Efficacy of *Cyperus rotundus* extract against cryptosporidiosis and toxoplasmosis in murine infections

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Background
Apicomplexa is a phylum of single-celled, obligate intracellular protozoan parasites that are among the most common morbidity-causing diseases worldwide. This phylum contains a variety of intestinal protozoa of medicinal and veterinary interest, such as *Cryptosporidium* and *Toxoplasma*. These parasites can be acquired orally, before infecting or infiltrating the intestinal epithelium. Nitazoxanide (NTZ) is the only FDA-approved medicinal therapy currently in use. The conventional pharmacological therapies for toxoplasmosis include pyrimethamine and sulfadiazine; nevertheless, they have major limitations. The use of medicinal plants for treatment and to reduce dependence on chemical drugs has become an important goal for therapeutic research.
Objective
Intending to develop alternative therapeutic options to address these health problems, we examined the efficacy of an ethanol extract of *Cyperus rotundus*, which has been demonstrated to have antiparasitic and hepatoprotective effects against *Cryptosporidium* and *Toxoplasma* in mice, with the goal of developing alternative therapeutic options to treat these health problems.
Materials and methods
A total sample of 72 male mice was used for the experiment, the animals were separated into two groups of 36 mice each: the first group was used to examine the activity of ethanol extract of *C. rotundus* against *Cryptosporidium*, and the second group was used to examine its activity against *Toxoplasma*. Each experimental model was divided into six subgroups of six mice each: the first group was noninfected nontreated, the second infected nontreated, third infected and treated with the standard drug, fourth and fifth infected and treated with *C. rotundus* at 250 and 500 mg/kg body weight, respectively, and the sixth infected and received a combination of half doses of both drugs [*C. rotundus* (250 mg/kg/day) and half dose of the standard drug (NTZ or Spiramycin)]. The parasitological parameters and reduced glutathione, super oxide dismutase, and malondialdehyde levels in the liver homogenates were used to determine the infections and medication impacts.
Results and conclusion
The results showed a promising finding that ethanol Egyptian herbal extract of *C. rotundus* and its combination with the standard drugs NTZ and Spiramycin have a promising antiparasitic and hepatoprotective activity against murine cryptosporidiosis and toxoplasmosis, respectively. The combined therapies resulted in the highest effectiveness of standard medications.

Keywords:
Cryptosporidium oocysts, *Cryptosporidium* trophozoite, *Cyperus rotundus* extract, hepatoprotective activity, *Toxoplasma tachyzoites*

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Introduction

Cryptosporidium parvum and *Toxoplasma gondii* are pathogenic protozoan parasites belonging to the phylum *Apicomplexa*. *Cryptosporidium* was the second most prevalent cause of moderate-to-severe diarrhea in children under the age of 2 years [1] Furthermore, in immunocompromised persons, it might be a life-threatening infection [2]. There is no vaccine, and the sole FDA-approved medication, Nitazoxanide (NTZ), has been demonstrated to have effective limits in many patient groups known to suffer high-illness risk [3].

T. gondii is the causative protozoan agent of toxoplasmosis, a widespread illness that is found all over the world [4]. Toxoplasmosis is associated with behavioral and neurochemical alterations [5–7]. *Toxoplasma* uses a variety of survival mechanisms, including intracellular parasitism and immunological

disruption, to evade the host's immunological response, making vaccine development exceedingly challenging [8]. The usual medication therapies for toxoplasmosis include pyrimethamine and sulfadiazine; however, these medicines cannot eliminate *T. gondii bradyzoites* [9] and several failure cases have also been documented indicating the presence of drug-resistant strains [10].

Herbal extracts are now seen to be a promising source for the development of novel medications. These herbal extracts possess a wide range of bioactive components that have a particular physiological impact on the human body, such as tannin, flavonoids, alkaloids, and phenolic substances, and may serve as possible substitutes for many synthetic drugs [11]. Since ancient times, Egypt has been known for its herbal medicine. The trends in the use of traditional alternatives to pricey pharmaceuticals, either alone or as complements to the chemical pharmaceuticals in treatment protocols, were discovered [12].

Cyperus rotundus belongs to the *Cyperaceae* family and distributes all over the world [13], including Egypt [14,15]. Antibacterial [16], antiviral [17], insecticidal [18], antiplasmodial [19], and antihelmintic [20] actions have been reported for *C. rotundus*, in addition to anti-inflammatory [21], antidiabetic [22], antidiarrheal [23], cytoprotective [14], antioxidant [24], cytotoxic and apoptotic [25], and antipyretic and analgesic activities [26].

