



Antiparasitic effect of limonene, a monoterpene compound found in the essential oils against *Giardia lamblia*

Ali Kharazmkia¹, Javad Ghasemian Yadegari¹, Mohamad Mohamadian², Rehman Ali³, Hossein Mahmoudvand^{4*}

¹Razi Herbal Medicines Research Center, School of Pharmacy, Lorestan University of Medical Sciences, Khorramabad, Iran

²Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran

³Department of Zoology, Faculty of Biological Sciences, Kohat University of Science and Technically Kohat-26000, Khyber Pakhtunkhwa, Pakistan

⁴Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran

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ABSTRACT

Introduction: The present work aimed to assess the anti-parasitic effects of limonene (LMN) against *Giardia lamblia* trophozoites and cysts.

Methods: Anti-Giardia activities of LMN (25, 50, and 100 µg/mL) were assessed against *G. lamblia* cysts and trophozoites for 15-360 minutes. The effects of LMN on the apoptosis stimulation *G. lamblia* parasites were determined by colorimetric protease assay.

Results: *Giardia* trophozoites were more sensitive to LMN than cysts. The best effect of LMN was seen at 100 µg/mL. LMN markedly triggered caspase-3 activity by 10.2%, 25.3%, and 36.1%, respectively ($P < 0.001$).

Conclusion: We found the potent *in vitro* anti-giardia activity of LMN against *G. lamblia* parasites with the maximum activity at 50 and 100 µg/mL. However, additional surveys are necessary to reveal the specific efficacy, mechanisms, and safety of LMN.

Implication for health policy/practice/research/medical education:

This work revealed the potent *in vitro* anti-Giardia activity of limonene (LMN) against *G. lamblia* with the maximum activity at 50 and 100 µg/mL. We also showed that induction of apoptosis could be considered as one of the cellular mechanisms of action of limonene; nevertheless, additional surveys are necessary to consider it as a new drug.

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Introduction

Giardia lamblia is a flagellum protozoan of the human intestine that has been reported all over the world (1). The prevalence of its infection called "giardiasis" varies from 1% to 25% in various parts of the world; whereas it is estimated that 280 million human infections occur worldwide each year (2). Giardiasis is mostly found in tropical and sub-tropical climates and can cause watery gastrointestinal disorders, and malabsorption, especially in children (3). Currently, a number of synthetic drugs, e.g., metronidazole, furazolidone, and quinacrine are prescribed for treating giardiasis. However, recent reports have shown that these drugs are accompanied by several adverse side effects, e.g., neurotoxicity, gastrointestinal disorders, unpleasant taste in the mouth, nausea,

leukopenia, as well as carcinogenic and mutagenic effects (4). Hence, it is essential to discover an effective anti-Giardia agent with minimal complications and maximum efficacy against giardiasis.

The custom of herbs and their derivatives for giardiasis therapy is increasing in recent years due to little or no side effects (5). Limonene (LMN, C₁₀H₁₆), a monoterpene hydrocarbon, is one of the most frequent combinations found in the essential oils of aromatic herbs (6). LMN has several pharmacological properties, e.g., anti-inflammatory, analgesic, antioxidant, and anti-cancer effects (7). In addition, its antimicrobial effects against various pathogenic bacteria, fungi (e.g., *Candida* spp), viruses (e.g., COVID-19), and parasites (e.g., *Plasmodium* spp) (8) have been reported. The present work aimed to

*Corresponding author: Hossein Mahmoudvand,
Email: dmahmodvand@gmail.com

assess the anti-parasitic effects of LMN against *Giardia lamblia* trophozoites and cysts.

Materials and Methods

Giardia lamblia cyst collection and isolation

Cysts were obtained from fecal specimens of giardiasis patients who were referred to health centers in Khorramabad city, Iran. The specimens were then examined through direct and formalin-ether methods (9). In the next step, the sucrose 0.85 M gradient technique was used to concentrate *Giardia* cysts (10). The specimens were then diluted with distilled water (12:1) and shaken for 5 minutes to obtain an aqueous solution. The upper phase was gradually added to sucrose solution (0.85 M). After centrifuging, the cysts were obtained in the mid layer by means of a Pasteur pipette and were used to at 1×10^5 cysts/mL.

Giardia lamblia trophozoites collection

Giardia trophozoites were obtained based on the technique defined by Bingham and Meyer (11) by the aqueous hydrochloric acid solution and TYI-S-33 media supplemented with 20% fetal calf serum, pen/strep (500 µg/mL).

Anti-*Giardia* effects of limonene

To perform the in vitro anti-*Giardia* activity, LMN at 25, 50, and 100 µg/mL (100 µL) was mixed with cysts and trophozoites suspension (100 µL) in micro-tubes and the mixture was kept at 37 °C for 15-360 minutes (11). Following the incubation time and removing the supernatant, 0.1% eosin stain solution (50 µL) (Sigma-Aldrich, Germany) was mixed with the residual cysts and trophozoites. After preparing the smears, the viable cyst and trophozoites were checked by a light microscope (400x magnification).

