Bioactivity guided fractionation of *Moringa oleifera* Lam. flower targeting *Leishmania donovani*

Manoj Kumar Singh¹, Joydeep Paul¹, Tripti De¹ & Tapati Chakraborti²*

¹Infectious Disease Division, CSIR-Indian Institute of Chemical Biology (IICB), Jadavpur, Kolkata-700 032, West Bengal, India
²Department of Biochemistry and Biophysics, University of Kalyani, Kalyani-741 235, West Bengal, India

Received 01 February 2015; Revised 21 May 2015

Leishmaniases is a group of diseases caused by the protozoan parasite belonging to the genus *Leishmania*. At least 20 species of *Leishmania* are known to infect humans transmitted by female sandflies, *Phlebotomus* spp. *Leishmania donovani* causes visceral leishmaniasis, considered most lethal among the common three forms of leishmaniasis. Lack of appropriate vaccines, emergence of drug resistance and side effects of currently used drugs stress the need for better alternative drugs, particularly from natural sources. Here, we conducted *in vitro* and *in vivo* experiments to study the efficacy of different parts of *Moringa oleifera* Lam. against *Leishmania donovani* promastigotes. The flower extract of *M. oleifera* (MoF) was found to be the most potent antileishmanial agent when compared to other parts of the plant like leaf, root, bark and stem. It imparted significant reduction in parasite number in infected macrophages. The bioactivity guided fractionation of MoF showed ethyl acetate fraction (MoE) as the most active and gave significant parasite reduction in the infected macrophages. Further, growth kinetics studies revealed loss of *L. donovani* promastigotes viability in the presence of MoE in both time and dose dependent manner. *In vivo* experiment in Balb/c mouse model of leishmaniasis supported the *in vitro* findings with a remarkable reduction of the parasite burden in both liver and spleen.

**Keywords:** Antileishmanial activity, Black fever, Drumstick tree, Dum dum fever, Immunomodulation, Kala-azar, Visceral leishmaniasis

Leishmaniasis is the second deadliest parasitic disease after malaria caused by the protozoan parasite *Leishmania*¹. This has attained considerable importance during the last decade due to increased number of cases worldwide, including non-endemic countries²,³, and also its co-infection with HIV⁴,⁵. Approximately, 12 million people are affected by various forms of leishmaniasis worldover, and about 350 million people are at risk of contracting leishmaniasis⁴,⁵. About 0.2-0.4 million new visceral leishmaniasis (VL) and 0.7-1.2 million cutaneous leishmaniasis (CL) cases have been reported to occur each year. Of the 98 countries endemic for leishmaniasis, 6 countries viz., Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan account for >90% of global VL cases⁶.

Leishmania is a dimorphic protozoan parasite that lives as motile flagellated form in the gut of the vector female sand fly *Phlebotomus* spp., and as non motile round shaped amastigote form inside the host macrophages¹. A recent work of Majumdar *et al.*⁷ has highlighted the role of Toll like receptor 2 and CC chemokine receptor 5 in the entry of the parasite *Leishmania donovani* into the host macrophages. Many of the drugs being clinically used to treat leishmaniasis are based on pentavalent antimony compounds, developed five decades ago. The toxicity of these agents, their side effects and the growing resistance have become a matter of concern. Alternative drugs, such as amphotericin B and pentamidine, also have unpleasant side effects⁸-¹². Efforts for alternative non toxic, cost effective drug against leishmaniasis have led to development of a few potential drugs from natural resources¹³-¹⁶ including amphibians¹⁷. Works on development of an effective vaccine against leishmaniasis for use in human though resulted in some products, their efficacy has not been proved till now¹⁸,¹⁹. Mandal *et al.*²⁰ have designed a colorimetric β-lactamase assay for high-throughput screening of amastigotes of *L. donovani* and thereby
checking the efficacy of antileishmanial drugs or vaccines.

