Annexure 1

List of Presentations and Publications
Publications:


Communicated Manuscripts:


Presentations:


Ethanopharmacological communication

Anti-inflammatory effect of a methanolic extract of leaves of Dregea volubilis

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Nitric oxide

ABSTRACT

Aim of study: The aim of this study was to establish the anti-inflammatory activity of the methanolic extract of Dregea volubilis leaves (MEDV) with its fractions and to delineate the possible mechanism of action for MEDV.

Materials and methods: The anti-inflammatory activities of MEDV along with its petroleum ether and chloroform fractions were evaluated in a carrageenan induced model of acute inflammation. The effect of MEDV on lipopolysaccharide induced production of nitric oxide (NO) in macrophages was also studied.

Results: MEDV (100, 200 and 400 mg/kg body weight) significantly reduced carrageenan induced paw edema; chloroform fraction was most potent (66%, p<0.001). MEDV was non-toxic up to 125 μg/ml in mouse peritoneal macrophages wherein it (0-100 μg/ml) reduced lipopolysaccharide induced NO secretion (p<0.001). MEDV (100, 200 and 400 mg/kg body weight) significantly reduced carrageenan induced production of NO (p<0.001).

Conclusion: MEDV possesses significant anti-inflammatory activity. Chloroform fraction of MEDV showed best anti-inflammatory activity.

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1. Introduction

Inflammation generally occurs in response to tissue injury and is associated with the release of different mediators like bradykinin, nitric oxide (NO), vasoactive amines (histamine, serotonin, adenosine), interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) and eicosanoids (prostaglandins, thromboxanes, leukotrienes, lipoxins (Bennett and Brown, 2003). The subcutaneous injection of carrageenan causes extravasation of plasma (Szolcsanyi et al., 1998) resulting in increased exudation of water and plasma proteins along with infiltration of neutrophils. Furthermore, it is accompanied with an increased formation of arachidonic acid metabolites via the cyclooxygenase and lipoxygenase enzyme pathways (Gamache et al., 1986). Nitric oxide, synthesized by inducible nitric oxide synthase (iNOS) also contributes towards the inflammatory response and it therefore may be envisaged that a compound capable of inhibiting excessive production of NO could possess potential anti-inflammatory activity (Sarkar et al., 2005). Lipopolysaccharide (LPS), derived from the cell wall of gram-negative bacteria, activates multiple signaling pathways in macrophages and enhances production of inflammatory mediators (Chao et al., 2000). The currently available anti-inflammatory agents are effective but possess several side effects (Bennett and Brown, 2003) and therefore, it is imperative that these synthetic drugs can be replaced with compounds that are equally efficacious, but less toxic and comparatively free from side effects.

In Ayurveda, Dregea volubilis is extensively used to treat inflammation, piles, leucoderma, asthma, tumors, urinary discharge etc. (Kirtikar and Basu, 1935). Dregea volubilis, (L.f.) Benth. ex Hook. f. (Family: Asclepiadaceae) [Synonym: Wattakaka volubilis (L.f.) Stapf; Asclepias volubilis Lf.] is a large twining shrub having woody vines is widely distributed in India, Sri Lanka, Myanmar, Indonesia, Thailand and China. Dregeinin D and kaempferol have been isolated from its leaves whereas dregeosides, hyperoside, dregevinin A and P as also drebyosogenin were isolated from its seeds, stem and roots respectively (Anonymous, 1976; Yoshimura et al., 1983, 1985). Furthermore, Sabu et al. (2002) isolated three novel polyoxypregane glycosides doodibilioside A, B, C along with dregevinin D and P from the flowers. Additionally, Biswas et al. (2009) isolated an anti-inflammatory pentacyclic triterpenoid taraxerol from fruits of the plants. Similar attempts were made by Divya et al. (2008) and Nandi et al. (2009) with other extracts of the plant. The purpose of the present work was to investigate the anti-inflammatory activity of a methanolic extract from leaves of Dregea volubilis (MEDV) and...
its fractions as also establish the possible mechanism of action of MEDV.

2. Materials and methods

2.1. Plant material

Fresh leaves of *Dregea volubilis* were collected from Ramchandrapur, District of South 24 Parganas, West Bengal, India, in November (2008) and identified by Botanical Survey of India, Howrah, India. The voucher specimen was deposited in the herbarium of Division of Pharmaceutical Chemistry and Medicinal Chemistry, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, for future reference. The shade dried leaves were powdered and stored in air-tight containers.

2.2. Preparation of extract

The powdered leaves were extracted in a Soxhlet apparatus using methanol (CDH, India). The solvent was removed under vacuum and the crude extract (MEDV, 27%) was stored in a desiccator. Crude MEDV (20.0 g) was suspended in water and partitioned with petroleum ether (40-60 °C), chloroform and ethyl acetate; the fractions were individually vacuum concentrated. Crude MEDV, petroleum ether fraction (MEDVPF, 8%, w/w) and the chloroform fraction (MEDVCF, 4%, w/w) were suspended in 1% Tween 80 prior to each animal experiment, whereas for the cytotoxicity assay, MEDV (500 mg) was solubilized in DMSO. The MEDVPF and MEDVCF fractions were taken based on phytochemical similarity with the parent extract.

2.3. Phytochemical screening

Preliminary phytochemical screening was performed for alcohols, steroids, carbohydrates, tannins, oils, proteins, triterpenoids, deoxy-sugar, cadenoloids, flavonoids, cyanogenic and coumarin glycosides (Khandelwal, 2006).

2.4. Test animals

Wistar rats of either sex, weighing 120-140 g, were used. They were acclimatized for one week under controlled conditions of temperature (25 ± 2 °C), light/dark cycle of 12 h each and fed a standard diet with water ad libitum. The study had received prior approval from the Institutional Animal Ethical Committee, Pharmacy College, Azamgarh, India (937/c/06/CPCS/A).

2.5. Toxicity study

Acute toxicity and determination of LD₅₀ (upstairs and downstairs method) was performed as previously described (Ghosh, 2008). Further toxicity study was done with rat for a single fixed dose of 2 g/kg bodyweight (b.w.) according to the OECD guidelines (No. 420).

2.6. Carrageenan induced model of acute inflammation

The acute inflammatory response was measured using carrageenan (0.1 ml, 1%, w/v, in normal saline, Winter et al., 1962) wherein rats were divided into seven groups (n=6 per group). One hour prior to carrageenan injection, the control group (Group I) received 1% Tween 80, groups II, III and IV received crude MEDV (100, 200 and 400 mg/kg b.w.); groups V and VI received MEDVPF and MEDVCF (100 mg/kg b.w.), respectively while Group VII received indomethacin (10 mg/kg b.w. in 1% Tween 80, Sarkar et al., 2005). p.o. The paw volumes were measured immediately before and 1-24 h following carrageenan injection using a plethysmometer with slight modifications, according to Roy et al. (1980). The percentage inhibition was calculated according to Sarkar et al. (2005).

2.7. Preparation of mouse peritoneal macrophages

Peritoneal macrophages were collected as previously described (Sarkar et al., 2005) and macrophage viability (>95%) confirmed by the Trypan blue dye exclusion technique.

2.8. Cytotoxicity assay

The viability of macrophages against MEDV was determined after incubating macrophages (2 × 10⁶ cells/200 μl in RPMI phenol red-free medium, Medium A) in 96-well tissue culture plates. Cells were incubated with MEDV (0-500 μg/ml) at 37 °C, 5% CO₂ for 48 h. At the end of drug exposure, MTS [(3-(4,5-dimethyl thiazole-2-yl)-5-(carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H tetrazolium)] assay was performed (Barltrop et al., 1991; Sarkar et al., 2008). DMSO, the vehicle, was non-toxic up to 0.5%, and therefore all experiments were performed at a conc. lower than 0.5%.

2.9. Measurement of nitric oxide (NO) production in macrophages

Peritoneal macrophages (2 × 10⁶ cells/ml in Medium A) were seeded in 6 well tissue culture plates and incubated at 37 °C, 5% CO₂ for 1-4 h for adherence. Cells were then treated with LPS (10 μg/ml) in the presence or absence of MEDV (0-100 μg/ml) for 48 h, after which the standard Griess assay was performed (Hibbs et al., 1988). The nitrite levels were assayed using a standard curve generated with sodium nitrite as standard and N-monomethyl l-arginine (NMMA, 100 μM) was used to confirm the assay specificity.

2.10. Statistical analysis

All results were expressed as mean ± S.E.M. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey test using computerized GraphPad Prism version 4.03, Graph Pad Software Inc.

3. Results

3.1. Phytochemical screening

MEDV was positive for saponins, steroids and cadenoloids.

3.2. Toxicity study

In the LD₅₀ study, all mice survived with a dose up to 2.0 g/kg b.w., p.o. No death was also observed with rats at 2.0 g/kg b.w. There were no signs of toxicity, change in color of skin, eye and mucous membrane as also no changes were observed in their respiratory rate or behavior pattern during the 14 days observation period. There was no tremor, convulsion, diarrhea observed during the study period.

3.3. Anti-inflammatory activity

MEDV (100, 200 and 400 mg/kg b.w.) caused a dose-dependent decrease in pedal inflammation as compared to control (Table 1), activity being maximal at the 5th hour causing 31% (p < 0.05), 35% (p < 0.01) and 48% (p < 0.001) inhibition respectively. The petroleum ether (MEDVPF, 100 mg/kg b.w.) and chloroform fractions (MEDVCF, 100 mg/kg b.w.) were more effective than MEDV (60% and 66%
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema volume in ml (X inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>MEDV 100 mg/kg</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>MEDV 200 mg/kg</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>MEDV 400 mg/kg</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>MEDV 100 mg/kg</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>MEDV 200 mg/kg</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Indomethacin 10 mg/kg</td>
<td>0.29 ± 0.02</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (n=6).

**References**


Barhop, J.A., Greene, T.C., Coy, J.G., Coy, J.G., 1991. 5-(3-Carbomethoxyphenyl)-2-(4-sulphophenyl)-1,3,4-oxadiazole (SDF) and related analog of 5-(3-sulphophenyl)-2- demethylbenzamide (SDF) reducing to purple water soluble formazans as cell-viability indicators. Bioorganic Medical Chemistry Letters 1, 611-614.


Anti-inflammatory effect of allylpyrocatechoi in LPS induced macrophages is mediated by suppression of iNOS and COX-2 via the NF-kappa B pathway. International Immunopharmacology 8, 1264–1271.


