

Extracts of *Artemisia annua* leaves and seeds mediate programmed cell death in *Leishmania donovani*

Mohammad Islamuddin,¹ Abdullah Farooque,² B. S. Dwarakanath,² Dinkar Sahal³ and Farhat Afrin¹

Correspondence

Farhat Afrin

farhatafrin@jamiahamdard.ac.in or

afrin_farhat@yahoo.co.in

¹Parasite Immunology Lab., Department of Biotechnology, Jamia Hamdard (Hamdard University), New Delhi 110 062, India

²Institute of Nuclear Medicine and Allied Sciences, Timarpur, Delhi 110 054, India

³International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India

Leishmaniasis is one of the major tropical parasitic diseases, and the condition ranges in severity from self-healing cutaneous lesions to fatal visceral manifestations. There is no vaccine available against visceral leishmaniasis (VL) (also known as kala-azar in India), and current antileishmanial drugs face major drawbacks, including drug resistance, variable efficacy, toxicity and parenteral administration. We report here that n-hexane fractions of *Artemisia annua* leaves (AAL) and seeds (AAS) possess significant antileishmanial activity against *Leishmania donovani* promastigotes, with GI₅₀ of 14.4 and 14.6 µg ml⁻¹, respectively, and the IC₅₀ against intracellular amastigotes was found to be 6.6 and 5.05 µg ml⁻¹, respectively. Changes in the morphology of promastigotes and growth reversibility analysis following treatment confirmed the leishmanicidal effect of the active fractions, which presented no cytotoxic effect on mammalian cells. The antileishmanial activity was mediated via apoptosis, as evidenced by externalization of phosphatidylserine, *in situ* labelling of DNA fragments by terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) and cell-cycle arrest at the sub-G₀/G₁ phase. High-performance thin-layer chromatography (HPTLC) fingerprinting showed that the content of artemisinin in crude bioactive extracts (~1.4 µg per 100 µg n-hexane fraction) was too low to account for the observed antileishmanial activity. Characterization of the active constituents by GC-MS showed that α-amyrinyl acetate, β-amyrine and derivatives of artemisinin were the major constituents in AAL and cetin, EINECS 211-126-2 and artemisinin derivatives in AAS. Our findings indicate the presence of antileishmanial compounds besides artemisinin in the n-hexane fractions of *A. annua* leaves and seeds.

Received 2 July 2012

Accepted 31 August 2012

INTRODUCTION

Leishmaniasis, a complex spectrum of diseases, is currently a global health problem, occurring in cutaneous, mucocutaneous or visceral forms. Visceral leishmaniasis (VL) or kala-azar, caused by *Leishmania donovani*, is often associated with marked suppression of the host's cell-mediated immune response, leading to severe morbidity and mortality if left untreated (Haldar *et al.*, 1983). VL is also considered as an opportunistic infection among immunocompromised

patients around the world (Albrecht *et al.*, 1996). Treatment of VL relies mainly on pentavalent antimonials and second-line drugs such as amphotericin B and pentamidine. However, resistance to antimonial chemotherapy, coupled with its high cost, toxicity and parenteral route of administration, is a matter of great concern in endemic regions of Bihar, India (Sundar *et al.*, 2000). In view of the ongoing challenge to introduce new antileishmanial drugs, traditional medicinal plants are anticipated to provide valuable leads (Ahua *et al.*, 2007; Sarkar *et al.*, 2008; Misra *et al.*, 2009; Mokoka *et al.*, 2011). According to the World Health Organization, approximately 80% of the world's inhabitants rely on traditional medicines for their healthcare (WHO, 2000). *Artemisia annua* L. (Asteraceae), a traditional medicinal plant, has been used extensively as an antimalarial (Willcox *et al.*, 2006; Atemnkeng *et al.*, 2009) and anticancer agent. The efficacy of artemisinin (one of the constituents of

Abbreviations: AAL, n-hexane fraction of *Artemisia annua* leaves; AAS, n-hexane fraction of *A. annua* seeds; FITC, fluorescein isothiocyanate; PCD, programmed cell death; PI, propidium iodide; PS, phosphatidylserine; TdT, terminal deoxynucleotidyltransferase; TUNEL, TdT-mediated dUTP nick end labelling; VL, visceral leishmaniasis.

Two supplementary tables are available with the online version of this paper.

A. annua, *A. indica* and *A. dracuncululus*) against promastigotes and amastigotes of *L. donovani* was reported by Sen *et al.* (2007). The antileishmanial activity of ethanolic extracts of leaves of *A. indica* was investigated in exponential-phase promastigotes from six strains responsible for cutaneous, mucocutaneous or visceral leishmaniasis and the IC₅₀ ranged from 210 to 580 µg ml⁻¹ (Ganguly *et al.*, 2006). The action of flavonoids from *A. annua* as antioxidants and their potential synergy with artemisinin against malaria and cancer have been observed by Ferreira *et al.* (2010). In this study, we delineated the putative mechanisms that contribute to the leishmanicidal activity of bioactive fractions from *A. annua* leaves and seeds with the aim of developing an effective herbal remedy for the treatment of VL that may complement or synergize with conventional drugs against VL.

METHODS

Materials. M199 medium, propidium iodide (PI) and RNase were obtained from Sigma-Aldrich, fetal bovine serum (FBS) from Gibco-BRL, DMSO from SRL and methanol from Merck. Annexin V–fluorescein isothiocyanate (FITC) and ApoDirect kits were from Roche Inc. All other chemicals were from Sigma-Aldrich unless stated.

Parasite culture. *L. donovani* strain AG83 was a kind gift from Dr Nahid Ali (IICB, Kolkata, India) and was maintained *in vivo* in BALB/c mice. Promastigotes were maintained in medium M199 supplemented with 10% heat-inactivated FBS, supplemented with (ml⁻¹) 100 U sodium penicillin G and 100 µg streptomycin sulfate, at 22 °C, and subcultured every 72 h in the same medium at a mean density of 2 × 10⁶ cells ml⁻¹ (Afrin *et al.*, 2002).

Plant material and extraction. Fresh *A. annua* leaves and dried seeds with floral parts were collected from the Herbal Garden of Jamia Hamdard, washed and air-dried in the shade at room temperature. The dried plant materials were ground separately in an electric grinder. Powdered leaves and seeds were extracted with solvents of increasing polarity (n-hexane, ethanol and water). The residue from the n-hexane extraction was dried and extracted successively with ethanol and water. All the fractions were concentrated to dryness under reduced pressure at 35 °C using a rotary evaporator. The semisolid paste from the organic solvents was further concentrated to dryness in a vacuum desiccator. The aqueous extract was lyophilized, and all the extracts were stored at -20 °C until use. Stock solutions of 25 mg ml⁻¹ were prepared aseptically in DMSO (cell culture grade), except the aqueous extract (which was prepared in 20 mM PBS, pH 7.2), and diluted further in culture medium (1 mg ml⁻¹) to achieve a final DMSO concentration not exceeding 0.2%.

