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# EFFECT OF SOME MEDICINAL HERBS VERSUS NITAZOXANIDE ON CRYPTOSPORIDIUM PARVUM INFECTED IMMUNOSUPPRESSED MICE

By
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**Abstract**

Generally cryptosporidiosis is endemic more or less worldwide and is one of the commonest causes of persistent diarrhea among children, especially immunosuppressed ones. The study evaluated the potential therapeutic effects of some natural herbal agents versus Nitazoxanide® in treating *Cryptosporidium parvum.*

A total of sixty Albino mice were immunosuppressed and then infected with *C. parvum* oocysts and were divided into 6 groups. Three infected groups were treated with Asafoetida, Curcumin, and *Artemisia annua*, compared to Nitazoxanide treated, negative, and positive controls. Assessment of treatment was microscopy by counting oocytes stool, histopathology of ileocaecal region andplasma clearance of alpha 1 anti-trypsin by ELISA.

Results showed that Asafoetida, artemisinin, and curcumin eliminated infection in 50, 55, & 55% of treated mice respectively. Nitazoxanide remained gold standard (80%) successive rate.

**Keywords:** Mice, Cryptosporidiosis, Artimesinin, Asafoetida, Curcumin, Nitazoxanide.

## Introduction

*Cryptosporidium* species are intracellular gastrointestinal protozoa cause risky zoonotic diarrhea worldwide (El Bahnasawy *et al,* 2018). In Egypt, so many authors dealt with cryptosporidiosis from different aspects as (El-Sibaei *et al,* 2003, El shazly *et al,* 2017; El-Badry *et al,* 2017; Fahmy *et al,* 2021; Imam *et al,* 2022 and others) The main symptoms are watery diarrhea, stomach cramps or pain, dehydration, nausea, vomiting, fever and weight loss, but some people don't have symptoms at all (CDC, 2021). The parasite infiltrates the enterocytes at the brush border leading to inflammatory diarrhea; increased transepithelial permeability, villous atrophy, crypt hyperplasia, cell death; and somalabsorption (Di Genova and Tonelli, 2016). Increased intestinal permeability and local inflammatory response were mechanisms and protein-losing enteropathy occurred as a diverse disorder associated with excessive loss of serum protein through the GIT. Protein loss by radioactive chromium chloride (I.V.) or labeled human sera albumin was limited due to requisite radioactive and long exposure period (Levitt, 2017). Fecal clearance of plasma alpha 1-antitrypsin (A1AT) measured of protein leakage into the intestinal tract, the 54 KDa anti-inflammatory glycoprotein secreted mainly by the liver and plays an important role in tissue repair and lymphocyte activation, which degradation by digestive enzymes so used as an endogenous marker for losing protein from the intestinal tract (Karatas and Bouchecareilh, 2020).

Nitazoxanide® was a FDA-approved drug sold under brand name Alinia among others, is a broad-spectrum antiviral medication that is used in medicine for treatment of various helminthic, protozoal, and viral infections (Reference ID: 3956992). It was not approved for immunosuppressed hosts, but it can reduce diarrhea, and alleviate the symptoms without eliminate of cryptosporidiosis underlining the importance of finding new drugs those patients (Sparks *et al*, 2015).

Medicinal plants have been successfully used as an alternative source of drugs for treatment of microbial diseases worldwide long ago (Abouel-Nour *et al,* 2017). Asafoetida (*Ferula asafoetida*) or dung of devil, belongs to Umbelliferae family, which aqueous extract, resin extract from stems and roots of *Asafoetida* essential oil were used as an anthelmintic, antispasmodic, carminative, laxative, mucolytic, expectorant, and sedative (Mahendra and Bisht, 2012).

Curcumin belongs to ginger Zingiberaceae family, tuberous rhizomes, or underground

stems, acts as an antioxidant and anti-inflammatory and against *Helibacter pylori* and many intestinal & extra-intestinal protozoa, such as *Cryptosporidium*, *Giardia*, *Leishmania*, and *Trypanosomes* (Sarkar *et al*. 2016).

