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In vitro efficacy of *Ziziphus vulgaris* L. and *Camellia sinensis* L. extracts against *Giardia duodenalis* cysts

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Abstract

There is an increasing interest in exploring plant extracts as potential new treatment options for these ailments. *Ziziphus vulgaris* L. and *Camellia sinensis* (L.) Kuntze are medicinal plants widely used in different traditional medical practices, especially in Iranian traditional medicine, for addressing a range of health issues. Although their biological properties are well-established, their effectiveness against *Giardia duodenalis* cysts is still uncertain. This research aimed to assess the impact of *Z. vulgaris* and *C. sinensis* extracts on *G. duodenalis* cysts *in vitro* and to compare their effectiveness with metronidazole. Cysts were extracted from stool samples and concentrated using a 0.85 M sucrose solution. Extracts of *Z. vulgaris* and *C. sinensis* were prepared at concentrations of 25.00, 50.00, and 100 mg mL^{-1}. The impacts of these extracts at different concentrations were evaluated at 10, 15, 30, 60, and 180 min, and the results were compared to control groups. The collected data were documented and statistically analyzed. The findings revealed that *Z. vulgaris* extract at a concentration of 100 mg mL^{-1} and *C. sinensis* extract at a concentration of 50.00 mg mL^{-1} showed similar effectiveness to metronidazole in eliminating *Giardia* cysts. In addition, the cytotoxic effects of *Z. vulgaris* and *C. sinensis* extracts, in comparison with metronidazole, indicated a rise in fatality rates with prolonged exposure times and higher extract concentrations. Therefore, it could be concluded that extracts of *Z. vulgaris* and *C. sinensis* were as effective as metronidazole for killing Giardia cysts *in vitro*.

Introduction

Giardia duodenalis (also referred to as *Giardia intestinalis*) is an important protozoan parasite that affects a wide range of vertebrates, including humans and pets.[1] Humans, particularly children, commonly become infected through fecal-oral transmission, occurring when individuals consume infectious cysts present in contaminated water and food. The incubation period for the infection can range from 9 to 15 days after ingesting these cysts.[2] Symptoms may vary from none at all to severe acute watery diarrhea, nausea, abdominal pain and weight loss.[3]

Metronidazole, widely recognized as flagyl, is the most commonly prescribed medication for giardiasis. It is a nitroimidazole derivative effective against anaerobic bacteria and protozoa. Other medications like furazolidone and quinacrine are also utilized for this condition.[4] While metronidazole has been successfully employed to treat infections caused by protozoa, bacteria and fungi for an extended period, recent studies have raised alarms regarding its potential carcinogenic and teratogenic effects observed in lab rats. Furthermore, there are signs of developing drug resistance in certain giardia strains. Individuals taking metronidazole have reported various side effects, such as headaches, nausea, dizziness, peripheral neuropathy and brief neutropenia.[4,5] Considering the side effects linked to chemical treatments, researchers are increasingly looking into natural and non-chemical alternatives for parasite elimination.[6]

Green tea, obtained from the dried leaves of *Camellia sinensis*, contains several biologically active compounds such as polyphenols, methylphenols, essential oils, proteins, vitamins and amino acids.[7] Previous studies have mainly concentrated on isolating polyphenols from *C. sinensis*[2,3] and evaluating their possible antioxidant, antiviral and anticancer effects.[7-9] The most significant component present in *C. sinensis* is catechins, which play a crucial role in its biological benefits. These advantages include lowering blood lipid levels, decreasing inflammation and demonstrating antibacterial, antimalarial, anticancer and antioxidant effects.[6] Catechins,

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part of the flavan-3-ol group, have attracted considerable attention due to their potential therapeutic properties. Their potent antioxidant and antiviral abilities could help alleviate the damaging impacts of various diseases.[10,11]

Ziziphus vulgaris, also referred to as jujube, belongs to the Rhamnaceae family. This plant is extensively cultivated in countries such as Burma, Iran and various regions of India.[12] Phytochemical studies reveal that *Z. vulgaris* is rich in bioactive compounds including cyclopeptide alkaloids. Multiple research efforts indicate that these cyclopeptide alkaloids play a role in the antiplasmodial and anti-mycobacterial effects of the plant.[13] Furthermore, Ziziphus is known to contain terpenoids, alkaloids, steroids, polysaccharides and glycosides from the saponin group.[13] A study conducted by Rahimi *et al.*, found that the extract of *Z. vulgaris* exhibited *in vitro* anti-*Trichomonas gallinae* effects,[13] suggesting it has potential as a candidate for the development of new antiparasitic medications.

