



Potential therapeutic and prophylactic effects of Asafoetida in murine cryptosporidiosis

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Abstract *Cryptosporidium parvum* is an important coccidian parasite that could infect the intestine, respiratory and biliary tracts of man and animals. This study aims to test the potential therapeutic and prophylactic effects of a natural herbal agent (Asafoetida) versus the nowadays drug of choice (Nitazoxanide). Fifty bred female, white Albino mice of CDI strain were divided into 5 groups; **group I (GI)**: immunosuppressed, infected with *C. parvum* and treated with Asafoetida, **group II (GII)**: immunosuppressed, prophylactically treated with Asafoetida for 7 days prior to infection, **group III (GIII)**: immunosuppressed, infected and treated with Nitazoxanide, **group IV (GIV)**: immunosuppressed and infected (Positive control), **group V (GV)**: immunosuppressed and non infected (Negative control). Parasitological and histopathological examinations of the stool, ileocaecal and liver specimens were performed for the study groups. **GI** showed reduction of the mean oocyst count in stool with improvement of the pathological changes at the ileocaecal region with preservation of hepatic architecture. Results of **GI** were better than **GII** and **GIV** but not as good as **GIII**. **GII** showed the least improvement among the test groups. **GIII** showed the best response between the test groups. **GIV** show no

statistical significant difference between the mean oocyst count in the mice stool at the time of infection and 7 days after infection. It was therefore concluded that Asafoetida is a promising natural therapeutic and prophylactic agent against cryptosporidiosis while, Nitazoxanide is the best chemotherapeutic agent against cryptosporidiosis.

Keywords *Cryptosporidium parvum* · Asafoetida · Nitazoxanide

Introduction

Cryptosporidium parvum (*C. parvum*) is an obligate intracellular coccidian protozoon parasite causing a disease called cryptosporidiosis. In developing countries, it is considered to be the most significant waterborne organism (Haque et al. 2003). It accounts for the majority of parasitic intestinal infections worldwide (Doganci et al. 2002). Nevertheless, many infection outbreaks occurred in the developed countries, treated water in swimming and wading pools had strong associations with such outbreaks (Craun et al. 2005). *Cryptosporidium* can maintain viability and infectivity in moist and cool situations for several months (Dawson et al. 2004). Many strains of *Cryptosporidium* were reported in human patients, such as *C. parvum*, *C. hominis* and *C. bovis* (Helmy et al. 2013). *Cryptosporidium* spp. infection in animals leads to an economic loss and becomes an important source for zoonotic infection. Clinically, *C. parvum* may precipitate severe life-threatening diarrhea in immunocompromised hosts (Guk et al. 2005). Moreover, a case control study was performed in United Kingdom and Sweden, revealed that *Cryptosporidium* infection may lead to persistent symptoms in immunocompetent patients (Hunter et al. 2004 and

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Insulander et al. 2013). Post infectious irritable bowel syndrome was strongly suggested to be the cause of the persistent symptoms (Marion et al. 2006 and Khaldi et al. 2009). Another study reported reactive arthropathy following cryptosporidiosis (Hay et al. 1987). Nitazoxanide (Nanazoxid) is the drug of choice for treatment of cryptosporidiosis as it helps to alleviate diarrhea by attacking the parasite metabolic processes. In immunocompromised patients, a combination of Azithromycin (Zithromax) along with one of the antiprotozoal drugs may be used (Rosenblatt 1992). Although, Nitazoxanide was an important innovative drug treatment for cryptosporidiosis in children, it has a limited efficacy in compromised or malnourished patients. This raises an important question about the proper management for these patients (Hayley et al. 2015). There are few available drugs against cryptosporidiosis like Azithromycin, Paromomycin, Roxithromycin and antiretroviral drugs (Gargala 2008). Unfortunately, these drugs are not effective to kill the infectious oocyst with probable side effects in addition to restricted availability in developing countries. Furthermore, many reports about drug toxicity, resistance and failure made the prescription of such synthetic antimicrobials non preferable for doctors. So, it is a great challenge for the health professionals to find a novel, effective, safe and inexpensive drug alternatives (Rosenblatt 1992).

