Journal of Zoonotic Diseases
https://jzd.tabrizu.ac.ir/
Published by the University of Tabriz
Online ISSN: 2717-2910

Comparison of therapeutic effects of hydroalcoholic extract of Asafoetida with metronidazole in mice infected with *Giardia lamblia*

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Article type:
Original article

Keywords:
Therapeutic effects
Asafoetida,
Metronidazole
Giardia lamblia
Mice

Article history:
Received:
November 20, 2023
Revised:
November 28, 2023
Accepted:
January 12, 2023
Available online:
January 13, 2023

Abstract

Giardia lamblia is the protozoan agent of giardiasis and is one of the most common causes of diarrhea worldwide, especially in Iran. Nowadays, drugs such as metronidazole, furazolidone, tinidazole, and quinacrine are used to treat giardiasis. These drugs have different side effects, so research to find a drug with few side effects seems necessary. Asafoetida, an oleo gum resin obtained from an Iranian endemic herb, Ferula Assa-foetida has many healing properties, especially in the treatment of parasitic diseases. This study aimed to investigate the effect of Asafoetida hydroalcoholic extract on the *G. lamblia in vivo* in mice. In this experimental study, 25 mice were divided into 5 groups of 5 including negative control, drug control with metronidazole, and 3 experimental groups treated with *Asafoetida* extract at doses of 100, 200, and 400 mg/mL. Then, the effect of Asafoetida hydroalcoholic extract up to the tenth day was evaluated. In this study, the weight of mice in the groups treated with hydroalcoholic extract of Asafoetida was lower than the control group that did not take any drugs. The effect of plant extract against *G. lamblia* showed that the reduction in the number of cysts in the groups of 100, 200, and 400 mg/mL was equal to 71.8%, 80.9%, and 93%, respectively ($p<0.05$). The results of this study showed that Asafoetida has very good antigiardial effects on *in vivo* conditions and can be considered as one of the treatment options for the treatment of giardiasis.

Introduction

Giardiasis is a parasitic disease caused by a flagellate protozoan called *Giardia lamblia*. *G. lamblia* has a global distribution and with 280 million cases per year. It is the most common intestinal parasite in developed countries. In these countries, this protozoan is usually accompanied by diarrhea outbreaks. In the United States, *G. lamblia* is the cause of most outbreaks of water-borne diarrhea. The prevalence of infection in Europe is

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https://doi.org/10.22034/jzd.2024.17396
https://jzd.tabrizu.ac.ir/article_17396.html
Cite this article: Hariri D., Garedaghi Y. comparison of therapeutic effects of hydroalchoholic extract of Asafoetida with metronidazole in Mice infected with Ghiardia lamblia. Journal of Zoonotic Diseases, 2024, 8 (1): 452-459.
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between 2 and 17%. In Asia, Africa, and Latin America, about 200 million people have symptomatic giardiasis and about 500,000 new cases are reported annually in these areas. This protozoan is one of the most common parasitic protozoa in Iran and is an important cause of diarrhea, especially in children. The prevalence of this parasite in our country is reported to be 19 to 22% on average (1). *G. lamblia* has two forms trophozoite and cyst. Cysts are an infective form of the parasite and are highly infective to humans. Cysts can survive in the environment for up to 3 months. Due to the resistance of cysts to chlorination of water, it is easier to spread through water and the disease is transmitted to humans mainly through contaminated water (2).

Among the most important ways of giardiasis transmission, the following can be mentioned: Person-to-person transmission through direct oral contact with feces, especially in dense communities such as kindergartens and among family members, transmission through contaminated food and in developing countries, transmission among homosexuals. *Giardia lamblia* is also known to cause chronic diarrhea in travelers (3).

