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Exploring the Role of *Cymbopogon citratus* Essential Oil as a Natural Inhibitor of Serine Palmitoyltransferase in *Leishmania donovani*

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Abstract

The *Leishmania* parasite is divided into two main divisions: the old-world *Leishmania* species and the new-world *Leishmania* species. The antileishmanial medications currently in use have a number of side effects, for instance, their toxicity to humans, liver issues, long-term treatment, and extreme cost. *Cymbopogon citratus* is a herbal medicine used as an anti-inflammatory, antibacterial, antifungal, antiprotozoal, and gastrointestinal disturbances remedy. Sphingolipids (SLs) in many eukaryotes, one of the important cell membrane components, and their metabolites interfere with the control of many vital functions in the cell, such as cell growth, apoptosis, and differentiation. In this study, the essential oil of *C. citratus* with concentration of 500 µg / ml is selected to test its activity against serine palmitoyltransferase (SPT) enzyme gene expression at different periods (24, 48, and 72 hours post-treatment). The results of the gene expression tested by the RT-qPCR technique showed downregulation of the SPT enzyme gene in treated parasites compared with untreated parasites (control). This study represents the first investigation to study the effect of the crude essential oil extracted from *C. citratus* on the SPT enzyme in *L.donovani* promastigote stage in Iraq.

Keywords: *Leishmania donovani,* Serine Palmitoyltransferase, *Cymbopogon citratus*, Sphingolipid, Gene expression.

استكشاف دور زيت نبات حشيشة الليمون *Cymbopogon citratus* كمثبط طبيعي لإنزيم سيرين بالميتويل ترانسفيراز في *Leishmania donovani*

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الخلاصة:

تقسم طفيليات الليشمانيا إلى مجموعتين رئيسيتين، ليشمانيا العالم القديم وليشمانيا العالم الجديد. تتميز العلاجات الحالية المستخدمة كعلاج مضاد لليشمانيا بآثار جانبية جسيمة تتمثل بسميتها للإنسان والعلاج طويل الأمد ومشاكل الكبد والتكلفة الباهظة *Cymbopogon citratus* . هو دواء عشبي يستخدم كعلاج مضاد للالتهابات ومضاد للبكتيريا والفطريات ومضاد للطفيليات واضطرابات الجهاز الهضمي.

تتداخل السفينجوليبيدات (SLs) في العديد من حقيقيات النوى، وهي أحد أهم مكونات غشاء الخلية ومستقلباتها، مع التحكم في العديد من الوظائف الحيوية في الخلية مثل نمو الخلايا وموت الخلايا المبرمج

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والتمايز .في هذه الدراسة، تم اختيار تركيز *C. citratus* البالغ 500 ميكروجرام / مل، لاختبار نشاطه ضد التعبير الجيني لإنزيم Serine palmitoyltransferase (SPT) في مدد زمنية مختلفة (24 و 48 و 72 ساعة بعد العلاج) .أظهرت نتائج التعبير الجيني التي تم اختبارها بتقنية RT-qPCR انخفاض في التعبير الجيني لإنزيم SPT في الطفيليات المعالجة عند مقارنتها بالطفيليات غير المعالجة (مجموعة السيطرة) .تمثل النتائج في هذه الدراسة أول بحث لدراسة تأثير الزيت العطري الخام المستخرج من *C. citratus* على إنزيم SPTفي الطور امامي السوط في *L.donovani* في العراق.

Introduction

Leishmania is an obligate intracellular parasite that infects mammalian macrophage cells [1]. *Leishmania* belongs to the Trypanosomatidae family and causes a disease called leishmaniasis [2]. The World Health Organization (WHO) considers leishmaniasis one of the nine most important infectious subtropical and tropical diseases [3]. Cutaneous *Leishmania* (CL) is the most endemic *Leishmania* species in Iraq [4], as well as Visceral *Leishmania* (VL) [5]. The available drugs for CL and VL include pentavalent antimonials, such as sodium meglumine antimoniate, stibogluconate, miltefosine, amphotericin, and paromomycin B (both amphotericin B deoxycholate and liposomal amphotericin B), as well as pentamidine isethionate [6].

These drugs cause many adverse effects, the major ones being nausea, abdominal pain, and anorexia. Due to these unwanted side effects, many infected individuals remain untreated. When the leishmaniasis infections remain untreated, they may lead to permanent disfigurement, co-infections, and, in some cases, death. For these reasons, modern drug development research focuses on finding alternative treatments characterized by greater specificity, fewer side effects, and lower cost than currently used chemical drugs. One such alternative treatment is the use of medicinal plants [7].

