





Leishmanicidal Effects of *Allium giganteum* Saponin-Rich Fraction on *Leishmania major*

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Abstract

Background and objectives: Leishmaniasis is caused by the genus of *Leishmania* and is one of the important health problems worldwide. Serious side effects, the lack of effective vaccines and the emergence of drug resistance are the major weak points of leishmaniasis treatment. The purpose of this study was to evaluate leishmanicidal effects of *Allium giganteum* saponin rich fraction, natural compounds with history of antimicrobial properties, on promastigotes and axenic amastigotes of *L. major* and macrophages cell line J774. **Methods:** The chloroform-methanol (9:1) extract of the flowers was fractionated by MPLC using an RP-18 column. The saponin-rich fraction was detected by TLC and H-NMR analyses and evaluated for leishmanicidal activity on *L. major* and macrophages cell line J774 using MTT assay at 24, 48 and 72 h of incubation. **Results:** At concentrations of 75, 100 and 150 µg/mL, over the time of 24 to 72 h, a significant decrease in the live parasite's rate was observed ($p < 0.05$). At 200 µg/mL concentration, all parasites were killed and maximum leishmanicidal effect was observed. The IC_{50} s for promastigotes and axenic amastigotes were 90.01 ± 13.42 µg/mL and 29.76 ± 17.91 µg/mL, respectively; the value for the J774 macrophage cell line was 33.17 ± 4 µg/mL. **Conclusion:** The results of this study showed the significant leishmanicidal effect of saponin rich fraction from *Allium giganteum* on promastigote and axenic amastigote of *L. major* and macrophage cell line in vitro. Complementary in vivo studies for evaluating the effects of the fraction on leishmaniasis in BALB/c mice is recommended.

Keywords: *Allium giganteum*; *Leishmania major*; MTT assay; saponin

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Introduction

Leishmaniasis includes a range of diseases caused by different species of obligate intracellular parasites of *Leishmania* and transmitted by the bite of infected sand flies. The disease has been identified as one of the 10 major parasitic diseases in the tropical regions by the WHO. Leishmaniasis has been reported in

cutaneous, mucocutaneous and visceral forms in more than 98 countries worldwide, with an annual incidence of 2 million cases of cutaneous and 500,000 cases of visceral leishmaniasis. Cutaneous leishmaniasis is prevalent in two clinical forms in Iran, wet (rural) caused by *L. major* and dry (urban) by *L. tropica* [1]. The

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highest incidence of cutaneous leishmaniasis has been reported from Afghanistan, Iran, Saudi Arabia, Syria, Palestine, Brazil and Peru [2].

Despite self-limiting of the cutaneous form, for several reasons including ugly scarring and chronic wound cases treatment is recommended. The first therapeutic line is pentavalent antimonial compounds (SbV). These compounds, by disrupting the functioning of the enzymatic system of phosphokinase, reduce energy production and result in the death of the parasite ultimately [3]. Today, the occurrence of drug resistance leads to the development of strains that are highly resistant to the first-line drugs [4]. In Iran, therapeutic failure has been observed in 12% of patients who were given antimonials to treat *L. tropica* infections. In the Mediterranean basin, the treatment efficiency of visceral leishmaniasis has been reported roughly 95% [5].

Other medications include amphotericin B, pentamidine, and myltheposin [4]. The limitations of the mentioned treatments such as high cost, painful injection, patient inaccessibility and also side effects reveal the need to discover effective, low-cost, with fewer side effect medications. Considering the importance of medicinal plants and the potential of natural compounds in the treatment of various diseases, a large part of today's research has been devoted to the therapeutic effects of herbs and their compounds [6,7]. Examples of natural compounds with anti-leishmaniasis effects, which have been investigated and documented in numerous studies are quinoline alkaloids (copsarine), isoquinoline alkaloids (limassin), saponins (alpha-hedrin) and naphthoquinones (lapakol) [8-10].

