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In vitro antileishmanial activity of *Aloe vera* leaf exudate: A potential herbal therapy in leishmaniasis

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Abstract *Aloe vera* has wide spread use in health products, and despite several reports on the whole plant and inner gel, little work has been performed on the leaf exudate. Our aim was to evaluate the *in vitro* efficacy of *Aloe vera* leaf exudate (AVL) in leishmaniasis. Irrespective of the disease manifestation, promastigotes from strains responsible for cutaneous, mucocutaneous, and visceral leishmaniasis were susceptible to AVL and their IC_{50} ranged from 100 to 180 μg/ml. In axenic amastigotes cultured from a *L. donovani* strain 2001 responsible for visceral leishmaniasis, the IC_{50} was 6.0 μg/ml. AVL caused activation of host macrophages evident by an increased release of members of reactive oxygen species that was attenuated by preincubation with free radical scavengers. Collectively, our data indicates that AVL, via its direct leishmanicidal activity which can be further enhanced by activation of host macrophages, is an effective antileishmanial agent meriting further pharmacological investigations.

Keywords *Aloe vera* · Leishmaniasis · Antileishmanial · Reactive oxygen species

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Abbreviations

AVL	*Aloe vera* leaf exudate
BSA	Bovine serum albumin
CL	Cutaneous Leishmaniasis
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
H_2DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
IC_{50}	50% inhibitory concentration
L-NAME	N^{ω}-L-arginine Methyl Ester
MCL	mucocutaneous Leishmaniasis
Medium A	RPMI 1640 (without phenol red) with 10 % FCS and 100 μg/ ml gentamycin
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NO	Nitric oxide
PBS	Phosphate buffered saline (pH 7.4)
ROS	Reactive oxygen species
SAG	sodium antimony gluconate
SOD	superoxide dismutase
VL	Visceral Leishmaniasis

Introduction

Leishmaniasis, a complex parasitic disease caused by parasites of the genus *Leishmania*, endemic in 80 countries, is a major global health problem threatening almost 350 million people worldwide with about 2 million new cases reported annually [1]. The disease is manifested in three forms, namely visceral, mucocutaneous or cutaneous; the severest is the visceral form caused mainly by *Leishmania donovani* and *Leishmania infantum* with an estimated 500,000 new cases reported annually [1]. Cutaneous leishmaniasis (CL) is the commonest presentation representing up to seventy-five percent of all new cases

is caused by *L. tropica* and *L. major* wherein lesions appear on the face, arms, and legs. Mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis* and *L. amazonensis* is manifested by nasal obstruction and bleeding and generation of painful mucosal lesions; visceral leishmaniasis (VL) by *L. donovani* and *L. infantum* is manifested primarily by hepatosplenomegaly, anemia, prolonged fever and can be fatal if left untreated.

For the last 70 years, sodium antimony gluconate (SAG) has been the mainstay of antileishmanial therapy. However, its use is associated with hazardous iatrogenic effects like severe cardiotoxicity accompanied by congestive heart failure, ventricular tachyarrhythmia, and ventricular fibrillation that could be fatal [2]. Further limitations are its variable efficacy [3] and of greater concern is the steady erosion in its efficacy especially in North Bihar, India [4]. The chemotherapy of VL also includes Amphotericin B and its lipid formulations, but limitations are their parenteral administration, toxicity and high cost [3]. Miltefosine (HePc), originally developed as an anticancer drug, is the first orally effective antileishmanial drug but is not without its share of toxicity and high potential for resistance [5].

