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Original Article

The in Vitro Effects of *Echinophora Cinerea* on Cell Line, *Giardia Lamblia* Cyst, and *Giardia Muris*

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Abstract

Background and Aim: Currently, *Giardia lamblia* is the most common parasitic protozoan in many countries, including Iran, and the most important cause of diarrhea among travelers, children, and homosexuals. Chemical drugs could be used to treat giardiasis. These drugs can have carcinogenic effects and can lead to drug resistance, which is being reported from different regions across the world, including Iran.

Materials and Methods: The present study was conducted to phytochemically analyze *Echinophora cinerea*, and to compare the in vitro effects of *E. cinerea* extract and metronidazole on *G. lamblia* and *Giardia muris*. In addition, this research investigates the in vitro effect of ethanolic *E. cinerea* extract on *G. lamblia* and *G. muris* cysts as well as the cytotoxicity of this extract on cell line from the SFIF-PI-44 (intestine of sheep) and IEC-18 (ileum of rat).

Results: α-phellandrene was found to be the most significant and abundant compound of *E. cinerea*. According to the findings, 4 and 8 mg/mL of *E. cinerea* caused the gradual destruction of *G. muris* cysts (6.83 and 7.89 cysts/h, respectively). Moreover, 4 and 8 mg/mL of the same extract caused the gradual destruction of *G. lamblia* cysts (7.92 and 7.89 cysts/h, respectively). Furthermore, the mean rate of cysts destruction was found to be 8.83 cysts/h by 8 mg/mL of *E. cinerea* extract.

Conclusion: Regarding the optimal effects of *E. cinerea*, further research seems necessary on the in vitro antigiardial effects of this plant.

Keywords: *Echinophora cinerea*, *Giardia lamblia* cyst, *Giardia muris*, In vitro

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Introduction

Parasitic diseases are considered one of the main health issues which impose heavy costs on communities. These infections are among the most widespread human diseases in the world. Currently, *Giardia lamblia*, a flagellated protozoan, is the most prevalent parasitic protozoan in many countries,

including Iran. *G. lamblia* is the cause of giardiasis, a human intestinal parasitic infection which is present in all countries (1). Giardiasis is transmitted from one person to another through contaminated food and water, and is the most important reason for diarrhea in travelers, children, and homosexuals (2). According to epidemiological data, the prevalence of this infection is 1%-20% worldwide. Giardiasis has been reported from different regions of Iran (3, 4).

The *Giardia* cyst transforms into trophozoite in small intestine, duodenum, or culture medium. This protozoan absorbs nutrients from the materials in the intestine and epithelial cells through sucking plates, and causes gastrointestinal diseases, particularly diarrhea and malabsorption. The symptoms of giardiasis usually persist for three weeks after the ingestion of the cysts (5). Metronidazole, tinidazole, ornidazole, shinidazole and nimorazole, mebendazole and albendazole, and furazolidone are some of the drugs used to treat giardiasis. The carcinogenic effects of these drugs have been confirmed in animal models (6).

Moreover, the resistance of giardia to chemical drugs has been reported from different regions of Iran and the world (7, 8). Many studies worldwide have been conducted on the use of medicinal plants for the treatment of giardiasis (9-11). There are debates about the side effects and drug resistance induced by the use of chemical drugs. Giardiasis can be transmitted through cyst and become prevalent, and *Giardia* trophozoite in patients is a significant issue. Hence, various approaches have been recommended to destroy the cysts and trophozoites. According to the World Health Organization, the use of medicinal plants is one of these approaches. In various studies, the biological effects of many species of medicinal plants have been examined.

Echinophora cinerea (*Khosharizeh* in Persian, locally called *Fialeh*) is a plant from family Apiaceae which reaches 30-100 cm in height. Four perennial herbaceous aromatic plants from genus Echinophora occur in Iran, of which *E. cinerea* and *Echinophora platyloba* occur exclusively in Iran and *Echinophora sibthorpiana* and *Echinophora orientalis* occur also in Anatolia, Armenia, Russia, Turkmenistan, Afghanistan, Balkan Peninsula, Krete, Cyprus, and Syria (12). *E. cinerea* is found abundantly in the highlands with an altitude of over 1500 m in Lorestan province, western Iran, especially Oshtorankouh, Kouh-e kala, Garinkouh, and Sefidkouh. Aerial parts of *E. cinerea* can be used (13, 14).

E. cinerea may be used as a food spice (15). This plant is a stomach tonic and has antibacterial, diuretic, and anti-cancer properties (16). Several studies have reported *E. cinerea* antifungal effects on certain fungi such as *Trichophyton rubrum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Microsporum canis*, and *Candida albicans* (17-19). Moreover, *E. platyloba* has been reported to be effective in relieving the pain caused by dysmenorrhea (20).

