

Comparing the Effect of Hydroalcoholic Extract of Rosemary and Metronidazole in Treating Infection Caused by *Giardia lamblia* in Mice under In vivo Conditions

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ABSTRACT

BACKGROUND AND OBJECTIVE: Giardiasis is one of the most common infections of the gastric tract in the world, which is caused by protozoa. Today, metronidazole, furazolidun, tinidazole and quinacrine are commonly used to treat this disease. These medications are associated with several adverse side effects and finding an alternative solution with fewer side effects seems necessary. The present study aims to analyze the effect of hydroalcoholic extract of rosemary (*Rosmarinus officinalis*) on the cysts of *Giardia lamblia* parasite under in vitro conditions.

METHODS: In this experimental study, 25 mice were categorized into 5 groups (n=5) including negative control group, positive control group (treated with metronidazole) and other three treatment groups (treated with 100, 200 and 400 mg/ml rosemary extract). Then, the effect of hydroalcoholic extract of rosemary was analyzed until the tenth day.

FINDINGS: According to the results of this study, the body weight of the mice in the groups treated with hydroalcoholic extract of rosemary (20.78 - 26.64) was less than control group (28.70 ± 0.69), which received no medication ($p < 0.0001$). Investigating the effect of rosemary extract on *Giardia lamblia* parasite demonstrated that decline in the number of cysts in 100, 200 and 400 mg/ml groups were 70.7, 80.6 and 94%, respectively ($p < 0.05$).

CONCLUSION: Results of the present study demonstrated that rosemary has proper in vivo effects and can be considered as a suitable alternative for treatment of Giardiasis.

KEY WORDS: Rosemary, Metronidazole, *Giardia lamblia*.

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Introduction

Giardia lamblia, also known as *Giardia intestinalis*, is one of the most common pathogenic protozoa of digestive system, which infects a wide range of vertebrates, including humans. The infection caused by this protozoan, which is one of the most common parasitic infections, is called Giardiasis (1). This parasite is the cause of steatorrhea or prolonged diarrhea in passengers and is observed in two forms of trophozoite and cyst (2, 3). This parasite is one of the most common intestinal parasites worldwide and 280 million people in the world get infected with this parasite every year (4).

The contamination level of this protozoan is 10–50% in developing countries (5–7). *Giardia* has a global distribution and is more prevalent in regions with tropical and sub-tropical climate; this parasite is one the most common intestinal parasites in Iran, particularly in the northern regions (8, 9). Humans being infected by cyst form through consumption of contaminated water and food and direct fecal-oral contact. Due to resistance of the cyst of this parasite to chlorination of water, its distribution through water in human body is more common; cysts can survive in the environment for 3 months (10, 11). The risk of this disease is present in all age groups, however, its incidence is higher among children (8–10). *Giardia* may cause acute infection or chronic infection as well as clinical symptoms such as acute watery diarrhea, abdominal cramps, malabsorption syndrome and weight loss in infants and children (12, 13).

Nowadays, drugs such as metronidazole, furazolidun, tinidazole and quinacrine are commonly used to treat giardiasis. Although the use of these drugs is beneficial in most cases, it is accompanied by several side effects including unpleasant taste in the mouth, digestive discomfort, nausea, headache, leukopenia, neurotoxicity, restlessness, seizure and dizziness and interferes with the process of treatment. On the other hand, the adverse, carcinogenic and mutagenic effects of some of these drugs have been proved after being used in some animal labs (14–17). Therefore, it is necessary to find a drug with fewer side effects and with positive effects against trophozoite and cyst. Plants are suitable substitutes for chemicals drugs; several studies have been conducted regarding the effects of various plants on *Giardia lamblia* (18, 19). Rahimi Esboei et al. analyzed the effect of three plants (*Sambucus ebulus*, *Artemisia annua* and *Allium paradoxium*) on *Giardia* parasite and found acceptable effects, while *Sambucus*

ebulus was more effective than the other plants (20 – 22). In a similar study, Gholami et al. analyzed the effect of chamomile on *Giardia lamblia* parasite, which had less anti – *Giardia* effect compared with the recent study (23). *Rosmarinus officinalis* is a plant of the *Lamiaceae* family in the form of small shrubs with aromatic leaves and small blue flowers. Breeding this plant is common in most parts of Iran (24). Several studies have been conducted about the role of *Rosmarinus officinalis* extract in the treatment of some diseases such as headache and circulatory disorders (25).