Medicinal plants are very important in medicine and play a vital role in the development and manufacturing of numerous new drugs. Due to their easy availability, safety, and non-toxic nature, herbal medicines play an important role in the treatment of many diseases [8]. From medicinal plants extraction, an enormous array of complex chemical compounds, including flavonoids, alkaloids, phenylpropanoids, sterols, and terpenoids, are produced, along with essential oils (EOs) [9]. According to Samya *et al*., [10], several medical plants have shown antileishmanial activity. One such plant that exhibits high toxicity against *Leishmania,* is *Cymbopogon citratus*, commonly known as Lemongrass [5].

C.citritus possesses diuretic, anti-pyretic , anti-inflammatory, and antispasmodic properties and is used in herbal therapy to treat gastrointestinal disorders. Several studies have demonstrated its antibacterial, antifungal, and antiprotozoal properties [11, 12].

According to Khasanah *et al.,* [13], the Gas Chromatography-Mass Spectrometry (GCMS) analysis determined E-citral, Z-citral, and β-myrcene as the primary components in lemongrass leaf waste essential oil. Oxygenated monoterpenes represented the largest compound group in the analysis. The essential oil composition included β-myrcene followed by minor amounts of monoterpenoids, sesquiterpenes, and ketones. Various pretreatments of lemongrass (*C. citratus* (DC.) Stapf) leaves included fresh leaves and room temperature drying for 48 h as well as 70 ºC cabinet dryer drying for 5 h before distillation. The pretreatment of lemongrass DC Stapf leaves with a fresh condition, 48-hour room temperature drying and 70 ºC cabinet dryer drying for 5 hours along with water and water steam distillation methods produced significant differences in Z-citral, E-citral, and total citral yield but failed to alter Z-citral, E-citral, and total citral content. The water steam distillation from fresh lemongrass leaves produced the greatest amount of citral.

An important factor that supports *Leishmania* and other members of the kinetoplastids phylum in surviving in the host bloodstream and inside the vector during their life cycle is their surface membranes. These membranes are covered by glycosylphosphatidyl-inositol (GPI)-anchored molecules, which are enriched with sphingolipids and sterols, collectively known as lipid rafts. Sphingolipids (SLs) include glycosphingolipids (GSLs) and sphingomyelin (SM) in mammals, whereas inositol phosphorylceramide (IPC) is the major sphingolipid in *Leishmania* species. IPC plays a critical role in parasite differentiation, morphology, infectivity, vesicular trafficking, proliferation, and virulence; consequently, IPC is considered a potential target for anti-parasitic drugs [14,15].

The production of SLs depends on the activity of the key, rate-limiting heterodimeric enzyme serine palmitoyltransferase (SPT). This study represents the first investigation using crude essential oil from *C. citratus* as a neutral anti-leishmanial therapy targeting SPT, the key enzyme in the SLs biosynthesis pathway of *Leishmania donovani* promastigotes.

Material and method

C. citratus Plant Collection and Essential Oil Extract

C. citratus plants were collected from the Department of Biology's herbal center, University of Baghdad / College of Science. To extract the essential oil, the aerial parts of the plant (leaves) were dried and cut into little pieces. The plant leaves weighed 250 gm placed in 1250 ml of distilled water. The procedure described in the European Pharmacopoeia [16] was employed for essential oil extraction, using hydro-distillation for three hours with a Clevenger apparatus. 1 % is the percentage of extracted oil relative to the dry weight of the plant material.

Dilution and Preparation of Essential Oil Concentrations.

Crude oil was extracted using the Clevenger apparatus [17], and a stock solution with a concentration of 10,000 µg/ml was prepared. From this stock, a desired concentration of 500 µg/ml was prepared by diluting the essential oil in 1% Dimethysullfaxide (DMSO) SDFCL S D fine-Chem. Limited/ india. The prepared solution was stored at room temperature and protected from light until it was used for gene expression studies.

In a previous study by Abdullah and Alqaisi [5], various concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/ml) were tested to determine their toxicity against *Leishmania donovani* promastigotes. The concentration of 500 µg / ml, which showed the greatest effect on the parasite, was selected to test its activity against SPT enzyme gene expression at different time points (24, 48 , and 72 hours post-treatment).

