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In vitro Antileishmanial Activity of Aegle marmelos Seeds

JK Gour[1], BB Mishra[2], V Kumar[1], N Singh[1], MR Gedda[1], VK Tiwari[2], Rakesh K. Singh[1]

Abstract

The protozoan parasite of the genus *Leishmania* is responsible for the infectious disease leishmaniasis. The disease has three clinical forms i.e. cutaneous, mucocutaneous and visceral leishmaniasis. The visceral form of the disease, caused by *L. donovani* complex affects millions of people around the world especially in Indian subcontinent & East Africa it is almost fatal, if left untreated. Generic pentavalent antimonials have been the mainstay for therapy in the endemic regions due to efficacy and cost effectiveness but the growing incidence of their resistance has seriously hampered their use. The second line drugs like amphotericin B and miltefosine, though they are not true antileishmanials, are being used effectively. However, high cost and probability of resistance development, necessitates the development of true and novel antileishmanial compounds. The future of higher plants as sources of new medicinal leads for use in investigation, prevention, and treatment of diseases is highly promising. This study discloses strong in vitro antileishmanial activity of seed extracts of *Aegle marmelos* (family- Rutaceae) on promastigote and amastigote forms of *L. donovani* parasites. The cytotoxicity on RAW 264.7 macrophage cell line further confirmed the usefulness of extract. In context to limited treatment options and growing resistance for available drugs, the seed extracts of *Aegle marmelos* offer a greater prospect towards antileishmanial drug discovery and development.

Keywords: Leishmania, drugs, cytotoxicity

Introduction

Leishmaniasis, a neglected tropical disease caused by 30 species of *Leishmania* parasite, is a major infectious disease prevailing in endemic areas of Asia, Africa, Southern Europe and South America. Nearly two million people are affected annually and 350 million men, women and children in 88 countries are vulnerable to attack by the pathogen.[1] The incidence of visceral form of disease, also known as black sickness or kala-azar has significantly increased during the past 5-6 years, and virtually occupying a pandemic status.

Chemotherapy has been the only effective method of treatment of visceral leishmaniasis (VL) as no vaccine has been developed so far. Sodium antimony gluconate (SAG) is the mainstay of treatment in soon after the fact that it is caused by Leishmania. However, during last decades the parasites has developed resistance against antimonials in many parts of world including State of Bihar (India) have been reported.[2,3] Treatment using second line drugs i.e. pentamidine, amphoterin-B and its liposomal formulations are limited due to high cost and serious toxic effects.[4] Paramomycin, sitamaquine and various antimycotic azoles are other drugs with considerable usefulness in the therapy are under clinical trials.[5] This urges the search of novel antileishmanial chemotherapeutics against the disease for its global effective control.

The plant *Aegle marmelos*, also known as *Bel*, is found distributed in India, Pakistan, Bangladesh and Sri Lanka.[6] In Ayurvedic system of medicine the plant is credited with numerous therapeutic potentials i.e. cardiotonic, aphrodisiac, hepatoprotective, neuro-

[1]Department of Medicine Biochemistry, Faculty of Science, BHU, Varanasi

[2]Department of Chemistry, Centre of Advanced Study, BHU, Varanasi

Corresponding author:

Rakesh K. Singh, Department of Medicine Biochemistry, Faculty of Science, BHU, Varanasi. Email: rakeshbhu@yahoo.com

protective, hypoglycemic, anti-inflammatory and antimicrobial activities.[7] In continuation to our efforts for linking the phytochemicals with their desired bioactivity, we now disclose the excellent anti-leishmanial activity of seed extracts of A. marmelos. The seed extracts were evaluated against the promastigotes and amastigotes of *Leishmania donovani*. The miltefosine was used as reference drug. The extracts were also assayed for cytotoxicity on RAW 264.7 macrophage cell lines.

Experimental

General experimental procedure

Dulbecco's modified eagle medium (DMEM) with L-glutamine (Lot No.720593) was purchased from GIBCO. Foetal Bovine Serum (FBS) was obtained from GIBCO (Lot No.41F0483K), penicillin-G (1653 unit mg^{-1}) (Lot No.038K05531), streptomycin sulfate salt (7651Umg^{-1}) (Lot No.088K06751), Gentamycin sulfate salt hydrate (Lot No.SZE6066X) were obtained from Sigma Aldrich.

