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Original article

Anti-parasitic Activity of Some Medicinal Plants Essential Oils on *Giardia lamblia* and *Entamoeba histolytica*, In Vitro

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Abstract

Background and objectives: Giardiasis and amoebiasis are two common diseases in human societies which make increasing problems for the health managing systems. In the present study, the antiparasitic activity of *Allium sativum*, *Artemisia sieberi*, *Zatraria multiflora*, *Chenopodium botrys* and *Eucalyptus globulus* essential oils on *Giardia lamblia* cysts and *Entamoeba histolytica* trophozoites were investigated in vitro. **Methods:** *Giardia lamblia* cysts and *E. histolytica* trophozoites were isolated from infected sample stools. The parasites were treated by four concentrations (0.2, 0.1, 0.01 and 0.001 µg/mL) of essential oils, separately for 30 min at 37 °C. **Results:** The essential oils at the concentration of 0.1 and 0.2 µg/mL indicated appreciate parasiticidal effect ($p<0.05$). *Eucalyptus globulus* had maximum efficacy on both *G. lamblia* and *E. histolytica* with mortality rates of 79.7% and 87.6% within 30 min. **Conclusion:** All essential oils showed potent anti-giardiasis and anti-amoebiasis activity. Also, *E. globulus* with the highest efficiency could be considered as anti-protozoa medication to use an adjunct or primary therapy.

Keywords: cysts; *Entamoeba histolytica*; essential oil; *Giardia lamblia*; herbal medicine

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Introduction

Gastrointestinal (GI) infections are one of the most common diseases that affect the quality of individuals' life. *Giardia lamblia* and *Entamoeba histolytica* are two types of protozoa that cause many digestive complications including diarrhea, dysentery, abdominal cramps, fever and infection. The disease is more prevalent in densely populated areas where hygiene requirements are not respected, especially in rural areas where domestic and wild animals are playing an essential role in the transmission of the parasites [1-5]. The cyst stage of the parasite is the transmission form which exists in the carrier animals stool. After ingestion of the cysts, they are transformed into the active form (trophozoite) in the GI tract and attach to the small intestine and cause GI disorders or are

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transmitted through the bloodstream and form abscesses in organs like the brain, eye, liver and other vital organs and tissues or even lead to death in some cases [6-8]. Some individuals have no symptom of the disease but transmit the infection to others. Considering the importance of the illness and its adverse effects on humans, many studies have been conducted to find the appropriate treatment, which has resulted in some successful medications. However, the genesis of the drug resistance and hazardous side effects persuaded scientists into thinking of finding new safe anti-parasitic drugs [9]; however, the disease remains a global disaster and a definitive solution has not been found yet. Today, medicinal herbs are the widespread supply of drugs and several studies has been performed on the effectiveness of medicinal herbs extracts on parasitic infections. In this research, five medicinal herbs essential oils; *Allium sativum L., Artemisia sieberi B., Zatraria multiflora L., Chenopodium botrys L., Eucalyptus globulus* Labill., has been chosen based on Iranian traditional medicine (ITM) books to examine the effects on *G. lamblia* cysts and *E. histolytica* trophozoites viability in vitro.

Material and Methods

Ethical considerations

The protocol of the research was approved by the Committee of the Shiraz University of Medical sciences (79/10).

Chemicals

All the reagents and solvents such as sucrose, NaCl, KCl, $MgCl_2$ and etc were purchased from Merck Co., Germany.

Plant material and essential oils preparation

Eucalyptus globulus (E1-217-41), *Z. multiflora* (E1-364-151), *A. sativum* (E2-21-51), *C. botrys* (E1-83-13), *A. sieberi* (E1-39-29) were collected from the plain area in Shiraz , Fars Province (2013), Iran and Professor Mohammad Azadbakht confirmed the scientific names. The essential oils of *A. sativum* Bulb, *A. sieberi*, *Z. multiflora*, *C. botrys* aerial parts and *E. globulus* leaves were prepared by the Clevenger apparatus.

Isolation of *G. lamblia* cysts

A highly purified *G. lamblia* cysts suspension was achieved by combining the sucrose flotation method with a simplified sucrose gradient protocol. Sixty stool samples containing *G. lamblia* were concentrated by 0.85 M sucrose method. The stool samples were diluted with normal saline and shaken for 5 min to form a suspension. The suspension was filtered and 3 mL was transferred 3 mL of 0.85 M sucrose. The solution was centrifuged at 600 rpm for 10 min. The cysts in the middle layer were slowly transferred to another tube and washed 3 times with normal saline. The washed cysts were carefully added to the top of a discontinuous density gradient consisting of two 3 mL layers of 0.85 M and 0.4 M sucrose [10].

