

Antiparasitic activity of plumericin & isoplumericin isolated from *Plumeria bicolor* against *Leishmania donovani*

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Background & objectives: The severe toxicity, exorbitant cost and emerging resistance of *Leishmania* species against most of the currently used drugs underscores the urgent need for the alternative drugs. The present study evaluates *in vitro* anti-leishmanial activity of *Plumeria bicolor* and its isolated compounds.

Methods: The *in vitro* anti-parasitic activity of chloroform extract of *Plumeria bicolor*, plumericin and isoplumericin were tested alongwith appropriate controls against promastigote and amastigote forms of *Leishmania donovani* using 96 well microtiter plate. The concentration used for assessing the anti-leishmanial activity of extract of *Plumeria bicolor* and both isolated compounds were 100 µg/ml and 15 µM, respectively. The viability of the cells was assessed by MTT assay. The cytotoxicity of these compounds was performed against J774G8 murine macrophage cells lines at the concentration of 30 µM.

Results: The *Plumeria bicolor* extract showed activity with the IC₅₀ of 21±2.2 and 14±1.6 µg/ml against promastigote and amastigote forms of *L. donovani*, respectively. Plumericin consistently showed high activity with the IC₅₀ of 3.17±0.12 and 1.41±0.03 µM whereas isoplumericin showed the IC₅₀ of 7.2±0.08 µM and 4.1±0.02 µM against promastigote and amastigote forms, respectively. Cytotoxic effect of the chloroform extract of *P. bicolor*, plumericin and isoplumericin was evaluated in murine macrophage (J774G8) model with CC₅₀ value of 75±5.3 µg/ml, 20.6±0.5 and 24±0.7 µM, respectively.

Interpretation & conclusions: Our results indicated that plumericin showed more potent activity than isoplumericin and might be a promising anti-leishmanial agent against *L. donovani*.

Key words Isoplumericin - *Leishmania donovani* - *Plumeria bicolor* - plumericin

Leishmaniasis is a major public health problem causing significant morbidity and mortality in Africa, Asia and Latin America. Approximately, 12 million people are affected worldwide, with an increasing incidence of 2 million new cases diagnosed every year and 350 million people at risk, despite all efforts being

made to fight the disease¹. Leishmaniasis is caused by species of *Leishmania*, a unicellular kinetoplastid protozoan flagellate, which is transmitted through the bite of female phlebotomine sandfly species. It presents mainly in 3 clinical forms; visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous

leishmaniasis (MCL), of which VL is the most severe form of the disease, lethal if untreated and is caused by species of *Leishmania donovani* complex². VL is endemic in the tropical and sub-tropical regions of Africa, Asia, Southern Europe, South and Central America. India accounts for half of the total 500,000 VL or kala-azar infections that are recorded annually worldwide³.

In the absence of any effective vaccine, the only mean to treat and control leishmaniasis is affordable medication. Most of the drugs currently being used for leishmaniasis, suffer from one or other limitations like exorbitant cost, difficult to administer, high toxicity or development of resistance^{4,5}. Therefore, there is an urgent need for safe, more effective and economically feasible drugs for the treatment of leishmaniasis. In this context, medicinal plants hold promise as sources of novel therapeutic agents⁶. WHO and the U.S. Food and Drug Administration (FDA) have recognized the importance of natural products and a number of compounds derived from nature are at various stages of multicenteric clinical trials all over the world. But not much scientific data are accumulated on their safety, purity and standardization; hence their efficacy and toxicity investigations are required⁷.

Plumeria is a genus of shrubs and trees of the family *Apocynaceae*. The various *Plumeria* species are used for the cure of rheumatism, diarrhoea, blennorrhoea, venereal disease⁸ and reported to exhibit significant antibacterial, antifungal, antiviral⁹ and anti-cancerous¹⁰ activity.

In the present study, we undertook an evaluation of the anti-leishmanial activity of the chloroform extract from the stem bark of *Plumeria bicolor*, commonly known as "Champa". The active antileishmanial compounds were purified by bioactivity-guided fractionation of the extract, which were identified by mass spectral analysis. The *in vitro* anti-proliferative effect of the chloroform extract, its fractions and purified active compounds was evaluated against *L. donovani*.

