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Article type : Original Article

Immunotherapeutic Effects of *Glycyrrhiza glabra* and Glycyrrhizic Acid on *Leishmania major* Infected BALB/C Mice

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Disclosures

The authors declare that there is no conflict of interest regarding to the concept and publication of this article.

Conflicts of Interests

The authors declare no conflict of interest regarding to the concept and publication of this article.

Acknowledgments

This article has been extracted from an MSc thesis compeleted by Shima Sheikhi in School of Medicine, *Shahed University of Medical Sciences* (Ethical code: IR.Shahed.REC.1396.114). This study was financially supported by Immunoregulation Research Center, Shahed University, Tehran, Iran, and Center for Research and Training in Skin Diseases and Leprosy, *Tehran University of Medical Sciences, Tehran, Iran.*

Data Availability Statement

The data supporting of study are not publicly available but are available through corresponding authors upon reasonable request.

Author contribution

The authors helped to accomplish of the study with unsparing efforts. The corresponding Authors have planned the research, supervised the experiments and led the manuscript preparation and submission. Author 1 and author 5 carried out the experiments. Author 3 performed the plant extraction and phytochemical section. Every author contributed in the manuscript preparation.

Abstract

Aim: The objective of the current study was to investigate the Immunotherapeutic activities of the hydroalcoholic extract of *Glycyrrhiza glabra* (HEG) and glycyrrhizic acid (GA) in the treatment of *Leishmania major* (*L. major*) infection BALB/c mice.

Methods: The effect of HEG, GA was checked *in vitro* on growth of *L. major* promastigote and amastigote using MTT assay and microscopic counting, respectively. For *in vivo* experiment, *L. major* infected BALB/c mice were treated intraperitoneally with HEG, GA, Meglumine antimonite, or phosphate buffer saline as a negative control for one month. Then, the cytokine response (IFN-γ and IL-4) to *Leishmania* antigens was evaluated using ELISA method. The parasite burden of the lymph node was assessed at the end of treatment.

Results: HEG, and GA significantly inhibited growth of of *L. major* promastigotes and amastigotes. Although the lesion development, parasite burden in the lymph nodes, level of IFN-γ and the ratio of IFN-γ/IL-4 in HEG, GA and Meglumine antimonite treated mice were significantly higher compared with the negative control group, but there was no difference between the HEG, GA and Meglumine antimonite group.

Conclusion: The hydroalcoholic extract of *G. glabra* and glycyrrhizic have shown therapeutic and immunomodulatory effects on *L. major* infected BALB/c mice.

Keywords: *Glycyrrhiza glabra*, Glycyrrhizic acid, *Leishmania major*, Immunomodulatory

1. Introduction

Cutaneous Leishmaniasis (CL) is a protozoan skin disease caused by different species of *Leishmania* in the old world. CL is the most common form of leishmaniasis. Treatment of CL is difficult in different parts of the world, Iran is among 9 countries with the most reported(1). Clinical form of CL depend upon the causative species *Leishmania*, and the host immune response (2-4) .Experimentally, it has been shown that induction of Th1 type of response plays a critical role in leishmaniasis control. IFN-γ which is secreted mainly by Th1 cells, up-regulates macrophages Nitric Oxide (NO) generation and NO inhibits parasite replication, while induction

of Th2 response is associated with secretion of interleukin IL-4, IL-10, IL-13 which is associated with suppression of IFN-γ mediated macrophage activation (5-8).

Pentavalent antimonial derivatives (meglumine antimoniate or Meglumine antimonate , and sodium stibogluconate or Pentostam) remain the first-line treatment for CL .It has been demonstrated that Meglumine antimonate is responsible for oxidative stress-derived DNA damage in leishmania infection with reduced side-effects and more availability (9, 10).Although, many other modalities are used as the second-line treatment. In general, on the one hand, treatment of CL is recognized as a challenging obstacle, which is accompanied by side effects, drug resistance, long period of injections, and sometimes low efficacy(11-14) . Despite the possibility of developing vaccines against leishmaniasis, no vaccine has been manufactured yet(15, 16). Therefore search for effective modalities against CL is required. Nowadays, there is a great interest to research on the effects of the herbal medicines due to safety, low cost and immunomodulatory properties on various diseases including CL (17-20).

