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RESEARCH ARTICLE

Stachys lavandulifolia Vahl. exhibits promising *in vitro* and *in vivo* antileishmanial activity against *Leishmania major* infection

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ABSTRACT

This study aimed to consider the *in vitro* and *in vivo* effects of the *Stachys lavandulifolia* methanolic extract (SLME) (2.5, 5, 10, 25, 50, 100 µg/mL) against *Leishmania major* infection. The *in vitro* antileishmanial effects of SLME was studies on promastigote and amastigote forms of *L. major*. The effect of SLME on the nitric oxide (NO) and apoptosis, secretion of Th1/2 cytokines, and infectivity rate in macrophages cells were also studies. The cytotoxicity of SLME on human (THP-1) and murine (J774-A1 cell) macrophage cells was investigated through the measuring the 50% cytotoxic concentrations (CC_{50}). Moreover, the *in vivo* effects of SLME for healing the cutaneous leishmaniasis (CL) lesions in infected BALB/c mice studied by assessing the lesions size and the parasite load during four weeks of treatment. The calculated 50% inhibitory concentration (IC_{50}) values for SLME and meglumine antimoniate (MA) against the promastigote stage were 23.4 and 71.1 µg/mL, respectively. For amastigote stage, the IC_{50} values for SLME and MA were 39.3 µg/mL and 44.3 µg/mL, respectively. Followed by 28 days' topically therapy with SLME at doses of 50 and 100 mg/kg/day, the CL lesions size as well as parasite load were significantly ($p<0.001$) reduced; such that the recovery percentage of the infected mice was 80% and 97% after treatment with SLME at the dose of 50 and 100 mg/kg, respectively. SLME also markedly induced the NO production and apoptosis; whereas decreased infection rate in macrophage cells. After incubation of infected macrophages with SLME, the level interferon gamma was meaningfully ($p<0.001$) elevated as a dose-dependent response; in contrast, release of interleukin 10 (IL-10) and IL-4 markedly ($p<0.001$) decreased. The CC_{50} value for SLME against THP-1 and J774-A1 cell was 996.4 µg/mL and 741.3 µg/mL, respectively. The calculated selectivity index of >10 for SLME and MA confirmed their specificity to amastigotes and the low toxicity for macrophages. Our results showed the potent effects of SLME in eliminating and controlling *Leishmania* parasites in both *in vitro* and *in vivo* assays. Based on the current experimental study, SLME can be suggested as an alternative medicine for the isolation and production of a new agent for treating CL caused by *L. major*. Although, we found some cellular mechanisms of SLME against *Leishmania* parasites, but, additional surveys are necessary to specify the accurate mechanisms of action, toxicity, and its efficacy mainly in human subjects.

Keywords: Leishmaniasis; herbal medicines; promastigote; amastigote; apoptosis.

INTRODUCTION

Cutaneous leishmaniasis (CL) is reported from most parts of the world and is spread in different tropical and subtropical regions from deserts to rainforests and from villages to cities (Burza *et al.,* 2018; Vaselek, 2021). CL in Saudi Arabia is endemic generally in the Al-Hassa Oasis and Al-Qassim provinces and in the rural regions around Riyadh; so that, 26,300 cases of CL were reported from 2006 to 2016 (Al-Tawfiq & AbuKhamsin, 2014; Alanazi *et al*., 2016). The ineffectiveness of carrier and reservoir control methods, the cost of treatment, the side effects of treatment with antimicrobial compounds, the long duration of existing treatments and their failure to respond, justify the search for an effective vaccine against leishmaniasis (Prasanna *et al*., 2021). However, no effective and reliable vaccine has been developed for this disease and the fight against this disease has always been considered in the national planning of countries and despite national and international investments (Ghaffarifar *et al*., 2013), not only has this disease not been eradicated, but always with the emergence of new foci, this disease is becoming more prevalent around the world (AlMohammed *et al*., 2021).

In recent years, the treatment of CL has faced many difficulties because of the appearance of resistance to standard drugs, which are mainly pentavalent compounds (Roatt *et al*., 2020). Reports from physicians also indicate recurrence, lack of improvement, adverse effects of medications, and the occurrence of dangerous side effects

in patients (Albalawi *et al*., 2021a). The emergence of resistant strains has results in finding of new anti-leishmaniasis agents such as miltefosine, amphotericin B, ketoconazole, paromomycin and other chemical compounds, but none of these drugs are without side effects (Siadat *et al*., 2020). Also, the toxicity of these agents and the stability of their side effects, even after dose adjustment and long-term treatment, are among their disadvantages (Ponte-Sucre *et al*., 2017). On the other hand, these treatments are not suitable, especially in rural areas, due to their high cost and lack of access to them (Ponte-Sucre *et al*., 2017).

Today, the use of herbs and their derivatives because of the minimal toxicity, lower cost, and high effectiveness and accessibility (Ullah *et al*., 2020; Kumar *et al*., 2021). In recent years, the *in vitro* and *in vivo* anti-leishmanial activities of several plants, e.g. *Pistachio* species, *Ziziphus spina-christi*, *Myrtus communis*, *Trichodesma africana* and *Pergularia tomentosa* have been reported, which show that the use of these plants can lead to wound healing in animal models (Bahmani *et al*., 2015; Bahmani *et al*., 2017); however, final acceptance of the use of medicinal herbs for CL treatment is still held up by variable results of investigations that are seldom sufficiently powered.

Stachys lavandulifolia Vahl. belongs to the Lamiaceae family is a famous herb which is mainly grown in Saudi Arabia, Syria, Iraq, and central Asia (Tundis *et al*., 2014). The herb is applied in traditional medicine to reduce pain, improve gastrointestinal symptoms, and treat infections (Minae *et al*., 2015). On the other hand, *S. lavandulifolia* because of having the high content of phenolic, flavonoid, and terpenoides compounds displayed various pharmacological effects, e.g. wound healing, antibacterial, antiviral, anti-parasitic, anti-inflammatory, antioxidant, neuroprotective, and hepatoprotective effects (Nasri *et al*., 2011; Pirbalouti & Koohpyeh, 2011; ʿican *et al*., 2012; Tomou *et al*., 2020). Since this plant has potential medicinal and therapeutic effects, we aimed to assess *in vitro* and *in vivo* antileishmanial activity against *L. major* infection.

MATERIALS AND METHODS

Chemicals

In this study, RPMI1640 medium, 10% fetal bovine serum (FBS), Folin Ciocalteau reagent (FCR), filter paper, aluminum chloride ($AlCl_3$), Catechin, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], sulfoxide dimethyl (DMSO), cytokine (IFNγ, IL-10, and IL-4) kits, caspase-3 like activity, Griess reagent, and lipopolysaccharide were purchased from SIGMA, St. Louis, MO, USA. Meglumine antimoniate (Glucantime, MA) was prepared from Sanofi-Aventis, S ᴛo Paulo, Brazil. along with Interferon gamma (IFN-ᴛ) was purchased from Chemicon, Millipore. All solutions and reagents were prepared to the best degree.

Plant collection

Plant materials (leaves) were procured from a shop in Riyadh city, Saudi Arabia, and identified by a botanist at the Department of Basic Science. The herb herbarium samples (No. 28-2020) were archived in Department of Basic Science, Almaarefa University, Saudi Arabia.

Ethics

The Ethical Committee for Animal Experiments of Almaarefa University, Saudi Arabia (No. IRB06-25012022-09) approved the current work.

