



Therapeutic Effect of *Scrophularia striata* Ethanolic Extract against Localized Cutaneous Leishmaniasis Caused by *Leishmania major* (MRHO/IR/75/ER)

Malihe ZAHIRI¹, *Mehdi MOHEBALI^{1,2}, Mahnaz KHANAVI³, Mousa SAHEBGHARANI⁴, Abedin SAGHAFIPOUR¹, Jamileh ESMAEILI⁴, Homa HAJJARAN¹, Amir Ahmad AKHAVAN¹, Seyed Mahdi REZAYAT^{4,5}

1. Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran
3. Dept. of Pharmacognosy and Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
4. Dept. of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
5. Dept. of Pharmacology and Toxicology, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran

*Corresponding Author: Email: mohebali@tums.ac.ir

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Abstract

Background: We evaluated the effect of the ethanolic extract of *Scrophularia striata* on the Iranian strain of *Leishmania major* (MRHO/IR/75/ER) both in vitro and in vivo conditions

Methods: The effective dose (ED) of ethanolic extract of *S. striata* were determined using MTT assay on the growth of promastigote forms of *L. major* in axenic culture media. Then, the ED₅₀ of *S. striata* on mice peritoneal macrophages was determined using calculation of amastigote forms on mice peritoneal macrophages. For in vivo experiments, the therapeutic effects of various concentrations of *S. striata* on infected BALB/c mice was studied. A total of 75 infected mice were randomly divided into five groups: two groups (10% and 50% of *S. striata*) as experimental and three as control (ethanol 50%, Glucantime® and no treatment). The efficacy were determined by comparing the diameters of lesions and the microscopically examinations.

Results: The effect of *S. striata* extract (0/625%, 1/25%, 2/5%, 5%, 10%, 20% and 50%) on peritoneal macrophages of Balb/c mice infected with *L. major* in tissue-culture slides was assessed. *S. striata* extract (10%) removed the *L. major* amastigotes-infected macrophages significantly after 24 h ($P < 0.05$). The higher concentrations of *S. striata* ethanolic extract (20%, and 50%) had highly toxic effects on macrophages, resulted in the disintegration of the cytoplasm of macrophages after 48 and 72 h. In concentration 10% of *S. striata*, more than 85% of *L. major* amastigotes-infected macrophages were damaged without cytotoxicity effects on macrophages. The higher concentrations had toxic effects on cultured macrophages.

Conclusion: *S. striata* ethanolic extract 10% had anti leishmanial effects in both in vivo and in vitro.

Keywords: *Leishmania major*, Balb/c mice, *Scrophularia striata*, In vivo, In vitro

Introduction

Leishmaniasis is one of the Neglected Tropical Diseases (NTDs), which are a group of infection diseases in tropical and subtropical areas in 149 countries, mostly common in low-income population, that affect more than one billion people

(1). Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* transmitted to human by the bite of infected female Phlebotomine sandflies. According to WHO report on NTDs about 310 million are exposed to the risk of in-

fection with estimated 1.3 million new cases annually of which 300,000 are visceral and 1 million are cutaneous and mucocutaneous leishmaniasis. There is an increase in the number of the recorded cases, but the actual increase is unknown due to reporting is compulsory in just 34% of affected countries (1).

Visceral leishmaniasis, also known as kala-azar is the fatal form if left untreated with over 20,000 to 50,000 deaths annually. Cutaneous leishmaniasis (CL) is the most common form of the disease occurring mainly in 10 countries including Iran. CL is causing ulcers that heal spontaneously but remains permanent scars as deformation of the infected area. Zoonotic cutaneous leishmaniasis (ZCL) is caused by *L. major* and is more common in many rural parts of Iran (2).

The first choice in the treatment of leishmaniasis includes the pentavalent compounds of antimonial such as Glucantime® and Pentostam®. However, both drugs are challenging due to serious side effects on kidneys and liver, reports of ineffectiveness and resistance against some species of *Leishmania*, and high costs (3). Since there is no effective vaccine, improvement of existing medications and discovery of new drugs would be the main ways to treat leishmaniasis. In this regards, natural products from traditional medicinal plants, such as plant extracts can be good candidates to study as new antileishmanial drugs.

