BTC, Antinal, Antinal@Cu-BTC and Septrin@Cu-BTC respectively, showed increase in survival rate to 100% at the 21 day PI (Table 4).

Table 2. *Cryptosporidium* oocysts shedding in experimentally infected groups.

Days	Infected groups								*F* value
	Non-treated	Cup-BTC	NTZ	NTZ@	Antinal	Antinal@	Septrin	Septrin@	
2	92.1±0.71^{ab*}	90.4±1.3	86.1±1.41^{ab}	84.0±1.41^{c}	89.4±0.89^{a}	87.6±1.58^{ab}	91.7±1.79^{ab}	86.4±1.3^{bc}	2.14^{NS}
3	93.6±1.34^{b*}	91.1±1.1^{d}	88.8±1.3^{a*}	85.0±1.4^{d}	91.8±1.92^{b}	90.6±1.14^{a}	94.1±1.22^{a}	91.8±1.3^{a}	**3.65***
4	92.8±0.84^{bc}	90.2±1.1^{d}	87.3±1.82^{ab}	68.6±1.95^{f}	88.8±0.84^{d}	85.6±1.14^{ab}	95.2±1.92^{a}	86.8±0.84^{e}	**6.82****
5	94.8±0.84^{b*}	86.8±1.92^{a}	84.6±1.82^{a}	52.2±1.1^{d}	88.1±2.45^{b}	84.2±1.92^{a}	96.1±1.92^{a}	87.6±2.3^{c}	**4.85***
6	97.8±1.67^{c}	83.4±1.58^{a}	79.1±1.14^{a}	29.6±1.1^{d}	83.0±1.58^{b}	78.4±0.89^{a}	93.0±2.35^{a}	86.8±3.63^{c}	**5.53****
7	100.3±1.58^{c}	73.4±1.58^{a}	67.0±1.58^{a}	21.3±1.3^{e}	76.6±3.29^{b}	63.2±2.17^{a}	83.6±0.89^{a}	81.2±2.12^{d}	**7.3****
8	98.2±1.14^{d}	66.4±2.07^{b}	64.8±1.64^{b}	14.5±1.3^{f}	65.4±3.91^{c}	61.2±1.1^{a}	75.4±3.13^{b}	70.4±6.43^{e}	**336.7****
9	89.1±1.3^{d}	66.8±1.3^{d}	57.6±0.89^{b}	8.4±1.3^{f}	57.8±1.92^{c}	55.7±0.84^{a}	63.8±2.95^{b}	60.6±1.14^{e}	**94.5****
10	87.1±1.87^{c}	66.0±1.0^{b}	53.6±1.82^{a}	6.5±0.71^{d}	46.0±1.0^{b}	42.4±1.82^{a}	64.8±3.49^{a}	55.6±0.55^{d}	**113.4****
11	79.2±1.14^{d}	55.4±1.14	49.4±2.07^{b}	3.5±1.0^{f}	43.2±2.39^{c}	20.0±1.73^{b}	57.4±3.13^{a}	46.4±0.71^{e}	**524.3****
12	65.5±1.1^{c}	54.4±1.14	44.0±2.12^{a}	2.7±0.55^{d}	41.4±1.34^{b}	10.9±1.41^{a}	52.6±2.12^{a}	43.1±1.0^{d}	**414.5****
13	60.6±2.52^{d}	50.0±2.07^{b}	17.8±1.48^{c}	1.1±0.84^{e}	32.0±2.07^{b}	8.9±1.87^{a}	40.2±1.1^{c}	36.1±0.55^{e}	**792.7****
14	55.6±1.87^{c}	48.0±1.7^{a}	13.0±1.0^{c}	0.9±0.4^{d}	18.2±1.3^{b}	6.4±1.34^{a}	21.0±1.0^{c}	18.5±0.4^{d}	**1003****
15	32.5±1.48	24.2±1.8^{a}	11.2±0.84^{b}	0.6±0.5^{e}	12.5±1.0^{d}	3.8±0.89^{b}	16.1±0.84^{b}	9.3±0.5^{e}	**1031.3****
16	18.6±0.55^{a}	17.5±0.4^{d}	6.9±0.8^{c}	0.0±0.0^{d}	7.5±1.0^{b}	2.5±0.84^{a}	9.4±0.89^{c}	7.5±0.3^{d}	**664.71****
17	7.2±0.0^{c}	5.1±0.4^{b}	2.8±0.8^{ab}	0.0±0.0^{c}	4.1±0.89^{b}	0.1±1.1^{a}	5.0±0.7^{ab}	3.9±0.23^{c}	**31.1****
18	2.3±0.0^{c}	1.4±0.89^{c}	1.3±0.8^{ab}	0.0±0.0^{c}	0.4±0.89^{b}	0.0±1.1^{a}	2.0±0.7^{ab}	1.5±0.23^{c}	**168.8****