Preparing of extract

Three hundred grams of the dried leaves were extracted by percolation method using 70% methanol for 72 h at 21°C. Extract was then filtered by filter paper and concentrated in vacuum condition at 55°C using a rotary evaporator (Heidolph, Germany) and kept at –20°C until testing (Albalawi, 2021d).

Phytochemical and secondary metabolites analysis

The principal phytochemical examination of the SLME was studies to study the presence of flavonoids, tannins, saponins, alkaloids, and glycosides according to the prior studies using the following reagents and chemicals: alkaloids with Mayer and Dragendorff's reagents, flavonoids with the use of Mg and HCl, tannin with 1% gelatin and 10% NaCl solutions, glycosides with FeCl2 and H2SO4, and saponin with ability to produce suds. (Albalawi *et al*., 2022).

Total phenol content

FCR method utilized to measure the total content of phenolic compounds. Initially, 20 ᵸL of the extract solution was mixed in a test tube with 1/160 ml of distilled water and 100 µL of FCR. After 8 min, 300 µL of sodium carbonate solution (20% w / v) was added to the contents of the test tube. After shaking, the test tubes were placed in a water bath at 40°C and after 30 minutes, their absorption was read by a spectrophotometer at a wavelength of 760 nm (Singleton *et al*., 1999).

Total flavonoid content

$AlCl_3$ colorimetric approach was applied to measure the total content of flavonoids. Initially, extract was mixed with $AlCl_3$ (0.1%), ethanol (%95), potassium acetate (0.1%). Followed by 30 min incubation in room temperature, the absorbance of the combination was studied at 415 nm. The standard curve depicted by quercetin was used to presented the results in milligrams of quercetin per gram of extract (mg QE/ g DW) (Phuyal *et al*., 2020).

The tannin condensed contents

The contents of tannin were calculated based on the procedure explained by Broadhurst and Jones (Broadhurst & Jones, 1978). Briefly, one ml of the extract solution and Catechin as control was mixed with 5 mL vanillin-HCl and mixture was incubated for 3h. the optical density of the combination was then read at 510 nm; the findings were finally expressed as mg Catechin equivalent per gram dray weight (mg CE/g DW).

Parasite

L. major promastigotes (MHOM/TM/82/Lev) were provided from the cell bank of the Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Saudi Arabia. Promastigotes were preserved and proliferated in sterile falcons containing NNN and RPMI1640 medium enriched with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 10% FBS in an incubator at 25°C.

Cell culture

Human (THP-1) and murine (J774-A1) macrophage cells line were prepared from the cells bank of the Department of Biological Sciences, Faculty of Science and Humanities, Saudi Arabia. Cells were then stored and kept in cell culture flasks containing the RPMI1640, improved with penicillin (200 IU/mL), streptomycin (100 µg/mL) and 10% FBS in an incubator at 37°C with 5% CO_2.

Anti-proliferative effects on promastigote forms

Anti-proliferative effects of SLME on promastigote forms was studied by MTT assay according to the previous study (Albalawi *et al*., 2021b). Briefly, promastigotes in the logarithmic phase (1'10^6/ mL) were incubated with SLME concentrations (2.5, 5, 10, 25, 50, 100 µg/mL) in the 96 wells plates at 24°C for 72 h. After discarding the supernatant, and followed by adding the MTT powder (0.25 mg/ mL), the plate was incubated for four hours at 24°C. Then DMSO was added well to eliminate Formazan crystals. As a final point, the absorbance of plates was studied by an ELISA reader (BioTek-ELX800) at 540 nm. The non-treated promastigotes and those treated with MA (2.5, 5, 10, 25, 50, 100 µg/mL) were considered negative and positive controls, respectively.

Effect on the intracellular amastigote forms

The method was performed based on the previous study (Albalawi *et al*., 2021c). Briefly, THP-1 cells (1'10^5/mL) we were seeded in the 24-Well Lab-Tek plated (with 1 cm^2 coverslips put on their floor) at 37°C in 5% CO2 to. After 24h and followed by discarding the nonadherent cells, *L. major* promastigotes (1'10^6/mL) in the stationary phase at the ratio of 10:1 to THP-1 cells were transferred to the plates. Then, various concentration of SLME (2.5, 5, 10, 25, 50, 100 µg/mL) and MA (2.5, 5, 10, 25, 50, 100 µg/mL) were distinctly added to wells and incubated for 48 hours. After fixing the slides in methanol, they were stained with Giemsa and studied light microscopy. The number of amastigotes inside 100 macrophages was recorded, and the 50% inhibitory concentrations (IC_{50}) were determined. The non-treated cells containing amastigotes and those treated with MA were considered negative and positive controls, respectively.

Effect on the infectivity rate in macrophages

To do this examination, *L. major* promastigotes (1 ' 10^6/mL) were pre-treated with SLME (5 ϧg/mL, which has no significant toxicity effect on promastigotes) for two hours at 21°C. The treated promastigotes were washed and again exposed to THP-1 cells for 4 hours. Lastly, the slides were prepared and stained with Giemsa dye, and were examined under a light microscope by calculating 100 cells (Albalawi *et al*., 2021c). The non-pre-treated promastigotes and those treated with MA were considered negative and positive controls, respectively.

Induction of apoptosis in *Leishmania* parasites

The effect of SLME on induction of apoptosis was studied by evaluation of caspase-3 like activity in promastigotes treated with SLME at ¼, 1/3, and ½ IC_{50} based on the previous investigation. Briefly, promastigotes (1'10^6/mL) were incubated with SLME for 48h. After centrifuging the mixture at 1000, the cell deposition was lysed and were again centrifuged at 3000 rpm for 10 minutes. After adding 10 µL of caspase solution (pNA-DEVD-Ac) to the mixture of supernatant (5 µL) buffer (85 µL), the mixture was incubated for two hours at 37°C. As a final point, the absorbance of combination was measured at 405 nm with an ELISA reader (Albalawi *et al*., 2021b).

Effect of nitric oxide (NO) production

In this method, after incubating the THP-1 cells (1'10^5/mL) with various concentration of SLME (1/4 IC_{50}, 1/3 IC_{50}, and ½ IC_{50}) for 48 h, in a 96-well plate the supernatant of reaction (20 µl) was mixed with the nitrite assay buffer (80 µL), Griess reagent A (10 µL, Sigma-Aldrich) and B (10 µL) the amount of NO was recorded at 540 nm in an ELISA reader (BioTek-ELX800). The cells treated with the combination of lipopolysaccharide (LPS, 10 ng/mL) along with IFN-γ (10 U/mL) were considered as the positive control.

Evaluating the secreted cytokines in infected macrophages

To do this, THP-1 cells were exposed by promastigotes and treated with various concentration of SLME as defined in the earlier paragraph in attendance of LPS (2.5 µg/mL) for discharge of Th2 or PMA (25 ng/mL)-ionomycin (1.0 µg/mL) for Th1 cytokines overnight. The level of some released cytokines, e.g. IFNγ, IL-10, and IL-4 was determined in the cell-free suspension by commercial kits based on the producer instructions and the absorbance of suspension was read at 450 nm in ELISA reader.

Cytotoxic effects on THP-1 macrophages cells

The cytotoxicity effect of SLME was performed based on the previous study; briefly, THP-1 and J774-A1 cells (1'10^5/mL) were separately treated with concentrations of SLME in the 96 wells plates for 48h at 37°C with 5% CO2. Then, similar to the stage of anti-proliferative effects on promastigote forms, colorimetric MTT assay was carried out to study the cytotoxicity of SLME on macrophage cells. The 50% cytotoxic concentrations (CC_{50}) and subsequently the selectivity index (SI) according to the ratio CC_{50} for macrophage/IC_{50} for intracellular amastigotes were reported (Delavari *et al*., 2014).