*Artemisia annua* is an annual weedy herb belongs to Asteraceae family, Artemisinin and its derivatives is well known for their antimalarial activity, a wide range of antimicrobial and antiparasitic properties with varying efficacy (Loo *et al,* 2017).

The present study aimed to evaluate three medicinal herbs versus Nitazoxanide® in treating *Cryptosporidium parvum* in immunosuppressed Albino mice.

## Materials and Methods

Animals: Laboratory-bred male Albino mice, about 4–6 weeks old, weighing 20–25 g, were purchased, maintained under standard laboratory conditions on normal diet. All the animal experiments were performed following the guidelines approved by the Institutional Animal Care and Use Committee after the approval of the Institutional Ethical Committee. All experiments were performed in the biological unit, Faculty of Science, Fayoum University, in a well-ventilated plastic cage with clean woodchip bedding in conditioned room (27±2$^{o}$C) and given normal diet.

Experimental Design: Sixty immunosuppressed laboratory-bred Albino male mice were divided into 6 groups of 10 mice each. G1: infected and treated with Asafoetida, G2: infected and treated with Artemisinin, G3: infected and treated with Curcumin, G4: infected and treated with Nitazoxanide, G5: infected and untreated (Positive control), & G6: neither infected nor treated (Negative control)

Immunosuppression: Dexamethasone sodium phosphate was used for immunosuppression Mice were administered with 0.25ug/gm/day of orally using an esophageal tube for two weeks before the oral inoculation with *Cryptosporidium parvum* oocysts and was maintained during the whole experiment for all groups (Toulah *et al,* 2012).

Infection: *Cryptosporidium parvum* oocysts were kindly obtained from the Animal Research Institute, Egyptian Ministry of Agriculture, Giza Governorate. Mice were orally infected with *C. parvum* oocysts 1000 oocysts dissolved in 200µl of PBS by suitable esophageal tube.

Preparation of plant extracts: Three plants tested were Asafetida (dried latex, gum oleoresin exuded from rhizome or tap), Curcumin (an active compound from turmeric, *C. longa)*, and *Artemisia annua* purchased locally and identification was approved by the Botany Department, Fayoum Faculty of Agriculture.

For extraction, fine powder of each herb (50g) was obtained by electric blending and boiled in distilled water (200 ml) for 90 min, and filtered through What-man papers. The filtrated water extract was evaporated under reduced pressure and lyophilized to give a dry extract. The obtained dry residues were reconstituted in DMSO to give stock solutions of 100 mg/ml. The extracts were sterilized by filtration using Acrodisc (Gelman, 0.22µm size) and then preserved in the deep freezer (–20°C) till needed at a dose of 100 mg/kg for artemisinin and 300mg/kg for asafetida and curcumin (Abdelmaksoud *et al,* 2020).

Nitazoxanide was used as a controlled drug for 7 successive days post-infection as 500mg tablets (Minapharm, Egypt) tablets were crushed and dissolved in DMSO, and then diluted in an incubation medium to give concentrations of 250mg/ml (Pandy, 2020).

Parasitological examination: Stool samples were collected and subjected to parasitological examination after proper mixing and staining by Modified Zheil-Nelsen (MZN) stain (Henricksen and Pohlenz, 1981) using an oil immersion lens and for counting the oocyst in 10 high power fields by using 40X lens. A stool examination was done a week after parasitic inoculation to the confirm infection and 1 week after treatment.

A1AT clearance assay: Blood sample was drawn from saphenous or dorsal pedal vein

to sterile Eppendorph tube (Parasuraman *et al,* 2017). Alpha 1 Antitrypsin (AIAT) using ELISA (A1AT ELISA KIT mouse, Abcam). After 24 hours stool was collected, the volu me was measured. Aliquot was stored for up to 6 months at −20°C without preservative for A1AT (RIDASCREEN α1-Antitrypsin R-Biopharm) determined by using ELISA following the manufacturers' instructions. The A1AT clearance was calculated using the formula

A1AT clearance = (FxV)/S mL/24 hours where F = fecal concentration of A1AT; V = fecal volume; S= serum concentration of A1AT.