E. cinerea has also been reported to exert antinocardial effects on human pathogenic nocardia such as *Nocardia asteroides* and *Nocardia brasiliensis* (21). P-cymene, α-phellandrene, β-cymene, carvacrol, and some alkaloids are some of the chemical compounds extracted from *E. cinerea* by hydrodistillation and hydrodistillation-headspace microextraction (20). Furthermore, this plant contains saponins, flavonoids, and alkaloids (22). The important terpene and saponin of *E. cinerea* are trans-β-ocymene and CAY-1, respectively. CAY-1 has a terpenoid base and can exert bactericidal effects on the germinating conidia of *Aspergillus flavus*. *E. cinerea* can exert such effects in less than toxic doses for human cells (23). CAY-1, a saponin, seems to be responsible for bactericidal effects by exerting destructive effects on fungal well membrane continuity (24).

E. cinerea has different chemical compounds and therapeutic effects on various diseases. The aim of this study was to phytochemically analyze *E. cinerea* and to compare the in vitro effects of *E. cinerea* and metronidazole on *Giardia lamblia* and *Giardia muris*.

Materials and Methods

Investigation of in vitro Antiparasitic effects

To investigate the in vitro antiparasitic effects of *E. cinerea*, 4 and 8 mg/mL of *E. cinerea* extract were separately added to 1 mL suspension containing *G. lamblia* cysts (2000 cysts/mL) in a test tube (treatment) and the tube was kept at 37°C. Subsequently, the percentage of living cysts in the treatment tube was calculated every 60 min using eosin 0.1% until all the cysts were destroyed. To

eliminate the effects of factors effective on the cysts life (including time), we simultaneously treated some cysts with distilled water, rather than *E. cinerea* extract, in another tube (control tube), and the related data were recorded as the control data. To obtain more detailed findings on the *E. cinerea* effects on *cysts*, three similar tubes (containing *E. cinerea* extract+cysts) were simultaneously studied and the mean values were used for analysis.

Investigation of cytotoxic effects

The cytotoxic effects of *E. cinerea* on the cells of intestine of sheep (SFIF-PI 44) and ileum of rat (IEC-18) (Pasteur Institute of Iran) were investigated. In RPMI-1640, different dilutions of the cell lines were prepared with fetal bovine serum 2.5%. Lactate dehydrogenase diagnostic kit (Roche Applied Sciences, Germany) was used to assess the cytotoxicity of different dilutions on the cell lines. The procedures were conducted in triplicate and the mean values of three experiments were taken.

Results and Discussion

The Effect of *E. cinerea* on *G. muris* Cysts

According to the findings, treatment with 4 mg/mL of *E. cinerea* extract caused the gradual (6.83 cysts/h) destruction of the cysts, such that all the cysts inactivated after 24-hour treatment with *E. cinerea* extract, but in the control group, the corresponding mortality was 5% (mean; 0.49 cyst/h) with a statistically significant difference (t-test, $p<0.01$). Treatment with 8 mg/mL of *E. cinerea* extract caused the gradual (7.89 cysts/h) destruction of the cysts, such that all the cysts inactivated after 12-hour treatment with *E. cinerea* (Figure 1), but in the control group, the corresponding mortality was obtained 10% (mean; 0.78 cyst/h) with a statistically significant difference (t-test, $p<0.01$).

The Effect of *E. cinerea* extract on *G. lamblia* cyst

Treatment with 4 mg/mL of *E. cinerea* extract caused the gradual (7.92 cysts/h) destruction of the cysts, such that all the cysts inactivated after 12-hour treatment with *E. cinerea* extract, but in the control group, the corresponding mortality was 10% (mean; 0.75 cyst/h) with a statistically significant difference (t-test, $p<0.01$).

Treatment with 8 mg/mL of *E. cinerea* extract caused the gradual (8.83 cysts/h) destruction of the cysts, such that all the cysts inactivated after 6-hour treatment with *E. cinerea* extract (Figure 2), but in the control group, the corresponding mortality was derived 4% (mean; 0.53 cyst/h) with a statistically significant difference (t-test, $p<0.05$).

The results of cytotoxicity

After preparing 6, 7, 8, 9, and 10 mg/mL of *E. cinerea* extract, their cytotoxic effects on the SFI7-PI44 and IEC-18 cell lines was investigated. As shown in Table 1, *E. cinerea* extract exerted the lowest cytotoxic effect on the IEC-18 cell line, such that 80 and 100 mg/mL of this extract caused minimal changes in the cells under study, with no statistically significant difference ($p>0.05$).