***In vivo* effect on CL in BALB/c mice**

Animals

A total of 40 male BALB/c mice aging from 6 to 8 weeks were allocated into four groups containing 10 mice. Animals were kept in suitable condition (24 ± 1°C), lighting (12-h light/dark cycle), and relative humidity 40–70% and received a food and water *ad libitum*.

Establishment of CL in mice

CL was induced in mice via subcutaneous injection of 100 µl of *L. major* promastigotes in stationary phase (1'10^6 parasites/mL) into the tail of mice (Albalawi, 2021d).

Treatment of mice with CL

In the sixth week after infection when leishmaniasis lesions appeared in mice, mice were topically cured with SLME (50 and 100 mg/kg/day, the selection of these doses was according to the primary experiments and earlier study for 4 weeks. Infected mice in the negative and control groups were treated with the normal saline and MA (intralesional inoculation, 30 mg/kg/day), respectively (Figure 1).

Evaluating the *in vivo* antileishmanial effects on CL in mice

The *in vivo* antileishmanial effects of SLME on CL in mice were studies by evaluating the lesions size at the before treatment, 2nd week, and 4th week of treatment by a Vernier caliper. In addition, the load of parasite in the tested mice, at the before treatment, 2nd week, and 4th week of the SLME therapy was calculated by preparing a smear of the lesions, staining them with Giemsa dye and finally examining them with a light microscope (Albalawi *et al*., 2021c).

Statistical analysis

The examinations were repeated in three times, and the findings were indicated as mean ± standard deviation. Data analysis was done by SPSS 25.0 version software. Also, one-way analysis of variance (ANOVA) and Post Hoc Dunnett test were utilized to compare the findings between groups. For calculating the IC_{50} and CC_{50} values we used the Probit regression in SPSS software. Significance level $p<0.05$, and 95% confidence interval were considered

RESULTS AND DISCUSSION

By the *in vitro* anti-proliferative activity of SLME on promastigote forms, the findings showed that after three days' exposure of *L. major* promastigotes with SLME, with elevating the concentration, the growth rate of promastigotes markedly increased ($p<0.001$). The calculated IC_{50} value of SLME and MA for promastigote forms was 23.4 and 71.1 µg/mL, respectively (Table 1). By the *in vitro* activity of SLME on the intracellular amastigote forms, the findings indicated that after treatment of amastigote infected macrophages with SLME, the mean number of parasites noticeably reduced ($p<0.001$) in a dose-dependent response. The calculated IC_{50} value of SLME and MA was 39.6 µg/mL and 44.3 µg/mL, respectively (Table 1).

Previous studies revealed the antimicrobial effects of *S. lavandulifolia* against a wide range of pathogenic bacterial, fungal, viral, and parasitic strains (Tundis *et al*., 2014); for example, Barati *et al*. (2017) have reported that *S. lavandulifolia* aqueous and hexane extracts at dose 100 mg/mL considerably reduced the viability of *Giardia lamblia* cyst by 93% and 100%, respectively. In a study conducted by Sereshti *et al*. (2012) the result exhibited that watery and ethanolic extract of *S. lavandulifolia* at the concentrations of 10, 50, 100, 200, 500 and 1000 µg/ml markedly declined the viability of trophozites of *Trichomonas vaginalis in vitro*. Another study conducted by Asadi *et al*. (2012) the findings showed that *S. lavandulifolia* hydroalcoholic extract at the concentrations of

Table 1. The 50% inhibitory concentrations (IC_{50}) and 50% cytotoxic concentrations (CC_{50}) values determined for the *S. lavandulifolia* methanolic extract (SLME), compared with the meglumine antimoniate (MA) as well as the selectivity index (SI) against intramacrophage amastigote forms of *Leishmania major*. The findings were indicated as mean ± standard deviation (n=3)

Drug	Promastigote IC_{50} (µg/mL)	Amastigote IC_{50} (µg/mL)	CC_{50} (µg/mL) of the macrophage Cells		SI
			THP-1 cells	J774-A1 cell	
SLME	23.4 ± 2.01	39.3 ± 2.51	996.4 ± 15.6	741.3 ± 9.6	>20
MA	71.1 ± 3.15	44.3 ± 3.012	1026.2 ± 11.51	–	>2

Table 2. Effect of *S. lavandulifolia* methanolic extract (SLME) on inhibition of infection in macrophages in comparison with the meglumine antimoniate (MA). Mean ± SD (n = 3)

Promastigotes	% of infected macrophages	% of reduction
Non-treated	81.7 ± 5.35	–
SLME (5 µg/mL)	29.2 ± 3.15	64.2*
MA	24.3 ± 2.51	70.3

* p < 0.001 difference was statistically significant compared with the negative control.

Table 3. The effect of *S. lavandulifolia* methanolic extract (SLME) on nitric oxide (NO) production in human macrophage cell line (THP-1) in comparison with the positive (IFN-γ+LPS) and negative controls (non-treated). The findings are indicated as mean ± standard deviation (n=3)

Concentration (µg/mL)	NO production (nM)
¼ IC_{50}	6.11 ± 0.62
1/3 IC_{50}	17.4 ± 1.34*
1/2 IC_{50}	23.5 ± 1.51*
Non-treated	4.71 ± 0.26
IFN-γ+LPS	33.6± 3.32

* *p* < 0.001 difference was statistically significant compared with the negative control.

50 and 100 µg/ml had the potent antileishmanial effects against promastigotes of *Leishmania major in vitro*. However, this difference between our results and previous studies is probably due to some factors, e.g., study method, type of *Leishmania* species, type of extract, the place of the collected plant (Gharirvand Eskandari *et al*., 2020).

Considering the *in vivo* assay, the results showed that the mean diameter of the CL lesions was markedly decreased, such that the recovery percentage of the infected mice was 80% and 97% followed by 28 days' treatment with SLME at the doses of 50 and 100 mg/kg/day, respectively (Figure 2). In MA treated infected mice, the diameter of the CL lesions significantly decreased by 9.4 mm; whereas in mice treated with normal saline, the size of the CL lesions elevated by 8.6 mm. The microscopic assessments revealed that followed by 28 days' treatment with SLME at the doses of 50 and 100 mg/kg/day, the load parasites were significantly reduced when compared with the control group (Figure 3). Considering the wound healing activity of *S. lavandulifolia*, Pirbalouti & Koohpyeh, (2011) have reported that aqueous extract of *S. lavandulifolia* flowers considerably reduced (92%) in the wound size with significant tissue regeneration in the skin wound of male Wistar rats.

It has been previously proven that inhibition of infection rate in macrophage cells, as the main pathogenesis of *Leishmania* parasites, is one of the critical mechanisms in assessing new agents (34). Our findings revealed that pre-treatment promastigotes with SLME caused a considerable decrease (p<0.001) in the infection rate of macrophages in comparison with the control group. The infection rate in the SLME-treated (5 µg/ml) and non-treated macrophages was 81.7% and 29.2%, respectively (Table 2). Stimulation of programmed cell death (apoptosis) is considered as one of the central mechanisms in the inhibition and control of *Leishmania* parasites (Elmore, 2007). Between various apoptotic mediators, caspases and especially caspase-3 are mostly involved in the induction of apoptosis in *Leishmania*. (Zangger *et al*., 2002). Our results showed that in colorimetric protease assay SLME, mainly at 1/3 IC_{50} and ½ $IC_{50,}$ markedly provoked the induction of the caspase-3 activity by 19.2 and 32.6%, respectively (Figure 4).