Plant Material

Seeds of *A. marmelos* were purchased from suburb of Varanasi, India and a voucher specimen has been preserved at Department of Chemistry, Banaras Hindu University, Varanasi, India.

Extraction

The fully dried and finally powdered seeds of *A. marmelos* (200 g) were extracted with ethanol (5 L) for 38 hours at 60-70 °C using soxhlet apparatus. The ethanol extract was further in vacuo concentrated and the residue (8.5 g) was stored at -20 °C until used for antileishmanial bioactivity.

Culture of L. donovani parasites

The protozoan parasites *Leishmania donovani* (MHOM /IN/80/Dd8) promastigotes were maintained in vitro in liquid DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) and gentamycin 20 mg/mL, streptomycin 100 mg/mL, penicillin 100 UmL^{-1} (Sigma Chemicals, USA) at pH 7.2 in a BOD incubator at 25 °C. For culturing and maintenance of amastigote form of the parasites, same liquid medium were used but medium was acidified to pH 5.5 and incubated at 37 °C in humidified CO_2 incubator containing 5% CO_2. Biochemical characterization of axenic amastigotes was done by lectin agglutination test.[8] For testing the sample, stationary phase of the parasites were used. The parasite concentrations of 1x106 mL^{-1} were adjusted for the in vitro assays by using Neubaur's counting chamber.

Drug Reconstitution

For preparation of stock, residue of ethanol extract was dissolved in sterile Milli-Q water and stored at 4°C for a maximum of 3 days. Further, the reference drug Miltefosine was also dissolved in Milli-Q water to obtain a stock solution of 20 mg/mL. For working solutions, drug was diluted in complete DMEM in required concentration.

In vitro assay on promastigotes and amastigotes

For in vitro anti-leishmanial assay, working solutions of reference drug and ethanol extract were prepared. A twofold dilution of the drug and ethanol extract was setup in 96-well plate (NUNC, Denmark). Briefly, 200 µL aliquots of the reference drug and ethanol extract were dispensed in triplicate in the row of 96-well plate. 100 µL of liquid medium was dispensed in the remaining wells. From the drug and ethanol extract, 100 µL was aspirated and transferred to the successive well of the second row and so on, and so forth up to the second last row in order to have a twofold dilution. 100 µL of the parasite suspension at 106/mL was dispensed in drug and ethanolic extract as well as control rows to adjust concentrations (reference drug 0.005 to 0.000312 mg/mL and ethanol extract 25 to 1.5625 mg/mL). The last row was used as control well.

For assaying efficacy on promastigotes, the plate was incubated at 25 °C for 24 h in a BOD incubator. For assay on amastigotes, the plate was incubated at 37 °C in a CO_2 incubator containing 5% CO_2 and 95% humid air for 24 h. All the experiments were performed in triplicate. Parasites with no drugs were used as control. After 24 h, the live promastigotes were counted in Neubaur's counting chamber under microscope. Non-motile, dead and with distorted shape were excluded. The amastigotes were counted by trypan blue dye exclusion method to obtain number of live parasites.

Determination of IC_{50}

To determination the percentage inhibition, mean of control wells was equated to 100% survival and all other counts at different drug concentration were converted into percentages. Inhibitory concentration (IC_{50}), the concentration of drug that inhibits 50% of parasites growth, was determined by plotting a graph between percentage inhibitions versus different drug concentration using Sigma Plot version 11.

Assessment of Cytotoxicity

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to assess the cytotoxicity for cellular proliferation of reference drug and ethanol extract on RAW 264.7 macrophage cell line, obtained from National Centre for Cell Sciences, Pune. Briefly, in a 96 well tissue culture plate, macrophages $2x10^4$ cells/well in complete RPMI-1640 medium were seeded and plates were incubated in a humidified CO_2 incubator containing 5% CO_2 at 37 °C for 4 hours. The cells were washed with fresh RPMI-1640 medium (without serum) to remove non-adherent cells and 100 µL of fresh complete medium was dispensed. The 100 µL reference drug at different concentration starting from 200 to 6.25 µg/mL and ethanol extract at different concentration starting from 15 to 0.9375 mg/mL was dispensed in each well in a series of two fold dilution and plates were incubated for 72 hours at same parameters of humidity, CO2 and temperature. After completion of incubation, 50 µL of 0.5% MTT was added to each well and plates were further incubated for 4 hours to allow MTT to enter in cells for its reduction into insoluble purple colored formazan. After incubation, the plates were centrifuged at 5000 rpm for 10 min at 4 °C and the supernatant was discarded from each well. The insoluble formazan inside the cells was solubilised with 100 mL of DMSO, added in each well, and absorbance of the plate was read at 540 nm. The experiments were done in triplicate and cells without drug served as control. Results were expressed as percentage reduction in cell proliferation, compared with controls. The cytotoxicity concentration required to inhibit the 50% of cells proliferation (CC_{50}) was determined as above.