Allium genus (Amaryllidaceae) comprises important medicinal plants and has 900 species in many parts of the world. There are also about 135 species of this plant in Iran and they are rich sources of flavonoids, steroidal saponins and organosulphur compounds [11]. Steroidal saponins are high molecular weight glycosides that are made up of glyconi portion attached to the agglyconi (sapogenin). They have pharmacological effects including cardioprotective, anti-tumor, immunomodulator, lipid-lowering, anticoagulant, antifungal and anti-parasitic effects [12-14]. In addition to the use of this species in the treatment of leishmaniasis in the traditional way, the anti-leishmaniasis effect of these plants, especially *A. sativum* (garlic), as

well as the steroid saponins extracted from other natural sources, including Razmoseide A, and the combination of saponin steroid extracted from *A. paradoxum* has been proven in recent scientific studies [15]. The antibacterial effects of saponins against *Streptococcus bovis*, *Ruminobacter amylophilus* and antifungal effects against *Cryptococcus neoformans* and *Aspergillus fumigatus*, as well as anti-parasitic effects against *Trichomonas vaginalis*, have been also demonstrated. *Allium giganteum* grows in the eastern regions of Iran [16-18]. It is a herbaceous perennial plant, with swollen onions, variable height from 20 to 30 cm and a particular fragrance similar to the garlic family. Its leaves are thin, ribbon-shaped and flowers are spherical umbrella shaped white and purple in color [19]. Regarding the pharmacological and anti-parasitic effects of *Allium* plants, especially *A. giganteum*, this study was performed to fractionate the compounds in this plant and determine the effect of saponin-rich fraction on promastigotes and amastigotes. Also, since the location of the leishmania parasite in the vertebral host is macrophages, in this study the cytotoxic effect of the saponin-rich fraction on macrophage was also evaluated.

Materials and Methods

Ethical considerations

This research was an experimental study that evaluated the effect of the *A. giganteum* saponin-rich fraction on *Leishmania major* (MRHO / IR / 75 / ER) compared to Amphotericin B. The study protocol was approved by the ethical committee of Isfahan University of Medical Sciences, Isfahan, Iran with the code No: IR.MUI.REC.1395.3.926, 2017.

Chemicals

Glucose, sodium chloride, brain heart Infusin agar, ethyl acetate, methanol, bioethanol, hexane, chloroform, hydrochloric acid, sodium dodecyl sulfate (SDS) (Merck, Germany). RPMI (1640) (Bahar azma, Iran), fetal bovine serum and MTT (Sigma-Aldrich, USA), penicillin/streptomycin (Jaber Ebne Hayyan Pharmaceutical Company, Iran) were applied in the study.

Plant material and extraction

Allium giganteum flowers were collected from "Koohsorkhe" in Khorasan province, northeastern of Iran, May 2017, and dried under

shade. A plant specimen was prepared by by Mohammad Reza Joharchi, Ferdowsi University of Mashhad. A voucher specimen (No. 43213) was registered and deposited at the Herbarium of Department of Pharmacognosy; Faculty of Pharmacy, Isfahan University of Medical Sciences.

Dried flowers were powdered. Extraction was carried out by applying 4-stage maceration with *n*-hexane, chloroform, chloroform-methanol (9: 1) and methanol, respectively. Each step was repeated 4 times using 3 litres of mentioned solvents. Finally, all extracts were concentrated under vacuum at 40 °C using a rotary evaporator apparatus [20].

Fractionation

The chloroform-methanolic extract was fractionated by Medium Pressure Liquid Chromatography (MPLC). For this purpose, the glass column of the device containing silica gel RP-18 (Lichroprep® RP-18, 25-40 µm) was used as a fixed phase and linear gradient of water and methanol as the mobile phase.

Fifteen fractions were obtained. The fractions were investigated using the thin layer chromatography (TLC) SiO₂ silicagel plates; GF254, BAW solvent system (butanol: acid acetic: water at a ratio of 4: 1: 5) and Cerium(IV) sulfate reagent; based on the similarity of the compounds, seven final fractions were prepared, concentrated with a rotary evaporator apparatus and dried with the help of a freeze-dryer instrument.

The final fractions were completely dried after examination with TLC and in order to ensure the quiddity of the compounds, dissolved in 0.5 mL of methanol duotore solvent. The HNMR spectra were then prepared by means of a nuclear magnetic resonance (NMR) spectrometer. Due to the number and position of the protons in the HNMR spectra, the specific signaling patterns of saponin steroid compounds, as well as the results of the fraction analysis on TLC, the saponin-rich fractions were identified and the fractions were evaluated for anti-parasitic activity.

Cultivation of *L. major* promastigotes

Frozen promastigotes of *Leishmania major* (MRHO/IR/75/ ER) were obtained from the Department of Parasitology, School of Medicine, Isfahan University of Medical Sciences. Promastigotes were cultured in N.N.N (Novy-Nicole-Mc.Neal) medium at 24 ± 1 °C. Then, for

mass production, they were transferred to RPMI-1640 medium containing FBS (10%), streptomycin (100 µg/mL) and penicillin (100 µg/mL).