Among plants used in herbal medicine, *Glycyrrhiza glabra (G. glabra)*, also known as 'licorice', is a well-known plant species to the *Leguminosae* (also known as *Fabaceae*) family (21-23). It is routinely found in *Asia Africa,* and *Mediterranean* regions (22-24). Interestingly, the roots and rhizomes of this plant have been used as a drug for more than 4,000 years (25, 26). From phytochemical aspects, roots of *G. glabra* contain water-soluble compounds such as triterpenes, saponins, flavonoids, polysaccharides, pectin, amino acids, mineral salts, etc (27). Glycyrrhizic acid (GA) is the main component of *G. glabra*, constitute of 5%-20% of the root's weight (23, 28) and as a major therapeutic and immunomodulatory agent was extracted in the hydroalcoholic extract of Glycyrrhiza glabra(29, 30). Also the result of phytochemicals investigations of G. glabra roots revealed the presence of carbohydrates, phenolic compounds, flavonoids, proteins, saponins, sterols and tannins in methanolic extract(31).

G .glabra roots were used for treatment of gastritis, respiratory infections, peptic ulcers, dyspepsia, as an anti-inflammatory agent and alleviation of allergic reactions (26, 32, 33), and Inflammation-associated Corneal Neovascularization (34). Experimental and clinical studies showed an immunomodulatory and pharmacological effects of *G. glabra*, including anti-oxidant, anti-bacterial, anti-viral, anti-fungal, and anti-tumor activities (35-40). The results of study depicted that combination therapy of GA and sodium antimony gluconate in the treatment of *L. donovani* infection in BALB/c mice induced a significanthy lower parasitic burden, and up-

regulation of Th1 response (41). Another study conducted by the same group revealed that GA treatment of *L. donovani* infected macrophages, significantly increased the expression of IL-12 and TNF-α, and reduced IL-10 and TGF-β (42) .As it has been investigated in another study the immunomodulatory activities of aqueous roots of *G. glabra* when combined with zinc significantly enhance leukocyte count, antibody titer, and phagocytic index (43). Furthermore, the main component of *G. glabra* showed immunomodulatory effects (like anti-oxidant, anti-bacterial, anti-ulcerative, anti-tussive and expectorant (22, 44), anti-protozoa, anti-tumor (39), and anti-viral activities (21, 24), which accompanies with induction of free radicals (45), enhanced Th1 responses, activated macrophages, and NO generation, IgG2a/IgG1 ratio, as well as expression of CD86, CD40, and MHC-II markers on Dendritic Cells (DCs) (13, 38, 46, 47).

There are a wide range of molecular studies, investigating immunomodulatory properties of herbal compound such as *G. glabra* or GA aimed at leishmaniasis treatment due to the existence of immunostimulants like saponin triterpenoid agents in their alcoholic extracts (42, 48-51).

Present study is proposed to explore immunomodulatory and immunotherapeutic effects of hydroalcoholic extract of *G. glabra* (HEG) and GA on *in vitro* growth of *L. major* and *in vivo* effect on *L. major* infection in BALB/c mice.

2. Materials and Methods

2.1. Ethical Considerations

This study was reviewed by Research Ethical Committee of Shahed University of Medical Sciences (Tehran, Iran) (IR.Shahed.REC.1396.114).

