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ORIGINAL PAPER

Valeriana wallichii root extracts and fractions with activity against *Leishmania* spp

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Abstract Leishmanial diseases, posing a public health problem worldwide, are caused by *Leishmania* parasites with a dimorphic life cycle alternating between the promastigote and amastigote forms. Promastigotes transmitted by the vector are transformed into amastigotes residing in the host tissue macrophages. Presently, new antiparasitic agents are needed against *Leishmania donovani* and *Leishmania major*, the respective organisms causing visceral and cutaneous leishmaniasis, since the available treatments are unsatisfactory due to toxicity, high cost, and emerging drug resistance. Over the years, traditional medicinal flora throughout the world enriched the modern pharmacopeia. Hence, roots of 'Indian Valerian' (*Valeriana wallichii* DC) were studied for its antileishmanial activity for the first time. The methanol and chloroform extracts showed activity against *L. donovani* promastigotes and both promastigotes and amastigotes of *L. major*. The most active fraction, F3, obtained from the chloroform extract, showed IC_{50} at ~3–7 μg/ml against both the promastigotes and 0.3 μg/ml against *L. major* amastigotes. On investigation of the mechanism of cytotoxicity in *L. donovani* promastigotes, the 'hall-mark' events of morphological degeneration, DNA fragmentation, externalization of phosphatidyl serine, and mitochondrial membrane depolarization indicated that F3 could induce apoptotic death in leishmanial cells. Therefore, the present study revealed a novel and unconventional property of *V. wallichii* root as a prospective source of effective antileishmanial agents.

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Introduction

The leishmaniases are parasitic diseases caused by about 20 species of pathogenic protozoans belonging to the genus *Leishmania* and classified as one of the 'neglected tropical diseases' (NTD) with a huge global burden like any other serious ailment (Hotez and Pecoul 2010). At present, an estimated 12 million people in four continents, particularly the people living under low socio-economic conditions, are infected with this vector-borne kinetoplastid NTD with a wide range of clinical symptoms.

The dimorphic life cycle of *Leishmania* parasites alternates between the flagellated promastigotes, transmitted by phlebotomine sandfly vectors into the vertebrates, and the intracellular amastigotes formed in the mammalian host tissue. The clinical symptom of the disease is a consequence of the multiplication of the amastigotes inside the host macrophages. Thus, leishmaniasis is manifested as a visceral form (VL) in Brazil, Sudan, and the Indian subcontinent (where it is known as Kala-azar) and as the disfiguring cutaneous lesions (CL) which are endemic in some parts of South America, Africa, and the Middle-Eastern regions of Asia, while the mucocutaneous forms generally occur in South America only. Typically, *Leishmania donovani* and *Leishmania major* are the causative

organisms for VL and CL, respectively, together leading to nearly 2 million new cases (0.5 million for VL and 1.5 million for CL) per annum (WHO 2010). Furthermore, opportunistic co-infections with leishmaniases have occurred among the immuno-compromised people even from non-endemic areas around the world (Alvar et al. 1997).

The leishmanial diseases are problematic in terms of treatment. No vaccines are available, and the treatment depends on a limited range of drugs. Pentavalent antimonials are the oldest and foremost line of therapeutics, even with obvious drawbacks like prolonged intravenous regimen, toxicity, lack of efficacy, and high cost. However, the alternatives like pentamidine and amphotericin B also suffer from similar limitations. The situation is aggravated further due to the growing unresponsiveness of VL to the antimonials (Jha 2006). Even miltefosine, the newly introduced drug, has indicated potential resistance against VL (Sánchez-Cañete et al. 2009). Therefore, there is a desperate need to look for better drugs, which will be safer and less expensive. In fact, keeping in view the apparent lack of commercial interest to develop drugs for NTDs, a WHO manifesto has recently mandated an urgent action to find novel antikinetoplastid molecules (Hotez and Pecoul 2010). This situation prompted us to look for the presence of antileishmanial agents in medicinal plants possessing diverse biological activities and formulated as traditional remedies for various diseases (Harvey 1999).

Traditionally, medicinal plants found all over the world have taken care of health problems of the society at large and also furnished scientific leads for drug development. *Valeriana wallichii* DC (syn. *Valeriana jatamansi* Jones), also called 'Indian Valerian', is one such herbal ingredient of Oriental medicinal preparations widely accepted for the treatment of anxiety and emotional stress, insomnia, and nervous conditions. It is also known to be antispasmodic and stimulant and to improve liver function in gastrointestinal disorders (Nadkarni 1976). It is a member of the Valerianaceae family, consisting of about 200 species of *Valeriana* occurring throughout the world (Singh et al. 2006). Historically, in the Western cultures, too, Valerian roots were known as stimulant–tonic, antispasmodic, and calmative and used to alleviate sleep disorder, mental depression, hysteria, and low forms of fever. Therefore, considerable research was aimed at establishing the chemical and pharmacological basis of its sedative or tranquilizing function (Balderer and Borbely 1985; Leathwood and Chauffard 1985; Houghton 1999). However, no antiparasitic/cytotoxic activity of this plant has been reported so far, besides a few recent observations on its anticancer (Lin et al. 2009) and nematicidal (Kim et al. 2008) effects. During a routine screening of medicinal plant samples collected from different locations in India, *V. wallichii* root was tested for antileishmanial property, and it was found to inhibit the growth of both *L. donovani* and *L. major* parasites. In the present study, we describe a bioassay-guided analysis of the extracts leading to semi-pure 'active' fractions which showed marked cytotoxicity against the leishmanial cells, with evident morphological changes and induction of signaling events indicating apoptosis-like cell death. To the best of our knowledge, this is the first such study undertaken on *V. wallichii* roots.

Materials and methods

Plant material and preparation of extracts

Dried rhizomes were obtained from an herb shop in Kolkata, India, and identified as *V. wallichii* DC (syn. *V. jatamansi* Jones) roots at the Institute of Himalayan Bioresource Technology (IHBT), Palampur, Himachal Pradesh, India, through a morphological comparison with authentic reference samples. The voucher specimen of the herbarium collected from IHBT campus was preserved in our laboratory (IHBT Ref. No. 11666).