It noteworthy, the medicinal herbs were used for treatment of different parasitic infections all over the world so many years ago (Behnke et al. 2008). In the herbal medicine, Asafoetida was used as a mucokinetic, diuretic, sedative and antiparasitic agent. In traditional therapies, Asafoetida was used in a dose of 0.2–0.5 g daily (Mohammadi et al. 2009 and Sadraei et al. 2003). Different studies proved that the aqueous extract, resin extract from the stems and roots and essential oil of Asafoetida was effective treatment for intestinal worms, lead to inhibition of *Trichomonas vaginalis* growth in vitro, and decrease the *Schistosoma mansoni* worm load in the experimental animals respectively (Bhattarai 1992; Ramadan and Al Khadrawy 2003; Ramadan et al. 2004).

With this introduction in mind, this study aimed to evaluate the potential therapeutic and prophylactic effects of the methanol extract of Asafoetida on white albino mice that were experimentally immunosuppressed and infected with *C. parvum* and compare the therapeutic effect with Nitazoxanide.

Aim of the work

Evaluating the potential therapeutic and prophylactic effects of Asafoetida in murine cryptosporidiosis.

Materials and methods

Animals

Animal source and handling

Laboratory bred female, white Albino mice of CDI strain, about 4–6 weeks old, weighing 20–25 g, were obtained from Theodor Bilharz Animal house. Animal experiments were performed in the biological unit of Theodor Bilharz Research Institute (TBRI), in a well-ventilated plastic cage with clean wood-chip bedding in conditioned rooms (27 ± 2 °C) and away from direct sunlight, ensuring good sanitary condition. All applied experiments on animals were carried out according to the internationally valid guidelines after the approval of the institutional ethical committee.

Animal groups

Fifty laboratory bred white albino female mice were used in this study.

Mice were divided into 5 groups; 10 mice each:

- Group I immunosuppressed, infected and treated with Asafoetida
- Group II immunosuppressed and received Asafoetida for 7 days prior to infection (as prophylaxis)
- Group III immunosuppressed, infected and treated with Nitazoxanide
- Group IV immunosuppressed and infected (Positive control)
- Group V immunosuppressed and non infected (Negative control)

Immunosuppression

Mice were administered with 0.25 ug/g/day of dexamethasone sodium phosphate (Dexazone) orally by gavage using esophageal tube. Dexazone was given daily for 2 weeks prior to oral inoculation with *Cryptosporidium* oocysts and was maintained weekly during the whole experiment for all groups (Rehquel et al. 1998).

Infection

Cryptosporidium parvum oocysts were obtained from the Animal Research Institute in Giza governorate, Egypt. Mice were orally infected with *C. parvum* oocysts via gavage using esophageal tube. A pilot study was performed to calculate the proper infecting dose by oral infection of mice with different doses of oocysts followed by stool

examination 1 and 2 weeks after the infection; infecting each mouse by 500 oocysts didn't establish infection, increasing the dose to 1000 oocysts succeeded to establish infection but further increasing the dose to 5000 oocysts caused the death of the mice.

Accordingly, the infection dose for (**GI–GIV**) was 1000 *C. parvum* oocysts that were dissolved in 200µL of PBS.

Drugs administration

- Plant material

Asafoetida powder was purchased from a local market in Giza governorate, Egypt. The identification of the plant was authenticated at the Department of Botany, Faculty of Science, Cairo University, Egypt.

- Preparation of methanol extract of Asafoetida

The dried powder of Asafoetida was soaked overnight in absolute methanol at room temperature. The pooled extracts were filtered, evaporated under reduced pressure using rotatory evaporator (Ghareeb et al. 2018).

