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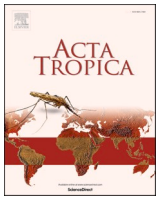

Promising effects of 1,8 Cineole to control *Giardia lamblia* infection: Targeting the inflammation, oxidative stress, and infectivity

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ABSTRACT

Reportedly, synthetic drugs such as metronidazole, furazolidone, tinidazole, and quinacrine are used for the treatment of giardiasis but are associated with adverse effects. In this study, we aimed to investigate the in vitro and in vivo effects of eucalyptol (ECT, 1,8 cineole) alone and in combination with metronidazole (MNZ) on *Giardia lamblia*. The effects of ECT on cell viability, plasma membrane permeability, and gene expression levels of adenylate cyclase (AK) and extracellular signal kinases 1 and 2 (ERK1 and ERK2) in trophozoites of *G. lamblia* were assessed. In vivo, the effects of ECT alone and in combination with MNZ were assessed on mice infected with *G. lamblia*. In addition, the gene expression of inflammatory genes (e.g., TNF-α, IL-1β, and IL-10) and antioxidant genes (catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 2 (GPX2)) was determined by real-time PCR. The IC_{50} values of ECT, MNZ, and ECT+MNZ on trophozoites were 30.2 μg/mL, 21.6 μg/mL, and 8.5 μg/mL, respectively. The estimated Fractional inhibitory concentration index (FICI) values for ECT and MNZ were 0.28 and 0.39, respectively. The application of ECT on *G. lamblia* trophozoites resulted in a dose-dependent increase in plasma membrane permeability, particularly at concentrations of ½ IC_{50} and IC_{50} ($P < 0.05$). The treatment of infected mice with various doses of ECT, mainly in combination with MNZ for 7 days, resulted in a significant decrease ($P < 0.001$) in the average number and viability of cysts. ECT, especially when combined with MNZ, caused a significant ($P < 0.001$) reduction in the expression of TNF-α and IL-6 genes, and an increase ($P < 0.05$) in the expression of IL-10 genes. ECT alone and mainly in combination with MNZ leads to a significant ($P < 0.001$) increase in the gene expression of CAT, SOD, and GPX genes. These findings demonstrate that the use of ECT in these doses, even for 14 days, does not have any toxic effects on the function of vital liver and kidney tissues. The study findings confirmed the promising effects of ECT against *G. lamblia* infection both in vitro and in vivo. Considering the possible mechanisms, ECT increases plasma membrane permeability and reduces the expression levels of infectivity-related genes. In addition, ECT suppresses inflammation and oxidative stress, controlling giardiasis in mice. More studies are needed to clarify these findings.

1. Introduction

Giardia lamblia (*G. lamblia*), also known as *Giardia intestinalis*, is a common pathogenic protozoan that infects a wide range of vertebrates, including humans and is a frequent cause of giardiasis (Carranza and Lujan, 2010). This parasite is responsible for causing fatty diarrhea (steatorrhea) or persistent diarrhea in travelers and exists in two forms: trophozoite and cyst (Robertson et al., 2010). It is considered one of the most common intestinal parasites worldwide, with an estimated 280 million annual infections. It is particularly prevalent in developing countries, where infection rates range from 10 % to 50 % (Minetti et al., 2016). *Giardia* is more common in temperate and tropical regions compared to cold regions, and it is notably prevalent in Iran, especially in the northern regions (Hooshyar et al., 2019). Human infection occurs through the ingestion of contaminated food and water, as well as direct fecal-oral contact. The resistance of the parasite's cysts to water

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chlorination contributes to its transmission through water. Infection can occur at any age, but it is more common in children (Cernikova et al., 2018). Clinical symptoms of *Giardia* infection include acute watery diarrhea, abdominal cramps, malabsorption syndrome, and weight loss. Both acute and chronic infections are observed (Muhsen and Levine, 2012).

As there is currently no reliable and safe vaccine available for the prevention of *Giardia* infection, chemotherapy using synthetic drugs is now regarded as the most optimal approach for treating giardiasis (Nash, 2001). Currently, drugs such as metronidazole, furazolidone, tinidazole, and quinacrine are used for the treatment of giardiasis (Escobedo and Cimerman, 2007). While these medications are generally effective, they are associated with adverse effects, including unpleasant taste, gastrointestinal discomfort, nausea, headache, leukopenia, neurotoxicity, restlessness, convulsions, and dizziness, which can hinder the treatment process. Furthermore, certain drugs have been demonstrated to have harmful, carcinogenic, and mutagenic effects in laboratory animals (Lalle and Hanevik, 2018). Consequently, there is a pressing need to identify a medication with minimal side effects.

In recent years, there have been reports on the therapeutic potential of certain herbs and their derivatives, including *Eucalyptus* spp., *Zataria* spp., and *Allium* spp., in treating *Giardia* infection (Alnomasy et al., 2021). However, the widespread use of herbal remedies for giardiasis is currently hindered by inconclusive research findings that are not always adequately substantiated (Alnomasy et al., 2021). 1,8 Cineole, also known as eucalyptol (ECT), is a monoterpene and a predominant constituent of various aromatic plant essential oils, such as eucalyptus, rosemary, croton, and salvia (Cai et al., 2021). It is widely used in the pharmaceutical industry for its roles as an antioxidant, anti-inflammatory, antimicrobial, antihypertensive, and analgesic agent. Furthermore, 8,1-cineole has been observed to temporarily disrupt intercellular lipids, thereby allowing otherwise impermeable substances to penetrate the skin (Azerad, 2014; Hoch et al., 2023). Despite the aforementioned cases, in this study, we decided to investigate the in vitro and in vivo effects of ECT alone and in combination with metronidazole (MNZ) on *G. lamblia*.

