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Therapeutic Efficacy of Ginger (*Zingiber officinale*), Ginseng (*Panax ginseng*) and Sage (*Salvia officinalis*) Against *Cryptosporidium parvum* in Experimentally Infected Mice

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CRYPTOSPORIDIUM parvum (*C. parvum*) is a worldwide zoonotic protozoan parasite infects most mammalian hosts causing a major health problem. The present study investigated the efficacy of ginger (*Zingiber officinale*), ginseng (*Panax ginseng*) and sage (*Salvia officinalis*) methanolic extracts on the progression of cryptosporidiosis in the experimental mice. Forty five mice experimentally infected with *C. parvum* were treated with medicinal plants extracts (ginger, ginseng and sage) as compared to the reference drug, Nitazoxanide (NTZ). Mice fecal smears were examined daily for 4 weeks post infection (PI). The results interpreted by oocysts count and histological examination of mice intestinal mucosa.The obtained results recorded that there was a statistically significant reduction in oocyst shedding in high dose ginger, ginseng and NTZ treated groups till no oocysts were found at days 21 and 23 PI, respectively. The infected non-treated, NTZ and low dose of both, ginseng and sage treated mice groups returned to excrete oocysts at low levels at day 27 PI while the other animal groups' feces were still negative for *C. parvum* oocysts. The histopathological examination showed that NTZ, high dose ginger and ginseng treated mice had more protective and curative effect on infected mice intestinal epithelium in comparison with other treatments used.In conclusion, these results proved the therapeutic efficacy of ginger, ginseng and sage medicinal plants against the *C. parvum* in experimentally infected mice, and that theginger extract had an obvious effect on infected mice than other treatments and such results could be adapted in similar infections in susceptible animals and man.

Keywords: *Cryptosporidium parvum*, Mice, Therapeutic efficacy, Ginger, Ginseng, Sage.

Introduction

The Apicomplexan *Cryptosporidium* is a universal obligatory enteric protozoan parasite that infects a wide range of vertebrate hosts associating with self-limited mild to severe diarrhea causing prolonged life-threatening disease [1]. The transmission of *Cryptosporidium* parasites might be food and waterborne through contaminated food and water supplies by the environmentally and chlorine-resistant oocysts which certainly given the parasite a more widespread recognition in humans and animals husbandry [2, 3]. Due to the ubiquitous distribution of the microorganism in the environment including a large variety of animal species, so there is high possibility of zoonotic transmission of infection

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from animal to human especially under poor hygienic conditions [4].

Cryptosporidium inhabited the microvilli of the epithelial surface of the gastrointestinal and respiratory tracts causing significant morbidity and mortality [5]. The main site of infection is the small intestine, although infection might be found throughout the gastrointestinal tract and extra-intestinal sites such as the lung [6]. *Cryptosporidium* is one of the few protozoans which showed a weak or no effectiveness through the chemotherapy treatment [7]. The lack of anti-cryptosporidial drugs could reflect the unique features of this parasite which separate it from other coccidian including its cellular location and its capacity for autoinfection [8]. *Cryptosporidium parvum* (*C. parvum*) was been the predominant species used for drug discovery investigations [9].

It has been known for centuries that aromatic plants, mainly their essential oils or components, can act versus many organisms including bacteria, viruses, fungi, protozoa, parasites and insects[10-12]. These extracts often interfere with central targets in parasites, such as DNA (intercalation, alkylation), membrane integrity, microtubules and neuronal signal transduction [13]. Ginger (*Zingiber officinale*) had historical medicinal uses dating back 2500 years in China and India [14]. Its pharmacological properties varied including antioxidant, anti-inflammatory, anticancer [15], antimicrobial [16], anthelmintic[17]and antiprotozoal activities [18,19]. Also, it had nematocidal, cestocidal, trematocidal, insecticidal, molluscicidal and anti-leech effects [20]. Ginseng roots (*Panax ginseng*) is considered as one of the most well-known oriental medicinal herbs that had been used in eastern Asian cultures for thousands of years [21]. Modern therapeutic studies claim a wide range of pharmaceutical activity of ginseng such as vitality, immune function, cancer, cardiovascular disease, cognitive and physical performance, and sexual function[22,23]. Sage(*Salvia officinalis*) is an evergreen short woody stems plant with grayish leaves, and blue to purplish flowers. It has a long history of medicinal and culinary use. Its leaves contain essential oils and tannins that can help to facilitate digestion with anticonvulsant, anti-fever, antiseptic, anti-diabetic, antibiotic, antifungal and anti-parasitic effects [24].