The clinical signs of giardiasis usually begin one to three weeks after eating the cysts, and the most important symptom of the disease is diarrhea, which is seen in 90% of symptomatic patients. Diarrhea may be acute and self-limiting or chronic and debilitating. The most common clinical symptoms of giardiasis in Iran include abdominal pain and bloating. Other symptoms include indigestion, epigastric pain, nausea, vomiting, and foul-smelling fatty stools. In chronic infections, fat malabsorption, lactose, iron, vitamins A and B_{12}, weight loss, and growth retardation have been reported, especially in children (4). Common drugs for the treatment of this disease include nitroimidazole compounds including metronidazole, tinidazole, ornidazole, shinidazole and nimurazol, benzimidazole compounds such as mebendazole and albendazole, and other drugs such as quinacarine and furazolidone and parmomycin, of which metronidazole is the drug of choice for the treatment of giardiasis (5). These drugs have unpleasant side effects such as a bad taste in the mouth, indigestion, nausea, headache, and leukopenia; also, some of these drugs can lead to neurotoxic effects, restlessness, seizures, and dizziness and disrupt the treatment process. In addition, the effects of mutagenicity, carcinogenicity, and some of their adverse effects on the fetus have been proven in laboratory animals. *G. lamblia* resistance to metronidazole, quinacrine, and parmomycin has been identified in laboratory and clinical trials in various parts of the world, and cases of drug resistance are increasing in patients (6, 7). Due to the side effects of anti-giardia drugs as well as increasing parasite resistance to common drug treatments, it is necessary to find new, effective, and safe drugs for the treatment of this disease (8). Extensive variety, an abundance of compounds with therapeutic properties, few side effects, and lack of drug resistance have made them can be used as an important source for the search for new drug compounds and the synthesis of effective drugs (9). Ferula assa-foetida is an important medicinal plant of Apiaceae. The gum derived from this plant, called Asafoetida, has been used in traditional medicine in the treatment of many diseases, especially parasitic diseases (10). In this study, the effect of Asafoetida hydroalcoholic extract on *G. lamblia* in mice was investigated.

Materials and methods

Preparation of plant and chemical constituents of Asafoetida

Ferula assa-foetida is a medicinal plant of the Umbelliferae family (Apiaceae) and native to Iran. It is used because of its high therapeutic value (11). The metabolites such as β-Pinene, α-Pinene, Propyl n- butyl disulfide, and 1,2-dithiolane are the main constituents of the essential oil of this plant. The other major components of the plant are (Z)-1-propenyl sec-butyl disulfide and β-Eudesmol, 2-isopropyl-5-methyl-9-methylene- and β-Maaliene

were characterized as the major constitutes. The essential oils of the plant were dominated by the volatile sulfur-containing compounds as well as sesquiterpene (Table 3).

Method of preparation of alcoholic extract of Asafoetida

150 g of dry Asafoetida powder was mixed with 600 ml of 90% ethanol (Merck, Germany) and placed on a shaker in a dark environment for 48 hours. The resulting mixture was filtered using Whatman No. 1 filter paper and divided into several plates. To evaporate the alcohol, the plates were incubated at 50 °C for several hours. The extracts in the plates were then completely dried at room temperature and exposed to air for several days. The resulting extract was weighed and stored at -70 °C (12).

Metronidazole

Metronidazole is an antibiotic for anaerobic bacteria and an antiparasitic. Metronidazole is effective for the treatment of guinea worm disease, giardiasis, trichomoniasis, and amoebiasis. It is on the World Health Organization's list of essential medicines, known in the health system for its high level of safety and effectiveness. Once absorbed into the cell, metronidazole is regenerated by intracellular reactions and converted to toxic metabolites. Metronidazole metabolites damage cellular DNA. Because regenerative reactions involving the production of these metabolites occur only in anaerobic cells, human cells and aerobic bacteria are largely immune to damage (Table 3).

Collection and extraction of G. lamblia cyst

G. lamblia cyst was collected from infected stool samples of patients with giardiasis referred to health centers. Cysts were observed in fecal samples by microscopic methods. Samples were concentrated by the 85% sucrose method. To isolate Giardia cysts, stool samples were diluted with distilled water in a ratio of 1 to 12. To make an aqueous solution, 20 ml of the diluted sample was poured into a container containing pearls and shaken for 5 minutes. The above suspension was removed and 5 ml of water was added to the resulting precipitate. Three ml of 85% molar sucrose was added to the obtained solution. The resulting suspension was centrifuged at 600 rpm for 10 minutes. The cysts were removed by Pasteur pipette and washed 3 times with normal saline. Eosin staining was used to measure the percentage of live cysts (13).