GI received 5% concentrations of the methanol extract of Asafoetida. The extract of the natural herb was given for 7 successive days post infection. **GII** got the infection after a 7 days course of the methanol extract of Asafoetida prophylactic administration (Farhadi et al. 2016).

This dose was selected according to pilot study that revealed very weak effect using a concentration of 2.5% and the same responses on using a concentration of 5% and 10%, so we preferred to use the minimal effective dose.

GIII received oral suspension of Nitazoxanide in a dose of 65 mg. The drug was administered for 7 successive days post infection. The doses were calculated by extrapolation of human therapeutic doses to animal doses according to the table of (Barnes and Paget 1965).

Parasitological examination

Stool samples were collected and subjected to parasitological examination after staining by modified Zheil Nelsen (MZN) stain according to (Henricksen and Pohlenz 1981) using the oil immersion lens ($\times 100$) to ensure that mice have been infected and to count *C. parvum* oocysts.

Oocyst count in stool was done in **GI**, **GII**, **GIII** and **GIV** 7 days post infection followed by sacrifice of the tested mice and for **GV** it was done at the same time as previous groups.

Histopathological examination

Sacrifice of all mice was done 7 days post infection and it was done at the same time for the mice of **GV**. The ileocecal region and liver were obtained and fixed in 10%

buffered formalin solution, embedded in paraffin wax blocks that were sectioned then stained in the pathology lab of TBRI, staining was done using Hematoxylin and Eosin (H&E) to assess the pathological changes and explore any abnormal proliferation pattern.

Statistical analysis

Data were analyzed using paired sample *t* test, *P* value is considered significant if < 0.0001 .

Ethical consideration

This study was approved by Scientific Research Ethics Committee of TBRI.

Results

Parasitological examination for the stool of different study groups

GI, **GII** and **GIII** showed highly significant statistical difference between the mean oocyst count in the stool of mice before and after treatment (**GI**: 91.4 and 25.10 respectively) (**GII**: 96.56 and 48.11 respectively) and (**GIII**: 92.2 and 3.0 respectively). The study revealed that **GI** showed improvement more than **GII** and **GIV** but less than **GIII**. **GII** showed the least improvement among the test groups. **GIII** showed the best response between the test groups with marked reduction of the mean oocyst count in the stool. **GIV** show no statistical significant difference between the mean oocyst count in the mice stool at the time of infection and 7 days after infection (Table 1).

Table 1 The results of parasitological examination of different test and control groups

	Mean	SD	<i>p</i> value*
GI before treatment	91.40	8.140	< 0.0001
GI after treatment	25.10	4.332	
GII at the onset of infection	96.56	13.116	< 0.0001
GII 7 days after the infection	48.11	8.223	
GIII before treatment	92.20	9.920	< 0.0001
GIII after treatment	3.00	1.054	
GIV at the onset of infection	93.20	11.698	0.001
GIV 7 days after the infection	91.30	11.926	