There is a paucity of data about using of medicinal plants for cryptosporidiosis control in human and animals especially in Egypt [25], therefore, the present study was aimed to explore the anti-cryptosporidium action of ginger, ginseng and sage methanolic extracts on *C. parvum* in experimentally infected mice and to evaluate their protective and curative capacity of these extracts on cryptosporidiosis.

Material and Methods

Plant extracts preparation

About one kilogram of plants, Ginger (*Zingiber officinale*) rhizomes, Ginseng (*Panax ginseng*) roots and Sage(*Salvia officnalis*) leaves, were obtained from a Shefaa Company, Cairo, Egypt, then grinded by using Moulinex® grinder, France. Then, they were macerated and extracted with absolute methanol, manually shaken for 30 minutes and allowed to stand with continuous shaking at a shaking water bath for 3 days. After that, solutions were filtered with sterile filter papers (Whatman No.1) into a clean conical flask and dried under reduced pressure in a rotary evaporator (Heidolph, Germany) at a temperature below 50°C and stored at 4°C until use. A solution of each extract in bi-distilled water containing 2-4 drops of $Tween_{80}$ was prepared in two doses, 300 and 100 mg/kg Body weight (Bwt) for ginger extract[26] and 100 and 50 mg/kg Bwt for ginseng and sage extracts [21,27].

Preparation of Cryptosporidium parvum oocysts

C. parvum oocysts were collected from naturally-infected calf's feces. Oocysts were concentrated by floatation in Sheather's sugar solution [28] and identified by modified Ziehl-Neelsen technique [29]. Sedimented oocysts were collected and stored in a 2.5% potassium dichromate solution at 4°C. Prior to infection, oocysts were concentrated and counted in a phosphate buffer saline (PBS) solution using a hemocytometer.

Animals

Male Swiss albino mice, aged two weeks, of 20–25 g Bwt were used. They were housed in well ventilated cages with perforated covers, supplied with standard pellet food and water obtained from a colony maintained in National Research Centre, Dokki, Egypt. Bedding was changed every day.

Standard drug of choice

A dose of 100 mg Nitazoxanide(NTZ, Cryptonaz®, Copad Pharma, Egypt) drug was orally administered to mice (once daily) for 5 days.

Experimental Design

Forty five male Swiss albino mice were used in the present study. Mice were divided into 9 equal groups, 5 mice for each. The first group was kept as negative control (neither infected nor treated). The 2nd group was inoculated orally with *Cryptosporidium* oocysts at a dose of $1x10^4$ oocysts/mouse[30]by gastric gavage, using a 23-gauge needle tipped with plastic tubing [31] and untreated. The 3rd group was infected and treated with 100mg NTZ. The 4th and 5th groups were infected and treated with ginger extract with doses of 300 and 100 mg/kg Bwt, respectively. The 6th and 7th groups were infected and treated with ginseng with doses of 100 and 50 mg/kg Bwt, respectively. The 8th and 9th groups were infected and treated with sage extract with doses of 100 and 50 mg/kg Bwt. Doses were administered daily by gastric tubes 1 h before meals and for 5 consecutive days. Animal fecal samples were collected and examined daily for 4 successive weeks.

Fecal analysis and oocyst shedding

Fecal samples of mice will be collected from day 3 post infection (PI) till the end of the experiment (4 weeks PI) and examined under microscope (Olympus,) for determination of the number of *C. parvum* oocysts output counted for each group in 50 fields (oil immersion).