2.2. Parasite and Animals

L. major (MRHO/IR/75/ER) was collected from infected mouse and cultured in NNN and sub-cultured in RPMI-1640 medium (Sigma) supplemented with 15% heat-inactivated Fetal Calf Serum (FCS) (Gibco), 200 U/mL of penicillin (Sigma), and 200 μg/mL of streptomycin (Sigma). Fifty female BALB/c mice (5–7 weeks old) were purchased from Pasteur Institute of Iran and kept in an air-conditioned room with a 12:12 h light: dark cycle at room temperature and fed with water and standard rodent pellets. Experiments were performed according to the Ethics Committee Regulations of Shahed University of Medical Sciences.

2.3. Preparation of *G. glabra* Extract

The GA powder (70%) was purchased from Sigma-Aldrich. Roots (or rhizomes) of *G. glabra* were purchased from Shirin Daru Shiraz Company, *Shiraz*, *Iran*. The licorice powder (1 g) was weighed and extracted by the ultrasonication method (30 min, 50°C) with 100 mL of aqueous methanol extraction solvent (50:50 v/v). The extract was concentrated under vacuum at 50°C using a rotary evaporator (Heidolph, Germany) and the solvent was removed in a hot air oven. The extract powder was stored at 4°C, dissolved in Phosphate-Buffered Saline (PBS) and used after sterilization through filtration through a 0.22µm sterile filter.

2.4. Cytotoxic activity on Macrophages *in vitro*.

BALB/c peritoneal macrophages were harvested and added at $4x10^5$ cells per well, in 96-well plates, and incubated for 2 hours at 37 ºC and 5% CO_2.Then the medium was substituted with RPMI-1640 supplemented with 10% FCS, 1% penicillin-streptomycin solution 1%, containing the HEG or GA (0-5000 μg/mL) and incubated for an 24-hour. MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Then, the medium and MTT were removed. Dimethyl Sulfoxide (DMSO) was added as solubilizing agent then, the absorbance (Optical Density (OD)) of each well was read immediately using an ELISA MICROPLATE READER with 540 nm wavelengths. (BioTek, Instrument, NC, USA). Macrophages with no drug treatment were used as control and culture medium with no macrophages and treatment was considered as blank. Also, the Selectivity Index (SI) was calculated by the ratio of cytotoxicity (CC50) of host cells/IC50 of promastigotes and amastigotes in macrophages. Dimethyl Sulfoxide (DMSO) was added as solubilizing agent and

2.5. Anti-promastigotes Assay

The effects of HEG and GA on promastigotes of *L. major* were assessed using colorimetric cell viability MTT assay. Practically, 5×10^5 promastigotes were harvested at logarithmic phase and plated to each well of the 96-well culture plate. Then, 20 μL of various concentrations of HEG (0-7500 μg/mL), GA (0-4000 μg/mL) or Meglumine antimonate (25-250 μg/mL) were added to each well and incubated at 26ºC for 24 and 48 hours. After adding MTT solution (5 mg/mL) Dimethyl Sulfoxide (DMSO) was added as solubilizing agent. Then, the absorbance (Optical

Density (OD)) of each well was read on an ELISA MICROPLATE READER, using 540 nm wavelength (BioTek, Instrument, NC, USA). Culture medium was used as blank and promastigotes with no drug treatment were considered as control.

2.6. Anti-amastigote Assay

Twenty four hours after treatment of the parasite-infected macrophages, the cytotoxcic effects of HEG and GA on the growth of amastigotes were examined through microscopic assays by counting the number of amastigotes in macrophages in all of the groups. 5×10^5 murine macrophages were distributed in each well of 6-chamber slides (Lab-Tek, Nalge Nunc International, NY, USA), and then were incubated for 24 hours at 37 ºC and 5% CO_2 atmosphere .In the next step, 2.5×10^6 promastigotes at stationary phase were added to the murine macrophages and incubated for an additional 4 hours. Then, free parasites were removed and the culture was treated with different concentrations of HEG, GA, or Meglumine antimonate and incubated for 24 hours. Finally, the slides were fixed with methanol and stained by Giemsa. 100 macrophages were checked using a light microscope and the number of infected macrophages and amastigotes per macrophages were recorded. The infected macrophages without drug treatment were considered as control and uninfected macrophages with no treatment were used as blank.