Parasite isolate

L.donovani isolate (MHOM/IQ/2005/MRU15) was kindly provided by the University of Baghdad, College of Science, Department of Biology, and Laboratory of Parasitology for graduate studies.

Leishmania donovani culture

Leishmania donovani promastigotes were activated in Novy-MacNeal-Nicolle (NNN) medium and subsequently transferred to RPMI-1640 medium supplement with 10% fetal bovine serum (FBS) (Gibco®/USA) and 1% antibiotics penicillin and streptomycin (Capricorn scientific GmbH /Germany). The cultures were maintained at 26°C under strict sterile conditions to achieve the logarithmic phase. The parasites were examined

microscopically before starting the experiment to detect the bacterial and fungi contamination, then the parasite count was determined using a Neubauer chamber, and promastigotes in the logarithmic phase were adjusted to a concentration of 2×10^6 promastigotes.

Gene expression

RNA Extraction and Purification

The following steps outline how RNA was extracted from *Leishmania* using the TRIzol™ Reagent (Thermo Scientific, USA) protocol:

A.Sample Lysis in TRIzol

This stage involved centrifuging 1.4 ml of parasite culture for two minutes at 13000 rpm in order to precipitate it. After the supernatant was disposed of, the pellet and 0.5 ml of TRIzol™ reagent were combined. The lysate was pipetted up and down multiple time times to homogenize it.

B- Three-phases separation

After adding 0.2 ml of chloroform to each tube containing the lysate, the tubes were tightly sealed. After two to three minutes of incubation, the mixture was centrifuged for ten minutes at 12,000 rpm. The mixture was divided into three phases at a pH 4: an upper colorless aqueous phase, interphase, and a lower organic phase. The RNA containing the aqueous phase was moved to a new tube.

C- RNA precipitation

The phase aqueous was incubated for ten minutes after 0.5 ml of isopropanol was added. The mixture was then centrifuged for ten minutes at 12,000 rpm at 4°C. At the tube's bottom, the RNA formed a white, gel-like pellet. The supernatant was then discarded.

D- RNA washing

For each tube, 0.5 ml of 70% ethanol was added. The mixture was briefly vortexed and centrifuged at 10000 rpm for five minutes. Ethanol was then aspirated, and the pellet was left to air-dry to remove ethanol residues.

E- RNA solubility

After being rehydrated in 50 µl of nuclease-free water, the RNA pellet was incubated at 55–60°C for 10–15 minutes in a water bath or heat block.

Determine RNA and cDNA Yields

Fluorescence Method

The concentration of the extracted RNA and the quality of the samples for use in subsequent processes were evaluated using a Quantus™ Fluorometer. To do this, 199 µl of diluted QuantyFlour Dye was combined with 1 µl of RNA. For Five minutes, the mixture was allowed to sit at room temperature in the dark. The parameters of RNA purity and concentration were then determined utilizing a NanoDrop.

Primer preparation

The gene ID within TriTrypDb (https://tritrypdb.org) is LdBPK_350320.1, and the gene is located on chromosome 35. Primers were designed using Geneious software (https://www.geneious.com/features/primer-design) and ordered from Macrogen Company / Korea in a lyophilized form as follows: SPT-F 5`-AAGCGCATCGTCATCATC-3`
SPT-R 5`-GCAGAGCCTTGTACTTCTTC-3 with annealing temperature 60°C. Nuclease-free water was used to dissolve the lyophilized primers, creating a stock solution with a final concentration of 100 pmol/µl. After that, 90µl of nuclease-free water was mixed with 10µl of the primer stock solution (stored at -20°C) to create a working primer solution, which had a final concentration of 10 pmol/µl.

Measurement of the gene expression of SPT

The absolute quantification method was used to assess the effect of the crude extract of *C. citratus* on *SPT* gene expression in *L. donovani* at 24, 48, and 72 hours. In a qPCR assay, a dilution series of known template copy numbers was used to create the standard curve. The standard curve, which was used to determine the template concentration (copies/ µl) of the samples, was produced by a linear regression of the log concentration (copies/ µl) vs CT values.