So far, there has been no research examining the anti-*Giardia* properties of *Z. vulgaris* and *C. sinensis* extracts. Consequently, this study evaluated the impacts of these extracts on *G. duodenalis* cysts *in vitro* as an alternative to metronidazole, with the intent of reducing the side effects linked to metronidazole. Furthermore, this research compared the effects of metronidazole with the extracts of *Z. vulgaris* and *C. sinensis* on *G. duodenalis* cysts *in vitro*.

Materials and Methods

Preparation of plant extract. The plants *Z. vulgaris* and *C. sinensis* were obtained from the Persian herbal market and validated by the University of Agriculture in Urmia, Iran. It is essential to mention that these extracts were also used in our earlier research.[13] All dried plant materials including green tea leaves and dried jujube fruit were placed in an electric blender (Moulinex, Paris, France) and ground into a fine powder. We combined 100 g of the powdered plant material with 500 mL of 70.00% ethanol and agitated the solution with a magnetic stirrer for 2 hr. The solution was then allowed to sit at room temperature for 24 hr. After this period, it was stirred again before undergoing filtration. The solvent was subsequently removed using a rotary evaporator. For future use, the semi-solid residual components were stored at a temperature of 4.00 ˚C.[30]

Gas chromatography-mass spectrometry analysis. The composition of the extract was examined using gas chromatography-mass spectrometry (Thermo Scientific™, Paris, France). The helium carrier gas was set at a flow rate of 0.50 mL *per* min with a split ratio of 1 : 20. The gas chromatography protocol began at an initial temperature of 40.00 ˚C which was then gradually raised to 250 ˚C at a rate of 80.00 ˚C *per* min. The injector and detector temperatures were kept steady at 250 ˚C. Individual compounds were recognized by comparing their relative retention times on a capillary column with those of known samples as well as by aligning their peak-to-peak mass spectra with existing data.[13]

Preparation of *G. duodenalis* cysts. Fecal samples containing *G. duodenalis* cysts were collected from freshly excreted feces of patients referred to laboratory at Urmia University of Medical Sciences and then transferred to the parasitology laboratory of the Urmia University. The sucrose density gradient technique was utilized to extract *G. duodenalis* cysts. To begin, 10.00 - 15.00 mL of distilled water was introduced to the stool samples harboring the target cysts to form a suspension. A damp gauze layer was then employed to filter the resulting suspension. The filtered suspensions were transferred into centrifuge tubes and spun for 5 min. The upper portions of these suspensions were collected and set aside. This procedure was conducted three times. Subsequently, 3.00 mL of 0.85 sucrose solution was added to the tubes, along with 3.00 mL of the prepared stool suspension. The tubes were then stored in a refrigerator at 4.00 ˚C and centrifuged for 10 min. This resulted in a four-layer structure, with the *G. duodenalis* cysts forming cloud-like rings in the middle layer. A Pasteur pipette was utilized to carefully extract these cloud layers and to transfer them into a different tube. Following this, 3.00 - 5.00 mL of distilled water was incorporated into the samples, and the tubes were subjected to another centrifugation for 5 min at 600 *g* to separate the sucrose solution. The layer at the top of the tubes was set aside, and the sediment underwent additional centrifugation for 5 min at 600 *g*. This step was repeated two more times. Ultimately, the upper layer was discarded, leaving behind the sediment that contained the purified cysts.[14]

***In vitro* assay.** To assess how effective *Z. vulgaris* and *C. sinensis* were in eliminating *G. duodenalis* cysts, three concentrations were evaluated along with various durations of exposure. The purified samples of cysts (500 µL) were treated with 500 µL of the extracts at each concentration (25.00, 50.00, and 100 mg mL^{-1}). Also, metronidazole was used at a concentration of 5.00 mg mL^{-1} for the same exposure durations of 10, 15, 30, 60, and 180 min. The viability of *G. duodenalis* cysts was examined with 0.10% Eosin vital staining, using a hemocytometer and an optical microscope. Non-treated cysts were considered as negative control groups in each experiment. All experiments were conducted in triplicate and the percentage of dead cysts was determined using the formula: $[D / (L + D)] \times 100$, where, L represents the number of living cysts and D denotes the number of dead cysts.[3,14]

Statistical analysis. Statistical analysis was conducted using SPSS Software (version 27.0; IBM Corp., Armonk, USA). One-way analysis of variance followed by Tukey's post-hoc test was employed to assess differences between the test and control groups. A significance level of less than 0.05 was considered statistically significant.