Anti-inflammatory effect of allylpyrocatechol in LPS-induced macrophages is mediated by suppression of iNOS and COX-2 via the NF-κB pathway

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Abstract

The crude ethanol extract of Piper betle leaf is reported to possess anti-inflammatory activity which has been suggested to be mediated by allylpyrocatechol (APC). In the present study, we have demonstrated the anti-inflammatory effects of APC (10 mg/kg, p.o.) in an animal model of inflammation. To investigate the mechanism(s) of this anti-inflammatory activity, we examined its effects on the lipopolysaccharide (LPS)-induced production of NO and PGE2 in a murine macrophage cell line, RAW 264.7. APC inhibited production of NO and PGE2 in a dose dependent manner as also decreased mRNA expression of iNOS, COX-2, IL-12p40 and TNF-alpha. Since nuclear factor-κB (NF-κB) appears to play a central role in transcriptional regulation of these proteins, we investigated the effects of APC on this transcription factor. APC inhibited LPS induced nuclear factor-kappaB (NF-κB) activation, by preventing degradation of the inhibitor kappaB (κB). Taken together, our data indicates that APC targets the inflammatory response of macrophages via inhibition of iNOS, COX-2 and IL-12 p40 through down regulation of the NF-κB pathway, indicating that APC may have therapeutic potential in inflammation associated disorders.

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1. Introduction

Inflammation is a complex stereotypical response of the body to cell damage and vascularized tissues. The inflammatory response is phylogenetically and ontogenetically the oldest defense mechanism that is controlled by cytokines, products of the plasma enzyme systems (complement, coagulation, clotting, kinin and fibrinolytic pathways), lipid mediators (prostaglandins and leukotrienes) released from different cells, and by vasoactive mediators released from mast cells, basophils and platelets [1]. A key player in inflammation is the macrophage whose wide tissue distribution makes them well suited to provide an immediate defense against foreign elements prior to leukocyte migration [2]. As macrophages participate in both adaptive immunity, via antigen presentation and IL-12 production, and innate immunity against bacterial, viral and fungal pathogens, they display a range of functional and morphological phenotypes.

Pharmacological options for the treatment of inflammatory diseases that are often chronic are associated with severe side effects, and therefore the search for less toxic yet equally efficacious compounds is an area of intense research [3]. Piper betle (Piperaceae), a perenni climber, has its ethno medicinal properties traced back to Sanskrit literature as early as 3000 B.C. Its medicinal properties as a carminative, aromatic, digestive and stimulant are described in the Susruta Samhita, a medical-scientific treatise on the indigenous Ayurvedic system of medicine [4]. The cytoprotective role of R. betle [5] as also its radioprotective [6], antimicrobial [7], antifungal [8] and anti-inflammatory activity [9] are well documented. However, the key phytoconstituents contributing to these pharmacological activities remains to be identified. Very recently, we have reported that the phenols, chevibetol (CHV), and allylpyrocatechol (APC), and their respective glycosides are the major constituents of R. betle leaves [9,10] but the phytoconstituent(s) responsible for the anti-inflammatory activity of R. betle have not been established. In this present study, we have examined the anti-inflammatory activity of allylpyrocatechol (APC), isolated from the R. betle leaf ethanol extract and investigated its biochemical mechanism of action.

2. Materials and methods

2.1. Materials

The R. betle leaves were collected from the local market and identified (collection no. 2610) taxonomically by the Botanical Survey of India, Indian Botanical Garer, West Bengal. The mouse monocyte-macrophage cell line RAW 264.7, was obtained from National Centre for Cell Sciences, Pune. N,1-naphtyl ethylene diamine dihydrochloride (Loba Chemie Pvt. Ltd., Mumbai, India), sulphanilamide, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS) (SRL, Mumbai, India), (4-3-dimethylazolid-2-yl)-5-(2-carboxyoxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS, Promega, Madison, Wisconsin, USA), RNeasy® Kit (Ambion, Austin, Texas, USA), Limulus amoebocyte lysate (LAL) assay kit (Bio Whittaker, Germany), one step RT-PCR kit (Olegen, Hilden, Germany), primers (Sigma, Genosys, USA), prostaglandin E2 EIA kit monoclonal (Cayman Chemical Company, Ann Arbor, USA), and antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were used. All other chemicals were obtained from Sigma Aldrich (St. Louis, Missouri, USA). The acute inflammatory response was measured using dextran (2% in 0.9% NaCl w/v) as the phlogistic agent [11]. The experimental animals were divided into 3 groups (n=6). One hour after administration of APC (10 mg/kg b.w., p.o.) or the positive control, indomethacin (10 mg/kg b.w., p.o.), dextran (0.1 ml) was injected s.c. in the sub-planteral region of the left hind paw. The left paw volumes were then measured plethysmometrically for three consecutive hours, and the % inhibition of edema formation was calculated using the formula [12]:

\[
\frac{(E_c - E_t)}{E_c} \times 100
\]

where \(E_c\) and \(E_t\) are the edema volumes of the control and the treated animals.
2.6. Cytotoxicity assay

RAW 264.7 cells (2 × 10^5/ml well) were seeded in 96 well tissue culture plates and allowed to adhere for 1–3 h at 37 °C, 5% CO₂. Following addition of APC (0–25 μg/ml), the cells were incubated for 48 h and the MTS–PMS assay was performed [13].

2.7. Determination of nitric oxide

The presence of nitrite, a stable oxidation product of NO accumulating in the culture medium was measured as an indicator of NO production by the Griess assay [14]. After incubating the RAW 264.7 cells (1 × 10^6/ml well) in 6 well tissue culture plates at 37 °C, 5% CO₂ for 1–3 h, they were treated with lipopolysaccharide (LPS, 10 μg/ml) in the presence or absence of APC (0–5 μg/ml) for 48 h. Equal volumes of supernatant and Griess reagent (mixture of 0.1% NED in water and 1% sulphanilamide in 5% phosphoric acid) were mixed, the mixture was kept in the dark at room temperature for 10 min and absorbances were measured spectrophotometrically at 546 nm. The nitrite levels in culture supernatants were evaluated using a standard curve, generated with sodium nitrite (0–100 μM).

The intracellular NO was measured using dianisidine fluorescein diacetate (DAFDA), a non-fluorescent dye that fluoresces on reaction with NO [15]. The RAW 264.7 cells (1 × 10^6 cells/ml/well) were incubated with APC (5 μg/ml) for 2 h at 37 °C, 5% CO₂ in the presence of LPS (10 μg/ml). The cells were then resuspended in sheath fluid, containing DAFDA (10 μM) and incubated further for 30 min at 37 °C, 5% CO₂. The reaction was terminated by placing the cells on ice for 10 min, and flow cytometry (FACS Calibur, Becton Dickinson) was carried out using forward scatter vs. side scatter to gate the macrophage population, and a FL1 histogram to quantify geometric mean fluorescence (GMFC) of viable macrophages. The analyses were done using BD Cell Quest Pro software [9].

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from RAW 264.7 cells, after treatment with LPS in the presence and absence of APC (2.5 or 5 μg/ml) using the RNeasy kit. Subsequently, RT-PCR was carried out with the one-step RT-PCR kit using RNA (125 ng/reaction) that was reverse-transcribed into cDNA and amplified, using gene specific primers for 1-actin (sense: 5′-TGGACATTGCTGGCATCAGAAGC-3′, anti-sense: 5′-TAAAGGACGACTGAGCACTGACGAGG-3′), anti-sense: 5′-CATGGCTTGGCCTGAGGAGTCTCTCAAGG-3′, anti-sense: 5′-GCCAGCATCCCTTGTGAGGATCTT-3′; IL-12 p40 (sense: 5′-CAGAAGCTAACCATCTCCTGGTTTG-3′, anti-sense: 5′-TCCGGAG- CTAGACTTTTACAGCTC-3′) IL-12 p40 (sense: 5′-TCCGGAG- CTAGACTTTTACAGCTC-3′) IL-12 p40 (sense: 5′-TCCGGAG- CTAGACTTTTACAGCTC-3′); TNF-a (sense: 5′-GGGCTTGCCCCTGGAAGTTTCTCTTCAAAG-3′, anti-sense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), iNOS (sense: 5′-GCAAAGCAATACTGCTGTTTGTGTT-3′, anti-sense: 5′-TCCGGAG-TAATTGGCTGCTCCACACG-3′); iNOS (sense: 5′-GGGAGACTATCAGAA-TAGATG-3′, anti-sense: 5′-ATGGTCACAGTAGATGTTTATACGTC-3′) and TNF-a (sense: 5′-GGCAAGGCTCCTTGGACGCTTTCACGTC-3′, anti-sense: 5′-ACATCCAGGCCTTCAGTGAAGCCG-3′). For reverse transcription, all samples were subjected to an initial incubation at 50 °C for 30 min followed by an initial PCR activation (95 °C for 15 min) as per the manufacturers instructions. For INOS, samples were submitted to 36 cycles of denaturing (94 °C for 1 min) followed by annealing (54 °C for 1 min) and extension (72 °C for 2 min). The reactions were carried out at 50 °C for 40 min, followed by 25 cycles of denaturating (94 °C for 45 s), annealing (55 °C for 40 s) and extension (72 °C for 30 s) [15]. With regard to 1-actin, IL-12 p40 and TNF-a, samples underwent 35 and 28 cycles of denaturing (94 °C for 30 s) respectively, annealing (58 °C for 45 s), and extension (72 °C for 30 s) [17,18]. After a terminal extension step at 72 °C for 10 min, RT-PCR products were resolved by electrophoresis on 1.5% agarose gels containing ethidium bromide (0.5 μg/ml), and visualized with the Molecular Imager ChemiDoc XRS System (Bio Rad, California, USA). The expression of iNOS, IL-12 p40, COX-2 and TNF-a was quantified by densitometry using Total Lab Nonlinear Dynamic Image analysis software (Nonlinear USA, Inc., Durham, NC).

2.9. Western blotting

RAW 264.7 cells were incubated with APC (2.5 or 5 μg/ml) in the presence and absence of LPS (10 μg/ml) for 24 h. The cells were lysed (50 mM Tris (pH 7.5), 50 mM EDTA (pH 7.5), 2% in phenylmethane sulphonyl fluoride (PMSF) in isopropanol, 2 mg/ml leupeptin in water, aprotinin and 1-mercaptoethanol) and the protein content was estimated [19]. Equal amounts of total cellular proteins (50 μg) were resolved on SDS-polyacrylamide mini gels (10%) and transferred to nitrocellulose membranes. Following blocking of non-specific binding sites with blocking buffer (20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 3% bovine serum albumin) for 1 h, membranes were incubated overnight with anti-phosphorylated e-I (1:1000 dilution in 0.5% BSA in TBS) [20]. The membranes were subsequently probed with alkaline phosphatase conjugated anti-rabbit IgG, and visualized using nitroblue tetratolizolium (50 mg/ml)/5-bromo-4-chloro-3-indolyl phosphate (15 mg/ml in H₂O).