Isolation of *E. histolytica* cysts

Forty stool samples containing *E. histolytica* cysts were collected. The trophozoites were cultured in Dr bohlave's locke medium and subcultured every 48 h [10]. The Dr bohlave's locke medium consists of 8g NaCl + 0.2 g $CaCl_2$ + 0.2 g KCl+ 0.01 g $MgCl_2$ + 2 g Na_2PO_4 + $NaHCO_3$+ 0.3 g KH_2PO_4 + distilled water up to 1000 mL.

Treatment of parasites with essential oils

The essential oils were diluted with DMSO. Four concentrationsof 0.2, 0.1, 0.01 and 0.001µg/mL of each essential oil were examined on 30 *G. lamblia* cysts samples. All concentrations with locke's medium were added to the test tubes, separately. The contents of the tubes were shaken to become a suspension and kept resting for 24 h at 4℃. Subsequently, 1.0×10^3 of *E. histolytica* trophozoites were added to the test tubes and then incubated at 37 ℃ for 30 min. The viability was proved by eosin coloring. Dead cysts absorbed eosin color, while live cysts were non-colored. Control group was treated by DMSO alone. All the experimental procedures were replicated three times.

Statistical analysis

All data have been presented as mean ± SD. The results were analyzed using SPSS software ver. 16 by one-way ANOVA test. The p- value less than 0.05 was considered as significant.

Results and Discussion

The yields of essential oils were 1.8% for *E. globulus*, 1.7% for *Z. multiflora*, 1.6% for *A. sativum*, 1.55% for *A. sieberi* and 0.75% for *C. botrys*. The parasiticidal effects of essential oils have been shown in tables 1 and 2. The results indicated that all the highest concentrations of

the essential oils showed the most considerable parasiticidal activity on cysts compared to the control group ($p<0.0001$).

There is a significant difference between the control group and all treatment groups at 0.1 and 0.2 µg/mL concentrations of essential oils ($p<0.05$). *Allium sativum* at the highest concentration 0.2 µg/mL, killed 67.46% of the cysts after after 30 min; similarly, 75.26% for *A. sieberi*, 68.43% for *Z. multiflora*, 77.73% for *C. botrys*, and 79.7% for *E. globulus* were recorded. It seemed that at same concentrations *E. globulus* essential oil showed better effects on *G. lambia* cysts while *A. sativum* showed the minimum effects on cysts (figure 1).

Eucalyptus globulus essential oil at the concentration of 0.2 µg/mL presented the highest lethal effect on *E. histolytica* trophozoites (87.6%) followed by *Z. multiflora* (84.7%), *A. sativum* (83.6%), *C. botrys* (68.3%) and *A. sieberi* (66.3%).

Eucalyptus globulus, C. botrys and *A. sieberi* in all tested concentrations and *Z. multiflora* and *A. sativum* from the concentration of 0.01 µg/mL, increased the mortality rate of *G. lamblia* cysts in a concentration dependent manner. *Eucalyptus globulus,* in all tested concentrations and *C. botrys, A. sieberi, Z. multiflora* and *A. sativum* from concentration of 0.01 µg/mL, increased the mortality rate of *E. histolytica* trophozites in a concentration dependent manner. The comparisons of different essential oils at all concentrations have been shown in figures 1 and 2. At the concentration of 0.2 µg/mL, *E. globulus* significantly showed better effects on mortality rate of cysts in comparison to *Z. multiflora* and *A. sativum* ($p<0.001$) followed by *C. botrys* in comparison to *Z. multiflora* and *A. sativum* ($p<0.001$). At the concentration of 0.2 µg/mL, *E. globulus* significantly reduced the viability of trophozoites in comparison to *C. botrys* and *A. sieberi* ($p<0.0001$). *Zatraria multiflora* significantly increased the mortality rate of trophozoites in comparison to *C. botrys* and *A. sieberi* ($p<0.0001$); also, the trophozoiticidal effect was significantly higher with *A. sativum* in comparison to *C. botrys* and *A. sieberi* ($p<0.0001$).

