

EFFECT OF CINNAMON AND GINGER METHANOLIC EXTRACTS ON MURINE INTESTINAL CRYPTOSPORIDIOSIS. IN-VIVO EVALUATION

By

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Abstract

Cryptosporidium species the Apicomplexa protozoan parasites, causing diarrheal disease in man and animals, due to the lack of a licensed vaccine and resistance to the available effective therapy. This study explored the curative and the prophylactic effect of the cinnamon and ginger methanolic extracts separately and combined with nanazoxide on the experimentally infected mice with *Cryptosporidium*. Ninety Swiss albino mice, weight of 23-25gm, aged 7-8 weeks were divided into nine groups to assess the therapeutic and the prophylactic effect of cinnamon and ginger methanolic extracts separately then combined with nanazoxide. The result was assessed parasitologically, histopathologically and by TEM. The best efficacy was three weeks post infection in all groups. Therapeutic effect of ginger was better than cinnamon extract, but combined ginger and nanazoxide gave the least oocyst shedding. The prophylactic dose of ginger and cinnamon methanolic extracts showed marked decrease in oocysts shedding and ginger was the best. Histopathological sections showed immune cells infiltration with a decrease in number of tissue parasite that was absent in the prophylactic groups given ginger extract. The results were confirmed by transmission electron microscopy explored a great improvement in the small intestinal brush border of the same groups.

Keywords: *Cryptosporidium*, cinnamon-ginger, transmission electron microscopy

Introduction

Cryptosporidium species (spp.) are protozoan parasites that cause diarrheal disease in humans and animals (Tzipori and Ward, 2002). In immunocompromised individuals, *Cryptosporidium* represents a serious health problem (Breurec *et al*, 2016). Nitazoxanide which is the traditional treatment for cryptosporidiosis is ineffective especially in immunocompromised individuals (Amadi *et al*, 2009).

Due to the lack of a licensed vaccine and the effective drugs for cryptosporidiosis with the emergence of resistance to the available therapeutics, it was indicated to search for effective anti-parasitic drugs (Ryan *et al*, 2016). A number of medicinal plant extracts have more antiparasitic effect than the currently used drugs (El-Sayed and Issa, 2008). Through the intervening with the process of intercalation or alkylation of the parasite DNA, the plant extracts have a remarkable

effect. Also, they can inhibit the membrane integrity and the neural signal transduction of the target parasite (El-Sayed *et al*, 2012).

Cinnamon is a popular spice, obtained from the inner bark of trees being a member of the genus *Cinnamomum*. It has different synonyms as *Cinnamomum zeylanicum* (CZ) and *Cinnamomum cassia* (CC) (Shen *et al*, 2002). Cinnamon has many active constituents including cinnamaldehyde compounds (bark), eugenol (leaf), camphor (root), volatile oils, tannins, mucilage, limonene, and saffrole that possess an antibacterial, antiseptic, antiviral, and antifungal properties (Gruenwald *et al*, 2010; Cahyana *et al*, 2015). It revealed that different extracts of cinnamon were effective against bacteria, yeast, *Leishmania*, and *Toxoplasma* as well (Senhaji *et al*, 2005). Its leaves exhibit an immunomodulatory, anti-inflammatory, anti-parasitic, and antioxidant properties (Anthony *et al*, 2005). It also improves the immune system

and reduces the risk of colon cancer (Wondrak *et al*, 2010).

Zingiber officinale Roscoe (Ginger) is commonly used as a nutrient remedy (Surh 1999). The oily resin of roots contains many bioactive components, as gingerol, shogaol, paradol, zingerone, zerumbone, 1-dehydro-(10)-gingerdione, terpenoids, and flavonoids. They have antioxidant activities, anti-inflammatory, antimicrobial, and liver-protecting activities (Arshad *et al*, 2014). Ginger can be used as an anti-parasitic for overcoming drug-resistance in parasitic diseases. It has an anti-protozoal effect against *Toxoplasma gondii* (Choi *et al*, 2013), *Giardia lamblia* (Mahmoud *et al*, 2014), *Blastocystis spp.* (Abdel-Hafeez *et al*, 2015), and *Trypanosoma spp.* (Kobo *et al*, 2014).