The rhizomes were pulverized, and three separate portions (10 g each) were refluxed for 2 h with 100 ml each of water, methanol, and chloroform, respectively, to obtain three extracts, viz., VwW, VwM, and VwC. The aqueous extract (VwW) was lyophilized, while the solvents were removed from VwM and VwC in vacuo. After thorough drying in vacuo, the yields (%*w*/*w*) for VwW, VwM, and VwC were recorded as 17.9%, 9.0%, and 1.1%, respectively, and preserved at 4°C. The process was repeated to get higher amounts of the extracts as per requirement of the experiments.

Bioassay-guided semi-purification of extracts for isolation of active fractions

The three extracts prepared from *V. wallichii* roots were subjected to assessment of antileishmanial activity (described below), and VwC was selected for further fractionation through preparative thin-layer chromatography. A saturated solution of VwC (120 mg) in dichloromethane was applied on glass plates (20×20 cm) coated with silica gel G (Merck, Mumbai, India) and chromatographed using a mixture of chloroform and methanol (4.8:0.2; *v*/*v*). Two distinct bands at R_f=0.7 and R_f=0.8, respectively, were observed, which were eluted with ethyl acetate to collect the fractions F2 (dark brown, 15 mg) and F3 (bright yellow, 37 mg).

In a separate experiment, VwM was subjected to a fractionation using preparative HPLC. For this purpose, the extract (about 85 mg) was dissolved in a mixture of methanol and water (3:1) and subjected to a ZORBAX Eclipse XDB-C18 column (150×4.6 mm; 5 μm—Agilent,

Waldbronn, Germany) on a Agilent 1100 HPLC System (Agilent, Waldbronn, Germany). Eleven fractions were achieved by using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) in conjunction with elution conditions of 10–90% B in 30 min by a flow of 1 ml/min.

Preparation of test samples for biological studies

The extracts (VwW, VwM, VwC), as well as the fractions, were dissolved aseptically in dimethyl sulfoxide (DMSO; analytical grade) to get the respective stock solutions. The final concentration of DMSO in the culture was adjusted to 0.1% (*v*/*v*), and cells treated similarly with DMSO alone were used as control.

Parasite culture and maintenance

An authentic strain of *L. donovani* promastigote (MHOM/IN/1983/AG83) was obtained from the Indian Institute of Chemical Biology, Kolkata, India (Mukherjee et al. 2009). Promastigotes were cultured under aseptic conditions in a BOD chamber (22±2°C) in Schneider's insect medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with streptomycin (150 μg/ml), penicillin G (100 μg/ml), gentamicin (150 μg/ml), and 10% heat-inactivated fetal bovine serum (HyClone, Logan, USA) at pH 7.2.

Studies on *L. major* were done at Würzburg. The cloned virulent *L. major* isolate (strain: MHOM/IL/81/FE/BNI) was maintained by passage in BALB/c mice. Promastigotes were grown in blood agar cultures at 27°C, 5% CO_2, and 95% humidity.

Antileishmanial activity against *L. donovani* and *L. major* promastigotes

The inhibitory activity of the plant extracts/fractions against *L. donovani* promastigotes was determined by colorimetric assay (Mosmann 1983) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sisco Research Laboratory, Mumbai). The promastigotes (5×10^5 cells/ml; 300 μl) were treated with and without the test samples at different concentrations and incubated at 22±2°C. The tests were performed at a concentration range of 1–500 μg/ml for the extracts and 1–20 μg/ml for the fractions, while amphotericin B was tested as a positive control (0.01–10 μM). After 72 h, the cells were harvested and re-suspended in PBS (500 μl) containing MTT (0.3 mg/ml). After keeping in darkness for about 18 h, the purple formazan crystals were dissolved in DMSO, and the optical density (O.D.) was measured at 570 nm in an ELISA reader (BIO-RAD; model 680, USA). The number of viable promastigotes is directly proportional to the amount of purple formazan produced through the reduction of yellow MTT by the dehydrogenase enzyme present in the inner mitochondrial membrane of the living cells. The percentage of growth inhibition was calculated as follows: % inhibition = [(O.D. of untreated control − O.D. of treated set)/O.D. of untreated control] × 100. The IC_{50} value as a measure of the inhibitory activity of the tested sample was determined from a dose–response curve with the help of Origin 5.0 software (Microcal Software, Inc., Northampton, MA, USA). Each assay was performed in triplicate, and the result was expressed as the mean of three independent experiments.

Antiparasitic activity against *L. major* promastigotes by alamarBlue® assay was conducted as described previously (Ponte-Sucre et al. 2006, 2007). Promastigotes were seeded into 96-well plates with a final cell density of 1×10^7/ml in phenol red-free RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; PAA, Pasching, Austria), gentamicin (50 μg/ml), penicillin G (100 U/ml), glutamine (2 mM), 2-mercaptoethanol (50 μM), and Hepes, pH 7.2 (10 mM) (final volume 200 μl), in the absence or presence of increasing concentrations of tested samples (0.032 to 100 μg/ml for the extracts and fractions) in duplicate wells. Amphotericin B (Sigma-Aldrich, USA) and pentamidine isethionate (Sigma-Aldrich, USA) were tested as reference compounds. The 96-well plates with *L. major* promastigotes were incubated at 27°C, 5% CO_2, and 95% humidity for 24 h. Finally, 20 μl of alamarBlue® (Trinova Biochem, Giessen, Germany) were added to each well 24 h after starting the incubation with compounds. AlamarBlue® is an indicator dye to quantitatively measure the cell viability. The blue oxidized form alamarBlue® (resazurin) is reduced by metabolically active cells to the red resorufin. At 72 h after compound addition, O.D.s were measured with an ELISA reader (model Multiskan Ascent; Thermo Electron Corporation, Dreieich, Germany) using λ=550 nm as test wavelength (resorufin) and λ=630 nm as reference wavelength (resazurin). The half-maximal inhibitory concentrations (IC_{50} values) were calculated by linear interpolation as previously described (Huber and Koella 1993). Each assay was performed in two independent experiments, and the results were expressed as the mean and standard deviation.