Therefore, in an attempt to develop a new therapy from Egyptian herbal extract for *C. parvum* and *T. gondii*, the parasitological and hepatoprotective activities of ethanol extract of *C. rotundus* against *C. parvum* and *T. gondii* in mice were evaluated. In addition, combinations of the extract with the validated anti-apicomplexan drugs may offer a viable therapeutic strategy to be evaluated to boost antiparasitic effects, minimize costs, enhance treatment quality, and reduce medication toxicity.

Materials and methods

Ethical and regulatory guidelines

The experimental animals' studies were conducted following internationally accepted guidelines, and the Ethical Committee for Animal Experimentation, Theodor Bilharz Research Institute, Egypt, while making efforts to abate animal pain.

Preparation of plant extract

Fresh *C. rotundus* samples were collected from Orman garden, Giza, Egypt. The rhizomes were identified by a botanical specialist and consultant at Orman botanic gardens. The plant was dried, powdered into a fine powder, and stored in secure containers, Theodor Bilharz Research Institute's Medicinal Chemistry Department. Five-hundred grams of dry powder was extracted with 85% ethanol for 1 week. The extract was then filtered and concentrated under a vacuum using a rotatory evaporator (BUCHI, Flawil, Switzerland). The crude ethanol extract was then collected and dried for future use [27,28].

Experimental animals and parasites

A total of 72 male albino mice, —5–6 weeks old, ~20–22 g, were obtained from Schistosome biological supply program (Theodor Bilharz Research Institute, Cairo, Egypt). To exclude parasite infection, their feces were examined for 3 consecutive days before the commencement of the experiments as determined using the formol–ether concentration method [29] and the modified Ziehl–Neelsen technique [30]. They were kept in a room with temperature, lighting, and relative humidity controls. They were fed conventional food and given unlimited access to water. The animals were separated into two groups of 36 mice each: the first group was used to examine the activity of *C. rotundus* against *Cryptosporidium,* and the second group was used to examine the activity against *Toxoplasma*. The parasitological parameters and oxidative stress in the liver were used to determine infection and medication impact.

The activity of *Cyperus rotundus* extract against *Cryptosporidium*

Except for the control group, mice were infected intraesophageally with 1×10^3 *Cryptosporidium* oocysts by gastric gavage [31]. The *Cryptosporidium* isolates utilized in this study originated from infected patients in Theodor Bilharz Research Institute Hospital. A modified formalin–ether-sedimentation procedure was used to concentrate the samples. To confirm the presence of oocysts, the stool samples were stained using a modified Ziehl–Neelsen staining method. Nanazoxid suspension (100 mg/5 ml) from Utopia Pharmaceuticals (Cairo, Egypt) was administered orally in a dosage of 200 mg/kg daily for 5 consecutive days to mice, based on prior studies [32].

Mice were divided into six subgroups of six mice each. The first subgroup was kept as negative control (neither infected nor treated). The second subgroup was infected but not treated. The third subgroup was infected and given 200 mg of NTZ. In the fourth and fifth subgroups, infected mice were given

Cyperus extract at a dosage of 250 and 500 mg/kg body weight, respectively. The sixth subgroup was infected and treated with the combination of *Cyperus* and NTZ (half dose of both, 250 mg/kg *Cyperus*+100 mg/kg NTZ). Doses were administered daily for 5 consecutive days [32]. On the 14th day after treatment, feces were collected from each mouse in the treated and control groups, and oocysts were counted/feces weight. Mice were sacrificed by rapid decapitation, and liver was collected for assessment of different oxidative stress-related biochemical parameters [glutathione (GSH), super oxide dismutase (SOD), and malondialdehyde (MDA)] using the spectrophotometer. Duodenal content was also examined for trophozoite count after scarification of mice, smeared on a slide and stained by modified Zeihl–Neelsen stain, and examined microscopically.

The activity of *Cyperus rotundus* extract against *Toxoplasma*

Tachyzoites from the virulent RH strain of *T. gondii* were maintained in Swiss albino mice by intraperitoneal passages at the laboratory, Department of Parasitology (Theodor Bilharz Research Institute), Egypt. *Tachyzoites* were extracted from the ascites' fluid of infected mice on the fourth day of infection [33]. Debris and host cells were filtrated through a sheet of glass-wool fibers. The filtrate was washed three times and diluted with phosphate buffer saline, pH 7.4. After counting in a hemocytometer, suspensions were adjusted to 2×10^2 tachyzoites/ml with saline and 0.4-ml aliquots were injected intraperitoneally into mice as Grujić *et al*. [34] described.