Growth kinetics assay. Promastigotes of *L. donovani* AG83 (2 × 10⁶ cells ml⁻¹) were incubated in the presence of test extracts (n-hexane, ethanol and aqueous extracts of *A. annua* leaves and seeds), artemisinin and artesunate in M199 containing 10% FBS (complete medium) at a concentration of 100 µg ml⁻¹. Pentamidine (100 µg ml⁻¹) served as the reference drug, while 0.2% DMSO, which represented the highest concentration in the test extracts, was used as a solvent control. Parasites in medium alone were taken as a control. Viable parasites were enumerated for 7 days using a phase-contrast microscope under a 40 × objective (Afrin *et al.*, 2001).

Reversibility assay. To confirm the leishmanicidal effect of active fractions of *A. annua* leaves and seeds, treated and untreated parasites after 7 days of incubation were washed twice with incomplete

medium and finally resuspended in complete medium and cultured at 22 °C for a further 96 h. The viability of the parasites was ascertained microscopically (Afrin *et al.*, 2001).

Analysis of cellular morphology. Morphology of the promastigotes was evaluated after 96 h of treatment with the test extracts, pentamidine or 0.2% DMSO and photomicrographs were taken at ×400 magnification under phase-contrast microscopy (Dutta *et al.*, 2007b).

Determination of GI₅₀. To determine the GI₅₀ (concentration of extract that inhibited growth of parasites by 50%), promastigotes at a density of 2 × 10⁶ cells ml⁻¹ were incubated in triplicate with or without the most active fractions of n-hexane extracts of *A. annua* leaves (AAL) and seeds (AAS) at serial threefold dilutions starting from 100 µg ml⁻¹ for 96 h. Pentamidine served as the reference drug and artemisinin as a known bioactive compound of *A. annua*.

Antileishmanial activity in an ex-vivo macrophage–amastigote model. To evaluate the effects of AAL and AAS on intracellular *L. donovani* amastigote forms, macrophages from the J774 cell line (2.5 × 10⁵ cells ml⁻¹) were allowed to adhere to glass coverslips in RPMI 1640 medium supplemented with 10% FBS and cultured for 12 h at 37 °C in 5% CO₂. Adherent macrophages were infected with stationary-growth-phase promastigotes using a cell to parasite ratio of 1:10 and incubated at 37 °C for 12 h. Subsequently, non-phagocytosed parasites were removed by gentle washing, and infected macrophages were incubated with AAL and AAS (0–100 µg ml⁻¹) for an additional 48 h. The coverslips were fixed in methanol and Giemsa-stained for microscopic evaluation of amastigote viability. At least 100 macrophages per coverslip were analysed, and the concentration that decreased growth of amastigotes by 50% (IC₅₀) was determined (Dutta *et al.*, 2008).

Visualization of phosphatidylserine (PS) exposure by annexin V–FITC/PI binding of cells. Externalization of PS in the outer membrane of extract-treated promastigotes was measured by double staining for annexin V and PI according to Paris *et al.* (2004) with minor modifications. Briefly, exponential-phase promastigotes (2 × 10⁶ cells ml⁻¹) were incubated with AAL or AAS (the most active fractions) at a concentration of 100 µg ml⁻¹ for 72 h. Pentamidine served as the reference drug, while parasites without any treatment were taken as a control. Untreated and treated parasites were washed twice in cold PBS and centrifuged at 14 000 g for 10 min. The pellet was resuspended in annexin V–FLUOS labelling solution (100 µl, containing both annexin V and PI), according to the manufacturer's instructions. After 15 min of incubation in the dark at 26 °C, 400 µl incubation buffer was added and mixed and the fluorescence signal was acquired using a BD LSR-II flow cytometer and analysed using FLOWJO software.

In a parallel experiment, promastigotes stained with annexin V–FITC and PI were analysed using a fluorescence microscope (not shown).

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay. DNA fragmentation was detected by incorporating FITC–dUTP catalytically at the 3'-hydroxy end of the fragmented DNA using an *in situ* cell-death detection kit (Roche Inc.) according to the manufacturer's instructions. Briefly, exponential-phase promastigotes (2 × 10⁶ cells ml⁻¹) were incubated for 72 h with 100 µg AAL or AAS or pentamidine (positive control) ml⁻¹ or with medium alone (parasite control) or 0.2% DMSO (solvent control). After 72 h, the cells were washed, fixed with 4% paraformaldehyde on ice for 1 h, washed with PBS and incubated with 3% H₂O₂ in methanol for 10 min at 25 °C. This was followed by washing with PBS, and the cells were then permeabilized with freshly prepared, chilled 0.1% Triton X-100 for 5 min on ice. Cells were washed twice with PBS, after which 50 µl reaction mixture containing terminal deoxynucleotidyltransferase (TdT) and FLUOS-labelled dUTP was added and the mixture was

incubated for 1 h at 37 °C, washed and finally resuspended in PBS for acquisition in a BD LSR-II flow cytometer and subsequent analysis using FLOWJO software. Histogram analysis of FL-1H (x-axis; FITC fluorescence) was recorded to show the shift in fluorescence intensity compared with the unstained cell population used as a negative control (Dutta *et al.*, 2007a).

In a parallel experiment, stained samples were visualized under a high-resolution fluorescence microscope, and images were captured and processed (Misra *et al.*, 2008).

Cell cycle analysis. The potency of the extracts for damaging DNA was detected through flow cytometry by PI staining, which binds DNA stoichiometrically (Dutta *et al.*, 2007b). Exponential-phase promastigotes (2×10^6 cells ml⁻¹) were treated with AAL or AAS (100 µg ml⁻¹) for 72 h at 22 °C, washed twice with PBS, fixed with 80% chilled ethanol and kept at 4 °C for 24 h. The cells were then washed with PBS and the pellet was resuspended in 500 µl DNase-free RNase (200 µg ml⁻¹) for 1 h at 37 °C. The cells were stained with PI (50 µg ml⁻¹) and incubated in the dark for 20 min at 4 °C. Data acquisition was carried out using a BD LSR-II flow cytometer and analysed using FLOWJO software.

Cytotoxicity to mammalian cells. Macrophages were collected by peritoneal lavage from starch-stimulated mice. Peritoneal cells were collected in RPMI 1640 medium (incomplete), pelleted by centrifugation at 2500 r.p.m. for 10 min at 4 °C, washed twice and finally resuspended in complete medium. Macrophages at a cell density of 8×10^5 ml⁻¹ were incubated with AAL or AAS at varying concentrations for 48 h in a CO₂ incubator (5% CO₂, 37 °C). Pentamidine as a reference drug and 0.2% DMSO as a solvent control (which represented the highest concentration of DMSO required to dissolve the test extracts) were also used. Macrophages without any treatment were taken as a control. Cells were observed under a phase-contrast microscope and viability was ascertained after trypan blue staining.

Phytochemical estimation of extracts by high-performance thin-layer chromatography (HPTLC). The artemisinin content in the different fractions (n-hexane, ethanol and aqueous) of *A. annua* leaves and seeds was analysed by HPTLC (Widmer *et al.*, 2007). Different fractions were applied to a silica-coated TLC plate (10 × 20 cm). The plate was air-dried and developed with benzene/ethyl acetate/glacial acetic acid (9:0.8:0.2) as the mobile phase in a Camag twin-trough chamber. Standard artemisinin was used to generate a calibration curve for quantification. Densitometric determination of artemisinin was carried out after derivatization with anisaldehyde reagent. Scanning and quantification of spots were performed at 450 nm in absorbance/reflectance mode with a Camag TLC scanner 3 using Win CATS 1.3.2 software.