2.10. PgE₂ assay

Prostaglandin E₂ (PGE₂) was measured using a commercially available kit. Briefly, RAW 264.7 macrophages (8 x 10^5/well) were incubated with LPS (10 μg/ml) in the presence of APC (2.5 or 5 μg/ml) for 24 h. The PGE₂ levels in supernatants (50 μl) were calculated as described in Materials and methods. Each value represents the mean±S.D. of at least three experiments. *p<0.001 and †p<0.005 as compared to controls.

2.11. Statistical analysis

The results are expressed as mean±S.D. Statistical analysis was evaluated using Graph Pad Prism software and Student's t test, p<0.05 was considered as statistically significant.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Anti-inflammatory activity of APC in dextran-induced model of acute inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>%Edema volume (ml) (X Inhibition)</td>
</tr>
<tr>
<td>1st hour</td>
<td>2nd hour</td>
</tr>
<tr>
<td>Control</td>
<td>0.78±0.09</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.2±0.03</td>
</tr>
<tr>
<td>APC (10 μg/kg bw)</td>
<td>0(33.33%) (76.50%) (83.87%)</td>
</tr>
<tr>
<td>APC (10 μg/kg bw)</td>
<td>0.33±0.13</td>
</tr>
<tr>
<td>(58.06%) (68.75%) (74.19%)</td>
<td></td>
</tr>
</tbody>
</table>

*Acute inflammation was induced using dextran; edema volume (ml) was measured plethysmometrically for 3 h and % Inhibition was calculated as described in Materials and methods. Each value represents the mean±S.D. of at least three experiments. *p<0.001 and †p<0.005 as compared to controls.
3. Results

3.1. Isolation and characterization of allylpyrocatechol

The fraction eluting with 10% ethyl acetate/hexane furnished CHV (0.16% w/w of the extract) and APC (yield: 0.9% w/w of the extract) as light yellow oils, which were then characterized from their respective spectral data. The chemical structures of CHV and APC are shown in Fig. 1 and following are their spectral data:

(i) 3-Hydroxy-4-methoxyallylbenzene (chevibetol, CHV): HPLC ret. time: 9.5 min (single peak); IR (film): 3509, 3070, 3003, 1638, 1509, 995, 865, 760 cm⁻¹; ¹H NMR (CDCl₃): δ 3.28 (d, J=6.5 Hz, 2H, ArCH₂), 3.85 (s, 3H, OCH₃), 5.01–5.09 m (m, 2H, olefin), 5.58 (broad, 1H, Ar-OH), 6.63–6.79 (m, 3H, ArH); ¹³C NMR (CDCl₃): δ 36.1, 55.7, 114.8, 118.2, 122.4, 130.4, 132.2, 141.4, 147.5; MS (m/z, rel. Int.): 56 (37), 66 (39), 78 (67), 92 (100), 103 (43), 105 (40), 121 (31), 132 (25), 150 (26), 165 (M+1, 37).

(ii) 3,4-Dihydroxyallylbenzene (allylpyrocatechol, APC): HPLC ret. time: 6.7 min (single peak); IR (film): 3398, 1606, 1444, 964, 863, 789 cm⁻¹; ¹H NMR (CDCl₃, 200 MHz): δ 3.28 (d, J=6.5 Hz, 2H), 4.99–5.08 (m, 2H), 5.36 (broad s, 2H), 5.80–6.01 (m, 2H); ¹³C NMR (CDCl₃, 50 MHz): δ 38.1, 55.7, 114.8, 118.2, 122.4, 130.4, 132.2, 141.4, 147.5; MS (m/z, rel. Int.): 56 (37), 66 (39), 78 (67), 92 (100), 103 (43), 105 (40), 121 (31), 132 (25), 150 (26), 165 (M+1, 37).

Figure 2 Effect of APC on NO production. A: RAW 264.7 cells (1 x 10⁶/ml/well, 1) were incubated with LPS (10 µg/ml, 2) in the presence of APC (2.5 µg/ml, 3) or 5 µg/ml, 4] at 37 °C, 5% CO₂ for 48 h and assayed for levels of extracellular NO as described in Materials and methods. Each point represents the mean ± S.D. of NO₂ (µM) of at least three experiments in duplicate. B: RAW 264.7 cells (1 x 10⁶/ml/well, 1) incubated with LPS (10 µg/ml) in the absence (--) or presence of APC (5 µg/ml, ---) at 37 °C, 5% CO₂ for 2 h and assayed for levels of intracellular NO as described in Materials and methods. The data is a representative profile of three experiments. Inset: RAW 264.7 cells were incubated with LPS, in the absence (1) or presence of APC (5 µg/ml, 2). The results are the mean ± S.E.M. of the geometric mean fluorescence channel (GMFC) of at least three experiments in duplicate. C: RAW 264.7 cells (1) were treated with LPS (10 µg/ml, 2) in conjunction with APC (2.5 µg/ml, 3) or 5 µg/ml, 4) for 24 h. RNA was isolated and subjected to RT-PCR as described in Materials and methods. RT-PCR products of β-actin and iNOS were resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of iNOS (■) was quantified densitometrically with Total Lab software. The results are representative of three experiments.
The LAL assay confirmed that endotoxin is practically absent in APC.

3.2. APC reduces dextran-mediated acute inflammatory response in rats

Dextran, an established phlogistic agent induces an inflammatory response resulting in edema. Accordingly, reduction of edema volume by a test compound gives a measure of its anti-inflammatory potential. Initially, both CHV and APC were tested for their activity against dextran-induced inflammation. As APC showed a more pronounced reduction in dextran-induced paw edema, it was selected for this study. Compared to control animals, APC (10 mg/kg b.w., p.o.) effectively reduced the edema by 74.19%, which was comparable with indomethacin (10 mg/kg b.w.), an established anti-inflammatory drug that caused 83.87% reduction in edema volume (Table 1). APC (10 mg/kg b.w., p.o.) effectively reduced the edema by 74.19%, which was comparable with indomethacin (10 mg/kg b.w.), an established anti-inflammatory drug that caused 83.87% reduction in edema volume (Table 1).

3.3. APC causes reduction of nitric oxide (NO) production and inhibition of iNOS

LPS, a major cell wall component of gram-negative bacteria induces a strong innate and adaptive immune response and was used to elicit increased NO synthesis. As the half-life of NO is very short, we used nitrite production as an indicator of NO released by LPS activated macrophages. Compared to normal macrophages, administration of LPS increased NO production >4 fold, the mean ± SD being 66.32 ± 0.73 vs. 16.17 ± 0.14, p < 0.005 (Fig. 2A). APC reduced LPS-induced NO production in a dose-dependent manner, as with 2.5 and 5 µg/ml, the production of NO decreased by 69.31% (6 fold, 20.33 ± 0.11, p < 0.001), and 82.92% (7 fold, 11.33 ± 0.33, p < 0.001) respectively as compared to LPS-treated macrophages (Fig. 2A). DMSO, the vehicle control, had no effect on NO production (61.54 ± 1.71) which reconfirmed its immunological inertness. Furthermore, APC itself caused minimal change in production of NO in resting macrophages. In parallel, a cell viability assay was performed which confirmed that the observed reduction in NO production by APC was not associated with loss of cell viability (data not shown).

This was corroborated by measurement of intracellular NO using DAF-DA. Compared to background fluorescence, LPS increased intracellular NO from basal levels by 76.50%, mean ± SEM of GMFC being 155.46 ± 7.49 vs. 88.08 ± 2.97, p < 0.05, (Fig. 2B). APC (5 µg/ml) effectively reduced the GMFC to 80.08 ± 2.58, p < 0.05, comparable with naive macrophages.

To further elucidate the mechanisms involved in inhibition of NO generation by APC in LPS-activated macrophages, we analyzed its effect on LPS-induced iNOS gene expression in macrophages. RAW-264.7 cells in normal conditions express non-detectable levels of iNOS mRNA, which increased markedly after 24 h of LPS stimulation (Fig. 2C). With the addition of APC (0–5 µg/ml), a dose dependent inhibition of iNOS expression was demonstrated indicating that APC modulated expression of iNOS.

Figure 3 Effect of APC on expression of pro-inflammatory cytokines. A: RAW 264.7 cells (1) were treated with LPS (10 µg/ml, 2) in conjunction with APC [2.5 µg/ml, (3) or 5 µg/ml, (4)] for 24 h. RNA was isolated and subjected to RT-PCR as described in Materials and methods. RT-PCR products of β-actin and IL-12 p40 mRNA were resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of IL-12 p40 was quantified densitometrically with Total lab software. The data is a representative profile of three experiments. B: RAW 264.7 cells (1) were treated with LPS (10 µg/ml, 2) in conjunction with APC [2.5 µg/ml, (3) or 5 µg/ml, (4)] for 24 h. RNA was isolated and subjected to RT-PCR as described in Materials and methods. RT-PCR products of β-actin and TNF-α mRNA were resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of TNF-α was quantified densitometrically with Total lab software. The data is a representative profile of three experiments.
Anti-inflammatory effect of APL in LPS induced macrophages mediated by iNOS/CGX-2 via NF-κB

dependent manner (Fig. 3A). In case of TNF-α, with the addition of LPS, a small increase in the expression of TNF-α occurred, which was slightly attenuated by APL (Fig. 3B).

3.5. APC reduces release of inflammatory mediators in macrophages

Prostaglandin E₂ (PGE₂), the most important inflammatory product of cyclooxygenase-2 (COX-2) was quantified in supernatants of LPS-treated RAW 264.7 macrophages [22]. PGE₂ has a very short half-life and is converted to 13,14-dihydro-15-keto PGE₂, which is more stable, and its measurement indicates the amount of PGE₂ (pg/ml) present. Compared to unstimulated macrophages, addition of LPS dramatically increased the PGE₂ level by 55 fold, mean±SD being 132.5±10.6 vs. 7312.5±1361.6 respectively, p<0.002. With the addition of APC (2.5 or 5 pg/ml) a dose dependent reduction in PGE₂ was observed, being 14.63 fold (93.16%, 500.0±14.1, p<0.002) and 22.5 fold (95.56%, 325.0±91.9, p<0.002) respectively (Fig. 4A).

In order to determine the mechanism by which APC reduced LPS-induced PGE₂ production, we studied the ability of APC to influence LPS-induced expression of COX-2. The addition of LPS resulted in a clearly defined increase in COX-2 expression that was markedly attenuated in a dose dependent fashion when treated with APC (Fig. 4B) corroborating that APC induced a decrease in COX-2 which translated into a dramatic decrease in PGE₂.

