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Anti-Giardial Activity of Chloroformic Extract of *Tanacetum parthenium* and *Artemisia annua in vitro*

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Abstract

Background: Giardiasis is a one of the most prevalent intestinal parasitic diseases in human, treatment of this disease through medicinal plants is very important since parasite resistance to chemical drugs exists. Thus, in this study, the *in vitro* anti-giardial activity of chloroformic extract of *Tanacetum parthenium* and *Artemisia annua* on cyst and trophozoite of *Giardia lamblia* were separately investigated.

Materials and Methods: In this experimental study chloroformic extracts of *Artemisia annua* and *Tanacetum parthenium* on cyst and trophozoite of *G. lamblia in vitro* were prepared in 1, 10, 50 and 100 mg⁄ml concentrations for 5, 10, 30, 60 and 180 min. Purified cysts were used for encystations and culture in TYI-S-33 medium. Then, 2 ml of each solution was placed in test tubes, to which 10,000 washed cysts and trophozoites were added. The contents of the tubes were gently mixed and incubated. The percentages of dead parasites were determined by counting 500 cysts. Non treated parasites were considered a control group in each experiment and the viability of the parasites checked with Eeosin staining and statistical analysis were done.

Results: The results showed that chloroformic extracts of *A. annua* at 100 mg/ml concentration affected on Gardia cyst 86% and 100% trophozoite after 1 hour. *T. parthenium* at 50mg/ml concentration killed cysts (83%) and trophozite (100%) after 1 hour, respectively. *T. parthenium* chloroformic extract had a better effect on cyst and trophozoite of Giardia at 50 mg/ml after 1 hour exposure than *A. annua* extract.

Conclusion: According to this study, A. *annua* and *T. parthenium* chlorofomic extracts could be considered as a more effective anti-giardial agent. Chloroformic extract of *T. parthenium* was also shown the anti-giardial activity compared with *A. annua* and control groups at all exposure times. Therefore, in the future research using these plants are recommended against Giardia in low concentration in the *in vivo*, also to find fractions of the pharmacological effects of these plants.
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Keywords: *Giardia lambelia*; Chlorofomic extract; *Artemisia annua*; *Tanacetum parthenium*; Cyst; Trophozoite

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Introduction

Giardiasis is a one of the most prevalent intestinal parasitic infection in human, caused by the flagellate protozoan *Giardia lambalia*, with worldwide distribution and high rates in Mediterranean countries as well as in north of Iran (1-3). The emergence and spread of Giardiasis in human population is anincreasing problem around the world, especially in developing countries. Giardiasis has been also included as "Neglected Disease" by WHO, but it is not reported by many countries of the world. Based

on research and increasing knowledge of the Giardia genome will improve our understanding of contamination source, transmission routes and features contributing to human illness (4-7).

Cyst and trophozoite are two distinct forms of the parasite; cysts are dormant forms responsible for the transmission of Giardiasis. They are excreted from an infected host with the feces and are exceptionally hardy and capable of tolerating extremes of pH and temperature. Transmission to humans usually occurs through the ingestion of cysts in contaminated water or food or via direct fecal-oral contact. The trophozoites are the vegetative form of Giardia (1-2, 5).Prevalence rates range from 2-7% in developed countries and 20-30% in most developing countries. Prevalence of *G. lamblia* in Iran almost is 10.9%. The CDC estimates that there are upwards of 2.5 million cases annually (5). There is no abundant evidence for the pathogenic potential of *Giardia* but they are able to colonize and rapidly replicate in the gastrointestinal tract as well as cause gastrointestinal symptoms. Children are more frequently affected than adults, although all ages are may display symptoms ranging from mild diarrhea, Flatulence, anorexia, crampy abdominal pains, and epigastric tenderness to steaorrhea and full blow malabsorption syndrome and extra intestinal symptoms, such as fever, maculopapular rash, pulmonary infiltrates, lympha denopathy, poly-arthritis and urticarial (2-3).

For the treatment of Giardiasis Metronidazole as the first choice and other drugs; Tinidazole, Albandazole, Furazolidone are also used (8- 11). Undesirable side effects and failures in treatment and drug resistance are frequently reported (5-9). For example side effects of Metronidazole include nausea, loss of appetite, vomiting, diarrhea, lethargy, weakness, anemia, blood in the urine, head tilt, seizures, disorientation, and stumbling. It may also cause liver disease, which can result in yellowing of the gums, skin, and eyes. Damage to nerves is also possible, and the mutagenic effects of this drug in some bacteria and animal models were confirmed (8, 12- 14).