*Moringa oleifera* (Moringaceae), commonly known as drumstick tree is a South Asian tree, is a multipurpose medicinal plant. Almost each and every part of the plant has some medicinal value. Various parts of this plant such as the leaves, roots, seed, bark, fruit and flowers act as cardiae and circulatory stimulants; possess antibacterial, anticancer, anti-diabetic, antiepileptic, antifungal, antihypertensive, anti-inflammatory, antioxidant, antipyretic, antispasmodic antitumor, anitulcer, cholesterol lowering, diuretic, hepatoprotective, immunomodulatory and radioprotective activities; and play a role as neurotransmitter. Also, it ameliorates fluoride toxicity and used in the treatment of urolithiasis. These diverse properties of this plant led us to test its efficacy against *L. donovani* parasite. Here, we have evaluated the in vitro efficacy of different parts of *M. oleifera* plant against *L. donovani*. Further, in vitro bioactivity guided fractionation was done with the most active part. An in vivo efficacy of the most active fraction was also done in murine model of visceral leishmaniasis.

**Materials and Methods**

**Bioactivity guided extraction and fractionation of *M. oleifera***— Crude powdered plant material (15 g) was extracted thrice for 48 h with 100 ml aqueous:methanol solvent (1:1) each time. Methanol solvent of the crude extracts obtained from root, bark, stem, seed, leaves and flower were dried under reduced pressure in rotary evaporator. The left over aqueous part was dried in lyophilizer. For fractionation, 1 g of the most active part i.e. the *M. oleifera* flower extract (MoF) was dissolved in 10 ml of water and fractionated successively with 10 ml of chloroform, ethyl acetate and saturated n-butanol with each step repeating ten times. The chloroform fraction (MoC), ethyl acetate fraction (MoE) and butanol fraction (MoB) were further dried under reduced pressure in rotary evaporator. The left over aqueous fraction (MoA) was dried by lyophilization. All the yields were weighed and kept for further use. Aqueous:methanol solvent yielded 26% (w/w) crude extract. Further, solvent-solvent extraction yielded 2.8% (w/w) MoC, 4.56% (w/w) MoE, 15.94% (w/w) MoB and 51.22% (w/w) MoA, respectively.

**Animals, parasites and animal infection**— Experimental BALB/c mice (irrespective of sex, originally bought from Jackson Laboratory, Bar Harbor, Maine), reared in the CSIR-Indian Institute of Chemical Biology facility were used with prior approval of the animal ethics committee of the Institute (Accreditation Number 147/1999/CPCSEA; internal approval number II/C/IAEC/TD/2005). Parasites were maintained in golden hamsters and promastigotes obtained after transforming amastigotes from infected spleen, were maintained in M199. For infection, 4-6 wk old animals were injected with $1 \times 10^7$ (in 100 µl PBS) second passage stationary phase promastigotes through intra-cardiac (i.c.) route. To detect progressive infection, the infected animals were sacrificed and the stamp or impression smear of the cut surface of the spleen or liver was taken on the slide. The parasite load was determined after fixing the stamp smears with ice cold methanol (Merck), staining with Wright’s Giemsa and observing under bright field oil-immersion microscope. Splenic and hepatic parasite burden in infected animals were expressed as *Leishmania donovani* units (LDU), where LDU is equal to the number of parasites per 1000 nucleated host cell multiplied by organ weight in gram.

$$LDU = \frac{\text{No. of intracellular amastigotes}}{1000 \text{nucleated cells} \times \text{organ wt. (g)}}$$

**Growth kinetics**— Growth kinetics was done to find out the inhibitory effect of *M. oleifera* on the parasite growth. *L. donovani* (1st passage) were taken and washed thrice in PBS and the concentration adjusted to $3 \times 10^6$ cells per ml of M199. One ml of medium containing parasites was taken in 24-well plate and different concentrations i.e. 25, 50 and 100 µg/ml of MoE were added. One set with no treatment was kept as control. Parasites were kept at 22°C on shaker with mild speed and the counts were taken at different intervals of time in a hemocytometer up to 24 h using trypan blue exclusion method.

