

# Pro-apoptotic effect of the landrace Bangla Mahoba of *Piper betle* on *Leishmania donovani* may be due to the high content of eugenol

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In the absence of effective and safe treatment for visceral leishmaniasis or Kala-azar – a devastating parasitic disease caused by *Leishmania donovani* – the search for anti-leishmanial agents from natural resources in common use is imperative. Recently, the comparative *in vitro* anti-leishmanial activity of methanolic extracts from two landraces of *Piper betle* – *P. betle* landrace Bangla Mahoba (PB-BM) and *P. betle* landrace Kapoori Vellaikodi (PB-KV) – has been reported. Here, the putative pathway responsible for death induced by the effective extract of PB-BM methanolic extract in promastigotes, as well as the intracellular amastigote form of *L. donovani*, was assessed using various biochemical approaches. It was found that PB-BM was capable of selectively inhibiting both stages of *Leishmania* parasites by accelerating apoptotic events by generation of reactive oxygen species targeting the mitochondria without any cytotoxicity towards macrophages. The study was extended to determine the presence or absence of activity of the methanolic extract of PB-BM and PB-KV on the basis of differences in essential oil composition present in the extract assessed by GC and MS. The essential oil from PB-BM was found to be rich in eugenol compared with that from PB-KV. The anti-leishmanial efficacy of PB-BM methanolic extract mediated through apoptosis is probably due to the higher content of eugenol in the active landrace. This observation emphasizes the need to extend studies related to traditional medicines from bioactive plants below the species level to the gender/landrace level for better efficacy and reproducibility.

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## INTRODUCTION

Leishmaniasis comprises a group of infectious diseases with worldwide distribution causing severe morbidity or/and fatality, of which visceral leishmaniasis (VL) (or Kala-azar) caused by the protozoan parasite *Leishmania donovani* is the most devastating. For various reasons, current therapies are not proving very effective. The first line of drugs for VL, such as pentavalent antimonials, sodium stibogluconate and meglumine antimoniate, have variable efficacy and have severe side effects (Thakur *et al.*, 1984). Amphotericin B and pentamidine, the second-line drugs used clinically,

**Abbreviations:** FITC, fluorescein isothiocyanate; FIU, fluorescence intensity unit; GFP, green fluorescent protein; H<sub>2</sub>DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; IC<sub>50</sub>, concentration inhibiting cell growth by 50%; KI, Kovats index; MFI, mean fluorescence intensity; PB-BM, *Piper betle* landrace Bangla Mahoba; PB-BME, PB-BM essential oil; PB-BMM, PB-BM methanolic extract; PB-KV, *Piper betle* landrace Kapoori Vellaikodi; PB-KVE, PB-KV essential oil; PB-KVM, PB-KV methanolic extract; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labelling; VL, visceral leishmaniasis.

have limited efficacy and are very toxic (Iwu *et al.*, 1994). Moreover, cases of drug resistance are also on the rise (Croft, 2001). Due to these problems, interest in the study of ethano-medicines as a source of new chemotherapeutic compounds with comparable or better activities and minimal side effects has increased in recent years. India is rich in traditional medicinal plant species, providing opportunities to explore and exploit this resource for various diseases and metabolic disorders. *Piper betle* Linn. (*Piperaceae*) is a well-known medicinal plant grown widely in the humid climate of South-East Asia. Globally, more than 600 million people consume *P. betle* daily in one form or the other. It is also known to be dioecious with more than a hundred landraces reported. Differences between landraces in terms of leaf shape, size and chlorophyll content along with characterization based on random amplification of polymorphic DNA have been reported (Kumar *et al.*, 2006; Verma *et al.*, 2004). The leaves are known for their antimicrobial, anti-inflammatory and antifungal activities (Ramji *et al.*, 2002). The leaves inhibit carcinogen-induced tumours of the oral cavity and

mammary tissue (Rao, 1984). Protective single/combined treatment with betel leaf and turmeric against methyl(ace-toxymethyl)nitrosamine-induced hamster oral carcinogenesis has been observed. In addition, the leaves exert chemopreventive effects against lung and forestomach tumours induced by *N'*-nitrosornicotine and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (Padma *et al.*, 1989).