Scrophularia striata Boiss (*S. striata*) is a member of flowering plant of *Scrophulariaceae* family. Members of this family can be found in temperate climate areas such as tropical mountains of central Europe, central Asia, North America and especially in the Mediterranean area (4). Many species of *Scrophularia* have been used as medical herbs in Asian countries for treatment of diseases such as various inflammatory diseases, eczema, wounds, ulcers, abdominal pain, and cancer (4, 5). In Iran, this plant grows and gathers in northeastern regions. *S. striata* is traditionally used for the treatment of infectious diseases, wound and burn healing (6). Previous research studies showed the antibacterial (7-9), analgesic (10), nephroprotective (5), nitric oxide suppressive

(11), and anticancer (4, 12) properties of *S. Striata*.

The aim of this study was to evaluate the in vitro and in vivo antileishmanial effect of *S. striata* ethanolic extract and to compare it with a reference drug Glucantime® against Iranian *Leishmania* strains (MRHO/IR/75/ER).

Materials and Methods

In vitro studies

Mouse peritoneal macrophages

The macrophages of peritoneal fluid of male BALB/c mice were collected and resuspended at 5×10^4 /ml in RPMI 1640 supplemented with 15% FBS. Cells were plated in 8-chamber Lab-Tek tissue-culture slides, and adherent macrophages were infected with late logarithmic promastigote parasites at the parasites-to macrophage ratio of 5:1. After 2 h of incubation at 34 °C, extracellular parasites were removed by washing and fresh medium containing the different fixed-ratio solutions extract was thirsty at concentrations of 0/625%, 1/25%, 2/5%, 5%, 10%, 20%, and Glucantime® 1.25, 2.5, 5, 10, 20 µM. Each point was tested in triplicate. Each 5-ml ampoule of Glucantime® contained 1.5 g meglumine antimoniate salt corresponding to 0.405 g of pentavalent antimony. The tissue culture slides were incubated for 3 days, then fresh Glucantime and *S. striata* extract was added, and the slides were incubated for an additional 72 hrs. The slides were fixed and stained with Giemsa 10%. Three slides were used for each concentration. The percentage of infected macrophages and the number of parasites per infected cell were evaluated by microscopic examination of at least 100 macrophages. The ED₅₀ is defined in this study as the effective dose of *S. striata* extract and Glucantime® that reduces the survival of *Leishmania* parasites by 50%. ED₅₀ values were determined by liner regression analysis.

Cell viability measurements by MTT assay

MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is a colorimetric

assay used to measure the reduction of MTT dyes (tetrazolium) into formazan by mitochondrial enzymes in viable cells. Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula:

$$\text{Viable cells (\%)} = (AT-AB)/(AC-AB) \times 100$$

where AC is the absorbance of the untreated samples, AT is the absorbance of the treated samples, and AB is the absorbance of the blank. All values are means of triplicate wells. Results were expressed as the concentration of the inhibited parasite growth by 50% (IC50: half maximal inhibitory concentration).

In vivo studies

In vivo study was carried out on Male Balb/c mice weighing 23–30 gr (6-8 weeks old) purchased from Animal Breeding Stock Facility of Razi Institute, Karaj, Iran. The animals were housed in a temperature-controlled room ($24 \pm 1^\circ\text{C}$) in standard polycarbonate cages, four-five mice in each cage on a 12-h light/dark cycle with free access to food and water. All behavioral experiments were performed between 9:00 AM and 2:00 PM. All procedures were carried out in accordance with institutional procedures for animal care and use. The animals were randomly assigned to different groups, plus each mouse was used only once and each treatment group consisted of 12–15 animals. Additionally, all efforts were made to reduce animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Male BALB/c mice were infected with subcutaneously 2×10^6 *L. major* promastigotes (MHRM/IR/75/ER) in the tail base. The weight and diameter of lesions were measured before treatment. Impression smears were prepared from lesions; fixed, and stained with Giemsa 10% stain in water. The mice were randomly divided to five groups, that four (15 mice per group) and one group (5 mice) for animal Laboratory control. The groups included:

Group 1: Control [1] mice with locally ethanol 50%.