2.7. Experimental Design

Fifty BALB/c mice (5–7 weeks old) were infected by subcutaneous injection of *L. major* amastigotes obtained from infected BALB/c spleen homogenates in the hind footpad. Four weeks later, the mice with lesions and nodules were randomly divided into 5 groups (10 mice per group) and treated intraperitoneally daily for one month as follows: 200 or 600 mg/kg of HEG, GA (200mg /kg), Meglumine antimonite (160 mg/kg), or PBS as a negative control.

2.8. Lesion Development

The lesions' diameter were measured weekly in two dimensions, using a digital caliper before and during the treatment procedure (D and d). The size (mm) was presented according to the formula $S = (D + d)/2$ (52).

2.9. Parasite Load

A Limiting Dilution Assay (LDA) in the inguinal lymph node cells culture was used for the evaluation of parasite burden. Briefly, inguinal lymph nodes of sacrificed mice were removed and 10-fold serial dilutions of lymph node cell suspension (12 serial dilutions) were prepared in Schneider medium supplemented with 12% heat-inactivated FCS (Gibco), and penicillin-streptomycin solution 1%. Then, 100 µL of each serial dilution was added to a 96- culture plate, 8 wells for each dilution. After 7 days incubation at 26ºC, the number of negative wells (absence of motile parasites), and positive wells (presence of motile parasites) were checked using an inverted microscope. The frequency of viable *L. major* parasites was computed using the LDA program (53).

2.10. Cytokine Assay

To evaluate the immune responses, the splenocytes were isolated from different experimental groups and cultured for 72 hours at 37 ºC and 5% CO_2 atmosphere, in the presence of Soluble *Leishmania* Antigen (SLA; 10µg/mL). The levels of IL-4, and IFN-γ in the supernatants of cultured splenocytes were titrated using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The assay was performed according to the manufacturer's instruction commercial kit (R&D Systems) for quantitative detection of mentioned cytokines in the supernatants of cell culture.

2.11. Nitric Oxide Quantification

Macrophages from the peritoneum of different groups of mice were isolated. Single-cell suspension was prepared and 4×10^5 cells were added to each well of a 96 plate in the presence of 10 µg/mL of SLA and were incubated for 24 hours at 37ºC and 5% CO_2. Then, the supernatants were collected and an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-[1-

naphthyl] ethylenediamine dihydrochloride in 5% H_3PO_4) was added. The plates were incubated for 10 minutes at room temperature and then, the absorbance of each well was read immediately on an ELISA MICROPLATE READER (Garni Company) at 540 nm. The nitrite levels were measured using the standard curve of sodium nitrite.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS) version 22.0 computer software (SPSS Inc., USA) was used for the biostatistical analysis of acquired data. The Kolmogorov-Smirnov test was applied for the determination of data distribution. Parametric one-way Analysis of Variance (ONE-WAY ANOVA) and Non-parametric Mann Whitney *U* test was used for comparison between the groups and control group. All of the experiments were carried out in triplicate. Statistical significance was considered at p value <0.05.

3. Results

3.1. Cytotoxic activity on Macrophages in vitro.

The results of colorimetric cell viability MTT assay for the evaluation of the biological effects of HEG and GA on murine macrophages showed that the average CC50 of HEG on macrophages was 2,500 ± 0.018 µg/mL and CC50 value of GA was 5,000 ± 0.068 µg/mL(Table1). Using different dosages of HEG (from 0 to 1,000 µg/mL) and GA (from 0 to 2,000 µg/mL) did not significantly inhibit the macrophages growth.