Method of measuring the lethal effect of extracts on G. lamblia cysts

To determine the lethal effect of the extracts, cysts were stained with 0.1% eosin. In this method, an equal volume of a mixture of the extract and Giardia cyst is mixed with eosin dye. After 10 minutes of staining, under microscopic examination, the dead cysts turn red, while the living cysts do not absorb the dye and are completely free colors (14).

Animals and laboratory conditions

25 laboratory mice, about two weeks-old and weighing approximately 20 grams, were purchased from Tabriz University of Medical Sciences. All ethical principles of work were observed by the ethical protocol of working with laboratory animals approved by the Research Ethics Committee of the University of Medical Sciences (https://ethics.research.ac.ir/IR.IAU.TABRIZ.REC.1402.282). Mice were infected with 20,000 *G. lamblia* cysts by gavage. To ensure that the mice were infected with the parasite, the mice's feces were examined for 11 consecutive days. The formalin-ether method was used to determine the infection of mice with fecal cysts (15). Then, the animals were divided into 5 groups; Negative control (Mice infected with Giardia cyst and untreated), Drug control (Mice infected with Giardia cyst treated with metronidazole 10 mg/kg), and 3 experimental groups including; Experiment 1 (Infected mice treated with Asafoetida extract at a concentration of 100 mg/mL), Experiment 2 (Infected mice treated with Asafoetida extract at a concentration of 200 mg/mL), Experiment 3 (Infected mice treated with Asafoetida extract at a concentration of 400 mg/mL). Doses of 1 mg/mL Asafoetida extract with concentrations (100, 200,

and 400) were administered by gavage to mice in experimental groups for 10 consecutive days per kg body weight.

To determine the number of live *Giardia lamblia* cysts from eosin staining. /1 % was used. In such a way that cysts that take color are considered dead and cysts without color are considered alive.

Statistical analysis

Data analysis was performed using SPSS 22 software. The ANOVA and T-tests were performed with $p < 0.05$ considered significant.

Results

Evaluation of parasite infection in mice

Observations showed that cysts appear in the feces of mice up to 11 days after intragastric inoculation and the mice become infected with the parasite. In some infected mice, symptoms such as; isolation, dullness and tangling of body hair, and loose stool consistency were observed.

Evaluation of mice weight before and after infection and treatment

There was no significant difference in the mean weight of mice before the intervention ($p>0.001$). However, after the intervention, there was a significant difference between the different groups ($p<0.0001$). Weight increased in all groups, which was significant compared to the beginning of treatment in the negative control group, groups 1 and 2, but not in the positive control group ($p>0.001$) and group 3 ($p>0.001$) before and after the intervention (Table 1). After the therapeutic intervention, the highest mean weight was in the negative control group and the lowest mean was in the positive control group and also in the treatment group 3. After the intervention, the mean weight of the negative control group was significantly different from the positive control and other groups ($p<0.0001$). The mean weight of group 3 with positive control was not significantly different after the intervention, but there was a significant difference between each of the three groups after the intervention. In terms of mean weight, group 3 and the drug were similar in function.

Table 1. The mean weight of mice in different treatment groups compared to positive and negative control groups.