Group 2: Control [2] infected mice but non-treated

Group 3: Control [3] infected treated with Glucantime® 60 mg/kg injected IP daily for 28 days.

Group 4: Infected treated with *S. striata* extract 10% administered locally daily for a period of 28 days.

Group 5: Infected treated with, *S. striata* extract 50% administered locally daily for a period of 28 days.

The diameters of lesions were measured one, two and 8 weeks after the beginning of the treatment. The slides were prepared before and after treatment, stained with Giemsa 10% and examined by light microscopy ($\times 1000$). The efficacy were determined by comparing the diameters of lesions and the microscopically examinations in the interventional and control groups.

Statistical analysis

Statistical significance between groups was analyzed by Student's *t* test using SPSS version 15 (Chicago, IL, USA). One- or two way analyses of variance (ANOVAs) followed by post hoc Tukey's tests was used to analyze the data where appropriate. Tests of homogeneity of variance were used to ensure normal distribution of the data. Values of $P < 0.05$ were considered statistically significant.

Ethical approval

This study was reviewed by the Ethics Committee of Tehran University of Medical Sciences in accordance with Helsinki Declaration and guidelines and approved with Code No: 17192).

Results

The effect of S. striata ethanolic extract and Glucantime® on Balb/c mice peritoneal macrophage

Fig. 1 shows the effect of *S. striata* extract (0/625%, 1/25%, 2/5%, 5%, 10%, 20% and 50%) on peritoneal macrophages of Balb/c mice infected with *L. major* in tissue-culture slides. *S. striata* extract (10%) removed the *L. major*

amastigotes-infected macrophages significantly after 24 hours ($P < 0.05$). On the other hand, the higher concentrations of *S. striata* ethanolic extract (20%, and 50%) have toxic effects on macrophages. These concentrations resulted in the disintegration of the cytoplasm of macrophages after 48 and 72 h. Fig. 2 shows The ED_{50} of Glucantime as standard drug against CL caused by *L. major* was calculated 7.2 μM according to the liner regression.

Fig.3 shows the viability of promastigotes of *L. major* (MRHO/IR/75/ER) treated for 24, and 72 hrs with *S. striata* extracts (0.625%, 1.25%, 2.5%, 5%, 10%, 20% and 50%) were further analyzed by MTT assay. Formazan production was different between *S. striata* extracts and control groups. Interestingly, the ED_{50} of Glucantime® and *S. striata* ethanolic extract was 25 μM , and 12.5% after 24 h, respectively, and 2.5% for *S. striata* ethanolic extract after 72 h according to the liner regression.

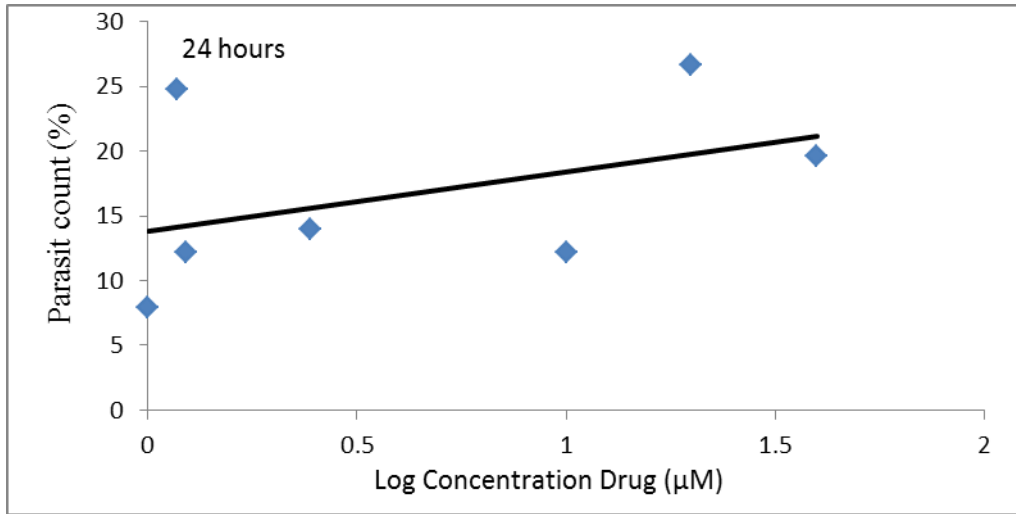


Fig. 1: Effect of logarithmic concentrations of *S. striata* ethanolic extract on *L. major* amastigotes in peritoneal macrophages of Balb/c mice