Antileishmanial activity against *L. major* amastigotes

The screening of extracts and fractions against *L. major* amastigotes residing in macrophages was done according to Lang et al. 2005. A 1.801-kbp fragment of firefly luciferase (LUC)-coding region was cut from pGL4.13 (Promega, Mannheim, Germany) by *Nco*I/*Xba*I and subsequently cloned into *Leishmania* expression vector pLEXSY-hyg2 (Jena bioscience, Jena, Germany) with marker gene for selection with hygromycin B (HYG) and cut with *Nco*I/*Nhe*I. After linearization by Swa I LUC, HYG was

integrated into 18s rRNA locus of nuclear DNA of *L. major*. Virulence of LUC-transgenic *L. major* was maintained by passage in BALB/c mice. Promastigotes were grown in blood agar cultures at 27°C, 5% CO_2, and 95% humidity and with the addition of 50 μg/ml hygromycin B. Bone marrow-derived macrophages generated as previously described using L929 supernatants (Schleicher and Bogdan 2009) were seeded (2×10^5 cells/ml, 200 μl) into a white 96-well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated for 4 h at 37°C to promote adhesion. LUC-transgenic promastigotes of *L. major* were harvested, re-suspended in RPMI-1640 medium, and finally added to each well. The infection ratio of macrophages with LUC-transgenic *L. major* promastigote was adjusted to 1:15. These co-cultures were incubated for 24 h at 37°C, 5% CO_2, and 95% humidity to ensure infection and differentiation to amastigotes. After washing twice with RPMI-1640 medium, amastigotes-infected macrophages were incubated in the absence or presence of increasing concentrations of test samples (0.001 to 100 μg/ml for the extracts and fractions) for further 24 h at 37°C in duplicate wells. Then, 25 μl Britelite™ (PerkinElmer, Waltham, MA, USA) was added in each well and luminescence was measured with a luminometer (PerkinElmer). The half-maximal inhibitory concentrations (IC_{50} values) were calculated by linear interpolation as previously described (Huber and Koella 1993). Amphotericin B and pentamidine were tested as reference compounds. Each assay was performed in two independent experiments, and the results were expressed as the mean and standard deviation.

Cytotoxicity assay ex-vivo

Peritoneal exudate cells (1×10^6 cells/ml) were isolated from BALB/c mice, seeded in a 35-mm plate (Tarsons, India) with RPMI-1640 medium (supplemented with 10% heat-inactivated FCS), and kept in a humidified atmosphere of 5% CO_2 at 37°C for adherence. After 24 h, cells were treated with VwM, VwC, F2, and F3, respectively, at a single dose of 100 μg/ml. Following incubation for 48 h, the viability of the macrophages was determined by the MTT-reduction assay as described before (Basu and Hazra 2006). The cytotoxicity of the test samples was estimated as the percentage of growth inhibition with respect to the untreated control cells (considered as 100% viable).

Determination of cellular morphology in *L. donovani* promastigotes

Aliquots of *L. donovani* promastigotes (5×10^6 cells/ml) were treated with 25 μg/ml of F3 and incubated at 22±2°C. At intervals of 4, 6, 24, and 48 h, the treated cells were harvested, re-suspended in PBS (pH 7.4), and placed on glass slides for observation under an optical microscope (Olympus CH 20i, Singapore) at a magnification of ×40. The cellular morphology was recorded through CAMEDIA Master 4.0 program and images were processed using Adobe Photoshop 5.5 (Adobe Systems, Inc., Mountain View, CA, USA) software. At least 20 microscopic fields were observed for each sample.

Determination of annexin V-fluorescein isothiocyanate/propidium iodide binding of *L. donovani* promastigotes

Double-staining for annexin V-fluorescein isothiocyanate (FITC)/iodide (PI) of *L. donovani* promastigotes was performed according to the manufacturer's instruction, using an annexin V-FITC staining kit (Sigma-Aldrich Co., St. Louis, MO, USA) as done previously (Mukherjee et al. 2009). Briefly, promastigotes (1×10^6 cells/ml) were treated with or without VwC at different concentrations and incubated at 22±2°C. After 48 h, cells were harvested, washed in PBS, and re-suspended in 100 μl binding buffer (1x). After incubation for 1 h, 4 μl FITC and 5 μl PI were added to each set and kept in the dark at room temperature for 20 min. Subsequently, treated and untreated cells were analyzed on a flow cytometer (Becton Dickinson, San José, CA, USA) using a 515 nm filter for FITC fluorescence (FL-1H) and a 623 nm filter for PI detection (FL-2H), and a dot plot of FL-1H (*X*-axis; FITC fluorescence) versus FL-2H (*Y*-axis; PI fluorescence) was recorded.

Flow cytometric analysis of cell cycle

L. donovani promastigotes (1×10^6 cells/ml), untreated or treated with VwC, were incubated for 24 h at 22±2°C. Cells were then harvested, washed in PBS, fixed in 100 μl chilled 70% ethanol, and incubated overnight at −20°C. After two washes in PBS (pH 7.4), promastigotes were re-suspended in PBS, and ethidium bromide (50 μg/ml) and RNase A (50 μg/ml) were added before incubation at room temperature in darkness for 20 min. The fluorescence intensity was analyzed with a flow cytometer (Becton Dickinson, San José, CA, USA) using a 595 nm filter. The results were recorded as histogram to show the shift in percentage of treated cells in sub-G1 region with respect to the untreated control (Mukherjee et al. 2009).

DNA laddering experiment

Fragmentation of DNA into nucleosomal bands, as a function of apoptotic cell death, was studied by DNA laddering assay as described before (Mukherjee et al. 2009). Promastigotes (1×10^6 cells/ml), cultured for 24 h in the presence or absence of different concentrations of F3 fraction, were harvested and re-suspended in digestion

buffer [500 μl; 10 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris–HCl (pH 8.0), and 0.5% sodium lauryl sulfate] to which 0.5 mg/ml proteinase K was added. The mixture was incubated for 3 h at 37°C in the presence of 0.1 mg/ml RNase A. The DNA material was extracted by phenol/chloroform (1:1) treatment and precipitated by adding 3 M sodium acetate and ice-cold ethanol (100%) to the aqueous phase. Following overnight incubation at −20°C, the material was centrifuged (14,000×g) and the pellet was collected, air-dried, and re-suspended in Tris–EDTA buffer (50 μl, pH 8.0). DNA aliquots (10 μg) were electrophoresed on 2% agarose gel containing ethidium bromide (0.5 μg/ml), using Tris–acetate–EDTA (pH 8.0) running buffer, and photographed under UV light.