GC-MS analysis of AAL and AAS. The chemical composition was analysed and active compounds from AAL and AAS were characterized by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu QP2010 with a DB-5 column (30 m, film 0.25 µm, ID 0.25 mm). The temperature of the column was increased programmatically from 45 to 270 °C at 5 °C min⁻¹, and the injector or detector temperature for the analysis was about 250 °C. Helium was used as the carrier gas at a flow rate of 1.5 ml min⁻¹. The mass spectrometer was operated in electron impact ionization mode at 70 eV. Identification of chemical constituents was based on matching recorded mass spectra with those obtained from the WILEY8.LIB and NIST08.LIB library spectra provided with the software of the GC-MS system.

Statistical analysis. Antileishmanial activity against promastigotes and amastigotes was expressed as GI₅₀ and IC₅₀, respectively, following linear regression analysis. All values reported are

means ± SEM of samples in triplicate and are from one of three independent experiments. $P \leq 0.05$ were considered significant.

RESULTS

In vitro leishmanicidal activity of test extracts by growth kinetics assay

All fractions of *A. annua* leaves and seeds were assayed for their cytotoxicity against *L. donovani* promastigotes. The test extracts exhibited time-dependent killing of promastigotes at a concentration of 100 µg ml⁻¹. The n-hexane extracts of seeds (AAS) and leaves (AAL) exhibited the highest cytotoxicity. No viable parasites were observed after 2, 3 or 5 days of incubation with pentamidine, AAS and AAL, respectively. In the case of artemisinin (100 µg ml⁻¹), the growth of parasites initially slowed down, but eventually increased after 96 h compared with the control. No cell death was observed in artesunate or the solvent control (Fig. 1). Untreated parasites also proliferated at a normal rate.

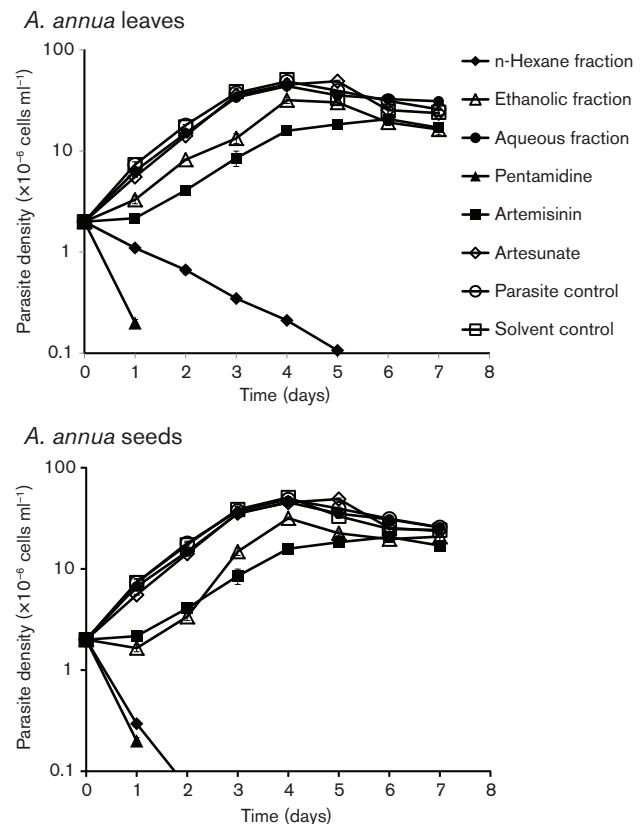


Fig. 1. Analysis of antipromastigote activity of *A. annua* leaf and seed fractions. Exponential-phase promastigotes (2×10^6 cells ml⁻¹) of *L. donovani* were incubated with 100 µg each fraction ml⁻¹ for different times, as described in Methods.

Alteration of cellular morphology of extract-treated promastigotes

Visual inspection by phase-contrast microscopy revealed the onset of cell shrinkage and cytoplasmic condensation upon treatment of promastigotes with AAL or AAS ($100 \mu\text{g ml}^{-1}$) (Fig. 2), marked by complete circularization of almost all cells at 96 h post-treatment. Similar morphological changes were observed in pentamidine-treated parasites. Microscopic study indicated that the effect was leishmanicidal rather than leishmanistatic.

Growth reversibility assay of extract-treated promastigotes

To confirm the lethal effect of AAL and AAS for promastigotes, treated and untreated parasites (from the growth kinetics study) were washed and resuspended in fresh medium and their viability was ascertained microscopically after 96 h of incubation. No viable parasites were detected after incubation with AAL or AAS, as was also observed with pentamidine, confirming their leishmanicidal effect. Artemisinin-treated parasites reverted to late-exponential phase and resembled untreated, control parasites (Fig. 3).

Evaluation of the GI_{50} of n-hexane fractions against promastigotes

The viability of promastigotes after treatment with AAL or AAS was evaluated using a modified GI_{50} assay. Treatment of promastigotes with n-hexane fractions demonstrated dose-dependent inhibition of parasite growth, with GI_{50} achieved at 14.4 and $14.6 \mu\text{g ml}^{-1}$ for AAL and AAS, respectively. The established antileishmanial drug pentamidine, used as a positive control, showed a similar trend in dose-dependent parasite killing, with a GI_{50} of $1.1 \mu\text{g ml}^{-1}$ (Fig. 4). Exposure to DMSO (0.2%), representing the highest concentration of diluent present in the test extracts ($100 \mu\text{g ml}^{-1}$), showed no loss of parasite viability.

Antiamastigote activity of AAL and AAS

In *Leishmania* infection, the promastigotes transform into amastigotes within the phagolysosomal vacuoles of macrophages. Accordingly, the antileishmanial activity of AAL and AAS was tested against intracellular amastigotes in *L. donovani*-infected J774 macrophages. Giemsa-stained coverslips were analysed microscopically for the presence of phagocytosed amastigotes within the macrophages. The IC_{50} of AAL and AAS for *L. donovani*