Therefore, in the recent studies has been paid to extracts derivates from plants used in traditional medicine, because in the current decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs (15-17). Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in developed and in developing countries. In the similar studies has been suggested to identify the compounds responsible for the anti-giardial activities in the chloroform leaf extracts of P. betle and P. chaba (17-19).

According to current research and so, isolated bioactive compounds from plants were utilized against a wide range of microorganisms particularly parasites. Among these plants, *Allium* and *Artemisia spp.* has an undeniable portion and a plenty of researches were carried out in order to study on impact of this massively valuable plant. *Artemisia annua*, also known as annual wormwood or Sweet Annie, belongs to the family Asteraceae. Its activity against malaria parasites in primate models was demonstrated (20-21), but Artemisinin, by Chinese scientists were isolated and characterized of the active anti-malarial principle in 1972 (20, 22). *T. parthenium* is a perennial herbaceous essential oil bearing plant belongs to Asteraceae family. The species of genus *Tanacetum* have been used as medicinal plants for over 2000 years (23).

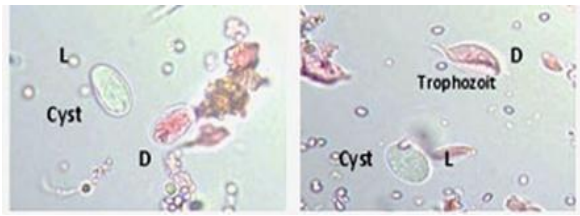

Figure 1. Cyst and Trophozoite of *G. lamblia*, died (D) and viable (L) cyst excluded the eosin stain

However, a combination treatment for Giardia, as part of the control approach to protozoan infections should be investigated further (15, 21, 24). Due to high prevalence of Giardiasis, plan diversity and the geographic situation of the Mazandarn province, north of Iran, the use of medicinal plants in the treatment of parasitic infections especially intestinal parasitic is are necessary. Thus, in this study, the *in vitro* anti-giardial activity of chloroformic extract of *T. parthenium* and *A. annua* on cyst and trophozoite of *G. lamblia* were investigated separately.

Materials and Methods

Purification of G. lamblia cysts

G. lamblia cysts were collected from faces samples of patients from different hospitals of Mazandaran University of Medialcal Sciences. All specimens were processed immediately after arrival, ordinarily within 48 h after excretion. By combining the sucrose flotation method a highly purified cyst suspension was achieved by a simplified sucrose gradient method. Stools were broken up in tap water and filtered through a 300 urn filter. 3 ml of the fecal suspension were layered on 3 ml of 0.85M sucrose and centrifuged

at 600 rpm for 10 min at 4 °C. The cysts at the sucrose-water interface were aspirated with a Pasteur pipette and washed 3 times with water. Washed cysts were carefully added to the top of a discontinuous density gradient, consisting of two 3-ml laves of O-85 and 0.4M sucrose. After centrifugation at 600 rpm for 10min at 4 °C cysts concentrated at the 0.85-0.4M sucrose interface were collected and washed again. Purified cysts were resuspended in distilled water and stored at 4 °C for a maximum of 3 ysprior to use (11, 29).

Excystation of G. lamblia cysts and Culture methods

At this stage Bingham and Meyer (1979) method were used for excystation procedure (25). One volume of purified cyst suspension was added to 9 volumes of induction solution, aqueous hydrochloric acid (pH 2), and the preparation was incubated at 37 °C for one hour. After centrifugation at 600 rpm for 10 min at room temperature, the supernatant was removed and the pellet was inoculated into excystation medium. Initial experiments demonstrated that survival of most of the primary cultures could be favorably affected by the addition of bile and by increasing the serum component to 20% (26). Therefore the excystation medium used consisted of filter-sterilized TYI-S-33 culture medium, with bovine bile added (11, 27), supplemented with 20% heat inactivated fetal calf serum. Screw capped borosilicate glass culture tubes containing 8 ml of the medium, supplemented with penicillin (500 iu/ml) and streptomycin (500 ug/ml), and were incubated at 37 °C in a slant.

Table 1. Effect of chloroformic extract of *Artemisia annua* on *G. lamblia* cyst and trophozoite at different times.