Because of having flavonoid (diosmetin, diosmin, gancoenyl, luteolin, hypsidoline and apigenin), terpenoid (Triterpenoid Oleanolic acid, Ursolic Acids and ditropan carnosol) and phenolic (Caffeic, Chlorogenic, labiatic, Neochlorogenic, and Rosemaric Acid) compounds, the extract of this plant has therapeutic properties including anti-asthma, anti-parasitic, anti-cancer, anti-baldness and other properties (17 – 25). Therefore, considering the confirmed positive effects of several compounds of these plants on various diseases, this study was conducted to analyze the effect of hydroalcoholic extract of *Rosmarinus officinalis* on the cysts of *Giardia lamblia* parasite in mice under in vitro conditions.

Methods

Extraction: *Rosmarinus officinalis* plant was obtained from Mazandaran Research Center of Medicinal Plants and the genus and species of the plants were identified. After washing the plant, the leaf of the plant was dried under suitable conditions. Extraction was done using percolation method in this study. 750 mg powdered plant was prepared. Extraction was done using percolation method and chloroform solvent. Finally, 5.5 g extract was prepared and 100, 200 and 400 µg/ml concentrations of the extract were prepared using dimethyl sulfoxide (DMSO) and the obtained extract was kept at 4 °C (28).

***Giardia lamblia* cyst collection and extraction:** *Giardia lamblia* cysts were collected from infected stool samples of patients with Giardiasis who referred to medical centers of Mazandaran Province. The cysts in stool samples were observed using two methods of microscopy and formalin-ether. The samples were concentrated by 85% w/v sucrose method. For isolating *Giardia* cyst, of stool samples were diluted by distilled water based on 1 to 12 ratio. To prepare watery solution,

20 cc of the diluted sample was poured into the container containing perl and was shaken for 5 minutes. The suspension was filtered and 5 ml water was added to the remaining sediment. 3 ml 85% M sucrose was added to the solution. The obtained suspension was centrifuged for 10 minutes at 600 rpm. The cysts were removed by Pasteur pipettes and washed for 3 times using normal saline. Eosin staining 0.1% was used to measure the percentage of living cysts (29).

Animals and In vitro conditions: 25 BALB/c mice (around 2 weeks old) with an approximate weight of 20 ± 3 g were purchased from Pastor Institute of Amol City. The experiments were performed according to ethical principles based on Ethics committee recommendations for laboratory animals approved by Ethics Committee of Mazandaran University of Medical Sciences (MU-94-2374). The mice with infected with 2×10^4 *Giardia lamblia* cysts by gavage. In order to make sure the mice are infected with the parasite, the stool of mice were evaluated for 11 consecutive days. Formalin – ether method was used to determine whether the stool of mice are infected to the parasite. Then, the animals were divided into 5 groups (n=5); negative control (mice infected to *Giardia lamblia* cyst that did not undergo treatment), drug control (mice infected to *Giardia lamblia* cyst and treated with metronidazole) and 3 treatment groups including Test 1 (the infected mice treated with 100 µg/ml *Rosmarinus officinalis*), Test 2 (the infected mice treated with 200 µg/ml *Rosmarinus officinalis* extract) and Test 3 (the infected mice treated with 400 µg/ml *Rosmarinus officinalis* extract). 1 mg/l *Rosmarinus officinalis* extract with 100, 200 and 400 concentrations per kilogram of body weight were administered to the mice in the treatment groups by gavage for 10 consecutive days. Eosin staining 0.1% was used to measure the percentage of living cysts. The cysts that absorbed the color were dead and the cysts that did not absorb the color were considered as living (30).