3.2. Anti-promastigote Assay

Cytotoxic effects of HEG and GA on promastigote forms of *L. major* were assessed by MTT assay. Treatment with HEG and GA significantly inhibited the growth of promastigotes of *L. major* at least in the concentrations of 250, and 500 µg/mL, respectively. As shown in Table 1, the average IC50 values 24 hours after treating with HEG, GA and Meglumine antimonate on promastigote of *L. major* were 1250 ± 0.018, 3000 ± 0.017, and 50 ±0.043 µg/mL, respectively.

Additionally, the average IC50 values 48 hours after treating with HEG, GA and Meglumine antimonate were 1,000 ± 0.016, 3,000 ± 0.017, and 25 ± 0.009 μg/mL, respectively.

3.3. Anti-amastigote Assay

The growth inhibitory effect of HEG and GA was examined by microscopic counting the number of amastigotes in infected macrophages (Figure 1). The IC50 values of HEG, GA and Meglumine antimonate on amastigote form of *L. major* were 500 ± 0.058, 1000 ± 0.12, and 25± 0.028 μg/mL, respectively.

3.4. Lesion Development Assay

The footpad swelling of different groups of mice was measured for five weeks using a digital caliper (Figure 2). The mean lesion size in mice received HEG, GA or Meglumine antimonite was significantly decreased when compared to the PBS group (Figure 3). Also, there was a significant increase in the lesion development in negative control mice after five weeks and no significant difference was observed among the treated groups.

3.5. Parasite Load

The numbers of viable parasites in the draining lymph nodes of infected mice were measured using LDA assay (33). As shown in Figure 4, the mice in the control group (receiving PBS), showed a significantly higher load of the parasites while a group of mice receiving HEG, GA and Meglumine antimonate significantly presented a lower parasite load in the lymph node compared to the control group (Figure 4). Also there was no significantly difference between herbal drugs and Meglumine antimonite.

3.6. Cytokine Assay

The results of IFN-γ and IL-4 levels in the supernatants of cultuterd splenocytes in the different groups of mice depicted that the level of IFN-γ was significantly higher in groups treated with GA, Meglumine antimonate and HEG (600 mg/kg) compared to the negative control (Figure 5a),but there was no significant difference between herbal drugs and Meglumine antimonate. The amount of IL-4 (Figure 5b) was lower in the treated groups. The ratio of IFN-γ /IL-4 for each

mouse in the groups receiving HEG, GA, and Meglumine antimonate was significantly higher in comparison with negative group .In addition there was no significant difference between herbal treatments and Meglumine antimonate ($p<0.05$) (Figure 5c).

3.7. Nitric Oxide Quantification

The NO levels were quantified in the supernatant of cultured macrophages of different groups using Griess assay. The NO production of macrophages in the mice of control groups (receiving PBSshowed the lowest levels. Although the mean value of NO in other treated groups was higher in comparison with the group received PBS, NO value in GA and HEG groups (600 mg/kg dose) was significantly higher in comparison with other groups ($p<0.05$). (Figure 6).

4. Discussion

As one of the unresolved challenges in health system, treatment of CL is an untackled obstacle, being accompanied with side effects and drug resistance (22, 54, 55). Hence, there is an imperative need to exploiting knowledge toward more effieicient approaches for treatment of leishmaniosis (56). Therefore, the development of medicinal plants that possess dual anti-parasitic and immunomodulatory properties is helpful against leishmaniasis. In this research, we investigated the anti-*Leishmania* and immunomodulatory effects of hydroalcoholic *G. glabra* root extract and GA on *L. major* infection in BALB/c mice. *In vitro* cultures results indicated that HEG and GA considerably inhibited the growth of both forms of *L. major*. IC50 of HEG for the *L. major* was comparatively less than the GA which might be caused by other chemical components in the extract. FurthermoreIC50 of HEG for the *L. major* was less than aqueous extract (57) .Similarly, in another study, Licorice extracts have been displayed to inhibit the promastigote growth of *L. chagasi* and *L. mexicana* (58). The cytotoxicity of GA on the amastigote form of *L. donovani* was less than the current findings on *L. major* (23), using the salts of GA 70% instead of GA 98% and differential virulence of the *Leishmania* strains may be accounted for contrasting. It has been reported that licorice extract has promising antimicrobial properties (35, 36).