Axenic cultivation of amastigotes of *L. major*

Promastigotes were first cultured in RPMI-1640 medium enriched with 20% FBS at 26 °C for 72 h. They were then transferred to RPMI-1640 medium at pH 5.5 and cultured for 4 to 6 passages at the same temperature. The parasites were then transferred to 37 °C and 5% CO₂. Promastigotes were then transformed into amastigotes after 24 h under environmental conditions [21].

Macrophage culture

J774 macrophage cell line was purchased from Pasteur Institute of Tehran and cultured in cell culture flasks containing RPMI-1640 medium with 100 penicillin U/mL and 100 µg/mL streptomycin supplemented with 20% FBS incubated at 37 °C and 5% Co₂.

Evaluation of the leishmanicidal activity of saponin-rich fractions using MTT assay

Fifty µL of parasite suspension containing 1×10⁶ of logarithmic promastigotes were transferred to each test wells in a 96- well plate. Then 50 µL of 2, 20, 50, 75, 100, 150, 200 µg/mL concentration of saponin-rich fraction was added to the wells. For the preparation of negative control wells, 50 µL of culture medium and for positive control, 50 µL of amphotericin B solution with a concentration of 0.6 µg/mL were used. Since a small amount of DMSO was used to dissolve the test fractions, a well containing 50 µL of 0.5% DMSO solution was considered. Plates were incubated at 24 ± 1 °C and the percentage of live promastigotes in each well was determined by the MTT assay at 24, 48 and 72 h.

Cytotoxicity of saponin-rich fractions activities against J774 macrophages and amastigotes were determined using the same protocol as for promastigotes with two modifications. The final cell concentrations were 2 × 10⁶ /mL for J774 macrophages. The plates were incubated at 37 °C. To perform MTT assay, 10 µL of 5% MTT solution was added to each well and was incubated for 4 h at 37 °C and 5% CO₂ in a dark condition. Then, 100 µL of 10% SDS solution (in 0.01 N hydrochloric acid) was added to each well and incubated again for 24 h under the mentioned condition [22].

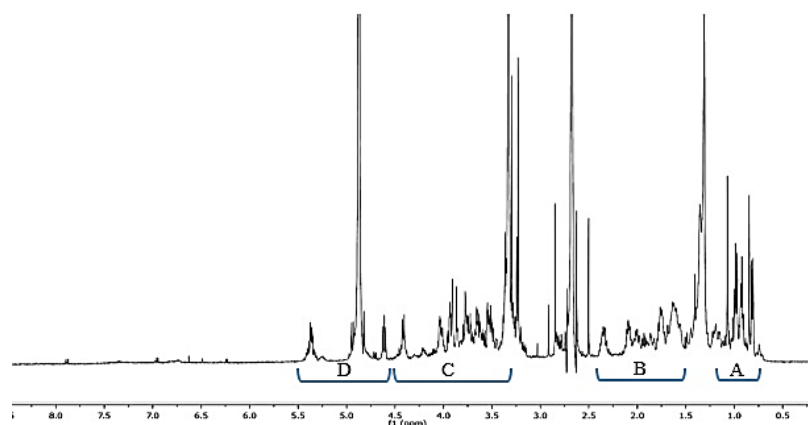


Figure 1. HNMR spectrum of the 7th fraction of chloroform-methanol extract of *Allium giganteum* (CD₃OD solvent, 400 MHz); A: two long singlets and two long doublets arising from four methyl groups of the structure; B: overlapped signals of steroidal structure protons; C: overlapped signals of sugar protons; D: signals of sugar anomeric protons

Finally, the optical absorption of formazan crystals dissolved in the wells was measured by ELISA Reader (STAT FAX, USA) at 570 nm and according to the results, the IC₅₀ (inhibitory concentration), the concentration of the drug that killed 50% of the parasites, was determined.

Each part was tested triplicate in three independent experiments against *L. major* and macrophages, and the results were expressed as the mean value.

Statistical analysis

One-way ANOVA and independent t-test were used for statistical analysis. Kruskal-Wallis and Mann-Whitney U test were used to compare the multiple and two groups, respectively at 95% significance level using SPSS-16 software.

Results and Discussion

Chloroform-methanolic extract of *A.giganteum* was fractionated using MPLC. After comparing the eluent contents using TLC and merging the similar tubes, considering the R_f of steroid saponins (0.3-0.5) and their colour with cerium sulphate reagent, it was concluded that fraction 7 was a saponin rich fraction. This finding was confirmed by preparing HNMR spectrum and observation of characteristic signals of steroid saponins specially two long singlets and two long doublets between 0.5 to 1.2 ppm arising from four methyl groups of the structure, as well as overlapped signals (1.2 to 2.5 ppm) of steroidal structure protons, overlapped signals (3.2 to 4 ppm) of sugar protons and individual signals

within 4.2 to 5.2 ppm for sugar anomeric protons (figure 1) [23,24].