Statistical analysis: The data were analyzed using SPSS Ver. 22, ANOVA, Tukey's follow-up test and T-test, while $p < 0.05$ was considered significant.

Results

Investigating the infection of mice with the parasites:

Observations demonstrated that at most 11 days after intestinal inoculation, the cysts appeared in stool of mice and the mice were infected. In some infected mice, symptoms such as isolation, fatigue, disarrangement of body hair and loose stool could be observed.

Analyzing the weight of mice before and after

infection and treatment: Before intervention, the mean weight of mice was not significantly different ($p=0.993$). However, there was a significant difference between the mean weight of mice in different groups ($p < 0.0001$). The weight of mice increased in all groups and this weight increase was significant in negative control group, group 1 and group 2 compared to the beginning of the study. However, no significant difference was observed in mean weight of positive control (0.184) and group 3 ($p = 0.343$), before and after the intervention, despite the slight increase in weight (Table 1).

Table 1. The mean weight of the studied mice in the treatments groups compared with positive and negative control groups

Group	Before the intervention (Mean±SD)	After the intervention (Mean±SD)	P-value**
Negative control	20.28±1.71 ^A	28.70±0.69 ^A	0.001
Drug control (Metronidazole)	20.16±0.65 ^A	20.86±1.61 ^D	0.184
100 mg/ml Rosmarinus officinalis (group 1)	20.12±0.76 ^A	26.64±1.01 ^B	0.000
200 mg/ml Rosmarinus officinalis (group 2)	20.40±0.89 ^A	24.76±1.39 ^C	0.000
400 mg/ml Rosmarinus officinalis (group 3)	20.10±1.34 ^A	20.78±1.05 ^D	0.343
P-value*	0.993	<0.0001	

* Anova ** T-test. Similar letters in each column indicates lack of difference.

After the therapeutic intervention, the highest mean weight belonged to negative control group and the lowest mean weight could be observed in positive control group and the treatment group 3 (the infected mice treated with 400 µg/ml *Rosmarinus officinalis* extract). After the intervention, there was a significant difference between the mean weight of negative control and positive control and other groups ($p < 0.0001$). The mean weight of treatment group 3 was not significantly different from positive control after the invention, however, a significant difference was observed between the 3 treatment groups after the invention. In terms of mean weight, group 3 and drug group had similar function.

Table 2. Analysis of mean viability of the cysts in different treatment groups compared with negative and positive control groups, before and after the treatment

Group	Before treatment (Mean±SD)	After treatment (Mean±SD)
Negative control	100±0.00 ^A	100±0.00 ^A
Drug control	100±0.00 ^A	0.002±0.004 ^E
100 mg/ml <i>Rosmarinus officinalis</i> (group 1)	100±0.00 ^A	29.17±4.71 ^B
200 mg/ml <i>Rosmarinus officinalis</i> (group 2)	100±0.00 ^A	19.40±3.20 ^C
400 mg/ml <i>Rosmarinus officinalis</i> (group 3)	100±0.00 ^A	6.00±2.64 ^D

ANOVA and T-Test were similar ($p < 0.0001$). Similar letters in each column indicates lack of difference.

The level of infection to the parasite was similar in all groups before the intervention and an average of 100% living cysts was reported. After the intervention, the percentage of living cells in negative control group was still one hundred and in positive control group, 100% of parasites were dead. Investigating the treatment groups demonstrated that the mean percentage of living cysts in group 1, group 2 and group 3 were 29.17, 19.40 and 0.6, respectively. In other words, we can say that the mean dead cells at concentrations of 100, 200 and 400 mg/ml were 70.8, 80.6 and 94%, respectively. There was no significant difference between the groups before the intervention and they were completely matched. However, significant differences could be observed between the groups after the intervention ($p < 0.0001$).