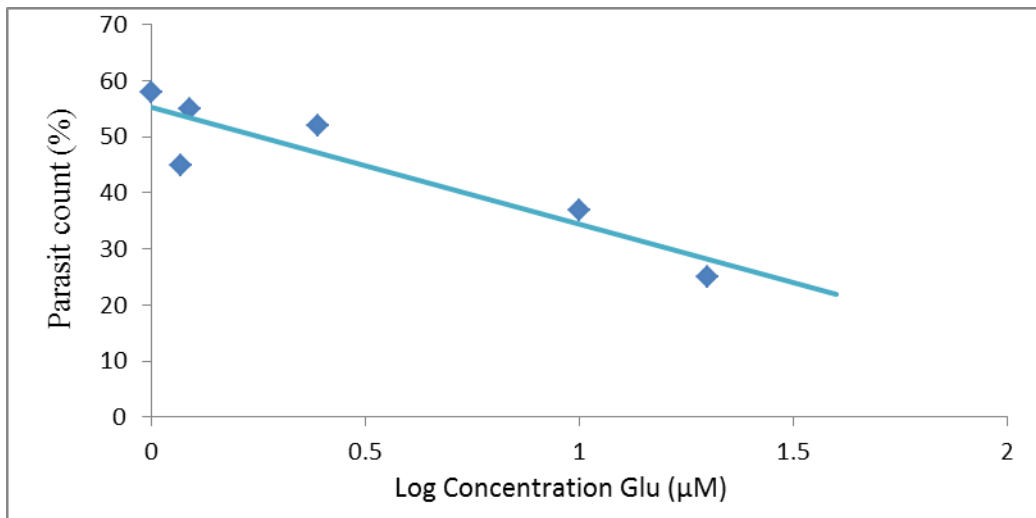


Fig. 2: Effect of logarithmic concentrations of Glucantime® on *L. major* amastigote in peritoneal macrophages of Balb/c mice

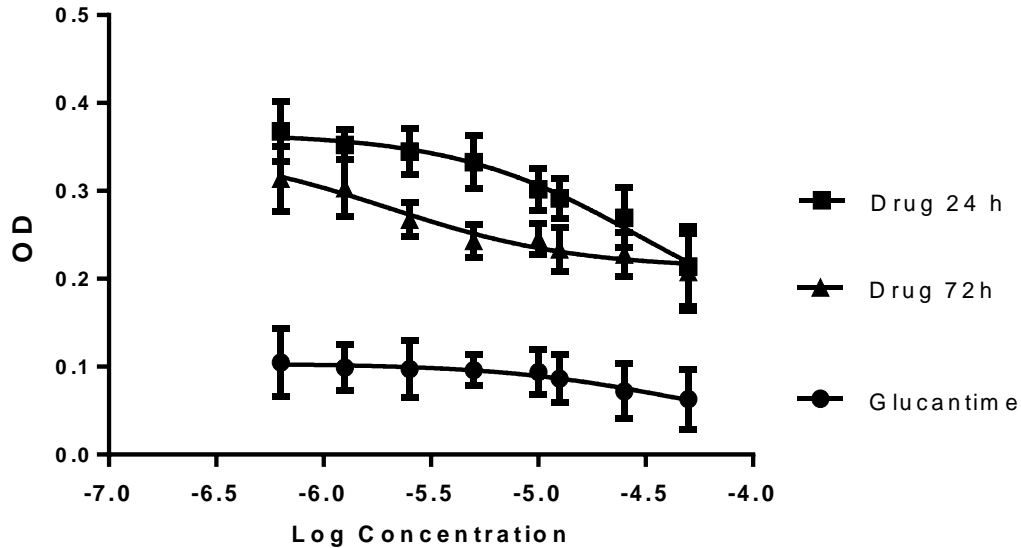


Fig. 3: Effect of logarithmic concentrations of glucantime® and *S. striata* ethanolic extract on promastigotes of *L. major* (MRHO/IR/75/ER) by MTT assay. The ED₅₀ of Glucantime® is determined 25 µM, and for *S. striata* ethanolic extract is 12.5%, and 2.5% after 24, and 72 h of incubation