**In vitro antileishmanial activity**— For infection, RAW 264.7 (Mouse leukemic monocyte macrophage cell line) was adhered on glass coverslips (1×10^5 cells/0.5 ml complete RPMI medium) for 24 h. Adherent cells were then infected with stationary-phase 2nd passage *L. donovani* promastigotes at a parasite: macrophage ratio of 20:1 and incubated for 24 h in 5% CO$_2$ at 37°C for optimum attachment and internalization of the parasites. The excess parasites were then removed by vigorous washing with FCS free media. The infected macrophages were again incubated with complete RPMI medium...
supplemented with 10% FCS in the presence or absence of extracts obtained from different parts of *M. oleifera* plant or different concentrations of MoE for 24 h. The coverslips were then fixed in pre-chilled methanol, stained with Giemsa and examined under oil immersion microscope. Degree of infection was expressed in terms of number of amastigotes/1000 macrophages.

**In vivo antileishmanial activity**— To determine the antileishmanial activity of MoE, BALB/c mice were infected with *L. donovani* parasites as given above. After two months of infection mice were treated with different concentrations of MoE as indicated. Spleen and liver parasite burden were determined as mentioned earlier in the section, “Animals, parasites, and animal infection”. 

**Statistical analysis**— Results were expressed as mean ± SD of the individual set of experiments. An unpaired two-tailed Student t-test was used for statistical analysis of the data. *P* values of <0.05 were considered statistically significant.

**Results**

**Flowers, the most potent antileishmanial part of *M. oleifera* plant**— Different parts of *M. oleifera* plant like bark, stem, seed, leaf, root and flower were evaluated for its antileishmanial activity. *L. donovani* infected RAW cells were treated with 100 µg/ml of 50% methanolic extract of each part of the plant as mentioned above. Bark and stem were found to be least active and provided 23 and 24% reduction in parasite number in infected macrophages, respectively. The flower extract was the most active and imparted 86% protection. The seed, leaves and root were also active and showed 54, 67 and 70% reduction in parasite number in infected macrophages, respectively (Fig. 1A).

**MoE, the most potent antileishmanial active fraction**— For bioactivity-guided fractionation of the most active part i.e. the flower extract, MoF was dissolved in water and fractionated successively with chloroform, ethyl acetate and saturated n-butanol for further purification of the active components. The ethyl acetate fraction of *M. oleifera* flower extract (MoE) was found to be most effective and showed 91% reduction in parasite number in infected macrophages at a dose of 50 µg/ml (Fig. 1B). The crude extract (MoF) at a concentration of 100 µg/ml showed 86% parasite reduction. Similarly, the other fractions were evaluated with a dose of 50 µg/ml each. The chloroform fraction (MoC) was found to be toxic at this concentration and the cells got lysed. The n-butanol (MoB) and the left over aqueous fraction (MoA) provided 84 and 73% of parasite reduction, respectively (Fig. 1B).

**Growth kinetics**— In growth kinetics experiment, MoE was evaluated against *leishmania* promastigote parasite. MoE showed activity against *L. donovani* promastigotes in both time and dose dependent manner. MoE treatment at a concentration of 25 µg/ml showed 97, 82 and 26% viable parasites after a time period of 6, 12 and 24 h, respectively. Similarly, treatment with MoE at a higher dose of 50 µg/ml showed 93%, 71 and 11% viable parasites at the same time intervals. Further, MoE treatment at 100 µg/ml concentration validated a similar observation and

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Fig. 1— *In vitro* parasite burden and percent reduction in parasite number in infected macrophages in presence/absence of (A) different parts of *Moringa oleifera* plant; and (B) different fractions of *M. oleifera* flower. [*1×10⁵*] RAW 264.7 cells in triplicates were exposed to the parasites in 1:20 ratio and incubated for 24 h, in 5% CO₂, at 37°C for optimum attachment and internalization of the parasites. The excess parasites were then removed by vigorous washing with FCS free media. The infected macrophages were again incubated with complete medium and FCS. These were further treated with/without100 µg/ml of crude extract or 50 µg/ml of each of the fractions as indicated in figure. Control-indicates infected macrophages without any treatment. Data represent the mean ± SD of three coverslips per group, and are representative of three individual experiments.* *P* <0.0001 compared with respective control groups; unpaired two-tailed Student’s t-test]
showed promastigote viability as 86, 64 and 6% after 6, 12 and 24 h, respectively (Fig. 2).