The results of the follow-up tests demonstrated that there was a significant difference between all groups; the worst and the best condition was observed in negative and positive control group, respectively. In treatment groups 1, 2 and 3, as the drug concentration increased, inhibition of the parasite increased. As such, group 3 showed the nearest result to the positive control group. All groups, except for negative control group, experienced significant decline in the number of living parasites after the intervention and this decline was significant in the mentioned groups ($p < 0.0001$).

Therefore, considering that the mean weight of positive control group and treatment group with 400 mg *Rosmarinus officinalis* were not different after the intervention and weight changes were not significant and in analysis of the level of living parasites, the lowest

percentage of living parasites compared with positive control, was observed in this group (400 mg *Rosmarinus officinalis*), we may confirm the acceptable effect of *Rosmarinus officinalis* plant.

Discussion

In this study, the chloroform extract of *Rosmarinus officinalis* at various concentrations was compared with metronidazole and acceptable effects were observed compared with this drug, which is a golden standard for the treatment of giardiasis. The medicinal treatment for controlling giardiasis is metronidazole, however, the toxic effects of this drug on human cells have been confirmed by several studies (31) and in recent years, researchers were looking for alternative treatments with fewer side effects.

Several studies have been conducted regarding the use of medicinal plants for treatment of different infections in Iran and other countries so far and researchers have reported numerous plants for the treatment of giardiasis. Among these plants, *Achyrocline satureioides*, *Eugenia uniflora*, *Foeniculum vulgare*, *Psidium guajava*, *Allium sativum*, *Trachyspermum ammi* and some other plants had acceptable effect on *Giardia lamblia* cysts and trophozoites (32–34).

Sajadi et al. assessed the anti – *Giardia* effect of lemon juice and vinegar and concluded that after 3 hours, vinegar extract had most anti – *Giardia* effect compared with other items (35). Moreover, Rahimi Esboei et al. in several studies investigated the anti – *Giardia* effect of *Chamomile*, *Allium ursinum* L, *Sium sisarum* and *Allium ampeloprasum* and reported almost acceptable effects (20 – 22). According to the studies, supplementary researches have not been conducted yet and the in vivo effects of this medicine are not completely recognized and it requires further investigation. Recent studies indicated that because of having effective compounds, *Rosmarinus officinalis* can be useful for treatment of infections such as bacterium, fungus and parasite (26).

Celiktas et al. analyzed the antibacterial effect of *Rosmarinus officinalis* on several bacteria including *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Clostridium pneumoniae*, *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus epidermidis* and demonstrated that *Rosmarinus officinalis* had desirable effects on the tested bacteria (36). Results of a study by Pintore et al. demonstrated that gram positive bacteria

are more sensitive than gram negative bacteria (37). Moreover, the anti-parasitic effects of *Rosmarinus officinalis* have also been analyzed in various studies and it was shown that *Rosmarinus officinalis* plant also has anti-parasitic potential. Awad et al. in Malaysia investigated the anti-trypanosome effects of *Rosmarinus officinalis*.

In this in vivo study, the effect of *Rosmarinus officinalis* on *Trypanosoma evansi* was investigated in rabbits and it was shown that 3.5 kg body weight concentration of *Rosmarinus officinalis* can reduce the clinical symptoms in the animal. Moreover, it was specified that this plant inhibits increased liver enzymes in the host compared with control group (38). In a similar study, Badr et al. investigated the effect of ether, ethyl acetate, methanol and water extract on *Giardia lamblia* parasite. In this study, ether, ethyl acetate and

methanol extracts had very good effects, while the water extract had acceptable effect on *Giardia lamblia* parasite (39). According to the results of the present study, it was found that *Rosmarinus officinalis* plant benefits from suitable anti – *Giardia* effects and considering medical history and herbal compounds, further studies are required to assess the effect of this medicine on human cells and the toxic effects of this medicine.

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