All medications were administered on the first day of infection and continued for 5 days. Mice were divided into six subgroups, as described in the *Cryptosporidium* model: the first was noninfected, the second was infected but not treated, and the third was infected and treated with Spiramycin (Phraonia Pharmaceuticals, Cairo, Egypt, in the form of one-and-a-half milligrams per tablet). The Spiramycin tablet was ground and the dose per mouse was calculated and adjusted to dissolve in 100 µl of saline for oral administration at a dose of 200 mg/kg [34], the fourth and fifth groups were infected and treated with *Cyperus* extract at doses of 250 and 500 mg/kg body weight [22], respectively. The sixth subgroup was infected and treated with the combination of *Cyperus* and Spiramycin (half dose of both, 250 mg/kg *Cyperus* +100 mg/kg Spiramycin). On the 14th day after infection, peritoneal fluid containing *T. gondii tachyzoites* was collected, and the mean number of *tachyzoites* was determined. The percentage reductions in the mean number of parasites in treated versus infected control mice were calculated as per the formula:

Reduction percent: %R=100 (C-T/C); C: the infected control subgroups, T: treated mice subgroups.

Biochemical analysis

The liver was homogenized in the appropriate buffer (1 g/10 ml) using a glass homogenizer. The homogenate was filtered and centrifuged for the analysis of antioxidant enzymes. Reduced GSH, SOD, and MDA levels in liver homogenates were determined using biodiagnostic assay kits according to the techniques of Beutler and Kelly [35], Marklund and Marklund [36], and Mihara and Uchiyama [37], respectively.

Statistical analysis

The Statistical Package for Graph Pad Prism application (San Diego, California, USA), version 6.0 for Windows, was used to analyze the obtained and confirmed data. To compare the different studied groups, quantitative data were presented as mean and SE and analyzed using the *F* test (analysis of variance) followed by Tukey's multiple-comparison test. Differences were considered as significant at *P* value less than 0.05.

Results and discussion

C. parvum and *T .gondii* are significant parasites of both humans and animals globally, necessitating the development of novel and effective therapies [38]. Medicinal plants are regarded as a valuable source for the discovery of novel antiparasitic drug leads [39–41] for cryptosporidiosis [42–44] and toxoplasmosis [45–48]. As a result, the current study was carried out to create novel anti-*Cryptosporidium* and anti-*Toxoplasma* treatments using our native medicinal plant *C. rotundus*. In this work, the anti-apicomplexan action of *C. rotundus* extract was markedly observed in mice in both monotherapy (500 mg/kg) and combination therapy against *Cryptosporidium* and *Toxoplasma*. A remarkable result to emerge from the data is that the most potent anti-*Cryptosporidium* and anti-*Toxoplasma* activity were recorded in the combination treatments of *Cyperus* with clinical drugs, NTZ and Spiramycin, respectively. The combined therapy resulted in the highest-percentage reduction in the number of *Cryptosporidium* trophozoites in intestinal contents; 72.6%, followed by NTZ treatment, which resulted in a decrease of 55.8% (Table 1). Stool analysis

demonstrated a significant reduction in the number of *Cryptosporidium* oocysts in the group treated with *Cyperus* (500 mg/kg) and combination group. The combined NTZ-*Cyperus*-treated group had the largest-percent reduction in oocyst count (85.4%), followed by *Cyperus* (500 mg/kg) (76.3%) and finally *Cyperus* (250 mg/kg) group (65.2%). The group that received NTZ had the least reduction (Table 2). Peritoneal fluid examination of *T. gondii*-infected mice indicated that the combination Spiramycin–*Cyperus* treatment induced the highest-percent reduction in *T. gondii tachyzoites* count (66.2%), followed by Spiramycin (64.7%) and finally *Cyperus* (500 mg/kg) group (55.5%). The *Cyperus* (250 mg/kg) group had the lowest decrease (31.4%) (Table 3). To the best of our knowledge, this study is the first investigation to identify *Cyperus* as an anti-apicomplexan compound with potent efficacy against parasites representing all branches of the apicomplexan phylogeny.

