

***In vitro* activity of *Albizia gummifera* (J.F. Gmel.) C.A. Sm. seed extract against promastigote stages of five *Leishmania* species known to cause human leishmaniasis**

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Abstract

Background: Modern drugs for treatments of leishmaniasis are expensive, bearing limited efficacy and significant toxicity as well as emergence of resistant parasite to them. Several medicinal plants are being used traditionally to treat *Leishmania* infections in Ethiopia. Therefore the discovery of safer and more efficacious drug from natural product is so essential.

Objective: To investigate the antipromastigote activity of crude extract of *Albizia gummifera* seed against five *Leishmania* species responsible to cause cutaneous and visceral leishmaniasis.

Methods: The plant materials were macerated and extracted using 70% ethanol. The extract of each plant was screened for its antipromastigote and hemolytic activities *in vitro*. Data analysis was done by using Graphpad Prism version 6 software. The criterion for activity was considered as an $IC_{50} < 100 \mu\text{g/ml}$.

Results: Haemolytic test of ethanol extracts *A. gummifera* showed LC_{50} value of $453.55 \pm 3.9 \mu\text{g/ml}$. The crude ethanol extract of *A. gummifera* showed the highest potency against promastigotes of *L. tropica*, *L. major*, *L. donovani*, *L. chagasi* and *L. aethiopica* species with IC_{50} values less than $10 \mu\text{g/ml}$. Among the different fractions of *A. gummifera*, the n-butanol fraction showed comparable anti-leishmanial activity (IC_{50} ranges between 0.18 and 0.28 $\mu\text{g/ml}$) with the standard drug, Amphotericin B, having IC_{50} between 0.24 and 0.29 $\mu\text{g/ml}$.

Conclusions: *A. gummifera* seed extract considered as a good candidate for further bioassay-guided fractionation and isolation of anti-leishmanial lead compound(s) that could be used for anti-leishmanial drug discovery.

Key words: anti-leishmanial, lead compound(s), crude extract, n-butanol, LC_{50} , IC_{50} values

Introduction

Leishmaniasis is a disease caused by a protozoan parasite of the genus *Leishmania*. It is transmitted by the vector *phlebotomine* sand fly. Globally it affects more than 12 million people and 350 million are at a risk of developing the disease with 2 million new cases occurring annually (WHO 2010). Clinically the disease has got three forms: cutaneous leishmaniasis (CL), visceral leishmaniasis or Kala-azar and mucocutaneous leishmaniasis (MCL) (Desjeux 2004). In Ethiopia, CL is endemic in the highlands of the country, mainly caused by *L. aethiopica* and occasionally by *L. tropica* and *L. major*. Visceral leishmaniasis caused by *L. donovani*, is found in the arid and semi-arid area of Ethiopia (Ashford *et al.* 1973; FMOH 2013; Hailu *et al.* 2006).

Modern drugs for current therapies against leishmaniasis are pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), Amphotericin B, miltefosine, pentamidine and paromomycin (Croft and Yardley 2002; Frezard *et al.* 2009). However, all of these drugs have limited efficacy and significant toxicity as well as resistance emerges by the parasite to them (Croft *et al.* 2005).

Moreover, there is no effective vaccine against human leishmaniasis (Handman 2001). Discovery for safer and more efficacious drugs is, therefore, essential. In this regard, medicinal plants claimed by traditional healers to treat leishmaniasis in Ethiopia may offer prospects for discovering new compounds with therapeutic properties. This study was, therefore, designed to scientifically validate the *in vitro* anti-leishmanial activities of five traditional medicinal plants.

Material and methods

Plant material: The medicinal plant, *Albizia gummifera* (J.F. Gmel.) C.A. Sm. seeds, was collected from its natural habitats in September and October 2010. The plant was identified and authenticated by a botanist at Traditional and Modern Medicine Research laboratory, Ethiopian Public Health institute (EPHI), the specimen was given with a voucher number of AG 2006 and was deposited in the herbarium of Traditional and Modern Medicine Research Directorate, EPHI, Addis Ababa. The Plant materials were then air dried in the plant processing unit of Traditional and Modern Medicine Research Laboratory of EPHI.

Extraction of plant materials: The air-dried and powdered plant material (100 g) was extracted with one liter of 70% ethanol by maceration. The resulting ethanol extract was filtered and evaporated using a rotatory evaporator and then lyophilized. Ethanol extract of *A. gummifera* was dissolved in distilled water and fractionated with different solvents: petroleum ether, chloroform, and n-butanol. Fractionated extracts of *A. gummifera*, with the above solvents, and the aqueous marc were used for subsequent investigation against anti-promastigote activity. The crude and fractionated extracts were stored at 2°C until used.