3.6. APC reduced IκB phosphorylation

In resting, unstimulated macrophages, the heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, IκB [23]. As it has been reported that LPS-mediated activation of NF-κB correlates with the hyperphosphorylation of κB-alpha and its subsequent degradation, we examined the phosphorylated levels of IκB by immunoblot analysis. As expected, treatment with LPS enhanced phosphorylation of IκB protein, evidenced by increased binding of phospho-specific IκB antibody, as compared to controls (Fig. 5). This was prevented by the addition of APC (2.5 or 5 pg/ml), which blocked at a transcriptional level, accounting for its inhibition of NO production.

3.4. APC inhibits expression of pro-inflammatory cytokines

Macrophages upon stimulation by LPS secrete several pro-inflammatory cytokines including IL-1β, IL-6, IL-12 and TNF-α [21]. Accordingly, we examined the effect of APL on LPS-induced expression of IL-12p40 and TNF-α. With regard to IL-12p40, a subunit of the heterodimeric cytokine, LPS induced a dramatic increase in its expression, which was decreased by APL in a dose dependent manner (Fig. 3A). In case of TNF-α, with the addition of LPS, a small increase in the expression of TNF-α occurred, which was slightly attenuated by APL (Fig. 3B).
phosphorylation and degradation of IκB protein, thus preventing nuclear translocation of NF-κB.

4. Discussion

For centuries, dietary and medicinal phytochemicals have been used as anti-inflammatory remedies, but identifying their active component(s) has only recently been initiated. The leaves of *P. betle* have been reported to possess a wide range of pharmacological activities, but the key phytoconstituents have not been pinpointed. The cytoprotective and anti-oxidant activity of APC was shown to cause attenuation of indomethacin induced gastric ulceration in rats [24], prompting further pharmacological elaboration of the underlying pathways involved.

Acute inflammation is a short-term process characterized by swelling, redness, pain, heat generation, and loss of function, caused by infiltration of plasma and leukocytes in the tissues. It occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed [25]. Dextran is a complex polysaccharide composed of many glucose units of various lengths. The paw edema induced by dextran is known to be mediated by histamine and serotonin, known mediators of inflammation [26]. The efficacy of APC (10 mg/kg b.w.) in reducing dextran-induced acute inflammation (Table 1) prompted us to delineate the underlying molecular mechanisms contributing to the observed anti-inflammatory action.

NO is an important inflammatory mediator and an important regulatory molecule for various physiological functions such as neurotransmission, vasodilatation and importantly for host-defense [27]. NO is generated during nitric-oxide synthase (NOS)-catalyzed conversion of L-arginine to citrulline. NOS exists in three isoforms, namely endothelial NOS (eNOS), neuronal NOS (nNOS), which are constitutive, and inducible NOS (iNOS), produced by activated macrophages [28]. Though NO plays a pivotal role in many body functions, its excess production especially in macrophages can lead to cytotoxicity, inflammation, carcinogenicity and autoimmune disorders [29,30]. Therefore, NO inhibitors are essential for prevention of inflammatory diseases. APC caused a dose dependent decrease in extracellular and intracellular production of NO (Fig. 2A and B), which was attributed to its ability to down regulate the mRNA expression of iNOS (Fig. 2C).

The pro-inflammatory cytokines are principal factors for aggravation of inflammatory diseases. Stimulation of macrophages by LPS released from gram negative bacteria leads to induction of several pro-inflammatory cytokines such as IL-1α, IL-6, IL-12 and TNF-α. Of these, IL-12 is a heterodimer composed of two subunits α and β, which are encoded by two separate genes. Increased NO production by iNOS in pathological states is also associated with up regulated IL-12 p40 levels [31]. Similarly, the enhanced expression of IL-12p40 by LPS was decreased by APC concomitant with reduction in NO production and iNOS expression, thus inhibiting LPS induced macrophage activation (Fig. 3A).

TNF-α is a cytokine possessing both growth stimulating as well as inhibitory properties and is mainly secreted by the macrophages, though others, like mast cells, adipose tissue, and fibroblasts also secrete TNF-α. It is involved in a wide variety of cellular reactions like proliferation, differentiation, apoptosis and inflammation [32]. During inflammation, it triggers a cascade of cytokines responsible for increased mobilization of macrophages to the site of inflammation and also increases vascular permeability. In our study, addition of LPS increased the production of TNF-α but the addition of APC caused a modest attenuation of LPS induced TNF-α production (Fig. 3B). Dehydroxymethylepoxyquinomicin, a NF-κB inhibitor, similarly inhibited secretion of pro-inflammatory cytokines amongst which IL-6 and IL-12 were the most susceptible and TNF-α the least [33].

Cyclooxygenases (COX) are enzymes responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostaclitins and thromboxanes). Increased generation of NO is known to activate COX, which in turn converts arachidonic acid to prostaglandins causing pain, swelling and stiffness [34]. COX exists in two isoforms, the constitutive, COX-1 and inducible, COX-2 and is produced in abundance by activated macrophages and other cells at the site of inflammation [35]. Taken together, inhibition of COX-2 can provide relief from symptoms of inflammation and pain. PGE2, a well studied prostanoid is produced in activated cells and tissues following activation of COX-2, and is mainly released by mast cells, keratinocytes or macrophages. It increases vascular permeability along with other vasoactive components such as histamine, bradykinin or NO resulting in edema, pain and hyperalgesia at the site of inflammation [36]. In our study, the dramatic increase in PGE2 production following stimulation by LPS was sharply attenuated by APC (Fig. 4A) indicating its strong anti-inflammatory potential that was further corroborated by the concomitant decrease in expression of COX-2 (Fig. 4B).

It has been shown that in LPS-activated macrophages, NF-κB is a critical factor for expression of various pro-inflammatory cytokines along with NO and COX-2 [37]. It is a transcription factor which in the inactive state remains sequestered in the cytoplasm by IκB, a protein containing many ankyrin repeats, by virtue of which it inhibits the nuclear activation sites of NF-κB [38]. When activated, IκB gets phosphorylated and is degraded to release NF-κB, which then enters the nucleus, binds to DNA, allowing transcription of iNOS mRNA and is subsequently accompanied by increased production of NO [39]. Therefore, inhibition of IκB phosphorylation, as observed with APC (Fig. 5), would inhibit the generation of NF-κB dependent cytokines (Fig. 3A), expression of iNOS (Fig. 2C) and COX-2 (Fig. 4B), thereby reducing inflammation (Table 1).

Taken together, our study has established that APC mediates its anti-inflammatory activity via inhibition of the NF-κB pathway. This suggests that developing drugs that interfere both with the signaling routes and expression of molecules involved in the inflammatory cascade could be harnessed in a range of diseases wherein inflammation plays a critical role.

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Anti-inflammatory effect of APL in LPS induced macrophages mediated by iNOS/COX-2 via NF-κB

Fellowships from University Grants Commission and Council for Scientific and Industrial Research, Govt. of India respectively.

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[35] Ackermann C, Kavanaugh A. Tumor necrosis factor as a thera-


In vitro and in vivo activity of *Aloe vera* leaf exudate in experimental visceral leishmaniasis

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Abstract The leishmanicidal activity of *Aloe vera* leaf exudate (AVL) has been demonstrated in promastigotes and axenic amastigotes, but its effectiveness in animal models has not been evaluated. The presence of alkaloids, triterpenes, cyanidines, proanthocyanidines, tannins, and saponins in AVL was identified. Its effectiveness in four *Leishmania donovani* strains was studied both in promastigotes (IC\textsubscript{50} ranged from 70-115 \(\mu\)g/ml) and amastigotes (IC\textsubscript{50} ranged from 3.1–11.4 \(\mu\)g/ml). In amastigotes, the killing by AVL was facilitated through its induction of nitric oxide in *leishmania*-infected macrophages. The safety index was good as AVL up to 300 \(\mu\)g/ml remained nontoxic to monocytes and macrophages. In a *L. donovani* BALB/c mouse model, oral or subcutaneous administration of AVL (15 mg/kg body weight \(\times\) 5 days) reduced parasitemia by >90% in the liver, spleen, and bone marrow without impairment of hepatic and renal functions. Collectively, we conclude that AVL shows promising antileishmanial activity and may provide a new lead agent in the treatment of Leishmaniasis.

Introduction

Leishmaniasis is a vector-borne complex parasitic disease caused by obligate intra-macrophage protozoan parasite *Leishmania* and threatens almost 350 million people worldwide with about two million new cases reported each year. It is manifested in visceral, mucocutaneous, or cutaneous forms, severest being the visceral form (VL) with an estimated 500,000 new cases annually (Desjeux 2004). Progress in developing a protective vaccine against leishmaniasis has to date not been effective. The Indian subcontinent, a major endemic area of VL, is currently facing an unprecedented increase in primary unresponsiveness to sodium antimony gluconate (SAG), the first line of treatment (Chappuis et al. 2007). The chemotherapy of VL includes Amphotericin B and its lipid formulations but its limitations include parenteral administration, toxicity, and high cost (Guerin et al. 2002). Paromomycin, Sitamaquine, and combinational therapies are under the process of evaluation but are not devoid of toxicity (Chappuis et al. 2007).
The World Health Organization (WHO) has estimated that approximately 80% of individuals rely on traditional medicines (Newman et al. 2000). The harmonization of traditional and modern medicine is one of the principle goals in the progressive playground of pharmacognosy (WHO 2000).

Aloe vera, a commonly used medicinal plant of family Liliaceae, is well established for its different medicinal properties like skin burns, antimetastatic, and antimicrobial activity (Ali et al. 1999; Reynolds and Dweck 1999; Ferro et al. 2003). The Aloe vera leaf exudate (AVL) was found to be effective against promastigotes responsible for cutaneous, mucocutaneous, and visceral leishmaniasis (Dutta et al. 2007b) which is mediated by inducing programmed cell death (Dutta et al. 2007a). In this study, we demonstrate the parasiticidal effect of AVL on promastigotes and amastigotes of Leishmania donovani irrespective of their antimonial sensitivity, with minimal toxicity in monocytes, macrophages, and BALB/c mice. The in vivo efficacy of AVL (oral and subcutaneous (sc)) was established, and lack of impairment of hepatic or renal function indicates that AVL could be developed as an effective oral herbal remedy against VL.