Histopathological examination: One week post-treatment, all mice were individually sacrificed. The ileocecal samples were fixed in 10% buffered formalin solution, embeded in paraffin wax blocks, sectioned and stained using hematoxylin and eosin to for histopathological study.

Statistical analysis: Data were collected, tabulated and analyzed by using Social Sciences (SPSS) version 18.0. Student's t test compared the means between two groups, & ANOVA was used to compare the means among three or more groups. P value was considered significant if it was < 0.05.

Ethical consideration: This study was approved by Scientific Research Ethics Committee of Faculty of Medicine, Fayoum University, which went with Helsinki Guidelines

## Results

Cryptosporidiosis caused severe diarrhea in immunosupressed mice, with marked decrease in activity, and seven of them died during experimental evaluation. A week post infection stool examination was done to confirm infection with oocysts/mouse were 97± 23oocyst/10Hpf.

Details are given in table (1) and figure (1)

Table 1: Parasitic examination, A1AT clearance oocyst count in stool and tissue sections among groups:

| Group | TTT | Number | *P value | A1AT clearance | P value | Oocyst post TTT /10 Hpf | **P value | oocysts/ 50 HPF | ***P value |
|---|---|---|---|---|---|---|---|---|---|
| G1 | Positive | 4 | 0.054 | 82.3 ±12.9 | 0.053 | 33.0± 3.3 | 0.018 | 44± 6 | 0.006 |
| | Negative | 4 (50%) | | 31.3 ±19.4 | | 0 | | 0 | |
| | died | 2 | | | | | | | |
| G2 | Positive | 4 | 0.031 | 67.6 ±10.3 | 0.023 | 30.0 ± 3.9 | 0.021 | 41 ±9 | 0.007 |
| | Negative | 5 (55%) | | 30.3 ±16.8 | | 0 | | 0 | |
| | died | 1 | | | | | | | |
| G3 | Positive | 4 | 0.031 | 85.8± 5.54 | 0.051 | 33.6 ± 18 | 0.017 | 55± 3 | 0.010 |
| | Negative | 5 (55 %) | | 32 ±11.4 | | 0 | | 0 | |
| | died | 1 | | | | | | | |
| G4 | Positive | 2 | 0.001 | 67± 33.9 | 0.015 | 18.0 ± 2.3 | 0.022 | 23± 6 | 0.001 |
| | Negative | 8 (80 %) | | 31.3± 19.4 | | 0 | | 0 | |
| G5 | Positive | 7 | | 95.3±11.8 | | 97 ± 23 | | 118 ±16 | 0.001 |
| | Negative | 0 | | -- | | 0 | | 0 | |
| | died | 3 | | -- | | | | | |
| G6 | Positive | 0 | | -- | | 0 | | 0 | |
| | Negative | 10 | | 12.7± 5.9 | | 0 | | 0 | |
| Total | Positive | 27 | | 87.6± 12.1 | 0.001 | | | | |
| | Negative | 26 | | 27.0±8.2 | | | | | |
| | died | 7 | | | | | | | |

*P value of herbs vs control. **A1AT C and oocyst post treatment, ***Oocysts in sections, and stools. High A1AT clearance in positive controls (protein losing enteropathy and malabsorption). After treatment, a significant reduction in mice treated with artemisinin and nitazoxanide association between oocysts, A1AT Clearance, pathological, and clinical manifestations.

Pathological findings were: A- Examination of ileocecal sections from negative control showed normal structure of villi, intact brush border, an average number of goblet cells without no inflammation and lymphoid follicle appear normal. B- Examination of ileocecal sections from the positive control showed so many apical oocysts with basophilic circular shaped with minute ulcers, many inflammatory cells, plasma cells , and eosinophils in lamina propria, Villuous atrophy (disturbed villus/crypt length ratios), goblet cell depletion and hyperplastic lymphoid follicles, associated with huge oocysts

excretion and marked elevated A1AT clearance these changes were in positive controls. C- Examination of small intestinal sections at ileocecal junction, all treated mice showed various degrees of healing with improvement in villous architecture as the partial villous blunting with scanty apical organisms, lymphoid follicles were more or less mildly enlarged with mild plasma cells and inflammatory cells invasion, associated with few oocysts and low A1AT clearance.