Change in mitochondrial membrane potential ($\Delta\psi_m$)

Alteration in mitochondrial membrane potential ($\Delta\psi_m$) after the drug treatment in *L. donovani* promastigotes was estimated fluorimetrically using the lipophilic cationic fluorescent indicator JC-1 as done before (Mukherjee et al. 2009). Briefly, promastigotes (2×10^6 cells/ml) were incubated at 22±2°C for 48 h in the absence or presence of graded concentrations of extract and fractions, respectively. Cells were harvested, washed, treated with 10 μg/ml JC-1, and incubated at 37°C for 30 min. After washing twice with PBS, fluorescence intensity was measured at 530 and 590 nm in a Perkin Elmer fluorescence spectrometer (Model No.: LS55) using an excitation wavelength of 485 nm. The ratio of intensity values obtained at 590 and 530 nm (relative fluorescence intensity = R.F.I.) was plotted as the relative $\Delta\psi_m$. Each assay was performed in duplicate, and the results were expressed as the mean of two independent experiments.

Intracellular reactive oxygen species generation

Intracellular reactive oxygen species (ROS) generation induced by VwC and F3 was measured using 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA). DCFH-DA is first converted to DCFH by intracellular esterase and then fluorescent DCF by ROS. Therefore, intracellular ROS generation can be monitored directly by measuring the intensity of DCF (Cathcart et al. 1983). Briefly, *L. donovani* promastigotes (2×10^6 cells/ml) were treated with or without extract and fractions of different concentrations, respectively, and incubated at 22±2°C for 3 h. Cells were then centrifuged, washed with PBS, and incubated with DCFH-DA (10 μM) at room temperature. After 30 min, fluorescence intensity of DCF in both treated and untreated cells was measured at 520 nm, using an excitation wavelength of 490 nm. Each assay was performed in duplicate.

Statistical analysis

Data are expressed as the arithmetic mean±standard deviation values. IC_{50} values were calculated using dose–response curves in Origin 5.0 software (Microcal Software, Inc., Northampton, MA, USA).

Results

Antileishmanial activity against *L. donovani* and *L. major* promastigotes

The three extracts of *V. wallichii* roots were evaluated for the antileishmanial activity through MTT-reduction assay of *L. donovani* promastigotes. It was found that VwM and VwC could positively inhibit the cell growth, showing 50% inhibition at approximately 45 and 10 μg/ml, respectively, the chloroform extract (VwC) being relatively more effective than the methanol extract VwM, while the water extract VwW did not produce a notable inhibition at the highest tested dose of 500 μg/ml. Similar observations were obtained by alamarBlue® assay on *L. major* promastigotes as well (Table 1).

Based on these findings, the methanol (VwM) and chloroform extracts (VwC) were fractionated. The separation of VwM via HPLC delivered 11 fractions, where the two most lipophilic late-eluting fractions, viz., F10 and F11, showed IC_{50} values of 8.2 and 8.9 μg/ml in the alamarBlue® assay on *L. major* promastigotes. VwC was separated by means of chromatography on silica gel plates to get two semi-pure fractions, viz., F2 and F3, both of

Table 1 Antileishmanial activity of the extracts and semi-purified fractions of *V. wallichii* root against *L. donovani* and *L. major* parasites (strain within parenthesis)

Tested sample	IC_{50} (μg/ml)[a]		
	L. donovani (MHOM/IL/81/FE/BNI)	*L. major* (MHOM/IN/1983/AG83)	
	Promastigote	Promastigote	Amastigote
VwW	>500	[b]	[b]
VwM	44.1±0.4	[b]	[b]
VwC	9.9±0.2	9.0±0.8	0.8±0.4
F2	8.4±0.5	40.1±0.4	1.3±0.7
F3	4.1±0.3	7.1±0.4	0.3±0.0
Amphotericin B	0.4±0.1[c]	0.3±0.1[c]	0.02±0.00[c]
Pentamidine	[b]	82.5±7.5[c]	0.96±0.11[c]

[a] IC_{50} values are mean±SD of three independent determinations

[b] Not determined

[c] IC_{50} values are in micromolars for standard drugs tested as positive control

which were subjected to the MTT assay on *L. donovani* promastigotes. The results clearly showed that the IC_{50} values of F3 was less than that of F2, indicating that F3 was more promising than F2, as shown in Table 1. A similar observation was obtained with the alamarBlue® assay on *L. major* promastigotes, revealing a pronounced difference between the IC_{50} values of F2 and F3 (Table 1). The antileishmanial potential of VwC, F2, and F3 was further confirmed through the significant inhibition of the growth of intracellular amastigotes of *L. major*, as IC_{50} values of these extracts and semi-pure fractions of *V. wallichii* roots were found to be in the low micromolar range (0.3–1.3 μg/ml).

Cytotoxicity assay ex-vivo

The cytotoxicity of the test samples was determined by the ex-vivo MTT-reduction assay on mouse peritoneal exudate cells by treatment with the extracts/fractions of *V. wallichii* root. It was found that the percentage inhibition of cell growth induced by 100 μg/ml each of VwM, VwC, F2, and F3 was 57.2%, 58.1%, 66.4%, and 63.2%, respectively. The water extract (VwW) was not screened for cytotoxicity as this fraction was not found to inhibit the growth of leishmanial promastigotes at the highest dose tested (500 μg/ml).

Determination of cellular morphology in *L. donovani* promastigotes

A visual inspection of *L. donovani* promastigotes treated with F3 revealed significant changes in cellular morphology beginning at the fourth hour of treatment (Fig. 1B). When compared to the untreated control promastigotes (Fig. 1A), substantial reduction in size and shape, with loss of flagella, could be observed. At the end of 48 h, complete rounding of shape, cytoplasmic condensation, and shrinkage of almost all the cells were observed (Fig. 1C–E).