Todays, it has been proven that CL is linked with the Th2-dominated cytokine response (e.g., IL-10 and IL-4) which results in suppressing of the immune system in the host. IL-10 and IL-4 are well-known as the principle immune suppressive cytokines related to the leishmaniasis. IFNᴛ as one of the main Th1 cytokines which has a critical role in controlling CL, where, commonly down regulated during CL (Panaro *et al*., 2001). Here, to assess whether the SLME displayed its antileishmanial effects was through the change of cytokine reaction of host, we study the cytokine release by the infected macrophages treated with SLME. As shown in Figure 5, after incubation of infected macrophages with SLME, the level IFNᴛ was markedly (p<0.001) elevated as a dose-dependent manner compared to untreated cells. In contrast, release of IL-10 and IL-4 markedly (p<0.001) decreased, after incubation of infected macrophages with SLME at ¼ IC50, 1/3 IC50, and ½ IC50. Macrophages are one of the most important immune cells involved in controlling and eliminating *Leishmania* parasites. These cells inhibit and eliminate the parasite by stimulating nitric oxide synthetase and subsequent secretion of nitric oxide (Panaro *et al*., 2001). Table 3 shows the effect of SLME on the NO release in THP-1 macrophages cells. The obtain results in the Griess reaction assay exhibited that the macrophages treated with SLME induced the NO production, whereas a remarkable (p<0.001) increase was observed at concentrations of 1/3 IC_{50} and ½ IC_{50} compared to the control group.

The principal phytochemical investigation of SLME exhibited the attendance of the alkaloids, flavonoids, glycosides, saponins, tannins, and terpenoids in this plant. The analysis and measurement of the contents of secondary metabolites displayed that the total flavonoid, phenolic, and tannin content was 16.82 (mg QE/g DW), 26.42 (mg GEA/ g DW), and 4.12 (mg CE/g DW), respectively. Concerning the antimicrobial activities of these secondary metabolites, previous studies confirmed the antibacterial (e.g., Gram-positive and Gram-negative bacteria), antifungal (e.g., *Candida* spp., *Aspergillus* spp., *Penicillium* spp.), antiviral (e.g., human immunodeficiency virus (HIV) and *Sindbis* virus), and antiparasitic (e.g., *Cryptosporidium parvum* and *Encephalitozoon intestinalis*, *Plasmodium falciparum*, *Leishmania* spp.) effects of the flavonoids and phenolic compounds (Lehane & Saliba, 2008; Ramɒrez-Macɒas *et al*., 2012; Naithani *et al*., 2008; Lucchini *et al*., 1990; Mead & McNair, 2006). In addition, previous investigations have indicated that the antimicrobial mechanisms of the flavonoids, and phenolic compounds are associated to their impact on the nucleic acid synthesis suppression, cytoplasmic membrane dysfunction, energy metabolism dysfunction,

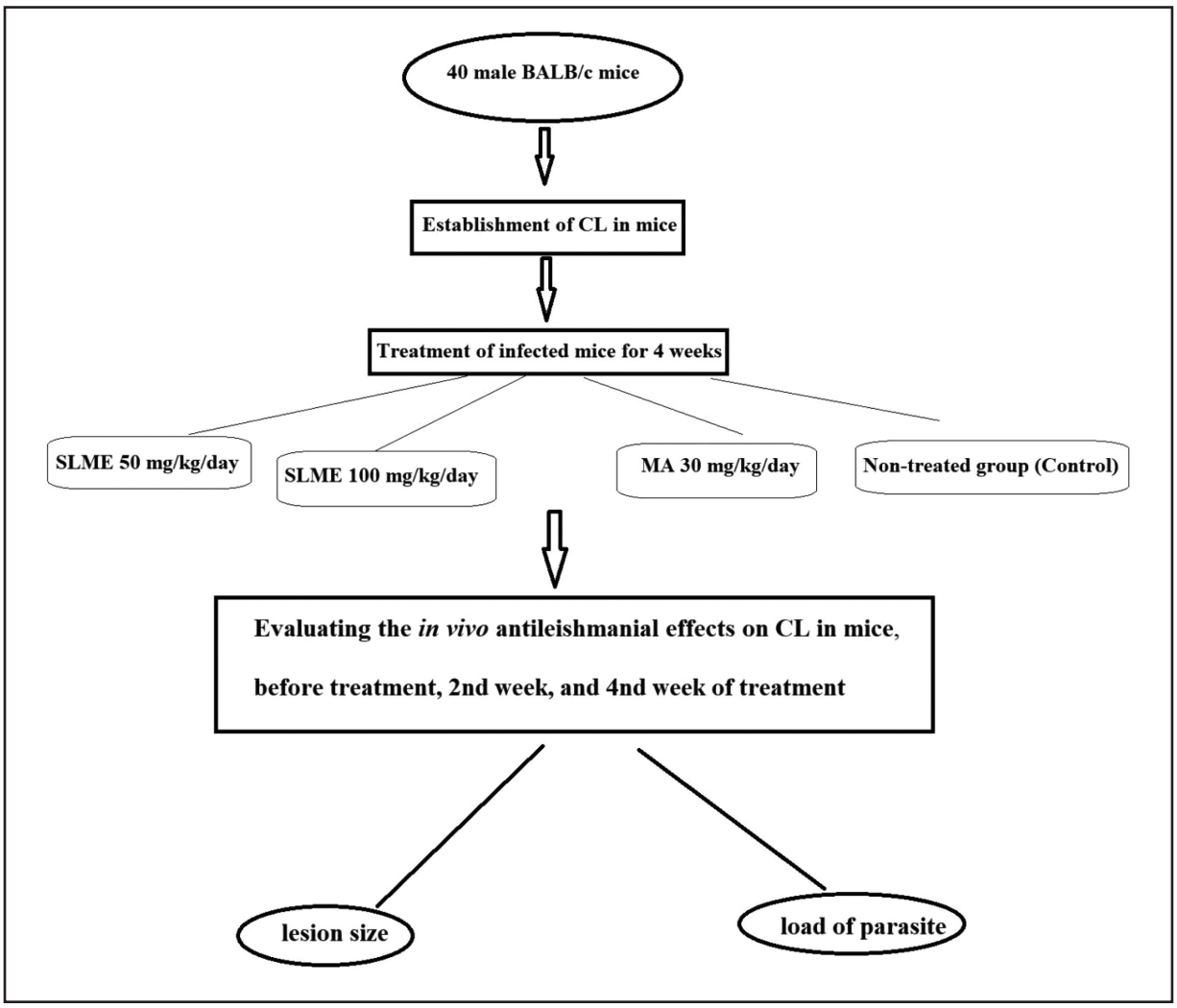

Figure 1. Flowchart of the study design in the present work.