Induction of the caspase-3 activity

The effect of LMN on the induction of the caspase-3 activity of trophozoites was measured through incubating

the trophozoites with various concentrations of LMN, based on the protease caspase-3 kit (Sigma-Aldrich, Germany) (12).

Statistical analysis

All examinations were done three times. The findings were investigated by the SPSS software, version 26.0 and were compared by One-way analysis of variance (ANOVA). $P < 0.05$ was reflected statistically significant.

Results

Effect of limonene on *Giardia lamblia* cysts

Figure 1 shows that LMN significantly ($P < 0.001$) reduced the viability of *G. lamblia* cysts. Based on the results, the maximum effect of LMN was reported at 100 µg/mL; this concentration after 120 minutes, destroyed *Giardia* cysts ($P < 0.001$). However, this compound at 50 µg/mL after 240 minutes destroyed all *Giardia* cysts ($P < 0.001$). Among the studied concentrations of LMN, the lowest efficiency was related to 25 µg/mL; after 360 minutes of incubation, it was able to eradicate 94.3% of *G. lamblia* cysts.

Effect of limonene on *Giardia lamblia* trophozoites

The findings revealed that *Giardia* trophozoites were more sensitive to LMN than cysts (Figure 2). Based on the results, the maximum effect of LMN was reported at 100 µg/mL; this concentration after 60 minutes, destroyed *Giardia* trophozoites ($P < 0.001$). LMN also at 50 and 25 µg/mL after 120 and 240 minutes killed all *Giardia* trophozoites ($P < 0.001$).

The caspase-3 activity of trophozoites

Figure 3 shows LMN at 2.5, 5, and 10 µg/mL markedly triggered caspase-3 activity by 10.2%, 25.3%, and 36.1%, respectively ($P < 0.001$).

Discussion

There are a number of pharmaceutical medications that are prescribed for treating of the *Giardia* infection, including nitroimidazoles (e.g., metronidazole, tinidazole)

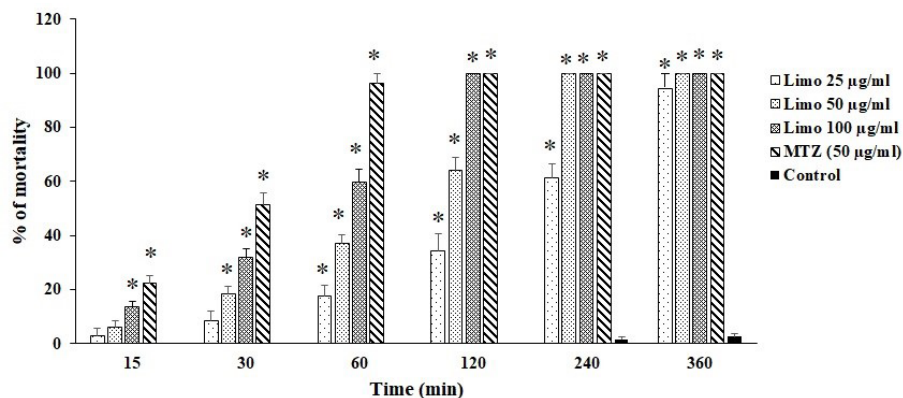


Figure 1. Effects of limonene on *Giardia lamblia* cysts in different time compared with the metronidazole (mean \pm SD). * $P < 0.05$, a significant difference compared with the control group. MTZ, metronidazole; Limo, limonene.

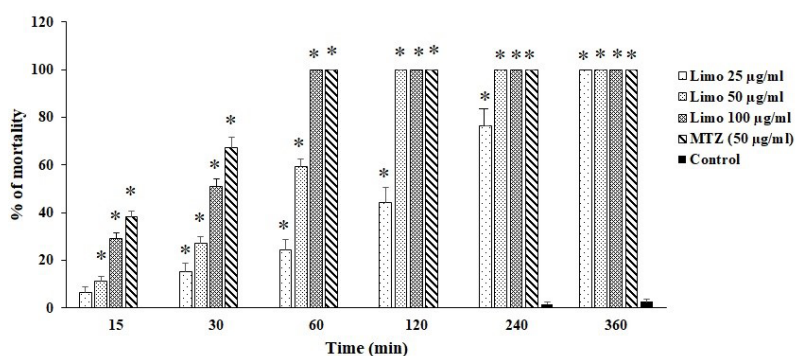


Figure 2. Effects of limonene on *Giardia lamblia* cysts in different time compared with the metronidazole (mean \pm SD). * $P < 0.05$, a significant difference compared with the control group. MTZ, metronidazole; Limo, limonene.

