Effect of *Caparis spinosa* root extract on promastigotes and amastigotes of *Leishmania major*

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ABSTRACT

This study was carried out to identify the efficacy of *Caparis spinosa* root extract on promastigotes and amastigotes of *Leishmania major*. *Caparis spinosa* extracts were prepared. Promastogotes of *L.major* (1×10^6 parasites/ml)were incubated at 26 ºC for 24,48 and 72hours in fresh medium(NNN), in absence or presence of different concentrations (0.1,0.3,0.5,0.7,0.9 mg/ml) of *Caparis spinosa* methanolic root extract. It was determined that anti-protozoal activity of Caparis extract (0.9 mg/ml) was similar to glucantime as a gold standard drug ($p<0.05$) and both were able to kill 97.8 % of promastigotes after72 hours. Microscopic observation showed that mainly complete lyses of promastigotes after treating with0.9 mg/ml of *Caparis spinosa* for72hours. Higher extract concentrations had more effect on promastigote population ($p<0.05$). It was demonstrated that *Caparis sponosa* root extract has positive effect on amastigotes of *leishmania major*. It was determined 0.7 mg/ml and 0.9 mg/ml Caparis root extract concentrations were more effective than other concentrations on amastigots of Leishmania in ulcers. The mean of ulcer size of the mice that received 50 µl of 0.7 mg/ml and 0.9 mg/ml Caparis root extract were less than mice receiving 0.1, 0.3 and 0.5. The mean of ulcer size of the mice that received 50 µl of 0.9 mg/ml *Caparis spinosa* root extract were significantly smaller than control group after six weeks($p<0.05$) ,but the mean of ulcer size in mice which had been received glucantime (150 mg/ml)and Caparis root extract were not significantly different ($p>0.05$). The results were suggestive that Caparis root extract had significantly similar effect in reduction of ulcer size as compared to glucantime ($p<0.05$).

Key words: *Caparis spinosa*; Promastogote; Amastigote; *Leishmania major*.

INTRODUCTION

Leishmaniasis is transmitted through the bites of infected female phlebotomine sandflies all over the world except Australia and Antarctica. It is a disease with diver's clinical symptoms which symptoms depend on both infecting species of Leishmania and host immune response. Cutaneous leishmaniasis affects the skin membranes. Skin sores usually start at the site of the sand fly bite [1].Some kind of drugs such as pentamidine and amphotricine B are used in treatment, which maybe very toxic [2]. As a result of extensive interest in medicinal plants or their extracts all over the world many herbal extracts have been extensively used by native populations to treat leishmaniasis [3]. In the present study, the antileishmanial activity of Caparis extracts root was tested to validate the antiprotozoal properties of this plant. Caparis is a genus in the family Capparaceae which is included in the Brassicacea in the universe APGII system. Capparis spieces occurs over a wide range of habitat in the subtropical and tropical zones.

MATERIALS AND METHODS

Preparation of plant extract:

The *Caparis spinosa* roots were dried in an oven with ventilation system at 30 °C. Then, the fluid extract was prepared by maceration for seven days using 80% ethanol as a solvent and 20% water, according to the Regulation Norm 309 (Regulation Norm, 1992). Solvent was evaporated, and the extract was lyophilized, dissolved in dimethyl-sulfoxide (DMSO, BDH, England) at 20 mg/ml and stored at 4°C.

Parasite culture (Promastigote)

Promastigotes of *Leishmania major* (MRHO/IR/75/ER strain) were maintained by RPMI-1640 medium supplemented with 10% bovine serum (FBS), 100 μg of Streptomycin/ml,

and 100 U of Penicillin/ml, with passage each 3 or 4 days at 26 ºC. Promastogotes of *L. major* (1×10^6 parasites/ml) were incubated at 26 ºC for 24, 48 and 72 hours in fresh medium, in absence or presence of different concentrations (0.1, 0.3, 0.5, 0.7, 0.9 mg/ml) of the methanolic extract of *Caparis spinosa*. The parasites were not used after 10 *in vitro* passages. Inactive *L. major* promastigotes in the stationary growth phase were added to the plate well. A negative control (with Glucantime: 150 mg/ml) was included in the study.