Promastigote cultivation: Standard strain of *Leishmania* parasites used in this study were: *L. donovani* (MHOM/50/68/15), *L. chagasi* (MCAN/84/CO9/0), *L. major* (MHOM/IR/72/NAD/MS), and *L. tropica* (IROS /NA /80 /HD3. *L. aethiopica* promastigote was isolated from patients with cutaneous leishmaniasis. The promastigotes were grown in Novy MacNeal Nicolle (NNN) medium and then cultured in RPMI-1640 supplemented with 10%FBS, (100U/100µg/ml) penicillin/streptomycin, and (2mM/ml) L-glutamine at pH 7.2, and incubated at 26°C in a dark environment.

Anti-promastigote assay: The assay was carried out in a 96-well micro-titer plate where the extracts were diluted three-fold with RPMI-1640 to obtain serial dilutions ranging from 1.2 to 900 µg/ ml in 100 µl of culture medium with each test concentration in duplicate. Then, 100 µl of suspensions containing 3.5×10^6 promastigotes /ml at logarithmic phase were added to each well. The plates were incubated at 26°C for 72 hours and growth of *Leishmania* promastigotes were determined using the Alamar blue assay (Mikus and Steverding 2000), then optical density (OD) of each plate was measured after 24 hours using ELISA reader fluorometrically at 544 nm an excitation wavelength and 590 nm as an emission wavelength. The IC₅₀ value was calculated using Graphpad prism version 6 software.

Red blood cell (RBC) lysis assay: Assay for hemolytic activity of the extract was carried out as described in

Tiuman *et al.* (2005). In brief, 200 µl of 4% red blood cell suspension (in sterile 5% glucose soln.), serial dilutions of the extracts (1.2 to 900 µg/ ml) and reference drugs (0.055 - 40µg/ml) were added, and the mixture was incubated at 37°C for 2 hours. The suspensions were centrifuged at 1,000 rpm for 10 min, and 100 µl of the supernatants was transferred to a 96-well plate, and absorbance was measured at 540 nm using ELISA reader. Hemolytic effects were expressed as percentage of the absorbance of the positive control and the 50% lytic concentrations (LC₅₀) were determined from sigmoidal dose–response curves with Graphpad prism 6.0 software. The negative control was the red blood cell suspension with 1.0 % DMSO and the positive control was saponin. These tests were performed in duplicates on two separate occasions.

Results

Anti-promastigote activity of the crude extracts of *A. gummifera*: Results of the anti-promastigote activity study showed that the crude ethanol extract of *A. gummifera* possessed a promising anti-promastigote activity with an IC₅₀ value less than 1.2 µg/ml against *L. tropica* (0.78 µg/ml), *L. major* (0.94 µg/ml) and *L. chagasi* (0.35 µg/ml) which are almost comparable to IC₅₀ value for control drug; 0.24 µg/ml, 0.27 µg/ml and 0.28 µg/ml, respectively (Table 1). However, the IC₅₀ values for *L. donovani* (8.65 µg/ml) and for *L. aethiopica* (9.21µg/ml) are much higher compared to IC₅₀ value for control drug; 0.29µg/ml for each (Table 1).

In vitro activity of the fractionated extracts of *A. gummifera* against promastigotes: N-butanol and aqueous fractions of *A. gummifera* had significantly high anti-promastigote activity. The effects of different fractions (petroleum-ether, n-butanol, chloroform and aqueous) of *A. gummifera* extract were also evaluated against promastigotes of *L. aethiopica* clinical isolate and reference strains of *L. donovani*, *L. major*, *L. tropica* and *L. chagasi*. Results indicated that n-butanol and aqueous fractions of *A. gummifera* had significantly high anti-promastigote activity.

Table 1: IC₅₀ values of medicinal plant ethanol extracts and reference drug against Leishmania species promastigotes

Plant species and Control drug	IC ₅₀ (µg/ml)				
	<i>L. tropica</i>	<i>L. major</i>	<i>L. donovani</i>	<i>L. chagasi</i>	<i>L. aethiopica</i>
<i>A. gummifera</i> (seed)	0.78	0.94	8.65	0.35	9.21
Amphotericin B	0.24	0.27	0.29	0.28	0.29

The n-butanol fractions had a respective anti-promastigote activity with IC₅₀ values of 0.22, 0.18, 0.28, 0.18 and 0.27 µg/ml against *L. tropica*, *L. major*, *L. donovani*, *L. chagasi* and *L. aethiopica*, respectively. Aqueous fractions with IC₅₀ values of 1.11 µg/ml against *L. tropica*, *L. major* and *L. donovani*; 1.41 and 2.83 µg/ml against *L. chagasi* and

L. aethiopica, respectively, were obtained. The chloroform fraction had IC₅₀ values of 17.91, 31.62, 31.62, 9.38 and 52.99 µg/ml against *L. tropica*, *L. major*, *L. donovani*, *L. chagasi* and *L. aethiopica*, respectively while the petroleum-ether fraction had IC₅₀ values of 80.95 µg/ml against *L. major* (Table 2).