The medicinal importance of *P. betle* along with its high level of consumption in areas endemic for leishmaniasis prompted us to evaluate the anti-leishmanial efficacy of this natural product. The *in vitro* anti-leishmanial activity of the methanolic extracts from leaves of *P. betle* landrace Bangla Mahoba (PB-BM) and *P. betle* landrace Kapoori Vellaikodi (PB-KV) has been evaluated previously against *L. donovani* promastigotes and intracellular amastigotes by our group, and a potent anti-leishmanial activity was observed only for PB-BM methanolic extract (PB-BMM) (Tripathi *et al.*, 2006). The current study investigated the anti-leishmanial mode of action of PB-BMM by using various biochemical assays. In addition, possible explanations for the different activities obtained for both PB-BMM and PB-KV methanolic extract (PB-KVM) are discussed based on the composition of their essential oils.

## METHODS

**Plant material and preparation of extract.** *P. betle* landraces were grown at the National Botanical Research Institute, Lucknow, India, by obligate vegetative propagation to eliminate the variability that can arise from crops grown from seed. Mature leaves of the landraces BM and KV were harvested, washed and patted dry in the shade until there was no change in their dry weight. The dried leaves were pulverized to a fine powder and extracted in methanol until there was no further green colour in the extract. Pooled extract was concentrated in a Rota Vapour and further dried under vacuum. Both plants yielded 50 g methanol extract (kg dry leaf powder)<sup>-1</sup>.

**Extraction of essential oils.** Distillation was carried out for about 12 h for 2 days. The oil was recovered in ether, dried and weighed. The PB-BM essential oil (PB-BME) and the PB-KV essential oil (PB-KVE) represented 0.20 and 0.10% of the fresh leaf weight, respectively.

**GC and GC-MS analyses of the essential oils.** GC analysis was carried out by using a PerkinElmer AutoSystem XL chromatograph equipped with a flame-ionization detector and a PE-5 column (60 m × 0.32 mm, 0.25 µm film thickness). The column oven temperature was maintained initially at 100 °C for 1 min and then programmed at an increase rate of 3 °C min<sup>-1</sup> up to 280 °C. The column head pressure was maintained at 10 p.s.i. with a split ratio of 1 : 30 and the carrier gas used was hydrogen. The injector and detector temperatures were 250 and 280 °C, respectively.

GC-MS analysis was carried out on a PerkinElmer XL system attached to a TerboMass. GC-MS analysis was performed on an Equity-5 column (60 m × 0.32 mm, 0.25 µm film thickness). The column oven temperature was initially held at 70 °C for 2 min and then programmed at an increase rate of 3 °C min<sup>-1</sup> up to 250 °C, with a 2 min hold. The column head pressure was maintained at 10 p.s.i. with a split ratio of 1 : 30. The carrier gas used was helium. Ionization was by electron impact at 70 eV, and the temperature of the injector,

source and interface was kept at 250 °C. Identification of the constituents in the oil was carried out by comparing Kovats index (KI) values and peak enrichment with authentic samples, and by comparing the mass spectra of eluted compounds with those in the (USA) National Bureau of Standards (NBS) and National Institute of Standards and Technology (NIST)/Wiley libraries.

**Parasites.** The promastigotes of *L. donovani* (MHOM/IN/80/Dd8) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup> at 26 °C (all from Sigma). Transgenic parasites expressing green fluorescent protein (GFP) (Singh & Dube, 2004) were cultured in the presence of 100 µg geneticin ml<sup>-1</sup> (G 418 sulphate; Sigma).

**Isolation of amastigotes from a macrophage cell line.** J774A.1 macrophages (10<sup>8</sup> cells) in 50 ml culture flasks (Nunc) were infected with promastigotes at an m.o.i. of 10 and incubated at 37 °C in 5% CO<sub>2</sub> for 8–12 h, after which the cells were washed three times with PBS (pH 7.2) and complete Dulbecco's minimal essential medium was added. Infected macrophages were harvested by centrifugation at 2000 g for 10 min. The pellet was resuspended in 1 ml PBS and passed repeatedly through a 26-gauge sterile needle to facilitate the release of amastigotes by forced bursting of the macrophages. The released amastigotes were purified using Percoll (Sigma) density-gradient centrifugation (Chang, 1980). Briefly, amastigotes released from the macrophages were centrifuged at 800 g for 10 min to remove tissue debris. The supernatant was centrifuged at 1600 g for 15 min at 4 °C. The pellet was resuspended in 5 ml 45% Percoll in 2 mM EDTA in PBS and layered over a cushion of 2 ml 90% Percoll. The gradient was centrifuged at 3500 g at 4 °C for 1 h in a swing-out rotor. The amastigotes were collected from the interphase of the 45–90% step gradient and washed with PBS.