The study aimed to explore the curative effect of cinnamon and ginger methanolic extracts separately and combined with nanazoxide and the prophylactic capability of these herbal extracts on the experimentally infected mice with *Cryptosporidium*.

Material and Methods

Stool samples were collected from patients suffering from diarrhea or any GIT troubles attending the outpatient clinics of Zagazig University Hospitals. Fresh fecal samples were collected and preserved in formalin 10%. The direct smear, the formol ether concentration technique and the permanent mount using modified Ziehl-Neelsen stain were done for each sample. Positive samples for *Cryptosporidium* were collected, preserved in potassium dichromate 2.5%, and then stored at 4°C.

Oocyst isolation: Fecal specimens were washed then filtrated. The filtered solution was centrifuged at 2500 rpm for 5 minutes twice for washing and removing potassium dichromate. To the sediment, 20ml of distilled water and 20ml of diethyl ether were added mixed and then centrifuged at 2500 rpm for 5 minutes and this step was done twice. The sediment was washed with distilled water and saturated water with sugar was added and was centrifuged at 2500 rpm

for 5 minutes. In this method, *C. parvum* oocysts were floated and gathered with a pipette and stored in distilled water with 0.5% sodium hypochlorite (Hadfield *et al*, 2015).

Mice: Ninety Swiss albino mice were all free from any parasitic infection, weight of 23-25gm, aged 7-8 weeks obtained from the Schistosome Biological Supply Program (SBSP) in Theodor Bilharz Research Institute (TBRI) Cairo, Egypt.

Immunosuppression: The synthetic corticosteroids (dexamethasone) were used for immunosuppression of mice in a dose of 20 mg/kg/day intramuscularly 3 times a week for 3 weeks for each mouse prior to the oocysts inoculation (Rasmussen and Healey, 1992). The mice continued to receive dexamethasone at the same dose throughout the experiment.

Infection: All mice were infected orally (intraesophageal) with prepared *Cryptosporidium* oocysts; after day 21 of dexamethasone administration. About 1000 oocysts in 200µl of phosphate-buffered saline (PBS) /mouse were given (Benamrouz *et al*, 2012).

Plant material: Dried *Cinnamomum verum* and dried rhizomes of *Zingiber officinale* were purchased from a local herbal shop (Cairo, Egypt). The plant samples were grounded to a fine powder with an electric mill for the extraction process.

Extraction of plants: Dried *Cinnamomum verum* (500gm) and dried of *Zingiber officinale* (500gm) was soaked separately in 85% methanol for one week then, the *Cinnamomum verum* extract was filtered several times via Whatman No.1 filter paper then concentrated via the Buchi Rotatory evaporator at 40°C to remove methanol completely; same process was repeated to *Zingiber officinale* extract.

Nanazoxide administration and medicinal plant preparation: Nanazoxide (obtained from Medizen pharmaceutical industries for Utopia pharmaceuticals) is given 7 days (PI) in a dose of 100mg/kg body weight (0.04mg /mouse/day) for seven consecutive days. Methanolic extract of both herbs was given

7 days (PI) in a dose of 20mg/kg/d for seven consecutive days.

Experimental design: This experiment was carried out in Theodor Bilharz Research Institute (TBRI) Cairo, Egypt. The infected immunocompromised mice were divided into eight groups each contain ten mice and there was one normal non-infected group: GI: infected control (infected not treated). GII: infected and treated with nanozoxide 0.04mg/mouse/day. GIII: infected and treated with methanolic extract of cinnamon plant 20mg/kg/d. GIV: infected and treated with methanolic extract of ginger 20mg/kg/day. GV: infected and treated with a combination of a half dose of nanozoxide and methanolic extract of cinnamon 20mg/kg/d. GVI: infected and treated with a combination of a half dose of nanozoxide and methanolic extract of ginger 20mg/kg/d. GVII: given prophylaxis with methanolic extract of cinnamon 20mg/kg/d. one week before infection then continuing its administration throughout infection and 1 week PI. GVIII: given prophylaxis with methanolic extract of ginger 20mg/kg/d. one week before infection then continuing its administration throughout infection and 1 week PI, & GIX: Control negative

Parasitological assessment (oocyst count in stool samples): Fresh fecal pellets from each mouse in the studied groups were collected separately and examined daily then the mean numbers of the oocysts were calculated at the 7th day PI (to assess infection establishment), 14th day PI (1-week post-treatment) and 21st day PI (2weeks post-treatment) before mice scarification, according to each group. Each sample was suspended in 10% formalin and homogenized. Approximately, 1 mg was prepared as a fecal smear to be stained with the modified Ziehl-Neelsen staining method. The stained fecal smear was examined microscopically and number of *Cryptosporidium* oocysts was counted.