Material & Methods

Plant material: The stem bark of *P. bicolor* was collected from the campus of the University of Rajasthan, Jaipur, and botanical identification was done at the Department of Botany, University of Rajasthan and the voucher specimen was submitted at the herbarium (voucher specimen no. RUBL-20603).

Preparation of plant extract: The methanol extract from the bark of *P. bicolor* was prepared at the All India Institute of Medical Sciences, New Delhi as described previously¹⁰, with slight modifications. The bark was dried in shade and grounded to fine powder. The powdered material (3 kg) was extracted with methanol (10×5 l) extensively for 72 h. The methanol extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator [Labmate (Asia) Pvt. Ltd. Model: RVC 2-18] at <40°C, which yielded a semi-solid brown mass. The concentrated mass was treated with acetonitrile to remove fats. Acetonitrile solvent was evaporated to dryness and the resulting mass (100 g) was stored at -20°C until further use.

Isolation and purification of active compounds: Fat free extract (100 g) was re-extracted with chloroform. The chloroform soluble portion afforded 30 g of extract after removal of the solvent. Half of the obtained extract was used for anti-parasitic activity and remaining half was subjected to repeated column chromatography for separation and isolation of pure compounds over silica gel column. For this, a column of 1.2 m in height with 5 cm in diameter and 800g silica gel G (60-120 mesh) was used. The column was eluted with different solvents using mixtures of benzene-chloroform-methanol in order of increasing polarity and P1-P8 fractions were collected. All these fractions were tested for their anti-parasitic activity against *L. donovani*.

Identification and structure elucidation of active compounds: The structures of the purified compounds from fraction P5, showing better anti-leishmanial activity than other fractions, were identified by mass spectrometry (Agilent 1100 Series LC/MSD API-ES spectrometer, Alpharetta, Georgia, USA), infrared analyses (FTIR-8400S Spectrophotometer, Shimadzu, Japan) and nuclear magnetic resonance (NMR) (JEOL AL-300 MHz Spectrometer, Tokyo, Japan) using ¹H NMR (300.40 MHz) and ¹³C NMR (75.45 MHz) analyses in CDCl₃ and by comparison of spectral data with those available in the literature.

Parasite and macrophage cell culture maintenance: The *L. donovani* promastigote (MHOM/IN/1998/KE16), isolated from a VL patient from Bihar in eastern India¹¹, was routinely maintained at 24°C in M-199 (GIBCO®, USA) medium containing penicillin (100U/ml), streptomycin (100µg/ml) (Invitrogen, USA) and supplemented with 10 per cent heat inactivated foetal calf serum (FCS; GIBCO®, USA). The infectivity of the parasite was maintained by periodic intravenous

inoculation of the promastigotes in BALB/c mice. Briefly; the promastigotes in their mid log phase were harvested by centrifugation at 4500 g at 4°C in a refrigerated centrifuge. Pellets were re-suspended in PBS (pH 7.4) for intravenous infusion. Six to 10 wk old BALB/c male mice with body weight of 25-30 g, were injected intravenously (iv) in the lateral tail vein with about 1×10^7 log phase promastigotes. After 30-35 days of inoculation, animals were sacrificed, spleen and liver harvested and parasites culture isolated in Novy-MacNeal-Nicolle (NNN) medium followed by mass culture in M199 medium. J774G8 murine macrophage cells were maintained in RPMI 1640 (GIBCO®) medium supplemented with 10 per cent FCS at 37°C in a humidified mixture of 5 per cent CO₂ atmosphere (NuAire, Inc., USA).