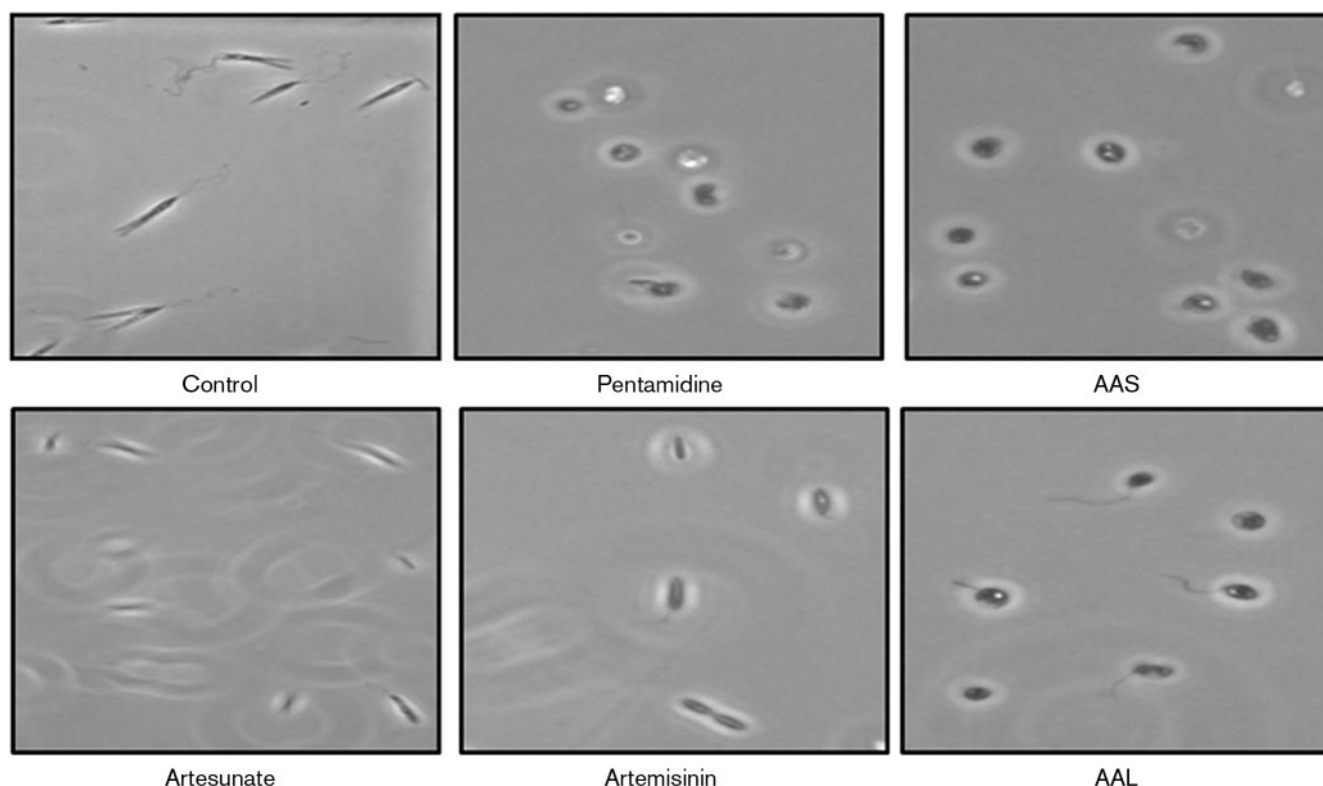


Fig. 2. Analysis of cellular morphology of AAL- and AAS-treated promastigotes. Exponential-phase promastigotes (2×10^6 cells ml^{-1}) were incubated with $100 \mu\text{g}$ AAL or AAS ml^{-1} for 96 h and analysed by light microscopy ($\times 400$ magnification), as described in Methods.

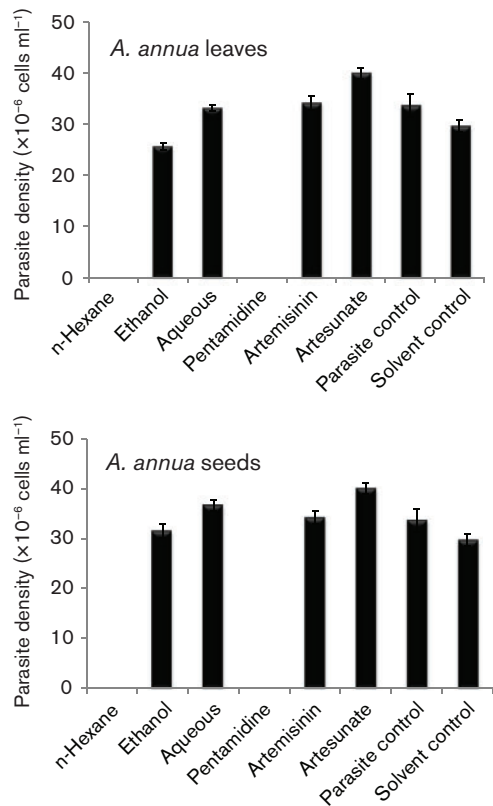


Fig. 3. Reversibility analysis of test extract-treated promastigotes revealed no reversion of growth in AAL-, AAS- or pentamidine-treated samples.

amastigotes was found to be 6.6 and 5.05 $\mu\text{g ml}^{-1}$, respectively, close to the IC_{50} of pentamidine (1 $\mu\text{g ml}^{-1}$) (Fig. 5).

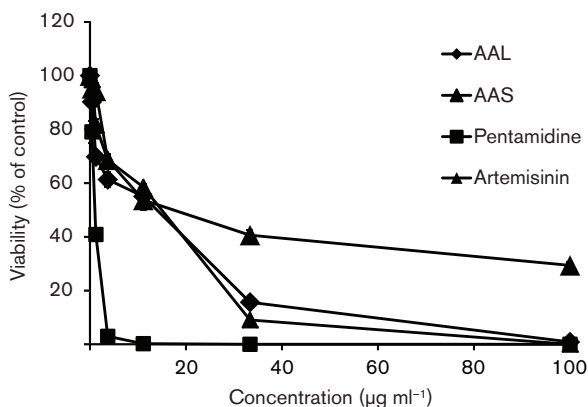


Fig. 4. Estimation of GI_{50} of AAL and AAS against promastigotes. Parasites (2×10^6 cells ml^{-1}) were incubated with serial threefold dilutions of AAL and AAS (starting at 100 $\mu\text{g ml}^{-1}$) for 96 h and viability was determined microscopically, as described in Methods.

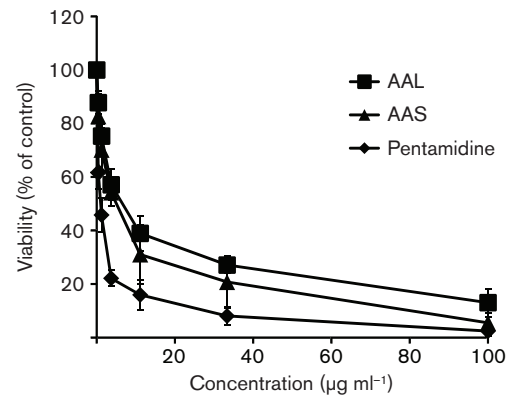


Fig. 5. Effects of AAL and AAS on *L. donovani* intracellular amastigote forms. After infection with promastigotes, J774 macrophages were incubated with serial threefold dilutions of AAL and AAS (starting at 100 $\mu\text{g ml}^{-1}$) for 48 h and viability was determined microscopically, as described in Methods.

Externalization of PS in promastigotes

In metazoan and unicellular cells, translocation of PS from the inner to the outer leaflet of the plasma membrane occurs during programmed cell death (PCD) (Mehta & Shaha, 2004; Sudhandiran & Shaha, 2003; Koonin & Aravind, 2002). Annexin V, a Ca^{2+} -dependent phospholipid-binding protein with affinity for PS, is used routinely to label its externalization. Since annexin V can also label necrotic cells following the loss of membrane integrity, simultaneous addition of PI, which does not permeate cells with an intact plasma membrane, allows discrimination between apoptotic cells (annexin V⁺, PI⁻), secondary necrotic cells (annexin V⁺, PI⁺), necrotic cells (annexin V⁻, PI⁺) and live cells (annexin V⁻, PI⁻). Accordingly, in order to study whether the mechanism of cell death triggered by AAL and AAS was via apoptosis or necrosis, treated and untreated promastigotes were double-stained with FLUOS-conjugated annexin V and PI. Significant proportions of promastigotes treated with AAL and AAS (3.1 and 6.6 %, respectively) stained positive for annexin V, compared with only 0.4 % in artemisinin-treated and untreated cells (Fig. 6). This was comparable to the level of apoptosis triggered by pentamidine (3.2 %), which served as a positive control, indicating that the leishmanicidal activity of AAL and AAS is via apoptosis.