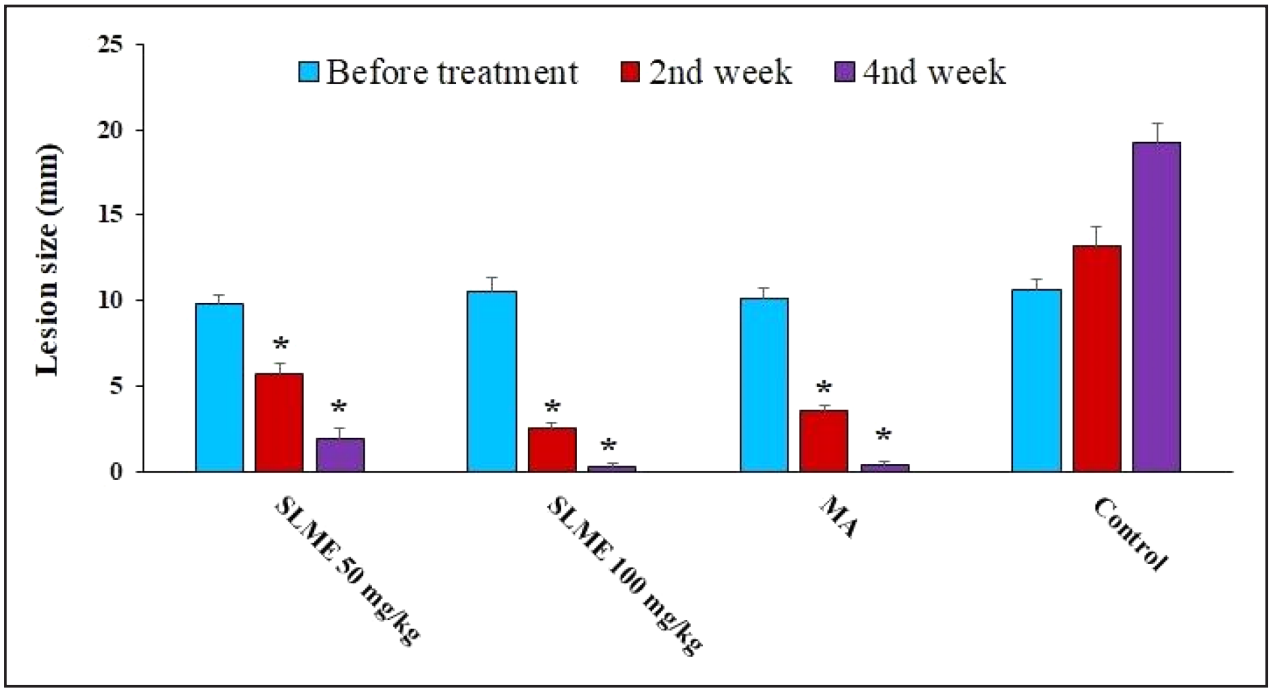

Figure 2. Effect treatment with various doses of *S. lavandulifolia* methanolic extract (SLME) on the lesions size in BALB/c mice infected by *L. major*. The findings are indicated as mean ± standard deviation. * $p < 0.001$ shows the difference was statistically significant in comparison with control. (n=10).

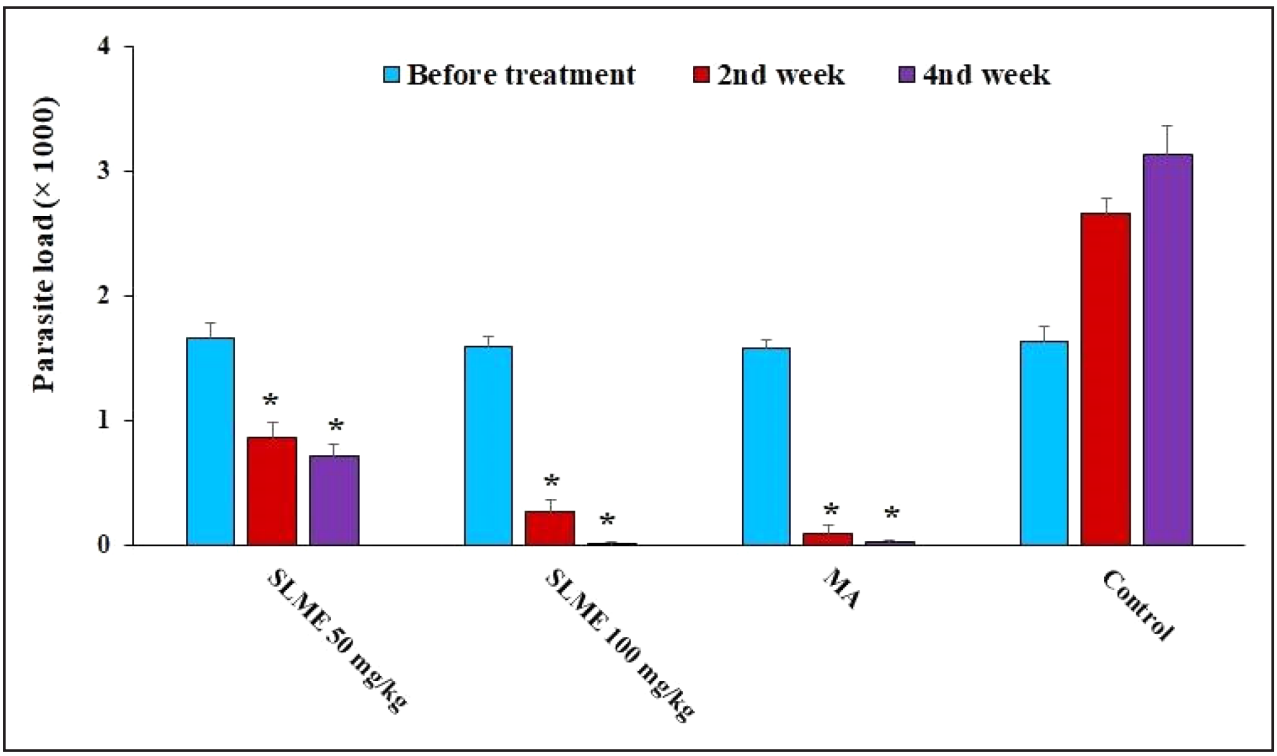

Figure 3. Effect treatment with various doses of *S. lavandulifolia* methanolic extract (SLME) on the mean number of parasites (parasite load) in BALB/c mice infected by *L. major*. The findings are indicated as mean ± standard deviation. * $p < 0.001$ shows the difference was statistically significant in comparison with control. (n=10).