Histopathological Studies

Specimens were collected from different parts of small intestine of mice of all groups at the end of experiment and fixed directly in 10% formalin. The fixed specimens were dehydrated, cleared, embedded in paraffin, sectioned at 4µm and stained with hematoxylin and eosin (H&E) staining [32].

Statistical analysis

Data of oocyst count were analyzed for the means and standard deviations. Significance of the results was evaluated using Analysis of variance (ANOVA) and Duncan using Statistical Package for Social Science (SPSS) [33] computer programs (2002).

Results

C.parvum oocyst shedding in infected non-treated, NTZ-treated and methanolic extracts of ginger, ginseng and sage treated groups

Examination of fecal smears for detection of *C. parvum* oocyst counts revealed that there was a gradual reduction in oocyst shedding in the infected non-treated group and continued till no oocysts found at day 24 PI. There was a statistically significant reduction in oocyst shedding in ginger (300 mg/kg) and NTZ treated groups till no oocysts were found at days 21 and 23 PI, respectively. Also, oocyst shedding in ginseng (100 mg/kg) treated group diminished gradually till reaching negligible numbers of oocysts at day 21 PI and reached no oocyst shedding at day 24 PI. Ginger extract (300 mg/kg) diminished *C. parvum* oocysts count significantly in experimentally infected mice than other treatments in almost all days PI. At day 25 PI, all examined feces of infected groups were negative for *C. parvum* oocysts. At day 27 PI, oocysts were seen again in feces of infected non-treated mice. Also, NTZ, ginseng (50mg/kg), sage (50mg/kg) treated mice retuned to excrete oocysts at low levels. Other animal groups' feces were still negative for *Cryptosporidium* oocysts (Table 1 & Fig. 1).

Histopathological examination

There are sharp indicative differences in the appearance of the intestinal epithelial mucosa of all mice groups at the end of experiment (4 weeks PI) (Fig.2). The villi of non-infected (negative control) mice showed normal structure without any pathological changes in the mucosa or the lamina propria (Fig. 2A), while, the appearance of villi of infected non-treated (positive control) mice showed evident histopathological alterations including shortening, atrophy and desquamation of epithelial lining layer in most villi, hyperplasia in goblet cells and congestion in the lamina propria. (Fig. 2B). Examination of intestinal epithelial sections prepared from mice treated with NTZ, high dose ginger and ginseng treated mice displayed less histopathological lesions and more histologic correction in comparison to control mice (Fig. 2C, D1 & E1). While the intestinal sections from low dose ginger and ginseng and also from high and low dose sage treated mice showed lower regaining to normal appearance and less marked correction in the intestinal pathological alterations in comparison with other treatments used (Fig. 2 D2, E2, F1 & F2).

TABLE 1. *Cryptosporidium* oocyst shedding in experimentally infected non-treated, NTZ-treated, ginger, ginseng and sage extracts treated mice.