In an ongoing search for better and cheaper leishmanicidal agents, plant-derived products are an attractive option [6,7]. According to The World Health Organization (WHO), approximately 80% of the world's inhabitants rely on traditional medicines for their health care [8]. Therefore, the harmonization of traditional and modern medicine is one of the principle goals in the progressive playground of pharmacognosy. *Aloe vera*, a commonly used medicinal plant of family *Liliaceae* is well established for its different medicinal uses ranging from treating skin burns to antimetastatic and antimicrobial activity [9–11]. The aim of this work was to identify a new leishmanicidal agent from the leafy exudate of *Aloe vera* (AVL). We report that the extract had a direct parasiticidal effect on promastigotes and axenic amastigotes. This extract modulated the immune function of murine peritoneal macrophages by increasing generation of members of reactive oxygen species (ROS). Taken together, AVL can be considered as a potential herbal remedy against VL that needs to be tested *in vivo*.

Materials and methods

Chemicals

M-199 medium and fetal bovine serum [FBS] were obtained from Gibco-BRL; dimethyl sulfoxide [DMSO] from SRL; H_2DCFDA (Dichlorofluoresceindiacetate) from Molecular Probes. All other chemicals were obtained from Sigma.

Parasite culture

Promastigotes of different species of *Leishmania* were routinely cultured at 22°C in M-199 medium supplemented with 10% heat-inactivated FBS and gentamycin (100 μg/ml). The strains included in this study are included in Table 1. Prior to experiments, promastigotes were transferred to a modified RPMI-1640 medium (without phenol red) containing 10% heat-inactivated FBS and Gentamycin, referred to as Medium A.

Axenic amastigotes

Promastigotes of a *L. donovani* strain 2001, isolated from an active kala-azar patient, were routinely cultured as described above. For transformation to axenic amastigotes, stationary phase promastigotes were subjected to a change in pH and temperature [12]. Accordingly, the promastigotes were transferred to RPMI 1640 (pH 5.5) containing KH_2PO_4 (114.6 mM), K_2HPO_4 (10.38 mM), $MgSO_4$ (0.5 mM), D-Glucose (4.0 mg/ml), MES monohydrate buffer (5.35 mg/ml), and 20% heat-inactivated FBS and kept at 37°C in presence of 5% CO_2. These transformed axenic-amastigotes were routinely maintained in the same medium in a similar environment.

Mammalian cell culture

Murine peritoneal macrophages were lavaged following starch induction (2% starch i.p., 2 ml/mouse) and kept in Medium A at 37°C in the presence of 5% CO_2.

Table 1 *Leishmania* strains used in this study

Strain	Designation	Species	Disease form	IC_{50} in promastigotes (μg/ml)
K 27	MHOM/SU/74/K27	*L. tropica*	Cutaneous	176
JISH 118	MHOM/SA/85/JISH 118	*L. major*	Cutaneous	102.5
LV 81	MORY/BR/72/M1824	*L. amazonensis*	Mucocutaneous	182
L 280	MHOM/PE/66/L 280	*L. braziliensis*	Mucocutaneous	177.5
MON 29	MHOM/ES/81/BCN 1	*L. infantum*	Visceral	167.5

Collection and preparation of the plant extract

Fresh succulent leaves of *Aloe vera* (*Aloe barbadensis Mill.*) were collected; its inner gel and outer leafy coat were manually separated. The outer leafy coat was then crushed in an electric grinder, lyophilized and stored at 4°C. Subsequently, the lyophilized leaf was dissolved in DMSO overnight, the solution was centrifuged at 2000 rpm×5 min and the resultant pellet was discarded. A stock solution (3.5 mg/ml) was stored at 4°C until used and used in all the experiments.

In-vitro effect of AVL on *Leishmania* promastigotes

Log phase promastigotes of five *Leishmania* species, resuspended in Medium A, were seeded in 96-well tissue culture plates (1×10^5/250 μl/well). The parasites were exposed to increasing concentrations of AVL (0–300 μg/ml) for 72 h at 22°C and their viability was evaluated using a modified MTT assay [13]. Briefly, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, 2.0 mg/ml, 0.05 ml/well) was added, and the wells were incubated in the dark for an additional 4 h at 37°C; the plates were then centrifuged (2000 rpm×5 min, 25°C); the supernatant was removed and the resultant pellet was dissolved in DMSO (200 μl). Absorbances of the resultant formazan, due to reduction of MTT by mitochondrial dehydrogenases of viable promastigotes was measured at 492 nm using a plate reader (Labsystems, Finland). Accordingly, the specific absorbance that represented formazan production was calculated by subtraction of background absorbance from total absorbance. The mean percentage viability was calculated as follows:

$$\frac{\text{Mean specific absorbance of treated parasites}}{\text{Mean specific absorbance of untreated parasites}} \times 100$$