Materials and methods

Materials

M-199 medium, Schneider’s medium, and fetal bovine serum (FBS) were obtained from Gibco-BRL, Grand Island, NY, USA; dimethyl sulfoxide (DMSO) from SRL, India; Thin layer chromatographic (TLC) plates from Merck, India; kits for estimation of serum alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum urea, and serum creatinine were from ‘Human Gesellschaft for Biochsmica and Diagnostica mbH’, Germany; Limulus amebocyte lysate (LAL) assay kit was from Bio Whittaker, Germany. All other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Parasites

Promastigotes of L. donovani were routinely cultured at 24°C in M-199 medium supplemented with 10% heat inactivated FBS and Gentamycin. The strains included in this study were two-typed strains AG83 (MHOM/IN/83/AG83); GE1F8R, a subclone of GEI (MHOM/IN/90/GEI) and two untyped strains 2001 and 39 isolated from Indian VL patients. AG83 and 2001 are SAG sensitive whereas GE1F8R and 39 are SAG unresponsive (Pal et al. 2001; Singh 2002; Dutta et al. 2005). Prior to experiments, promastigotes were transferred to a modified RPMI-1640 medium (without phenol red) containing 10% FBS and Gentamycin, referred to as Medium A.

Mammalian cell culture

Murine peritoneal macrophages were lavaged following starch induction (2% starch i.p., 2 ml/mice) and cultured in Medium A at 37°C, 5% CO₂. A human monocyte cell line, U937 was cultured in Medium A in the same environment.

Plant material and extraction

AVL was prepared as previously described (Dutta et al. 2007a, b). Briefly, fresh succulent leaves of Aloe vera were collected and crushed in an electric grinder (crude extract); the inner gelatinous portion and outer leafy coat were manually separated and similarly crushed. All three components, i.e., crude, gel, and leaf were individually tested for their antileishmanial activity. As the highest leishmanicidal activity was observed in the leaf fraction (Fig. 1a), it was lyophilized and stored at 4°C. This fraction was dissolved in DMSO (cell culture grade), referred to as leafy exudate of AVL and was used for all experiments.

Qualitative phytochemical analysis

TLC analysis of AVL was performed using silica gel TLC plates (Merck) which were sprayed with Dragendorff’s reagent and anisaldehyde-sulfuric acid to identify the presence of alkaloids and triterpenes, respectively; the presence of cyanidine, proanthocyanidine, saponin, tannin, phenol, flavans, and proanthocyanidol was tested by standard chemical tests (Harborne 1973).

Endotoxin assay

Presence of bacterial endotoxin in AVL was measured by LAL assay (US Pharmacopeia 1995) as per manufacturer’s instructions. Briefly, samples were incubated serially with LAL and chromogenic substrate, detection of endotoxin was measured by generation of p-nitroaniline at 405 nm and quantified against a standard curve of supplied bacterial endotoxin.

Analysis of in vitro antileishmanial activity

Log phase promastigotes resuspended in Medium A, were seeded in 96-well tissue culture plates (5 × 10⁴/250 μl/well). Freshly prepared crude, gel, and leaf extracts were added (0–30 mg/ml) and incubated for 72 h at 24°C. The viability of promastigotes was evaluated using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Dutta et al. 2005). Briefly, MTT was added

**Fig. 1** a Analysis of antileishmanial activity of different components of Aloe vera. L. donovani promastigotes, AGB3 (MHOM/IN/83/AG83) were incubated with increasing concentrations of Aloe vera crude extract (––), gel extract (empty squares) and leaf exudate (filled squares) as described in “Materials and methods.” Each point corresponds to the mean ± SD of three experiments in duplicates.

b Estimation of IC₅₀ of AVL in promastigotes of antimonial responsive and unresponsive strains. Promastigotes (5 × 10⁴ 250 µl⁻¹ well⁻¹) of AGB3 (MHOM/IN/83/AG83; empty circles), GE1FR (MHOM/IN/80/GE1FR; filled circles) and 39 (filled triangles) were incubated with increasing concentrations of AVL (0–300 µg ml⁻¹) 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.

c Evaluation of seasonal variation on antileishmanial activity of AVL. Promastigotes (5 × 10⁴ 250 µl⁻¹ well⁻¹) of antimonial responsive strain AG83 (MHOM/IN/83/AG83; empty circles) and antimonial unresponsive strain GE1FR (MHOM/IN/80/GE1FR; filled circles) were incubated for 72 h with increasing concentrations of AVL (0–300 µg ml⁻¹) that was collected during the rainy season (solid line) or in winter (broken line). Cell viability was evaluated by the MTT assay as described in “Materials and methods.” Each point corresponds to the mean ± SD of at least three experiments in duplicates.

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to each well and incubated in dark for an additional 4 h.

The resultant pellet was dissolved in DMSO and absorbances 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 leishmanicidal capability of AVL (0–280 µg/ml) in four parasite strains as well as probability of altered efficacy in different seasonally collected batches was similarly analyzed. The mean percentage viability was calculated as follows:

Mean specific absorbance of treated parasites × 100
Mean specific absorbance of untreated parasites

Accordingly, the 50% inhibitory concentration or the IC₅₀, i.e., the concentration that decreased cell growth by 50% was determined.

Measurement of antileishmanial activity in an ex vivo macrophage-amastigote model

Primed murine macrophages were seeded in 16-well tissue culture slides (5 × 10⁴/100 µl/well) and allowed to adhere overnight at 37°C, 5% CO₂. After removal of non-adherent macrophages, cells were infected overnight in the same environment with stationary phase promastigotes at a parasite:macrophage ratio of 10:1. Subsequently, non-phagocytosed parasites were removed by gentle washing, and infected macrophages were incubated with AVL (0–35 µg/ml) for an additional 72 h. The slides were fixed in methanol and Giemsa-stained for microscopic evaluation of amastigote viability. At least 100 macrophages/well were analyzed and IC₅₀ was calculated, considering equal proportion of DMSO treated cells as control.

Measurement of nitric oxide production by macrophages

Murine peritoneal macrophages (5 × 10⁴/250 µl/well), with or without infection with L. donovani promastigotes, were incubated with AVL (0–87.5 µg/ml) for 48 h at 37°C, 5% CO₂. The supernatants were collected and the amount of nitric oxide (NO) generated was measured using the Griess reaction (Bredt and Synder 1994). Briefly, equal volumes of culture supernatant, sulfanilamide, and N-1 napthylethylenediamine dihydrochloride were incubated at room temperature for 5 min; formation of azo compound was measured colorimetrically at OD₅₅₀. The concentration of NO was extrapolated from a standard curve of sodium nitrite.
Table 1 Qualitative phytochemical analysis of AVL

<table>
<thead>
<tr>
<th>Method</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC (spraying agent: Dragendorff’s reagent)</td>
<td>Pale yellow color obtained</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>TLC (spraying agent: anisaldehyde-sulfuric acid reagent)</td>
<td>Pale blue violet color obtained</td>
<td>Presence of triterpenes</td>
</tr>
<tr>
<td>Cyanidin test</td>
<td>Pale greenish to pale yellowish color obtained</td>
<td>Presence of cyanidine</td>
</tr>
<tr>
<td>Proanthocyanidine test</td>
<td>Pale red color was obtained</td>
<td>Presence of proanthocyanidine</td>
</tr>
<tr>
<td>Saponins test</td>
<td>Persistent foam was obtained; remained after 30 min of shaking</td>
<td>Presence of saponins</td>
</tr>
<tr>
<td>Tannins test</td>
<td>Greenish brown color was obtained</td>
<td>Presence of tannins</td>
</tr>
<tr>
<td>Phenols test</td>
<td>No blue or blue black color was obtained</td>
<td>Absence of phenols</td>
</tr>
<tr>
<td>Flavans test</td>
<td>No red coloration was obtained—the solution remained pale yellowish in color</td>
<td>Absence of flavans</td>
</tr>
<tr>
<td>Proanthocyanidols test</td>
<td>The solution did not change into red—no red coloration.</td>
<td>Absence of proanthocyanidols</td>
</tr>
</tbody>
</table>

In vivo evaluation of antileishmanial activity in a *L. donovani* BALB/c mouse model

Stationary phase promastigotes (MHOM/IN/83/AG83) were used to infect 3 to 4-week old BALB/c mice (1–2×10⁷ /animal) through tail vein. One-month post infection, parasitemia was confirmed in two arbitrarily selected animals; after which, mice were randomly grouped into four groups. Control mice received DMSO orally (5% in normal saline, Group A, n=5). AVL was administrated to two groups, namely Group B (oral) and Group C (sc); these two groups were further divided into three subgroups (n=5) to receive orally or sc one of three doses of AVL (5/15/45 mg/kg body weight (b.w.)) daily for five consecutive days. Another group (Group D) received SAG (20 mg/kg b.w. * 5 days, sc, Group D, n=5) and served as the positive control. One week post treatment, mice were killed—smeared of liver, spleen, and bone marrow were prepared, fixed in methanol, and Giemsa-stained to examine the parasite burden. At least 500 macrophages per slide were microscopically counted. Data is expressed as mean ± SD of amastigotes present per 100 host cells and the percentage of parasite removal at each point.

Promastigote transformation assay

To trace viable amastigotes in target tissues, liver, and spleen of mice that received AVL 15 or 45 mg/kg b.w. were dissected into small pieces and incubated at 24°C in Schneiders medium supplemented with 10% FCS. After 7 and 14 days, cultures were examined microscopically for presence of promastigotes.

Toxicity studies

In vitro cytotoxicity of AVL against mammalian cells

Murine peritoneal macrophages and a human monocyte cell line (U937) cultured in Medium A were exposed to increasing concentrations of AVL (0–0.3 mg/ml) in Medium A at 37°C, 5% CO₂ for 72 h. At the end of the experiment, the percentage cell viability was evaluated by a modified MTT assay (Dutta et al. 2005).

In vivo

Hepatic and renal functions of BALB/c mice were tested for evaluation of sub acute toxicity of AVL. Mice were
Murine peritoneal macrophages (1 * 10^6, empty squares) were incubated with AVL (87.5 µg ml^-1) in the absence ( ) or presence ( ) of Polymixin B (25 µg ml^-1) for 24 and 48 h as described in "Materials and methods." At the end of 24 and 48 h, culture supernatants were analyzed for NO content using Griess reagent. Similarly, murine peritoneal macrophages infected with L. donovani (1:10, ) were incubated with AVL (37.5 µg ml^-1) in the absence ( ) or presence ( ) of Polymixin B (25 µg ml^-1) for 24 and 48 h and similarly analyzed for NO production. Each point is the mean ± SD of at least three experiments in duplicates.

Orally fed increasing doses of AVL from 50 to 250 mg/kg b.w. in a stepwise manner (Ghosh 2005). The dose was increased by 50 mg every 2-3 days up to 250 mg/kg b.w. and, once this dose was achieved, it was repeated every 48 h for an additional 2 weeks. The animals were then killed, and hepatic functions were measured (ALP and SGOT) while renal function was evaluated by measurement of serum urea and creatinine levels using commercially available kits. Hepatic and renal functions were similarly evaluated in Leishmania-infected BALB/c mice as described above.