To prepare the standard curve, ten 0.2 ml tubes were set up. In each tube, 90 µl of nuclease-free water was added, followed by the addition of 10 µl from an untreated sample with a known template copy number for the SPT enzyme gene. The copy numbers of the specific gene were calculated based on the absorbance of the standard, DNA mass (bp), and known template concentration. The first tube contained a concentration of 24 $x10^{-10}$ copies/µl. A serial dilution was performed by transferring 10 µl from the first tube with 24 $x10^{-10}$ copies/µl down to the tube with 24 copies/µl, which was then used to calculate the copy number of the samples.

The main components of the qPCR mixture, including qPCR master mix, were prepared according to the manufacturer's instructions, and program of qPCR is programmed as follows, an initial denaturation for 10 minutes at 95 ºC, denaturation for 20 seconds at 95 ºC, annealing for 20 seconds at 60 ºC, finally an extension step for 20 seconds at 72 ºC, the previous steps were repeated for 40 cycles. Tri-replicate for each group was used in this study.

Statistical analysis

The least significant difference–LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means.

Results

L.donovani promastigotes were treated with the IC_{50} concentration of crude essential oil from *C. citratus* for different time periods (24, 48, and 72 hours). A real-time qPCR technique was used to evaluate the effect of the crude essential oil on the SPT enzyme.

In the absolute quantification method, a series of dilutions were prepared from the control, which had a known template copy number, to generate a standard curve. This curve, created by a qPCR machine, allowed for comparison with unknown sample copy numbers (Figure 1). The system's computational program utilizes dilution data to generate the curve, providing an accurate curve shape and corresponding calculations (Table 1).

Significant differences ($P \leq 0.05$) were found by statistical analysis between the control group (untreated *L. donovani* promastigotes) and the treated promastigotes exposed to the IC_{50} concentration of *C.citratus* crude essential oil within at the three different time points (24, 48, and 72 hours). The results demonstrated that the crude essential oil of *C.citratus* down-regulated the expression of the *SPT* gene in treated promastigotes compared to the untreated promastigotes.

The findings indicated the most pronounced effect of *C. citratus* crude essential oil in a concentration of 500 µg / ml occurred at 48 hours (0.239±0.091 copies/µl), followed by 24 hours (0.934±0.66 copies/µl). The effect was less significant at 72 hours (1.21±0.6 copies/µl), which was comparable to the untreated promastigotes (13.92±1.838/µl). These results are summarized in Table 2 and Figures 2 and 3.

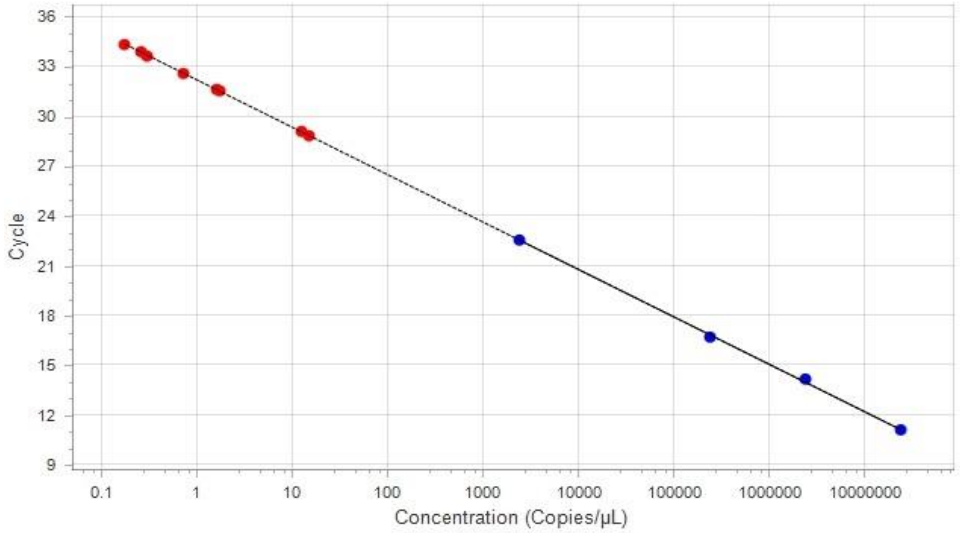

Figure 1: The standard curve illustrates the relationship between the concentrations (copies/µl) of the standard (known copy numbers, represented by blue points) and the unknown samples (represented by red points). The first two red points at the lower end of the curve represent the controls, while the remaining red points correspond to the treated samples.

Table 1: The equation that is used in determining the standard curve in the PCR machine.