Nuclear DNA fragmentation in AAS- and AAL-treated promastigotes by *in situ* labelling of DNA fragments via TUNEL assay

One of the hallmarks of apoptotic cell death is the degradation of nuclear DNA into oligonucleosomal units. DNA nicking resulting from exposure to AAL and AAS was detected using a TUNEL assay, in which the proportion of DNA nicks was quantified by measuring the binding of dUTP-FLUOS to the nicked end via TdT. Promastigotes

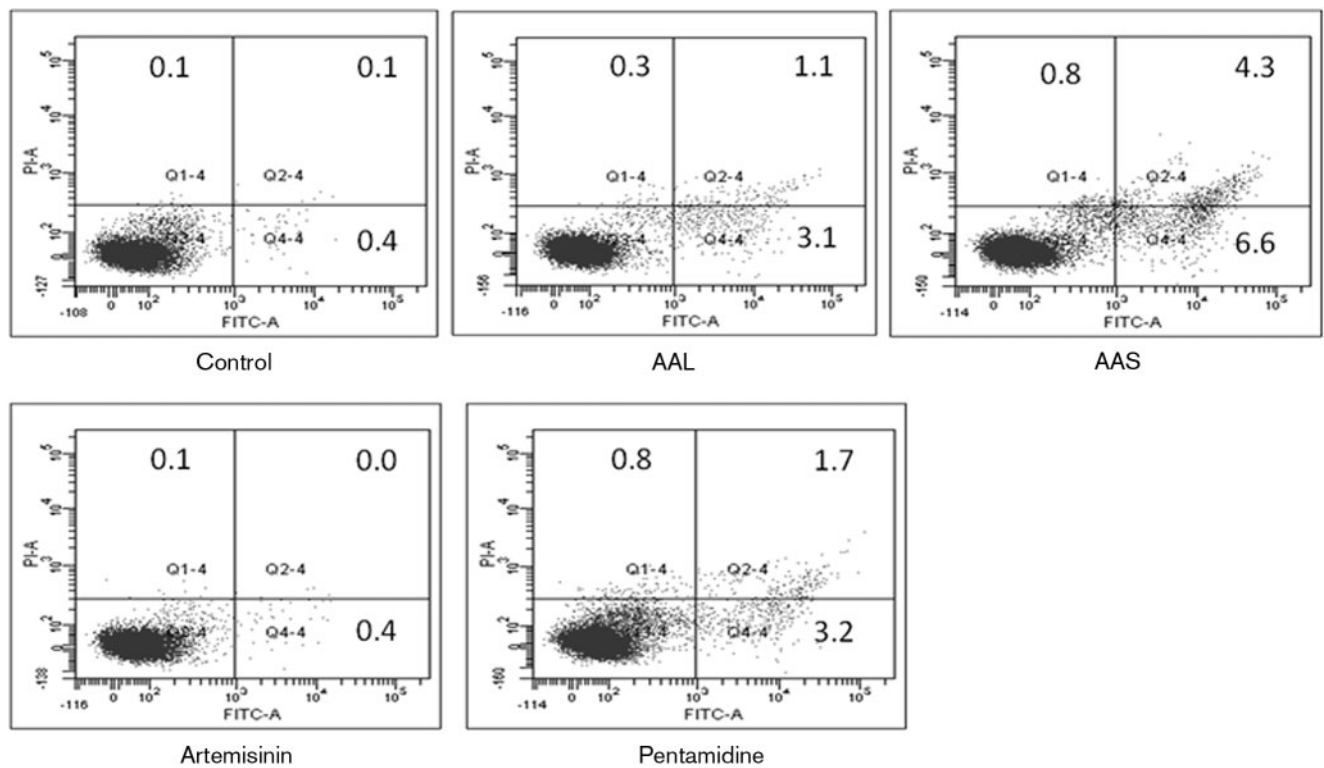


Fig. 6. Externalization of PS in AAL- and AAS-treated promastigotes. Parasites were incubated with AAL, AAS, artemisinin or pentamidine ($100 \mu\text{g ml}^{-1}$) for 72 h, co-stained with PI and annexin V-FITC and analysed by flow cytometry as described in Methods. A representative profile of three independent experiments is shown.

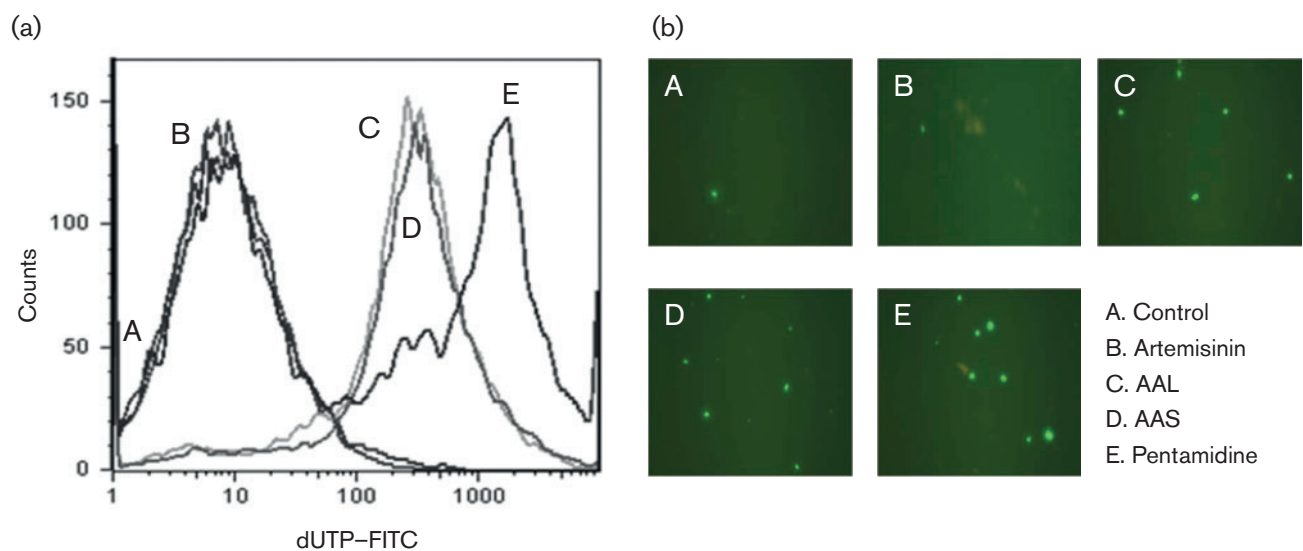


Fig. 7. Analysis of TUNEL positivity in AAL- and AAS-treated promastigotes. Exponential-phase promastigotes were incubated with AAL or AAS ($100 \mu\text{g ml}^{-1}$) for 72 h. Cells were fixed, stained with dUTP-FITC in the presence of TdT and analysed by flow cytometry (a) or observed under a fluorescence microscope (b), as described in Methods.