Table 2: IC₅₀ values of *A. gummifera* tested in petroleum ether, n-butanol, chloroform and aqueous fractions against Leishmania promastigotes

Fractions of <i>A. gummifera</i>	IC ₅₀ (µg/ml)				
	<i>L. tropica</i>	<i>L. major</i>	<i>L. donovani</i>	<i>L. chagasi</i>	<i>L. aethiopica</i>
Petroleum ether	> 100	80.95	>100	>100	8.56
N- butanol	0.22	0.18	0.28	0.18	0.27
Chloroform	17.91	31.62	31.62	9.38	52.99
Aqueous	1.11	1.11	1.11	1.41	2.83

Red blood cell (RBC) lysis assay

Extracts of *A. gummifera* and one reference drug used for anti-promastigote activity study were evaluated for their hemolytic effects. Haemolytic test for *A. gummifera* (seed) showed LC₅₀ values to be

453.55±3.9 µg/ml, while the LC₅₀ for standard anti-leishmanial drug; Amphotericin B, was 45 µg/ml. The negative control (1% DMSO) did not cause hemolysis while the positive control (1% saponin) showed 100% hemolysis (Table 3).

Table 3: Haemolytic property of traditional medicinal plant ethanol extracts and reference drug tested on sheep RBC

Study plants and Control drug	Blood hemolysis LC ₅₀ (µg/ml) Mean ± SD
<i>A. gummifera</i>	453.55 (±3.9)
Amphotericin B	45

Discussion

Leishmaniasis is a common parasitic problem in the world. The currently used treatments for leishmaniasis are pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), Amphotericin B, miltefosine, pentamidine and paromomycin. On the other hand, all of these drugs have limited efficacy and significant toxicity as well as resistance emerges by the parasite against them. The high LC₅₀ values determined for the plant extract show its safety nature. This is in agreement with previous report (Ghebreselassie *et al.* 2011) where aqueous leaf extract of *M. stenopetala* on mice treated with doses

of up to 900 mg/kg did not show any morphological changes in liver cells. Moreover, acute toxicity study on the aqueous extracts of *A. gummifera* (seed) in mice showed an oral LD₅₀ value of 2.50 g per kg animal body weight and an IP LD₅₀ value of 250 mg per kg animal body weight (Debella *et al.* 2007). The very low toxicity shown in both *in vitro* and *in vivo* studies indicated that the tested medicinal plants extracts may not be toxic and do not affect red blood cells. According to Bero *et al.* (2011), the *in vitro* anti-leishmanial activity of a given crude extract or compound must have an IC₅₀ value of ≤20 µg/ml to possess a promising activity, whereas IC₅₀ values

between 21 and 50 µg/ml, 50 and 100 µg/ml are considered to have moderate and low activity, respectively and that with an IC₅₀ > 100 µg/ml is considered inactive.

Accordingly, the crude ethanol extract and n-butanol and aqueous fractionation of *A. gummifera* had a promising anti-promastigote effect against *L. tropical*, *L. major*, *L. donovani* and *L. aethiopica* compared to other test plants. This is similar to the report on *in vitro* anti-leishmanial activity of the same medicinal plant species from Kenya: *A. gummifera* ethanol extract with IC₅₀ value of 10 µg/ml against *L. donovani* promastigotes (Muhammad *et al.* 2011). Thus, this medicinal plant may be a promising source of lead compounds for drug development against *Leishmania* parasites.

According to report of Debella (Debella, 2002) the n-butanol and aqueous partition products of *A. gummifera* fractions contains favonoids, saponins, phenols, phenolic glycosides and carbohydrates, which are very polar compounds. These constituents had stronger activity against *Leishmania* parasites compared to chloroform fractions which contains non-polar alkaloids and diterpenes. This indicates that the strong anti-promastigote activity of *A. gummifera* seed fractions could be due to the presence of the above mentioned compounds. The petroleum-ether fractions which contain fats and sterols, however, were found to be the least active in the present study. The anti-promastigote activities of *A. gummifera* n-butanol extracts against promastigotes of the five *Leishmania* spp. observed in this study exhibit the potential of this plant in the development of a new anti-leishmanial drug.

Conclusions

This *in vitro* study indicated that *A. gummifera* (seed) ethanol extract and n-butanol and aqueous fractions have an anti-promastigote activity with low toxicity profile. These results need, however, be confirmed using amastigotes stages of the parasite.

Conflict of interest

Authors declare that there is no conflict of interest.

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