Group (G)	Before intervention Mean ± SD	After the intervention Mean ± SD	*p*-value**
Negative control	20.24 ± 1.63^{A}	28.46 ± 0.67^{A}	0.001
Drug control (Metronidazole)	20.19 ± 0.62^{A}	$28.52 \pm 1/63^{D}$	0.181
Asafoetida 100 mg/mL (G1)	20.16 ± 0.81^{A}	26.34 ± 1.03^{B}	0.000
Asafoetida 200 mg/mL (G2)	20.44 ± 0.72^{A}	24.66 ± 1.28^{C}	0.000
Asafoetida 400 mg/mL (G3)	20.11 ± 1.21^{A}	21.84 ± 1.08^{D}	0.341
p-value*	0.995	< 0.0001	

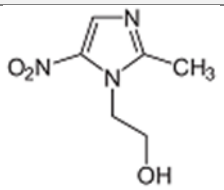
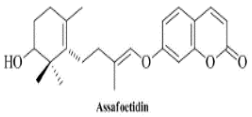
*Anova **T-test, the same letters in each column indicate no difference.

Table 2. Evaluation of the mean survival rate of Giardia excreted cysts in different treatment groups compared to positive and negative control groups before and after treatment.

Group (G)	Before intervention Mean ± SD	After the intervention Mean ± SD
Negative control	100 ± 0.00^{A}	100 ± 0.00^{A}
Drug control (Metronidazole)	100 ± 0.00^{A}	0.002 ± 0.03^{E}
Asafoetida 100 mg/mL (G1)	100 ± 0.00^{A}	28.20 ± 0.04^{B}
Asafoetida 200 mg/mL (G2)	100 ± 0.00^{A}	19.11 ± 2.05^{C}
Asafoetida 400 mg/mL (G3)	100 ± 0.00^{A}	7.00 ± 1.03^{D}

Anova and *t*-test, they were quite similar ($p<0.0001$). The same letters in each column indicate no difference.

Table 3. Antiparasitic compounds used in mice infected with *Giardia lamblia*.

Antiparasitic compounds	Molecular formula	Mode of action	Drug administration		Chemical structure
			Route	Dose	
Metronidazole	C6H9N3O3	Metronidazole inhibits nucleic acid synthesis by forming nitroso radicals, which disrupt the DNA of microbial cells.	Oral	10 mg/kg	
Asafoetida	C26H34O6	Asafoetida decreased counts and viability of Giardia. The degree of the inhibitory effect was dependent on the concentration and time of incubation with asafoetida extracts	Oral	100-400 mg/mL	

Before the intervention, the condition was similar in all groups in terms of parasite infection, so that in all groups, an average of 100% of live cysts were reported. After the intervention, the percentage of live cysts in the negative control group was still 100% and in the positive control group, almost all cysts were dead and the mean of live cysts was reported to be zero. In other words, 100% of the parasites in this group died. In the investigation on treatment groups, it was observed that the average percentage of live cysts in the first group was equal to 28.20, in the second group was 19.10 and in the third group was 7%. In other words, the average percentage of dead cysts at concentrations of 100, 200, and 400 mg/mL were 71.80%, 80.90%, and 93%, respectively (Table 2). There was no difference between the groups before the intervention and the wrists were full, but there was a significant difference between the groups after the intervention (p<0.0001).

Analysis by *t*-test showed that all groups had significant differences with the worst situation in the negative control group and the best situation in the positive control group. In treatment groups 1, 2, and 3, the rate of parasite inhibition increased with increasing doses of the drug. The closest result to the positive control group was observed in group 3.

All groups except the negative control group showed a significant reduction in live parasite numbers before and after intervention (p<0.0001). Therefore, considering the mean weight of the two positive control treatment groups and the Asafoetida 400 mg treatment group, there was no difference after the intervention and the weight changes were not significant also, in the investigation on live parasites, the lowest number of live parasites compared to the positive control group, in this group (receiving Asafoetida 400 mg) was observed, we can point to the acceptable effect of Asafoetida.