**Activity against promastigotes and intracellular amastigotes.** Exponential-phase transgenic GFP-expressing promastigotes (10<sup>6</sup> cells ml<sup>-1</sup>) were added to a 48-well culture plate (CellStar) and treated with different concentrations of miltefosine (a standard antiprotozoal drug used for the treatment of VL, used here as a reference drug), as well as with PB-BMM and PB-KVM. Untreated cells served as a control. J774A.1 macrophages (10<sup>5</sup> cells per well) cultured in 24-well plates were infected with late-exponential-phase GFP-expressing promastigotes at an m.o.i. of 10 and incubated at 37 °C in 5% CO<sub>2</sub> for 8–12 h. Wells were washed to remove non-phagocytosed parasites. Cells supplemented with complete medium were treated with different concentrations of extract, as well as with miltefosine. Both treated promastigotes and intracellular amastigotes were removed at different time intervals (48–72 h), washed in PBS and analysed by acquiring 10 000 cells in a FACSCalibur flow cytometer equipped with a 15 mV 488 nm air-cooled argon laser with excitation at 488 nm and emission at 515 nm, using CellQuest software (Becton Dickinson). Inhibition of parasite growth was determined by comparing the mean fluorescence intensity (MFI) of drug-treated parasites with that of untreated parasites.

**Cytotoxicity assay.** The *in vitro* cytotoxicity of PB-BMM against J774A.1 macrophages was assessed by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Cells (10<sup>5</sup> ml<sup>-1</sup>) were incubated with various concentrations of PB-BMM at 37 °C in 5% CO<sub>2</sub> for 48 h. MTT was added at a concentration of 400 µg ml<sup>-1</sup> and the cells were further incubated for 3 h at 24 °C. The cells were centrifuged at 1000 g, the pellets were dissolved in DMSO and the absorbance was read at 540 nm. The mean percentage of viable cells after treatment was calculated relative to the control, and results were expressed as the concentration inhibiting cell growth by 50% (IC<sub>50</sub>).

**DNA condensation study by propidium iodide (PI) staining.**

DNA condensation following treatment with  $11.2 \mu\text{g PB-BMM ml}^{-1}$  ( $\text{IC}_{50}$ ) was observed by staining untreated and treated promastigotes with PI as described by Rybczynska *et al.* (2001). Briefly, untreated and treated promastigotes were fixed in 4% paraformaldehyde on to poly-L-lysine-coated slides. Slides were washed twice with PBS to remove non-adherent cells. Adherent cells were permeabilized with 0.2% Triton X-100 for 1 min and washed twice with PBS. They were then incubated with  $10 \mu\text{g PI ml}^{-1}$  for 2 min. Cells were then observed using a high-resolution fluorescence camera (Leica DFC320) mounted on a Leica DM5000B microscope and images were processed as described above. At least 20 microscopic fields were observed for each sample.

**Double staining with annexin V and PI.** Externalization of phosphatidylserine on the outer membrane of treated and untreated promastigotes as well as on intracellular amastigotes was measured by the binding of annexin V and PI as described by Mehta & Shaha (2004). Briefly, parasites were incubated with  $10 \mu\text{g PB-BMM ml}^{-1}$  for different time periods. Treated and untreated promastigotes and purified amastigotes (Chang, 1980), suspended in annexin V binding buffer (BD Biosciences), were incubated with annexin V and PI following the manufacturer's instructions for 15 min in the dark at  $20\text{--}25^\circ\text{C}$ . Acquisition and analysis were carried out on a FACSCalibur flow cytometer using CellQuest software.