All data expressed as mean±SD; **@:** Loaded with Cu-BTC; ***:** Significant (P<0.05); ****:** Significant (P<0.001); **NS:** Not significant; **^{a,b,c}:** Significance between columns, **^{d,e,f}:** Significance inside each column.

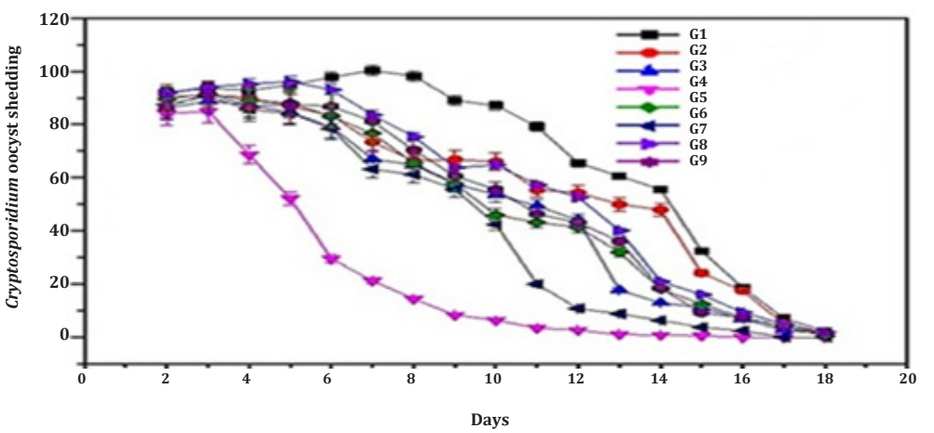

Fig. 3. *Cryptosporidium* oocysts shedding in experimentally infected non-treated, Cu-BTC, NTZ, NTZ@Cu-BTC, Antinal, Antinal@ Cu-BTC, Septrin, and Septrin @Cu-BTC.

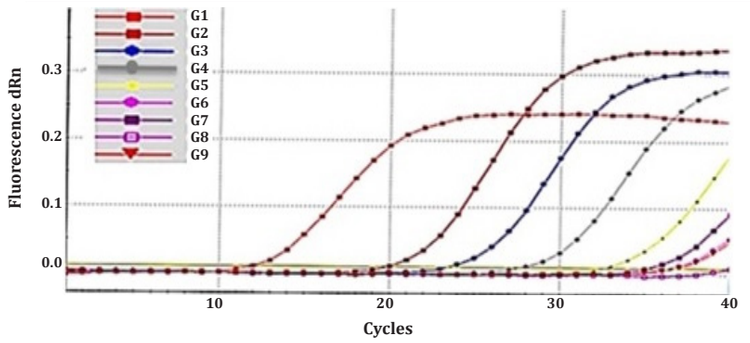

Fig. 4. Amplification plots of RT-PCR show the positive and negative cases. **G1:** Positive control (infected with *Cryptosporidium* spp. and non-treated); **G2:** Infected and treated with Cu-BTC; **G3:** Infected and treated with Septrin; **G4:** Infected and treated with Antinal; **G5:** Infected and treated with Septrin@Cu-BTC; **G6:** Infected and treated with NTZ; **G7:** Infected and treated with Antinal@Cu-BTC; **G8:** Negative control (non-infected non-treated); **G9:** Infected and treated with NTZ@Cu-BTC.