Discussion

So far no research has been conducted on the *in vivo* effect of Asafoetida on different forms of *Giardia* parasite in mice and our research is the first study in this regard. However, Rezaiemanesh et al. (2012) studied the lethal effect of aqueous and alcoholic extract of Asafoetida on *G. lamblia* cyst *in vitro*, and their results showed that the lethal effect of alcoholic extract was greater than that of aqueous extract (15). It has acceptable effects compared to metronidazole, which is the gold standard for the treatment of giardiasis. A therapeutic drug to

control giardiasis is the synthetic metronidazole, and its toxic effects on human cells have been confirmed in various studies, and also in recent years, researchers have been looking for an alternative drug. Ferula Assa-foetida is a plant native to Iran and Afghanistan and is exported from these regions to other countries of the world. Asafoetida has been used in traditional medicine to treat parasitic diseases, bloating, stomach pain, asthma, anorexia, epilepsy, and influenza. New research has shown that this herbal compound has therapeutic properties such as antifungal, cancer prevention, antioxidant, anthelmintic, antispasmodic, and anti-diabetic properties (16).

So far, many studies have been done on the use of medicinal plants in the treatment of various infections in our country and other parts of the world, and researchers have reported many plants for the treatment of giardiasis. Among these, plants *Eugenia uniflora*, *Achyrocline satureioides*, Garlic, apricots, *Psidium guajava*, *Foeniculum vulgare*, etc., had an acceptable effect on *G. lamblia* cysts and trophozoites (17). Sajadi et al. who evaluated the anti-Giardia effect of lemon juice and vinegar, concluded that vinegar extract had the highest anti-Giardia effect after 3 hours compared to the others (18, 19). Also, in various studies, Rahimi et al. examined the anti-Giardia effects of chamomile and reported almost acceptable effects (20). According to the studies, no additional studies have been done yet and the effects of these drugs *in vivo* conditions are in a veil of ambiguity and need additional studies. Recent studies suggest that Asafoetida may be effective in killing infectious agents such as bacteria, fungi, and parasites due to its active ingredients (21). In the study of Kumar et al., it was found that in the ethanolic extract of Asafoetida, compounds such as ferulic acid and amblyfron have a lethal effect on the snail intermediate host of the fasciola (22). The effect of other Asafoetida compounds such as galbanic acid and amblyperin against Leishmania and some pathogenic bacteria and viruses has been determined (23). Also, in various studies, the antiparasitic effects of Asafoetida have been studied and it has been shown that Asafoetida has antiparasitic potential (24). Awad et al. investigated the *in vivo* anti-trypanosomal effects of Asafoetida on rabbits infested with *Trypanosoma evansi* and it was shown that this plant can reduce clinical symptoms in animals (25). It has also been shown that this plant inhibits the increase of liver enzymes in the host relative to the control group. Badr et al. investigated the effect of ether, ethyl acetate, methanolic, and aqueous extracts on *G. lamblia*. In this study, ether, ethyl acetate, and methanolic extracts had a very good effect on it, but aqueous extract had an acceptable effect on *G. lamblia* (26, 27, and 28). According to the results of this study, it is suggested that pharmacological studies be performed to identify the effective compounds of this plant compound and experimental studies on other animal models and volunteers for clinical evaluations. As cysts are transmitted by giardiasis, Asafoetida can be used to prevent transmission; Asafoetida can also potentially prevent the onset of infection and the symptoms due to the infection in humans with the phenomenon of changing cyst-to-trophozoite (excystation) at the beginning of the small intestine. Based on the findings of this study, Asafoetida has been shown to possess good anti-Giardia properties. Moreover, given its history of medicinal use and herbal composition, there is a necessity to investigate the effects of this drug on human cells in future studies and to examine its potential toxicity.

Conclusion

The results of this study showed that Asafoetida has very good effects on *in vivo* conditions and can be considered as one of the treatment options for the treatment of giardiasis. The low side effects of Asafoetida and the lack of drug resistance have made it an important resource for searching for new drug compounds and synthesizing effective drugs.

Ethical approval

In this study, ethical considerations have been fully observed.

Acknowledgments

This article has been written using the results of the Master's degree student thesis that has been approved in the Islamic Azad University of Tabriz Medical Sciences Unit, With ID (https://ethics.research.ac.ir/IR.IAU.TABRIZ.REC.1402.282) in the ethics committee.

Thanks to the Vice Chancellor for Research of Islamic Azad University, Tabriz Medical Sciences Unit in Iran for their support and assistance.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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