Animals / Days PI	Infected, Non-Treated	NTZ-Treated	Ginger Treated		Ginseng Treated		Sage Treated		F-Value
			300mg/kg	100mg/kg	100mg/kg	50mg/kg	100mg/kg	50mg/kg	
Day 3	81.4±0.55^{a}	79.8±0.84^{b}	81±0.7^{ab}	81.6±0.55^{a}	81.8±0.84^{a}	^{a}81.6±0.89	81.6±1.95^{a}	81.6±0.89^{a}	2.14^{NS}
Day 4	84.4±1.14^{a}	83±1^{ab}	82±1^{b}	84.8±1.64^{a}	83.2±1.64^{ab}	84.2±1.1^{a}	84.4±1.52^{a}	85±1.87^{a}	*2.74
Day 5	84.5±1^{bc}	84.6±1.14^{bcd}	83.2±0.84^{cd}	85.4±1.34^{bc}	83.8±0.84^{cd}	86.2±1.3^{b}	88.2±1.1^{a}	85.8±1.79^{b}	** 8.94
Day 6	85.6±1.34^{c}	88±0.71^{ab}	86±1.41^{c}	88±1.41^{ab}	86.4±1.3^{bc}	88.2±1.79^{ab}	89.4±0.89^{a}	88±1.58^{ab}	*4.96
Day 7	87.6±1.14^{c}	90.6±1.34^{b}	81±1^{d}	93.8±1.3^{a}	93.8±1.3^{a}	94±1.22^{a}	91.8±1.92^{b}	93.6±1.14^{a}	**58.53
Day 8	93.4±1.14^{c}	94.8±0.84^{bc}	70.6±1.95^{f}	96.6±1.82^{ab}	83.8±0.84^{e}	97.2±1.92^{a}	88.8±0.84^{d}	96.6±1.14^{ab}	**217.3
Day 9	96.2±1.3^{a}	87.8±0.84^{b}	55.4±1.14^{d}	96.6±1.82^{a}	75.6±2.3^{c}	97.2±1.92^{a}	88±2.45^{b}	97.2±1.92^{a}	**336.7
Day 10	99.2±0.84^{a}	73.4±1.67^{c}	24.6±1.14^{d}	99.4±1.14^{a}	71.8±3.63^{c}	98±2.35^{a}	83±1.58^{b}	99.4±0.89^{a}	**940.4
Day 11	100.6±1.5^{a}	65±1.58^{c}	20.2±1.3^{e}	101±1.58^{a}	53±2.12^{d}	99.6±0.89^{a}	72.6±3.29^{b}	101.8±2.17^{a}	**1193.4
Day 12	96.8±1.1^{a}	44.4±1.14^{d}	13.2±1.3^{f}	84.8±1.64^{b}	30.4±6.43^{e}	85.4±3.13^{b}	65.4±3.91^{c}	96.2±1.1^{a}	**544.3
Day 13	90.6±1.52^{a}	26.8±1.3^{d}	8.2±1.3^{f}	84.6±0.89^{b}	14.6±1.14^{e}	84.8±2.95^{b}	57.8±1.92^{c}	89.8±0.84^{a}	**2414.5