2. Materials and methods

2.1. Chemicals

The ECT compound with a purity exceeding 99 %, TYI-S-33, Sytox green, Eosin powder, fetal calf serum, and MTT (3-(4,5-Dimethylthiazol-2-yl) powder were provided from Sigma Aldrich in Germany. Additional reagents were sourced from reputable companies and were of superior quality.

2.2. Collecting and isolating G. lamblia cysts

Cysts of *G. lamblia* were obtained from fresh fecal specimens of individuals diagnosed with giardiasis referred to the general hospitals in Khorramabad, located in Lorestan province, Iran. The stool samples were placed in sterile plastic containers with normal saline and then transferred to the parasitology laboratory of the Department of Clinical Laboratory Sciences at Lorestan University of Medical Sciences. The samples were assessed using two direct methods and formalin-ether with light microscopy (Ghasemian Yadegari et al., 2022). The cysts were concentrated using the 0.85 M sucrose gradient method as described previously (Kharazmkia et al., 2023). The cysts were then subjected to vital staining with 0.1 % eosin to determine their survival percentage. Where dead and viable cysts appeared colorless and pink, respectively. The number of cysts was then adjusted to 1×10^5 cysts/mL in distilled water using a hemocytometer.

2.3. Obtaining the of G. lamblia trophozoites

Trophozoites of *G. lamblia* were obtained by inducing excystation of the cysts using an aqueous hydrochloric acid suspension (pH = 2), followed by transfer to filter-sterilized TYI-S-33 culture medium supplemented with 20 % inactivated fetal calf serum, bovine bile, streptomycin (500 μg/ml), and penicillin (500 U/mL), and maintained at 37 °C (Bingham and Meyer, 1979; Ghasemian Yadegari et al., 2022).

2.4. In vitro effects of ECT on G. lamblia trophozoites

The cell viability MTT assay was used to determine the effects of ECT on *G. lamblia* trophozoites (Chabra et al., 2019). To do this, 0.1 mL of tachyzoites (10^5/mL) were exposed to ECT alone at concentrations of 5–100 μg/mL, metronidazole (MNZ) at concentrations of 5–100 μg/mL, and the combination of MNZ at concentrations of 5–100 μg/mL and ECT at a concentration of 15 μg/mL in 96-well plates for 72 h at 37 °C. Then, the MTT solution (5 mg/mL) was added to the wells and incubated under similar conditions for 4 h. Subsequently, dimethyl sulfoxide was added as a stop solution, and the optical density of the wells was measured at 570 nm by an ELISA reader (LX800; Biotec, USA) (Chabra et al., 2019). The experiments were conducted in triplicate, and the 50 % inhibitory concentrations (IC_{50}) values were determined by the Probit test in SPSS software.

2.4.1. Evaluating the synergic effects

To assess the synergistic effects of ECT in combination with MNZ, we examined the Fractional inhibitory concentration index (FICI) by the following formula (Odds 2003):

$$FICI = (IC_{50}\text{in combination})/(IC_{50}\text{drugalone})$$

FICI $\leq$ 0.5 displayed the synergistic effects; whereas 0.5 < FICI < 1 and FICI < 4 exhibited the additive and antagonistic effects, respectively (Odds 2003).

2.4.2. Effects of ECT on plasma membrane permeability

The effect of the ECT on the permeability of the plasma membrane of *Giardia* trophozoites was investigated by the SYTOX ® Green assay. The study included the exposure of parasites to Triton X-100 and normal saline as positive and negative controls, respectively. The assessment of plasma membrane permeability was conducted over a 4-hour period using a microplate reader (BMG, CLARIOStar, Germany) (Ghasemian Yadegari et al., 2022).

2.4.3. The expression level of genes related to pathogenesis by real-time PCR

The impact of ECT at concentrations of 1/3 IC_{50}, 1/2 IC_{50}, and IC_{50} on the expression levels of adenylate cyclase (AK), extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) was investigated using real-time PCR. The procedure for isolating RNA from *G. lamblia* trophozoites treated with varying concentrations of ECT was conducted following the guidelines provided by the Qiagen extraction kit (Qiagen, Germany). Consequently, cDNA was synthesized using random primers following the manufacturer's protocol of the cDNA synthesis kit (Qiagen, Germany), and the resulting cDNA was used for SYBR Green real-time PCR. The thermal cycling program for the PCR reaction involved an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 95 °C for 1 min and 60 °C for 30 s. The $2^{-\Delta\Delta Ct}$ was determined using the iQTM5 optical system software (Bio-Rad, Hercules, CA), with beta-actin serving as the housekeeping gene. The oligonucleotide primers used for PCR are specified in Table 1 (Eligio-García et al., 2017).

Table 1
The primers were used for real-time PCR.

Gene	Sequence (5′–3′)
ERK1	F: CAAGATTCGAGCTGCCATCA
	R: GCAGTGTGCGTTGGCAATAA
ERK2	F: AAACAAAGTGCCGTGGAACAG
	R: GGATGGGCCAAAGCTTCTTC
AK	F: CAACGGACATGCTATCTGAG
	R: CCACTGCACAGTAAGTGCTG
IL-1β	F: AACCTGCTGGTGTGTGACGTTC
	R: CAGCACGAGGCTTTTTTGTTGT
TNF-α	F: TGAACTTCGGGGTGATCGGT
	R: GGTGGTTTGTGAGTGTGAGGG
IL-10	F: GCTGGTTATTGTGCTGTCTC
	R: CTCTAGGAGCATGTGGCTCT
GPx	F: CCAAGAGAACTGCAAGAACGA
	R: CAGGACA CGTCATTCCTACAC
SOD	F: GGTGCCCTGGAGCCCTA
	R: ATGCGAAGTCTTCCACTGTC
CAT	F: GGA CGC TCA GCT TTT CAT TC
	R: TTGTCCAGAAGAGCCTGGAT
β-actin	F: GTGACGTTGACATCCGTAAAGA
	R: GCCGGACTCATCGTACTCC