Determination of annexin V-fluorescein isothiocyanate/ propidium iodide binding of *L. donovani* promastigotes

Externalization of the membrane-associated phospholipids phosphatidylserine, a characteristic of cells in early apoptosis, was studied by annexin V binding of promastigotes following treatment with different concentrations of VwC. Simultaneous application of the DNA binding dye PI and analysis of the stained promastigotes by flow cytometry was used to discriminate the necrotic or late apoptotic cells from the early apoptotic ones. The percentage of early apoptotic cells (annexin V positive and PI negative; lower

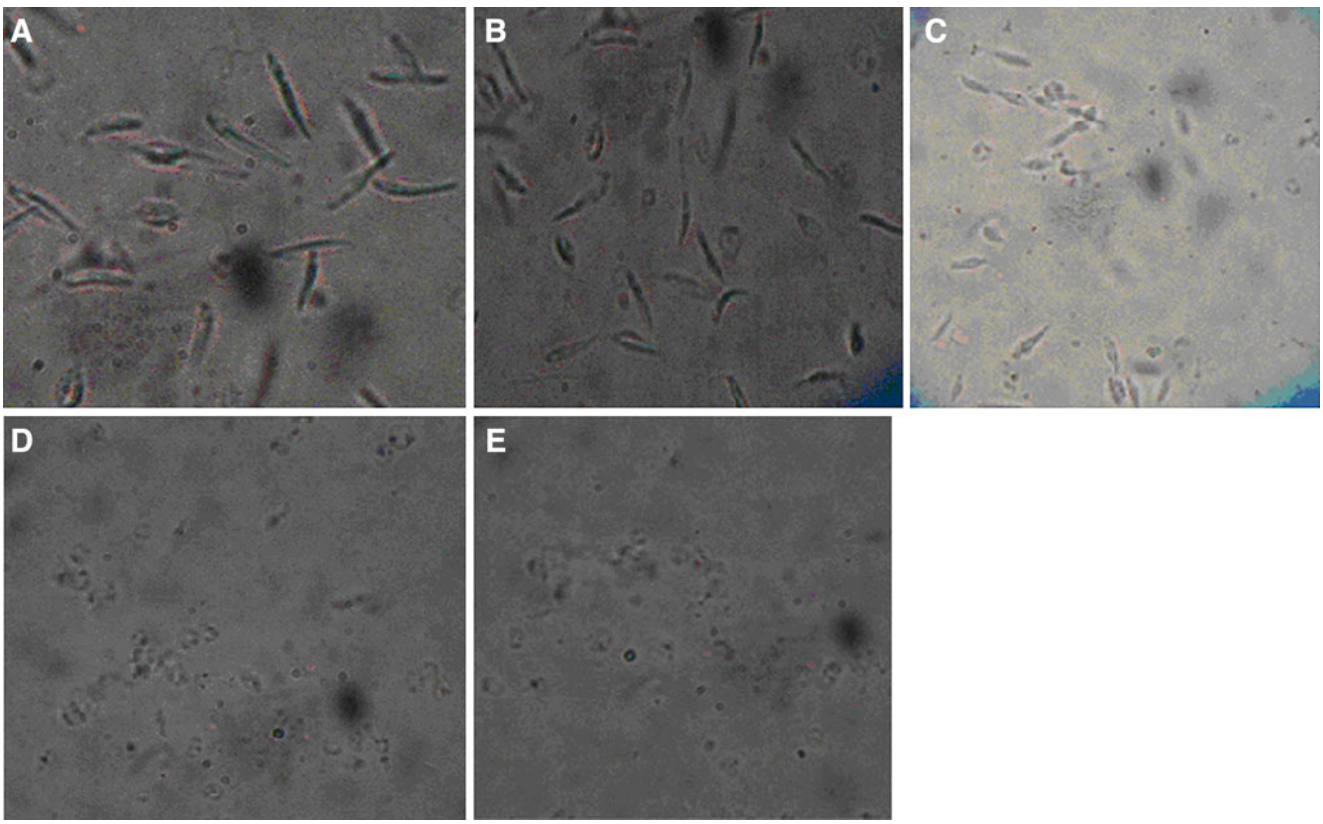

Fig. 1 Cellular morphology of *L. donovani* promastigotes (AG 83) after exposure to 25 μg/ml of F3: untreated control (**A**); promastigotes exposed for 4 h (**B**), 6 h (**C**), 24 h (**D**), and 48 h (**E**)

right quadrant) increased from 0.5% in untreated control to 5.5% and 7.3% following treatment with 10 and 25 μg/ml of the VwC, respectively (as shown in Fig. 2).

Flow cytometric analysis of cell cycle

Flow cytometry analysis after cell permeabilisation and labeling with ethidium bromide (EtBr) was used to quantify the percentage of pseudohypodiploid *L. donovani* promastigotes. EtBr binds to cellular DNA and the DNA fragmentation in apoptotic cells gives a fluorescence signal in a region lower than G1 cells (sub-G1 peak). Following 24 h incubation with 10 and 25 μg/ml of VwC, ~50–60% of the cells were found to be in the sub-G1, while only ~11% of the untreated control cells were found to be in the same zone (Fig. 3).

Oligonucleosomal DNA fragmentation in *L. donovani* promastigotes

Oligonucleosomal DNA fragmentation analysis of *L. donovani* promastigotes was carried out following treatment of the parasites with different doses of F3. After 24 h of incubation with 25 μg/ml of F3, DNA laddering profiles (Fig. 4) clearly demonstrated fragmentation of the genomic DNA of the promastigotes. Even at a lower dose (10 μg/ml), the fragmentation pattern was visible (lane 3), although it was not so pronounced at 5 μg/ml of F3 (lane 2; Fig. 4).

Change in mitochondrial membrane potential ($\Delta\psi_m$)

Alteration in the mitochondrial membrane potential ($\Delta\psi_m$) in *L. donovani* promastigotes was studied by treatment with extracts and fractions, respectively, at a concentration range of 5–500 μg/ml. Spectrofluorimetric data presented in Fig. 5 showed more or less gradual decrease in relative fluorescence intensity (RFI) with increasing concentrations of the tested samples. The fraction F3, at 500 μg/ml, could demonstrate a decrease in $\Delta\psi_m$ comparable to the positive control camptothecin (50 μM).

Intracellular reactive oxygen species generation

ROS generation in *L. donovani* promastigotes following treatment with either VwC or F3 was determined by spectrofluorimetric probe DCFH-DA. A moderate generation of ROS (~2.0 times) was evident at the highest dose of treatment, i.e., 500 μg/ml, for 3 h, although the lower doses did not show much effect with respect to the untreated control set. Camptothecin, a positive control in this experiment, could generate marginally more (~2.5-fold) ROS at a dose of 5 μM in comparison to the untreated control set (Fig. 6).