**p* value calculated using paired sample *t* test

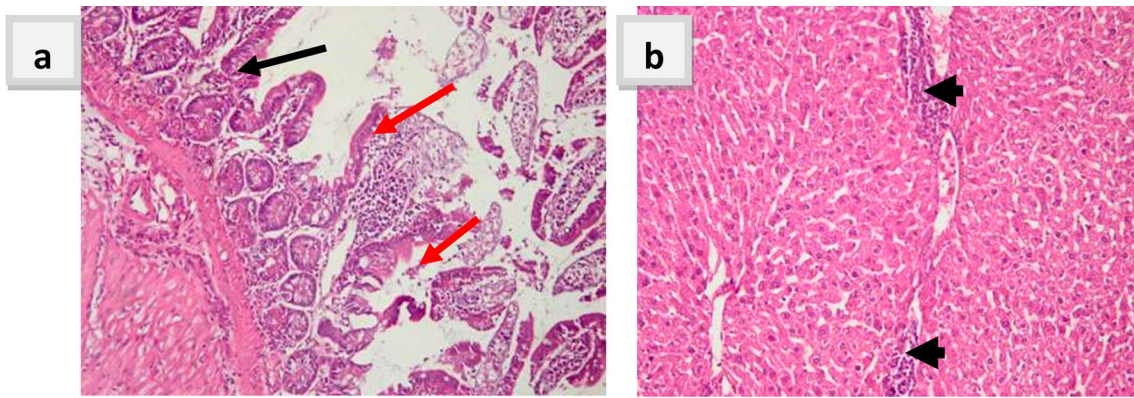


Fig. 1 **a** Section of small intestine in GI showing partial villous blunting with partial healing and focal ulceration of intestinal mucosa “red arrows” and mild decrease in the ratio between villous height to crypt length “black arrow” (H&E stain $\times 200$). **b** Section of liver in

this group showing preserved architecture and slightly expanded portal tracts showing mild lymphocytic infiltrate “black arrow heads” (H&E stain $\times 200$) (colour figure online)

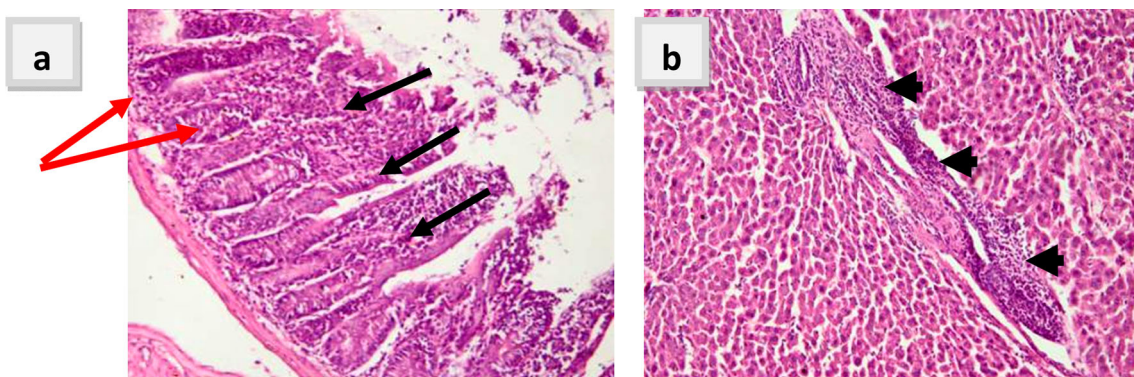


Fig. 2 **a** Section of small intestine in GII showing partial healing of the intestinal mucosa with areas of ulceration “black arrows” moderate goblet cell depletion and moderate inflammatory infiltration

in the lamina propria “red arrows” (H&E stain $\times 200$). **b** Liver section of this group with “black arrow heads” showing moderate lymphocytic infiltrate of portal tracts (H&E stain $\times 200$) (colour figure online)