Parasite culture (Amastigote)

Leishmania major (MRHO/IR/75/ER strain) was cultured in NNN media and transferred to the enriched medium of RPMI1640 for mass production. It was given passage four times and Leptomonade forms were elevated to concentration of 1×107. *L. major* was injected subcutaneously at the base of tail of 70 inbred (BALB/c) mice female at the age of 8 weeks. The mice were assigned to the following groups: control group without receiving any extract, five groups receiving 0.1, 0.3, 0.5, 0.7, and 0.9 concentrations and one group were injected with Glucantime as gold standard drug for leishmaniasis. After appearance of the nodule at the site of parasite injection every day use of *Caparis* root extract for the groups initiated and the lesion size were monitored weekly using vemiehcolise, respectively.

This trend continued till the death of the last mice in the 15 week. Data were collected and analyzed with the statistics of ANOVA.

Statistical analysis

All experiments were performed in triplicate. The mean and standard error of at least three experiments were determined. Statistical analysis of the differences between mean values obtained from experimental groups was done by means of students test P value of 0.05 or less were considered significant **.**

RESULTS

Effect of C*aparis spinosa* root extract on *L. major* promastigotes

The time course of the viability of *L. major* promastigotes in the absence or presence of *Caparis spinosa* root extract was shown in table 1. It was determined that anti-protozoal activity of *Caparis* extract (0.9 mg/ml) was similar to Glucantime as a gold standard drug ($p<0.05$) and both were able to kill 97.8 % of promastigotes after 72 hours. The effect of 0.9 mg/ml *Caparis* extract was significantly equal to Glucantime ($p<0.05$).Microscopic observation showed mainly complete lyses of promastigotes after treating with 0.9 mg/ml of *Caparis spinosa* for 72 hours. Extracts with higher concentration had more effect on promastigote population ($p<0.05$). The methanolic extract showed anti protozoal activity against developmental stages of *L. major* after 24, 48 and 72 hours of culture in fresh medium incubation. These results were corroborated by lyses of promastigote cells.

Microscopic observation showed mainly complete lyses of promastigotes after treating with 0.9 mg/ml of *Caparis spinosa* for 72 hours. Extracts with higher concentration had more effect on promastigote population ($p<0.05$). The methanolic extract showed inhibitory activity against developmental stages of *L. major* after 24, 48 and 72 hours of culture in fresh medium incubation. These results were corroborated by lyses of promastigote cells.

Table1. Effect of different concentrations of *Caparis spinosa* root extract on the number of promastigotes of Leishmania

Extract concentrations and control	**Number of parasites**			
	Beginning of culture	**24h**	**48h**	**72h**
Culture medium (Negative control)	380000	410000	420000	530000
DMSO+PBS buffer	400000	390000	410000	500000
Glucantime (Positive control)	450000	50000	20000	10000
0.1mg/ml extract	480000	440000	410000	400000
0.3mg/ml extract	475000	410000	380000	200000
0.5mg/ml extract	470000	110000	90000	50000
0.7mg/ml extract	460000	70000	25000	13000
0.9mg/ml extract	440000	48000	19000	10500

Effect of *Caparis spinosa* root extract on *L. major* amastigotes

In the next stage effect of *Caparis spinosa* root extract on amastigotes of *Leishmania* was studied. It was demonstrated *Caparis spinosa* root extract has positive effect on amastigotes of *Leishmania major*. It was determined that 0.7 mg/ml and 0.9 mg/ml *Caparis* root extract concentrations were more effective than other concentrations on amastigotes of *Leishmania* in ulcers. Also it was shown a statistically significant difference between control group and treatment groups ($p<0.05$). The mean of ulcer size of the mice that received 50 µl of 0.7 mg/ml and 0.9 mg/ml *Caparis* root extract were less than mice receiving 0.1, 0.3 and 0.5. The mean of ulcer size of the mice that received 50 µl of 0.9 mg/ml *Caparis spinosa* root extract were significantly smaller than control group after six weeks ($p<0.05$), but there was not significant difference between the mean of ulcer size in mice which had been received Glucantime (120 mg/kg/day) and *Caparis* root extract ($p>0.05$). The results were suggestive that *Caparis* root extract had significantly similar effect in reduction of ulcer size as compared to Glucantime ($p<0.05$).

DISCUSSION

Leishmaniasis is one of the most important infectious diseases in the world. There are wide differences in the clinical features of leishmaniasis. Cutaneous leishmaniasis is self-healing while visceral leishmaniasis is fatal. There is a general lack of effective drug for treating leishmaniasis. Pentavalent antimonial drugs are the first line treatment for leishmaniasis in all of world. Then Amphotricin B and Pentamidine were used as alternative drugs [4]. These drugs are expensive and they also have side effect. In addition resistance to these compounds has become a serious problem. Therefore alternative medicines and herbal medicines were approved by scientist in the world.