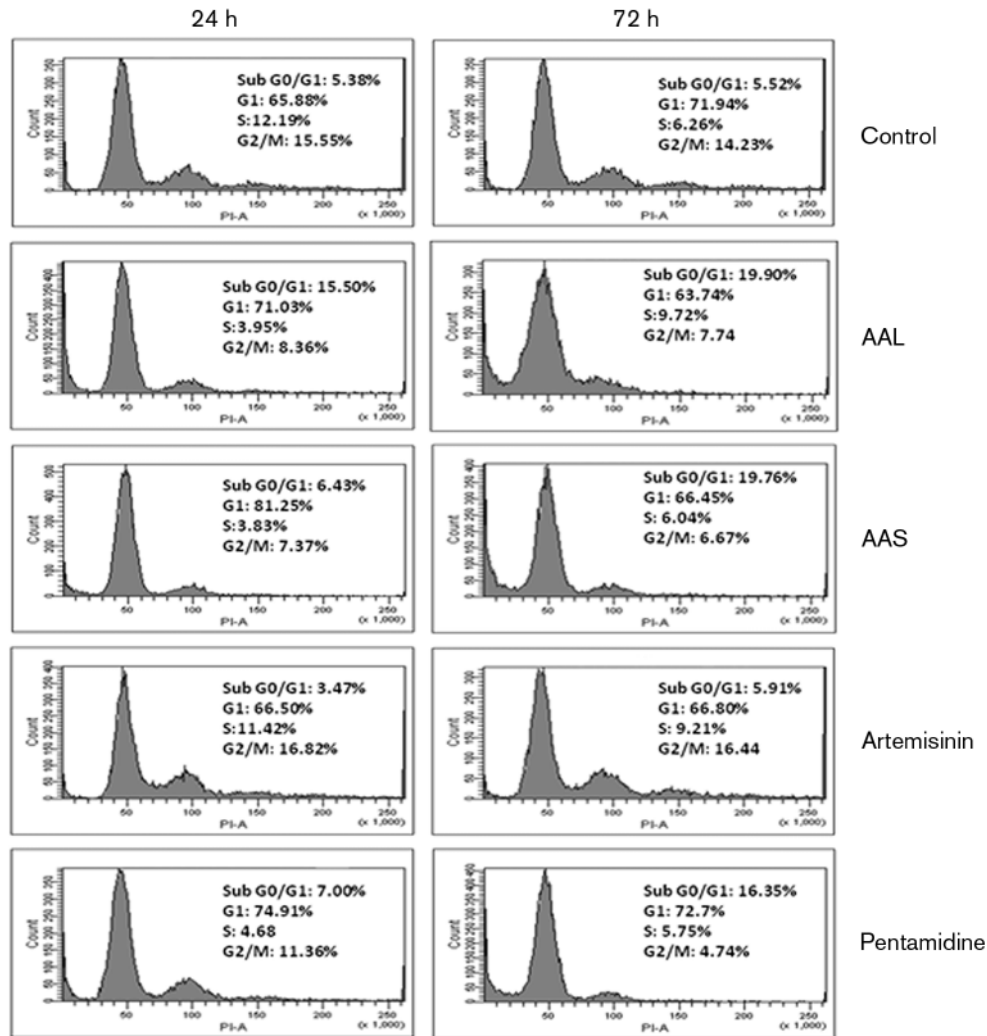


Fig. 8. Analysis of the cell-cycle status of AAL- and AAS-treated promastigotes. Exponential-phase promastigotes were incubated with AAL or AAS ($100 \mu\text{g ml}^{-1}$) for 24 or 72 h, fixed in chilled methanol and probed with PI and fluorescence signal was acquired using a LSR-II flow cytometer for subsequent analysis with FLOWJO software, as described in Methods.

treated with $100 \mu\text{g AAL or AAS ml}^{-1}$ for 72 h appeared yellowish green under the fluorescence microscope, representing incorporation of dUTP labelled with FLUOS, indicating nicking of DNA (Fig. 7b).

The occurrence of DNA nicking was also detected by quantifying the binding of dUTP-FLUOS to the nicked end via TdT, as the proportion of DNA nicks is directly proportional to the fluorescence obtained from dUTP-FLUOS. Treated promastigotes after 72 h showed an increase in nuclear DNA fragmentation, as evidenced by dUTP-FLUOS binding. The mean fluorescence intensity of untreated (control) and treated (AAL, AAS, artemisinin and pentamidine) fractions was found to be 611, 13083, 15296, 433 and 24289, respectively (Fig. 7a), providing strong evidence for apoptosis in *L. donovani* promastigotes.

AAL and AAS induced apoptosis, causing an increase in the sub-G₀/G₁ population

Flow-cytometric analysis after cell permeabilization and labelling with PI was used to quantify the percentage of pseudohypodiploid cells. In a given cell, the amount of PI correlates with the DNA content and, accordingly, DNA fragmentation in apoptotic cells translates into a sub-G₀/G₁ peak (Sarkar *et al.*, 2008). Promastigotes treated with AAL or AAS ($100 \mu\text{g ml}^{-1}$) for 24 or 72 h caused cell-cycle arrest at sub-G₀/G₁ phase, as analysed by flow cytometry (Fig. 8). The proportion of cells in the sub-G₀/G₁ phase was 5.52% in the control, which increased to 19.90, 19.76 and 16.35%, respectively, in promastigotes treated with AAL, AAS and pentamidine. Only a slight increase (5.91%) in the sub-G₀/G₁ cell population was observed upon treatment with artemisinin.

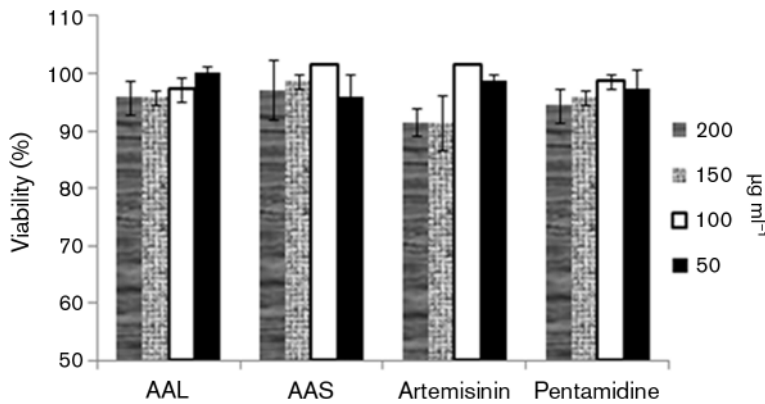


Fig. 9. Absence of adverse toxicity of test extracts on mammalian macrophages. Macrophages from the peritoneal cavity of mice were incubated for 72 h at 37 °C in a CO₂ incubator with increasing concentrations of test extracts or pentamidine and viability was ascertained.

This increase in the proportion of cells in the sub-G₀/G₁ phase corroborated our findings that AAL and AAS induced apoptosis in promastigotes, causing DNA fragmentation.