The 50% inhibitory concentration or the IC_{50} *i.e.*, the concentration that decreased cell growth by 50% was determined by graphical extrapolation.

Measurement of anti-leishmanial activity of AVL in axenic amastigotes

Axenic amastigotes were seeded in 96 well tissue culture plates (3×10^6/250 μl/well) in the presence of increasing concentrations of AVL (0 to 100 μg/ml) and incubated for 96 h at 37°C in the presence of 5% CO_2 and cell viability was analyzed microscopically by trypan blue exclusion. The IC_{50} of axenic amastigotes *i.e.*, the concentration of AVL that decreased the cell number by 50% was determined by graphical extrapolation.

Generation of reactive oxygen species (ROS) by AVL in macrophages

The generation of intracellular reactive oxygen species (ROS) by macrophages in the presence of AVL was identified using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a non-fluorescent dye that is converted to a fluorescent dye dichlorofluorescein (DCF) in the presence of free radicals [14]. Briefly, murine peritoneal macrophages were incubated with AVL (87.5 μg/ml) for 2 h at 37°C, 5% CO_2. To pin-point the reactive oxygen species generated by AVL, the macrophages were pre-incubated for 1 h, with superoxide dismutase (SOD, 100 units/ml) and catalase (500 units/ml) or N^{ω}-L-arginine methyl ester (L-NAME, 100 μM) or both, followed by incubation with AVL. After fixing the cells in paraformaldehyde (1%), the nonspecific binding sites were blocked with PBS–BSA (2%) and probed with H_2DCFDA (10 μM) for 30 min. After removal of the excess dye by washings with PBS, the cells were analyzed on a flow cytometer (FACS Calibur, CellQuest software, Becton Dickenson, USA).

Results and discussion

Leishmaniasis comprises a wide disease spectrum manifested in three forms namely cutaneous, mucocutaneous, and visceral. To study the *in vitro* leishmanicidal efficacy of AVL across the entire disease spectra, five parasitic strains were used that included K 27 and JISH 118 isolated from patients with CL, LV 81 and L 280 isolated from patients with MCL while MON 29 and 2001 were isolated from patients with VL.

AVL showed anti-promastigote activity

The promastigote form of the parasites can be easily maintained *in vitro* following isolation from patients, therefore allowing for rapid, preliminary screening of anti-leishmanial compounds. The anti-promastigote efficacy of AVL was evaluated by the MTT assay wherein the conversion of MTT to formazan by mitochondrial dehydrogenases is an indicator of cell viability; accordingly, a decrease in formazan production directly correlates with decreased cell viability. Screening of anti-promastigote activity with AVL (0 to 300 μg/ml) revealed a dose-dependent anti-promastigote activity in all the strains (Fig. 1), and the IC_{50} of K 27, JISH 118, LV 81, L 280 and MON 29 was 176 μg/ml, 102.5 μg/ml, 182 μg/ml, 177.5 μg/ml, and 167.5 μg/ml respectively (Table 1). The maximal concentration of DMSO used was 8% and caused minimal cell death (data not shown). It therefore can be assigned that AVL possesses a direct