### Results and discussion

#### Qualitative phytochemical analysis

Phytochemical analysis of AVL assigned the presence of alkaloids, triterpenes, cyanidine, proanthocyanidine, saponins, and tannins (Table 1). Many plant-derived compounds have been identified for their leishmanicidal activity (Kayser et al. 2003). Alkaloids, triterpenes, and saponin-like compounds individually or synergistically have been reported to have leishmanicidal activity (Kayser et al. 2003). Triterpenoid saponins from Maesa balansae (Myrsinaceae) have been shown to be effective against Leishmania infantum (Maes et al. 2004) as also Indole alkaloids from Peschiella australis have been demonstrated to have antileishmanial activity (Maes et al. 2004). The phytochemical analysis of AVL revealed the presence of similar compounds that possibly contribute to the observed antileishmanial activity (Table 1). However, their individual contributions can only be confirmed by bioassay-guided fractionated studies.

AVL-mediated antipromastigote activity is irrespective of antimonial sensitivity

Preliminary screening of antipromastigote activity with individual components of Aloe vera crude, gel, and leaf exudate demonstrated that the leafy exudate or AVL was the most effective (Fig. 1a). Subsequently, AVL was tested in four strains isolated from Indian VL patients of which two (GE1FR and 39) were from antimonial unresponsive patients. IC50 of AVL in these two strains was comparable with the antimonial sensitive strain 2001 and AG83. A dose-dependent antipromastigote activity was observed in all four strains and the IC50 of AG83, GE1FR, 2001, and 39 was comparable being 110, 115, 70, and 95 µg/ml, respectively (Fig. 1b).

### Table 2 Effect of AVL on hepatic, splenic and bone marrow parasitemia in a L. donovani BALB/c mouse model

<table>
<thead>
<tr>
<th>Group (n=5)</th>
<th>Hepatic burden</th>
<th>Splenic burden</th>
<th>Bone marrow burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control</td>
<td>1063±21.6 (-)</td>
<td>214.7±4.5 (-)</td>
<td>481±23.4 (-)</td>
</tr>
<tr>
<td>AVL, 5 mg/kg (oral)</td>
<td>562±25.5 (47.1)</td>
<td>24±6.1 (88.5)</td>
<td>220±28.01 (54.3)</td>
</tr>
<tr>
<td>AVL, 15 mg/kg (oral)</td>
<td>53.4±19.6 (94.8)</td>
<td>10.7±0.5 (95.6)</td>
<td>63.67±8.98 (86.8)</td>
</tr>
<tr>
<td>AVL, 45 mg/kg (oral)</td>
<td>36.6±11.2 (96.6)</td>
<td>0.27±0.15 (99.9)</td>
<td>6.33±0.58 (98.7)</td>
</tr>
<tr>
<td>AVL, 5 mg/kg (sc)</td>
<td>210±8.0 (80.2)</td>
<td>38±2.6 (82.3)</td>
<td>89.33±7.6 (81.4)</td>
</tr>
<tr>
<td>AVL, 15 mg/kg (sc)</td>
<td>213.7±25.4 (79.9)</td>
<td>18.8±2.2 (95.1)</td>
<td>58.67±2.5 (87.8)</td>
</tr>
<tr>
<td>AVL, 45 mg/kg (sc)</td>
<td>20±8.5 (98.1)</td>
<td>0.25±0.05 (99.9)</td>
<td>36±1.41 (92.5)</td>
</tr>
<tr>
<td>SAG, 20 mg/kg (sc)</td>
<td>27.6±11.5 (97.4)</td>
<td>92±8 (57.1)</td>
<td>261±10.6 (45.7)</td>
</tr>
</tbody>
</table>

Mice (n=5) received a daily dose of AVL for five consecutive days as described in "Materials and methods." The parasite burden (mean ± SD) was estimated by counting at least 500 host cells in each slide and the percentage removal was calculated.

---


Fig. 3 AVL-induced increase in NO production is not LPS mediated. Murine peritoneal macrophages (1 * 10^6, empty squares) were incubated with AVL (87.5 µg ml^{-1}) in the absence ( ) or presence ( ) of Polymixin B (25 µg ml^{-1}) for 24 and 48 h as described in "Materials and methods." At the end of 24 and 48 h, culture supernatants were analyzed for NO content using Griess reagent. Similarly, murine peritoneal macrophages infected with L. donovani (1:10, ) were incubated with AVL (87.5 µg ml^{-1}) in the absence ( ) or presence ( ) of Polymixin B (25 µg ml^{-1}) for 24 and 48 h and similarly analyzed for NO production. Each point is the mean ± SD of at least three experiments in duplicates.

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To evaluate whether the efficacy of AVL had a seasonal variation, samples were collected during the rainy season (June–Aug) and in winter (Nov–Jan). There was no variation in the leishmanicidal activity when tested both in the antimonial responsive strain, AG83 or an antimonial unresponsive strain, GE1F8R (Fig. 1c).

VL has traditionally been treated with pentavalent antimony, but the alarming increase in the incidence of antimonial unresponsiveness ranging from 34–64% in the Indian subcontinent stresses the need for alternative chemotherapy (Sundar 2001). As the IC\textsubscript{50} of AVL in all four strains was comparable irrespective of their responsiveness to antimony (Fig. 1b), it indicates that AVL and SAG possibly do not share a common programmed cell death mechanism responsible for mediating their antileishmanial activity and could potentially be applied in antimonial unresponsive VL patients.

Both antimonial unresponsive and sensitive amastigotes were sensitive to AVL (ex vivo).

Giemsa-stained slides were analyzed microscopically for the presence of phagocytosed amastigotes within macrophages. The IC\textsubscript{50} of AVL in SAG sensitive strains, AG83, and 2001 was 11.4±0.05 and 3.4±0.04 μg/ml, respectively, while in the SAG resistant strains GE1F8R and 39, the IC\textsubscript{50} was 9.2±0.07 and 3.1±0.06 μg/ml, respectively. The IC\textsubscript{50} values of AVL in amastigotes of these four strains ranged from 3–11 μg/ml which is comparable with the reported IC\textsubscript{50} of known antileishmanial compounds like Pentostam (4.9–50 μg/ml), Mileforosine (13.6 μM; Paris et al. 2004), SAG (154 μg/ml; Roberts and Rainey 1993) but higher than Amphotericin B (0.013–0.018 μg/ml) and its lipid formulations (0.2–2.6 μg/ml; Yardley and Croft 2000).

AVL increased nitric oxide production in macrophages

Analysis for nitrite and nitrate production in culture supernatants of pre-primed murine non-parasitized macrophages indicated that their basal NO production was 3.0±0.2 μM. Following a 48-h incubation with AVL, the NO production increased marginally to 4.0±0.35 μM (Fig. 2). However, in parasitized macrophages, a consistent decrease in NO production occurred in all four strains ranging from 0–2.5 μM, which is in agreement with disease progression (Fig. 2). With the addition of AVL (0–87.5 μM) to these parasite-laden macrophages, a dose-dependent increase in NO production occurred (Fig. 2) that was distinctly higher than untreated, infected macrophages. NO production at the highest concentration of AVL was 12.0±0.8 μM (Fig. 2). To eliminate the probability of microbial contamination being responsible for the increased NO production, cells were treated with AVL (87.5 μg/ml) in presence of polymixin B (25 μg/ml), a known inhibitor of lipopolysaccharide (LPS). The lack of any marked change in NO production in the presence of polymixin B confirmed that AVL independently caused increased NO production (Fig. 3). This was corroborated with the LAL assay that showed the amount of endotoxin to be 0.01 IU/ml indicating that AVL-induced NO production was not an endotoxin-mediated response.

Pre-primed murine peritoneal macrophages produced a basal level of NO that decreased with Leishmania infection owing to the down regulation of macrophage activity (Stenger et al. 1996; Sacks and Noben-Trauth 2002; Fig. 2). NO has been reported to cause extensive fragmentation of nuclear DNA in both axenic and intracellular

### Table 3 Effect of AVL on hepatic and renal functions in a sub acute toxicity BALB/c mice model

<table>
<thead>
<tr>
<th>Group</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53.87±7.96</td>
<td>87.46±1.30</td>
<td>17.01±12.28</td>
<td>0.83±0.02</td>
</tr>
<tr>
<td>AVL</td>
<td>53.29±4.99</td>
<td>87.92±13.62</td>
<td>18.05±2.63</td>
<td>0.8±0.03</td>
</tr>
</tbody>
</table>

*Mice (n=8) received AVL in a stepwise fashion as described in "Materials and methods." Enzyme estimations (mean ± SD) were done using commercial kits.

### Table 4 Effect of AVL on hepatic and renal functions of Leishmania donovani-infected BALB/c mice

<table>
<thead>
<tr>
<th>Group</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control</td>
<td>52.27±6.60</td>
<td>19.60±2.83</td>
<td>16.87±1.10</td>
<td>1.03±0.12</td>
</tr>
<tr>
<td>AVL 5 mg/kg (oral)</td>
<td>52.00±5.29</td>
<td>32.67±3.21</td>
<td>17.07±2.11</td>
<td>0.93±0.06</td>
</tr>
<tr>
<td>AVL 15 mg/kg (oral)</td>
<td>52.33±1.53</td>
<td>30.9±1.69</td>
<td>18.87±0.76</td>
<td>1.00±1.10</td>
</tr>
<tr>
<td>AVL 45 mg/kg (oral)</td>
<td>54.67±9.02</td>
<td>31.33±11.37</td>
<td>17.33±0.61</td>
<td>1.00±0.10</td>
</tr>
<tr>
<td>AVL 5 mg/kg (sc)</td>
<td>53.93±5.22</td>
<td>32.27±1.72</td>
<td>17.9±1.75</td>
<td>0.80±0.10</td>
</tr>
<tr>
<td>AVL 45 mg/kg (sc)</td>
<td>52.60±4.70</td>
<td>33.67±6.24</td>
<td>18.2±0.79</td>
<td>0.80±0.10</td>
</tr>
<tr>
<td>AVL 20 mg/kg (sc)</td>
<td>53.30±5.50</td>
<td>32.90±0.70</td>
<td>18.35±5.20</td>
<td>0.80±0.10</td>
</tr>
<tr>
<td>SAG 20 mg/kg (sc)</td>
<td>53.05±6.57</td>
<td>33.0±2.57</td>
<td>17.45±1.59</td>
<td>0.94±0.08</td>
</tr>
</tbody>
</table>

*Mice (n=5) received AVL daily for five consecutive days. Enzyme estimations (mean ± SD) were done using commercial kits.
amastigotes of *L. amazonensis*, and this fragmentation signal is regulated by non-caspase proteases of the proteasome (Holzmüller et al. 2002). Because AVL can promote caspase-independent programmed cell death in promastigotes (Dutta et al. 2007a) and the addition of AVL to parasite-infected macrophages triggered a dramatic increase in NO production (Fig. 2), it is likely that this enhanced NO mediates parasite elimination by inducing apoptosis. LAL assay revealed that endotoxin is practically absent in AVL; furthermore, Polymyxin B, a known competitive inhibitor of LPS (Holzmüller et al. 2002), did not reduce NO production by macrophages.