Among the different concentrations of saponin-rich fractions of *A. giganteum*, at concentrations of 1-50 µg/mL over 24 to 72 h there was no significant decrease in viable promastigotes and no leishmanicidal effects were observed. But at concentrations of 75, 100 and 150 µg/mL, a significant increase in leishmanicidal effects was observed ($p < 0.05$) over 24 to 72 h. Finally, at a concentration of 200 µg/mL, the saponin-rich fraction at 24 h had maximum leishmanicidal effects and all promastigotes were destroyed and this effect was repeated at 48 and 72 h. The results also showed that amphotericin B as the positive control showed complete leishmanicidal effects at all-time intervals. In contrast, in the negative control, no significant reduction of the parasite was observed. On the other hand, the logarithmic diagram of the results showed that the leishmanicidal effects of the extract was dose and time-dependent with increasing the extract concentration up to 200 µg /mL and increasing its effect time up to 72 h. Also, the effect of the leishmanicidal fraction at 200 µg /mL showed no significant difference with the effects of amphotericin B ($P < 0.05$) (figure 2).

The effect of this extract on axenic amastigotes was found to increase with increasing lethality from 5 µg/mL to 150 µg/mL and ultimately, the lethality was 100% at 200 µg/mL, but this effect was not time -dependent and no significant difference was observed between the three tested times.

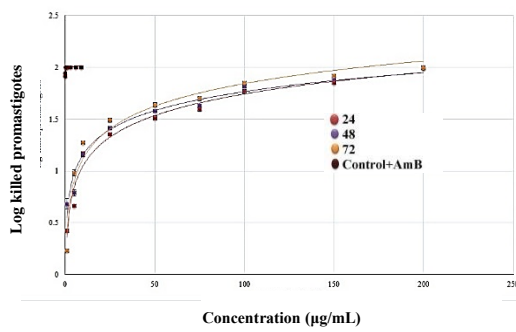


Figure 2. Leishmanicidal effects of saponin-rich fractions of *Allium giganteum* on promastigotes of *Leishmania major* at 24, 48 and 72 h incubation.

During the time, the lethal effect of the fractions isolates on J774 macrophage cell line was increased from concentrations of 5 µg/mL to 75 µg/mL ($p < 0.05$). Finally, at concentrations of 100, 150 and 200 µg/mL, the percentage of lethality was not significantly different at three-time points since at concentrations more than 100 µg/mL, 100% of cells were killed (figure 3).

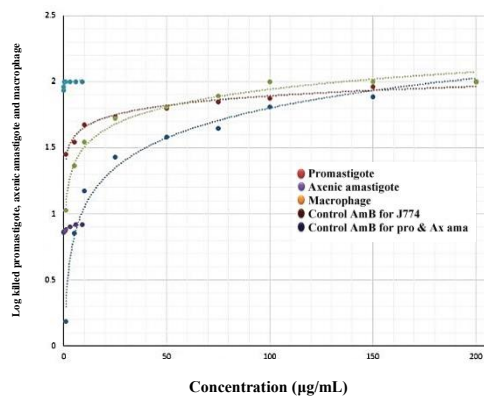


Figure 3. Leishmanicidal effects of saponin-rich fractions of *Allium giganteum* on promastigote, axenic amastigote and J774 macrophage cell line at 24, 48, and 72 h of incubation

The IC_{50} values of the saponin-rich fraction of *A. giganteum* on promastigotes in 24, 48 and 72 h didn't show any significant difference ($p < 0.001$). For amastigotes and macrophage cell line, there was no significant difference at different times ($p < 0.001$). The IC_{50} values have been provided in table 1.

Table 1. The IC_{50} (µg/mL) of saponin-rich fractions of *Allium giganteum* exposed to promastigote and amastigote and J774 macrophage cell line

Examined cells	$IC_{50} \pm SD$ (µg/mL)		
	72 h	48 h	24 h
Promastigote	62.85±3.89	75.31±5.12	90.01±13.42
Amastigote	12.86±2.24	15.69±3.14	29.76±17.91
Cell line J774	22.49±2.82	29.08±7.04	33.17±4.00

Leishmaniasis is more than an infectious disease and is a health problem in most countries of the world especially in tropical and subtropical regions. Iran is one of the areas with a significant prevalence of this disease [1,23]. Today, due to the limitations of existing treatments, the need to discover and expand effective drugs with cost-efficacy and fewer side effects is greater than ever. Given the importance of medicinal plants and the potential of natural compounds in the treatment of various diseases, many researches have been devoted to study the plant extracts. Numerous studies have demonstrated the anti-leishmanial effects of some plant like *Ferulago angulata* [24], *Prangos asperula* [24], *Artemisia aucheri* [25], *Medicago lupulina* [26] and *Allium sativum* [27] and *Ziziphora tenuior* [28].