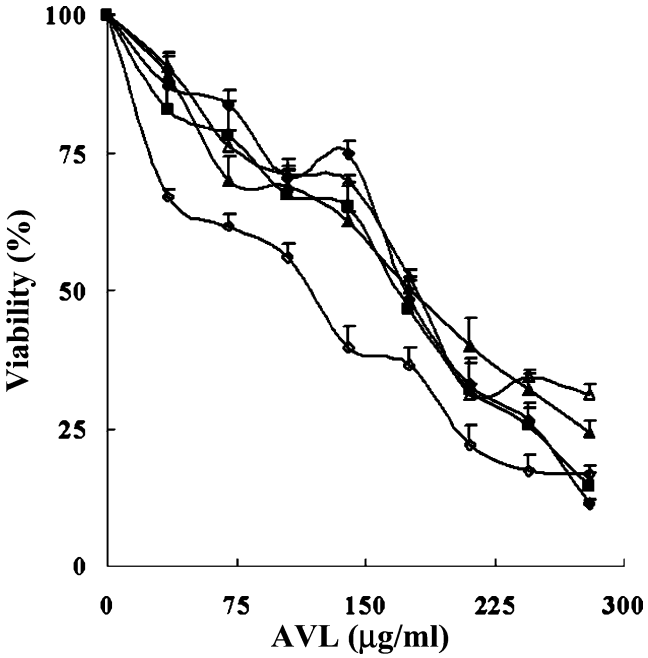

Fig. 1 Estimation of IC_{50} of AVL in promastigotes isolated from patients with cutaneous, mucocutaneous, and visceral leishmaniasis. Promastigotes (1×10^5/250 μl/well) of MHOM/SU/74/K27 (K 27, -♦-), MHOM/SA/85/JISH118 (JISH 118, -◇-), MORY/BR/72/M1824 (LV 81, -▲-), MHOM/PE/66/L280 (L 280, -Δ-) and MHOM/ES/81/BCN 1 (MON 29, -■-) were incubated with increasing concentrations of AVL (0–300 μg/ml) for 72 h and the MTT assay was performed as described in Materials and methods. Each point corresponds to the mean ± SD of at least three experiments in duplicates

parasiticidal effect on *Leishmania* promastigotes, irrespective of the species, suggesting its efficacy in all forms of Leishmaniasis.

AVL was effective against *L. donovani* axenic amastigotes

In the mammalian host, promastigotes transform into amastigotes after invading macrophages. Therefore, amastigotes represent the parasite form responsible for disease pathogenicity and are, understandably, the biologically relevant form. Anti-amastigote activity can be evaluated either in an *ex vivo* amastigote-macrophage model or an *in vivo* animal model. However, the mandatory technical expertise and high cost precludes its widespread applicability. Studies using axenically grown amastigotes are becoming an acceptable alternative as they combine the advantages of being a rapid primary screen using the clinically relevant stage of the parasitic life cycle [15]. Axenic amastigotes manifest all seven proteins of the amastigote-specific A2 gene family, down regulate synthesis of lipophosphoglycan (LPG), and possess amastigote like metabolic activities [16]. Screening of leishmanicidal activity of AVL (0 to 100 μg/ml) in axenically cultured amastigotes revealed a dose-dependent increase in anti-axenic amastigote activity in a *L. donovani* strain 2001 responsible for VL (Fig. 2), and the IC_{50} of AVL was 6.0 μg/ml. The potency of AVL to kill *leishmania* axenic amastigotes was over 25 fold higher than in promastigotes. It may be envisaged that AVL will be equally effective in amastigotes; such studies are ongoing.

AVL increased generation of reactive oxygen species (ROS) in macrophages

The activation of the macrophage is a primary mechanism to eliminate the *leishmania* parasite that is possibly accelerated by toxic metabolites of oxygen that includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO) etc. [17]. However, the parasite deviously deactivates these effector functions of the macrophage, thus allowing itself to survive and persist within the macrophage. To achieve this, the parasite employs multiple strategies leading to disease progression [18]. Macrophage inactivation inhibits IL-12 production which regulates macrophage effector molecules such as inducible nitric oxide synthase [18]; and therefore, activation of macrophages can be viewed as a *de novo* therapeutic strategy in Leishmaniasis.