2.5. Effect of ECT on animal model of giardiasis

2.5.1. Animal

A total of 64 male BALB/c mice, aged 40–60 days and weighing 20–25 g, were used in this study. The mice were housed under standard conditions, including a temperature of 24 ± 1 °C, a 12-hour light/dark cycle, and a humidity range of 40–70 %, with access to water and food ad libitum. This experimental research was developed and approved by the Animal Ethics Committee of Lorestan University of Medical Sciences, Khorramabad, Iran (IR.LUMS.REC.1401.221).

2.5.2. Induction of animal model of giardiasis

To induce giardiasis in the animal model, 0.2 mL of *G. lamblia* cysts (20,000 cysts) was orally administered to the mice in each experimental group. The infection was monitored by daily microscopic examination of the mice from the day of inoculation until the presence of *G. lamblia* cysts was identified in their feces by the formalin-ether technique (Safar Harandi et al., 2007).

2.5.3. Experimental design and treatment

The animals were also managed according to the Guidelines for the Care and Use of Laboratory Animals. They were inadvertently divided into 8 groups, each consisting of 8 mice including:

i Infected mice orally treated with normal saline for 1 week
ii Infected mice orally treated with MNZ (15 mg/kg/day) for 1 week (Albalawi et al., 2023)
iii Infected mice orally treated with ECT (10 mg/kg/day) for 1 week
iv Infected mice orally treated with ECT (15 mg/kg/day) for 1 week
v Infected mice orally treated with ECT (10 mg/kg/day) + MNZ (7.5 mg/kg/day) for 1 week
vi Infected mice orally treated with ECT (15 mg/kg/day) + MNZ (7.5 mg/kg/day) for 1 week
vii Non-infected mice orally treated with ECT (10 mg/kg/day) for 2 weeks
viii Non-infected mice orally treated with ECT (15 mg/kg/day) for 2 weeks

2.5.4. Parasitological examination

After a 7-day treatment, fecal samples from mice were analyzed by the formalin-ether method to detect *G. lamblia* cysts and calculate the reduction ratio. Additionally, the viability of excretory cysts was assessed using 0.1 % eosin vital staining. Cysts showing eosin coloration were considered non-viable, while those without this coloration were deemed viable. Concurrently, the body weight of the infected mice was monitored during the study period (Al-Megrin, 2017).

2.5.5. Collecting intestinal tissues

After the treatment period, mice from each group (groups i to vi) were euthanized using a combination of ketamine and xylazine (100 mg/kg). The duodenum tissue was excised, longitudinally incised, and rinsed with a 0.9 % sodium chloride solution to remove any remnants of the digestive contents (Khedr et al., 2021). Next, the epithelial layer of each section of the intestine was removed, rapidly frozen in liquid nitrogen, and preserved at −80 °C for RNA extraction purposes.

2.5.6. The expression level of inflammatory and antioxidant related genes by real-time PCR

The total RNA was extracted using the Qiagen extraction kit (Qiagen, Germany). Afterwards, the cDNA was synthesized using random primers following the manufacturer's protocol of the cDNA synthesis kit (Qiagen, Germany). The resulting cDNA was then used for SYBR green Real-time PCR with inflammatory genes (e.g., TNF-α, IL-1β, and IL-10) and antioxidant genes (catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 2 (GPX2)) as listed in Table 1 (Shaapan et al., 2021). Initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 95 °C for 1 min and 60 °C for 30 s. The $2^{-\Delta\Delta Ct}$ value will be determined using the iQTM5 optical system software (Bio-Rad, Hercules, CA), with beta-actin serving as the housekeeping gene. The oligonucleotide primers used for PCR are specified in Table 1.

2.6. Sub-acute toxicity of ECT in the healthy mice

The sub-acute toxicity of ECT on the liver and kidney function of healthy mice was determined in groups vii and viii. After 14 days of treatment, the mice were euthanized using ketamine-xylazine (100 mg/kg) anesthesia (Mahmoudvand et al., 2017). Subsequently, their abdomens were opened, and blood samples were collected from their hearts (Mahmoudvand et al., 2019). After centrifuging the blood samples, the obtained serum samples were examined by diagnostic biochemical kits from Pars Azmoon, Iran to evaluate the serum levels of kidney function indicators (blood urea nitrogen (BUN) and creatinine (Cr)) and liver function indicators (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) (Ezzatkhah et al., 2021; Mahmoudvand et al., 2016).

2.7. Statistical analysis

In order to enhance precision, the in vitro experiments were replicated thrice. The gathered data underwent analysis utilizing SPSS software version 26.0 and one-way ANOVA. A significance level of $P < 0.05$ was ultimately established to indicate a statistically significant variance.

3. Results

3.1. In vitro and the synergic effects of ECT on G. lamblia trophozoites

The data shown in Fig. 1 indicate that ECT alone and in combination with MNZ meaningfully ($p < 0.001$) declined the viability and growth rate of *G. lamblia* trophozoites in a dose-dependent manner when compared to the control group. The IC_{50} values of ECT alone, MNZ alone, and ECT+ fixed MNZ concentration (15 μg/mL) on trophozoites were 30.2 ± 2.15, 21.6 ± 1.85, and 8.5 ± 0.66 μg/mL, respectively. The estimated FICI values for ECT and MNZ were 0.28 and 0.39, respectively, demonstrating the synergistic effects of ECT in combination with MNZ.