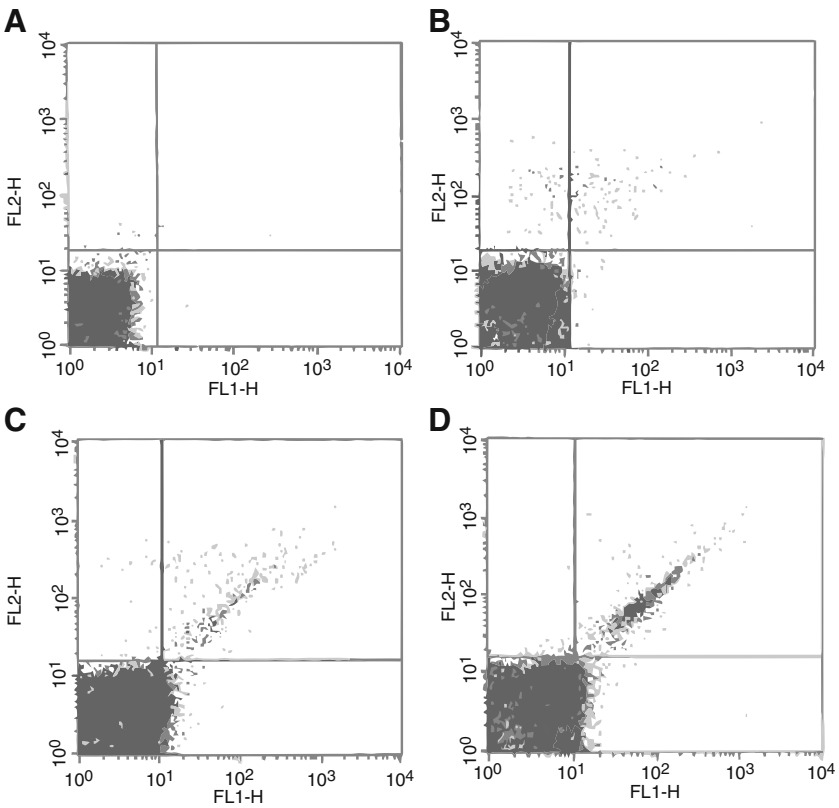

Fig. 2 Flow cytometric analysis of phosphatidyl serine externalization of *L. donovani* promastigotes. Cells treated with **C** 10 and **D** 25 μg/ml of VwC are presented in a dot-plot analysis along with the untreated control (**B**). The unstained population is also shown as an internal intensity control (**A**)

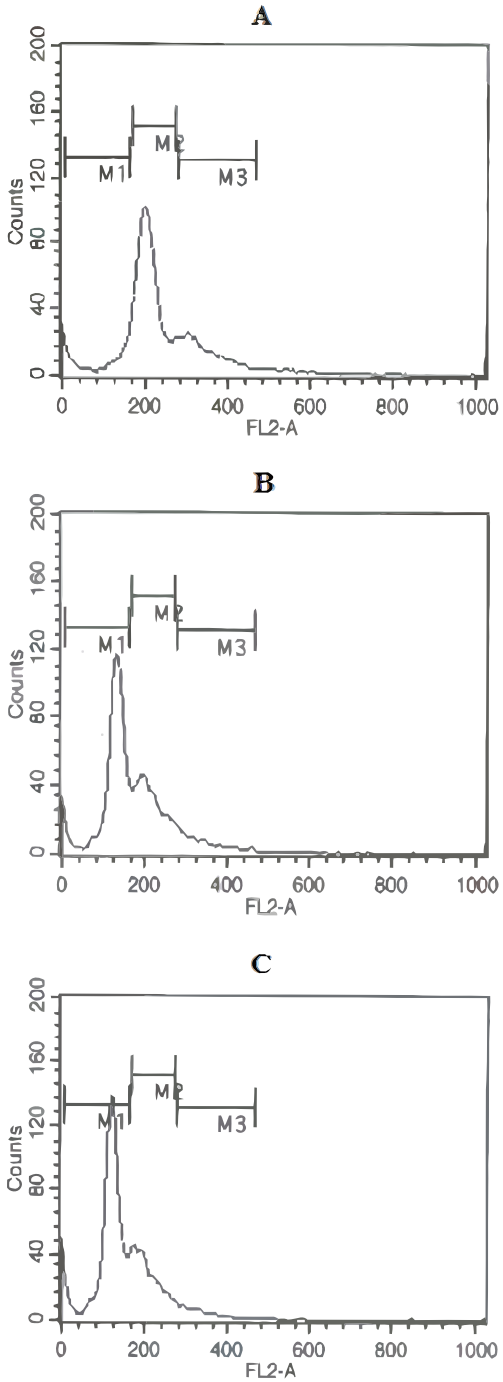

Fig. 3 Flow cytometric analysis of DNA content in *L. donovani* promastigotes: untreated **A**, treated with 10 μg/ml **B,** and treated with 25 μg/ml **C** of VwC, respectively. The amount of DNA in pseudohypodiploid cells is found in the sub-G1 peak region shown as *M1*

Discussion

Leishmanial diseases, being highly endemic in developing countries, constitute a worldwide health problem particularly since the emergence of AIDS. In the absence of a vaccine, there is an urgent need for more effective drugs to replace or supplement those in current use (Santos et al. 2008). Traditionally, different cultures around the world have been using their native plants for the treatment of systemic forms of leishmaniasis through oral administration of the crude extracts, while the cutaneous infections would be treated with topical preparations of the same (Chan-Bacab and Pena-Rodriguez 2001). Recently, several indigenous medicinal plants have been investigated for prospective antiparasitic properties (Sharma et al. 2009; Santin et al. 2009). Therefore, we have been extensively exploring plant resources to look for effective antileishmanial agents and serendipitously observed this activity in the root extract of an Indian species of Valerian, viz. *V. wallichii*.

In our first study on the *V. wallichii* root, the extracts prepared with the non-aqueous solvents were found to exhibit marked cytotoxicity towards promastigotes of both *L. donovani* and *L. major*, as well as the amastigotes of *L. major*. The chloroform extract (VwC) was subjected to bioassay-guided PTLC, which furnished a couple of semi-pure fractions showing significant antileishmanial activity with IC_{50} values lower than 10 μg/ml. F3, the most active fraction, was found to cause a progressive alteration in the cellular morphology of *L. donovani* promastigotes observed in a light microscopic study during 4–48 h of incubation. Further, the cytotoxicity of the 'active fractions' was found to be significantly less against the uninfected BALB/c mouse macrophages incubated with the test samples in the ex-vivo assay. Therefore, the selective killing of parasites by the plant-derived samples compared to the host

