

THE POTENTIAL CONVENTION OF GARLIC AND BLACK SEED DIFFERENT EXTRACTS AS AN EFFECTIVE TREATMENT OF *CRYPTOSPORIDIUM* SPP. : AN EXPERIMENTAL STUDY

By

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Abstract

The study investigated the effect of garlic (*Allium sativum*) and black seed (*Nigella sativa*) extracts products as treatment of *Cryptosporidium* infection in experimental mice in comparison to Nitazoxanide (NTZ). Forty-eight male Swiss Albino mice divided into 8 experimental conditions; G1-8, the first seven groups received 1.5×10^5 *Cryptosporidium* spp. oocysts on 1st infection day. The first group included mice treated with garlic juice; in G2, G4 & G5, mice were treated with black seed oil, alcoholic extract of black seed and alcoholic extract of black ariel parts respectively. In G3 mice were treated with garlic & black seed oil mixture, while G6 mice treated were with Nitaxanoide. Besides G7 were infected untreated (+ ve control); and G8 were neither-infected nor treated (- ve control); From 2nd day post-infection, pooled fecal samples of each group were collected daily to assess each extract efficacy as compared with NTZ and both +ve and -ve controls by calculating oocyst excretion pattern. The comparison between number of oocysts shedding rates among different treatment groups showed that the efficacy of NTZ was the highest (76.1%) followed by *N. sativa* oil (75.8%) followed by mixture of garlic and black seed oil (75.4%), alcoholic black seed extract (75.1%); alcoholic arial extract (74.7%) and the lowest was garlic juice (73.1%). Although both *A. sativum* and all *N. sativa* extract products showed a great degree of efficacy in reducing *Cryptosporidium* spp. oocysts excretions in the experimental animals compared to positive control, but without significance differences between them and preparation of both garlic, black seed and mixture with NTZ.

Keywords: *Allium sativum*; *Nigella sativa*; extracts; Nitaxanide; *Cryptosporidium* spp.

Introduction

Cryptosporidium is one of the opportunistic prevalent coccidian protozoan parasite (Fayer, 2008). Clinical features of cryptosporidiosis varied from abdominal pain, and anorexia (Fayer *et al*, 2000). The symptoms severity may wax and wane in patients according to the oocyst shedding intensity and host immunity (Current and Garcia, 1991).

Cryptosporidiosis drugs resistance to is one of most biological features as 200 regimen were tested (Miyamoto and Eckmann, 2015). Some exhibit promising effects but none was able to consistently control clinical signs or completely eradicate the infection. Some drugs reduced oocyst shedding, and also reduced environmental contamination

exposure and infection of susceptible hosts (Shahiduzzaman and Dausgies, 2012).

Cryptosporidiosis clinical course depended largely on the host immune status, benefit of chemotherapeutic agents or immunotherapy against cryptosporidiosis. (Chen *et al*, 2007). Nitazoxanide (NTZ) is one of the chemotherapeutic option, available as a treatment for cryptosporidiosis; with a broad-spectrum parasitocidal agent approved by US FDA for treating diarrhea caused by *Cryptosporidium* in children and adults (Carey *et al*, 2003). NTZ treated diarrhea within 3 to 4 days and reduced oocyst shedding (Abdel Megeed *et al*, 2015). But, NTZ showed limited ability to reduce oocyst shedding in a mouse model and in a pig model high dose (150-250mg/

kg/day) reduced oocyst shedding and diarrhea (Theodos *et al*, 1998).

The ban on drugs due to resistance development, demanded alternative cryptosporidiosis treatment using phytogetic (Nouzarian *et al*, 2011) and herbal products received great reputation acquired more acceptability by consumers as natural products (Abdel-Hady *et al*, 2014). Toghyani *et al*. (2011) reported that the beneficial effects of bioactive plant ingredients in hosts included improvement of endogenous digestive enzyme secretion, activate immune responses, enhancement of appetite and food intake, with anti-parasitic, antioxidant (Tonkal and Morsy, 2008), antibacterial, and antiviral agents (Okello and Kang, 2019)

The plant essential oils were recognized as novel anti-parasitic agents which components have activity against gastrointestinal parasites as nematodes (Benchaar *et al*, 2008), cryptosporidiosis *parvum* (Abouel-Nour *et al*, 2016). Abd El-Aziz *et al*. (2015) found that onion (*Allium cepa*) and cinnamon (*Cinnamomum zeylanicum*) oils affected *Cryptosporidium* spp. oocysts shedding due to *A. cepa* and *C. zeylanicum* activity that helped in parasite elimination (Wei and Shibamoto, 2010). Extracts of garlic, pine-bark, blue berry, and curcumin showed anti-cryptosporidiosis action (Kim and Healey, 2001; Abu El Ezz *et al*, 2011; Toulah *et al*, 2012).

