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


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
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., Zatrraria 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, MgCl₂ 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 CaCl₂ + 0.2 g KCl+ 0.01 g MgCl₂ + 2 g Na₃PO₄ + NaHCO₃+ 0.3 g KH₂PO₄ + distilled water up to 1000 mL.

Treatment of parasites with essential oils
The essential oils were diluted with DMSO. Four concentrations of 0.2, 0.1, 0.01 and 0.001 µg/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 °C. Subsequently, 1.0 × 10⁵ of E. histolytica trophozoites were added to the test tubes and then incubated at 37 °C 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. lamblia 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 trophozoites 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<0.001). Zatraria multiflora significantly increased the mortality rate of trophozoites in comparison to C. botrys and A. sieberi (p<0.0001); also, the trophozoitcidal 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 of 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

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Concentration (µg/mL)</th>
<th>0.2</th>
<th>0.1</th>
<th>0.01</th>
<th>0.001</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mortality rate (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>79.7±3.5</td>
<td>54.4±0.5</td>
<td>43.5±6.0</td>
<td>18.7±5.0</td>
<td>5.0±0.0</td>
<td></td>
</tr>
<tr>
<td>Zatraria multiflora</td>
<td>68.4±1.2</td>
<td>45.3±4.3</td>
<td>35.3±6.3</td>
<td>13.9±4.9</td>
<td>8.0±0.0</td>
<td></td>
</tr>
<tr>
<td>Allium sativum</td>
<td>67.4±3.6</td>
<td>43.4±4.2</td>
<td>33.6±6.4</td>
<td>12.8±4.6</td>
<td>8.0±0.3</td>
<td></td>
</tr>
<tr>
<td>Chenopodium botrys</td>
<td>77.7±3.8</td>
<td>51.9±4.5</td>
<td>41.0±5.5</td>
<td>17.2±4.5</td>
<td>6.0±0.6</td>
<td></td>
</tr>
<tr>
<td>Artemisia sieberi</td>
<td>75.2±3.9</td>
<td>50.8±4.5</td>
<td>39.5±5.5</td>
<td>15.8±4.8</td>
<td>6.0±0.9</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Concentration (µg/mL)</th>
<th>0.2</th>
<th>0.1</th>
<th>0.01</th>
<th>0.001</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mortality rate (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>87.6±3.2</td>
<td>69.4±3.0</td>
<td>49.6±0.4</td>
<td>19.6±2.9</td>
<td>7.0±0.6</td>
<td></td>
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<tr>
<td>Zatraria multiflora</td>
<td>84.7±3.1</td>
<td>61.3±0.3</td>
<td>40.3±5.3</td>
<td>11.0±0.9</td>
<td>7.0±0.6</td>
<td></td>
</tr>
<tr>
<td>Allium sativum</td>
<td>83.6±2.2</td>
<td>60.6±3.2</td>
<td>40.8±6.4</td>
<td>11.8±2.6</td>
<td>9.0±0.5</td>
<td></td>
</tr>
<tr>
<td>Chenopodium botrys</td>
<td>68.3±3.8</td>
<td>40.5±2.5</td>
<td>20.9±3.5</td>
<td>7.2±4.5</td>
<td>9.0±0.0</td>
<td></td>
</tr>
<tr>
<td>Artemisia sieberi</td>
<td>66.3±4.7</td>
<td>30.5±3.7</td>
<td>21.4±3.2</td>
<td>9.1±2.9</td>
<td>9.0±0.6</td>
<td></td>
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</tbody>
</table>
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. lambia 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. lambia 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 IC₅₀ <30 µg/mL. Dorstenia contrajerva, Senna villosa and Ruta chalepensis were effective against G. lambia with IC₅₀<38 µg/mL in vitro [12]. Chloroform extract of Artemisia annua at the concentration of 100 mg/mL exhibited 86% fatal effect on G. lambia cyst and the maximum efficacy (100%) on trophozoites after 1 h in vitro. Tanacetum parthenium (50 mg/mL) killed 83% of G. lambia cysts and 100% of trophozoites 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. lambia 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 1. The mortality rate of Giardia lambia 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.
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Figure 2. The mortality rate of *Entamoeba histolytica* trophozoites 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<0.001 significant as compared to *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, Acrrolein), *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.

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