Day 14	84±2.12^{a}	23±1.87^{c}	6±0.71^{d}	83.6±1.82^{a}	7.6±0.55^{d}	83.8±3.49^{a}	47±1^{b}	83.4±1.82^{a}	**1792.7
Day 15	58.4±1.82^{b}	20.4±1.14^{d}	3±1^{f}	57.4±2.07^{b}	6±0.71^{e}	75.4±3.13^{a}	43.2±2.39^{c}	59±1.73^{b}	**1023
Day 16	53±2.12^{a}	19.8±1.1^{c}	2.6±0.55^{d}	52±2.12^{a}	3±1^{d}	52±2.12^{a}	41.4±1.34^{b}	54±1.41^{a}	**1041.3
Day 17	32±1.87^{c}	19.4±0.89^{d}	2±0.71^{e}	31.1±1.6^{c}	2.4±0.55^{e}	31.2±1.64^{c}	36.6±1.82^{b}	42.6±1.67^{a}	**564.71
Day 18	18.6±1.34^{c}	15.4±2.52^{d}	1.2±0.84^{e}	17.8±1.48^{c}	1.6±0.55^{e}	18.2±1.1^{c}	25.4±2.07^{b}	32±1.87^{a}	**221.1
Day 19	13.4±0.89^{c}	13±1.87^{c}	0.8±0.4^{d}	13±1^{c}	0.8±0.4^{d}	13±1^{c}	15.2±1.3^{b}	18.6±1.34^{a}	**168.8
Day 20	12.2±1.64^{ab}	9.2±1.48^{c}	0.6±0.5^{c}	11.2±0.84^{b}	0.6±0.5^{c}	11.2±0.84^{b}	7±1^{d}	13.4±0.89^{b}	**117.6
Day 21	7.2±0.84^{b}	6.6±1.14^{b}	0±0^{d}	7±1^{b}	0.4±0.3^{d}	6.6±1.14^{b}	4.8±1.1^{c}	12.2±1.64^{a}	**66.7
Day 22	2.8±1.1^{c}	6.4±0.55^{a}	0±0^{d}	2.6±0.89^{c}	0.4±0.3^{d}	2.4±0.89^{c}	4±1^{b}	7.2±0.84^{a}	**51.7
Day 23	2.6±0.89^{ab}	0±0^{c}	0±0^{c}	1.8±0.8^{ab}	0.4±0.23^{c}	2±0.7^{ab}	1.6±0.89^{b}	2.8±1.1^{a}	**13
Day 24	2±1.2^{a}	0±0^{c}	0±0^{c}	2±1.2^{a}	0±0^{c}	1.2±0.4^{abc}	1.4±1.1^{ab}	2±1.2^{a}	*3.9
Day 25	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	--
Day 26	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	--
Day 27	6.2±0.84^{a}	1.8±0.84^{b}	0±0^{c}	0±0^{c}	0±0^{c}	0.4±0.04^{c}	0±0^{c}	0.4±0.2^{c}	**47.1
Day 28	15±1.58^{a}	0.6±0.3^{b}	0±0^{b}	0±0^{b}	0±0^{b}	0.4±0.04^{b}	0±0^{b}	0.6±0.3^{b}	**450