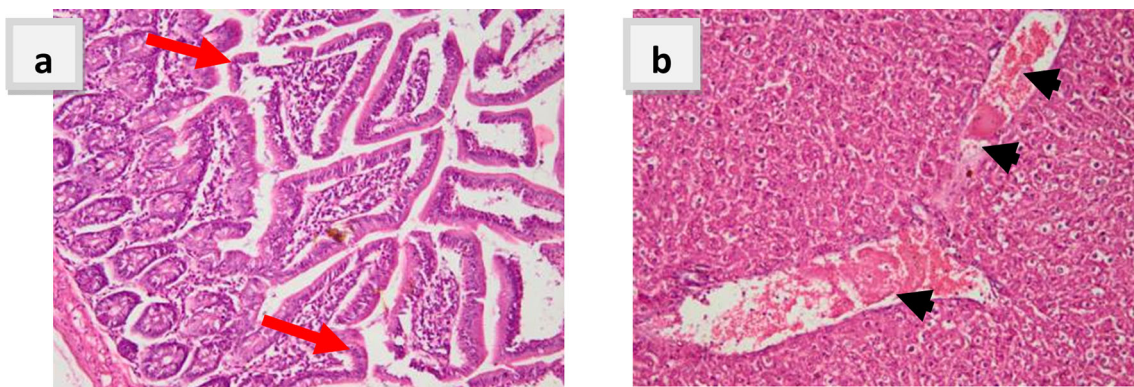


Fig. 3 **a** Section of small intestine in GIII revealed healing of intestinal surface epithelium with minute surface erosions “red arrows” (H&E stain $\times 200$). **b** Sections examined from liver in this

group showed preserved hepatic architecture and slightly congested sinusoids and central veins “black arrow heads” (H&E stain $\times 200$) (colour figure online)

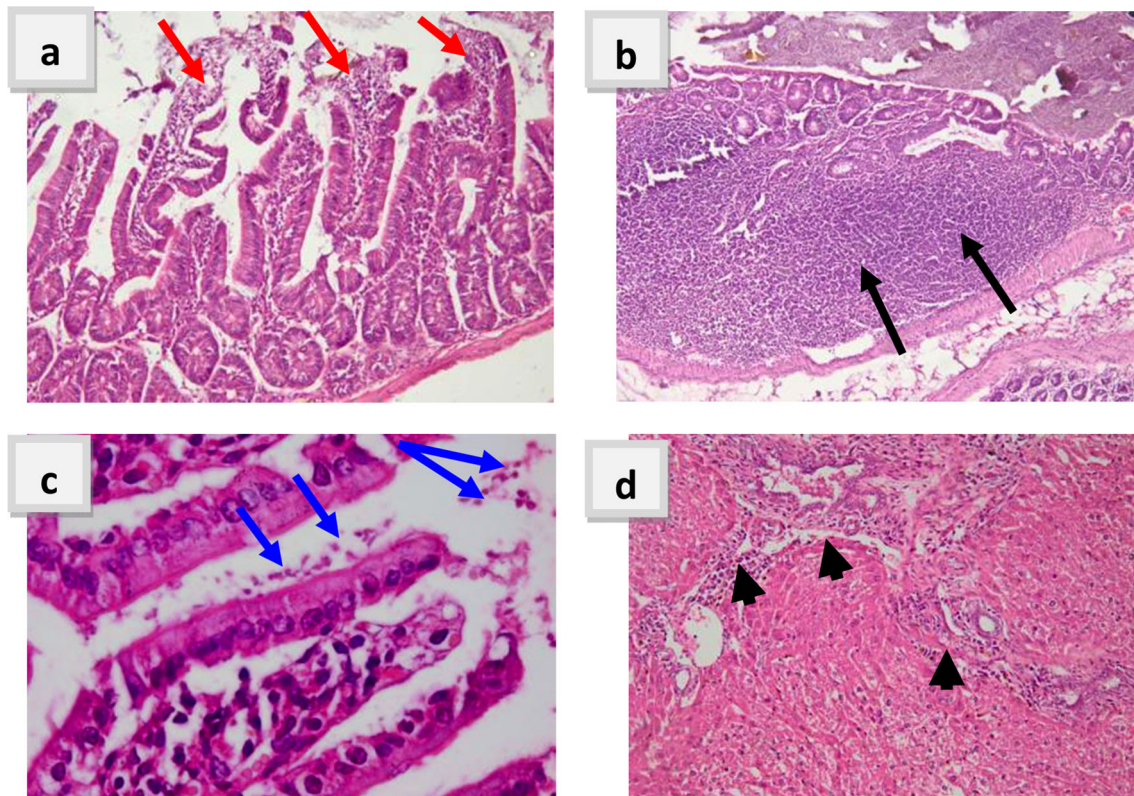


Fig. 4 **a** Section of small intestine from **GIV** showing significant villous blunting and ulceration “red arrows” (H&E stain $\times 200$). **b** Marked inflammatory infiltrate in the lamina propria with lymphoid follicle hyperplasia “black arrows” (H& E stain $\times 100$). **c** *Cryptosporidium* oocysts (tiny purple stained structures) detected in the