Histopathological examination: The jeju-

num and proximal 2 cm of the ileum were fixed in 10% neutral buffered formalin followed by immersion in xylene then impregnated in paraffin. 4-mm thick section was taken from each block to be stained with hematoxylin and eosin for evaluation, (Bancroft and Stevens, 1990).

Transmission Electron Microscope: 1- Thin 1 mm section of each sample was prepared, 2- Sample was fixed in Cacodylate 0.2M+Gluteraldehyde 4% in PH 7.4 & 4c for 2 hours, and 3- Sample was washed in Caccodylate 0.2M+ Saccharose 0.4M three times in a washer for 30min. then left till the next day, Post-fixation in 2% Osmic acid (1 Vol.)+ Caccodylate 0.3M (1Vol.) at PH 7.4 and 4c.

Statistical analysis: Data were analyzed by (SPSS; version 20 for windows). All data were expressed as mean \pm standard deviation. Difference between groups was calculated using Student's *t*-test.

Ethical considerations: Informed consent was taken from all patients before taking fecal samples. The experimental animal studies were conducted in accordance with the international valid guidelines and they were maintained with convenient conditions. The study was approved by the University Ethical Committee.

Results

A highly statistically significant difference was between all studied groups at different follow up periods PI. Within each week PI, the difference between the all studied groups was highly significant. As the best results were obtained on the third week PI, the therapeutic effects of cinnamon and ginger extracts on mean numbers of oocysts (19 ± 1.49 & 14 ± 1.39 respectively), were highly significant compared with nanozoxide (27 ± 2). But, combination between nanozoxide and either cinnamon or ginger (11.4 ± 1.07 & 9 ± 1.49 respectively), gave the best results among treated groups. As to prophylactic effect, the ginger extract (0.80 ± 0.07) was better than cinnamon extract (1.8 ± 0.77).

Histopathological changes (Fig. 1b, c) showed the developmental stage of *C. parvum* in the affected intestinal villi, sub-epithelial cell edema, atrophy and sloughing of the upper tips of some villi with inflammatory cells infiltration, and focal cystic dilation present inside the intestinal lumen of the infected group. GVII given a prophylactic dose of cinnamon methanolic extract, showed a moderate inflammatory cellular infiltration in submucosa (Fig. 1 d). GVIII given a prophylactic dose of ginger methanolic extract gave a mild inflammatory cellular infiltration in submucosa but no *C. parvum* (Fig. 1e).

The TEM of ultrathin section of murine

small intestinal epithelial cells revealed the luminal surface of columnar cells with *Cryptosporidium* trophozoite incarcerated in between the microvilli with restoration of the normal villi architecture in GVIII given the prophylactic doses of the ginger extract (Fig 2 e) when compared with the infected control GI, that showed a complete loss of microvilli, presence of *Cryptosporidium* meront containing merozoites, degenerated cells with dark small nuclei, rarified cytoplasm, degenerated organelles, and the mature *Cryptosporidium* oocyst with clear double wall (Fig. 2 c, d).

The details were given in table (10 and figures (1 & 2)

Table 1: Effect of different plants extracts on mean numbers of oocysts in groups

Variable	One week PI (n=10)	Two weeks PI (n=10)	Three weeks PI (n=10)	P
Group I	80.8 ± 2.34	81.5 ± 3.5	82 ± 2.6	0.645 (NS)
Group II	77 ± 4.05	40.8 ± 2.52	27 ± 2	<0.001 (HS)
Group III	76 ± 4.5	36.8 ± 2.25	19 ± 1.49	<0.001 (HS)
Group IV	74.6 ± 4.45	30 ± 1.49	14 ± 1.39	<0.001 (HS)
Group V	77.8 ± 4.07	20 ± 1.49	11.4 ± 1.07	<0.001 (HS)
Group VI	74.2 ± 5.09	17 ± 2.10	9 ± 1.49	<0.001 (HS)
Group VII	45.2 ± 5.59	9 ± 1.39	1.8 ± 0.77	<0.001 (HS)
Group VIII	40.4 ± 4.97	6 ± 2.2	0.80 ± 0.07	<0.001 (HS)
P#1:	0.001 (HS)	<0.001 (HS)	<0.001 (HS)	