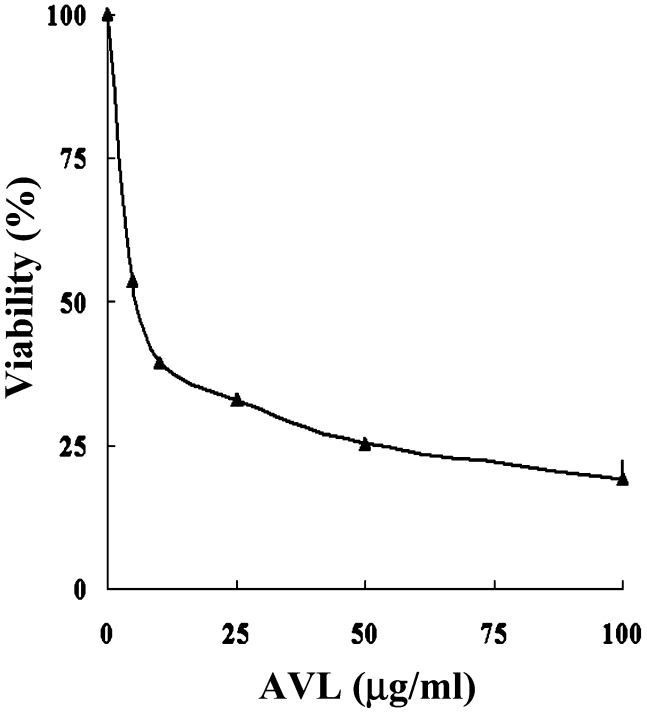

Fig. 2 Estimation of IC_{50} of AVL in axenic amastigotes. Axenic amastigotes (3×10^6/250 μl/well) of 2001 (-▲-) were incubated with increasing concentrations of AVL (0–100 μg/ml) for 96 h and cell viability was measured as described in Materials and methods. Each point corresponds to the mean ± SD of at least three experiments in duplicates