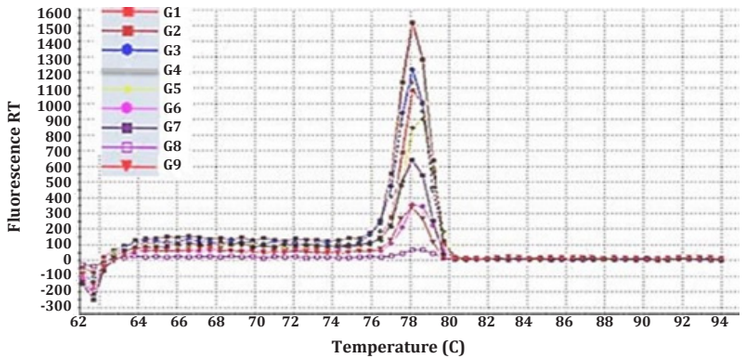

Fig. 5. Dissociation curve analysis of the amplicons obtained from RTPCR. **G1:** Positive control (infected with *Cryptosporidium* spp. and non-treated); **G2:** Infected and treated with Cu-BTC; **G3:** Infected and treated with Septrin; **G4:** Infected and treated with Antinal; **G5:** Infected and treated with Septrin @Cu-BTC; **G6:** Infected and treated with NTZ; **G7:** Infected and treated with Antinal@Cu-BTC; **G8:** Negative control (non-infected non-treated); **G9:** Infected and treated with NTZ@Cu-BTC.

Table 3. The Ct and Tm of both the standard (G1 and G8) and the treated samples (G2-G7 and G9).

Well name	Mice group	Ct	Final	Tm
G1	Positive control (infected, non-treated)	12.03	+	78.15
G2	Infected, treated with Cu-BTC	19.84	+	78.15
G3	Infected, treated with Septrin	19.94	+	78.15
G4	Infected, treated with Antinal	23.41	+	78.15
G5	Infected, treated with Septrin@Cu-BTC	27.70	+	78.10
G6	Infected, treated with NTZ	32.65	+	78.67
G7	Infected, treated with Antinal@Cu-BTC	36.25	+	78.15
G8	Negative control (non infected, non treated)	No Ct	-	78.67
G9	Infected, treated with NTZ@Cu-BTC	36.74	+	78.10

Ct: cycle threshold; Tm: melting temperature.

Table 4. Survival rates of G1-G9 mice on 7th, 14th, 21st days PI.

Groups	Number of dead mice days PI (n=10)			Survival rate (%)
	7th	14th	21st	
G1	0	0	0	100
G2	0	0	2	80
G3	0	1	0	90
G4	0	0	0	100
G5	0	0	0	100
G6	0	0	0	100
G7	0	0	0	100
G8	0	1	1	80
G9	0	0	0	100

G1: Non-infected, non-treated (control negative); **G2:** Infected, non-treated (control positive); **G3:** Infected, treated with Cu-BTC; **G4:** Infected, treated with NTZ; **G5:** Infected, treated with NTZ@Cu-BTC; **G6:** Infected, treated with Antinal; **G7:** Infected, treated with Antinal@Cu-BTC; **G8:** Infected, treated with Septrin; **G9:** Infected, treated with Septrin@Cu-BTC; **PI:** Post infection.

DISCUSSION

Cryptosporidium-infected individuals, particularly immunosuppressed patients, are prone to complications due to *Cryptosporidium* infection in both industrialized and developing nations. Aside from the numerous negative effects of the available therapeutics, the majority of immunodeficient patients do not benefit from the available medications. Consequently, there is a critical need for novel effective medications[28,29]. Consequently, Octaarginine proved to improve NTZ efficacy as an anti-cryptosporidial therapeutic[30]. In the current investigation, experimental mice infected with *Cryptosporidium* were treated for the first time with Antinal and Septrin, either alone or added to Cu-BTC. The *C. parvum* oocyst isolation and counts from all infected and treated groups (from the 2nd to 9th group) showed that Antinal@Cu-BTC and NTZ@Cu-BTC treated groups experienced a statistically significant decrease in oocyst shedding. Complete disappearance of oocysts in NTZ@Cu-BTC treated group was on 16th to 18th d PI, and Antinal@Cu-BTC on 16th d. Additionally, oocyst shedding in the Cu-BTC, uncombined Antinal, and uncombined NTZ administered groups rapidly decreased until insignificant amounts of oocysts were present at days 16 to 18 PI.

Treatment of experimentally infected mice, by NTZ@Cu-BTC and Antinal@Cu-BTC considerably reduced the number of *C. parvum* oocysts compared to other treatments on practically all PI days. At 16th d, the examined stools of NTZ@Cu-BTC group was negative for *C. parvum* oocysts followed by Antinal@ Cu-BTC which was negative at 18th d PI. Septrin@Cu-BTC treated mice showed significant decrease in oocyst count in comparison with control positive group and insignificant value in comparison with other treated groups.