In vitro anti-promastigote activity: The chloroform extract was initially dissolved in dimethyl sulphoxide (DMSO; Sigma, USA) and further diluted with the M-199 medium after a sterilizing filtration. To examine the anti-leishmanial activity of the extract and purified compounds, logarithmic phase promastigotes of *L. donovani* (1×10^6 cells/ml) were seeded in 96-well microtiter plate in presence of the extract (100 µg/ml) and compounds (15 µM) and then incubated at 24°C for 48 h. After 48 h, the activity of extract and purified compounds was evaluated on parasite growth in the point of mobility of parasites and cell morphology, microscopically. The viability of parasites was also assayed colorimetrically by the mitochondrial oxidation of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay as described previously^{2,12} with minor modifications. Briefly, MTT was dissolved in PBS (5 mg/ml) and sterilized by filtration (0.22 µm). MTT (400 µg/ml) was added to the plate and incubated for 4 h at 24°C. Finally, 100 µl of SDS-HCl (10% SDS in 0.01 N HCl) in each well, was added to dissolve the MTT formazan produced. The absorbance was measured at 570 nm with an ELISA reader and correlated with the number of living promastigotes, adequately standardized for each plate. Concentration of DMSO (0.5%) was maintained in all experiments as control and the growth in medium containing 0.5 per cent DMSO was taken as 100 per cent growth for comparison, while amphotericin B was used as the reference standard drug². Each assay was performed in duplicate with three independent experiments. The anti-promastigote activity was expressed as the IC₅₀ after 48 h of incubation. The IC₅₀ value was calculated with a sigmoid dose-response curve.

In vitro anti-amastigote activity: In order to evaluate the effect of extract and purified compounds on intracellular amastigotes, J774G8 macrophage cells were used. J774G8 (5×10^5 cells/ml) macrophages were plated onto 13-mm coverslips in 24-well plates for 1 h at 37°C in a 5 per cent CO₂ atmosphere. Non adherent cells were removed, and the macrophages were further incubated overnight in RPMI 1640 medium supplemented with 10 per cent FCS. Adherent cells were infected with *L. donovani* promastigotes (logarithmic growth phase) at a parasite/macrophage ratio of 10:1 and incubated for 1 h at 37°C in 5 per cent CO₂. Free promastigotes were removed by extensive washing with PBS (pH 7.2). After 24, 48, and 72 h, infected macrophages were treated at the different concentrations of extract and purified compounds. However, our observations did not find any significant difference in the results read at 48 and 72 h. Therefore, further assay readings were taken at 48 h. After 48 h, the monolayer was washed with PBS at 37°C, fixed in methanol, and stained with Giemsa. At least 200 macrophage cells per experiment were inspected by bright-field microscopy. The survival index was calculated by multiplying the percentage of macrophages with internalized parasites and the mean number of internalized parasites per macrophage². These tests were performed in duplicate with three independent experiments.

Cytotoxicity assay: It was done as described previously¹³. Briefly, J774G8 mammalian macrophage cells were maintained in RPMI 1640 medium supplemented with 10 per cent FCS at 37°C in a humidified mixture of 5 per cent CO₂ atmosphere. Macrophages (1×10^6 cells/ml) were seeded in 96-well microtiter plate in presence of isolated compounds, which were 2-fold serially diluted over six concentrations (30 to 0.93 µM/ml) in RPMI 1640 medium containing 10 per cent FCS, and then incubated for 48 h at 37°C in a humidified mixture of 5 per cent CO₂ atmosphere. The control wells without any extract or compounds (untreated cells) were used as control and considered as 100 per cent viable cells. The cell viability was determined using the MTT assay. Each assay was performed in duplicate with three independent experiments.

Statistical analysis: Data represented the mean ± SD of duplicate samples from three independent assays. The IC₅₀ values were calculated using dose-response curves in Graph Pad Prism 3.0 software (CLa Jolla, California, USA).

Results

Bioactivity-guided fractionation: The anti-leishmanial activity of all P1-P8 fractions was seen (data not shown). The activity was concentrated on fraction P5, which was obtained on eluting the column with chloroform and methanol (8:2 v/v). The solvent was removed and compounds A and B were obtained at different melting points and crystallized with methanol which gave white crystals.

Isolation and characterization of active compound A: Compound A was crystallized with methanol as white crystals (35% yield) and its melting point was 196-198°C and molecular formula was $C_{15}H_{14}O_6$. It was characterized as isoplumericin^{10,14-16}.