Furthermore, natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoan and other parasites [5]. Only few laboratories are involved in drug evaluation and development against these devastating diseases, particularly against leishmaniasis, which has been considered as a “neglected disease” [6]. In this sense, the potential of plant extracts as a source of anti leishmanial drugs has been demonstrated. Several studies about screening of plants extracts against *Leishmania* have been reported [7-9].

In Brazil one study described the screening of extracts obtained from 19 species of plants used in Brazilian traditional medicine for treatment of a variety of diseases. The extracts were tested against axenic amastigote and promastigote forms of *Leishmania (L.) amazonensis* and epimastigote forms of *Trypanosoma cruzi* in vitro at a concentration of 100 µg/ml *Baccharis trimera*, *Cymbopogon citratus*, *Matricaria chamomilla*, *Mikania glomerata*, *Ocimum gratissimum*, *Piper regnellii*, *Prunus domestica*, *Psidium guajava*, *Sambucus canadensis*, *Stryphnodendron adstringens*, *Tanacetum parthenium* and *Tanacetum vulgare* showed significant effects against one or both parasites, with a percentage of growth inhibition between 49.5 and 99%. The extracts showed no cytotoxic effect on sheep erythrocyte [10]. Sharif in 2006, showed that methanolic extract of *Artemisia aucheri* inhibited the parasite multiplication at dose of 150, 300 and 450 µg/ml at 48 and 72 hours of culture. Doses of 600 and 750 µg/ml showed the same effect at 24, 48 and 72 hours of culture ($p<0.05$). Methanolic extract of *Camellia sinensis* showed inhabitation of parasite multiplication when administered at doses of 150, 300, 450, 600 and 750 µg/ml at 72 hours ($p<0.05$) [11]. Also, Mirzaie showed that *Peganum harmala*, or Syrian Rue, has pharmacologically active compounds including several alkaloids with anti protozoal properties, which are found especially in the seeds and the roots. In this research, *Leishmania major* were cultured in vitro, then by using a MTT assay, the biological activity of *P. harmala* extract in comparison to potassium antimonyl tartrate [Sb(III)] on *L. major* promastigotes was assessed. For *P. harmala* extract and Sb (III), the concentration-response curve was plotted, from which IC50 values were determined. Both *P. harmala* extract and Sb (III) inhibited the growth of promastigote forms of *L. major* in vitro after 72 h. of incubation and had an IC50 of 1832.65±89.72 µg/ml and 17.87 ± 2.05 µg/mL, respectively. Statistical analysis of the results of the different concentrations of *P. harmala* extract and Sb (III) showed that there was no significantly difference between *P. harmala* extract and Sb (III) ($P>0.05$) but with a concentration increase