Orally and sc-administered AVL was leishmanicidal in a *L. donovani* BALB/c mouse model

The oral administration of AVL to *L. donovani*-infected mice at the lowest concentration (5 mg/kg b.w.) caused a 47.1% and 54.3% decrease in parasitemia in the liver and bone marrow, respectively, while the decrease in splenic burden was greater, being 88.8%. At 15 mg/kg b.w., over 95% of parasites were removed from liver and spleen and 86.8% from the bone marrow (Table 2). The highest concentration of AVL (45 mg/kg b.w.), used in this study, caused more than 97% decrease in parasitemia in all three organs examined (Table 2). With SAG, parasite elimination in liver, spleen, and bone marrow was 97.4%, 57.1%, and 45.7%, respectively (Table 2).

With regard to sc administration of AVL, the lowest dose (5 mg/kg b.w.) eliminated more than 80% parasitemia from all three organs examined (Table 2). At 15 mg/kg b.w., an 80% decrease in parasite burden was observed in liver, while in spleen and bone marrow, the percent elimination was 96% and 87.8%, respectively. The highest dose of AVL (45 mg/kg b.w.) caused more than 98% parasite removal from liver and spleen, while 92.5% burden was eliminated from the bone marrow (Table 2). Although successful transformation was achieved in control cultures, no promastigote transformation occurred from AVL-treated tissues (15 and 45 mg/kg b.w.).

The in vivo studies using AVL clearly indicates its efficacy when administered orally or subcutaneously. At its lowest concentration (5 mg/kg b.w., sc), AVL was more effective than SAG (20 mg/kg b.w., sc). With higher doses, both routes of administration resulted in removal of >95% parasitemia in all three affected organs, i.e., liver, spleen, and bone marrow suggesting that AVL holds great promise as an effective herbal remedy in VL.

Toxicity studies

As AVL contained saponin, known to be toxic to monocytes, it was important to test its toxicity in monocytes and macrophages. In vitro studies done with murine peritoneal macrophages and a human monocyte cell line U 937 exposed to AVL, (300 μg/mL) showed minimal cell death, being 12.2±0.5% and 18.4±0.5%, respectively.

Although *Aloe vera* leaf and gel are widely used, toxicity of the leafy exudate has not been studied and, therefore, hepatic and renal toxicity of AVL in BALB/c mice was evaluated. Following 1 month of AVL consumption (orally), up to a maximum of 250 mg/kg b.w., hepatic and renal functions of experimental mice were comparable with normal mice as evidenced from ALP, SGOT, serum urea, and serum creatinine levels (Table 3). AVL (up to 45 mg/kg b.w.) proved to be non-toxic in BALB/c mice used in antileishmanial screening (Table 4). Taken together, AVL has shown promising potential as a potent herbal remedy for VL and may provide potent lead compounds further strengthening the chemotherapeutic arsenal against VL.

Acknowledgements The work received financial support from Council of Scientific and Industrial Research and Department of Biotechnology, Government of India. Avijit Dutta received a Senior Research Fellowship from Indian Council of Medical Research. We gratefully acknowledge the receipt of strains from the Leishmania Bank (Dr. Swadesh Datagupta, Indian Institute of Chemical Biology, Kolkata) and Dr. Neelee Singh, Central Drug Research Institute, Lucknow.

References


Effect of *Aloe vera* on nitric oxide production by macrophages during inflammation

**D. Sarkar, A. Dutta, M. Das, K. Sarkar, C. Mandal, M. Chatterjee**

*Abstract*

Objective: To demonstrate the mechanism of action mediating the acute and chronic antiinflammatory activity of leafy exudate of *Aloe vera* (AVL) in animal models of inflammation.

Materials and Methods: The acute antiinflammatory activity of AVL was evaluated using carrageenan and dextran as phlogistic agents while its chronic antiinflammatory effect was investigated in a complete Freund's adjuvant-induced model of arthritis. The degree of inflammation in all models was measured plethysmographically. The effect of AVL on nitric oxide production in mouse peritoneal macrophages was measured by the Griess reagent.

Results: AVL (25 mg/kg) significantly reduced carrageenan and dextran-induced pedal edema in rats by 61.9% and 61.7%, respectively. In the Freund's adjuvant-induced model of chronic inflammation, AVL showed chronic antiinflammatory activity but failed to decrease the arthritic index indicating the absence of antiarthritic activity. AVL (10 pg/ml) caused a decrease in NO production in macrophages without causing toxicity.

Conclusion: AVL possesses acute and chronic antiinflammatory activity, which is partly mediated by reduced production of NO, which in turn prevents the release of inflammatory mediators.

**Keywords:** Antiinflammatory activity, acute inflammation, chronic inflammation, arthritis, inflammatory mediators.

**Introduction**

Inflammation is generally considered as an essentially protective response to tissue injury caused by noxious physical, chemical or microbiological stimulus. It is a complex process involving various mediators, such as prostaglandins, leukotrienes and platelet activating factor. The major macrophage derived inflammatory mediators such as pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), contribute to the development of inflammatory diseases. Thus, inhibition of the excessive production of TNF-α and NO could be employed as criteria to evaluate potential antiinflammatory compounds. The current management of inflammatory diseases is limited to the use of antiinflammatory drugs whose chronic administration is associated with several adverse effects. Plant-derived products are slowly emerging as a viable alternative because they are cheap, abundantly available and relatively less toxic.

The genus *Aloe* belongs to the *Asphodelaceae* family, *Alooideae* subfamily, and comprises about 420 species of succulent plants. They are indigenous to southern and eastern Africa and Madagascar but have been introduced in other tropical countries, the Mediterranean area and the West Indies. Aloe has been used as a folk medicine for 3000 years and in the last decade *Aloe vera* is being extensively used in health drinks, topical creams, toiletries and cosmetics. From the leaves of *Aloe* plants, three types of commercial products are obtained namely, the dried exudate, excreted from the aloin cells present in the zone of the vascular bundle, the gel, a mucilaginous juice present in the centre of the leaf and the oil, extracted by organic solvents. The dried exudate is used as a bitter in alcoholic beverages while the oil, comprising the fatty fraction of the leaf is used primarily in the cosmetic industry as a pigment carrier and soothing agent. Studies have shown that the gel component has antiinflammatory activity that is mediated through antibradykinin activity and inhibition of prostaglandin production. However, no studies have been undertaken with regard to the antiinflammatory activity of the leaf exudate. Accordingly, in this study, we have evaluated the antiinflammatory potential of *Aloe vera* leaf exudate as also demonstrated that this antiinflammatory activity is mediated partly via reduction of nitric oxide production in macrophages.
Materials and Methods

Animals
Male albino Wistar rats (100-120 g) and Swiss albino mice (av. wt, 25-30 g) were used. All animals were housed under standard conditions of temperature (25±5°C). A 12-h light/dark cycle was maintained and the animals were provided with standard pellet diet and water ad libitum. They were acclimatized for one week and then subdivided for different experimental schedules. The experiments were carried out after getting approval from the Institutional Animal Ethics Committee.

Chemicals and drugs
Naphthyl ethylene diamine dihydrochloride [NED (Loba Chemicals)], sulphanalminide, zymosan, phenazine methosulphate [PMS (SRL)], MTS: {3-(4, 5 dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4- sulphophenyl)-2H tetrazolium (Promega), Indomethacin (Innecin 25, E.M. Pharmaceuticals Pvt. Ltd., India), dexamethasone (Dexona, Cadila Healthcare Ltd., India), Complete Freund’s Adjuvant, Carrageenan and dextran were obtained from Sigma, MO, USA.

Preparation of plant extract
Fresh succulent leaves of Aloe vera were collected, the inner gel component removed and the leafy exudate homogenized in an electric grinder. This was subsequently lyophilized and stored at 4°C. It was reconstituted in dimethylsulfoxide (DMSO, 50 mg/ml), labelled as AVL and used for all experiments.

Tests for acute inflammation
Carrageenan or dextran-induced edema
Animals were divided into four groups (n = 6). In all the groups, acute inflammation was induced by injection of either 0.1 ml of freshly prepared carrageenan (1.0 %) in 0.9 % w/v NaCl solution or 0.1 ml of dextran (2%) in 0.9 % w/v NaCl solution into the subplantar region of the hind paw of rats. AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally administered one hour before injection of the phlogistic agent. The paw volumes were measured plethysmographically (using AVL in different doses (0- 25 mg/kg, b.w.) and indomethacin (2.5, 5 and 10 mg/kg, b.w., as a standard reference) were orally


diagnosis of NO production was calculated as follows1101

\[
\frac{R_i - R_e}{R_e} \times 100
\]

where \( R_i \) is the edema volume of the control group and \( R_e \) is the edema volume of the treated group.

Test of chronic inflammation
Freund’s complete adjuvant induced arthritis model
Rats were divided into three groups (n = 6). Experimental arthritis was induced according to the method of Newbould et al. Briefly, 0.1 ml of Freund’s complete adjuvant was injected intradermally into the plantar aspect of the hind paw of each animal. Animals were administered AVL (25 mg/kg, b.w., orally) and dexamethasone (0.1 mg/kg, b.w., orally, as a standard reference) for the initial 13 days. The degree of inflammation was measured plethysmographically; accordingly, edema formation and the percentage of inhibition was calculated as described above on days 1, 3, 5, 9, 13 and 21 and the primary and the secondary lesions were measured.