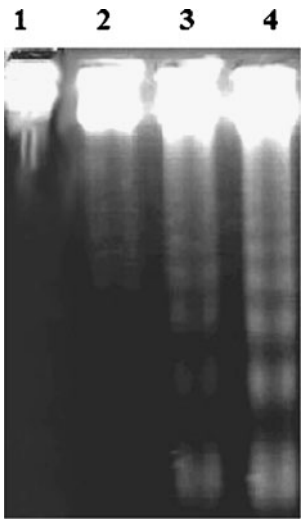

Fig. 4 Determination of apoptotic cell death by DNA fragmentation in *L. donovani* promastigotes. *Lane 1*, DNA from untreated promastigotes; *lanes 2*, *3*, and *4*, DNA from promastigotes treated with 5, 10, and 25 μg/ml of F3, respectively

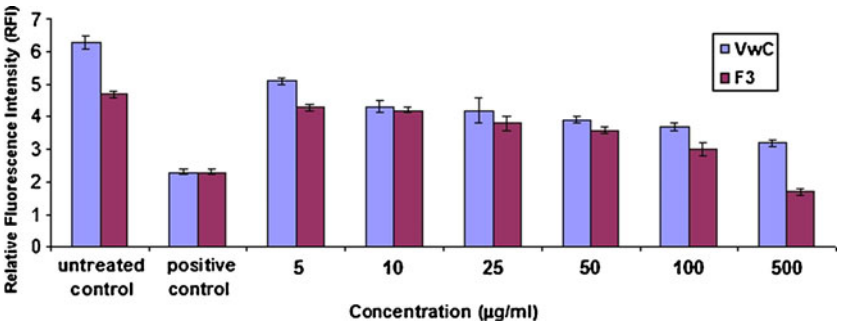

Fig. 5 Analysis of the mitochondrial membrane potential of *L. donovani* promastigotes following treatment with VwC and F3. Treated/untreated promastigotes (2×10^6 cells/ml) were exposed to different doses of VwC and F3 and stained with potentiometric probe JC-1 (10 µg/ml). Decrease in relative $\Delta\psi_m$ values are expressed as the ratio of fluorescence intensity measured at 590 nm (for J-aggregates) versus 530 nm (for J-monomer). Camptothecin (50 µM) served as a positive control

macrophage cells prompted us to evaluate the fraction F3 for its mechanism of cytotoxicity against *Leishmania* cells.

Thus, a gel electrophoretic study revealed ladder-like fragmentation of the genomic DNA of *L. donovani* promastigotes, which was a 'hall-mark' event to clearly indicate an apoptosis-like mode of cell death induced by F3 at a dose of 25 µg/ml. This was further corroborated by the flow cytometric analysis of phosphatidyl serine externalization of the promastigotes treated with 25 µg/ml of VwC. Fluorimetric assessments using specific probes (JC-1 and DCFH-DA) were carried out on *L. donovani* promastigotes to evaluate (1) mitochondrial membrane depolarization and (2) intracellular ROS generation caused by VwC as well as F3. *L. donovani* promastigotes responded positively to these studies, more or less, although at comparatively higher concentrations (100–500 µg/ml) of VwC and F3. Camptothecin, a potent antileishmanial plant-derived alkaloid, was used as positive control for both experiments.

Again, two of the semi-pure fractions obtained through HPLC of the methanol extract (VwM, which was relatively less effective than the chloroform extract, VwC) were also found to show a promising value for IC_{50} (~8–9 µg/ml) when tested against *L. major* promastigotes (data not shown in Table 1). Therefore, in addition to F3, these fractions would also be subjected to further analysis in the next phase of our study to identify the 'active' antileishmanial compound(s) in *V. wallichii*.

Valerian roots have been used for hundreds of years for its sedative and antispasmodic properties. Also, the reputation of *Valeriana* spp. in modern rational phytotherapy is mainly for treating nervous tension and temporary sleeping problems (Barnes et al. 2002). It is rather intriguing that neither *V. wallichii* nor any of its phytochemical constituents has been reported for any cytotoxic application, except for toxicity of the essential oil components (e.g., α-pinene, β-pinene, camphene, *p*-cymene, limonene, borneol, patchouli alcohol, etc.) against nematodes, termites, and fungi (Kim et al. 2008; Zhu et al. 2003; Letchamo et al. 2004). Apparently, there is no ethnopharmacological information about the application of *V. wallichii* and other species of *Valeriana* specifically against leishmaniasis. It might be speculated that this was due to the fact that these herbs are generally found to grow mostly in the mountainous (1,300–3,300 m) terrain of the Himalayas (Singh et al. 2006) and

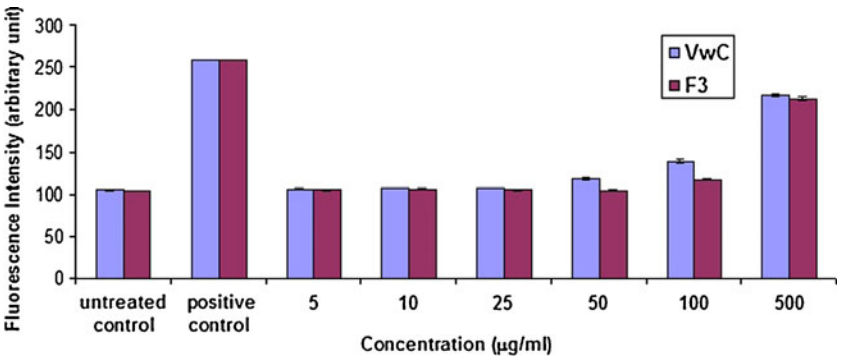

Fig. 6 Determination of ROS generation in *L. donovani* promastigotes induced by VwC and F3. Treated or untreated promastigotes (2×10^6 cells/ml) were exposed to different doses of VwC and F3 and stained with fluorimetric probe DCFH-DA (10 µM). The amount of intracellular ROS generation is expressed by measuring the intensity at 520 nm. Camptothecin (5 µM) served as a positive control

not native to the endemic regions of leishmanial diseases in the Indian subcontinent. Further in this context, we should also consider the wide-ranging diversity of the phytochemical constituents in *Valeriana* genus due to the variation in the agroclimatic conditions of its habitat ranging from tropical to alpine (Mathela et al. 2005). Therefore, it would be our next endeavor to elucidate the composition of active semi-pure fractions of VwM and VwC, particularly F3 as described above, through suitable hyphenated analytical techniques involving HPLC and GC so that the antileishmanial activity could be traced to the molecular level. Incidentally, we have also observed that the chloroform extract of *V. officinalis* root (procured from Germany) showed about 25-fold weaker antileishmanial activity (IC_{50} value ~225 μg/ml) in comparison to that of *V. wallichii* against *L. donovani* promastigotes. However, in order to assess the clinical relevance of these findings, further extension of these studies needs to be undertaken on the amastigote forms of the *Leishmania* spp.