Results

Gas chromatography-mass spectrometry analysis. An analysis conducted through gas chromatography-mass spectrometry pinpointed essential chemical constituents in the analyzed extracts. In the *Z. vulgaris* extract, the major compounds consisted of catechin (22.67%), quercitrin (19.83%) and kaempferol (14.44%) as presented in Table 1. For the *C. sinensis* extract, the most noteworthy components were caffeine (24.07%), naphthalene (12.36%) and squalene (11.34%) as detailed in Table 1.

Table 1. Major chemical compounds in *Ziziphus vulgaris L.,* and *Camellia sinensis (L.) Kuntze extracts* identified by GC-MS

Extracts	Major compounds	Amount (%)
Ziziphus vulgaris	Quinic acid	11.45
	Gallic acid	8.74
	Catechin	22.67
	Epicatechin	2.08
	Quercitrin	19.83
	Naringin	5.34
	Acacetin	2.13
	Kaempferol	14.44
	3,4-di-O-caffeoylquinic acid	1.22
	1,3-di-O-caffeoylquinic acid	4.56
Camellia sinensis	Squalene	11.34
	Methyl stearate	3.05
	Methyl linolenate	7.43
	Caffeine	24.07
	Naphthalene	12.36
	α-bulnesene	2.52
	Eremophilene	1.22
	γ-muurolene	1.38
	n-hexadecanoic acid	9.32
	Methyl hexadecanoate	0.43

Assessment of mortality rate. Using 0.10% Eosin vital staining to test the vitality of Giardia cysts made it possible to tell the difference between live cysts which remained unstained and dead cysts which stained bright red (Fig. 1).

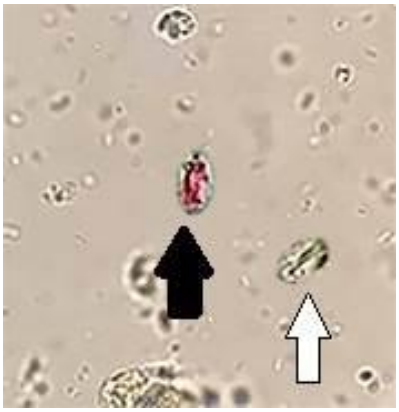

Fig. 1. Vital staining of Giardia cysts using 0.10% Eosin: viable unstained cysts (white arrow) and dead stained cysts (black arrow) at 400 ×.

The anti-*Giardia* properties of varying concentrations of *Z. vulgaris* and *C. sinensis* extracts (25.00, 50.00, and 100 mg mL^{-1}) across different exposure times (10, 15, 30, 60, and 180 min) are detailed in Tables 2 and 3.

Table 2. Mortality rates (%) at different exposure times of treated *G. duodenalis* cysts by different concentrations of *Z. vulgaris* extract.

Concentration	Time (min)				
	10	15	30	60	180
Control*	71.00	80.00	91.00	98.00	100
25.00 (mg mL^{-1})	21.00	40.00	55.00	69.00	79.00
50.00 (mg mL^{-1})	39.00	57.00	68.00	79.00	89.00
100 (mg mL^{-1})	51.00	68.00	79.00	88.00	98.00

* Metronidazole (5.00 mg mL^{-1}).

Table 3. Mortality rates (%) at different exposure times of treated *G. duodenalis* cysts by different concentrations of *C. sinensis* extract.

Concentration	Time (min)				
	10	15	30	60	180
Control*	71.00	80.00	91.00	98.00	100
25.00 (mg mL^{-1})	28.00	39.00	55.00	71.00	82.00
50.00 (mg mL^{-1})	43.00	64.00	76.00	87.00	98.00
100 (mg mL^{-1})	68.00	78.00	85.00	94.00	100

* Metronidazole (5.00 mg mL^{-1}).

The average findings from three experiments on the impact of *Z. vulgaris* extract at different concentrations and durations on *G. duodenalis* cysts indicated that the addition of the extract notably decreased the number of viable cysts. The greatest reduction was observed at a concentration of 100 mg mL^{-1} after 180 min. With longer exposure times, the mortality rate of *G. duodenalis* cysts rose for each concentration of 25.00, 50.00, and 100 mg mL^{-1} ($p < 0.05$). Specifically, the mortality rates recorded after 10 min were 21.00, 39.00, and 51.00%, respectively. These rates climbed to 79.00, 89.00, and 98.00% after 180 min (Fig. 2). The study on *C. sinensis* extracts showed that exposing the cysts for 180 min at a concentration of 100 mg mL^{-1} resulted in total mortality of all cysts. Extending the exposure time at different concentrations significantly increased the mortality rate ($p < 0.05$; Fig. 2). Additionally, the research indicated that extracts of *Z. vulgaris* and *C. sinensis* were as effective as metronidazole for killing Giardia cysts *in vitro*. The higher mortality rate was linked to longer exposure times and greater concentrations of the extracts ($p < 0.05$).