Primary lesions refer to the edema formation in the injected hind paw that peaks 3-5 days after injection of the phlogistic agent and is measured on day 5 by calculating the percent inhibition of the edema volume of the injected paw using the formula described above. Secondary lesions are immunologically mediated changes characterized by inflammation of the non-injected sites (hindleg, forepaw, ears nose and tail) decrease in weight and occur after a delay of 11-12 days. Accordingly, secondary lesions were evaluated by calculating the percent inhibition of paw volume of the non-injected right paw over control on day 21 and using an arthritic index as the sum of scores according to the method of Schonholzen.18

Reso

<table>
<thead>
<tr>
<th>Lesion site</th>
<th>Nature of lesion</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear</td>
<td>a. Absence of nodules and redness.</td>
<td>0</td>
</tr>
<tr>
<td>b. Presence of nodules and redness.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nose</td>
<td>a. No swelling of connective tissue.</td>
<td>0</td>
</tr>
<tr>
<td>b. Intense swelling of connective tissue.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tail</td>
<td>a. Absence of nodules.</td>
<td>0</td>
</tr>
<tr>
<td>b. Presence of nodules.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Forepaw</td>
<td>a. Absence of Inflammation.</td>
<td>0</td>
</tr>
<tr>
<td>b. Inflammation of at least one joint.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hind paw</td>
<td>a. Absence of Inflammation.</td>
<td>0</td>
</tr>
<tr>
<td>b. Slight inflammation.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>c. Moderate inflammation.</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>d. Marked inflammation.</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Preparation of mouse peritoneal macrophages
To elicit activated peritoneal macrophages, mice were injected i.p. with starch [2% in phosphate buffered saline (PBS), 0.02 M, pH 7.2, 2 ml/mice]. After three days, the peritoneal cells comprising principally macrophages were lavaged with sterile PBS (10 ml). The exudate was centrifuged at 4000 rpm for 10 min and the resultant cell pellet washed in PBS and finally resuspended in complete RPMI-1640 phenol red free medium supplemented with 10% fetal calf serum (FCS)18. Macrophage viability (>95%) was confirmed by the Trypan blue dye exclusion technique.

Measurement of nitric oxide (NO) production in macrophages
Macrophages obtained by peritoneal lavage as described above were seeded (5 x 10⁶/ml) in RPMI 1640 phenol red free medium supplemented with 10% FCS in Petri dishes (35 mm diameter) and incubated at 37°C, 5% CO₂, for 4 hrs. AVL (0 - 10.0 µg/ml) was added and incubated for an additional 48 hrs. At the end of 48 h, 0.5 ml of the supernatant was withdrawn and nitrite accumulation as an indicator of NO production was measured using Griess reagent. Briefly, 0.5 ml of Griess reagent [mixture of 1:1 of naphthylethylenediamine dihydrochloride (0.1% in water) and sulphanalminide (1% in 5% phosphoric acid)] was added to 0.5 ml of supernatant and incubated in the dark at room temperature (25-30°C) for 10 min. Finally, absorbance at 546 nm was measured spectrophotometrically and a standard curve using sodium nitrite was used to calculate concentrations of nitrite. To demonstrate specificity, macrophages were incubated in the presence of N-monomethyl arginine (L-NMMA), 0.1mM an established inhibitor of NO production.
Antiinflammatory activity of AVL

Cytotoxicity assay

To determine the viability of macrophages in the presence of AVL or DMSO, they were seeded in 96-well tissue culture plates (1 x 10^5 cells/ml of RPMI 1640, supplemented with 10% FCS, 0.2 ml per well) and incubated with either AVL (0 - 10 mg/ml) or DMSO (0-1%) alone for 48 hrs. At the end of drug exposure, the MTS assay was performed. Briefly, MTS [3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium] was prepared (2 mg/ml in PBS) and stored in dark at -20°C. Another stock solution of phenazine methosulphate (PMS) was prepared (0.02 mg/ml in PBS) and stored -20°C. Just before use, MTS and PMS were mixed in the ratio of 20:1 and 20 μl of the solution was added to each well. The plate were then incubated at 37°C for 3 hrs and absorbances measured at 490 nm using a Multiskan ELISA reader (BioRad, USA).

Statistical analysis

Data were expressed as mean ±SEM and statistically assessed using one-way ANOVA followed by Tukey test; P<0.05 was considered significant.

Results

AVL possesses strong acute antiinflammatory activity in carrageenan and dextran-induced animal models of inflammation

AVL at 25 mg/kg, b.w., showed significant antiinflammatory activity in both carrageenan and dextran models. In the control group, paw volume was maximum at the third hour after which the paw volume decreased gradually and therefore readings up to the third hour were recorded (Tables 1 and 2). However, the antiinflammatory activity of AVL was sustained up to the sixth hour (data not shown). Indomethacin served as a positive control and was initially tested at three different concentrations of 2.5, 5.0 and 10.0 mg/kg, b.w. With 2.5 and 5.0 mg/kg, b.w., a maximum of 19.53% and 21.65% reduction in paw edema, respectively, was obtained. However, with 10 mg/kg, b.w., a marked reduction of 75% was obtained and accordingly, this concentration was selected for all experiments.

Chronic antiinflammatory activity of AVL in a complete Freund's adjuvant-induced model of arthritis

A significant decrease in the primary lesion as evident on day 5 was observed with both AVL (33.3%, P<0.05) and the dexamethasone treated group (57.66%, P<0.007) (Table 3, Figure 1) as compared to controls. With regard to the secondary lesions, it could only be partially evaluated as no edema formation was evident in the contralateral hind paw of control animals. On the 21st day, a significant decrease in edema volume was observed in the injected paw of both AVL (52.7%, P<0.01) and dexamethasone-treated group (63.51%, P<0.005).

With regard to the mean articular index, the score of AVL treated animals was not different from that of the control group being 2.0 vs. 1.8, respectively, indicating that AVL does not possess antarthritic activity. As expected, the dexamethasone-treated group showed profound antarthritic activity since it decreased the articular index almost 10-fold being 0.17.

AVL-treated macrophages showed decreased nitric oxide (NO) production

The formation of NO is classically assayed by measuring nitrite (NO2-) production, a primary, stable and nonvolatile breakdown product of NO. The Griess reagent uses sulphanilamide and NED under acidic conditions to yield an azo compound that can be measured spectrophotometrically. AVL caused a 43.04% decrease in NO production in murine macrophages as compared to control values being 51.0 μM vs. 51.0 μM. The marked decrease in NO production in the presence of L-NMMA (8.75 μM) confirmed specificity of the reaction.

Toxicity of AVL in macrophages

As AVL decreased NO production in macrophages, it was relevant to confirm that this decrease was not due to AVL or DMSO induced cell death but specifically by macrophage
A: Macrophages were incubated with DMSO (0 - 1%) in 96 well plates at 37°C and 5% CO₂ for 48 h. Each point is the mean ± SEM of three experiments in duplicates, *P<0.0001 as compared to control (one-way ANOVA followed by Tukey test).

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg, b.w.)</th>
<th>*Edema volume (ml)/% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td>AVL</td>
<td>25</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(17.14%)</td>
<td>(33.33%)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.1</td>
<td>0.33 ± 0.02**</td>
</tr>
<tr>
<td></td>
<td>(52.86%)</td>
<td>(57.69%)</td>
</tr>
</tbody>
</table>

One-way F 20.11 12.55 9.175 7.950 3.565
ANOVA df 2,15 2,15 2,15 2,15 2.15
P 0.0001 0.01 0.002 0.004 0.056

*Edema volume and % inhibition was measured as described in Materials and methods. Values are expressed as mean±SEM; *P<0.05; **P<0.01; ***P<0.005 as compared to control (Tukey test), n = 6 in each group.

Arthritis was established using complete Freund’s adjuvant and the animals received no drug (–♦–), AVL (±, 25 mg/kg b.w.) or dexamethasone (–▲–, 0.1 mg/kg b.w.). The degree of inhibition of edema formation was measured plethysmographically on days 3, 5, 9, 13 and 21. Each point is the mean ± SEM of two experiments (n=6 in each group). *P<0.05; **P<0.01; ***P<0.005 as compared to control (one-way ANOVA followed by Tukey test).

Discussion

The inhibition of carrageenan-induced inflammation in rats is an established model to screen compounds for potential anti-inflammatory activity. In the present study, AVL was evaluated for its efficacy in reducing inflammation.

inactivation. Accordingly, macrophages were incubated with AVL or DMSO and cell viability was determined by the MTS assay. Since this conversion can only be accomplished by viable cells, the amount of formazan is a direct measure of the number of cells present. It was found that DMSO up to 0.5% was nontoxic to macrophages (Figure 2A, P=0.72). However, with 1.0% DMSO, a significant increase in toxicity was observed (Figure 2A, P<0.0001). AVL up to a concentration of 0.1 mg/ml showed no decrease in viability (Figure 2B, P=0.31) but with higher concentrations (>0.25mg/ml), a significant decrease in viability was observed (Figure 2B, P<0.0001). Accordingly, all experiments were performed using a maximum of 0.1% DMSO or 0.1 mg/ml AVL.

B: Macrophages were seeded in 96 well plate with AVL (0-1 mg/ml) for 48 h at 37°C and 5% CO₂. Each point is the mean ± SEM of three experiments in duplicates, *P<0.0001 as compared to control (one-way ANOVA followed by Tukey test).
antinflammatory activity. According to Vinegar et al. (1987),\textsuperscript{1} the development of carrageenan-induced edema is biphasic; the first-phase occurs within one hour of carrageenan administration and is attributed to the release of cytoplasmonic enzymes, histamine and serotonin, from the mast cells. The second phase (1.0 h) is mediated by an increased release of prostaglandins in the inflammatory area and continuity between the two phases is provided by kinins. With regard to dextran-induced edema, it has been reported to be mediated mainly by histamine and 5-HT released by the mast cells.\textsuperscript{234} Taken together, it suggests that as AVL possesses potent acute antinflammatory activity (Tables 1 and 2), it is mediated possibly due to inhibition of the synthesis and/or release of mediators of inflammation, principally the prostaglandins. This can only be defined after developing models of inflammation using specific mediators.

The immunologically mediated complete Freund’s adjuvant arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis.\textsuperscript{235} This method in rats was originally developed by Pearson et al. (1963),\textsuperscript{14} wherein an injection of complete Freund’s adjuvant in the rat hind paw induces inflammation. It has been proposed that bacterial peptido-glycan and muramyl dipeptide are important non-specific immunogenic contributory components. In this model of immunologically mediated chronic synovial inflammation and arthritis, macrophages play a central role. After activation they are capable of synthesizing mediators such as PGE\textsubscript{2} and cytokines such as TNF-\alpha and IL-1. In turn, these synthetic products induce the production of a variety of enzymes which initiate cartilage and bone destruction.\textsuperscript{236, 237}

NO is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues.\textsuperscript{238} Nitric oxide, in macrophages, is produced as a free radical by iNOS by catalyzing the oxidation of guanidino nitrogen of L-arginine, thereby converting L- arginine to L-citrulline.\textsuperscript{239} NO is an important signaling and effector molecule in inflammation and immunity as it is known to couple with superoxides to form peroxynitrite. These, in turn, induce the production of prostaglandin endoperoxide synthase from monocytes/macrophages resulting in enhanced synthesis of prostaglandins, established mediators of inflammation.\textsuperscript{240} The reduced NO production by macrophages in the presence of AVL suggests that AVL by decreasing the release of NO, inhibits the release of inflammatory inhibitors such as prostaglandins resulting in suppression of inflammation. This can be confirmed by measuring the expression of iNOS in macrophages; such studies are ongoing.

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References