According to the findings, *E. cinerea* extract exerted the smallest cytotoxic effect on the cells under study, hence it could be used in medicine and pharmaceutical industries to treat giardiasis with minimal cytotoxic and side effects.

A comparison between the effects of the extracts and metronidazole

Table 1 shows the comparison of the effects of the extracts with those of metronidazole. In a study on *Echinophora stibthorpiana*, another plant from this family, methyl eugenol was reported to be the most abundant compound (26). Moreover, α-phellandrene was found to be the most abundant compound in the analysis of *Echinophora chrysantha* (27). In the light of the sufficient amounts of terpenes in *E. cinerea*, this plant could have many pharmaceutical and nutritional uses. In the *E. cinerea* essential oil extracted by hydrodistillation, P-cymene concentration was found to be 34.43%, α-phelandrene 21.88%, and α-pinene 3.31% and by headspace solvent microextraction, α-phellandrene concentration was 40.64%, Z-β-ocymene 17.28%, p-cymene 12.84%, α-pinene 5.18% (29).

Moreover, the volatile compounds of *E. cinerea* aerial parts have been investigated in Iran using GC and GC-MS. Twenty-seven compounds were identified, of which α-phellandrene (40.6%), α-pinnene (16.5%), β-phellandrene (9.8%), p-cymene (7.5%), linalool (5.4%), and citronellol (4.8%) were the most important ones (Sajjadi *et al*, 2012). In a study, 19 compounds were found in *E. cinerea* flowering shoots, gathered in Fars province. α-phellandrene (61.4%), β-phellandrene (10.7%), α-pinene (9.6%), and p-cymene (6.1%) were found to be the most important compounds of the

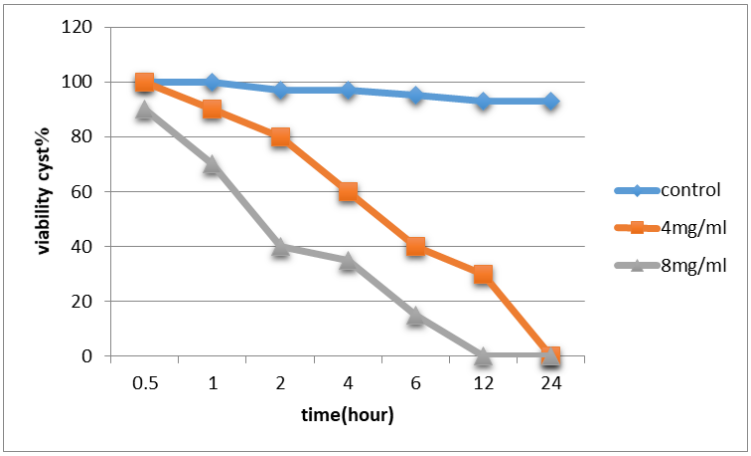

Figure 1. Mean changes in the proportion of survived *Giardia muris* that survived in the presence of *Echinophora cinerea* extract at different concentrations over time.

essential oil (30).

To the best of our knowledge, this study is the first research conducted on the effect of *E. cinerea* extract on *G. lamblia*. Therefore, there are no similar studies to make a comparison of the findings. However, an effort has been made to interpret the findings on metronidazole with reference to the works investigating the therapeutic effects of this drug. A study compared the effects of different concentrations of *Thymus vulgaris* essential oil, boiled extract, and sacculated extract (1:2, 1:4, 1:10, 1:50, and 1:100 total concentrations) with those of metronidazole at similar doses on *Giardia* cysts.

The findings demonstrated that the mean bactericidal effect of total concentrations of *T. vulgaris* essential oil was 91.1% (range; 77.8%-97.8%), which was higher compared to the mean bactericidal effects of metronidazole, 89.4% (range; 83.3%-95.6%), *T. vulgaris* boiled extract, 7% (range; 0%-20%), and *T. vulgaris* sacculated extract, 13.8% (range; 8%-22%) (31).

A study investigated the effects of the extracts of six plants, namely *Punica granatum*, *Artemisia campestris*, *Eucalyptus camaldulensis*, *Cuminum cyminum*, *Mangifera indica*, *Achillea santolina* at 31.25, 62.5, 125, 500, 1000, and 2000 mg/mL concentrations with reference to metronidazole (7.87, 15.75, 31.25, 62.5, 125, 250, and 500 mg/mL) on *G. lamblia* trophozoites in a culture medium. *C. cyminum* and *M. indica* were found to exert no significant effects on the destruction of Giardia cysts, such that 2000 mg mL of these two extracts caused 25% and 75% decrease in the number of cysts, respectively. However, the study did not discuss the findings on the