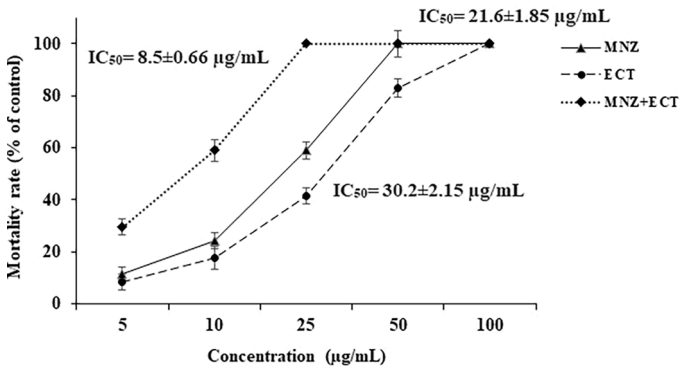

Fig. 1. In vitro effects of eucalyptol (ECT) at concentrations of 5–100 µg/mL, metronidazole (MNZ) at concentrations of 5–100 µg/mL, the combination of ECT at concentrations of 5–100 µg/mL with MNZ at 15 µg/mL on *G. lamblia* trophozoites by the cell viability MTT assay after 48 h incubation. The experiments were done in triplicates and that the 50 % inhibitory concentrations (IC_{50}) values were determined with the Probit test in SPSS software. Data are presented as Mean±sd.

3.2. Effects of ECT on plasma membrane permeability

The results indicated that treating *G. lamblia* trophozoites with ECT led to a dose-dependent increase, primarily at concentrations of ½ IC_{50} and IC_{50} ($P < 0.05$), in plasma membrane permeability, as demonstrated by elevated fluorescence levels (Fig. 2).

3.3. The expression level of genes related to pathogenesis by real-time PCR

As illustrated in Fig. 3, the gene expression of AK and ERK2 in the *G. lamblia* trophozoites group exhibited a dose-dependent decrease after exposure to ECT, particularly at concentrations of ½ IC_{50} and IC_{50}, compared to the group treated with normal saline ($p < 0.01$). The findings also indicated that the gene expression of ERK1 showed a reduction, although the decrease was not statistically significant compared to the control group.

3.4. Effect of ECT on animal model of giardiasis

After 7 days of administering ECT (alone and in combination with MNZ) to infected mice, the formalin-ether method was used to analyze mice feces for the presence of *G. lamblia* cysts and to determine the percentage reduction using eosin staining. As depicted in Fig. 4A, treatment with various doses of ECT, mainly in combination with MNZ (7.5 mg/kg/day) for 7 days, resulted in a significant decrease ($P < 0.001$) in the average number of cysts. Reductions of 97.2 %, 75.0 %, 90.4 %, 100 %, and 100 % were observed in groups treated with MNZ (15 mg/kg), ECT 10 mg/kg + MNZ, ECT 15 mg/kg + MNZ, ECT 10 mg/kg + MNZ, and ECT 15 mg/kg + MNZ, respectively. Additionally, Fig. 4B illustrates that the viability of *Giardia* cysts significantly declined ($P < 0.001$) after treatment with various doses of ECT, particularly in combination with MNZ for 7 days. The reduction in viability was observed at 96.0 %, 45.7 %, 87.1 %, and 100 % for MNZ (15 mg/kg), ECT 10 mg/kg + MNZ, ECT 15 mg/kg + MNZ, ECT 10 mg/kg + MNZ, and ECT 15 mg/kg + MNZ, respectively.

3.5. The expression level of inflammatory genes by real-time PCR

According to the results obtained from real-time PCR analysis by the $2^{-\Delta\Delta CT}$ method, it was noted that mice infected with *G. lamblia* showed increased levels of TNF-α and IL-6 gene expression and decreased levels of IL-10 gene expression ($p < 0.05$). However, when ECT was administered at doses of 10 and 15 mg/kg alone, and especially in combination with MNZ (7.5 mg/kg/day), there was a significant ($P < 0.001$) reduction in the expression of TNF-α and IL-6 genes and an increase ($P < 0.05$) in the expression of IL-10 genes (Fig. 5).

3.6. The expression level of antioxidant related genes by real-time PCR

In mice infected with *G. lamblia*, we observed a significant suppression ($p < 0.05$) in the gene expression levels of antioxidant enzymes CAT, SOD, and GPX. Conversely, when ECT was administered at doses of 10 and 15 mg/kg alone, and especially when combined with MNZ, there was a significant ($P < 0.001$) elevation in the gene expression of CAT, SOD, and GPX genes (Fig. 6).

3.7. Sub-acute toxicity of ECT in healthy mice

The results of the analysis of biochemical parameters of liver and kidney function showed that after receiving ECT at doses of 10 and 15 mg/kg for 2 weeks, despite some changes in these parameters, no significant difference was observed compared to the control group that received normal saline (Fig. 7).