The evident anti-apicomplexan action of *C. rotundus* extract discovered in the present study might be attributed to its functional bioactive components, such as alkaloids, flavonoids, saponins, tannins, and triterpenoids [27,49–51]. Alkaloids disrupt the

Table 1 The mean number and the percentage of reduction of *Cryptosporidium* trophozoites in intestinal content 14 days post-treatment

Groups	Number of trophozoites/HPF (mean±SE)	Percentage of reduction in the number of trophozoites	*F* test
Infected nontreated	18.83±1.276		
Infected treated with Nitazoxanide	8.33±0.88[a]	55.75	*F*=40.27, *P*<0.0001
Infected treated with *Cyperus* 250 mg/kg	14.00±0.68[a,b]	25.65	
Infected treated with *Cyperus* 500 mg/kg	9.33±0.42[a]	50.44	
Infected treated with Nitazoxanide +*Cyperus*	5.17±0.70[a,b,#]	72.56	

F, value for analysis of variance test. [a]Significant compared with an infected group. [b]Significant compared with infected treated with Nitazoxanide. [#]Nonsignificant compared with Nitazoxanide.

Table 2 The mean number and the percentage of reduction of *Cryptosporidium* oocyst in stool 14 days post-treatment

Groups	Number of oocyst (mean ±SE×10^3)	Percentage of reduction in the number of oocysts	*F* test
Infected nontreated	12.92±0.62		
Infected treated with Nitazoxanide	4.60±0.34[a]	64.24	*F*=119.1, *P*<0.0001
Infected treated with *Cyperus* 250 mg/kg	4.50±0.45[a,#]	65.21	
Infected treated with *Cyperus* 500 mg/kg	3.07±0.26[a,b]	76.26	
Infected treated with Nitazoxanide +*Cyperus*	1.89±0.17[a,b]	85.35	

F, value for analysis of variance test. [a]Significant compared with an infected group. [b]Significant compared with infected treated with Nitazoxanide. [#]Nonsignificant compared with Nitazoxanide.

Table 3 The mean number and the percentage of reduction of *Toxoplasma* tachyzoites in peritoneal fluid 14 days post-treatment

Groups	Number of tachyzoites (mean ±SE×10^3)	Percentage of reduction in the number of tachyzoites	*F* test
Infected nontreated	4.65±0.224		
Infected treated with Spiramycin	1.64±0.0874[a]	64.73	*F*=49.79, *P*<0.0001
Infected treated with *Cyperus* 250 mg/kg	3.19±0.284[a,b]	31.40	
Infected treated with *Cyperus* 500 mg/kg	2.07±0.154[a,b]	55.48	
Infected treated with Spiramycin +*Cyperus*	1.57±0.0943[a,#]	66.24	

F, value for analysis of variance test. [a]Significant compared with an infected group. [b]Significant compared with infected treated with Spiramycin. [#]Nonsignificant compared with Spiramycin.

parasite's amino acid metabolism and/or DNA synthesis of the parasite [52]. Some flavonoids have been shown to suppress *C. parvum* [53,54] and *T. gondii* [55,56] by damaging cell membranes and inhibiting DNA, RNA, and proteins synthesis, or inhibiting microorganisms' reproduction [53–57]. Furthermore, saponins affect the permeability of parasite cell membranes and promote cytotoxic action [58], resulting in parasite degeneration [59]. Tannins have antiparasitic activity as they inhibit parasite metabolism [60–62]. Terpenoids have been shown to have anti-apicomplexan activity [63,64], and their recognized actions include cell membrane instability, inhibition of key parasite enzymes with the resulting ultrastructural changes, and cell death [65].

Our findings support the notion that *C. parvum* and *T. gondii* cause oxidative stress in experimentally infected mice. This finding was approved by a significant rise in hepatic MDA levels in *C. parvum* and *T. gondii*-infected mice, as well as a significant drop in hepatic SOD and GSH levels. Previous findings related to *C. parvum* [66–69] and *T. gondii* infections [70–72] are consistent with our findings. Another promising finding was that *C. rotundus* treatment improved oxidative damage generated by *Cryptosporidium* and *Toxoplasma* by increasing antioxidant contents (SOD and GSH) and reducing MDA levels in liver tissue (Tables 4 and 5). Our findings are in line with earlier studies that confirmed the antioxidant activity of *C. rotundus* extract in vitro and in vivo [14,24,27,73–77]. *C. rotundus* was safe up to 2 g/kg body weight and did not cause toxicity to the host cells in vitro and in vivo [49,78–80].