	1mg/ml (%)		10mg/ml (%)		50mg/ml (%)		100mg/ml (%)		Control (%)	
	cyst	Trophozoite	cyst	Trophozoite	cyst	Trophozoite	cyst	Trophozoite	cyst	Trophozoite
1 min	67	65	69	67	71	67	73	72	3	9
5 min	69	78	75	79	77	84	79	86	3	9
30 min	75	83	79	87	82	88	78	92	5	9
1 hour	77	89	79	92	84	96	86	100	7	11

Preparation of plants chloroformic extract

The plants were dried under shade, and powdered mechanically using a commercial electrical blender. To obtain the chloroformic extract, 70g of dry powder was added to 350 ml of pure chloroform and mixed gradually for 1 h using a magnetic stirrer. The obtained solution was left at room temperature for 24 h. The solution was stirred again and filtered. Then, the solvent was removed by evaporation in a rotating evaporator at 40 °C. The remaining semisolid material was freeze-dried and placed into a sterile glass container and stored at 4 °C for further use (11, 28).

Effectiveness of plants extracts on cysts and trophozoites of G. lamblia

Four concentrations (1, 10, 50, and 100 mg ml^{-1}) of the plants choloroformic extract were applied for 5, 10, 30 and 60 min. To make the extracts solution at 1, 10, 50 and 100 mg ml-1 concentrations, 0.01, 0.1, 0.5, and 1 g of each dried extract were dissolved in 10 ml of normal saline, respectively. Then, 2 ml of each solution was placed in test tubes, to which 10,000 washed cysts and trophozoites was added. The contents of the tubes were gently mixed and incubated at 37 °C for 5, 10, 30, and 60 min. At the end of each incubation time the upper phase was carefully removed so as not to interrupt the parasites. Two milliliters of 0.1% eosin stain added to the remaining settled cysts and trophozoites and mixed gently. The upper portion of the solution was discarded after 15 min of incubation and the remaining pellet smeared on a glass slide, covered with a cover glass and examined under a light microscope. The percentages of dead parasites were determined by counting 500 cysts. Non treated parasites were considered a control group in each experiment, which was performed in triplicate. The third group was given metronidazole (0.2 mg/ml) as above and the fourth group was given normal saline alone (Control group). (DMSO)

Viability tests

Eeosin stain with the concentration of 0.1% (1 g of eosin powder in 1000 ml distilled water) was used to check the viability of the parasites. Fifteen minutes after exposure to the stain the cysts and trophozoites with no absorbed dye were considered potentially viable, otherwise, they were (Figure 1) (11).

Statistical analysis

Statistical analysis was performed by means of one-way ANOVA (analysis of variance) considering a

level of significance of 95% (P<0.05), with SPSS software.

Results

The results of different concentrations of chloroformic extract of *A. annua* and *T. parthenium* against *G. lamblia* cyst and trophozoite in different times are summarized in Tables 1 and 2. Chloroformic extracts of *A. annua* at 10mg/ml and 100 mg/ml concentration had affected on Giardia cyst 96%, 99% and trophozoit (100%) after 3 hour, whereas the chloroformic extract of *T. parthenium* at 1mg/ml and 10mg/ml concentration killed 97%, 99% on cyst and at 1mg/ml 100% on trophozite after 3 hour, 50 mg/ml effected 100% trophozite after one hour, respectively. Therefore, *T. parthenium* chloroformic extract had a better effect on cyst and trophozoite of Giardia at 10 mg/ml after 3 hour exposure than *A. annua* extract. Choloroformic extract of *A. annua* could maximum effect 100% on trophozoite of Giardia in 1 mg/ml concentration after 3 hour and 100 mg/ml concentration after one hour.

Table 2. Effect of chloroformic extract of *T. parthenium* on *G. lamblia* cyst and trophozoite at different times.

	1 mg/ml (%)		10 mg/ml (%)		50 mg/ml (%)		100 mg/ml (%)		Control	
	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite	Cyst	Trophozoite
1 min	46	40	48	49	56	51	58	53	3	8
5 min	69	53	71	56	73	58	75	58	3	9
30 min	74	70	76	73	78	75	80	78	3	9
1 hour	79	98	81	99	83	100	87	100	4	9

The results of present study indicated that chlorofomic extracts of *A. annua* showed a high activity effect on trophozoite in 100mg/ml at one hour and 99% on cysts in 100mg/ml after 3 hour exposure. *T. parthenium* revealed a high activity on trophozoite of Giardia at 50mg/ml after 1 hour (100%) and on cyst after 3 hour (Tables 2). No statistically significant differences (P> 0.05) were observed between chloroformic extract of the *A. annua* and *T.parthenium* at concentration of 1mg/ml, 10mg/ml and 50mg/ml on cyst after 3 hour and trophozoite of Giardia, but at concentrations of 50 and 100 after one hour (P< 0.05). As a result, chloroformic extract of *T. parthenium* in 10 mg/ml and *A. annua* in 100 mg/ml concentrations on the cyst and trophozoite of Giardia had a greater effected at 3 hour. *T. parthenium* in 50 mg/ml after one hour on trophozoite of Giardia was affected, whereas, similar effected was found using *A. annua* on 100 mg/ml after one hour.