Discussion

Herbal therapies have become increasingly popular because they are natural, cost-effective and environmentally sustainable. Recently, there has been heightened interest in utilizing natural anti-parasitic extracts for treating various ailments. Certain herbal extracts are emerging as practical alternatives to traditional medications due to their reduced side effects,

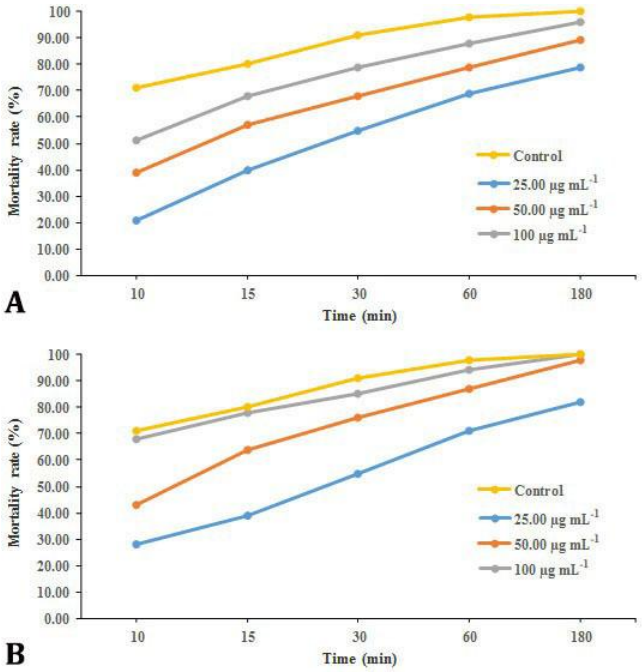

Fig. 2. Comparison of the mortality rate of **A)** *Ziziphus vulgaris* and **B)** *Camellia sinensis* extract in different concentrations and exposure times.

lower prices and easy accessibility. Studies exploring the connection between medicinal plants and parasites indicate that specific compounds in plant extracts, especially phenolic compounds, may hinder membrane development and respiration in parasites as well as enhance fluidity and permeability.[15]

Many studies have explored the antigiardial properties of various plants such as *Allium sativum*,[3] *Artemisia annua*,[16] *Tanacetum parthenium*,[17] *Eucalyptus radiata*,[18] *Ageratum conyzoides*,[19] *Origanum vulgare*,[20] *Satureja khuzestanica*,[3] *Zingiber* officinale,[21] and *Sambucus ebulus*[22] in treating giardiasis. The majority of these investigations focused more on *in vitro* analyses rather than *in vivo* applications, attributed to reasons such as cost-effectiveness, speed and the high throughput capacity of *in vitro* screening methods. These *in vitro* evaluations assessed anthelmintic activity by examining the hatching, development and motility of the parasites directly without influencing the internal physiological functions of the host.[23] One benefit of *in vitro* research is that once dependable results are achieved, the extracts or compounds can be examined further through *in vivo* studies.[24] Nevertheless, it is crucial to recognize that compounds or extracts that show efficacy *in vitro* may not necessarily perform with the same efficacy *in vivo*.[25] Plant extracts have been demonstrated to inhibit the proliferation and attachment of *G. duodenalis* to the intestinal and digestive systems.[26,27] Our study focused on investigating the anti-giardia properties of *Z. vulgaris* and *C. sinensis* extracts *in vitro*. To our knowledge, this represented the first study assessing the *in vitro* effects of *Z. vulgaris* and *C. sinensis* on *G. duodenalis* cysts.

Our results revealed a dose-dependent and time-dependent anti-Giardia response from *Z. vulgaris* and *C. sinensis* extracts *in vitro*. These findings were in agreement with those of Fallahi *et al.*, who noted comparable dose and time-dependent effects from olive leaf, *Satureja khuzestanica* and *Allium sativum* extracts.[4] Furthermore, a study by Dyab *et al.*, reported similar *in vitro* anti-*Giardia* effects achieved with extracts *of Zingiber officinale* and *Curcuma longa.*[21]