intestinal lumen and along brush border of the villi “blue arrows” (H&E stain $\times 1000$ “oil immersion”). **d** Section of liver in **GIV** showing expanded portal tracts with marked inflammation and moderate fibrosis “black arrow heads” (H&E stain $\times 200$) (colour figure online)

Histopathological examination of sections from ileocaecal junction and livers of the study groups

• GI

Examination of small intestinal sections at ileocaecal junction revealed partial improvement of the histopathological changes which follow cryptosporidiosis infection in the form of mild villous blunting with mild decrease villous height to crypt length ratio. There was partial healing of intestinal mucosa with focally ulcerated mucosa, mild goblet cell depletion and mild inflammatory infiltration in the lamina propria. Sections examined from liver revealed preserved architecture with slightly expanded portal tracts showing mild lymphocytic infiltrate as shown in Fig. 1.

• GII

Examination of small intestinal sections at ilocecal junction revealed mild improvement of the histopathological changes that follow cryptosporidiosis infection

including partial villous blunting and broadening with moderate decrease in villous height to crypt length ratio. There were ulcerations in the intestinal mucosa with partial healing, moderate goblet cell depletion and moderate inflammatory infiltration in the lamina propria. Sections examined from liver revealed preserved architecture with expanded portal tracts showing moderate lymphocytic infiltrate as shown in Fig. 2.

• GIII

Examination of sections from ileocaecal junction revealed significant improvement of the histopathological changes following cryptosporidiosis infection including restoration of the normal villous architecture with preserved villous height to crypt length ratio. The mucosal lining becomes intact with healing of the surface epithelium, preserved brush border of the villi and minimal surface erosions. Goblet cells were preserved with no depletion and minimal inflammatory infiltration in the lamina propria was detected. No *cryptosporidium* oocysts could be detected. Examination of liver sections showed preserved architecture and slightly congested sinusoids as shown in Fig. 3.

