



Comparative Investigation of the Inhibitory and Lethal Effects of Trigonelline and Glucantime Compounds on the Promastigote and Amastigote Stages of *Leishmania major*

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Abstract

Background and aims: The search for naturally derived pharmaceuticals with enhanced efficacy and reduced side effects for the treatment of leishmaniasis, a zoonotic infectious disease, has become a significant focus of research. This study aimed to evaluate the inhibitory and lethal effects of trigonelline, a natural alkaloid, in comparison to glucantime, a synthetic drug. Additionally, the study sought to assess the potential synergistic effects of these compounds on the promastigote and amastigote stages of *Leishmania major* (MRHO/IR/75/ER) under in vitro conditions.

Methods: In this experimental study, *L. major* promastigotes and amastigotes were cultured alongside mouse macrophages (J774) and treated with the compounds under investigation. Cell viability was assessed using the trypan blue exclusion test, while metabolic activity was measured through the MTT colorimetric assay. Additionally, the inhibition of amastigotes within macrophages was evaluated using Giemsa staining.

Results: Trigonelline, as a natural compound, significantly reduced the survival rate of promastigotes at concentrations of 25, 50, 100, and 200 µg/mL ($P < 0.001$). Furthermore, trigonelline enhanced the anti-leishmanial efficacy of glucantime against both the promastigote and amastigote stages at lower doses ($P < 0.001$). The cytotoxicity (CC₅₀) for macrophages treated with glucantime was determined to be 349.1 µg/mL, while for trigonelline, it was 1863.3 µg/mL. In the case of the combination treatment, the CC₅₀ was found to be 476.3 µg/mL.

Conclusion: Trigonelline demonstrated a synergistic effect with glucantime, enhancing its anti-leishmanial activity. Given its antioxidant properties, lower cytotoxicity to macrophage cells, and potential to reduce the required dosage of glucantime in combination therapy, trigonelline represents a promising option for the treatment of leishmaniasis.

Keywords: Leishmaniasis, Anti-parasitic effects, Synergistic effects, Macrophages, Inflammation, Natural compounds

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Introduction

Leishmaniasis is a significant non-communicable parasitic disease that poses severe health impacts and mortality, affecting over 80 countries, primarily in tropical and subtropical regions. It is recognized as the third most important disease among the six priority infectious and tropical diseases identified by the World Health Organization (WHO), owing to its substantial global public health burden (1-3). This disease is caused

by the obligate intracellular protozoan *Leishmania* and is transmitted to humans through the bites of infected sandflies. Approximately 350 million people are at risk of contracting leishmaniasis worldwide (4,5). Leishmaniasis manifests in several forms, including cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis. Annually, it is estimated that there are between 1 to 1.5 million new cases of cutaneous leishmaniasis and approximately 0.5 million cases of

visceral leishmaniasis (6). Mucocutaneous leishmaniasis, which is confined to specific regions in Central and South America, affects fewer individuals (7). In Iran, cutaneous leishmaniasis is prevalent in both rural and urban settings, with additional foci of Mediterranean-type visceral leishmaniasis (8,9).

The management of leishmaniasis poses significant challenges due to factors such as high medication costs, large required dosages, drug resistance, side effects, and the scarcity of new, affordable treatments (10). Current first-line therapies include glucantime and pentostam, both of which have been in use for over 60 years. Additionally, other medications such as miltefosine, pentamidine, and amphotericin B are utilized, despite their associated toxicity and the emergence of resistance (11). Given these challenges, there is an urgent need for effective, cost-efficient treatments with minimal side effects (12).

Plant-derived products are increasingly being investigated for their therapeutic potential due to their lower costs, reduced side effects, and quicker availability. These natural compounds have demonstrated promise in the treatment of various diseases, including leishmaniasis (13). Trigonelline, an alkaloid present in several plants such as coffee, fenugreek, soybeans, chickpeas, and alfalfa, is recognized for its diverse medicinal properties. These properties include antimicrobial effects against bacteria, viruses, and parasites, as well as anti-cancer, migraine-relieving, disinfectant, anti-diabetic, and cholesterol-lowering activities. Additionally, traditional Chinese medicine has utilized trigonelline for its therapeutic benefits (14-17).

This study aims to investigate the anti-leishmanial effects of trigonelline in comparison to glucantime and to evaluate their potential synergistic effects on the promastigote and amastigote stages of *L. major*.

Materials and Methods

Materials and reagents

Dimethyl sulfoxide (DMSO), MTT reagent, Trypan blue dye, and Giemsa stain were procured from Merck (Darmstadt, Germany). Glucantime was obtained from Sanofi-Aventis (France). All reagents used were of analytical grade and were utilized according to the manufacturer's instructions. Trigonelline was purchased from Sigma-Aldrich (Munich, Germany) and prepared at the desired concentrations using distilled water. Glucantime was prepared from ampoules containing 1.5 grams of the active ingredient, which was dissolved in 5 milliliters of solution to achieve the required treatment concentrations.