In vivo experiments revealed strengthened anti-*Leishmania* activities of HEG and GA by GA by reducing footpad lesion size and parasite burden in the lymph node. These results are in

accordance with the results of previous studies where a decreased footpad lesion of BALB/c mice, a significantly increased collagen contents, and inclined number of the fibroblasts were reported due to the immunomodulatoey properties of aqueous licorice extract. In addition, increased capillary buds of the wounds in rats, promisingly accelerated wound healing (59). Besides, GA has previously shown modulatory properties for the reduction of parasite burden in *L. donovani* infected mice (22-23).

It has been proven that induction of Th2 pattern is related to susceptibility, whereas, development of Th1 is accompanied by resistance in *L. major* infection in a murine model (6). Thus we have assessed the cytokines released in the supernatants of cultured splenocytes stimulated with *Leishmania* antigens. The data revealed that BALB/c mice treated with GA, 600 mg/kg dose of HEG and Meglumine antimonate significantly induced IFN-γ production ($p<0.05$). Although the lower level of IL-4 secretion was not significant, the up-regulation of the IFN-γ/IL-4 ratio displayed shifting Th2 toward Th1 response by the generation of more IFN-γ, being crucial for the effective immune responses against leishmaniasis. Moreover, NO production was significantly augmented in the peritoneal macrophages of groups of mice treated with GA and 600 mg/kg dose of HEG. On the other hand, HEG and GA treatment were found to be responsible for making an increase in the induction of IFN-γ and NO, verifying that parasite reduction was mediated via NO production (60). Our results were in accordance with several previous reports. Co-administration of GA and Sodium Antimony Gluconate (SAG) in *L. donovani* infected mice enhanced secretion of several pro-inflammatory cytokines (such as IL-12, IFN-γ, and TNF-α), and decreased secretion of several anti-inflammatory cytokines IL-4, IL-10, and TGF-β. Although, there was no significant decrease in the secretion of IL-10, TGF-β, and IL-4 after solely treatment with GA. So, if licorice extract and GA along with Meglumine antimonate are used, maybe there is a significant reduction in the secretion of IL-4 (22). Usage of GA in *L. donovani* infected macrophages boosted the expression of TNF-α and IL-12, concurrent with reduction in the secretion of IL-10 and TGF-β (23). Administration 18β-glycyrrhetinic acid coused a 11-fold increase in NO production in mouse peritoneal macrophages infected with Promastigote of *Leishmania donovani* and elimination of parasite load in infected BALB/c mice. Cytokine assay showed switching from Th2 to Th1, induced by NF-κB activation (61). Simalrly to our results, there is differentiated immune responses to induction of Th1 patterns.

Immunomodulation is a process in which the immune responses are modified. *G. glabra* and its component have been described to possess immunomodulatory and adjuvant features. Results from an experimental study showed that the aqueous root extract of *G. glabra* (at the dose of 1.5 g/kg) combined with zinc has been shown to significantly increase in leukocyte count, and phagocytic index when compared to the control group (25). Oral administration of aqueous methanolic extract of *G. glabra* (roots) against sporulated oocysts of mixed *Eimeria* species in broiler chickens can be totally served as an immunomodulant with significantly higher cell-mediated, and humoral responses (in comparison with PBS treated ones) In case of cell-mediated response, results of our study are in aaccordance with this study (62). Purified Glycyrrhiza polysaccharides enhanced the secretion of NO, IL-6, IL-1, and IL-12 in macrophages (25). In a study, it was shown that 18β-glycyrrhetinic acid induced the highest secretion of IFN-γ, and IgG2a/IgG1 ratio when compared to the control groups in an experimental *Candida albicans* infection (26). GA was able to up-regulate IL-12 production and MHC-II, CD86, and CD40 markers expression on DCs and augmented proliferation of T cells and generation of IL-10 and IFN-γ cytokines and diminished IL-4 secretion (38). In another study, increased IFN-γ level and inhabitant of ovalbumin-induced increases eosinophil count, as well as IL-13, IL-4, IL-5 levels in the bronchoalveolar fluid by GA were reported (27). Treatment of H22 hepatocarcinoma tumor-bearing mice with Glycyrrhizae polysaccharide inhibited tumor progression (63).