## Discussion

Cryptosporidiosis is one of the major causes of diarrhea in immunocompetent hosts and the cause of severe debilitating diarrhea in immunosuppressed ones causing malabsorption and weight loss, transmitted mainly by fecal-oral route through contaminated water and food is the principal mode of transmission.

Medicinal plants serve as a great source of drug innovation. Asafoetida could eliminate the infection in 50 % of animals with a significant reduction in mean oocyst count. It was long used as an antihelminthic to expel parasites from human and domestic animals.

Ramadan *et al.* (2004) in Egypt found that the powder form is superior to the oil form that led to a reduction in the number of eggs and worms of *Schistosoma mansoni* in a murine model. Farhadi (2016) in Iraq reported that *F. asafoetida* gave in-vitro anti-cestodes andanti-nematodes effect on infected rats. Also, Tavassoli *et al.* (2018) in Iraq reported that hydroalcoholic extract of *F. asafoetida* as 10, 50 & 100mg/ml killed more than 90% of *Strongylus* larvae in horses, but *A. sativum* extract at concentration of 50 & 100mg /ml killed over 95% of them ($P<0.05$). As to the anti-protozoal efficacy of *F. asafoetida* against *Leishmania* spp. inhibited 100% growth of cultured promastigote (Bafghi *et al,* 2014). Also, Alnomasy *et al.* (2021) in Saudi Arabia reported active of *F. asafoetida* as *Giardia lamblia* in vitro with 100% efficacy. Besides, Abdelmaksoud *et al.* (2020) in Egypt found that *F. asafoetida* efficacy in both prophylaxes rate of 70% prevention and treatment success rate of 65% in cryptoscoridiosis infected mice.

In the present study, artemisinin eliminated the infection in 55 % of treated animals with a significant reduction in mean oocysts' count with A1AT clearance. It is an FDA-approved drug against severe malaria and it showed moderate in-vitro effect against *Leishmania* amastigotes with 83.3% cure rate on mice. But, a mild inhibitory effect of artemisinin on *Toxoplasma gondii* was reported in vitro (Hencken *et al,* 2010). Islamuddin *et al.* (2014) in Iran reported artemisinin efficacy in visceral leishmaniasis with 90% decrease in parasite in liver and spleen without any toxicity was detected with a dose of 200mg/kg/day. Olivera *et al.* (2015) in France reported lethal activity against *Trypanosoma cruzi* and *T. bruci* in culture. Mesa *et al.* (2017) in Brazil treated cutaneous leishmaniasis with a dose of 500mg/kg/day for one month duration obtained 100% cure in infected mice. Other authors reported various efficacy against *Eimeria tenella* (Blake *et al,* 2015), *Acanthamoeba castellanii* (Lorenzo-Morales *et al,* 2015), *Naegleria fowleri* (Rice *et al,* 2015), and *Giardia lamblia* (Golami *et al,* 2016). Despite being an efficient anti-protozoa, there was no available data as to its effect on cryptosporidiosis, only *Artemisia spicigera* was tested against infection in immunosuppressed mice and clearance of the infection, due to lack of infected models and unavialability of the active artemisinin component (Tasdemir *et al,* 2015), as well the high toxicity of artemisinin in a dose of 200mg/kg (Fayer *et al,* 1994), and in the present this double toxic dose was not used.

In the present study, curcumin is the active ingredient of turmeric, eliminated the cryptosporidiosis infection in 55 % of the infected mice. Curcumin is known as an antioxidant, anti-inflammatory, and anti-parasitic with a wide range of domestic and medicinal uses. Asadpour *et al.* (2017) in Iran confirmed the anti-cryptosporidial and antioxidant

activity of curcumin against *C. parvum* and added that compared its efficacy with a dose of 4.3mg/kg/day against the paromomycin, which was slightly more efficacy. Ur Rahm an *et al.* (2022) in China found that curcumin controlled the *Cryptsporodium* infection by modulating gut microbiota and innate immune-related genes, which may be linked to the anti-Cryptosporidium mechanisms of curcumin.