Garlic (*A. sativum*) showed good prophylactic and treatment action against cryptosporidiosis (Abdel Megeed *et al*, 2015). Amino acids as arginine, one of the active chemical constituents of garlic and organosulphate compounds as aliin and allicin, enzymes as allinase, minerals and vitamins A, B1 & C. *allicin* (Diallylthiosulphinate) a sulphate compound in garlic bulb showed physiological activity of dietary garlic (Ayaz *et al*, 2008).

Nigella sativa (kalvangi) black seeds were reported in medical treatment of hepatic and gastrointestinal disorders (ascites, jaundice, fever, flatulence, dysentery, diarrhea) and others including cough, paralysis, conjunctivitis, skin diseases intrinsic hemorrhage, am-

enorrhea and local anesthetic action (Warrier *et al*, 2004), and anti-coccidian action (Baghdadi and Al-Mathal, 2011). *Nigella* oil contained saponin, nigellidine and nigellone and its seeds contained thymoquinone, monoterpenes such as p-cymene & α -pinene (Boskabady, 2002). Practically, *N. sativa* treated *C. parvum* infected calves (Nasir *et al*, 2013).

The study aimed to evaluate the effect of garlic (*A. sativum*) and black seed (*Nigella sativa*) extracts as compared with the conventional treatment for cryptosporidiosis in experimentally infected male mice.

Material and methods

Experimental infection: *Cryptosporidium* oocysts were collected from feces of naturally infected diarrheic calves of one day to 12 months of age from Assuit Veterinary field as proved by the modified acid fast staining method. Positive sediment samples were stored with 2.5% potassium dichromate solution at 4°C till needed (Abbassi *et al*, 2000). Discontinuous sucrose density gradient flotation technique was done (Arrowood and Stirling, 1987) to isolate and purify the oocysts. Purified ones were incubated with antibiotics (penicillin 5000Iu/ml, neomycin 1mg/ml & amphotericin B 50 μ g/ml) at 37°C for 12hrs to kill contaminated microbial (Obiad *et al*, 2012). Oocysts number was estimated in the concentrated stock by hemocytometer and light microscope at 40X (Gaafar, 2007). Inoculum per mouse was 1.5 \times 10⁵ oocysts on 1st infection day (Suresh and Rehg 1996).

Garlic preparation: Garlic was given to the mice as crude juice by separating fresh garlic bulbs, peeled, and washed with distilled water. The resultant bulbs (500gm) were crushed in a mortar to get uniform consistency. Distilled water was added and pasted to get 1g/ml aqueous solution. Raw garlic juice was stored at 20°C until needed (Masamha *et al*, 2010). Stock solution was diluted by distilled water to have the working solution and dose was 50mg/kg (Riad *et al*, 2009).

Black seed preparation: *N. sativa* seed was extracted (Al-Naggar *et al*, 2003). *N. sativa* seeds (0.5kg) were purchased from a local

market, grounded and extracted with *n*-hexane by soxhlet apparatus to get 250ml pure oil after solvent evaporation (Mahmoudvand *et al*, 2014).

Alcoholic extract of black seed: Five kg powdered seeds were defatted with *n*-hexane and extracted in methanol, and evaporated to dryness in a vacuum with a rotatory evaporator to get (160g) of the methanolic extract (MENS). MENS administered to mice as 1.25 g/kg (Mahmoudvand *et al*, 2014).

Aerial parts extract: Seven kg of *N. sativa* dried aerial parts were extracted with methanol to get 100g dried extract of which a stock solution was prepared and given to mice as 1.25 g/kg (Farah *et al*, 2004).

Nitaxanide preparation: Mice received Cryptonaz (Copad Pharma, Egypt) orally in a dose of 25ml (500mg) every 12hrs (Abdel Megeed *et al*, 2015)

Animals: Forty-eight male Swiss Albino mice, aged 3-5 weeks, weighed 25-30gm, were obtained from the animal house, Faculty of Medicine, Assuit University. Direct wet saline smear, iodine and Sheather's sugar flotation method were used to exclude any parasites (Garcia and Brucker, 1997). Mice were divided into 8 groups of 6 mice each.