4. Discussion

At present, drugs such as metronidazole, furazolidone, tinidazole, and quinacrine are used for the treatment of giardiasis (Escobedo and Cimerman, 2007). Although these medications are generally effective, they are linked to adverse reactions such as unpleasant taste, gastrointestinal discomfort, nausea, headache, leukopenia, neurotoxicity,

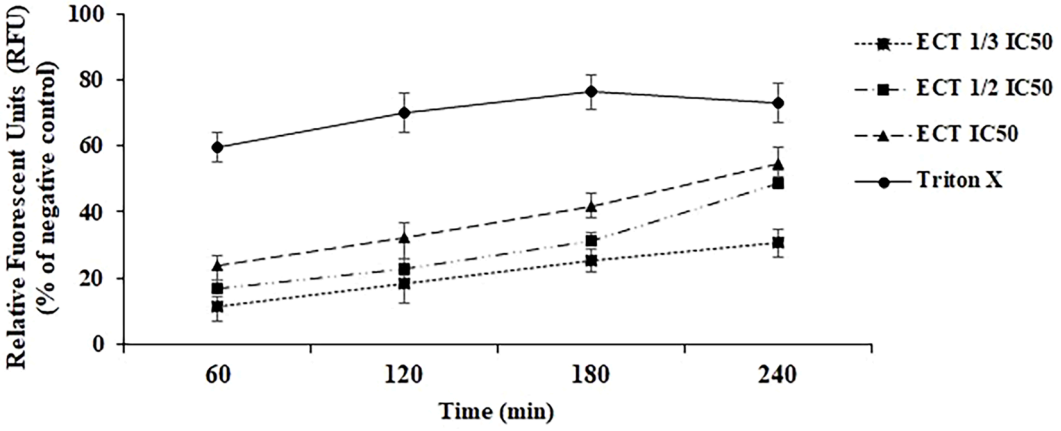

Fig. 2. Effects of eucalyptol (ECT) at 1/3, ½, and 50 % inhibitory concentrations (IC_{50}) on plasma membrane permeability of G. lamblia trophozoites by the SYTOX ® Green assay over a 4-hour period using a microplate reader. ($n = 3$), Mean±sd.

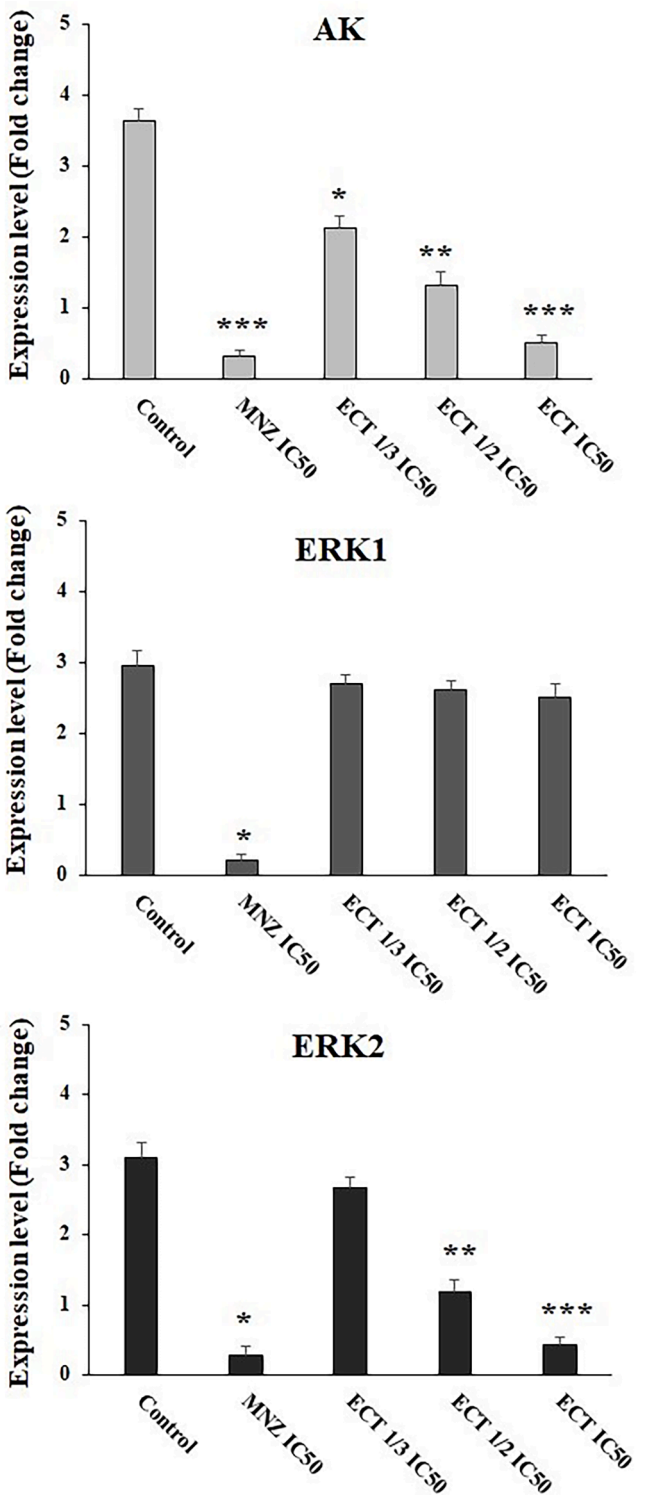

Fig. 3. Effects of eucalyptol (ECT) at 1/3, ½, and 50 % inhibitory concentrations (IC50) on gene expression level of adenylate cyclase (AK) and extracellular signal kinases 1 and 2 (ERK1 and ERK2) by the SYBR green Real-time PCR using the iQTM5 optical system software (Bio-Rad, Hercules, CA). * $p < 0.05$ compared to control normal saline; ** $p < 0.01$ compared to control normal saline; *** $p < 0.001$ compared to control normal saline; + $p < 0.05$ compared to MNZ alone.