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5. Conclusion

According to the difficulties in treatment of leishmania infection, it seems that there should a great research interest toward more efficient therapeutic approaches, including novel understandings on immunomodaltion-based therapeutic strategies. Present data indicated that licorice extract and GA indicated their anti-*Leishmania major* and immunostimulatory activites. It is believed that there is an imperative need for investigations of a wide range of natural products as immunomodulants, and various immune responses by more cytokines in more comprehensive in vitro and in vivo experiments to reach the most efficient therapeutic approaches individually or as a part of combination therapy.

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Table1. *In vitro* effects of HEG and GA on murine macrophages, promastigote, and amastigote of *L. major*

Drug	Macrophages CC50[a] (µg/mL)	Promastigote IC50[b] (µg/mL)	Amastigote IC50 (µg/mL)	Amastigote SI[C]
Hydroalcoholic extract of *G. glabra*	**2500 ± 0.018**	**1250 ± 0.018**	**500 ± 0.058**	**5**
Glycyrrhizic acid	**5000 ± 0.068**	**3000 ± 0.017**	**1000 ± 0.12**	**5**

[a] 50% Cytotoxic Concentration, [b] 50% Inhibitory Concentration, [c] Selectivity Index (CC50/IC50).

Figure Legends

Figure 1. Giemsa sained pictures of intra-macrophagic amastigotes after treatment with G. glabra extract, GA, and Meglumine antimonite. (A) Macrophages infected with *L. major* (5 parasites/cell), (B) Infected macrophages treated with 500 µg/mL of HEG, (C) Infected macrophages treated with 25 µg/mL Meglumine antimonate , (D) Infected macrophages treated with 1000 µg/mL of GA.

Figure 2. Lesion in foodpat of infected mice before and after treatment in different groups. a) lesion in foodpat of mice after one month, b)mice before treated with 200(mg/kg) HEG, c) mice before treated with 600(mg/kg) HEG, d) mice before treated with GA, e) mice before treated with Meglumine antimonite, f) mice before treated with PBS, g) mice after treated with200(mg/kg) HEG, h) mice after treated with600(mg/kg) HEG, i) mice after treated with GA, j) mice after treated with Meglumine antimonite, k) mice after treated with PBS.

Figure 3. Mean of lesion development in different groups. The mean of lesion size in mice received HEG, GA and Meglumine antimonite was reduced significantly compared with the negative control group from 3 to 5 weeks after treatment. Differences were considered statistically significant at $P < 0.05$.

Figure 4. The logarithm of the parasite burden in the lymph node of groups. The number of parasites decreased significantly in the mice treated with HEG, GA and Meglumine antimonate compared with the negative control (*P<0.05).

Figure 5. IFN-γ (a) and IL-4 (b) levels produced by splenocytes in the presence of SLA from experimental groups. A) The level of IFN-γ was significantly increased (P<0.05) in GA, Meglumine antimonate , and 600 mg/kg dose of HEG groups compared with the negative control, B) no significant IL-4 production was seen in all groups, C) The IFN-γ / IL-4 in HEG, GA and Meglumine antimonate injected mice were enhanced significantly (*P<0.05).

Figure 6. NO generation in the supernatant of macrophages from experimental groups in the presence of SLA. GA and 600 mg/kg dose of HEG groups were exhibited a significant increase in NO production (*P<0.05).