Result and Discussion

Leishmaniasis is a serious infection disease that affects million of people among several tropical and subtropical countries of the world. It is transmitted by the vector sandfly to the mammalian host[9] The main problem to treat this disease is acquiring the resistance, toxicity and variable efficacy between strains or species to most of drugs. Therefore, chemotherapy by using the natural products is the best a way to check the problem, particularly those plants that are used in traditional medicine[10]. A large number of natural products have been identified as antileishmanial agents that include chalcones,

Table 1: In vitro antileishmanial activity (IC_{50}) and cytotoxicity (CC_{50}) of ethanol extract obtained from *Aegle marmelos* seeds.

	Ethanol Extract (mg/mL)	Miltefosine (mg/mL)
IC50 value		
L. donovani promastigotes	9.473 ± 0.424	1.893 ± 0.083
L. donovani amastigotes	12.508 ± 0.344	3.047 ± 0.023
CC50 value		
RAW 264.7 macrophage cell line	8.164 ± 0.806	0.0124 ± 0.00042

[a] IC50 Concentration of test sample that inhibited 50% growth of promastigotes
[b] IC50 Concentration of test sample that inhibited 50% parasite growth of axenic amastigotes
[c] CC50 Concentration of test sample that inhibited 50% decrease in cell proliferation

lactones alkaloids, saponins, terpenes, indoles and acetogenins etc[11].

The present study showed the antileishmanial activity of the ethanolic extract of *A. marmelos* seeds on *L. donovani* strain (MHOM/IN/80/Dd8) promastigote and amastigote forms of the parasites that are similar to other antileishmanial agents isolated form other plants[12]. The negative control, corresponding to the parasite in the presence of ethanol that showed no detectable inhibition of growth, change in morphology or motility of the parasites. The in vitro antileishmanial activity in terms of IC_{50} for promastigotes and amastigotes, and CC_{50} for RAW 264.7 macrophages, determined after 12h exposure to different concentration of ethanol extract and reference drug miltefosine, are presented in Tables 1.

The results showed that the ethanolic extract was active at a dose of 1.5625 mg/mL after 24 h, causing around 2% inhibition in the growth promastigotes which increased with the duration of time, which was 15% at the end of 72 h at the same concentration. With increase in concentration of extract the activity was also increased with time that showed reduction in motility of promastigotes and inhibition in growth goes upto 100% with at a concentration of 25mg/mL for promastigotes. Similarly, with increase in concentration the inhibition rate was increased with increase in concentration that was confirmed by counting the amastigotes after staining with trypan blue exclusion dyes.

The results demonstrate that the promastigotes are more susceptible than amastigotes. Since, the test sample of A. marmelos is in crude form rather than in a purified form so, the active compound or agent present in the extract acts only in when the extract to be used is present in higher concentration. Further, analysis, fractionation, isolation and purification of

Figure 1. (a) Effect of Ethanol extract on L. donovani Promastigotes, IC50 = 9.473 ± 0.424 mg/mL

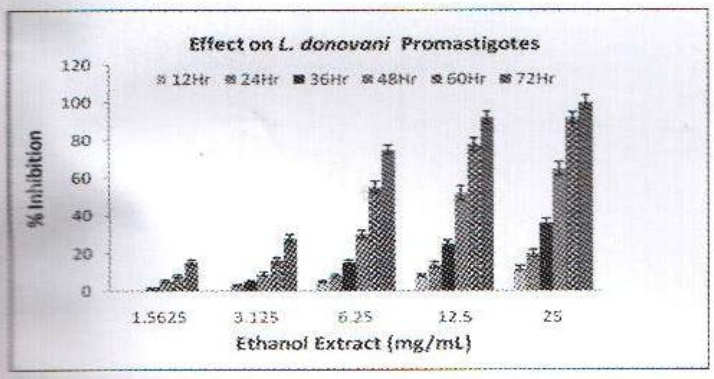


Figure 1. (b) Effect of Ethanol extract on L. donovani Amastigotes, IC50 = 12.508 ± 0.344 mg/mL