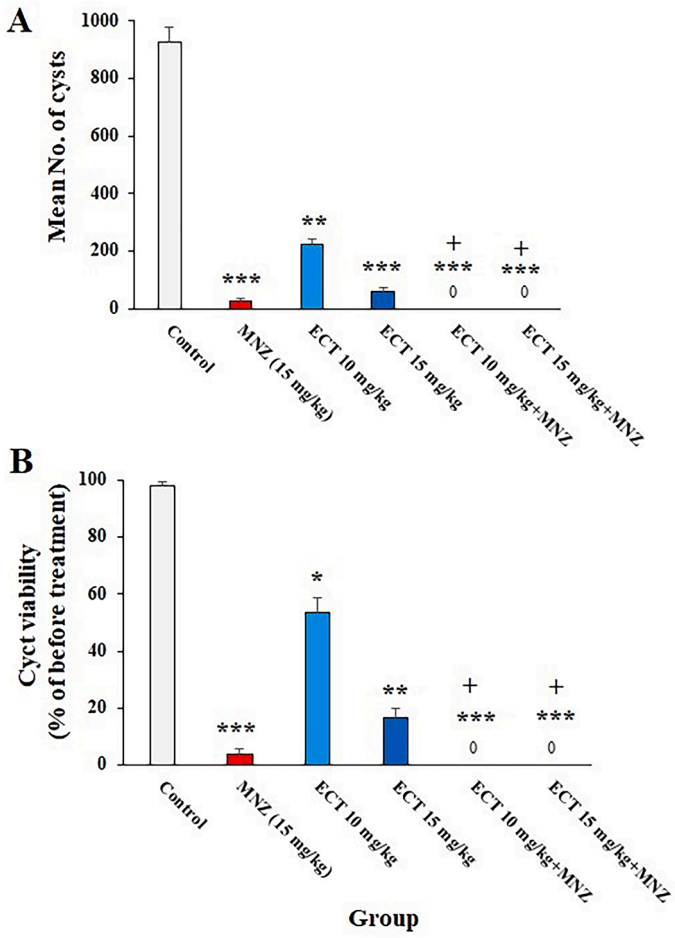

Fig. 4. In vivo effects of eucalyptol (ECT) and metronidazole (NNZ) alone and in combination on mean number of cysts of *Giardia lamblia* (A) and their viability (B) in mice infected with giardiasis after a 7-day treatment by the formalin-ether and vital 0.1 % eosin staining, respectively. * $p < 0.05$ compared to control normal saline; ** $p < 0.01$ compared to control normal saline; *** $p < 0.001$ compared to control normal saline; + $p < 0.05$ compared to MNZ alone.

restlessness, convulsions, and dizziness, which may hinder the therapeutic process. Additionally, certain drugs have been shown to have detrimental, carcinogenic, and mutagenic effects in laboratory animals (Lalle and Hanevik, 2018). Therefore, there is an urgent need to identify a medication with minimal adverse effects. In recent times, there have been studies on the potential anti-*Giardia* properties of essential oils derived from various herbs, including *Allium* spp., *Artemisia* spp., *Zataria* spp., *Eucalyptus* spp., *Ziziphora* spp., *Ferula* spp., and *Lavandula* spp., conducted in laboratory settings (in vitro) and animal models (in vivo). Nevertheless, the widespread use of herbal treatments for giardiasis is currently limited due to inconclusive research findings that may lack adequate statistical power, toxicity concerns, uncertainty regarding their mechanisms of action, and ambiguity surrounding their synergistic effects with common drugs (Alnomasy et al., 2021).

This study aimed to investigate the in vitro and in vivo effects of ECT alone and in combination with metronidazole on *G. lamblia*. Our results showed that the combination of ECT with MNZ significantly reduced the viability and growth rate of *G. lamblia* trophozoites in a dose-dependent manner compared to the control group. We also indicated that the FICI values for ECT and MNZ were 0.28 and 0.39, respectively, demonstrating the synergistic effects of ECT in combination with MNZ. By conducting an in vivo assay, we discovered that administering ECT, primarily in combination with MNZ, led to a significant decrease in the number and viability of *Giardia* cysts after 7 days. Prior research has

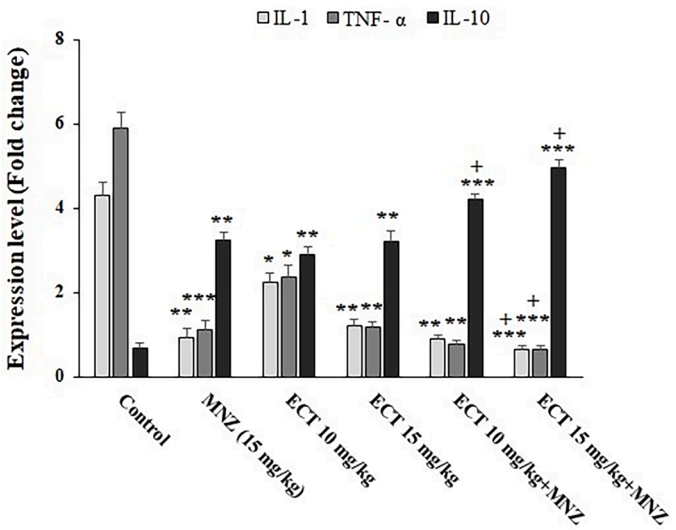

Fig. 5. In vivo effects of eucalyptol (ECT) and metronidazole (NNZ) alone and in combination on gene expression level of inflammatory (e.g., TNF-α, IL-1β, and IL-10) by SYBR green Real-time PCR using the iQTM5 optical system software (Bio-Rad, Hercules, CA). * $p < 0.05$ compared to control normal saline; ** $p < 0.01$ compared to control normal saline; *** $p < 0.001$ compared to control normal saline; + $p < 0.05$ compared to MNZ alone.