1- Infected treated group; includes 6 subgroups (G1- G6), G1: Mice treated with garlic juice, G2: Mice treated with black seed oil, G3: Mice treated with garlic & black seed oil mixture, G4: Mice treated with black seed alcoholic extract, G5: Mice treated with black ariel parts alcoholic extract, G6: Mice treated with Nitaxanoide. 2- G7: infected untreated group (control positive). 3-G8: uninfected & untreated group (control negative). Each mouse from G1 to G7 was experimentally infected with 10^5 oocysts through tuberculin syringe connected to a polyethylene tube after water overnight deprivation

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

Statistical analysis: Data were collected, tabulated and analyzed using Statistical Package for Social Science (SPSS) computer programs version 16. Variables were compa-

(Abdou *et al*, 2013). After confirmation of infection, treatment of each mouse of different groups started on day 3 post-infection and continued daily to 19th day last experimental day. Treatment ingestion was performed by gastric gavage (Riad *et al*, 2009).

Feces from each group including control were collected daily separately till 19th day according to the group to which they were assigned. Two gm from each sample was divided into 2 parts: 1st preserved in 10ml sodium acetate formalin (SAF) solution and homogenized for staining and the 2nd preserved in 10ml 2.5% potassium dichromate solution and homogenized for oocysts counting (Sayed *et al*, 2016).

Infection monitoring: a- After inoculation, all mice were daily observed for general health status, mortalities, dropping consistency and color. Pooled fecal samples from each group daily collected on the 2nd day for assessing the prepatent period and oocyst excretion pattern (Sayed *et al*, 2016). Fecal pellets were collected and fecal smears were daily examined for 19 days using Modified Ziehl-Neelsen stain to detect *Cryptosporidium* oocysts (Henriksen and Pohlens, 1981). Efficacy of natural extracts and nitazoxanide were assessed by daily measuring of oocysts shedding count in feces to the experimental end. b- Oocyst shedding count: Oocyst number per gram feces started on 4th day post-infection (PI) to the 19th day. Daily output of oocysts in the feces per gram (OPG) of each group were counted (Suresh and Rehg, 1996). Number of oocysts was calculated as: (Total no. of oocysts counted \times dilution factor)/(tested stool volume in gm) until the 19th day PI. (Sayed *et al*, 2016).

Natural exextracts efficacy: The efficacy of of the extract were done according to Eger-ton (1963) by using the following formula:

red by Chi-square test. A probability level of $P < 0.05$ was chosen to specify significant differences, and differences between oocysts shedding among treated and control groups

were tested by two-way ANOVA test. P-value < 0.05 reflected significant differences.

The Ethics Committee of the Assiut University approved this study as it met the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for International Organizations of Medical Sciences. The experimental animals were upheld under appropriate conditions at the animal house, Faculty of Medicine, Assiut University, Egypt.

Results

By comparing the oocyst shedding among garlic juice, black seed oil, mixture, nitaxanide (NTZ) and control +ve from the day 4th to 19th day, where the oocyst shedding with black seed oil was somewhat low (2000) as compared to the high shedding rate in +ve control (54000) and (6000) with NTZ. The oocyst shedding with garlic juice was (6000) and the garlic juice & black oil combination was (4000) on the same day.

There were high significant differences between all groups and preparation of both garlic, black seed and mixture with +ve control (P = 0.0000). Comparison between the effect of garlic juice and NTZ on *Cryptosporidium* oocyst output showed oocyst shedding in garlic juice and NTZ treated groups gave a higher rate in garlic juice group on days 4 & 5, then declined in days 7 & 12 as well by the end of day 18 & 19, without significance differences between garlic juice & NTZ, P = 0.984 (Fig. 1). Comparison between effect of black oil and NTZ on *Cryptosporidium* oocyst output showed that oocyst shedding output in Black seed oil group was higher in 4th day than NTZ, and became similar in day 5, 7, 8, 9, 10, 14 and 16 and lowered in the end in day 17, 18 & 19, without significance differences between black oil and NTZ, P = 1.000 (Fig. 2).

Comparison between effect of garlic juice & black oil mixture and NTZ on *Cryptosporidium* oocyst output showed that oocyst shedding output between garlic juice & black oil mixture and NTZ, on day 4, 5 & 6 was higher in garlic juice & black oil mixture

groups, then became lower on 7th day with oocyst numbers similar on day 9, 10, 12 & 14 to become lower again on the days 15, 16, 17, 18 till 19th day, without significance differences between garlic juice & black oil mixture and NTZ, P-value = 1.000 (Fig. 3).