of *P. harmala* extract or Sb (III), optical density decreased significantly, while inhibitory percentage increased. The different concentrations resulted in different optical densities or inhibitory percentages ($P<0.05$) so that *P. harmala* extract is effective against *L. major* [12]. Fatahi examined the effect of *Rubia tinctorum* extract on cutaneous leishmaniasis in BALB/c Mice. They found that the mean of lesion size of the mice that received 40, 60 and 80% *R. tinctorum* extracts Showed no statistically significant difference compared the mean of the lesion size of the mice in control group ($P>0.05$) [13]. In Iran, it was shown effect of *Alkanna tincturia* and *Peganum harmala* extracts on *Leishmania major*. The results indicated that both extractions can inhibit the growth of promastigotes, and in concentrations of 40 µg/ml of *P. harmala*, 200 µg/ml of *A. tincturia*, and 20 µg/ml of equal combination of *P. hamala* and *A. tincturia* is inhibitory concentration (IC50) for parasites growth. By adding these concentrations of the extracts to the infected macrophages in the culture, their effects were separately evaluated. The mean of amastigotes number in macrophages in the culture with *P. harmala*, *A. ticturia*, combination and control groups were 0.7, 0.7, 0.6, 2.3 amastigotes per macrophage [14]. Singh investigated the anti-leishmanial properties of 30 medicinally important plants from the VL endemic area of Bihar, India and compared them to two available anti-leishmanial drugs (Sodium Antimony Gluconate and Amphotericin B) and two plant lectins (Phytohemagglutinin and Concanavalin A) on *Leishmania donovani* promastigotes in vitro at 24 and 48 h after initiation of culture. They found eight plant extracts in addition to Phytohemagglutinin and Amphotericin B that significantly inhibited the growth of promastigotes ($p<0.03$). Also, it was determined minimum effective concentrations as well as effect on axenic amastigotes viability and the cell cytotoxicity on human peripheral blood of four (*Agave americana, Azadirachta indica*, *Eclipta alba* and *Piper longum*) of the eight plant extracts that induced significant promastigotes killing ($p=0.00098$). Effect-based dose finding analysis revealed that the threshold concentration of *A. americana* required to eliminate *L. donovani* after 24h was 0.05 mg/ml. *A. indica* and *P. longum* plant extracts eliminated *L. donovani* promastigotes after 48 h at concentrations of 0.1 and 0.5 mg/ml, respectively. *E. alba* eliminated the promastigotes at a concentration of 0.5 mg/ml within 24h. The axenic amastigote killing response was 1.90-, 2.52- and 1.3-fold higher than the promastigote killing response with *A. indica*, *A. americana* and *E. alba* plant extracts, respectively. *A. americana* and *A. indica*, respectively, led to approximate 2.5- and 1.3-fold declines in mitochondrial dehydrogenase activity compared with control. *E. alba* stimulation resulted in an up-regulation of dehydrogenase activity ($p<0.05$). The CSA from *P. longum* was found to be least cytotoxic; the observed difference in mitochondrial activity was insignificant ($p<0.05$) [15]. Feily studied on ethanolic extract of the root of *Echinacea purpurea* prepared from Zardband Pharmaceutical Co. for its direct leishmanicidal activity in *Leishmania* culture. *L. major* promastigotes (stationary phase) were cultured in different concentrations of the extract (0.5–125 mg/ml) for 30 min. Then the extract was removed and cell viability was determined during 120 h. LD50 for the promastigotes were determined as 22.3, 16.7, 3.66, 1.98 and 1.23 mg at 8, 16, 24, 48 and 72 h respectively. The results showed the irreversible leishmanicidal activity of the *E. purpurea*. Findings showed that all concentrations of the extract had anti leishmanial effect and they're useful for target cells [16]. Study of Soudi ,showed that ethanolic extract of *Green tea* has significant leishmanicidal activity against *L. major* promastigotes in different concentrations. There was a concordance in anti leishmanial effect of the ethanol extract with increasing of dosage (3, 6, 12, 24, 48, 96 mg/ml). In comparison with Glucantime the mean alive promastigotes in 12 mg/ml concentration of green tea was almost as same as 85 mg/ml Glucantime and higher green tea extract concentrations were higher effective than Glucantime. Study revealed a novel pharmacological activity against promastigotes of *L. major* and suggests that green tea extract has the potential of being used in leishmaniasis [17]. In Cuba , 48 alcoholic extracts from 46 Cuban plants were evaluated by an *in vitro* bioassay against *L. amazonensis*. The three most potent extracts against the amastigote stage of *L. amazonensis* were from *Hura crepitans*, *Bambusa vulgaris*, and *Simarouba glauca* [18]. By Mostafa ,antihelminthic activity of *Caparis spinosa* extract on *Lumbricus*

terrestris was carried out. This study showed that use of the aerial parts of *C. spinosa* as both the ethanolic and water extracts have antihelminthic activity. Furthermore, both type of extracts displayed significant antihelminthic properties at high concentrations and antihelminthic activities in a dose-dependent manner giving short time of paralysis and death of worm [19]. Camacho screened methanolic and aqueous extracts derived from 43 plant species, selected either from ethnobotanical or chemotaxonomical data, for their anti protozoal activity against *Leishmania donovani* and *Trypanosoma brucei brucei*. The cytotoxic activity against KB cells was also determined. IC_{50} value of eight extracts against *L. donovani* was less than 10 µg/ml. The most active was *Triclisia patens* with an IC_{50} value of 1.5 µg/ml against *L. donovani*. *Annona purpurea* and *Alstonia macrophylla* had IC_{50} values below 10 µg/ml against *Trypanosoma brucei brucei*. *Annona purpurea* was the most cytotoxic against KB cells [20].

As it was described, anti leishmanial effect of many plant groups in some parts of the world were determined. Over the last 15 years, interest in herbal medicines has increased worldwide in both developed and developing countries. Our results revealed a new pharmacological activity against *L. major* and suggest that *Caparis spinosa* root extract have the potential of being use as application in wound healing and also methanolic extract of *Caparis spinosa* showed inhibitory activity against development stages of *L. major* after 24, 48, 72 hours of culture in fresh medium incubation. The results presented able to be used in laboratory synthesis for development of new leishmanicidal agents using this plant.

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