The effects of *S. striata* ethanolic extract on lesions size in Balb/c mice infected by *L. major*

Table 1 shows the effects of *S. striata* ethanolic extract on lesions size in Balb/c mice infected by *L. major*. The mean size of the lesions in four infected groups was measured before treatment and after four weeks of treatment in Balb/c mice. As shown in Table 1, after four weeks treatment

with *S. striata* extract 10% the size of lesions was decreased significantly compared with the size of lesions before treatment ($P < 0.05$). On the other hand, the size of lesions of non-treated group was significantly increased during four weeks in comparison with control group ($P < 0.05$). While, *S. striata* extract 50% and Glucantime had not effect on the size of lesions after four weeks treatment, significantly ($P > 0.05$).

Table 1: The effects of *S. striata* ethanolic extract on the lesions size on infected Balb/c mice by *L. major*.

Groups	The mean size of the lesions before treatment	The mean size of the lesions after 4 weeks treatment	Significance
Control (non-treated) group	9.61±4.2	16.68±7.6	$P < 0.05$
Control (alcohol 50%)	14.97±3.41	19.22±4.65	$P > 0.05$
Glucantime® treated group	13.2 ± 3.6	7.4±3.5	$P > 0.05$
<i>S. striata</i> ethanolic extract 10%	10.6±5	5.58 ± 4	$P < 0.05$
<i>S. striata</i> ethanolic extract 50%	11.55 ± 2.60	14.05 ± 4.6	$P > 0.05$

The effect of *S. striata* ethanolic extract and Glucantime on the percent of infected macrophages

As shown in Fig. 4, the percent of infected

macrophages of Balb/c mice infected by *L. major* before and after four weeks treatment in Glucantime® and *S. striata* extract 10% inhibited the growth of amastigotes in infected macrophages

of skin lesions of Balb/c mice after four weeks treatment, significantly ($P < 0.001$, and $P < 0.01$, respectively). While, the percent of amastigotes

slides of non-treated group and *S. striata* extract 50% group did not have any changes after four weeks treatment, significantly.