**Estimation of reactive oxygen species (ROS) levels.** To evaluate the generation of ROS in promastigotes and infected macrophages following treatment with PB-BMM, the cell-permeant probe 2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ ) was used (Duranteau *et al.*, 1998).  $\text{H}_2\text{DCFDA}$  is a non-polar compound that readily diffuses into cells, where it is hydrolysed to the non-fluorescent derivative dichlorodihydrofluorescein and is thereby trapped within the cells. In the presence of a proper oxidant, dichlorodihydrofluorescein is oxidized to the highly fluorescent 2',7'-dichlorofluorescein. Cells treated with PB-BMM (at the  $\text{IC}_{50}$ ) for different time periods were resuspended in  $500 \mu\text{l RPMI 1640}$  and labelled with  $10 \mu\text{M H}_2\text{DCFDA}$  for 15 min in the dark. Cells were analysed for intracellular ROS by using a FACSCalibur flow cytometer with CellQuest software.

**Measurement of nitric oxide (NO) production in infected macrophages.** NO detection was carried out as follows. Briefly, treated and untreated cells were incubated with the fluorescent probe 4,5-diaminofluorescein-2 diacetate ( $1 \mu\text{M}$ ; Molecular Probes) following the manufacturer's protocol for 30 min in the dark and acquired on a FACSCalibur flow cytometer (excitation 488 nm, emission 535 nm). Data were analysed by CellQuest software and results were expressed as MFI.

**Mitochondrial membrane potential determination.** The mitochondrial membrane potential ( $\Delta\Psi_m$ ) was monitored by using JC-1 dye as a probe (Dey & Moraes, 2000). JC-1 is a cationic mitochondrial vital dye that is lipophilic and becomes concentrated in the mitochondria in proportion to  $\Delta\Psi_m$ : more dye accumulates in mitochondria with a greater  $\Delta\Psi_m$  and ATP-generating capacity (Sudhandiran & Shaha, 2003). The dye exists as a monomer at low concentrations (emission 530 nm) but forms J-aggregates (emission 590 nm) at higher concentrations. Briefly, both treated promastigotes and isolated amastigotes were collected after treatment with PB-BMM for various time periods and incubated for 7 min with  $10 \mu\text{M JC-1}$  at  $37^\circ\text{C}$ , washed and resuspended in medium. The ratio of fluorescence at 590 to 530 nm was considered to be the relative  $\Delta\Psi_m$  value.

**DNA fragmentation assay.** Fragmentation of chromatin to units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gel is an established hallmark of programmed cell

death or apoptosis (Bortner *et al.*, 1995). Total cellular DNA from treated and untreated isolated amastigotes was isolated by a published procedure (Das *et al.*, 2001). Briefly, pellets of untreated and treated cells ( $10^7$ ) were treated with sarcosyl detergent lysis buffer [50 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauryl sarcosine] and proteinase K ( $15.6 \text{ mg ml}^{-1}$ ) and incubated overnight at  $50^\circ\text{C}$ . The lysates were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at  $16\,000 \text{ g}$  for 5 min. To the upper phase, 0.3 M sodium acetate and 100% ethanol (twice the volume) were added, and the mixture was kept overnight at  $-20^\circ\text{C}$ . The sample was centrifuged at  $16\,000 \text{ g}$  for 10 min. The DNA pellet was washed with 0.5 ml 70% ethanol and solubilized in TE [10 mM Tris/HCl (pH 8.0), 1 mM EDTA]. RNase A ( $0.3 \text{ mg ml}^{-1}$ ) treatment was carried out for 1 h at  $37^\circ\text{C}$ . Extracted DNA was quantified spectrophotometrically at 260/280 nm. A total of  $10 \mu\text{g DNA}$  was mixed with tracking dye and run on a 1% agarose gel containing ethidium bromide in TAE buffer [40 mM Tris/acetate (pH 8.0), 1 mM EDTA]. Gels were run for 2 h at 50 V and visualized under UV light.