Also, many studies evaluated curcumin as many anti-parasites against many protozoa such as *Leishmania* where its efficacy exceeded cure more than pentamidine (Saleheen *et al,* 2002)**.** Also, it showed some inhibitory activity on the *Plasmodium* species in culture (Mimche and Taramelli, 2011)**.** It was reported that curcumin interfering with the tubular structure of *Giardia lamblia* trophozoites proliferation by perturbing microtubules **(**Gutiérrez-Gutiérrez *et al,* 2017), and down-regulated the virulence factor gene in *Entamoeba histolytica* and thus attenuated its pathogenicity (Rangel-Castañeda *et al,* 2019). But, studies on other protozoa such as *Trichomonas vaginalis, and Toxoplasma gondii* showed insignificant results. Its effect on helminths was investigated by Magalhães *et al.* (2009) who reported that in-vitro exposure to *Schistosoma* worms led to the death of all worms, and Kourosh *et al.* (2018) in Iran worked on *Fasciola* species found that Curcumin as the major compound extracted from C. longa serves for the various therapeutic and preventive purposes. Boonhok *et al.* (2022) in USA reported that curcumin inhibited cyst formation in surviving trophozoites, which may result from its effect on mRNA expression of the key *Acanthamoeba* ATG-related genes.

In the present study, nitazoxanide® the only FDA-approved drug for cryptosporidiosis eliminated the infection in 80% of infected mice with a significant decrease in A1AT clearance and oocysts count in stools of infected mice. Also, the histopathological sections showed much improvement regarding inflammatory cell infiltration, goblet cells' restoration, and healing of the minute ulcers. Rossignol *et al.* (2001) in USA reported that cryptosporidiosis diarrhea was treated in the majority patients received the nitazoxanide within 3 or 4 days of treatment initiation, and that nitazoxanide reduced the duration of both diarrhea (P<.0001) and oocyst shedding (P<.0001).

However, Amadi *et al.* (2002) in Zambia reported that the cryptosporidiosis treatment in HIV infected children proved to be difficult and unsatisfactory with no drugs having demonstrable efficacy in controlled trials except nitazoxanide. They reported no significant cure in children with cryptosporidiosis despite high dose and longer treatment duration, with neither eradication nor any clinical symptom reduction. Also, Abubakar *et al.* (2007) in UK reported that cryptosporidiosis was a common cause of gastroenteritis and was associated with risky lifethreatening illness among the immunocompromised individuals They found that nitazoxanide led to significant evidence of oocyst clearance compared with placebo with a RR of 0.52 (95% CI 0.30, 0.91), but the effect was not significant for HIV-seropositive patients, however, the nitaxozanide reduced parasitic load and might be useful in immunocompetent individuals. But, O'Connor *et al.* (2011) in USA concluded that as there was no effective specific therapy or vaccine available for cryptosporidiosis for immunocompromised, it was imperative to continue investigation into *Cryptosporidium* biology and host immune responses to develop novel and effective prophylactic and therapeutic strategies to prevent and treat this disease in those who are at the greatest risk of acquiring it and suffering its consequences.

## Conclusion

The three herbs showed somewhat a promising result in eliminating cryptosporidiosis with a significant reduction in the oocysts' count in stool and in ileocaecal sections with A1AT clearance. However, the nitazoxanide remains the drug of choice in eliminating the infection even in the immunosuppressed pat-

ients. Fecal antitrypsin concentration proved a valuable marker for mucosal disorders associated with abnormal transmucosal serum protein loss.

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**Explanation of figures**

Fig.1: *Cryptosporidium parvum* unstained
Fig. 2: A- Section in normal ileum showed healthy mucosa, goblet cell sand long villi G 6 ((H&E stain ×200). B- Mild affection of the mucosa with short stunting villi and inflammatory cell infiltration in treated mice (Healing Stage in Gs 1,2,3,4 (H&E stain ×200). C- Mucosal ulceration with infilteration of inflammatory cells seen in positive control (H&E stain ×200). D- Section in terminal ileum stained with (H&E x1000) with many mucosal, apical, basophilic, circular-shaped organisms (arrow), with epicellular invasion and blunting villi