Isolation and characterization of active compound B: It was obtained after eluting the column with chloroform and methanol (8:2) which was crystallized with methanol. It gave white crystals (65% yield) and its melting point was 210-212°C. The molecular formula was $C_{15}H_{14}O_6$. It was characterized as plumericin^{10,14-16}.

Anti-promastigote activity: For *in-vitro* anti-leishmanial assay, extract was dissolved in DMSO. The control experiments were performed using DMSO at the same concentration (0.5%) which was used to test the extract. The tested concentration of DMSO did not affect the growth of parasites. Moreover, there were no observable cytopathological changes found as compared to the control group cells, the chloroform extract of *P. bicolor*; isoplumericin and plumericin were used to evaluate the anti-leishmanial activity. Chloroform extract of *P. bicolor* was found to be active with the IC_{50} of 21 ± 2.2 $\mu\text{g/ml}$ (Fig. 1) against promastigote form of *L. donovani*. The isoplumericin and plumericin showed significant activity with an IC_{50} of 7.2 ± 0.08 μM and 3.17 ± 0.12 μM , respectively (Figs 2 & 3). The plumericin showed more potent activity than isoplumericin. Amphotericin B was used as a reference standard drug which showed IC_{50} of 0.08 ± 0.02 μM against promastigote form. At the concentration of 6 $\mu\text{M/ml}$ and higher, plumericin induced clear cytopathological changes in promastigote form of *L. donovani* such as ovoid cells, loss of flagellum, granulation, and rounding of the cells and some other morphological changes in parasites such as decreased mobility of promastigotes, round to oval shaping, decrease in size with dense cytoplasm and enlarged nuclei (Fig. 4).

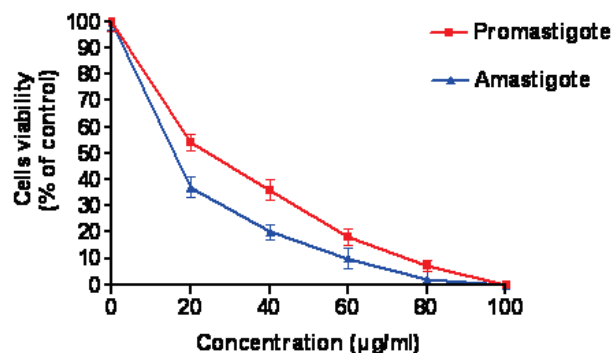


Fig. 1. Anti-leishmanial activity of chloroform extract from the bark of *Plumeria bicolor* against *L. donovani*.

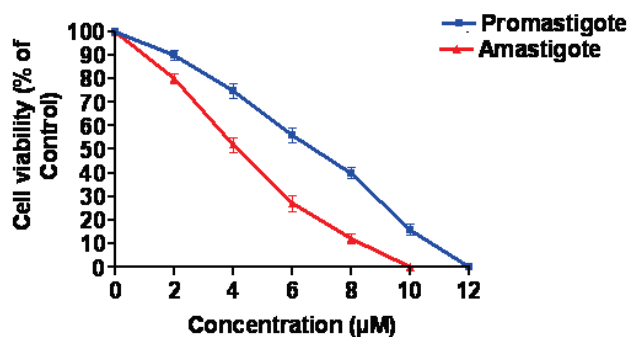


Fig. 2. Anti-leishmanial activity of isoplumericin against *L. donovani*.

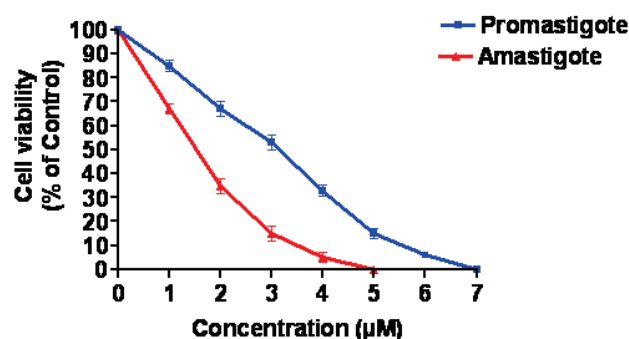


Fig. 3. Anti-leishmanial activity of plumericin against *L. donovani*.