Standard Curve	
Equation	y = -2.86 x + 32.22
Efficiency	1.24
R^2	0.9994

Table 2: The differences in the concentration (copies/µL) of the *SPT* gene between the controls and the treated samples with the crude essential oil of *C. citratus* show a down-regulation of the *SPT* gene in *L. donovani* promastigotes over three different time points (24, 48, and 72 hours). The Cq (cycle quantification) represents the cycle at which gene replication begins and is used to plot the curve on the Y-axis.

Time (hr.)	Cq	Calculated Concentration (Copies/µL)	Mean ± SD
T1 24	31.63	1.605	0.934±0.66 bc
T2 24	33.87	0.2629	
T1 48	33.7	0.3037	0.239±0.091 c
T2 48	34.38	0.1744	
T1 72	31.55	1.71	1.21±0.67 ab
T2 72	32.63	0.7186	
Control1	28.83	15.22	13.92±1.838 a
Control 2	29.07	12.62	
LSD value	--	--	0.944 *
Means having with the different letters in same column differed significantly. * (P≤0.05).			

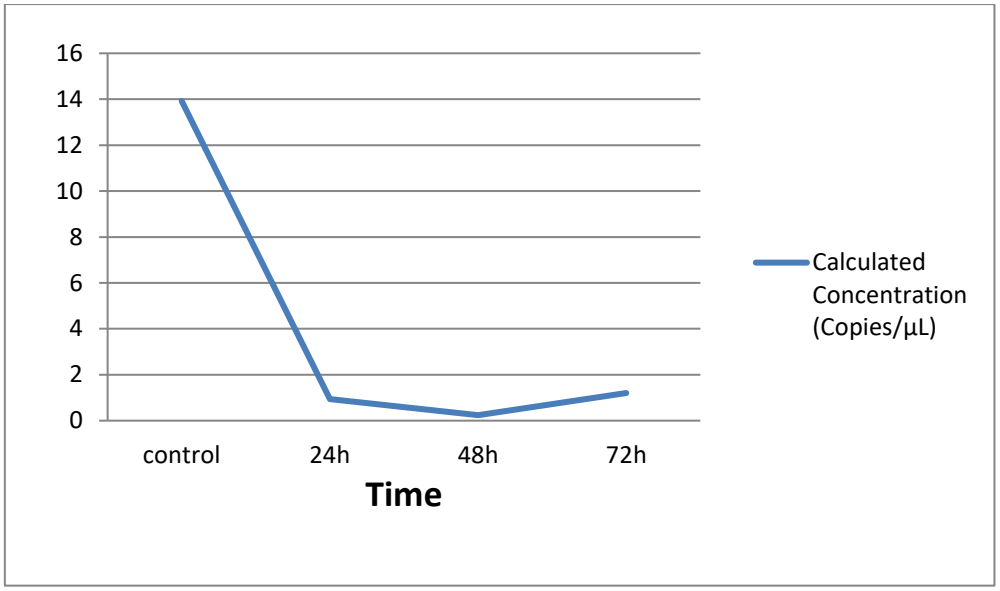

Figure 2: The down-regulation effect of *C. citratus* essential oil on SPT enzyme in *L.donovani* promastigotes