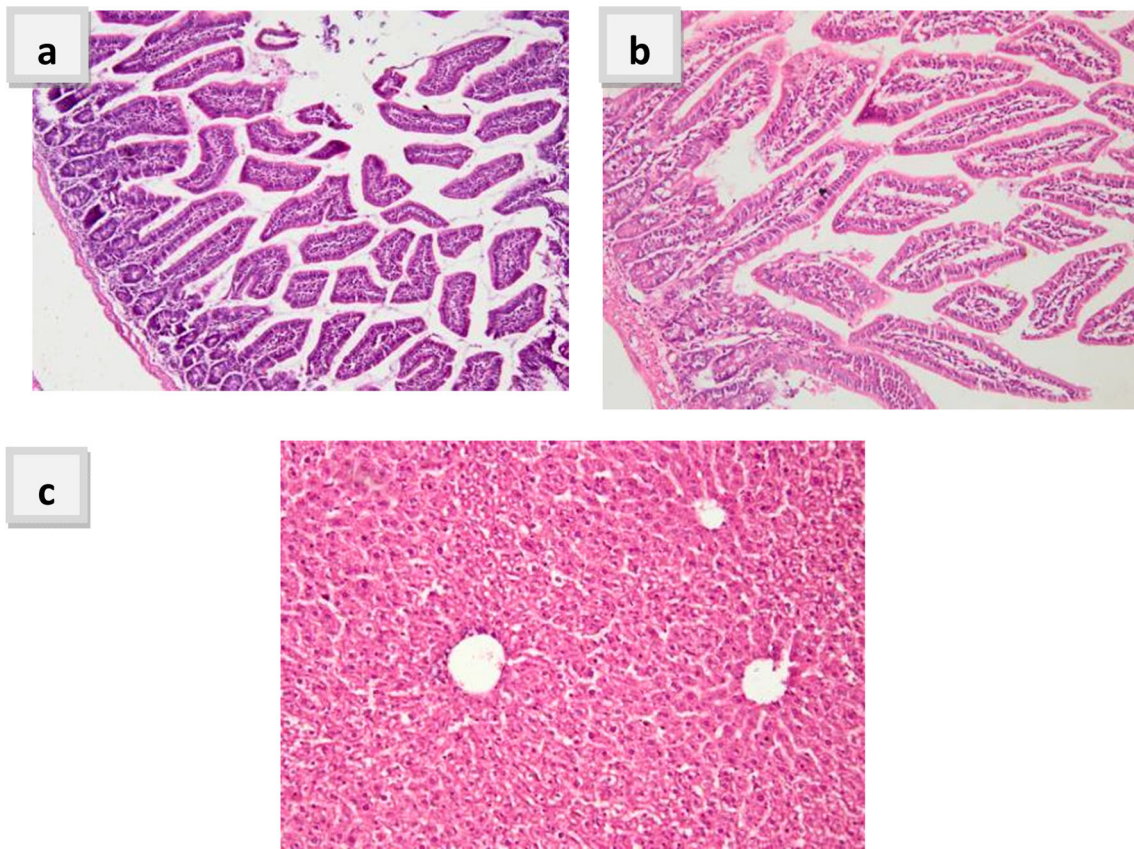


Fig. 5 a, b Section of small intestine of GV exhibiting normal villous architecture with intact brush border (H&E stain $\times 100$ and $\times 200$). c Section of liver of GV showing preserved hepatic architecture (H&E stain $\times 200$)

• GIV

Examination of sections from ileocaecal junction revealed significant histopathological changes in small intestinal villi architecture as a result of cryptosporidiosis infection. These changes were in the form of variable grades of villous atrophy with shortened broad villi and decreased ratio between villous height to crypt length. Mucosal ulceration and goblet cell depletion were detected together with inflammatory infiltrate of lamina propria formed mainly of lymphocytes, plasma cells and macrophages with lymphoid follicles hyperplasia. *Cryptosporidium* oocysts were detected along the brush border of the villi and in the intestinal lumen. Examination of liver sections revealed slightly congested sinusoids, scattered foci of spotty necrosis and interface activity with expanded portal tracts showing moderate lymphocytic infiltration forming porto-central bridges with interface activity and moderate fibrosis as shown in Fig. 4.

• GV

Examination of sections from ileocaecal junction revealed normal architecture of small intestinal villi with

average length and width. The brush border was intact, well formed and goblet cells are of average number. Examination of liver sections revealed preserved hepatic architecture with hepatocytes arranged in regular plates around central vein with portal tracts showing normal structures Fig. 5.

Discussion

Cryptosporidium parvum although causes self-limiting diarrhea in immunocompetent patients, it leads to severe and life-threatening diarrhea in immunocompromised hosts (Guk et al. 2005).

The side effects and the increasing resistance to the available antiparasitic drugs used in the treatment of *C. parvum* make the researchers face a great challenge to find a natural herbal alternative that is not toxic and easily obtained in adequate quantities to replace the standard drugs in the market (Rayan et al. 2005).