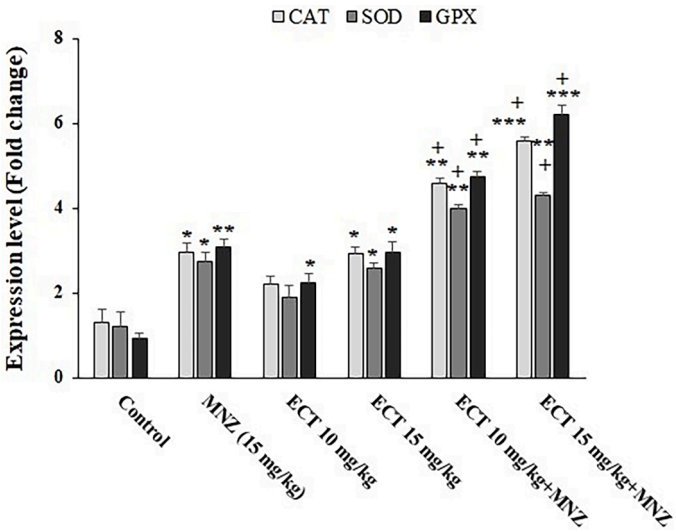

Fig. 6. In vivo effects of eucalyptol (ECT) and metronidazole (NNZ) alone and in combination on gene expression level of antioxidant (catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 2 (GPX2)) genes by SYBR green Real-time PCR using the iQTM5 optical system software (Bio-Rad, Hercules, CA). * $p < 0.05$ compared to control normal saline; ** $p < 0.01$ compared to control normal saline; *** $p < 0.001$ compared to control normal saline; + $p < 0.05$ compared to MNZ alone.

demonstrated that natural small molecules can serve as drug delivery systems to improve bioavailability, reduce toxicity, increase drug concentration, and prolong systemic circulation. This can enhance the efficacy of conventional drugs (Fu et al., 2023). With respect to the antiparasitic effects of ECT-rich herbs, AlGabbani et al. (2023) demonstrated that *Eucalyptus camaldulensis* essential oil, rich in ECT, at a concentration of 0.1 mg/mL, caused 100 % mortality in *G. lamblia* and *Entamoeba histolytica* trophozoites compared to the control group. Dehghani-Samani et al. (2019) revealed that *Eucalyptus globulus* essential oil exhibited promising anti-giardial activity (73.55 %) against cysts of *G. lamblia* clinical isolates after 480 min of exposure. Nosratabadi

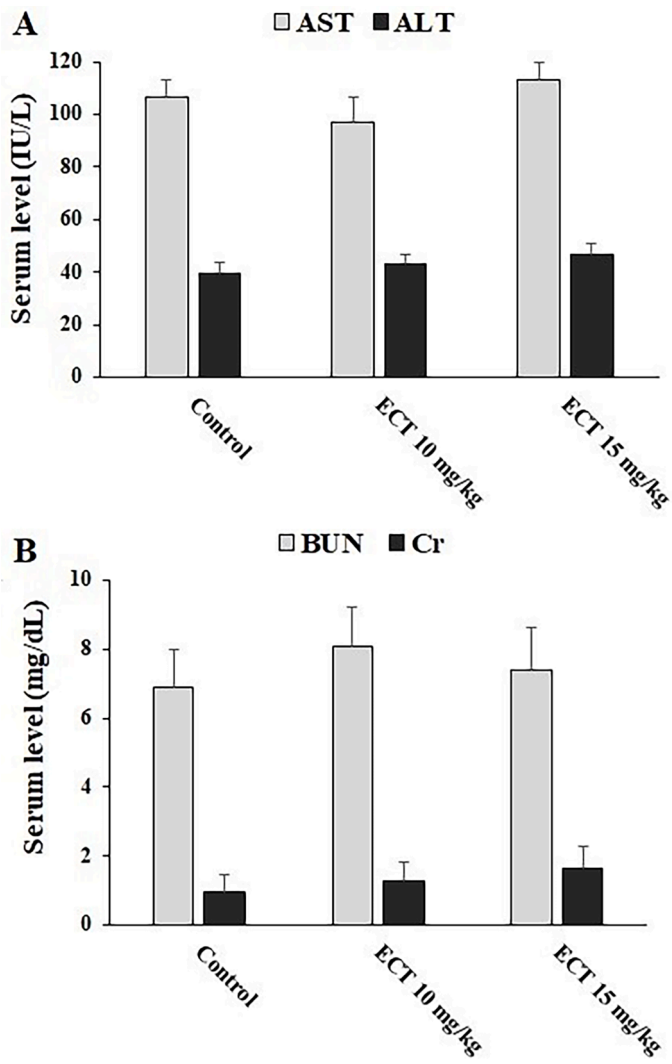

Fig. 7. The sub-acute toxicity of eucalyptol (ECT) on the liver and kidney function of healthy mice through evaluating the serum levels of (A) liver function indicators (Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and (B) kidney (blood urea nitrogen (BUN) and creatinine (Cr)) using diagnostic biochemical kits (Pars Azmoon, Iran).

et al. (2015) reported that treating Leishmania major promastigotes with *E. camaldulensis* methanolic and aqueous extracts for 72 h resulted in promising antileishmanial effects, with IC_{50} values of 586.2 and 1108.6 μg/mL, respectively. Hassani et al. (2013) also reported that the ethyl acetate and diethyl ether extracts of *E. camaldulensis*, at concentrations of 12.5 and 25 mg/ml after 24 h of exposure, resulted in 100 % mortality in *Trichomonas vaginalis* trophozoites. Reviews have also reported the anti-giardial effects of various essential oil derivatives, such as carvacrol, cinnamaldehyde, eugenol, limonene, linalool, and piquerol, with IC_{50} values ranging from 2.42 to 342.2 μg/mL. These effects are achieved through membrane damage, cytoplasmic leakage, inhibition of cysteine proteases, and induction of apoptosis-like death (Menezes and Tasca, 2023).