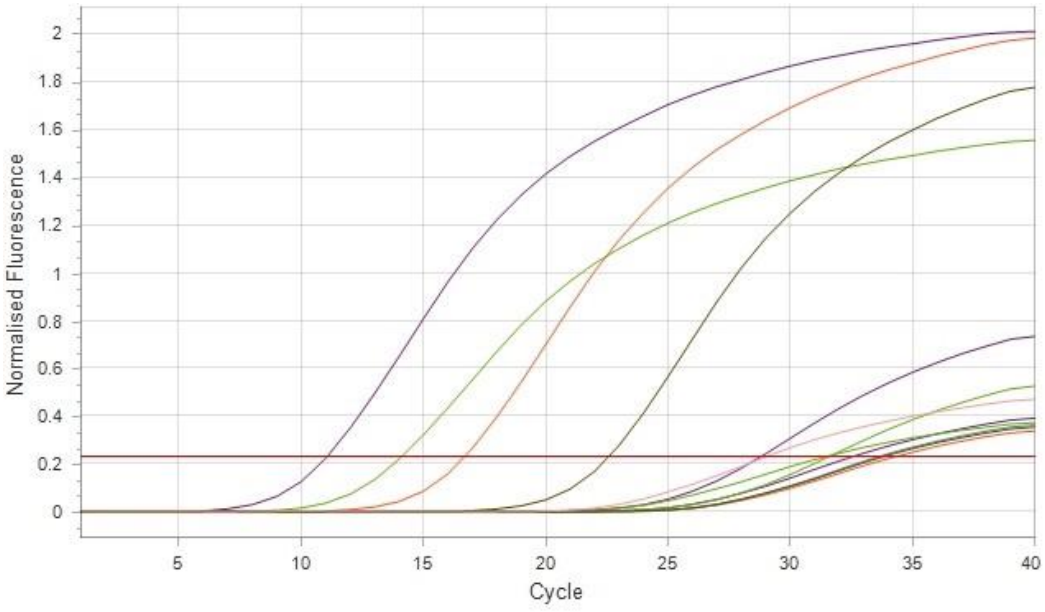

Figure 3: The amplification plot illustrates the differences in the expression of the *SPT* gene, where the first four lines represent the controls and the remaining lines correspond to the treated samples.

Discussion

Leishmania and other protozoa belonging to the Trypanosomatidae family are among the eukaryotic organisms that have the sphingolipid pathway (SLs). SLs have essential components of the cell membrane and are involved in several vital functions. These include controlling critical cellular processes such as cell growth, endocytosis, differentiation, apoptosis, and oncogenesis. Additionally, they act as mediators of cell signaling. In *Leishmania,* SLs primarily exist as inositolphosphorylceramide (IPC), which is abundantly present, constituting approximately 5-10% of the membrane lipids [18].

Denny *et al.,* [19] highlighted the potential of targeting the protozoan inositolphosphoryl-ceramide (IPC) pathway, particularly the IPC synthase enzyme, as an attractive anti-

protozoan drug target with minimal toxic side effects, as this enzyme is not found in mammals. Similarly, Sundar *et al.,* mentioned that the SLs pathway is an important factor in the pathogenesis of *Leishmania* parasites and can serve as an effective drug target [20]. Vermelho *et al.,* studied the sphigolipid biosynthesis pathway in *T. cruzi* parasite, focusing on IPC synthase and ceramide as potential therapeutic targets against the Chagas disease [21]. Furthermore, Brown *et al.,* suggested that inhibiting the Inositol Phosphorylceramide Synthase enzyme in the SLs pathway of *Leishmania major* could provide new therapeutic options for Leishmaniasis [22].

Dos Santos *et al.,* [23] demonstrated the inhibition of IPCs in *Leishmania* parasites by using tamoxifen as a chemotherapy agent, which acts by inhibiting the IPC synthase enzyme. The serine palmitoyltransferase (SPT) enzyme is crucial as the first key enzyme in the SLs biosynthesis pathway. It is responsible for the initial step in IPC formation in *Leishmania* and the production of other SLs in various organisms. This enzyme is vital for maintaining the viability and infectivity of *Leishmania* parasites and is considered an attractive druggable target [24].
Essential oils consist of a wide variety of small hydrophobic molecules, including phenylpropanoids, monoterpenes, and sesquiterpenes. These essential oils have demonstrated anti-leishmanial activity *in vitro* and *in vivo* against promastigote and amastigote forms of *Leishmania* spp. Among these, the essential oil from *C. citratus* shows promise as a source of new drugs against *Leishmania* [25].

As described in previous studies, the common feature of plant volatiles is their hydrophobic nature. Many of these components target the cell membrane as their primary site of action [26]. Additionally, Zarenezhad *et al.,* indicated that *C. citratus* essential oil affects the morphology, apoptosis, and cell membrane of *Leishmania*. Since SLs are important cell membrane components, this may explain the observed effect of crude essential oil on SPT gene expression [27].

