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 cytoxicity of SLME on human (THP-1) and murine (J774-A1 cell) macrophage cells was investigated through the measuring the 50% cytotoxic concentrations (CC₅₀). 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 (IC₅₀) values for SLME and meglumine antimoniate (MA) against the promastigote stage were 23.4 and 71.1 µg/mL, respectively. For amastigote stage, the IC₅₀ 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 CC₅₀ 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 (Roat et al., 2020). Reports from physicians also indicate recurrence, lack of improvement, adverse effects of medications, and the occurrence of dangerous side effects
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 FeCl3 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% 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

AlCl3 colorimetric approach was applied to measure the total content of flavonoids. Initially, extract was mixed with AlCl3 (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 3 h. the optical density of the combination was then read at 510 nm; the findings were finally expressed as mg Catechin equivalent per gram dry 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 (I774-A1) macrophage cell lines 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% CO2.

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 x 10⁶/mL) were seeded in the 24-Well Lab-Tek plated (with 1 cm² 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 x 10⁶/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 (IC₅₀) 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 x 10⁶/mL) were pre-treated with SLME (5 µg/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 IC₅₀ based on the previous investigation. Briefly, promastigotes (1 x 10⁶/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 x 10⁶/mL) with various concentration of SLME (1/4 IC₅₀, 1/3 IC₅₀, and ½ IC₅₀) for 48 h, in a 96-well plate the supernatant of reaction (20 µL) was mixed with the nitrate 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 IFNγ (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 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 x 10⁶/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 (CC₅₀) and subsequently the selectivity index (SI) according to the ratio CC₅₀ for macrophage/IC₅₀ 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 x 10⁶ 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 (intraleisional 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 IC₅₀ and CC₅₀ 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,

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 Sereshni 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 trophozoites 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
The 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. (Zannger et al., 2002). Our results showed that in colorimetric protease assay SLME, mainly at 1/3 IC₅₀ and ½ IC₅₀ markedly provoked the induction of the caspase-3 activity by 19.2 and 32.6%, respectively (Figure 4).

Today, 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. IFN-γ 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 anti-leishmanial 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 IFN-γ 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 ½ IC₅₀ and ⅔ IC₅₀, markedly provoked the induction of the caspase-3 activity by 19.2 and 32.6%, respectively (Figure 4).

Table 1. The 50% inhibitory concentrations (IC₅₀) and 50% cytotoxic concentrations (CC₅₀) 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)

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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)

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* 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)

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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

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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.
inhibition of bacterial virulence factors, exhibition a synergistic effect with existing synthetic drugs, etc (Chusnie & Lamb, 2005; Bourar 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 its 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 µg/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.

**ACKNOWLEDGEMENTS**

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**Conflicts of Interest**
The authors declare no conflict of interest in this study.

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*Figure 5.* The level of cytokines IFN$\gamma$, 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).

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