Anti-amastigote activity: The chloroform extract of *P. bicolor* and purified isoplumericin and plumericin treatment of macrophages infected with amastigote form showed that the extract and both purified compounds inhibit the growth of parasites (Fig. 5). After 48 h, the percentage of macrophages with internalized parasites was higher for the control than for the macrophages infected and treated with the extract and both compounds. The mean number of internalized

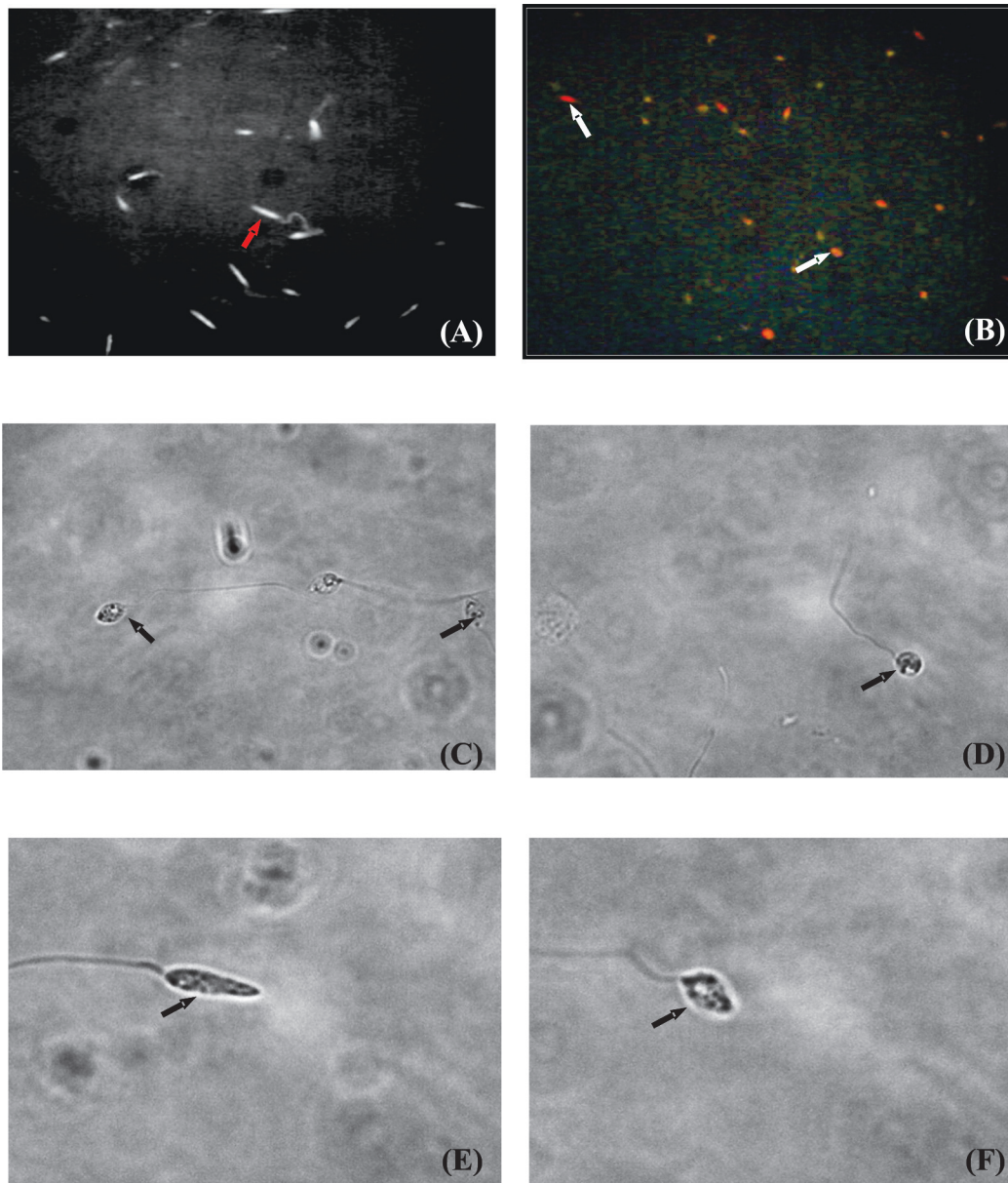


Fig. 4. PI stained promastigotes were photographed using a microscope with an Olympus microphotographic system (A) untreated cells at 400X using a light microscope, (B) plumericin treated cells detected in the orange range of 562-588 nm band pass filter at 400X using fluorescence microscope, and (C, D, E & F) plumericin treated cells showing deformities at 400X using a light microscope. Arrows show (A) live cells, (B) dead cells, (C-F) deformed cell morphology.