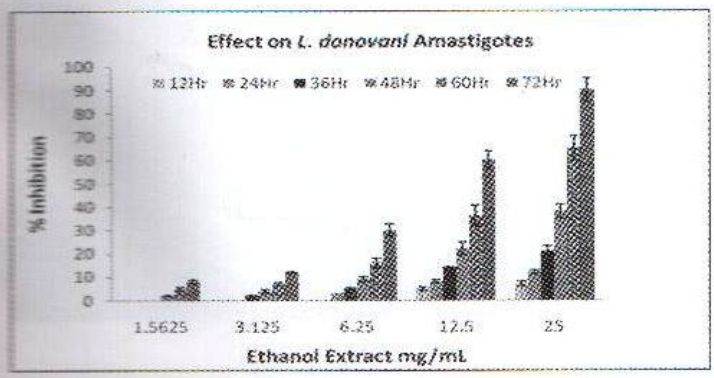


Figure 2. (a) Effect of Miltefosine on L. donovani promastigotes, IC50 = 1.893 ± 0.0834 mg/mL

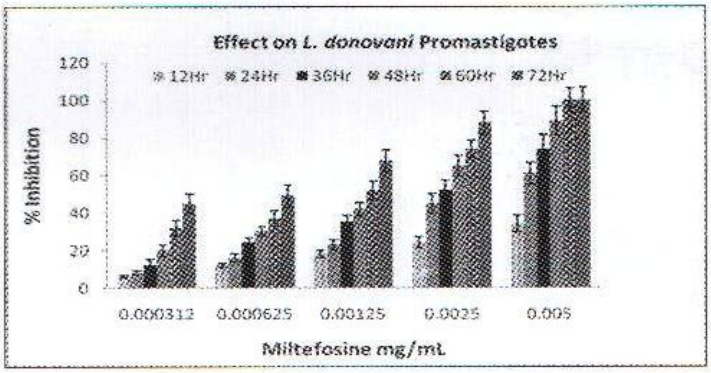
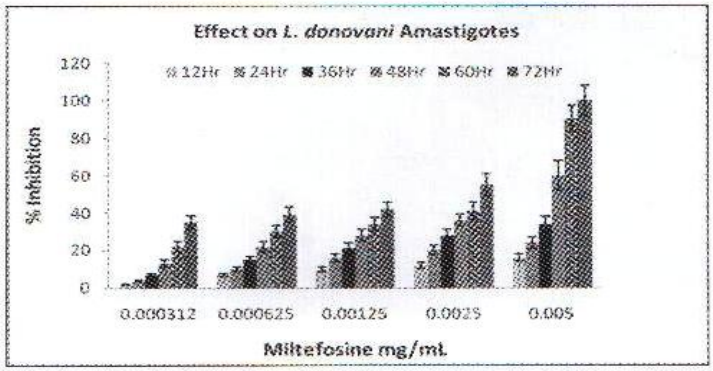


Figure 2. (b) Effect of Miltefosine on L. donovani Amastigotes, IC50 = 3.047 ± 0.023 mg/mL



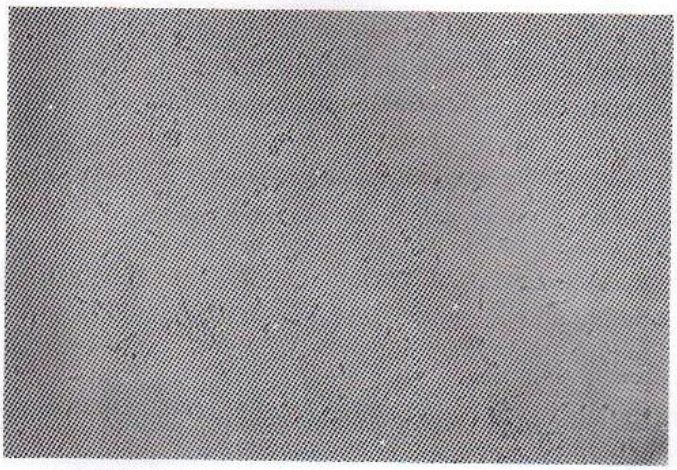
crude extract are needed for identification of its active antileishmanial agents.