According to the results of the present study, treatment with the methanol extract of the natural herb *Asafoetida* could effectively reduce *Cryptosporidium* parasites in

experimentally infected mice and improve the histopathological changes of small intestinal villi and preservation of liver architecture with slightly expanded portal tracts showing mild lymphocytic infiltrate following cryptosporidiosis. The antiprotozoal properties of Asafoetida were previously assessed against other protozoal parasites. The effects of Asafoetida herbal extract against *Leishmania major* promastigotes was tested, and revealed an important anti-leishmanial effect of this herb (Barati et al. 2010). This is in congruence with authors who proved reduction of the *Trichomonas vaginalis* parasite load by 90% within 1 h of exposure by giving the herbal extract of Asafoetida at the concentration of 2 mg/ml (Sarkari et al. 2009). Lethal effects against *Giardia* cysts were also reported by the aqueous and alcoholic extracts of Asafoetida (Rezaie-manesh and Shirbazou 2012). The antihelminthic effects of Asafoetida were tested by many authors who proved the anti-trematode effects of Asafoetida through the oral administration of Asafoetida essential oil. It notably reduced the number of eggs and worms in infected rats with *Schistosoma mansoni* (Ramadan et al. 2004). In another study, the anti-cestode effects of Asafoetida against *Hymenolepis nana* were proved through administration of 17.7 mg/ml of Asafoetida extract for 2 weeks with health improvement in around 90% of the infected rats (Maraghi and Soghra 1991). This is similar to a study that proved a significant decrease in the number of *Hymenolepis nana* in the experimental animals receiving the methanol extract of Asafoetida compared to control subjects (Youssefi et al. 2012). Nevertheless, the herbal extract fails to show a significant effect against nematode *Syphacia obvelata*, which exclude the antinematode effect of this medicinal herb (Farhadi et al. 2016).

The prophylactic doses of methanol extract of Asafoetida showed significant reduction of the mean oocyst count in the stool of infected mice 7 days after the infection, mild improvement of the histopathological changes at the ileo-caecal region, preserved liver architecture with expanded portal tracts showing moderate lymphocytic infiltrates. A review and meta-analysis declared that Nitazoxanide led to important evidence of oocyst clearance compared with placebo. The effect was not significant for HIV-seropositive participants. The absence of effective treatment for immunocompromized patients made an alarm for the importance of preventive interventions (Abubakar et al. 2007).

Nitazoxanide showed the best parasitological and histopathological responses in this study by significantly reducing the mean oocyst count in the stool of infected mice 7 days after treatment and also restore the normal villous and liver architectures following cryptosporidiosis. Nitazoxanide (NTZ) was described as a drug with broad activity against protozoa and helminthes. NTZ and its two

metabolites, tizoxanide (TZ) and tizoxanide-glucuronide (TZglu) were proved to suppress the growth of *C. parvum* in a concentrations < 10 mg/L (Theodos et al. 1998; Gargala et al. 2000 and Cai et al. 2005). Activity in the intestine is the role of NTZ and TZ. TZglu is excreted in high concentrations in bile and it is believed to be responsible for the activity of the drug against cholangitis due to disseminated cryptosporidiosis in immunocompromised patients. Clinical and parasitological cures from cryptosporidiosis were reported in immunocompetent adults, adolescents and children that were treated by a 3 days course of NTZ (Rossignol 2001; Rossignol 2006 and Amadi et al. 2002). Fifty-nine percent of adult AIDS patients with cryptosporidial diarrhea showed a persistent clinical response while on treatment with NTZ. Reports stated that doses up to 3000 mg/day or for long duration of treatment were non safe (Rossignol 2006).

Conclusion

Results of the present research indicated that the methanol extract of Asafoetida has promising therapeutic and prophylactic effects against *C. parvum*. Menthol extract of Asafoetida is cheap, easily obtained in suitable quantities and help to avoid the adverse reactions caused by chemotherapeutic drugs. Furthermore, complementary research regarding the side effects of this medicinal herb or its compounds will present it as useful therapeutic and prophylactic agent against *C. parvum* for the immunocompromized patients.

Funding None.

Compliance with ethical standards

Ethical consideration The study was approved by the Research Ethics Committee, Theodor Bilharz Research Institute (TBRI). All the animal experiments were performed according to the national regulations for the Animal Ethics rules.

Conflict of interest None.

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