Several studies have shown that ECT exhibits potent antimicrobial effects against Gram-positive bacteria, Gram-negative bacteria, viruses, and fungal strains (Cai et al., 2021). Previous studies showed the potent anti-*Giardia* effects of some terpene compounds such as geraniol (Dai et al., 2016), limonene (Andrade-Ochoa et al., 2021), linalool (de Almeida et al., 2007), thymol and carvacrol (Andrade-Ochoa et al., 2021) against the *G. lamblia* parasites with IC_{50} values ranging from 31.9 to 300 μg/mL. In terms of the antimicrobial mechanisms of action of

monoterpenes, studies have reported that these compounds exhibit efficacy by affecting cell membrane permeability, disrupting DNA synthesis, causing leakage of proteins and nucleic acids, producing reactive oxygen species, inducing oxidative stress, and triggering apoptosis (Trombetta et al., 2005).

Currently, a primary cellular mechanism for inhibiting pathogenic microorganisms involves disrupting and impacting the permeability of the plasma membrane (Houthaeve et al., 2022). The results indicated that the application of ECT on *G. lamblia* trophozoites resulted in a dose-dependent increase in plasma membrane permeability. It has been proven that the AK gene is involved in cyclic nucleotide biosynthesis and intracellular signal transduction. Additionally, two members of the mitogen-activated protein kinase (MAPK) family, ERK1 and ERK2, are the genes involved in the differentiation of trophozoites from cysts (Murtagh et al., 1992; Ellis et al., 2003). Here, we found that the gene expression of AK and ERK2 in the *G. lamblia* trophozoites group exhibited a dose-dependent decrease after exposure to ECT, whereas the gene expression of ERK1 showed a reduction, although the decrease was not statistically significant when compared to the control group. This suggests that ECT suppressed the process of DNA synthesis in *G. lamblia* and effectively reduced the formation of cysts in a laboratory setting, indicating its potential to diminish the parasite's ability to infect.

Studies have shown that *G. lamblia* triggers an inflammatory reaction, resulting in the production of IL-6 and TNF-α through various signaling pathways, such as nuclear factor κB p65 (NF-κB p65), p38, and ERK pathways (Pu et al., 2021). Previous studies have demonstrated that ECT exhibits anti-inflammatory effects by reducing dextran sodium sulfate (DSS)-induced colonic inflammation in mice. This reduction is achieved through the downregulation of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and IL-17 (Venkataraman et al., 2023). Furthermore, Lima et al. (2013) reported that the treatment of mice with acute pancreatitis using ECT significantly reduced inflammation by decreasing the levels of cytokines TNF-α, IL-1β, and IL-6, while increasing the level of IL-10. Our findings revealed that in mice infected with *G. lamblia*, when ECT was administered at doses of 10 and 15 mg/kg alone, and especially when combined with MNZ, there was a significant reduction in the expression of TNF-α and IL-6 genes, and an increase in the expression of IL-10 genes. The results suggest that ECT may control giardiasis in mice by suppressing inflammatory responses.

Previous evidence indicates that human infections have the potential to disrupt the natural balance within the host by causing an imbalance between the increased generation of reactive oxygen species (ROS) and decreased antioxidant host responses. This imbalance collectively leads to an increase in redox stress (Gain et al., 2023). Reportedly, parasitic infections, such as giardiasis, have been suggested to serve as a catalyst or enhancer of oxidative stress, especially in children with weakened immune systems (Mastronicola et al., 2016). Therefore, finding a new drug to treat these infections, especially one with high antioxidant potential, can be considered a novel strategy for controlling and treating giardiasis. We found that in mice infected with *G. lamblia*, there was a significant increase in the gene expression of CAT, SOD, and GPX genes. The results suggest that ECT may control giardiasis in mice by suppressing inflammatory responses. These results suggest the promising antioxidant effects of ECT for the treatment of giardiasis.

Referring to the ECT's toxicity profile, a previous study reported that in healthy mice, subacute toxicity was observed at doses of 600 mg/kg body weight and higher, as evidenced by decreased body weight and the development of liver and kidney lesions; whereas, no evidence of chronic or genotoxic effects associated with 1,8-cineole has been detected (De Vincenzi et al., 2002). The analysis of biochemical parameters related to liver and kidney function revealed that after receiving ECT at doses of 10 and 15 mg/kg for 2 weeks, there were some changes in these parameters. However, no significant difference was observed compared to the control group that received normal saline. These findings demonstrate that the use of ECT in these doses, even for 14 days, does not have any toxic effects on the function of vital liver and kidney tissues. As the main limitations of the study, we can point to the lack of clarity about the direct effects of the ECT on the *G. lamblia* parasite in the animal and a more detailed examination of the toxicity of the ECT in the animal

5. Conclusion

The study findings confirmed the promising in vitro and in vivo effects of ECT against *G. lamblia* infection. We also found the synergistic effect of ECT with MNZ; suggested that ECT combined with MNZ may be a promising strategy to combat against *G. lamblia* infection. Considering the possible mechanisms, ECT increased plasma membrane permeability and decreased the expression levels of infectivity-related genes. In addition, ECE suppresses inflammation and oxidative stress to control giardiasis in mice. More studies are needed to clarify these findings.

CRediT authorship contribution statement

Leila Masoori: Writing – review & editing, Methodology, Investigation, Conceptualization. **Amal Khudair Khalaf:** Writing – review & editing, Validation, Investigation. **Fatemeh Ezzatkhah:** Validation, Methodology, Investigation, Data curation. **Rafael Balaña-Fouce:** Validation, Investigation. **Hossein Mahmoudvand:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest in the present study.

Data Availability

No data was used for the research described in the article.

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