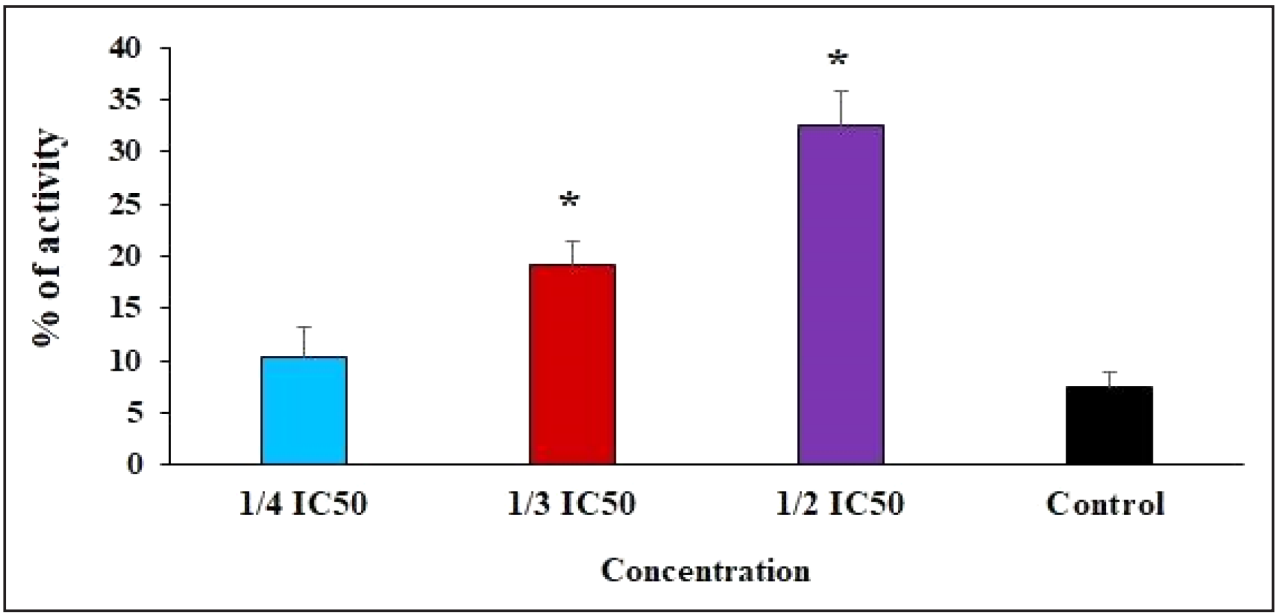

Figure 4. The effect of *S. lavandulifolia* methanolic extract (SLME) on Caspase-3-like activity of *L. major* promastigotes by the colorimetric protease methods. The findings are indicated as mean ± standard deviation. (n=3). * $p < 0.001$ shows the difference was statistically significant in comparison with control.

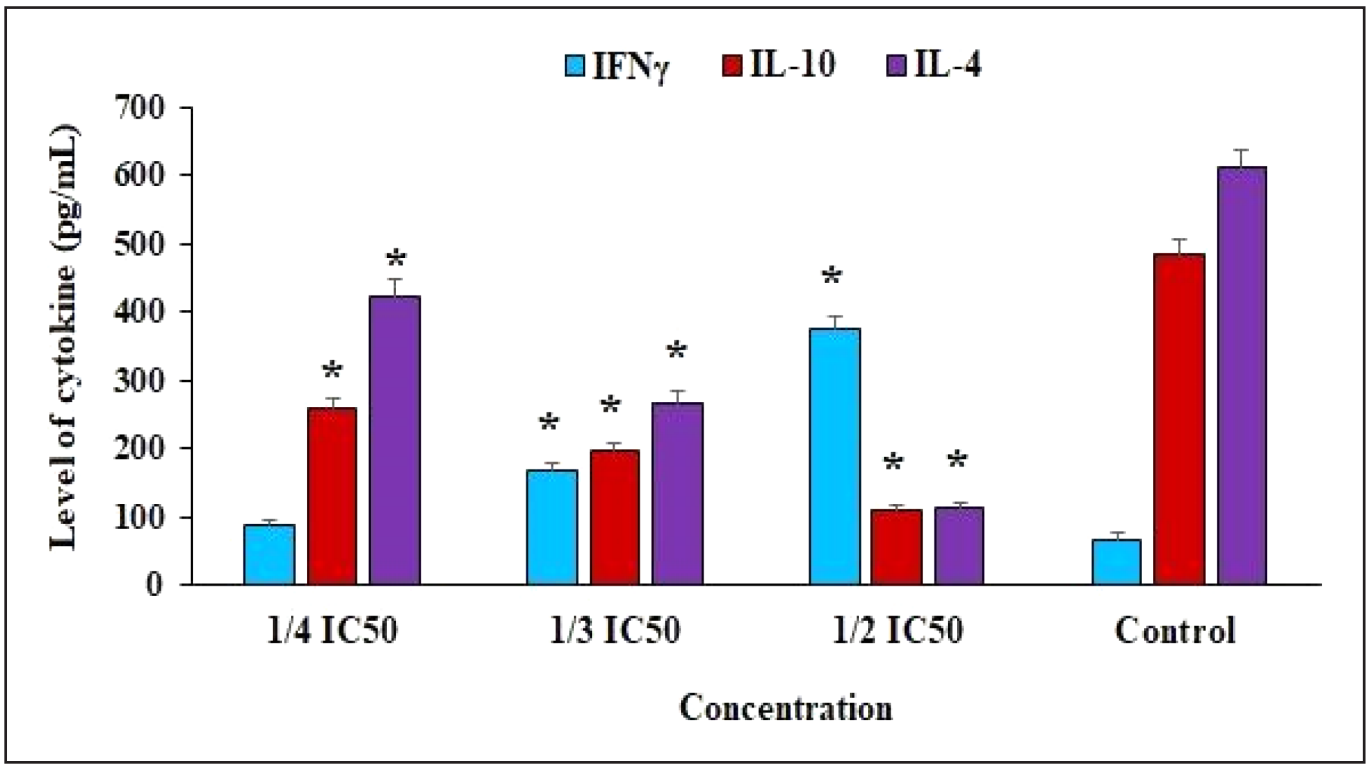

Figure 5. The level of cytokines IFNγ, IL-10 and IL-4 by the infected macrophages treated with *S. lavandulifolia* methanolic extract (SLME) at ¼ the 50% inhibitory concentrations (IC_{50}), 1/3 IC_{50}, and ½ IC_{50}. * $p < 0.001$ shows the difference was statistically significant in comparison with control. The findings are indicated as mean ± standard deviation (n=3).

inhibition of bacterial virulence factors, exhibition a synergistic effect with existing synthetic drugs, etc (Chusnie & Lamb, 2005; Bouarab Chibane *et al*., 2019; Kharazmkia *et al*., 2022; Mahmoudvand *et al*., 2022; Yadegari *et al*., 2022). On the other hand, investigations have demonstrated that the flavonoids and phenolic compounds through the strengthens the immune system mostly cellular immune system through the mTOR pathway signaling activity, stimulating immune cells (e.g., macrophages, and natural killer cells), regulation of cytokine excretion, phagocytosis, triggering of macrophages, and production of immunoglobulins are able to control and eliminate the microbial infections (Mendes *et al*., 2019; Chiang *et al*., 2003). Hence, it may be proposed that *S. lavandulifolia* exhibited it's *in vitro* and *in vivo* antileishmanial effects through the direct effect on parasites and also indirect mechanisms particularly strengthens the immune system mainly the cellular immune system.

Concerning the cytotoxicity effects of SLME, we found that the CC_{50} value for SLME against THP-1 and J774-A1 cell was 996.4 µg/mL and 741.3 µg/mL. The calculated SI above 10 for SLME and MA showed their specificity to intracellular amastigotes and low toxicity on macrophages (Table 1). In line with our results, Khanavi *et al*. (2012) have reported that *S. lavandulifolia* methanolic extract showed the low toxicity on the colon carcinoma (HT-29), breast ductal carcinoma (T47D), colorectal adenocarcinoma (Caco-2), and Swiss mouse embryo fibroblast (NIH 3T3) cell lines with CC_{50} values more than 1000 ϧg/mL.

CONCLUSION

Our results showed the potent effects of SLME in eliminating and controlling *Leishmania* parasites as well as improving the lesions of in BALB/c mice infected by *L. major*. Although, the findings of the current investigation revealed some possible antileishmanial mechanisms of SLME, such as prompting NO production, apoptosis, and effect on the infectivity rate in macrophages, nevertheless, further survives are necessary to specify the precise mechanisms of action, toxicity, and its efficacy mainly in human.

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Conflicts of Interest

The authors declare no conflict of interest in this study.