The present research indicated that extracts from *Z. vulgaris* and *C. sinensis* exhibited notable anti-*Giardia* effects after 180 min at a concentration of 100 mg mL^{-1}, resulting in mortality rates of 100 and 98.00%. Dyab *et al.*, investigated the impact of a dichloromethane extract from *Curcuma longa* on *Giardia* cysts and found that an extract concentration of 50.00 mg mL^{-1} eliminated 85.00% of cysts after 60 min.[21] Rahimi-Esboei *et al.*, studied extracts from *Artemisia annua* and discovered that the hydroalcoholic extract displayed the most significant anti-*Giardia* activity at concentrations of 50.00 and 100 mg mL^{-1} after durations of 3 and 24 hr.[28] Likewise, Rezaiemanesh and Shirbazou, observed a potent anti-*Giardia* effect from the ethanolic extract of *Ferula assafoetida*, with concentrations of 20.00 mg mL^{-1} achieving 100% cyst mortality after 4 hr.[29] In a separate study, Safarnezhad Tameshkel *et al.*, reported that 84.30% of cysts were eradicated within 60 min when exposed to over 50.00 mg mL^{-1} of methanolic extract from *Satureja hortensis*.[18] Furthermore, Rahimi-Esboei *et al.*, demonstrated that the hydroalcoholic extract from *Artemisia annua* possessed anti-*Giardia* properties at a concentration of 10.00 mg mL^{-1} after 3 hr of exposure.[22] The differences in findings across various studies could be attributed to the variations in plant species, types of chemical fractions, concentrations and exposure times.[30,31]

In this research, caffeine (24.07%) was recognized as the main ingredient of the *C. sinensis* extract. Several studies have suggested it possessed antiparasitic properties. Results showed that both *C. sinensis* extract and caffeine demonstrated anti-*Acanthamoeba*, anti-*T. gallinae* and antileishmanial effects.[13,32,33] Previous investigations have shown that *C. sinensis* and its components have antimicrobial capabilities against multidrug-resistant gram-positive and gram-negative bacteria.[34] Moreover, *C. sinensis* was found to be effective in inhibiting *Leishmania amazonensis*,[35,36] diminishing exposure to *Haemonchus contortus* worms[37] and preventing both the promastigote and amastigote stages of *Leishmania braziliensis*.[38] Additionally, *C. sinensis* inhibited *Babesia* spp., *Eimeria* spp., *T. gallinae*, and *Trypanosoma cruzi*.[13,39-41] Research by Fakae *et al.*, revealed that beers produced from *C. sinensis* displayed amoebic activity against *Acanthamoeba* trophozoites and effectively prevented the encystment of the parasite. Their results indicated that *C. sinensis* could

potentially provide inhibitors for the growth and encystation of *A. castellanii*.[42]

The current study investigated the effectiveness of *Z. vulgaris* in assessing the mortality rate of *Giardia* cysts. Our results showed that higher concentrations of the extract led to an increase in the mortality rate of *Giardia* cysts. This finding was consistent with the research by Rahimi *et al.*, which noted a dose and time-dependent anti-*T. gallinae* effect from *Z. vulgaris* extract.[13] The enhanced efficacy of *Z. vulgaris* on Giardia cysts may be linked to its chemical composition. In our research, we discovered catechin (22.67%) as one of the components in the jujube extract, along with smaller quantities of other compounds. Prior studies have also demonstrated the antiparasitic effects of catechin, quercitrin and kaempferol which are key constituents found in jujube, as highlighted in our study.[13,41,43-45] Additionally, our findings corroborated earlier research that explored the antiparasitic, antifungal and antimicrobial effects of jujube cyclopeptide alkaloids, tannins and saponins.[7,15] Other studies have confirmed that jujube extract is effective *in vitro* against Acanthamoeba species and malaria.[44] The amoebicidal action of *Z. vulgaris* is associated with its capacity to trigger apoptosis, as mentioned by Dodangeh *et al*.[45] Moreover, prior research has pointed out that the apoptosis-inducing effect of *Z. vulgaris* provides notable advantages compared to other commonly used medications such as metronidazole. The antiparasitic effects of *Z. vulgaris* may be linked to its ability to induce apoptosis,.[45] However, further mechanistic studies are needed to confirm this effect in *Giardia duodenalis*.

The findings of this study indicated that extracts from *C. sinensis* and *Z. vulgaris* might be promising candidates for new anti-*Giardia* treatments owing to their antiparasitic traits. While our results demonstrated promising anti-Giardia effects of *Z. vulgaris* and *C. sinensis* extracts *in vitro*, these findings need validation through *in vivo* studies to assess their therapeutic potential, safety, and pharmacokinetics in animal models. Future research will focus on *in vivo* evaluations to address these limitations and explore the practical application of these extracts as antiparasitic agents.

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Conflict of interest

The authors declare that there is no conflict of interest.

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