Taken together, it may be concluded that an 'Indian Valerian', viz., *V. wallichii* DC (syn. *V. jatamansi* Jones), a well-known traditional medicinal herb, would be a viable source for finding a good therapeutic agent against both the visceral and cutaneous forms of leishmaniasis. Further, the active principle(s), when identified, would provide novel 'leads' to design alternative treatments for these diseases.

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Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

Conflicts of interest The authors declare that they have no conflicts of interest.

References

Alvar J, Cañavate C, Gutiérrez-Solar B, Jiménez M, Laguna F, López-Vélez R, Molina R, Moreno J (1997) *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev 10:298–319

Balderer G, Borbely A (1985) Effect of valerian on human sleep. Psychopharmacology 87:406–409

Barnes J, Anderson LA, Phillipson JD (2002) Herbal medicines. A guide for healthcare professionals, 2nd edn. Pharmaceutical, London, pp 468–476

Basu S, Hazra B (2006) Evaluation of nitric oxide scavenging activity, in vitro and ex vivo, of selected medicinal plants traditionally used in inflammatory diseases. Phytother Res 20:896–900

Cathcart R, Schwiers E, Ames BN (1983) Detection of picomole levels of hydrogen peroxide using a fluorescent dichlorofluorescein assay. Anal Biochem 134:111–116

Chan-Bacab MJ, Pena-Rodriguez LM (2001) Plant natural products with leishmanicidal activity. Nat Prod Rep 18:674–688

Harvey AL (1999) Medicines from nature: are natural products still relevant to drug discovery? Trends Pharmacol Sci 20:196–198

Hotez PJ, Pecoul B (2010) "Manifesto" for advancing the control and elimination of neglected tropical diseases. PLoS Negl Trop Dis 4:e718

Houghton PJ (1999) The scientific basis for the reputed activity of Valerian. J Pharm Pharmacol 51:505–512

Huber W, Koella JC (1993) A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. Acta Trop 55:257–261

Jha TK (2006) Drug unresponsiveness & combination therapy for kala-azar. Indian J Med Res 123:389–398

Kim J, Seo SM, Lee SG, Shin SC, Park IK (2008) Nematicidal activity of plants essential oils and components from coriander (*Coriandrum sativum*), Oriental sweetgum (*Liquidambar orientalis*) and valerian (*Valeriana wallichii*) essential oils against pinewood nematode *Bursaphelenchus xylophilus*. J Agric Food Chem 56:7316–7320

Lang T, Goyard S, Lebastard M, Milon G (2005) Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice. Cell Microbiol 7:383–392

Leathwood PD, Chauffard F (1985) Aqueous extract of valerian reduces latency to fall asleep in man. Planta Med 2:144–148

Letchamo W, Ward W, Heard B, Heard D (2004) Essential oil of *Valeriana officinalis* L. cultivars and their antimicrobial activity as influenced by harvesting time under commercial organic cultivation. J Agric Food Chem 52:3915–3919

Lin S, Shen YH, Li HL, Yang XW, Chen T, Lu LH, Huang ZS, Liu RH, Xu XK, Zhang WD, Wang H (2009) Acylated iridoids with cytotoxicity from *Valeriana jatamansi*. J Nat Prod 72:650–655

Mathela CS, Chanotiya CS, Sammal SS, Pant AK, Pandey S (2005) Compositional diversity of terpenoids in the Himalayan *Valeriana* genera. Chem Biodivers 2:1174–1182

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Mukherjee P, Majee SB, Ghosh S, Hazra B (2009) Apoptosis-like death in *Leishmania donovani* promastigotes induced by diospyrin and its ethanolamine derivative. Int J Antimicrob Agents 34:596–601

Nadkarni KM (1976) Indian materia medica, 3rd edn. Popular Prakashan, Bombay, pp 1260–1262

Ponte-Sucre A, Vicik R, Schultheis M, Schirmeister T, Moll H (2006) Aziridine-2,3-dicarboxylates, peptidomimetic cysteine protease inhibitors with antileishmanial activity. Antimicrob Agents Chemother 50:2439–2447

Ponte-Sucre A, Faber JH, Gulder T, Kajahn I, Pedersen SE, Schultheis M, Bringmann G, Moll H (2007) Activities of naphthylisoquinoline alkaloids and synthetic analogs against *Leishmania major*. Antimicrob Agents Chemother 51:188–1894

Sánchez-Cañete MP, Carvalho L, Pérez-Victoria FJ, Gamarro F, Castanys S (2009) Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. Antimicrob Agents Chemother 53:1305–1313

Santin MR, dos Santos AO, Nakamura CV, Dias Filho BP, Ferreira IC, Ueda-Nakamura T (2009) In vitro activity of the essential oil of *Cymbopogon citratus* and its major component (citral) on *Leishmania amazonensis*. Parasitol Res 105(6):1489–1496

Santos DO, Coutinho CE, Madeira MF, Bottino CG, Vieira RT, Nascimento SB, Bernardino A, Bourguignon SC, Corte-Real S, Pinho RT, Rodrigues CR, Castro HC (2008) Leishmaniasis treatment—a challenge that remains: a review. Parasitol Res 103(1):1–10

Schleicher U, Bogdan C (2009) Generation, culture and flow-cytometric characterization of primary mouse macrophages. Methods Mol Biol 531:203–224

Sharma U, Velpandian T, Sharma P, Singh S (2009) Evaluation of anti-leishmanial activity of selected Indian plants known to have antimicrobial properties. Parasitol Res 105(5):1287–1293

Singh N, Ap G, Singh B, Kaul VK (2006) Quantification of valerenic acid in *Valeriana jatamansi* and *Valeriana officinalis* by HPTLC. Chromatographia 63:209–213

WHO (2010) TDR news item. http://apps.who.int/tdr/svc/news-events/news/ntd-elimination

Zhu BC-R, Henderson G, Yu Y, Laine RA (2003) Toxicity and repellency of patchouli oil and patchouli alcohol against Formosan subterranean termites *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). J Agricult Food Chem 51:4585–4588