SD: Standard deviation

HS: Highly significant (P<0.01)

Student's t-test

Discussion

Cryptosporidiosis is one of the most common infectious diarrheal illnesses in immunocompromised individuals worldwide. In developing countries, *Cryptosporidium* spp. is the second leading cause of infectious diarrheal diseases in children under the age of 5 years (Gebretsadik *et al*, 2018).

In this study, prophylactic and curative effects of methanolic extracts of ginger & cinnamon in a dose of 20mg/kg/ day for *Cryptosporidium* infected mice compared to the nanazoxide assessed by the oocyst shedding rate, histopathological examination was confirmed by the TEM.

In the present study, oocyst shedding was decreased in groups III, IV, V, VI respectively. The best results were obtained on the third week PI, in which, the therapeutic effects of cinnamon and ginger extracts on the oocysts mean numbers (19±1.49 & 14±1.39

respectively), were significantly lower than that of nanazoxide (27±2). Ginger gave better results than cinnamon. The combination of ginger and nanazoxide gave the lowest oocyst shedding. These results may be due to the anti-oxidant action of ginger which helps in the elimination of parasites (Sadhana and Gupta, 2013). A complete eradication of *C. parvum* oocysts on the 9th day PI was reported by Abouel-Nour *et al*. (2016), when ginger and metronidazole were used in the experimentally infected mice. Failure of the parasitic growth, its sexual development and the loss of oocyst development may be the mechanism of action. The difference in results may be due to different drugs. Choi *et al*. (2013) concluded that ginger extract has an effect against *Toxoplasma gondii* by inactivation of the apoptotic proteins within parasitized host cells, in addition to the blockage of inflammatory cytokine secretion.

Also, the ginger was approved to be safe for human uses (Rong *et al.*, 2009). Maximum oocyst shedding was seen in the infected not treated ones, which agreed with Certad *et al.* (2007) and Abdou *et al.* (2013).

The highest oocysts shedding reduction rate was observed in treated prophylactically with methanolic extract of cinnamon and treated prophylactically with methanolic ginger extract after 1 & 2 weeks, reached the best on the 3rd week PI (1.8 ± 0.77 and 0.80 ± 0.07 , respectively). This may be due to the anti-oxidant effect of cinnamon and ginger that helped in the parasites elimination (Sadhana and Gupta, 2013). Abu El Ezz *et al.* (2011) reported that both of onion (*Allium cepa*) and cinnamon (*C. zeylanicum*) oils induced a significant reduction in *C. parvum* oocysts count.

The general biological action of cinnamon on extracts induced lipid peroxidation, generation of anti-proliferative, the antioxidant effects and detoxication of enzymes (Kumar *et al.*, 2006). Also, the secretory system of *Toxoplasma gondii* treated with cinnamon extracts suffered a drastic disorganization and vesiculation (Melo *et al.*, 2011).

Ginger anti-inflammatory effects may be due to inhibiting prostaglandin and leukotriene synthesis (Srivastava and Mustafa, 1992). The 6-gingerol was found to inhibit the production of proinflammatory cytokines such as TNF- α , interleukin (IL)-1 β , & IL-12, produced by macrophages (Tripathi *et al.*, 2007). Majority of scientific evidences suggested that ginger and its components have anti-inflammatory effects in vitro and in vivo as well.