Giardia lamblia and *E. histolytica* are two types of protozoan parasites in which are considered as the main cause sof diarrhea in all parts of the world with different infection rates. These parasites are more common in tropical regions and places with a high population density and low health facilities [1-5,8]. Considering that failure in the treatment of human giardiasis and amebic disease is increasing phenomenon [13], World Health Organization (WHO) has suggested for using various natural products in the treatment of parasitic disease [14]. The needs for finding medicines with lower costs, higher efficacy and fewer side effects has remained a problem [15,16].

Table 1. The mortality rate (mean ± standard deviation) of *Giardia lamblia* cysts in the presence of different concentrations of essential oils at 37 ° C for 30 min of exposure

Essential oil	Concentration (µg/mL)				
	0.2	0.1	0.01	0.001	Control
	mortality rate (mean ± SD)				
Eucalyptus globulus	79.7±3.5	54.4±.05	43.5±6.0	18.7±5.0	5.0±0.0
Zatraria multiflora	68.4±3.2	45.3±4.3	35.3±6.3	13.9±4.9	8.0±0.0
Allium sativum	67.4±3.6	43.4±4.2	33.6±6.4	12.8 ±4.6	8.0±0.3
Chenopodium botrys	77.7±3.8	51.9±4.5	41.0±5.5	17.2±4.5	6.0±0.6
Artemisia sieberi	75.2±3.9	50.8±4.5	39.5±5.5	15.8±4.8	6.0±0.9

Table 2. The mortality rate (mean ± standard deviation) of *Entamoeba histolytica* trophozites in the presence of different concentrations of essential oils on at 37 ° C for 30 min of exposure

Essential oil	Concentration (µg/mL)				
	0.2	0.1	0.01	0.001	Control
	mortality rate (mean ± SD)				
Eucalyptus globulus	87.6±3.2	69.4±3.0	49.6±0.4	19.6±2.9	7.0±0.6
Zatraria multiflora	84.7±3.1	61.3±0.3	40.3±5.3	11.0±0.9	7.0±0.6
Allium sativum	83.6±2.2	60.6±3.2	40.8±6.4	11.8±2.6	9.0±0.5
Chenopodium botrys	68.3±3.8	40.5±2.5	20.9±3.5	7.2±4.5	9.0±0.0
Artemisia sieberi	66.3±4.7	30.5±3.7	21.4±3.2	9.1±2.9	9.0±0.6