In vivo antileishmanial activity by MoE—In vivo antileishmanial activity was evaluated in BALB/c mice model of VL. *L. donovani* Infected mouse were divided into five groups, each having 5 mice. Group I, vehicle control (treatment with PBS); and groups II-V, treatment with 10 mg/kg of MoF, MoE, MoB and MoA, respectively. Extract and fractions were fed orally for 10 consecutive days starting on 60th day after infection. Animals were sacrificed after treatment and the liver and spleen parasite load was determined for all groups. Parasite burden was calculated as given in materials and methods. LDU of treated group was compared with the untreated group and the percent protection was calculated. Results of the in vivo experiments were similar in trend with the in vitro experiments. The percent protection by MoE was the highest (almost 93% in both liver and spleen) (Fig. 3 A and B). Liver percent protection in presence of MoF, MoB and MoA was found to be 89, 78 and 67%, respectively. Similarly, spleen percent protection by MoF, MoB and MoA was found to be 88, 82 and 71%, respectively (Fig. 3 A and B).

Discussion

Natural products represent a rich source of potential chemical entities for the development of new effective drugs for neglected diseases13-16,21-32. Scientific evaluation of medicinal plants have made it possible the use of some metabolite like quinines, alkaloids, terpenes and flavonoids from them for the treatment of diseases caused by protozoan parasites. Advantages such as safety without much adverse side effects and low cost value have promoted the use of natural extracts as effective antileishmanial drugs. Here, we have reported the efficacy of *M. oleifera* plant against *L. donovani* parasite both in an in vitro and in vivo model.

*M. oleifera* represents a wide range of medicinal importance but still its efficacy against *L. donovani* parasite is least explored. Many of the medicinal properties of the plant like anticancer and antitumor24,35-37, hepatoprotective25,37, antiparasitic38,39 and immunomodulation28 encouraged us to test its effectiveness against *L. donovani* parasite.

Earlier, the flower extract of *M. oleifera* has been reported to have antibacterial activity against both
gram positive and gram negative bacteria\textsuperscript{40-42}. In the present study, we demonstrated flower extract of \textit{M. oleifera} to be most effective compared to its other parts viz., bark, stem, seed, root and leaves.

Growth kinetic study has shown that the extract is active against promastigotes and indicated that MoE can target parasite metabolism and inhibit its growth. Flowers of \textit{M. oleifera} consist of different classes of metabolites such as alkaloids, flavonoids, steroids and glycosides\textsuperscript{43,44}. Different compounds belonging to each of this group of metabolites are reported to have antileishmanial activities\textsuperscript{16,45-47}. MoE is also effective in killing the parasites within the infected macrophages\textsuperscript{48}. Overall, these findings indicate the efficacy of MoE as a potent antileishmanial component with probable role of immunomodulation in the host cells during \textit{L. donovani} infection.

**Conclusion**

In the present study of bioactivity-guided extraction and fractionation of the \textit{Moringa oleifera} flower, we have demonstrated that ethyl acetate fraction of the flower extract has antiparasitic action both \textit{in vitro} and \textit{in vivo} against \textit{Leishmania donovani}. Various metabolites reported from the flowers of \textit{M. oleifera} could be attributed to the leishmanicidal activity. This advocates the role of MoF in killing \textit{L. donovani} parasites. Separation and characterization of active principles, involvement of immunomodulatory and other mechanisms responsible for antileishmanial activity are the future scope of the study of antileishmanial therapeutics.

**Acknowledgement**

Authors thank the Council of Scientific & Industrial Research (CSIR), New Delhi for supporting the work.

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