In our study the encouraging results for treatment with NTZ-Cu-BTC is in agreement with previous work by Metawae *et al*.[31] who described nearly complete clearance of histopathological alterations in *Cryptosporidium* infected and treated groups, when comparing treatment with NTZ alone to NTZ loaded silica.

The number of *Cryptosporidium* oocysts was dramatically reduced by both NTZ@Cu-BTC and NTZ alone, and the clinical cure proved most effective with the former. Notably NTZ is a curative antibacterial drug used to treat infections of the digestive tract[9].The two nitroheterocyclic antibiotics nifuroxazide (Antinal) and nitrofurantoin, which have structural similarities with nifurtimox, are among the most promising ones since they also have potent anti trypanosomal activity. Nifuroxazide showed activity comparable to that of the recommended medication pentamidine[32]. Additionally, nifuratel (antibacterial, antiprotozoal, and antifungal), nitrofurazone and furazolidone, as well as nifurtimox (antiprotozoal), are frequently employed[33]. To produce an anti-pathogenic impact clinical 5-Nitrofurans (NFs) are activated by azoreduction, followed by nitroreduction, which is catalyzed by azoreductases and nitroreductases (NTRs)[34].

When parasite NADPH-cytochrome P450 reductases are present, nitroreduction takes place when the nitro group is reduced by type I or type II NTRs. Under anaerobic conditions, type I NTRs catalyze the reduction of 5-NFs to create anti-pathogenic hydroxylamine. Reactive oxygen and nitrogen species (ROS/RNS), which are produced during aerobic nitroreduction catalysed by type II NTRs, cause oxidative stress in pathogens, and ultimate death. Due to the number of 5-NFs functions, these medications can be used repeatedly to treat serious illnesses[34]. In the present study the Cu-BTC infected and treated group had higher effects than other groups because Cu-NPs have effective antiparasitic abilities[35].

Initially, nifuroxazide was prescribed as an oral antibiotic to treat colitis or traveler's diarrhea. Studies have shown that this medication is efficient at inhibiting the constitutive phosphorylation of the STAT-3 signal transducer and activator of transcription 3 in multiple myeloma (MM), which lowers the expression of the STAT-3 target gene Mcl-1[36] Additionally, the immunology of bovine cryptosporidiosis provides further evidence that STAT-3 down-regulation reduces the number of dendritic cells[37]. Typically, a vehicle or carrier is needed to administer these insoluble bioactive substances, which enhances activity, selectivity, and dispersion[38]. Many different carbon-based nanoparticles have been used to transport drugs and drug combinations, but they must be able to specifically target receptors in order to improve their effectiveness and reduce any side effects[39].

Our results related the antiprotozoal properties of Antinal@Cu-BTC and NTZ @Cu-BTC to their active ingredients. Additionally, the drug containing nanoparticles may hasten the blockade or compete for receptor sites on the gut surface, resulting in decreased *C. parvum* colonization[40]. This could account for the Antinal@Cu-BTC and NTZ@Cu-BTC apparent anti-cryptosporidial activity in addition to their previously noted anti-cryptosporidial and antiprotozoal properties[37]. The amplification plot showed that the Ct value decreased over time when compared to the untreated control (G1); this finding confirms that the medications utilized in the current investigation had an anti-*Cryptosporidium* impact.

In conclusion, it is determined that the Antinal@ Cu-BTC and NTZ@Cu-BTC have significant anti-cryptosporidial activity, reducing oocyst shedding, and protecting healthy animals from infection. The best results were for NTZ@Cu-BTC which showed 98.3% confirmed high performance. This conclusion suggests

that these compounds may be used to treat similar infections in humans and animals that are susceptible to them. Additionally, it is the first time the more potent efficacy of Antinal, particularly when loaded on Cu-BTC nanoparticles, has been demonstrated.

Author contribution: Soliman NA proposed the study topic and designed the study. Naguib MM, Ali HSM contributed with Abd El-Razik KA in performing the parasitological sections. Barakat AMA, Abdelhameed RM prepared Cu-BTC, and performed the drug toxicity. All authors contributed in writing the manuscript and accepted the authorship and the final version before publication.