Parasite and cell line preparation

In this experimental study, *L. major* (MRHO/IR/75/ER) promastigotes and amastigotes were obtained from the Leishmaniasis Research Center at Kerman University of Medical Sciences. J774-A1 mouse macrophage cells were sourced from female BALB/C mice provided by the

Pasteur Institute of Iran.

Experimental design and sample size

The sample size and experimental design were established based on similar studies and statistical guidance. Both developmental stages of *L. major* (promastigotes and amastigotes) were exposed to five concentrations of trigonelline and glucantime (12.5, 25, 50, 100, and 200 µg/mL) in triplicate (18). Data were collected through systematic observations and recorded in standardized checklists.

Culture and treatment procedures

Promastigotes and amastigotes of *L. major* and J774-A1 macrophages were cultured under standard conditions (18). Cell viability was assessed using the Trypan blue exclusion test. Metabolic activity was evaluated using the MTT colorimetric assay, while the anti-amastigote effect within macrophages was examined using Giemsa staining. For the viability assessment, cells were mixed with Trypan blue dye in equal proportions, and the total number of cells was counted using a Neubauer chamber. The viability was calculated using the following formula:

$$\text{Viability} = (\text{Number of live cells} / \text{Total number of cells}) \times 100$$

MTT assay

Promastigote cells of *L. major* were treated with trigonelline, glucantime, or their combination at various doses. The cells were plated at a density of 5×10^5 cells per well in a 96-well plate. After 72 hours of treatment, 100 µL of MTT solution (0.5 mg/mL in filtered phosphate-buffered saline [PBS]) was added to each well. The plates were then incubated for 4 hours at 37 °C, followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was carefully removed, and the purple formazan crystals were dissolved in 100 µL of DMSO. The plates were then covered with aluminum foil and shaken for 15 minutes to facilitate color development.

Giemsa staining

To evaluate the effects against amastigotes, J774-A1 macrophages were cultured and counted. Slides containing 50 000 macrophages in 100 µL were incubated at 37 °C in a 5% CO₂ atmosphere for 4 hours. Following the removal of the supernatant, promastigotes (250 000 per 100 µL) were added to the slides, while control slides received no parasites. The slides were then incubated for 24 hours. Drug dilutions were added to the slides under sterile conditions, with controls including macrophages without parasites or drugs (negative control) and macrophages with parasites but no drugs (positive control). After 72 hours, the slides were fixed with methanol, stained with Giemsa, and examined under a microscope. The number of infected macrophages and the number of amastigotes per macrophage were counted in a total of

100 macrophages.

Data analysis

Data were analyzed using SPSS version 20. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was employed to assess differences between groups. The half-maximal IC₅₀ and half-maximal CC₅₀ values were determined using the probit test in SPSS. Results are expressed as mean $\pm$ standard deviation, with statistical significance defined at $P < 0.05$.

Results

Activity against promastigotes

The survival rates of *L. major* promastigotes treated with various concentrations of glucantime and trigonelline were evaluated (Figure 1). Trigonelline significantly reduced the survival rate of promastigotes at all tested concentrations (25, 50, 100, and 200 $\mu\text{g}/\text{mL}$), with $P < 0.001$ for each concentration. Similarly, glucantime also significantly decreased promastigote survival at the same concentrations ($P < 0.001$ for all). Notably, the combination treatment of trigonelline and glucantime resulted in a significantly greater reduction in promastigote survival compared to individual treatments at each concentration ($P < 0.001$). All treatments with trigonelline, glucantime, and their combination at 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$ led to significantly reduced survival rates compared to the negative control group ($P < 0.001$).

Activity against amastigotes

The effects of trigonelline and glucantime on amastigotes were assessed at concentrations of 12.5, 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$. Both compounds significantly reduced the number of amastigotes within infected macrophages at concentrations of 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$ compared to the negative control ($P < 0.001$) (Table 1). Notably, the combination treatment demonstrated enhanced efficacy, with the number of amastigotes reaching zero at a concentration of 100 $\mu\text{g}/\text{mL}$.