In the present study, the leishmanicidal effects of *A. giganteum* saponin-rich fractions were investigated by MTT assay. This method used as an indicator of the growth and viability of the parasites against the drug's effects was first introduced by Mussman in 1983 and based on the conversion of tetrazolium salts into a color product (formazan) by the activity of succinate dehydrogenase present in mitochondria [29,30]. According to the findings of this study, saponin-rich fractions of *A. giganteum* demonstrated leishmanicidal effects against promastigotes and amastigotes of *L. major* as well as J774 macrophage line in vitro. It was found that these effects were concentration-dependent and increased with ascending concentration. The maximum effect was observed at 200 µg/mL and all parasites were killed in the first 24 h of incubation. Thus, the effect was not time-dependent and there was no significant difference between IC_{50} at 24, 48 and 72 h.

The parasite attacks and breaks in the vertebrate host macrophages and proliferates within them, so macrophages decompose and cause clinical signs and skin lesions. In this study, the effect of isolated fractions on macrophage cells was also examined. With respect to IC_{50} , it was found that the extract was effective at amastigotes at lower concentrations than macrophages and thus killed the parasites without damaging the host cells.

Allium plants are among the most important medicinal and edible plants worldwide that have been used for thousands of years. These plants are rich sources of potential medicinal compositions such as saponins, flavonoids, and sulfur compounds and are used throughout the

world to treat a variety of diseases, such as microbial and parasitic diseases [31]. Iranian traditional medicine, like other countries, has used herbs, especially garlic (*Allium sativum*) and its extracts to treat leishmaniasis [32]. Gamboa-Leon et al. accomplished a research on the anti-leishmanial activity of garlic extract against promastigotes of *L. mexicana*, they showed that the extract caused nitric oxide production by macrophages and led to parasite death [33]. Also, suggested that the garlic extract induced production of cytokines such as IL-12 and IFN γ by regulating the cell differentiation to Th1 and controled and healed leishmaniasis wounds [34]. The antiparasitic effects of steroid saponins as an important group of natural compounds with numerous potential therapeutic effects have been well documented in many studies. Dutta et al. have shown that the compound rasmozide-A, extracted from *Asparagus racemosus*, as a water-soluble saponin steroid, had significant leishmanicidal effects against *L. donovani* and was able to eliminate these parasites which is probably caused by activating apoptosis [15]. In the study of Rezaee et al., the leishmanicidal effects of a saponin steroid extracted from *A. paradoxum* was investigated and it was found to have significant effect against promastigotes of *L. major* [35]. Studies by Mimaki et al have led to the extraction and characterization of steroidal saponins alliogenin, agigenin and aginoside from *Allium giganteum*. These steroids are known as potent inhibitors of CAMP phosphodiesterase [36]. Panel et al. showed that the saponin-rich extract of *Thespesia populenis* showed a marked anti-inflammatory effect and reduced inflammation by inhibiting cyclooxygenase and prostoglobin [37].

The results of the present study demonstrated that the steroid-rich fraction of *A. giganteum* showed significant leishmanicidal effects against *L. major* which is probably because of apparent deformation of the parasite, restriction of flagellar motility, and ultimately parasitic wrinkle cell shrinkage and finally dissolving and tearing up the cell membrane. Given the traditional use of herbs in treatment of leishmaniasis and the results obtained in this study, further studies including evaluation of the effects of leishmaniasis in animal model as well as the isolation of compounds present in active fractions and evaluation of the effects of purified compounds will be valuable.

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Author contributions

Abbasali Eskandarain and Masoud Sadeghi Dinani supervised and coordinated the project; Simindokht Soleimanifard, Masoud Sadeghi Dinani and Omid Changiz performed experimental section; Simindokht Soleimanifard designed and was involved in preparation of the manuscript with the help of others.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

MPLC: medium pressure liquid chromatography; TLC: thin layer chromatography; FBS: fetal bovin serum; N.N.N medium: Novy-MacNeal-Nicolle medium; RPMI1640 medium: Roswell Park Memorial Institute Medium 1640