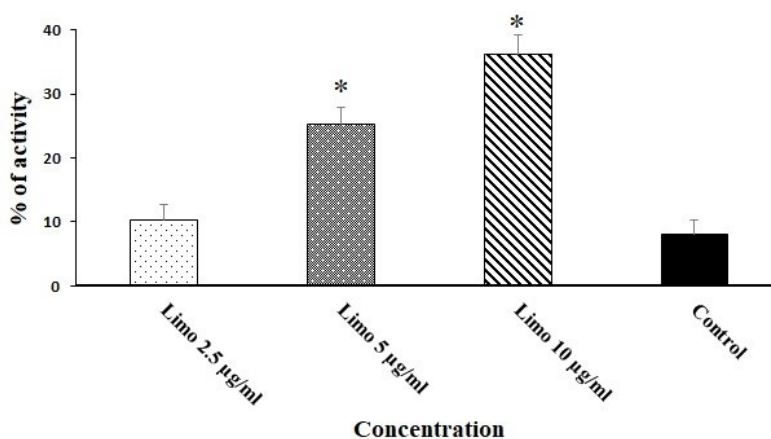


Figure 3. The effects of limonene on the caspase-3 activity of trophozoites (mean \pm SD). * $P < 0.05$, a significant difference compared with the control. Limo, limonene.

and benzimidazoles (e.g., mebendazole and albendazole), quinacrine, and furazolidone (4). These agents cause adverse effects such as neurotoxicity, gastrointestinal disorders, nausea, leukopenia, carcinogenic, and mutagenic effects, as well as the incidence of drug resistance (4,5). Essentially research investigations have focused on the search of novel and more effective drugs against giardiasis in endemic areas, especially by using natural products such as medicinal plants. Therefore, this work aimed to assess the antiparasitic effects of limonene against *G. lamblia*. Our findings revealed that *Giardia* trophozoites were more sensitive to LMN than cysts. Based on the results, the maximum effect of LMN was reported at 100 $\mu\text{g}/\text{mL}$; this concentration after 60 and 120 minutes, destroyed *Giardia* trophozoites and cysts ($P < 0.001$).

Previous studies demonstrated the potent antimicrobial properties of LMN on bacteria, fungi (e.g., *Candida* spp), viruses (e.g., COVID-19), and parasites (e.g., *Plasmodium* spp) (7,8). With respect to the antiparasitic effects of LMN, Arruda et al have reported potent *in vitro* leishmanicidal activity of LMN on promastigotes and amastigotes forms of *Leishmania amazonensis* with 50% inhibitory concentrations (IC_{50}) of 252 and 147 μM , respectively. They also showed that topically or

intrarectally therapy of *L. amazonensis*-infected mice with LMN significantly declined the parasite load and the size of the lesion (13). Moura et al have also reported that LMN by inhibiting isoprenylation, an important protein variation in eukaryotic cells, markedly reduced the parasites' development stage, the ring form to the trophozoite form; so that at the first cycle, 50% of the parasites killed (14). de Moraes et al revealed that LMN at 25 $\mu\text{g}/\text{mL}$ after 120 h significantly reduced the motility of *Schistosoma mansoni* adult worms and killed all worms *in vitro* (15).

Among the cellular mechanisms effective in controlling and eliminating microbial agents, the programmed cell death (apoptosis) is well-known as one of the critical molecular mechanisms (16). Caspases are principal mediators involved in the apoptosis of cells. Among caspases, caspase-3 activity shows a main role in assessing the rate of cell apoptosis (17). In this investigation, we revealed that LMN at 2.5, 5, and 10 $\mu\text{g}/\text{mL}$ markedly triggered caspase-3 activity by 10.2%, 25.3%, and 36.1%, respectively ($P < 0.001$). In view of the antimicrobial mechanisms of LMN, although the precise mechanism actions of this monoterpene have not been confirmed; however, previous studies reported that monoterpeneoid

components displayed antimicrobial activity through various mechanisms, e.g., membrane extension, increased membrane fluidity and permeability, respiration inhibition, modification of ion transport processes, induction of apoptosis, and increasing the protein leakage (18-20).

Conclusion

The results of the current investigation presented the potent *in vitro* anti-giardia activity of LMN against *G. lamblia* with the maximum activity at 50 and 100 µg/mL. We also showed that induction of apoptosis can be considered as one of the cellular mechanisms of action of LMN; nevertheless, additional surveys mainly in animal models are necessary to reveal the specific efficacy, mechanisms, and safety of LMN.

Authors' contribution

AK supervised the study, HM, MM, JGY, and RA reviewed and contributed to data collection and preparation of the manuscript. The first draft was prepared by HM and AK. All authors read the final version and confirmed it for publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

This project was approved by the ethics committee of Lorestan University of Medical Sciences with the ethics ID IR.LUMS.REC.1401.065.

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