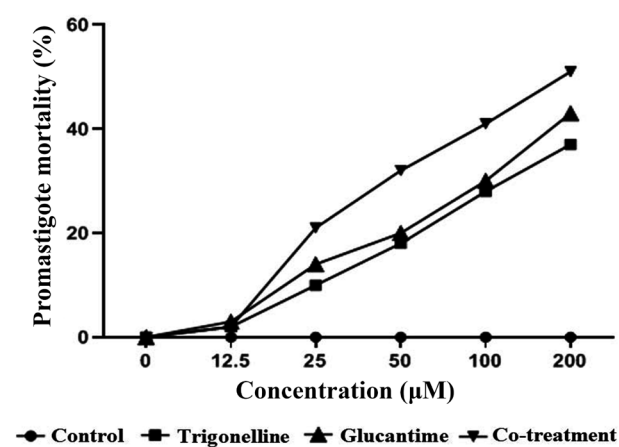


Figure 1. Mortality rates of *L. major* promastigotes treated with various concentrations of trigonelline and glucantime, both individually and in combination, compared to the control group

Cellular toxicity

Cellular toxicity was assessed for both amastigotes and promastigotes. The half-maximal IC₅₀ for trigonelline against amastigotes was 109.3 ± 12.1 $\mu\text{g}/\text{mL}$, while for glucantime it was 47.4 ± 8.3 $\mu\text{g}/\text{mL}$, indicating a significant difference between the two ($P < 0.001$) (Table 2). The IC₅₀ for the combined treatment was 36.3 ± 2.2 $\mu\text{g}/\text{mL}$, which was also significantly different from the individual treatments ($P < 0.001$).

For promastigotes, the half-maximal IC₅₀ for trigonelline was 412.3 ± 21.3 $\mu\text{g}/\text{mL}$, while for glucantime it was 318.5 ± 28.6 $\mu\text{g}/\text{mL}$, indicating a significant difference between the two ($P < 0.001$). The IC₅₀ for the combined treatment was 217.6 ± 8.4 $\mu\text{g}/\text{mL}$.

The CC₅₀ values for macrophages were 349.1 $\mu\text{g}/\text{mL}$ for glucantime, 1863.3 $\mu\text{g}/\text{mL}$ for trigonelline, and 476.3 $\mu\text{g}/\text{mL}$ for the combined treatment. The Selectivity Index (SI) values for glucantime, trigonelline, and their combination were 6.53, 14.69, and 13.1, respectively. All SI values exceeded the safety threshold ($\text{SI} \geq 1$), indicating that none of the treatments exhibited lethal effects.

The combination index (CI) for the trigonelline and glucantime combination was 0.966, suggesting a synergistic effect, as the value is less than 1.

Discussion

This study aimed to evaluate the inhibitory and lethal effects of trigonelline, a natural alkaloid, in comparison to glucantime, a synthetic drug, as well as their synergistic effects on the promastigote and amastigote stages of *L. major* (MRHO/IR/75/ER) under in vitro conditions. The investigation into the effects of trigonelline, glucantime, and their combination on the growth and proliferation of promastigote and amastigote cells of *L. major* revealed that increasing drug concentrations, particularly in combination treatments, enhanced the anti-leishmanial properties against both stages of the parasite. Notably, the cytotoxic effects on mouse macrophages did not show a significant increase. Additionally, the SI calculated for trigonelline was found to be favorable. While various studies have evaluated the impact of glucantime on the promastigotes of different *Leishmania* species (19-22), there is limited research on the anti-parasitic effects of trigonelline. However, studies have investigated its anti-inflammatory effects, antimicrobial properties, anti-cancer impacts, and blood sugar-lowering effects (23-27).

This study demonstrated that trigonelline at concentrations of 25, 50, and 100 $\mu\text{g}/\text{mL}$ effectively inhibited the growth of *L. major* promastigotes compared to the control group. Similarly, glucantime also exhibited inhibitory effects on the parasite at these concentrations. However, the use of combined concentrations of glucantime and trigonelline significantly enhanced the anti-parasitic effectiveness of this combination, suggesting that it may exert its anti-parasitic effects at lower concentrations of glucantime. This indicates a

Table 1. Effect of different concentrations of trigonelline and glucantime, administered alone and in combination, on the mean number of amastigotes per macrophage

Treatments concentrations (µg/mL)	Trigonelline		Glucantime		Co-treatment	
	Mean±SD (µM)	P value	Mean±SD (µM)	P value	Mean±SD (µM)	P value
0	46.9±7.2	NS	46.9±7.2	NS	46.9±7.2	NS
12.5	32.9±6.9	NS	45.9±6.7	NS	44.9±5.6	NS
25	28.4±5.4	<0.001	22.4±3.6	<0.001	18.7±3.6	<0.001
50	24.1±4.2	<0.001	19.1±4.3	<0.001	9.1±1.4	<0.001
100	19.1±2.9	<0.001	14.8±3.4	<0.001	0	<0.001
200	0	<0.001	0	<0.001	0	<0.001

NS, Not Significant

Table 2. Cytotoxicity (IC50) of *L. major* promastigotes and amastigotes, and CC50 for macrophages treated with glucantime, trigonelline, and combination therapy

Drugs	Amastigote		Promastigote		Macrophage	Si
	IC50±SD (µM)	P value	IC50±SD (µM)	P value	CC50 (µM)	(Selectivity Index)
Glucantime	47.7±8.3	NS	318.5±28.6	NS	349.1	6.53
Trigonelline	109.3±12.1	<0.001	412.3±21.3	<0.001	1863.3	14.69
Co-treatment	36.3±2.2	<0.001	217.6±8.4	<0.001	476.3	13.1

CC50, Cytotoxicity concentration 50

potential synergistic effect between these two compounds. Nevertheless, further studies are needed to confirm this synergy definitively.