Cytotoxicity of bioactive fractions on mammalian macrophages

Peritoneal macrophages were isolated from mice to check for any adverse side effects of the bioactive fractions, using pentamidine as the reference drug. The cytotoxicity assay revealed that there was no adverse toxicity of AAL or AAS, even at 200 µg ml⁻¹, on mammalian macrophages (Fig. 9).

Determination of artemisinin content in AAL and AAS by HPTLC fingerprinting

The artemisinin content in AAL and AAS was quantified by HPTLC fingerprinting. The content of artemisinin in 100 µg AAL and AAS was 1.447 and 1.336 µg, respectively (Table S1, available in JMM Online).

Composition of AAL and AAS

The major chemical constituents of AAL and AAS that may have contributed to their leishmanicidal activity were identified by GC-MS analysis. α-Amyrinyl acetate

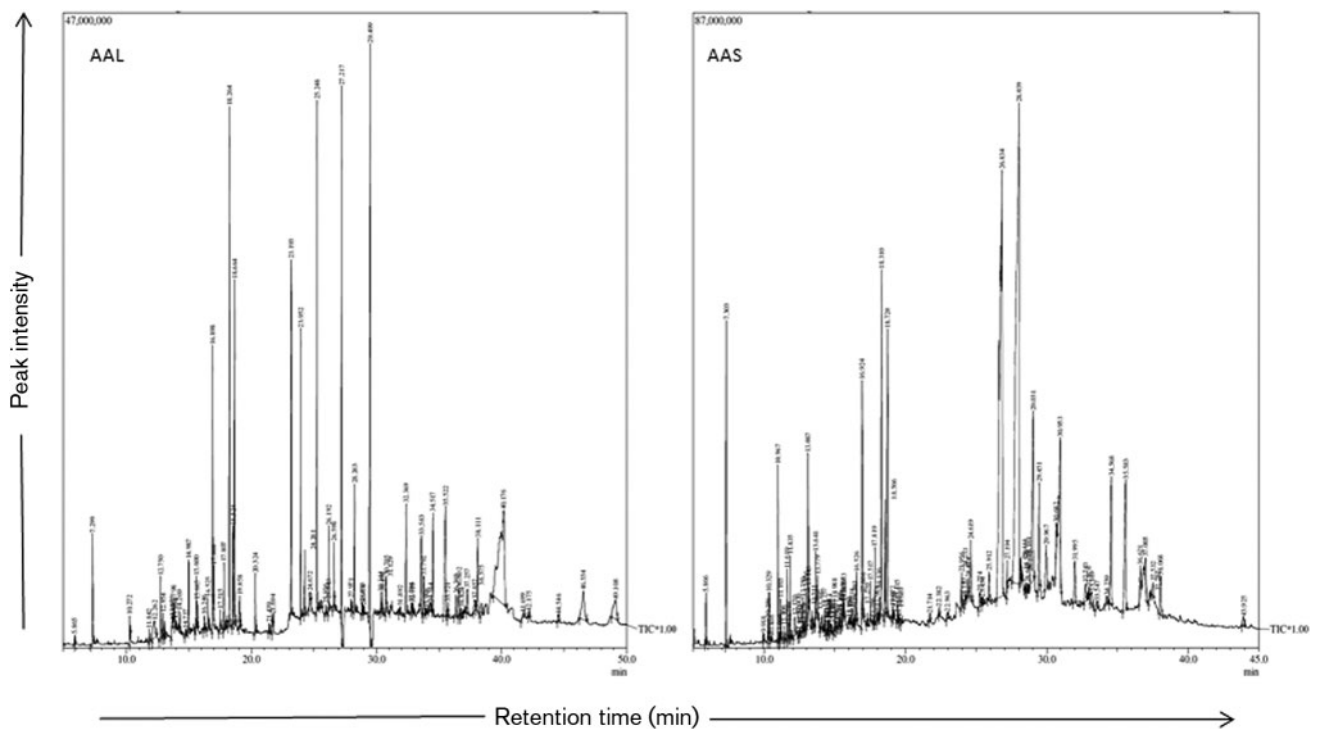


Fig. 10. Chromatogram of GC-MS analysis of AAL and AAS. GC-MS analysis was performed and chemical constituents were identified as described in Methods.

(28.17%), β -amyrine (20.87%), dihydroartemisinin 3-deoxy (4.91%), deoxyartemisinin (4.29%) and arteannuin (1.34%) were predominant in AAL, while cetin (15.23%), EINECS 211-126-2 (9.97%), dihydroartemisinin 3-deoxy (7.77%), deoxyartemisinin (5.15%) and arteannuin (1.45%) were revealed in AAS by comparison of mass spectral data and retention times (Fig. 10; Table S2).

DISCUSSION

The leishmanicidal activities of miltefosine (Verma & Dey, 2004), amphotericin B (Lee *et al.*, 2002) and camptothecin (Sen *et al.*, 2004) have been reported to be mediated by apoptosis. The efficacy of artemisinin against promastigotes of *L. donovani* was reported by Sen *et al.* (2007). However, in our study, we have shown that artemisinin and artesunate exhibit partial growth-inhibitory activity against *L. donovani* promastigotes at a concentration of 100 $\mu\text{g ml}^{-1}$, in contrast to the significant leishmanicidal effect mediated by n-hexane fractions of *A. annua* leaves (AAL) and seeds (AAS). To elucidate the mode of action of AAL and AAS against promastigotes of *L. donovani*, we used biochemical and morphological approaches and demonstrated that AAL and AAS induced PCD that shares several phenotypic features with metazoan apoptosis, as also reported by Debrabant *et al.* (2003). These apoptotic correlates include PS exposure, DNA nicking as evidenced by TUNEL assay and cell-cycle arrest at sub-G₀/G₁ phase. Taken together, we have demonstrated conclusively that AAL and AAS induce PCD in promastigotes that notably shares some, but not all, of the classical features of apoptosis observed in the higher eukaryotes. The bioactive fractions (AAL and AAS) had no observable cytotoxicity on mammalian macrophages, even at 200 $\mu\text{g ml}^{-1}$.

HPTLC fingerprint profiling showed that the content of artemisinin in the bioactive fractions was too low (~1.4 μg per 100 μg n-hexane fraction) to account for the observed antileishmanial activity. GC-MS analysis revealed α -amyrinyl acetate, β -amyrine, dihydroartemisinin 3-deoxy, deoxyartemisinin and arteannuin as the major constituents in AAL, while cetin, EINECS 211-126-2, dihydroartemisinin 3-deoxy, deoxyartemisinin and arteannuin were predominant in AAS. Synthetic dihydroartemisinin acetal derivatives (dimer and trimer) have also been reported to exhibit antileishmanial, antimalarial, anticancer and antifungal activities (Slade *et al.*, 2009). Our studies suggest that the leishmanicidal activity of AAL may be attributed to the presence of α -amyrinyl acetate, β -amyrine and derivatives of artemisinin (dihydroartemisinin 3-deoxy, arteannuin and deoxyartemisinin), while cetin, EINECS 211-126-2 and the artemisinin derivatives may have contributed to the observed effect in AAS. Our findings demonstrate promising antileishmanial activity of AAL and AAS mediated by PCD and, accordingly, the active constituents may have potential as a source of novel agents for the treatment of leishmaniasis.