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REFERENCES

1. Delling C, Daugschies A. Literature Review: Coinfection in young ruminant livestock—*Cryptosporidium* spp. and its companions. Pathogens 2022; 11(1):103.
2. Kumari S, Kulkarni P (Eds). Potential concern of foodborne pathogens in the food industry biological and chemical hazards in food and food products, In: Biological and Chemical Hazards in Food and Food Products. 1st edition, Apple Academic Press, 2022; 27-61.
3. Ghazy AA, Abdel-Shafy S, Shaapan RM. Cryptosporidiosis in animals and man: 1. Taxonomic classification, life cycle, epidemiology and zoonotic importance. Asian J Epidemiol 2015;8(3):48 .
4. Ryan U, Zahedi A, Feng Y, Xiao L. An update on zoonotic *Cryptosporidium* species and genotypes in humans. Anim (Basel) 2021; 11(11):3307.
5. Nasrin D, Blackwelder WC, Sommerfelt H, Wu Y, Farag TH, Panchalingam S, *et al*. Pathogens associated with linear growth faltering in children with diarrhea and impact of antibiotic treatment: The global enteric multicenter study. J Infect Dis 2021; 224(12):48–55.
6. Innes EA, Chalmers RM, Wells B, Pawlowic MC. A one health approach to tackle cryptosporidiosis. Trends Parasitol 2020; 36(3):290–303.
7. Rossignol JF, Kabil SM, El-Gohary Y, Younis AM. Effect of nitazoxanide in diarrhea and enteritis caused by *Cryptosporidium* spp. Clin Gastroenterol Hepatol 2006; 4:320–324.
8. Love MS, Choy RKM. Emerging treatment options for cryptosporidiosis. Curr Opin Infect Dis 2021; 34(5):455–462.
9. Husic-Selimovic A, Custovic N, Sivac N, Glamoclija U, Sukalo A, Mehic M. Nifuroxazide has better efficacy than probiotic treatment in adult patients with acute diarrheal syndrome. Mater Sociomed 2022; 34(4):267–271.
10. Abaza SM. Applications of nanomedicine in parasitic diseases. PUJ 2016; 9(1):1-6.
11. Kumar R, Sahoo GC, Pandey K, Das V, Das P. Study the effects of PLGA-PEG encapsulated amphotericin B nanoparticle drug delivery system against *Leishmania donovani*. Drug Deliv 2015; 22(3):383-288.
12. Petkar KC, Patil SM, Chavhan SS, Kaneko K, Sawant KK, Kunda NK, *et al*. An overview of nanocarrier-based adjuvants for vaccine delivery. Pharmaceutics 2021; 13:455.
13. Hang L F, Zhang T, Wen H, Liang LB, Li WM, Ma X F, *et al*. Controllable photodynamic performance *via* an acidic microenvironment based on two-dimensional metal-organic frameworks for photodynamic therapy. Nano Res 2021; 14:660-666.
14. Mohammad OS, El-Naggar HM, Abdelmaksoud HF, Barakat AM, Abdelhameed RM. The effect of *Nigella sativa* oil-and wheat germ oil-loaded metal organic frameworks on chronic murine toxoplasmosis. Acta Tropica 2023; 239:106823.
15. Mitchell DRG, Ball CJA. Quantitative X-ray diffraction and analytical electron microscopy study of service exposed 2.25Cr-1Mo steels. Mater Charact 2001; 47(1):17–26.
16. El-Shafey AAM, Hegab MHA, Seliem MME, Barakat AMA, Mostafa NE, Abdel-Maksoud HA. Curcumin@metal organic frameworks nano-composite for treatment of chronic toxoplasmosis. J Mater Sci Mater Med 2020; 31:1-13.
17. Mahmoud NE, Mahdi AA, Barakat AMA, Abdelhameed RM. Boosting vegetation, biochemical constituents, grain yield and anti-cancer performance of cultivated oat (*Avena sativa* L) in calcareous soil using oat extracts coated inside nanocarriers. BMC Plant Biology 2022; 22:1-15.
18. Thabrew MI, Hughes RD, McFarlane IG. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J Pharm Pharmacol 1997; 49(11):1132-1135
19. Su G, Burant CF, Beecher CW, Athey BD, Meng F. Integrated metabolome and transcriptome analysis of the NCI60 dataset. BMC Bioinformatics 2011; 12(1):1-7.
20. Aghamolaie, AS, Rostami Sh, Fallahi F, Tahvildar Biderouni A, Salehi N. Evaluation of modified Ziehl–Neelsen, direct fluorescent-antibody and PCR assay for detection of *Cryptosporidium* spp. in children faecal specimens. J Parasit Dis 2016; 40(3):958–963.
21. Gaafar MR. Efficacy of *Allium sativum* (garlic) against experimental cryptosporidiosis. Alexandria J Med 2012; 48(1):59-66.
22. HA Nahed, AT Hoda, IM Yomna. Effects of garlic on albino mice experimentally infected with *Schistosoma mansoni:* A parasitological and ultrastructural study. Trop Biomed 2009; 26(1):40-50.
23. Li X, Brasseur P, Agnamey P, Leméteil D, Favennec L, Ballet JJ, *et al*. Long-lasting anti-cryptosporidial activity of nitazoxanide in an immunosuppressed rat model. Folia Parasitol 2003; 50(1):19–22.
24. Lee YH, Lee DY, Shin DW. Prophylactic effects of trimethoprim-sulfamethoxazole in *Toxoplasma*-infected mice. Korean J Parasitol 1993; 31(4):363-370.