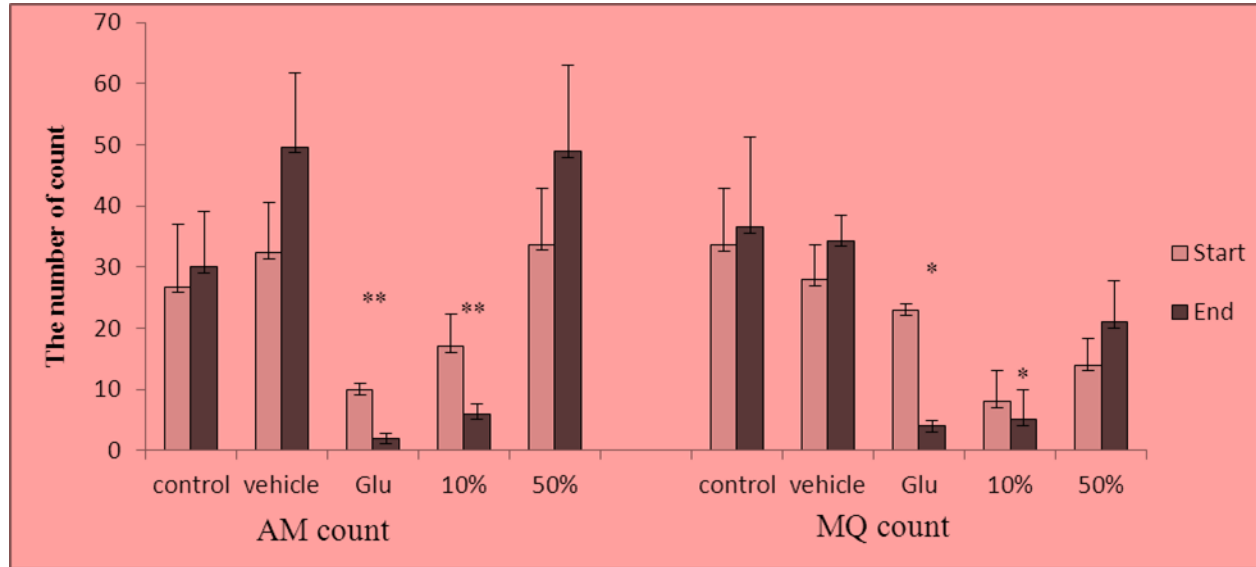


Fig. 4: Demonstrate the percent of amastigotes present in the slides of infected macrophages of lesions Balb/c mice infected by *L. major* before and after four weeks treatment. Glucantime® and *S. striata* extract 10% inhibited the growth of amastigotes in infected macrophages of lesions of Balb/c mice after four weeks treatment, significantly ($P < 0.001$, and $P < 0.01$, respectively). While, the percent of amastigotes slides of non-treated group and *S. striata* extract 50% group did not change after four weeks treatment, significantly

Discussion

ZCL caused by *L. major* is endemic and very common in about 15 rural areas of Iran, especially in the northeast, centre, and the southern parts of the country. About 70% of CL in Iran is caused by *L. major*. According to WHO reports, recently, the prevalence of CL is increased with its spread to new areas. The rate of unsuccessful treatment with Glucantime® has been increasing that maybe due to the resistance in Iran (1). In addition to the drug resistance, the various unpleasant side effects of Glucantime®, were caused researchers to find better substitutions for current standard medical treatment of CL. Traditional treatment of CL with herbal medicine is common in many endemic areas. The native medical plants with effective antileishmaniasis effects in Iran have been reviewed before (13). One of these medicinal plants is *S. striata* used for treatment of leishmaniasis in Ilam Province. The

antibacterial, anti-inflammatory and wound healing effects of different parts of *S. striata* have been demonstrated (7-9, 14-16). However, there are a few studies on antiparasitic effects of *S. striata* (17, 18). In both studies, the water extract of *S. striata* have been prepared to evaluate its anti-leishmaniasis effects on *L. major*.

Our results showed that the ethanolic extract of the concentration of 10% of *S. striata* could reduce the size of lesion of CL. The ED_{50} of *S. striata* extract was determined 12.5% and 2.5% after 24, and 72 h of incubation. Counting the number of parasites was indicated the significant difference between *S. striata* and control groups. *S. striata* could modulate the viability of Iranian strain of *L. major* promastigotes in Balb/c mice. Watery extract of *S. striata* could help to decrease of amastigote forms of *L. major* in the cultured mice macrophages but the investigation had not been completed in vivo conditions (18). In our experiments, *S. striata* extract 10% inhibited the

growth of amastigotes in infected macrophages of lesions and peritoneal of Balb/c mice, too. Interestingly, based on Shoohani et al. study, the hydro-alcoholic extracts of *S. striata* could stimulate collagen synthesis, faster wound contraction, decrease the inflammation, and bleeding on wound healing in rabbit. Statistical and histological studies showed that the best healing effect was obtained from *S. striata* 10% extract (19).

Components such as alkaloids, resin glycosides, iridoid, and cryptophilic acid have been isolated from different parts of *S. striata* (20). Five known components were isolated from the methanolic fraction extract of the aerial parts of *S. striata*: cinnamic acid, three flavonoids (quercetin, isorhamnetin-3-O-rutinoside and nepitrin) and one phenylpropanoid glycoside (acteoside 1) (21). The compounds, such as alkaloid, quinones, iridoids, terpenes, and indole analogues have been showed to have antileishmania activity in vitro. The exact mechanisms of action of these compounds have not been investigated yet, but alkaloids are able to interfere with the metabolism of aromatic amino acids in the parasite (22).

Conclusion

The ethanolic extract of *S. striata* affected parasitic proliferation phenomena. In addition, the extract can inhibit *L. major* proliferation. However, further investigations should be done to figure out the molecular basis of this procedure. Extract of *S. striata* are effective for the treatment of CL in Balb/c mice.

Ethical considerations

Ethical issues regarding plagiarism, informed consent, misconduct, data fabrication, double publication and/or submission, and redundancy have been completely observed by the author.

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