Comparison between effect of alcoholic black seed extract and NTZ on *Cryptosporidium* oocyst output showed that shedding in mice treated with alcoholic black seed extract was higher than NTZ treated one, on days 4, 5 & 6, became lower in days 7 & 12. Same result was on day 16 and another rise on days 17, 18 & 19 (Fig. 4), without significance differences between black seed alcoholic extract and NTZ, P = 0.999.

Comparison between effect of alcoholic black arial extract, NTZ and control +ve in *Cryptosporidium* oocyst output showed that shedding between black arial alcoholic extract and NTZ was higher in former treated group on days 4 & 6 to become lower on days 7, 9 & 12, with similar numbers on days 14, 15 & 19, without significance differences between alcoholic black arial extract and NTZ, P-value = 0.999 (Fig. 5).

Discussion

Plants active ingredients were used as non-conventional anti-parasitosis with more attention; due to the increasing resistance development to chemical drugs against parasites and vectors (Capela *et al.*, 2019), and parasites and microorganisms would continue to play their dangerous role in human welfare (Nicoletti, 2020).

In the present study, data showed that both garlic & black seed extracts have an antiprotozoal effect against cryptosporidiosis as indicated by reducing oocyst shedding, but without complete oocysts elimination. The mean oocysts count of the seven treated groups varied from the 2nd day to the 14th day post-treatment and then reduced gradually till the experimental end on the day 19th. Fluctuation phenomenon of oocyst shedding was reported by Hassan *et al.* (2019) who found a rebound increase of *Cryptosporidium* oocysts number and viability or rebound

phenomenon which included reinfection, autoinfection activation with thin-wall oocysts and/or inhibited stages reactivation. But, De Waele *et al.* (2012) reported that the patient could be infected with different *Cryptosporidium* species.

In the present study, garlic juice treated group showed a considerable reduction in *Cryptosporidium* oocysts mean count (73.1%), especially in the last five experimental days. Gaafar (2011) recorded the successful eradication of *Cryptosporidium* oocysts from the stool of infected immunocompetent mice received garlic for two days before infection and continued for two weeks latter. Also, Hazaa *et al.* (2016) reported that garlic decreased the mean of *Cryptosporidium spp.* oocysts count in experimentally infected mice, which started from the 2nd day post-treatment to the experimental end on 15th day post treatment. The mean oocysts count in (infected not treated) was high significantly ($P \leq 0.05$) along the whole experiment duration compared with treated groups.

In the present study, oocyst shedding pattern showed that garlic juice gave relatively similar effect as NTZ on the *Cryptosporidium* oocysts shedding. But, there was a high significance difference between garlic treated & infected untreated ones. This agreed with Wahba *et al.* (2003), who found that garlic extract caused a mild reduction of oocysts shedding in *C. baileyi* infected chickens, with significant difference in values of *Cryptosporidium* oocysts excretion patterns between garlic treated group and non-treated control group seven days post-treatment to the experimental end on 25th day. Abdel Megeed *et al.* (2015) also found that garlic successfully eradicated *Cryptosporidium* oocysts completely by the experimental end and concluded that garlic and NTZ had a great effect on *Cryptosporidium* oocyst output and hosts were *Cryptosporidium* free. Abdel-Hafeez *et al.* (2015) showed that garlic significantly lowered the oocysts shedding in a pattern similar to that of NTZ. However, Snyder *et al.* (2009) found that NTZ for treat-

ing *Cryptosporidium*-infected calves did not show expected good results, as neither decreased the clinical severity nor the oocysts shedding. Fareed *et al.* (1996) reported that garlic was used for treating cryptosporidial HIV patients with chronic diarrhea, complete healing occurred in some patients and partial healing in others.

The efficacy of garlic in treatment of experimental cryptosporidiosis could be explained by various mechanisms of action. Chemical contents of glucose and fructose might be responsible for *Allium sativum* anti-parasitic action by reducing *Cryptosporidium spp.* intestinal colonization (Harp, 1999). Richard (2001) reported that the garlic antiprotozoal effect belonged to allicin and some other compounds that acted as antimicrobial, antioxidative, enhance phagocytosis and increased the natural killer cells activity stimulating immune system and boost body's defense mechanism during treatment. Organosulfur compounds such as allicin, diallyldisulphide, S-allylcysteine, and diallyl trisulfide are the most important constituents of garlic playing important pharmacological roles by holding up the parasite mobility, food absorption, and reproduction (Azimi *et al.*, 2011).