parasites per macrophage treated with the extract and both compounds was markedly lower than that for the control. The survival index indicated that the extract and both compounds inhibited the parasite growth in the macrophages. Thus, the extract showed 50 per cent inhibition of cell survival (IC_{50}) at a concentration of $14 \pm 1.6 \mu\text{g/ml}$ (Fig. 1) and plumericin showed more potent leishmanicidal activity with IC_{50} of $1.41 \pm 0.03 \mu\text{M}$ than isoplumericin with IC_{50} of $4.1 \pm 0.02 \mu\text{M}$ (Figs 2 & 3). Amphotericin B was used as a reference

standard drug which showed IC_{50} of $0.06 \pm 0.01 \mu\text{M}$ against amastigote form of *L. donovani*.

Cytotoxicity assay: The cytotoxicity of both isoplumericin and plumericin was tested against murine macrophage (J774G8) model. After 48 h, the viability was checked by MTT assay. Cytotoxicity assay showed that the *P. bicolor* extract, isoplumericin and plumericin caused cytotoxic effect with CC_{50} value of 75 ± 5.3 , 20.6 ± 0.5 and $24 \pm 0.7 \mu\text{M}$, respectively.

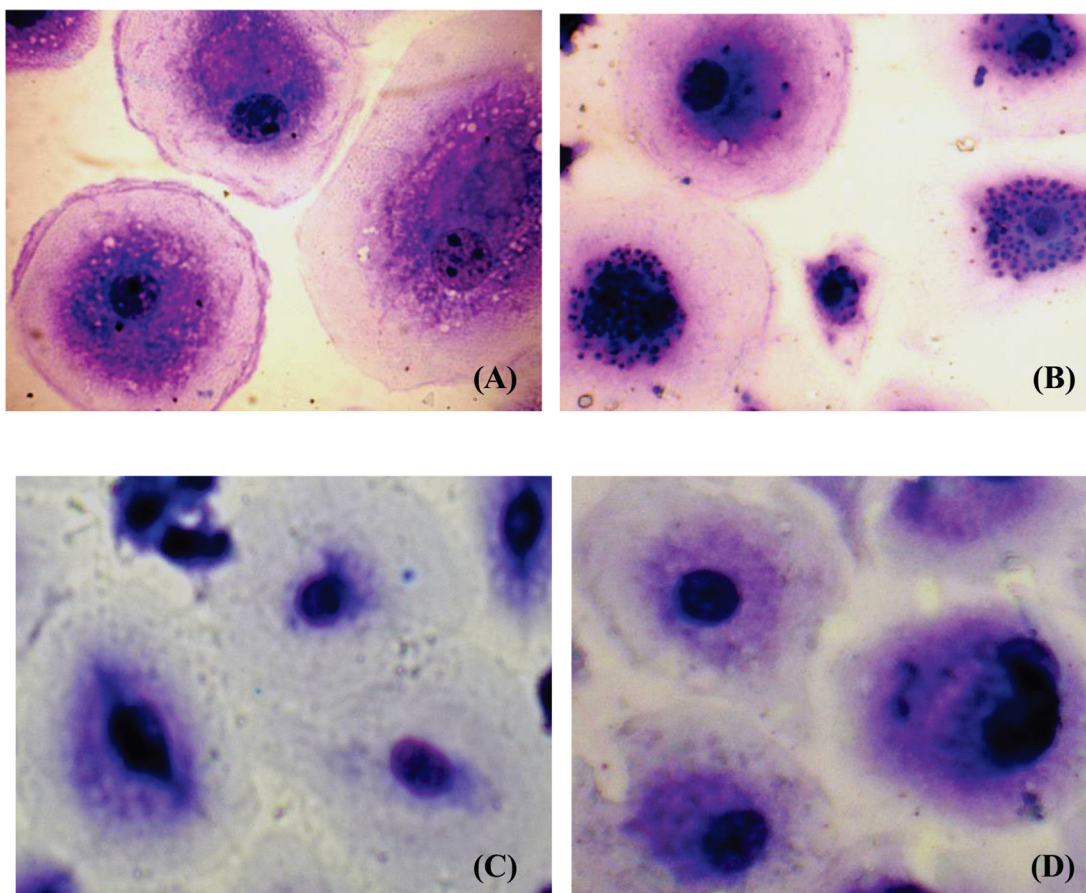


Fig. 5. Giemsa stained cells were photographed at 1000X magnification using a light microscope with an Olympus microphotographic system (A) Uninfected macrophages, (B) Untreated infected macrophages showing amastigotes within macrophages, and (C & D) amphotericin B and plumericin treated infected macrophages respectively, showing clearance of amastigotes.

Discussion

In the absence of new and the current therapeutics against leishmaniasis, herbal products could be an inspiration of new prototype for the drug development¹⁷. The biological and pharmacological activities of the plant iridoids have been reviewed, including antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, antirheumatic, hepatoprotective and antitumour activities^{18,19}. The genus *Plumeria* (*Apocynaceae*) is known as a source of iridoids. In this study, chloroform extract prepared from the bark of this plant showed significant anti-leishmanial activity. Two active leishmanicidal compounds were purified by bioactivity-guided fractionation of the extract, which were identified by spectroscopic analysis as isoplumericin and plumericin. Isoplumericin and plumericin had previously been isolated from different genera of Apocynaceae such as *Plumeria*, *Allamanda* and *Himatanthus*²⁰⁻²². Isoplumericin and plumericin

have been previously reported to have antifungal, anticancerous, antiviral and antibacterial actions²³. The anti-parasitic activity of plumericin from *Himatanthus sucuuba* (*Apocynaceae*) has been demonstrated against *L. amazonensis*, responsible for cutaneous leishmaniasis²⁴.