REFERENCES

Alanazi, A.D., Alyousif, M.S., Saifi, M.A. & Alanazi, I.O. (2016). Epidemiological studies on cutaneous leishmaniasis in Ad-Dawadimi District, Saudi Arabia. *Tropical Journal of Pharmaceutical Research* **15**: 2709-2712. https://doi.org/10.4314/tjpr.v15i12.24

Albalawi, A.E. (2021d). Antileishmanial activity of *Ziziphus spina-christi* leaves extract and its possible cellular mechanisms. *Microorganisms* **9**: 2113. https://doi.org/10.3390/microorganisms9102113

Albalawi, A.E., Abdel-Shafy, S., Khudair Khalaf, A., Alanazi, A.D., Baharvand, P., Ebrahimi, K. & Mahmoudvand, H. (2021c). Therapeutic potential of green synthesized copper nanoparticles alone or combined with meglumine antimoniate (glucantime®) in cutaneous leishmaniasis. *Nanomaterials* **11**: 891. https://doi.org/10.3390/nano11040891

Albalawi, A.E., Alanazi, A.D., Sharifi, I. & Ezzatkhah, F. (2021a). A systematic review of curcumin and its derivatives as valuable sources of antileishmanial agents. *Acta Parasitologica* **66**: 797-811. http://doi.org/10.1007/s11686-021-00351-1

Albalawi, A. E., Althobaiti, N. A., Alhasani, R. H., & Alnomasy, S. F. (2022). Anti-tumor effects and cellular mechanisms of *Pistacia atlantica* methanolic extract against Ehrlich solid tumor in mice. *Asian Pacific Journal of Tropical Biomedicine* **12**: 69-77. http://doi.org/10.4103/2221-1691.335695

Albalawi, A.E., Khalaf, A.K., Alyousif, M.S., Alanazi, A.D., Baharvand, P., Shakibaie, M. & Mahmoudvand, H. (2021b). Fe3O4@ piroctone olamine magnetic nanoparticles: Synthesize and therapeutic potential in cutaneous leishmaniasis. *Biomedicine & Pharmacotherapy* **139**: 111566. https://doi.org/10.1016/j.biopha.2021.111566

AlMohammed, H.I., Khudair Khalaf, A., Albalawi, A.E., Alanazi, A.D., Baharvand, P., Moghaddam, A. & Mahmoudvand, H. (2021). Chitosan-based nanomaterials as valuable sources of anti-leishmanial agents: A systematic review. *Nanomaterials* **11**: 689. https://doi.org/10.3390/nano11030689

Al-Tawfiq, J.A. & AbuKhamsin, A. (2004). Cutaneous leishmaniasis: a 46-year study of the epidemiology and clinical features in Saudi Arabia (1956–2002). *International Journal of Infectious Diseases* **8**: 244-250. https://doi.org/10.1016/j.ijid.2003.10.006

Asadi, M., Bahrami, S., Ansari, S.R. & Pakniat, N. (2012). Effect of hydroalcoholic extracts of Stachys lavandulifolia Vahl and Mespilus germanica leaves on *Leishmania major*. *Hormozgan Medical Journal* **15**: 279-284.

Bahmani, M., Abbasi, N., Hosseini, M. & Rafieian-Kopaei, M. (2017). Concise review: Medicinal plants are effective against leishmaniasis. *Biomedical Research and Therapy* **4**: 1775-1784.

Bahmani, M., Saki, K., Ezatpour, B., Shahsavari, S., Eftekhari, Z., Jelodari, M., Rafieian-Kopaei, M. & Sepahvand, R. (2015). Leishmaniosis phytotherapy: Review of plants used in Iranian traditional medicine on leishmaniasis. *Asian Pacific Journal of Tropical Biomedicine* **5**: 695-701. https://doi.org/10.1016/j.apjtb.2015.05.018

Barati, M., Fakhar, M., Gholami, S., Esboei, B.R. & Elmi, T. (2017). The evaluation of *Stachys lavandulifolia* leave extracts on cysts of *G. lamblia*, in vitro. *Journal of Archives in Military Medicine* **5**: e59529. https://doi.org/10.5812/jamm.59529

Bouarab Chibane, L., Degraeve, P., Ferhout, H., Bouajila, J. & Oulahal, N. (2019). Plant antimicrobial polyphenols as potential natural food preservatives. *Journal of the Science of Food and Agriculture* **99**: 1457-1474. https://doi.org/10.1002/jsfa.9357

Broadhurst, R.B. & Jones, W.T. (1978). Analysis of condensed tannins using acidified vanillin. *Journal of the Science of Food and Agriculture* **29**: 788-794. https://doi.org/10.1002/jsfa.2740290908

Burza, S., Croft, S.L. & Boelaert, M. (2018). Leishmaniasis. *The Lancet* **392**: 951-970. https://doi.org/10.1016/S0140-6736(18)31204-2

Chiang, L.C., Ng, L.T., Chiang, W., Chang, M.Y. & Lin, C.C. (2003). Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. *Planta Medica* **69**: 600-604. https://doi.org/10.1055/s-2003-41113

Chusnie, T.P.T. & Lamb, A.J. (2005). Antimicrobial activity of flavonoid. *International Journal of Antimicrobial Agents* **26**: 343-356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

Delavari, M., Dalimi, A., Ghaffarifar, F. & Sadraei, J. (2014). *In vitro* study on cytotoxic effects of ZnO nanoparticles on promastigote and amastigote forms of *Leishmania major* (MRHO/IR/75/ER). *Iranian Journal of Parasitology* **9**: 6-13.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology* **35**: 495-516. https://doi.org/10.1080/01926230701320337

Ghaffarifar, F., Jorjani, O., Sharifi, Z., Dalimi, A., Hassan, Z.M., Tabatabaie, F., Khoshzaban, F. & Hezarjaribi, H.Z. (2013). Enhancement of immune response induced by DNA vaccine cocktail expressing complete LACK and TSA genes against Leishmania major. *Apmis* **121**: 290-298. https://doi.org/10.1111/j.1600-0463.2012.02968.x

Gharirvand Eskandari, E., Setorki, M. & Doudi, M. (2020). Medicinal plants with antileishmanial properties: a review study. *Pharmaceutical and Biomedical Research* **6**: 1-16. http://doi.org/10.18502/pbr.v6i1.3422

İşcan, G., Demirci, B., Demirci, F., Göger, F., Kırımer, N., Köse, Y.B. & Başer, K.H.C. (2012). Antimicrobial and antioxidant activities of *Stachys lavandulifolia* subsp. *lavandulifolia* essential oil and its infusion. *Natural Product Communications* **7**: 1241-1244. https://doi.org/10.1177/1934578X1200700937

Khanavi, M., Manayi, A., Lotfi, M., Abbasi, R., Majdzadeh, M. & Ostad, S.N. (2012). Investigation of cytotoxic activity in four *Stachys* species from Iran. *Iranian Journal of Pharmaceutical Research* **11**: 589-593.