Many studies determined the effects of *N. sativa* seeds on parasitic and microbial diseases. Abu El-Ezz (2005) studied the effect of *N. sativa* on *Trichinella spiralis* in rats, Ayaz *et al.* (2007) studied its effect on *Hymenolepis nana* in naturally infected mice, El-Shenawy *et al.* (2008) studied its effect on *Schistosoma mansoni* infected mice, and Baghdadadi and Al-Mathal (2011) studied its effect *Eimeria stiedae* infected rabbits. They reported that *N. sativa* gave a potential successful anti-parasitic treatment.

No doubt, the solvent used in extraction, its properties, physiologic absorption degree, a preservative action and ability to do extract to complex or dissociate, factors determined biologically active compounds from extracted plants, methanol, ethanol, and water were the commonest solvents used to test plants antimicrobial activity (Rojas *et al.* 2006).

In the present study, extracts of *N. sativa* different parts (black seed oil, black seed alcoholic and extract of black ariel parts) were used to compare with NTZ effect on *Cryptosporidium* oocyst output. Black seed treated groups showed evident changes in *Cryptosporidium* oocysts shedding, with mean count gradually declined from 4th day post treatment to the experimental end on 19th day that was more clear in 10th & 11th day, but without significant differences among groups treated with d black seed extracts and NTZ.

Some authors studied effect of *N. sativa* extracts on other parasites. El-Shenawy *et al.* (2008) reported prophylactic and therapeutic effect on murine toxoplasmosis and schistosomiasis *mansoni*. Rayan *et al.* (2009) found that aqueous extract of *N. sativa* gave a potential therapeutic effect against *Dientamoeba fragilis*. Okeola *et al.* (2011) reported that *N. sativa* seeds gave a significant antioxidant and good phytotherapeutic action on *Plasmodium* spp. Fattahi *et al.* (2011) reported that black seed alcoholic extract gave good efficacy against cutaneous leishmaniasis in BALB/c mice. Mahmoud-vand *et al.* (2014) found that 50mg/ml *N. sativa* methanolic extract incubated for 10min. killed *E. granulosus* protozoa. But, Khan *et al.* (2013) and Nasir *et al.* (2013) reported that *N. sativa* extracts did not show good effect on oocyst shedding *C. parvum* in naturally infected dairy calves, which may be due to changes in drug formula or alcoholic extract or protozoa's susceptibility to drug action.

Haq *et al.* (1999) found that *N. sativa* stimulated both CD4 positive T-cells and macrophages creating an immunomodulatory approach in man and animals. Suthar *et al.* (2010) found that *N. sativa* oil inhibited the DNA synthesis by obstruction of histone deacetylase (HDAC) enzyme interacting with the chromosomes. Mansour (2000) found that *Thymo quinone* (TQ), the basic component of *N. sativa* seeds volatile oil gave significant cyto-protective action.

Parasitic treatment by black seed oil mixed with other medicaments or natural products

was given by Mahmoud *et al.* (2002) who showed encouraging outcomes of *N. sativa* in schistosomiasis treated as combined with praziquantel. Nilforoushadeh *et al.* (2010) reported that a combination of honey and *N. sativa* extract in cutaneous leishmaniasis patients with glucantime was more effective with clinical improved as compared to honey alone. Zaki and El-Amir (2018) reported that phenyl vinyl sulfone combined with *N. sativa* altered *Cryptosporidium* oocysts shedding than paromomycin treated one.

Conclusion

The present results hold perspective of finding a new therapeutic alternative to *Cryptosporidium* treatment. New and efficient natural products inhibited the *Cryptosporidium* growth without side effects may be very useful in the treatment.

Conflict of Interest: There was neither conflict of interest nor fund received.

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Explanation of figures

Fig. 1: *Cryptosporidium* oocysts shedding in mice treated with garlic juice and Nitaxanide in relation to +ve control.

Fig. 2: *Cryptosporidium* oocysts shedding in groups treated with black oil and Nitaxanide in relation to +ve control.

Fig. 3: *Cryptosporidium* oocysts shedding in mice treated with garlic juice & black oil mixture and Nitaxanide in relation to +ve control.

Fig. 4: *Cryptosporidium* oocysts shedding in mice treated with alcoholic black seed extract and Nitaxanide in relation to +ve control.

Fig. 5: *Cryptosporidium* oocysts shedding in mice treated with alcoholic black arjal extract and Nitaxanide in relation to +ve control.