ACKNOWLEDGEMENTS

This work received financial assistance from the Department of Biotechnology (DBT), Government of India. M. I. is the recipient of a Senior Research Fellowship from the DBT.

REFERENCES

- Afrin, F., Dey, T., Anam, K. & Ali, N. (2001). Leishmanicidal activity of stearylamine-bearing liposomes *in vitro*. *J Parasitol* **87**, 188–193.
- Afrin, F., Rajesh, R., Anam, K., Gopinath, M., Pal, S. & Ali, N. (2002). Characterization of *Leishmania donovani* antigens encapsulated in liposomes that induce protective immunity in BALB/c mice. *Infect Immun* **70**, 6697–6706.
- Ahua, K. M., Ioset, J. R., Ioset, K. N., Diallo, D., Mauël, J. & Hostettmann, K. (2007). Antileishmanial activities associated with plants used in the Malian traditional medicine. *J Ethnopharmacol* **110**, 99–104.
- Albrecht, H., Sobottka, I., Emminger, C., Jablonowski, H., Just, G., Stoehr, A., Kubin, T., Salzberger, B., Lutz, T. & van Lunzen, J. (1996). Visceral leishmaniasis emerging as an important opportunistic infection in HIV-infected persons living in areas nonendemic for *Leishmania donovani*. *Arch Pathol Lab Med* **120**, 189–198.
- Atemnkeng, M. A., Chimanuka, B., Dejaegher, B., Heyden, Y. V. & Plaizier-Vercammen, J. (2009). Evaluation of *Artemisia annua* infusion efficacy for the treatment of malaria in *Plasmodium chabaudi* infected mice. *Exp Parasitol* **122**, 344–348.
- Debrabant, A., Lee, N., Bertholet, S., Duncan, R. & Nakhasi, H. L. (2003). Programmed cell death in trypanosomatids and other unicellular organisms. *Int J Parasitol* **33**, 257–267.
- Dutta, A., Bandyopadhyay, S., Mandal, C. & Chatterjee, M. (2007a). *Aloe vera* leaf exudate induces a caspase-independent cell death in *Leishmania donovani* promastigotes. *J Med Microbiol* **56**, 629–636.
- Dutta, A., Ghoshal, A., Mandal, D., Mondal, N. B., Banerjee, S., Sahu, N. P. & Mandal, C. (2007b). Racemoside A, an anti-leishmanial, water-soluble, natural steroidal saponin, induces programmed cell death in *Leishmania donovani*. *J Med Microbiol* **56**, 1196–1204.
- Dutta, A., Sarkar, D., Gurib-Fakim, A., Mandal, C. & Chatterjee, M. (2008). *In vitro* and *in vivo* activity of *Aloe vera* leaf exudate in experimental visceral leishmaniasis. *Parasitol Res* **102**, 1235–1242.
- Ferreira, J. F. S., Luthria, D. L., Sasaki, T. & Heyerick, A. (2010). Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* **15**, 3135–3170.
- Ganguly, S., Bandyopadhyay, S., Bera, A. & Chatterjee, M. (2006). Anti-promastigote activity of an ethanolic extract of leaves of *Artemisia indica*. *Indian J Pharmacol* **38**, 64–65.
- Haldar, J. P., Ghose, S. K., Saha, K. C. & Ghose, A. C. (1983). Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. *Infect Immun* **42**, 702–707.
- Koonin, E. V. & Aravind, L. (2002). Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death Differ* **9**, 394–404.
- Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R. & Nakhasi, H. L. (2002). Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell Death Differ* **9**, 53–64.
- Mehta, A. & Shaha, C. (2004). Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. *J Biol Chem* **279**, 11798–11813.
- Misra, P., Khaliq, T., Dixit, A., SenGupta, S., Samant, M., Kumari, S., Kumar, A., Kushawaha, P. K., Majumder, H. K. & other authors

(2008). Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dihydrate: an approach for rational drug design. *J Antimicrob Chemother* **62**, 998–1002.

Misra, P., Kumar, A., Khare, P., Gupta, S., Kumar, N. & Dube, A. (2009). Pro-apoptotic effect of the landrace Bangla Mahoba of *Piper betle* on *Leishmania donovani* may be due to the high content of eugenol. *J Med Microbiol* **58**, 1058–1066.

Mokoka, T. A., Zimmermann, S., Julianti, T., Hata, Y., Moodley, N., Cal, M., Adams, M., Kaiser, M., Brun, R. & other authors (2011). *In vitro* screening of traditional South African malaria remedies against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*. *Planta Med* **77**, 1663–1667.

Paris, C., Loiseau, P. M., Bories, C. & Bréard, J. (2004). Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* **48**, 852–859.

Sarkar, A., Sen, R., Saha, P., Ganguly, S., Mandal, G. & Chatterjee, M. (2008). An ethanolic extract of leaves of *Piper betle* (Paan) Linn mediates its antileishmanial activity via apoptosis. *Parasitol Res* **102**, 1249–1255.

Sen, N., Das, B. B., Ganguly, A., Mukherjee, T., Tripathi, G., Bandyopadhyay, S., Rakshit, S., Sen, T. & Majumder, H. K. (2004). Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Cell Death Differ* **11**, 924–936.

Sen, R., Bandyopadhyay, S., Dutta, A., Mandal, G., Ganguly, S., Saha, P. & Chatterjee, M. (2007). Artemisinin triggers induction of cell-cycle arrest and apoptosis in *Leishmania donovani* promastigotes. *J Med Microbiol* **56**, 1213–1218.

Slade, D., Galal, A. M., Gul, W., Radwan, M. M., Ahmed, S. A., Khan, S. I., Tekwani, B. L., Jacob, M. R., Ross, S. A. & Elsohly, M. A. (2009). Antiprotozoal, anticancer and antimicrobial activities of dihydroartemisinin acetal dimers and monomers. *Bioorg Med Chem* **17**, 7949–7957.

Sudhandiran, G. & Shaha, C. (2003). Antimonial-induced increase in intracellular Ca^{2+} through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. *J Biol Chem* **278**, 25120–25132.

Sundar, S., More, D. K., Singh, M. K., Singh, V. P., Sharma, S., Makharia, A., Kumar, P. C. & Murray, H. W. (2000). Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis* **31**, 1104–1107.

Verma, N. K. & Dey, C. S. (2004). Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother* **48**, 3010–3015.

WHO (2000). *Traditional and Modern Medicine: Harmonizing Two Approaches*. Report of the Consultation Meeting, 22–26 November 1999, Beijing, China. World Health Organization West Pacific Region.

Widmer, V., Handloser, D. & Reich, E. (2007). Quantitative HPTLC analysis of artemisinin in dried *Artemisia annua* L.: a practical approach. *J Liquid Chromatogr Relat Technol* **30**, 2209–2219.

Willcox, M., Falquet, J., Ferreira, J. F. S., Gilbert, B., Hsu, E., de Magalhães, P. M., Plaizier-Vercammen, J., Sharma, V. P., Wright, C. W., Yaode, W. & RITAM *Artemisia annua* Task Force (2006). *Artemisia annua* as a herbal tea for malaria. *Afr J Tradit Complement Altern Medicines* **4**, 121–123.