The study also indicated that trigonelline exhibits anti-parasitic effects at concentrations comparable to those of glucantime. Notably, the percentage of macrophages infected with the parasite decreased after exposure to various concentrations of trigonelline, and the number of amastigotes per macrophage also declined compared to the control group. No studies were found that examined the effects of trigonelline on other parasites, including *Leishmania* or other members of the Mastigophora subclass. However, based on the results of this study, trigonelline at concentrations of 25, 50, 100, and 200 µg/mL demonstrates anti-leishmanial effects, with 100 µg/mL reducing the number of parasites in macrophages to zero. This suggests that higher concentrations may not be necessary for effective treatment.

The combined use of different concentrations of glucantime and trigonelline demonstrated synergistic effects on the survival of the intracellular parasite compared to the use of either drug alone. This finding is particularly significant, as it suggests that combining trigonelline with lower doses of glucantime can effectively eliminate intracellular forms of *Leishmania* more efficiently than glucantime alone. However, to further substantiate this finding, more detailed investigations on other *Leishmania* species would be beneficial.

The SI, calculated by comparing the effects of the drug on the disease agent (amastigote) and the host cell (macrophage), was found to be 14.69 for trigonelline, 6.53 for glucantime, and 13.1 for the combination treatment. All these values exceed 1, indicating that these treatments are within a safe range, consistent with previous studies demonstrating trigonelline's non-toxicity to host cells. Various studies have reported SI values for different

anti-parasitic agents, such as nicotinamide (SI: 3.9) and 6-gingerol (SI: 27.2) (28,29). The interaction of the combined treatment of glucantime and trigonelline showed a CI of less than 1, and the lower theoretical IC50 compared to the experimental IC50 indicated a synergistic effect between the two drugs. Previous research has also indicated that combination treatments against *Leishmania* can exhibit both synergistic and antagonistic effects (29-32).

Studies on 6-gingerol (another compound derived from ginger) and curcumin (the active ingredient in turmeric) have indicated increased apoptosis rates of *L. major* promastigotes, particularly in combination therapies (33,34). Additionally, research by Qin et al demonstrated that trigonelline exhibits schistosomicidal activity against *Echinococcus granulosus* and effectively inhibits the Nrf2 signaling pathway in *E. granulosus*. The Nrf2 protein is a critical factor in managing oxidative stress, and targeting Nrf2 could disable the antioxidant defense mechanism in *E. granulosus*, thereby preventing apoptosis by eliminating reactive oxygen species during oxidative stress (35). Nrf2 is considered a highly sensitive signaling molecule and can effectively counteract cellular apoptosis caused by oxidative damage (36).

Research has shown that parasites can suppress phagocytes and induce silent transfer into neutrophils through surface markers such as LPS and phosphatidylserine (37). This suppression leads to the initial prevention or reduction of inflammatory and pre-inflammatory cytokines, such as IL-1 and IL-12, thereby inhibiting Th1 cell activity. Conversely, the activation of cytokines such as IL-4 and IL-10 promotes the Th2 pathway, resulting in increased parasite proliferation and pathogenesis (38,39). IL-12 plays a crucial role in the formation of the immune response in leishmaniasis, with dendritic cells from susceptible mice producing lower

levels of this cytokine compared to those from resistant mice (40-42).

Conclusion

The data from this study indicate that trigonelline possesses anti-parasitic properties and inhibits the growth of both amastigotes and promastigotes of *Leishmania*. The recent findings show that the combined use of trigonelline and glucantime, particularly at lower doses of glucantime, effectively reduces the survival rate of this parasitic agent compared to the control group. When used in combination, this treatment demonstrates enhanced effects, making it a promising option for combination therapy due to its high antioxidant properties and lack of cytotoxicity to macrophage cells. Given that previous studies have not investigated the effects of trigonelline on *Leishmania* parasites, further research is warranted to evaluate the impact of this drug.

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Competing Interests

The authors declare that there are no conflicts of interest concerning the publication of this article.

Consent for Publication

Not applicable.

Data Availability Statement

The authors confirm that the data supporting the findings of this research are available within the article.

Ethical Approval

The protocol for this study was approved by the Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.MED.REC.1401.020).

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Human and Animal Rights

Not applicable.

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