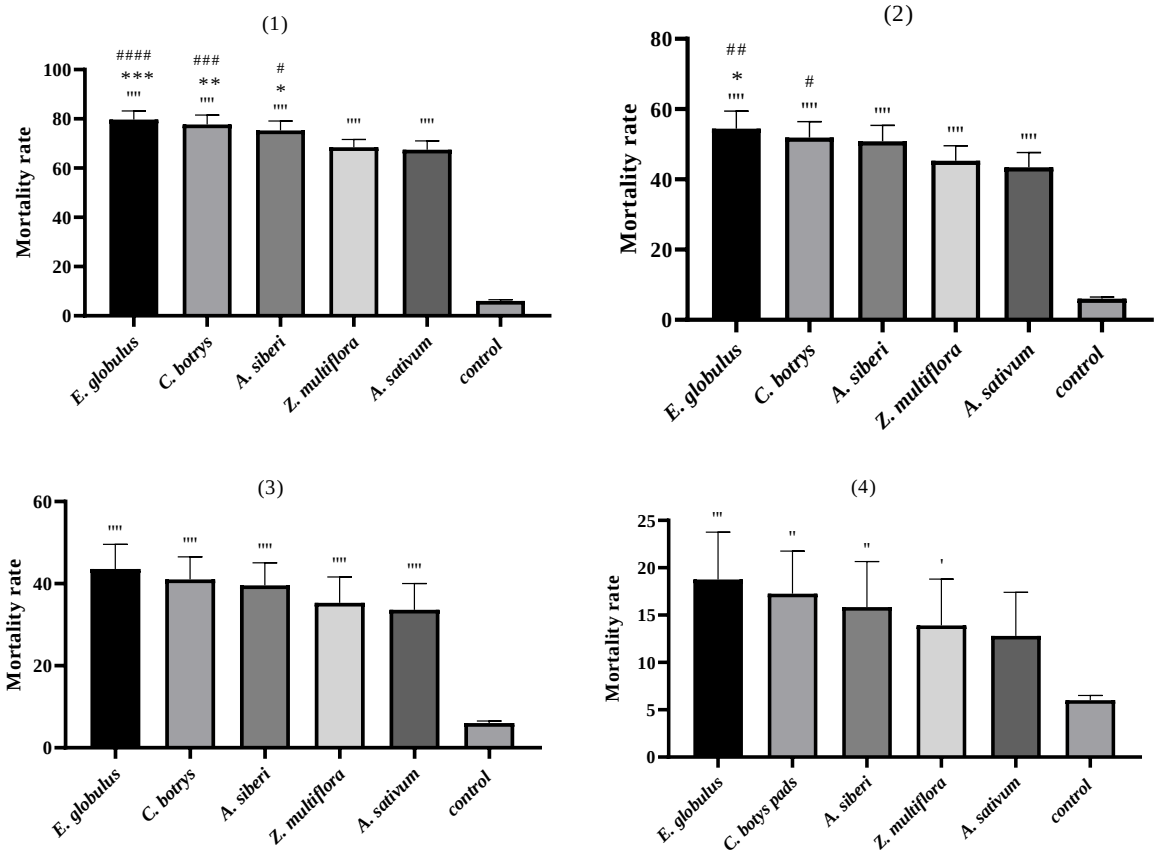

Figure 1. The mortality rate of *Giardia lamblia* cysts in the presence of (1) 0.2 µg/mL, (2) 0.1 µg/mL, (3) 0.01 µg/mL (4) 0.001 µg/mL of essential oils at 37 ° C for 30 min of exposure. Data are expressed as mean± SD. ""p<0.0001, '"p<0.001, "p<0.01, 'P<0.05 significant as compared to control. **** p<0.0001, *** p<0.001, **p<0.01, * p<0.05 significant as compared to *Zatraria multiflora.* #### p<0.0001, ### p<0.001, ## p<0.01, # p<0.05 significant as compared to *A. sativum*

Regarding this fact, many synthetic and natural compounds were studied at experimental or clinical trials stages. Anti-parasitic activity of various medicinal herbs has been previously investigated. For example, methanol extract of *Rubus coriifolius*, *Cuphea pinetorum* and *Helianthemum glomeratum* showed suitable effects in the treatment of *G. lamblia* and *E. histolytica* trophozoites in vitro. The high efficacy of *Cuphea pinetorum* was attributed to kaempferol and quercetin components [10]. Dichloromethane/methanol extract of *Geranium mexicanum* root was effective on *G. lamblia* at the concentration of 100 µg/mL and *E. histolytica* at the concentration of 79.2 µg/mL due to the flavonoid compounds, β-sitosterol 3-*O*-β-glucopyranoside and tyramine [11]; while *Annona cherimola*, *Punica granatum* and *Chiranthodendron pentadactylon* demonstrated acceptable effect on *E. histolytica* with IC_{50} <30 µg/mL. *Dorstenia contrajerva*, *Senna villosa* and *Ruta chalepensis* were effective against *G. lamblia* with IC_{50}<38 µg/mL in vitro [12].Chloroform extract of *Artemisia annua* at the concentration of 100 mg/mL exhibited 86% fatal effect on *G. lamblia* cyst and the maximum efficacy (100%) on trophozoites after 1 h in vitro. *Tanacetum parthenium* (50 mg/mL) killed 83 % of *G. lamblia* cysts and 100% of trophozites after 1 h [19]; whereas *Allium sativum* hydroalcoholic extract killed 40% of cysts at the concentration of 5 mg/mL (20). *Eucalyptus globulus* methanol extract mortality rate on *G. lamblia* cysts was 63.3% after 60 min at a concentration of 200 mg/mL [21].

Our previous work exhibited that fungal chitosan at the concentration of 400 µg/mL was very potent on *G. lambia* cysts [16]. Various researches have been conducted on extract of medicinal plants; however, rare studies have been designed on essential oils.