The most striking result to emerge from the data is that combination treatments have antioxidant activity against *Cryptosporidium* and *Toxoplasma* that is superior to standard drugs and even improve to the point of approaching that of healthy control. The hepatoprotective activity of *C. rotundus* extract may be attributed to the presence of flavonoids, alkaloids, terpenoids, and phenols [24,28,50,73]. Herbal medicines' antioxidant capabilities are beneficial in decreasing the toxicity of hazardous substances [81] or other drugs [82].

It appears that combining medicinal plants that contain biological bioactive compounds with synthetic

Table 4 Effect of *Cyperus rotundus* on oxidative stress parameters in liver of mice infected with *Cryptosporidium* 14 days post-treatment

Groups	GSH (mg/g protein) level (mean±SE)	Statistical analysis	SOD (mg/g protein) level (mean±SE)	Statistical analysis	MDA (mg/g protein) level (mean±SE)	Statistical analysis
Noninfected	96.20±1.66		9.71±0.22		2.63±0.11	
Infected	79.00±2.93*		8.27±0.27*		3.96±0.06*	
Infected treated with Nitazoxanide	85.38±1.45*	F=19.97, P<0.0001	9.12±0.12[a]	F=15.27, P<0.0001	3.08±0.18[a]	F=17.15, P<0.0001
Infected treated with *Cyperus* 250 mg/kg	77.98±1.72*		7.97±0.10*,b		3.75±0.11*,b	
Infected treated with *Cyperus* 500 mg/kg	81.40±0.92*		8.51±0.16*		3.11±0.14[a]	
Infected treated with Nitazoxanide+*Cyperus*	93.70±1.50[a],b		9.60±0.19[a]		2.86±0.13[a]	

F, *F* value for analysis of variance test; GSH, glutathione; MDA, malondialdehyde; SOD, super oxide dismutase. [a]Significant compared with infected. [b]Significant compared with infected treated with Nitazoxanide. *Significant compared with normal control.

Table 5 Effect of *Cyperus rotundus* on oxidative stress parameters in liver of mice infected with *Toxoplasma* 14 days post-treatment

Groups	GSH (mg/g protein) level (mean±SE)	Statistical analysis	SOD (mg/g protein) level (mean±SE)	Statistical analysis	MDA (mg/g protein) level (mean±SE)	Statistical analysis
Noninfected	99.43±2.79		9.49±0.25		2.78±0.16	
Infected	66.90±1.33*		7.45±0.29*		4.33±0.17*	
Infected treated with Spiramycin	83.87±1.34	F=8.89, P<0.0001	9.15±0.13[a]	F=18.94, P<0.0001	3.37±0.13*,a	F=19.69, P<0.0001
Infected treated with *Cyperus* 250 mg/kg	74.49±0.95*		7.98±0.083*,b		4.27±0.14*,b	
Infected treated with *Cyperus* 500 mg/kg	69.39±8.89*		8.13±0.148*,b		3.52±0.11*,a	
Infected treated with Spiramycin +*Cyperus*	84.54±2.65[a]		9.11±0.13[a]		3.17±0.11[a]	

F, *F* value for analysis of variance test; GSH, glutathione; MDA, malondialdehyde; SOD, super oxide dismutase. [a]Significant compared with infected. [b]Significant compared with infected treated with Spiramycin. *Significant compared with normal control.

conventional pharmaceuticals might boost activities, lower the costs, enhance treatment quality, and lessen medication-adverse effects. The mechanism behind the observed combined effects of *Cyperus* and the synthetic medicines NTZ and Spiramycin in the current investigation remained unknown. It might be related to these drugs' various synergistic modes of action.

Conclusion

In conclusion, the study supports the effectiveness of the Egyptian herbal extract *C. rotundus* after being combined with NTZ and Spiramycin in controlling the murine cryptosporidiosis and toxoplasmosis, respectively, than synthetic drugs. In addition to its antiparasitic effectiveness, *C. rotundus* extract and combined therapy exhibit hepatoprotective activity with a superior effect of the combined therapy than the standard drugs and even improve to the extent of approximating that of healthy control. More research on these combinations is needed since the encouraging results warrant further investigations.

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Conflicts of interest

There are no conflicts of interest.

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