All data expressed as Mean ± Standard Deviation. *: Significant differences at $P < 0.05$,**:Significant differences at $P < 0.001$ and N.S.: Non-significant. Means followed by different letters indicated significance. PI: post infection, NTZ: Nitazoxanide.

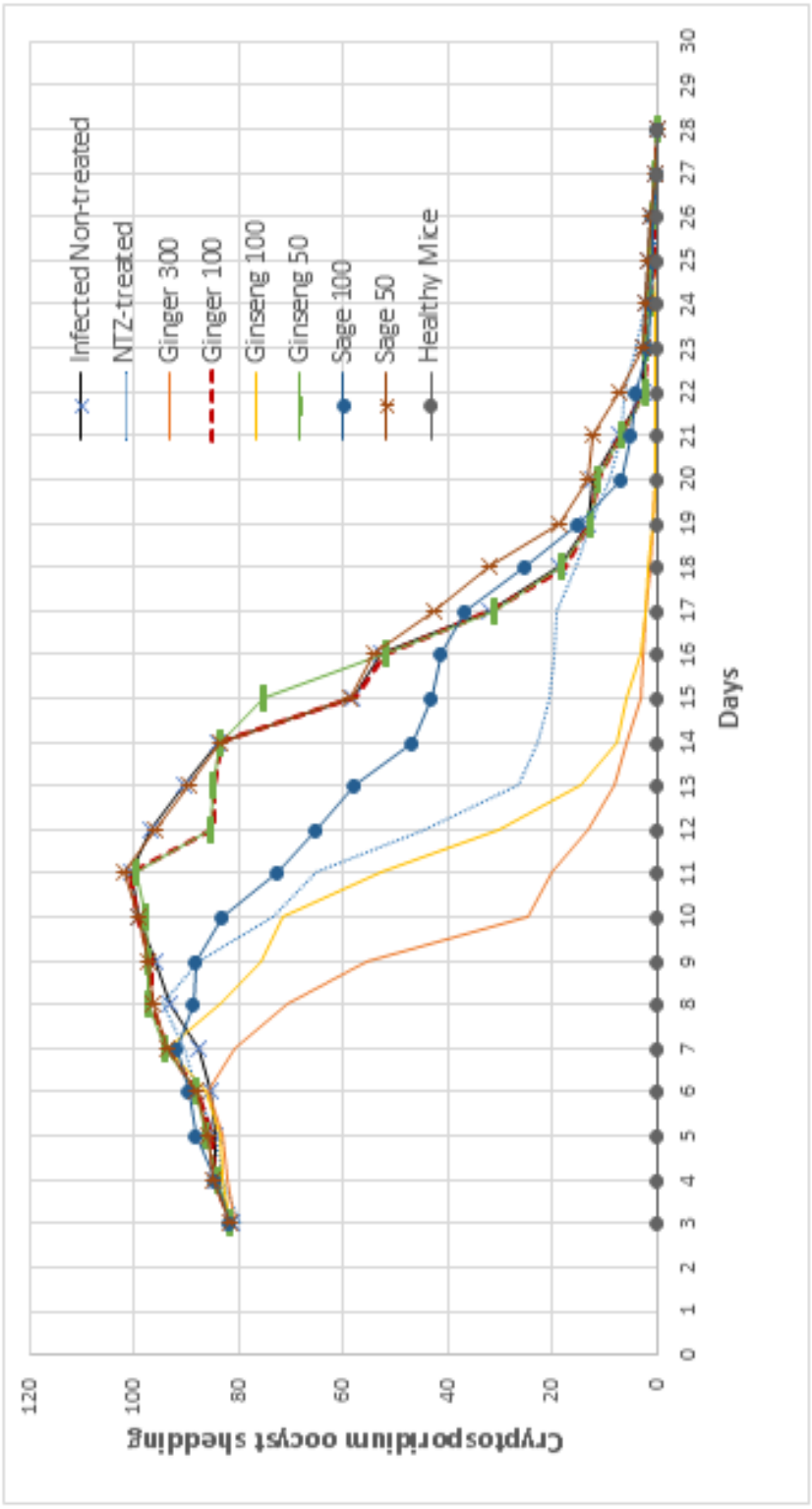

Fig. 1. *Cryptosporidium* oocyst shedding in experimentally infected non-treated, NTZ-treated, ginger, ginseng and sage extracts treated mice