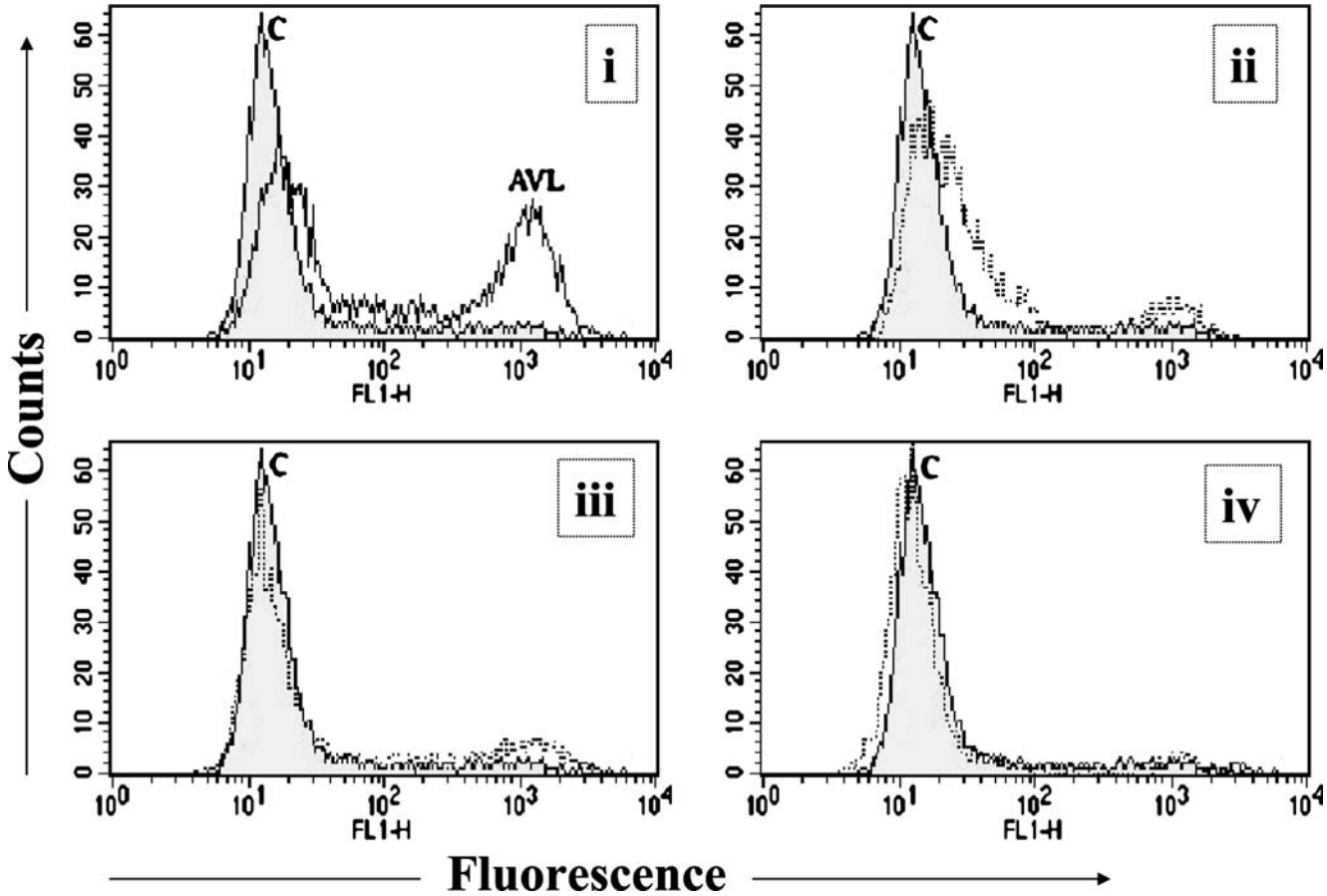

Fig. 3 Enhanced generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) within macrophages by AVL. Murine peritoneal macrophages were incubated with (i) AVL (87.5 μg/ml, -) for 2 h and following preincubation with inhibitors (- - -) namely (ii) L-NAME (iii) SOD, and catalase as also (iv) L-NAME, SOD, and Catalase as described in Materials and methods. After staining with H_2DCFDA, cells were subjected to flow cytometric analysis. In the plotted overlays, 'C' denotes the DCF-mediated fluorescence of control macrophages while 'AVL' denotes fluorescence of macrophages after 2 h AVL treatment

The increase in DCF mediated fluorescence following a 2 h incubation of macrophages with AVL (87.5 μg/ml) indicates that AVL causes activation of macrophages manifested in an enhanced ROS production (Fig. 3-i). There appear to be two subpopulations of macrophages; one subset where increased ROS production occurred whereas a second subset of macrophages was not activated by AVL.

To evaluate the contribution of NO in DCF-mediated fluorescence, macrophages were preincubated with L-NAME, a NO synthase inhibitor. The decreased activation of macrophages by AVL confirmed that AVL causes increased NO production (Fig. 3-ii). However, other ROS are also being generated as the fluorescence intensity remained slightly above control values.

A significant reduction in the fluorescence intensity of DCF following preincubation with SOD and catalase (Fig. 3-iii) also corroborated that AVL-induced production of H_2O_2 in macrophages. Furthermore, the preincubation of macrophages with L-NAME, SOD, and catalase decreased DCF fluorescence to levels comparable to baseline values (Fig. 3-iv). Collectively, the data indicates that AVL can activate macrophages resulting in the generation of ROS and reactive nitrogen species. In *L. donovani* amastigotes, reports indicated that ROS can induce a loss in mitochondrial membrane potential resulting in an apoptosis-like death [19]. Our data, therefore, suggests that AVL apart from its direct parasiticidal activity can also accentuate its anti-leishmanial activity via enhancement of ROS and RNS production in macrophages, thus making AVL a potential anti-leishmanial agent meriting further studies as a chemotherapeutic tool against Leishmaniasis.

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