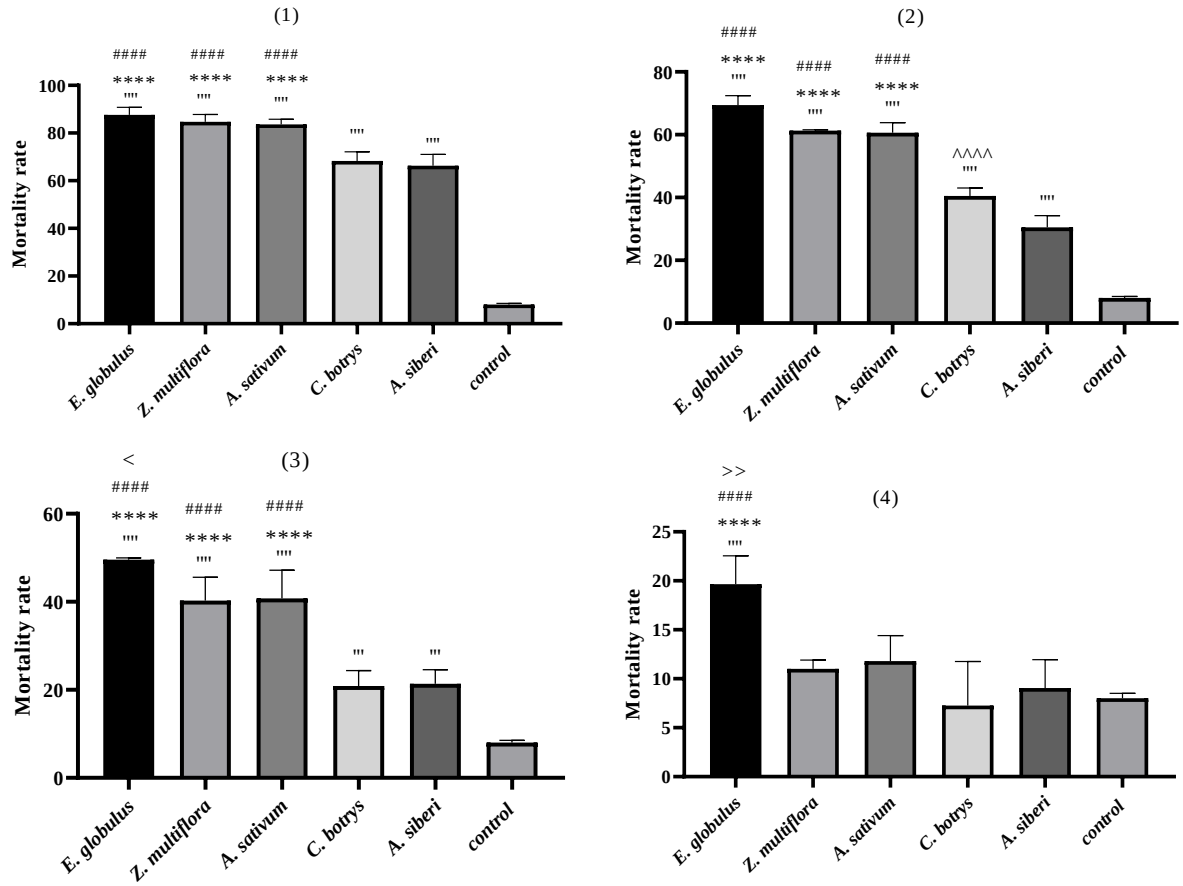

Figure 2. The mortality rate of *Entamoeba. histolytica* trophozites in the presence of (1) 0.2 µg/mL, (2) 0.1 µg/mL, (3) 0.01 µg/mL (4) 0.001 µg/mL of essential oils at 37 °C for 30 min of exposure. Data are expressed as mean± SD. ""p<0.0001, "'p<0.001 significant as compared to control. ****p<0.0001 significant as compared to *Chenopodium botrys.* #### p<0.0001 significant as compared to *A. sieberi.* <p<0.01 significant as compared to *Zatraria multiflora* and *Allium sativum*

Even though many reports have been carried out on the anti-parasitic activity of medicinal plants but concentration and time limitations and unavailability of some plants have been the main concerns.Many investigations reported the efficacy of natural components on the parasitic diseases. The active gradients of *A. sativum* (Allicin, Acrolein), *A. sieberi* (1,8-cineole, α-tujon, camphor), *Z. multiflora* (thymol, carvacrol, p-cymene, borneol, linalool, beta-pinene) , *C. botrys* (Ascaridole), *E. globulus* (eucalyptol, valeric aldehyde, butyric aldehyde, pinocarveol) have been reported to be responsible for their parasiticidal activity and many types of research proved their antimicrobial and aseptic effects [22].

In the present study, five essential oils were tested on *G. lamblia* cysts and *A. histolytica* trophozoites; among them, *E. globulus* showed the highest inhibitory effect on both parasites and can be suggested as adjunctive therapy along with chemical drugs. The main advantage of our study is the concentration and time savings in comparison to other researches.

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Author contributions

Mohammad Azadbakht: managed the project; Aroona Chabra, Taha Monadi, Fatemeh Akbari and Mohammad Hossein Motazedian participated

in the experimental work and writing the paper; Amir Saeedi Akbarabadi participated in the experiments

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

GI: Gastrointestinal; ITM: Iranian traditional medicine; WHO: World Health Organization