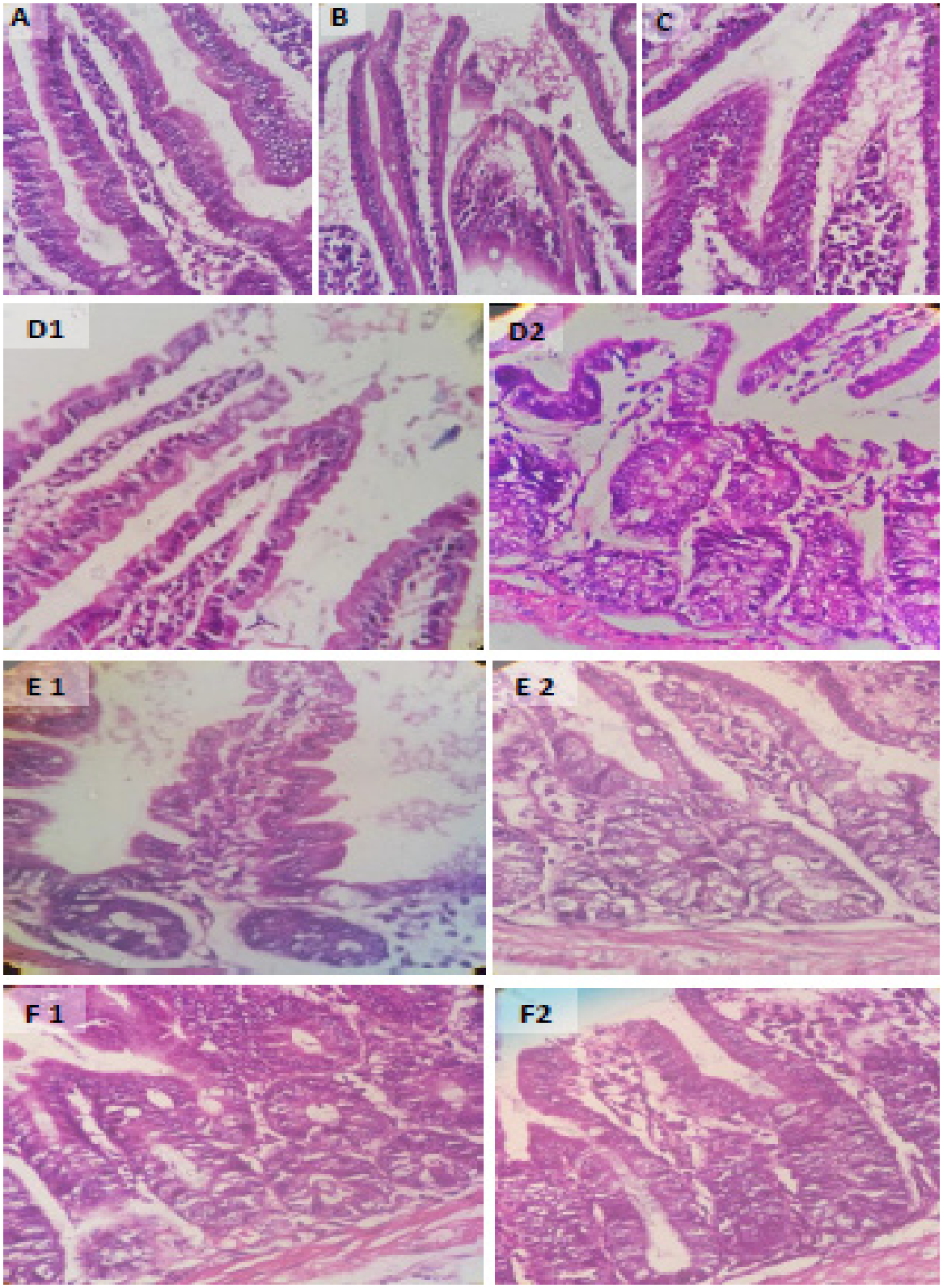

Fig.2. Histo-pathology of mice intestinal epithelia stained with H&E (X 100). *Cryptosporidium parvum* non infected (A), infected non-treated (B), infected NTZ treated(C), infected high dose ginger treated (D1), infected low dose ginger treated (D2), infected high dose ginseng treated (E1), infected low dose ginseng treated (E2), infected high dose sag treated (F1) and infected low dose sag treated (F1).

Discussion

In this study, significant dose-dependent reductions of fecal oocyst counts were detected with ginger, ginseng and sage extracts. High and low doses of ginger extract diminished *C. parvum* oocysts count significantly in experimentally infected mice than other treatments. Similar results about antiprotozoal effects of ginger extracts were recorded against *Giardia lamblia* trophozoites[34], *Toxoplasma gondii* both *in vitro* and *in vivo*[35], *Trypanosoma* [19] and *Blastocystis spp.*[36]. Moreover, it was found that ginger had the ability to increase digestive fluids, plus it could absorb and neutralize toxins and stomach acid[37]. Moreover, ginger extract caused inactivation of apoptotic proteins in infected host cells through the direct inhibition of *Toxoplasma gondii* and had anti-parasitic properties which inhibit inflammatory cytokine secretion *in vivo*[35].

The obvious effect of ginseng extract found in the present study on *C. parvum* infected mice might be due to that ginseng had been reported to maintain homeostasis of the immune system and to enhance resistance to illness or microbial attacks through the regulation of immune system [38]. Others, found that ginseng had been known as an immune modulator [39]. In addition, ginseng contains various pharmacological components such as polyphenolic compounds [40], that can interfere the energy generation mechanism by uncoupling the oxidative phosphorylation and also interfere with the glycoprotein of the cell surface of the parasites and cause death[41].Sage leaf contains tannic acid, oleic acid, ursonic acid, ursolic acid, cornsole, cornsolic acid, fumaric acid, chlorogenic acid, caffeic acid, niacin, nicotinamide, flavones, flavonoid glycosides, and estrogenic substance[42]. Amirmohammadi, et al. [24] found a significant reduction *(P<0.001)* in the number of eggs excreted by *Hymenolepis nana*, *Aspiculuristetraptera*, *Syphaciaobvelata* in mice and these results revealed that anti-parasitic effects of sage were reasonable and this plant might be used as antiparasitic natural product.