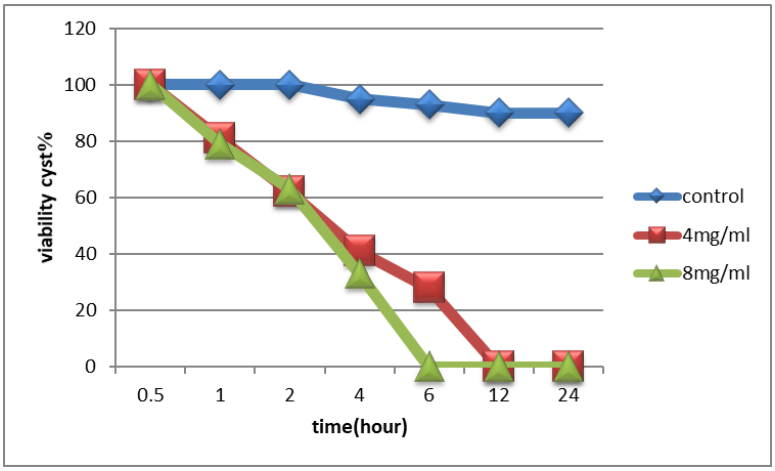

Figure 2. Mean changes in the proportion of *Giardia lamblia* cysts that survived in the presence of *Echinophora cinerea* extract at different concentrations over time.

Table 1: Central composite design with experimental conditions and extraction yield data.

Concentration(mg/ml)	Cytotoxicity							
	SFI7-PI-44				IEC-18			
	NC	MI	MO	MA	NC	MI	MO	MA
20	+	–	–	–	+	–	–	–
40	+	–	–	–	+	–	–	–
80	–	+	–	–	–	+	–	–
100	–	+	–	–	–	+	–	–

NC=Non-cytotoxic, MI=Mildly, MO=Moderately, MA=Markedly

effects of different concentrations of metronidazole on this parasite (32).

In a study on the in vitro effects of methanolic extracts of *Heracleum persicum*, *Satureja hortensis*, *Eucalyptus camadulensis* Dehnh on *G. lamblia* cysts, following the isolation of the cysts from stool and counting them, the researchers treated the cysts with 10, 100, and 200 mg/mL of the extracts under study for 30 and 60 min, and examined the destroyed and survived cysts using a microscope. The bactericidal effects of the extracts were found to be higher in 60-min treatment than 30-min. Sixty-min treatment with 200 mg/mL of *S. hortensis*, *E. camaldulensis, and H. persicum* extracts had 84.3%, 63.3%, and 44% bactericidal effects, respectively. Sixty-min treatment with 200 mg/mL of *S. hortensis* exerted the highest bactericidal effect on *Giardia* cyst, and 30-min treatment with 10 mg/mL of *H. persicum* extract exerted the lowest (27%) bactericidal effect (33).

A research that compared the effects of 0.05, 0.1, and 0.3 mg/mL of gold nanoparticles and those of similar concentrations of metronidazole on *Giardia* cyst, indicated that metronidazole could destroy 73%, 78%, 82%, 90%, and 99% of the cysts at 5, 15, 30, 60, and 180 min respectively (34). Fallahi *et al*. compared the in vitro effects of hydroalcoholic extracts of *Olea europaea* leaves, *Satureja khuzestanica*, and *Allium sativum* and metronidazole, a drug of choice for giardiasis, on *G. lamblia* cyst (35). They found that *O. europaea* leaves and *A. sativum* extract had the highest cytotoxic effects followed by *S. khuzestanica* extract and *A. sativum* extract.

Shahabi *et al*. investigated the effect of *Trachyspermum ammi* extract and essential oil on *Giardia* cyst, and demonstrated the MIC of *T. ammi* hydroalcoholic extract and essential oil to be 100 and 8 mg/mL respectively (36). Sadjjadi *et al.* found that that the *Vitis vinifera* vinegar could exert bacterial effects on *Giardia* cyst (37). Harandi *et al.* demonstrated in their study that chloroform *A. sativum* extract is effective on *Giardia* cyst (9). Hence, the use of certain herbal drugs that serve as alternatives to the drugs capable of causing side effects is of great significance.

Conclusion

The findings of the present study indicated that *E. cinerea* extract could exert remarkable antigiardial effects on *G. lamblia* cysts. Moreover, the findings demonstrated the sensitivity of *G. lamblia* cysts in the presence of *E. cinerea* extracts compared to *G. muris* cysts. Therefore, the long-term use of *E. cinerea* as a food or as a spice could lead to the provision of an environment that could prevent trophozoite survival, *G. lamblia* cyst formation in the intestine, and consequently transmission among people.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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