The likely gradual decrease in SPT expression may signify either a prolonged cellular reaction to a stressor or a feedback regulatory mechanism intended to adjust ceramide and sphingosine concentrations, both of which are recognized for their roles in influencing apoptosis and cell viability [28]. A slight reduction in SPT expression observed at the 24-hour could suggest the onset of the initial response phase, potentially involving early signaling events that affect transcription factors (such as ATF4 or CHOP) known to govern lipid metabolism in conditions of ER stress [29]. At 48 hours, the more evident down-regulation may align with the buildup of cellular stress or shifts in metabolism, leading to a greater suppression of the pathway. In this circumstance, changes in ceramide levels could attain functional significance, potentially affecting membrane integrity, autophagy, or apoptosis signaling [30].
After 72 hours, sustained or maximal down-regulation indicates either a long-term adaptation or a pathological outcome. This may encompass epigenetic alterations or ongoing activation of stress-response pathways (e.g., PERK-eIF2α-ATF4), leading to a prolonged reprogramming of genes involved in lipid biosynthesis [31]. A study conducted in [5] assessed the *in vitro* antileishmanial efficacy of C. citratus essential oil against *Leishmania donovani* promastigotes. The investigation revealed a time-dependent reduction in parasite viability at 24, 48, and 72 hours following treatment, yielding IC_{50} values of 640, 492, and 442 µg/ml, respectively. This gradual decrease indicates that extended exposure to the essential oil may amplify its inhibitory effects, likely through the sustained down-regulation of critical genes such as *SPT*.

Essential oils demonstrate hydrophobic characteristics, which result in poor solubility in aqueous media, thus creating obstacles to biological assays and therapeutic formulations. The polar aprotic solvent DMSO dissolves many organic substances, including essential oils, which enables researchers to use them in diverse studies [32]. DMSO regards cytotoxic at elevated concentrations, which may impact modified assay results or parasite survival rates. For this reason, the final concentration of DMSO must be controlled to remain at 0.1% (v/v) or below to prevent confounding effects in parasite cultures. So, the final concentration used in this study was 0.001%.

At present, there is an absence of direct evidence suggesting that the essential oil of *C.citratus* (lemongrass) or its main component, citral, influence the expression of the serine palmitoyltransferase (SPT) gene in *Leishmania* species. Nevertheless, numerous studies have indicated that the essential oil of C. citratus has considerable anti-parasitic effects against *Leishmania* and related protozoa, primarily operating through mechanisms that disrupt cellular and mitochondrial functions rather than through the modulation of specific gene expression [33].

Studies indicate that essential oils derived from C. citratus and citral are effective in inhibiting the proliferation of several species of *Leishmania*, specifically L. infantum, L. tropica, and L. major, with IC_{50} values between 25 and 52 μg/mL. Promastigotes that have been subjected to treatment display notable ultrastructural changes, including swelling of the mitochondria and kinetoplast, the presence of autophagosomal structures, disruption of the nuclear membrane, and condensation of chromatin. These changes suggest the induction of programmed cell death via apoptosis, evidenced by phosphatidylserine externalization, loss of mitochondrial membrane potential, and cell-cycle arrest at the G_0/G_1 phase [34].

Leishmania chagasi promastigotes exposed to C. citratus essential oil likewise produced dose-dependent growth inhibition and morphological changes, including cell swelling, lipid droplet accumulation, and increased acidocalcisome volume. Furthermore, aberrant-shaped cells with multi-septate bodies were seen, suggesting a possible influence on cytokinesis [35].

C. citratus essential oil showed anti-proliferative properties in studies involving Trypanosoma cruzi across all three evolutionary forms of the parasite. Treatment maintained the form of the plasma membrane while resulting in cytoplasmic and nuclear extraction. These results imply that the main component of the essential oil, citral, is in charge of the trypanocidal activity [33].

Citral combined with other essential oil components such as eugenol and thymol has also shown enhanced inhibitory effects on *Crithidia fasciculata* and *Trypanosoma cruzi*, suggesting possible synergistic interactions that raise cytotoxicity against these parasites [36].

The main compound of *C. citratus* essential oil, citral, has been found to produce pronounced ultrastructural changes in *Leishmania* promastigotes. Molecular docking analysis indicated that geraniol, another compound present in the EO, strongly binds to enzymes like UGPase, MetRS, and nicotinamidase in several species of *Leishmania*. These interactions indicate that EO might break the nucleotide sugar metabolism, protein synthesis, and the NAD^+ biosynthesis pathway [37].

Conclusion

While *C. citratus* essential oil exhibits significant anti-parasitic activity against *Leishmania* and related protozoa, current research does not provide evidence of its effects on specific gene expressions, such as the SPT gene. The observed anti-parasitic effects are primarily attributed to structural and functional disruptions within the parasite cells. Further studies are necessary to elucidate any potential gene expression modulation by *C. citratus*

essential oil in these organisms. Therefore, the observed effects on serine palmitoyltransferase (SPT) are part of a broader spectrum of metabolic disruptions caused by the EO, rather than a specific targeting of the sphingolipid pathway alone.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and that there is no funding for this manuscript.

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