The examination of intestinal epithelial section of non-infected, untreated (negative control) mice displayed normal histologic architecture of the epithelium while the examination of ileum section of infected untreated (positive control) mice were revealed visible histopathological alterations. The experimentally treated mice groups were retained their normal appearance with varying degrees of histopathological corrections. These results agreed with Abu El Ezz et al. [12], Abdelrahman et al. [43] and Abouel-Nour et al. [44]. In this study, the intestinal villi of infected ginger, ginseng high dose treated mice groups showed more histologic repair and normal tissue appearance in comparison to control and other treated groups. The markedly beneficial and corrected effect of ginger and ginseng in present study may be related to their anti-inflammatory, significant potential antioxidant and immunomodulatory competence[45]. Also, Ali et al.[46] proved the immunological capability of ginger due to its active constituents such as zingerone, paradol, gingerols and shogaols. Moreover, plant extract might block or compete for receptor sites on intestinal surface, thus, leading to reduction in *C. parvum* colonization[47]. This might explain the apparent anti-Cryptosporidial activity of the high dose of ginger extract besides its previously recorded anti-Cryptosporidial [44,48] and antiprotozoal activities [18,19].

Conclusion

It is concluded that the methanolic extracts of ginger, ginseng and sage possessing anti-cryptosporidial activity (*C. parvum*), diminished the oocysts shedding, protect the intestinal epithelial from deleterious effects of *C. parvum* and can protect the healthy animals from infection and such result could be adopted in similar infections in susceptible animals and man.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical considerations

The study was ethically cleared by ethical review board of National Research Centre. Ethical approval certificate is registered under number 16/233 .Experimental animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines.

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الكفاءة العلاجية للزنجبيل والجنسنج والمرمرية ضد الكريبتوسبوريديوم بارفوم في الفئران المعدية تجريبياً

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الكريبتوسبوريديوم بارفام (خَفِيَّاتِ الأَبْواغ) هو طفيل وحيوان اولي وحيد الخلية عالمى الانتشاريصيب معظم الثديات مسببا مشكلة صحية كبيرة. وقد فحصت الدراسة الحالية فاعلية كل من مستخلص الزنجبيل والجنسنج والمريمية الكحولي (بالميثانول) على تطور داء خَفِيَّاتِ الأَبْواغ في الفئران التجريبية. تم علاج ٤٥ فأر تجريبي معدي بخَفِيَّاتِ الأَبْواغ باستخدام مستخلاصات نباتات طبية وهي الزنجبيل والجنسنج والمريمية مقارنة بادوية مسجلة ومتعارف عليها (النيتازوكسانيد). تم الفحص اليومي لمسحات من براز الفئران لمدة 4 اسابيع بعد العدوى. وقد فسرت النتائج بعدد البويضات المتحوصلة والفحص النسيجي للغشاء المخاطى لامعاء الفئران. وقد سجلت النتائج وجود اختزال احصائي معنوي في اراقة البويضات المتحوصلة في مجموعات الجرعات العالية من الزنجبيل والجنسنج والنيتازوكسانيد حتى اختفت بعد ٢١ و ٢٣ يوم من العدوي تباعا. كما اوضحت النتائج ان المجموعة المعدية والتى لم تتلقى اى علاج ومجموعات الفئران المعالجة بالنيتازوكسانيد والجرعة المنخفضة من الجنسنج والمريمية عادت لافراز البيوضات المتحوصلة عند مستويات منخفضة عند اليوم ٢٧ بعد العدوى في حين ان براز المجموعات الاخرى للحيوانات كانت سالبة للبويضات المتحوصلة لخفيات الابواغ. كما اظهر الفحص النسيجي ان الفئران المعالجة بالنيتازوكسانيد والجرعة العالية للزنجبيل والجنسنج لها تأثير وقائي وشفائي على النسيج الطلائي لامعاء الفئران المعدية مقارنة بالمعالجات الاخرى. ونستخلص من النتائج السابقة وجود فاعلية دوائية للزنجبيل والجنسنج والمريمية ضد عدوى الفئران التجريبية بخفيات الابواغ، كما وجد ان مستخلص الزنجبيل له تأثير واضح على الفئران المعدية مقارنة بالعلاجات الاخرى وان مثل هذه النتائج يمكن ملائمتها في الحيوانات المعرضة لمثل هذه العدوى وكذلك الانسان.