Kharazmkia, A., Al-Abodi, H.R., Yadegari, J.G. Vahidi, A. & Mahmoudvand, H. (2022). Potential effects of alpha-pinene, a monoterpene commonly found in essential oils against Toxoplasma gondii infection; an in vitro and *in vivo* study. *Journal of Parasitic Diseases* 1-7. https://doi.org/10.1007/s12639-022-01514-1

Kumar, S., Mittal, A., Babu, D. & Mittal, A. (2021). Herbal medicines for diabetes management and its secondary complications. *Current Diabetes Reviews* **17**: 437-456. https://doi.org/10.2174/1573399816666201103143225

Lehane, A.M. & Saliba, K.J. (2008). Common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite. *BMC Research Notes* **1**: 26. https://doi.org/10.1186/1756-0500-1-26

Lucchini, J.J., Corre, J. & Cremieux, A. (1990). Antibacterial activity of phenolic compounds and aromatic alcohols. *Research in Microbiology* **141**: 499-510. https://doi.org/10.1016/0923-2508(90)90075-2

Mahmoudvand, H., Al-Abodi, H.R., Zolfagharkhani, P. & Ghasemian Yadegari, J. (2022). Anti-helminthic effects and cellular mechanisms of Astragalus ecbatanus extract against Echinococcus granulosus protoscoleces. *Journal of Parasitic Diseases* 1-8. https://doi.org/10.1007/s12639-022-01517-y

Mead, J.R. & McNair, N. (2006). Antiparasitic activity of flavonoids and isoflavones against *Cryptosporidium parvum* and *Encephalitozoon intestinalis*. *FEMS Microbiology Letters* **259**: 153-157. https://doi.org/10.1111/j.1574-6968.2006.00263.x

Mendes, L.F., Gaspar, V.M., Conde, T.A., Mano, J.F. & Duarte, I.F. (2019). Flavonoid-mediated immunomodulation of human macrophages involves key metabolites and metabolic pathways. *Scientific Reports* **9**: 14906. https://doi.org/10.1038/s41598-019-51113-z

Minae, B., Sardari, M., Sharifi, H., Abadi, M.S.R. & Sadeghpour, O. (2015). *Stachys lavandulifolia* Vahl. and its relation with marmazad activities in traditional manuscripts. *Iranian Red Crescent Medical Journal* **17**: e19932. https://doi.org/10.5812/ircmj.19932

Naithani, R., Huma, L.C., Holland, L.E., Shukla, D., McCormick, D.L., Mehta, R.G. & Moriarty, R.M. (2008). Antiviral activity of phytochemicals: a comprehensive review. *Mini Reviews in Medicinal Chemistry* **8**: 1106-1133. https://doi.org/10.2174/138955708785909943

Nasri, S., Ramezanghorbani, A. & Kamalinejad, M. (2011). Analgesic and anti-inflammatory effects of hydroalcoholic extract of *Stachys lavandulifolia* vahl S, aerial parts in male mice. *Armaghane Danesh* **16**: 161-171.

Panaro, M.A., Brandonisio, O., Sisto, M., Acquafredda, A., Leogrande, D., Fumarola, L. & Mitolo, V. (2001). Nitric oxide production by *Leishmania*-infected macrophages and modulation by prostaglandin E_2. *Clinical and Experimental Medicine* **1**: 137-143. https://doi.org/10.1007/s10238-001-8025-0

Phuyal, N., Jha, P.K., Raturi, P.P. & Rajbhandary, S. (2020). Total phenolic, flavonoid contents, and antioxidant activities of fruit, seed, and bark extracts of *Zanthoxylum armatum* DC. *The Scientific World Journal* **2020**: 8780704. https://doi.org/10.1155/2020/8780704

Pirbalouti, A.G. & Koohpyeh, A. (2011). Wound healing activity of extracts of *Malva sylvestris* and *Stachys lavandulifolia*. *International Journal of Biology* **3**: 174-179. https://doi.org/10.5539/ijb.v3n1p174

Ponte-Sucre, A., Gamarro, F., Dujardin, J.C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R. & Papadopoulou, B. (2017). Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLOS Neglected Tropical Diseases* **11**: e0006052. https://doi.org/10.1371/journal.pntd.0006052

Prasanna, P., Kumar, P., Kumar, S., Rajana, V.K., Kant, V., Prasad, S.R., Mohan, U., Ravichandiran, V. & Mandal, D. (2021). Current status of nanoscale drug delivery and the future of nano-vaccine development for leishmaniasis – A review. *Biomedicine & Pharmacotherapy* **141**: 111920. https://doi.org/10.1016/j.biopha.2021.111920

Ramírez-Macías, I., Marín, C., Díaz, J.G., Rosales, M.J., Gutiérrez-Sánchez, R. & Sánchez-Moreno, M. (2012). Leishmanicidal activity of nine novel flavonoids from *Delphinium staphisagria*. *The Scientific World Journal* **2012**: 203646. https://doi.org/10.1100/2012/203646

Roatt, B.M., de Oliveira Cardoso, J.M., De Brito, R.C.F., Coura-Vital, W., de Oliveira Aguiar-Soares, R.D. & Reis, A.B. (2020). Recent advances and new strategies on leishmaniasis treatment. *Applied Microbiology and Biotechnology* **104**: 8965-8977. https://doi.org/10.1007/s00253-020-10856-w

Sereshti, M., Yousofi Darani, H., Zebardast, N., Rafieian-Kopaei, M., Naeini, M. & Yousofi, H. A. (2012). Effect of ethanolic and watery extract of aerial parts of *Stachys lavandulifolia* on *Trichomonas vaginalis*, *in vitro*. *Journal of Medicinal Plants* **11**: 159-165.

Siadat, A.H., Zolfaghari, A., Shahmoradi, Z., Shariat, S. & Sohrabi, K. (2020). Application of laser for treatment of cutaneous leishmaniasis: a review of literature. *Lasers in Medical Science* **35**: 1451-1457. https://doi.org/10.1007/s10103-020-03006-1

Singleton, V.L., Orthofer, R. & Lamuela-Raventós, R.M. (1999). [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology* **299**: 152-178. https://doi.org/10.1016/S0076-6879(99)99017-1

Tomou, E.M., Barda, C. & Skaltsa, H. (2020). Genus *Stachys*: A review of traditional uses, phytochemistry and bioactivity. *Medicines* **7**: 63. https://doi.org/10.3390/medicines7100063

Tundis, R., Peruzzi, L. & Menichini, F. (2014). Phytochemical and biological studies of *Stachys* species in relation to chemotaxonomy: a review. *Phytochemistry* **102**: 7-39. https://doi.org/10.1016/j.phytochem.2014.01.023

Ullah, R., Alqahtani, A.S., Noman, O.M., Alqahtani, A.M., Ibenmoussa, S. & Bourhia, M. (2020). A review on ethno-medicinal plants used in traditional medicine in the Kingdom of Saudi Arabia. *Saudi Journal of Biological Sciences* **27**: 2706-2718. https://doi.org/10.1016/j.sjbs.2020.06.020

Vaselek, S. (2021). Canine leishmaniasis in Balkan – A review of occurrence and epidemiology. *Acta Tropica* **224**: 106110. https://doi.org/10.1016/j.actatropica.2021.106110

Yadegari, J.G., Khalaf, A.K., Saadatmand, M. & Mahmoudvand, H. (2022). Antiparasitic activity of Astragalus brachycalyx subsp. brachycalyx extract against hydatid cyst protoscoleces and its effect on induction of apoptosis: an *in vitro* and *ex vivo* study. *Journal of Herbmed Pharmacology* **11**: 428-34. https://doi.org/10.1007/s12639-022-01517-y

Zangger, H., Mottram, J.C. & Fasel, N. (2002). Cell death in *Leishmania* induced by stress and differentiation: programmed cell death or necrosis? *Cell Death & Differentiation* **9**: 1126-1139. https://doi.org/10.1038/sj.cdd.4401071