In the present study, plumericin showed more potent activity than isoplumericin against promastigote and amastigote forms of *L. donovani*. Microscopic evaluation of parasites treated with plumericin (6 μ M) for 24 h showed that approximately >40 per cent of the cell population became ovoid whereas control promastigotes retained classical morphology. At the end of 48 h, >70 per cent of the cells showed loss of flagellum, granulation, and round morphology with substantial reduction in size as compared to control promastigotes, suggesting cytoplasmic condensation and shrinkage, established marker of apoptosis²⁵, which may be the possible cause of cell death, an

effect exerted by plumericin. This compound caused a reduction in the macrophage infection and its effect on infected macrophages should be of great interest, since the radical group orientation influences the anti-parasitic activity of some compounds²⁶. The strong activity of the tetracyclic iridoid plumericin can be explained by the presence of an α -methylene γ -lactone moiety, susceptible to undergo a Michael-type addition with biological nucleophiles²⁷. Cytotoxicity assay showed that plumericin causes negligible cytotoxic effect against J774G8 murine macrophages at the concentrations used. This selectivity assay showed that the action of the isolated compound is specific for the protozoans and is not toxic for mammalian cells. The demonstration of the anti-leishmanial properties of plumericin adds another facet to the broad range of biological activities of these iridoids, which have recently been the challenging target of various synthetic approaches²⁸, because, most of the synthetic drugs like amphotericin B are known for their severe side effects such as nephrotoxicity. Intravenously administered amphotericin B has some common side effects with the first few doses which are associated with severe multiple organ damage in therapeutic doses²⁹.

This study shows a potent leishmanicidal activity of plumericin than isoplumericin which may provide promising lead against leishmaniasis. Further studies will be required to evaluate plumericin, whether it can be used as single anti-leishmanial compound or as fortifying agent with existing synthetic compounds for the development of anti-leishmanial agent. Also it would be very interesting to isolate or synthesize structurally related compounds in order to establish structure- activity relationships.

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