The present histopathological results of infected control showed sub-epithelial cell edema, atrophy and sloughing of the upper tips of some villi with inflammatory cells infiltration. These findings agreed with Gookin *et al.* (2002) and Mahmood *et al.* (2016). An explanation for the pathological changes was that *Cryptosporidium* displaced brush borders causing loss of epithelial cells which leads to shortening, sloughing and fusing of

the villi. A previous explanation was that *Cryptosporidium* toxins damage the epithelial cells (Tzipori, 2002). On the other hand, there was a remarkable decrease in the number of *Cryptosporidium* oocysts in the epithelial brush border in GVII & GVIII given prophylactic doses. The results were compatible with Harp *et al.* (1996) who reported that plant oils might block receptor sites on the surface of small intestinal villi, thus leading to a reduction in *Cryptosporidium parvum* colonization. Both extracts failed to restore the normal symmetrical architecture of ileal villi and mucosa completely. But, inflammatory cellular infiltration indicated that the immunity increased in the mucosa of the intestine. This may be attributed to the immune stimulant of cinnamon containing eugenol with the local antiseptic and anti-phagocytic properties (Wondrak *et al.*, 2010). This agreed with Lantier *et al.* (2013) who reported that immune cell induction and maintenance in infected intestine for protection against *C. parvum*. Also, Abouel-Nour *et al.* (2016) reported that supplementation of ginger to infected mice implied its potential antioxidant, anti-inflammatory, and immunomodulatory effects. Abu El Ezz *et al.* (2011) found that cinnamon oil improved the ileal villi, where the parasite colonized as the villi in treated mice retained their normal appearance.

In the present study, by TEM the small intestinal epithelial cells of the normal control had regular oval euchromatic nuclei, numerous apical microvilli, and the well-developed cell junctions, as well as a crypt containing goblet cells with numerous mucous granules that may coalesce. Regarding the infected untreated mice, there was a complete loss of microvilli, degenerated cells with dark small nuclei, rarified cytoplasm, and degenerated organelles, cryptosporidium meront containing merozoites and immature *Cryptosporidium* oocyst with a clear double-wall measuring 5 μ m was detected. Pohlenz *et al.* (1978) reported that many of the microvilli adjacent to parasite

are short; others are elongated; some rootlets of microvilli are missing. Microvilli of neighboring cells are normal shaped. *Cryptosporidium* trophozoite incarcerated in-between the microvilli with shortening of these adjacent microvilli and restoration of normal villi architecture away from the parasite was observed in mice received prophylactic doses of ginger extract. The intracellular immune cell infiltration, regeneration of MV & remained stages of parasite was detected in mice received prophylactic doses of cinnamon extract.

Conclusion

The study may be the first challenge to investigate the curative effect of ginger and cinnamon alone and combined with the nanazoxide and the prophylactic effect of these herbs against murine cryptosporidiosis using TEM. Prophylactic and curative effects of methanolic extracts of ginger and cinnamon in a dose of 20mg/kg/day gave variable but promising anti-cryptosporidial activity in infected mice. They could be used as a natural safe product for the preparation of a new therapeutic agent. Combination of nanazoxide and ginger or cinnamon gave good effects. The isolation and the purification of the bioactive components of them will be a future project.

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Explanation of Figures

Fig.1 a. Normal control group showing normal histological structure villi (black arrow), crypt of Lieberkuhn (red arrow) and muscular layers (astriks) (H&E $\times 100$) b, c infected group showing sub-epithelial cell edema (green arrow), atrophy and sloughing of upper tips of some villi (red arrow) with infiltration of inflammatory cells (black arrow) (H&E $\times 100$) d. Group VII treated prophylactically with methanolic extract of cinnamon showing moderate inflammatory cellular infiltration in submucosa (arrow)(H&E $\times 400$) e. Group VIII treated prophylactically with methanolic extract of ginger showing mild inflammatory cellular infiltration in submucosa (H&E $\times 400$).

Fig. 2 TEM of ultrathin section of murine small intestinal epithelial cells a, b: normal control murine small intestinal epithelial cells showing columnar cells with regular oval euchromatic nucleus (N), numerous apical microvilli (MV) and well developed cell junctions (arrows). A part of a crypt containing goblet cells (GC), with numerous mucous granules that may coalesce. c, d: small intestinal epithelial cells infected with *Cryptosporidium* (group I) showing complete loss of microvilli, *Cryptosporidium* meront containing merozoites, degenerated cells with dark small nuclei, rarified cytoplasm and degenerated organelles (O), mature *Cryptosporidium* oocyst with clear double wall. e: showing the luminal surface of columnar cells with *Cryptosporidium* trophozoite (T) incarcerated in-between the microvilli (MV) and restoration of normal villi architecture (group VIII) f: showing intracellular immune cell infiltration (arrows), regeneration of MV and completely destroyed parasite (C) (group VII).