The data are presented in mean ± standard deviation. The IC_{50} value of ethanol extract for promastigotes was 9.473±0.424 mg/mL (Figure 3), which was higher than miltefosine (1.893±0.083 mg/mL). For amastigotes, the IC_{50} for ethanol extract and miltefosine was found to be 12.508± 0.344 mg/mL (Figure 4), and 3.047±0.223 mg/mL, respectively. The observed CC_{50} for miltefosine was 0.0124 ± 0.00042 mg/mL (Table 1) and that of ethanol extract (8.164 ± 0.806 mg/mL).

Time-dependent dose relationship study demonstrated that ethanol extract inhibited 100% growth of promastigotes at concentrations 25 mg/mL after 60h, respectively (Figure 1a), whereas 90% growth of amastigotes was inhibited at 72 h by ethanol extract (Figure 1b). As the reference drug Miltefosine inhibits membrane ergosterols, thus severely damage the parasitic cell membrane. However, in our study, the promastigotes on treatment with ethanol extract were observed granulated after 48 hours possessing bleb formation, thus apoptosis like death pathway would be the probable mode of action. Also the ethanol extract demonstrated significant antileishmanial activity on both forms of the parasite. Further, analysis, fractionation, isolation and purification of crude extract are needed for identification of its active antileishmanial agents.

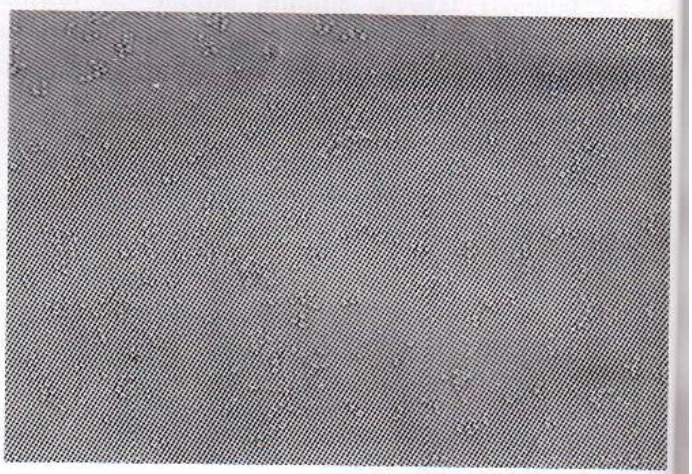
Figure 3. Effect of ethanol extract on L. donovani Promastigotes (a) Control (without treatment) (b) Effect at IC_{50}.

(a)

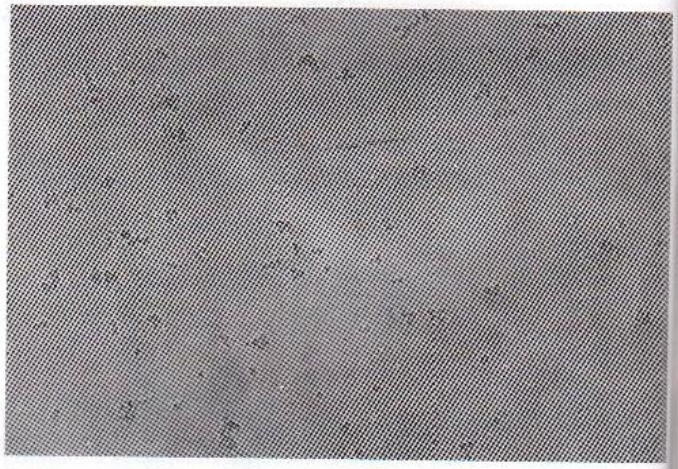


(b)

Figure 4. Effect of ethanol extract on L. donovani Amastigotes (a) Control (without treatment) (b) Effect at IC_{50}.



(a)



(b)

Conclusion

In the present study, antileishmanial activity of crude ethanol extract of A. marmelos against L. donovani has been demonstrated. Based on the selectivity index for the parasite, these active fractions are required to investigate for the identification of the active compound which may lead to the discovery of the new or leads to antileishmanial agent.

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