Discussion

G. lamblia is common intestinal protozoan parasites with severe symptoms of diarrhea and sickness can be infected infants, elderly and immuno-compromised individuals, (9, 20). The necessity to investigate new, safe, and effective agents for the treatment of Giardiosis is imperative due to the side effects and resistance of the reference drugs (9, 20, 30). Plants used for the treatment of gastrointestinal disorders, such as diarrhea and dysentery, raise the possibility of new alternative therapies (20). The present study evaluated the anti-giardial activity of chlorofomic aqueous extracts of 2 plants (A. *annua and T. parthenium*) used leaves and stems as edible and medicinal plants in Northern Iran. The results of our study indicated that chlorofomic extracts of *A. annua* and *T. parthenium* showed a high activity effect on trophozoite and cyst in 50-100mg/ml between 1-3 hour exposures. In this study we analyzed the anti-giardial activity of this plant and *T. parthenium* as new medical herbs, whereas, *A. annua* is well known for anti-Malarial and anti-Hemorrhoid activity (21, 23).

Many studies have investigated the *in vitro* and *in vivo* effect of various plants on *G. lamblia* cysts and trophozoites. (15-16, 29-30) showed the effects of different plants, fractions and/or purified compounds against *G. lamblia* cyst and trophozoite and comparing it with the present data (15-17). Rahimi-esboei et al. 2012 reported 97% and 100% anti-giardial activity for 100 mg/ml of *A. annua* extract on cyst and trophozoite *in vitro* (29).

Sadjjadi et al 2006 was shown that giardiacidal activity at 4 ˚C after 3 hours for lemon juice, Vinifer and vinegar were 18.9%, 12.8%, and 28.4%, at 24 ˚C, 28.3%, 16.2%, and 40.6%, respectively (15). Calzada et al. 2006 also reported the anti-protozoal activity of 27 crude methanolic extracts derived from 26 plants (24 genera in 20 families) used in Mexican traditional medicine for treatment of diarrhea and dysentery (30). They found that *Dorstenia contrajerva*, *Senna villosa* and *Ruta chalepensis* were the most active against *G. lamblia* with IC50 <38 g/ml. In other

researches *Geranium mexicanum*, *Cuphea pinetorum*, *Helianthemum glomeratum*,and *Rubus coriifolius* have been found to be effective on cyst and trophozoite of *G. lamblia in vitro* (16, 30).

Results of the present study demonstrate the *A. annua* and *T. parthenium* chlorofomic extracts could be considered as more effective than the majority of other plant extracts and their active principles. chloroformic extract of *T. parthenium* has also shown the anti-giardial effect to compare with *A.annua* and control groups at all exposure times. Obviously after 3 hour *A.annua* had a similar effect to *T. parthenium* in 1 mg/ml and 10 mg/ml concentration on trophozoite of Giardia. *T. parthenium* after one hour had 100% anti-giardial activity against cyst and against trophozoites in 10 mg/ml concentration. But as whole the effectiveness of *T. parthenium* was so more acceptable than *A. annua*. Therefore, in future research, using these plants is recommended against *in vivo* growth of Giardia with concentrations of 200 mg/ml and 400 mg/ml, also research to find fractions of the pharmacological effects of these plants.

Although, a rapid and complete giardiacidal effect with no local or systemic side effects and also low cost are some properties of an ideal anti-giardiacidal solution. From this point of view, no ideal giardiacidal agents have been described yet. The present study provides evidence of an inhibitory activity, ranging from moderately inhibited to killing by the choloroformic extract from *A. annua* and *T. parthenium* against *G. lamblia*. It is probably worthwhile to purify these active extracts to identify specific compounds responsible for the activities. This result enhances the ethno botanical uses of the plant as anti-diarrheal in cases associated with Giardiasis. To find an effective chemical composition, further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* study, drug formulation and clinical trials are recommended.

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