25. El-Sherbiny M, El-Sayed RM, Helal MA, Ibrahiem AT, Elmahdi HS, Eladl MA, *et al*. Nifuroxazide mitigates angiogenesis in Ehlrich's solid carcinoma: Molecular docking, bioinformatic and experimental studies on inhibition of Il-6/Jak2/Stat3 signaling. Molecules 2021; 26(22):6858.
26. Abu El Ezz NMT, Khalil FA, Abdelhamid EKA. Molecular epidemiology of cryptosporidiosis in pre-weaned cattle calves in Egypt. BJVM 2020; 23(1):1311-1477.
27. Nichols R, Campbell B, Smith H. Identification of *Cryptosporidium* spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. Appl Environ Microbiol 2003; 69(7):4183-4189.
28. Farthing MJ. Treatment options for the eradication of intestinal protozoa. Nat Clin Pract Gastroenterol Hepatol 2006;3(8):436-445.
29. Gargala G. Drug treatment and novel drug target against *Cryptosporidium*. Parasite 2008; 15(3):275-281.
30. Nguyen-Ho-Bao T, Ambe LA, Berberich M, Hermosilla C, Taubert A, Daugschies A. Octaarginine improves the efficacy of nitazoxanide against *Cryptosporidium parvum*. Pathogens 2022;11(6):6530.
31. Metawae AG, Bayoumy AM, Ali IR, Hammam OA, A Temsah KAT. Efficacy of nitazoxanide alone or loaded with silica nanoparticle for treatment of cryptosporidiosis in immunocompetent hosts. IJMA 2021; 3(2):1229-1239 .
32. Sztanke K, Maziarka A, Osinka A, Sztanke M. An insight into synthetic Schiff bases revealing antiproliferative activities in vitro. Bioorg Med Chem 2013; 21(13):3648-3666.
33. Ferreira LG, Andricopulo AD. Drug repositioning approaches to parasitic diseases: A medicinal chemistry perspective. Drug Discovery Today 2016; 21(10):1699-1710.
34. Zuma NH, Aucamp J, David DD. An update on derivatisation and repurposing of clinical nitrofuran drugs. Eur J Pharm Sci 2019; 140:105092.
35. Saad AA, Soliman MI , Azzam AM, Mostafa B. Antiparasitic activity of silver and copper oxide nanoparticles against *Entamoeba histolytica* and *Cryptosporidium parvum* cysts. J Egypt Soc Parasitol 2015;45(3): 593-602.
36. Misra SK, Wu Z, Ostadhossein F, Ye M, Boateng K, Schulten K. Pro-nifuroxazide self-assembly leads to triggerable nanomedicine for anti-cancer therapy. ACS Applied Materials Interfaces 2019; 11(20):18074-18089 .
37. Thomson S, Hamilton CA, Hope JC, Katzer F, Mabbott NA, Morrison LJ, *et al*. Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. Vet Res 2017;48(1):1-16.
38. Lu Y, Park K. Polymeric micelles and alternative nanonized delivery vehicles for poorly soluble drugs. Int J Pharm 2013; 453(1):198-214.
39. Kampert T, Misra SK, Srivastava I, Tripathi I, Pan D. Phenotypically screened carbon nanoparticles for enhanced combinatorial therapy in triple negative breast cancer. Cell Mol Bioeng 2017; 10(5):371-386.
40. Harp JA, Jardon P, Atwill ER, Zylstra M, Checel S, Goff JP, *et al*. Field testing of prophylactic measures against *Cryptosporidium parvum* infection in calves in a California dairy herd. Am J Vet Res 1996; 57(11):1586-1588.