***In situ* labelling of DNA fragments by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay.**

*In situ* detection of DNA fragments by a TUNEL assay was performed by using a DeadEnd fluorometric TUNEL system (Promega). Briefly, promastigotes and intracellular amastigotes ( $1 \times 10^6$ ) isolated from macrophages treated or not with PB-BMM were fixed in 4% formaldehyde and permeabilized with 0.2% Triton X-100 followed by incubation with buffer containing nucleotide mix following the manufacturer's protocol. Cells stained by TUNEL assay were analysed in a FACSCalibur flow cytometer. Green fluorescence, gated on forward and side light scatter, was determined using a band-pass filter ( $525 \pm 10 \text{ nm}$ ).

**Statistical analysis.** The data are presented as means  $\pm$  SD. The statistical significance of differences in percentage expression between treated and untreated groups was analysed by one-way analysis of variance using SigmaStat (version 2.03) software.

**RESULTS****Essential oil composition in PB-BM and PB-KV**

The amount of essential oil produced by PB-BM was higher than that obtained from PB-KV (0.2 and 0.1% of fresh leaf weight, respectively). The composition of the essential oils PB-BME and PB-KVE was studied by GC and GC-MS analyses, and the major constituents are shown in Table 1. PB-BME was found to be richer in eugenol and other phenols than PB-KVE (73.01 and 27.15% eugenol, respectively). *cis*-Methylisoeugenol and *trans*-isoeugenol co-elute in one peak but can be identified based on KI values, peak enrichment and comparison with the mass spectra of authentic samples. However, they were not detected by GC or GC-MS analysis in either of the essential oils.

**Determination of  $\text{IC}_{50}$  of PB-BMM against *L. donovani* promastigotes and intracellular amastigotes**

PB-BMM treatment showed a dose-dependent inhibitory effect on promastigotes and intracellular transgenic amastigotes. The  $\text{IC}_{50}$  values of PB-BMM for promastigotes and intracellular amastigotes were  $11.2 \pm 1.23$  and

**Table 1.** Major constituents of PB-BME and PB-KVE

Compound	KI value	PB-KVE (%)	PB-BME (%)
<i>n</i> -Hexanol	870	–	–
$\alpha$ -Thujene	931	0.05	–
Sabinene	978	0.29	–
$\beta$ -Myrcene	993	0.14	–
$\alpha$ -Phellandrene	1007	–	–
$\alpha$ -Terpinene	1019	0.04	–
<i>p</i> -Cymene	1029	0.04	–
Limonene	1032	–	–
$\beta$ -Phellandrene	1034	–	–
$\beta$ -Ocimene	1036	0.06	–
<i>cis</i> -Ocimene	1045	0.05	–
Linalool	1102	0.04	–
$\alpha$ -Thujone	1104	–	–
<i>trans</i> -Sabinenehydrate	1107	–	–
Decanal	1207	0.73	–
Geraniol	1255	0.24	–
Safrole	1295	46.28	–
Eugenol	1357	27.15	73.01
<i>cis</i> -Isoeugenol	1363	3.44	3.41
<i>cis</i> -Methylisoeugenol + <i>trans</i> -isoeugenol	1382	–	–
$\alpha$ -Copaene	1386	0.32	–
$\beta$ -Elemene	1396	0.07	–
Methyl eugenol	1407	2.84	–
$\beta$ -Caryophyllene	1431	2.56	–
$\alpha$ -Humulene	1461	1.43	–
$\gamma$ -Muurolene	1482	–	–
Germacrene D	1484	2.89	–
Eugenol acetate	1534	–	7.89
Methyl chavicol	1198	–	0.19
Chavicol	1253	–	1.93
$\delta$ -Cadinene	1526	–	1.71
Total		88.66	88.14

$9.31 \pm 0.53 \mu\text{g ml}^{-1}$ , respectively. This was comparable to the  $\text{IC}_{50}$  of the standard drug miltefosine (10 and  $5 \mu\text{g ml}^{-1}$ , respectively). No anti-leishmanial activity was observed for PB-KVM, even at a 50-fold higher concentration, against either form of the parasite. Thus, further studies were conducted using landrace PB-BMM.

### Cytotoxicity of PB-BMM

PB-BMM was found to be devoid of any cytotoxic effect towards macrophages, even at a concentration of  $100 \mu\text{g ml}^{-1}$ , which is many fold higher than the  $\text{IC}_{50}$  of PB-BMM against *L. donovani* promastigotes and intracellular amastigotes (data not shown).

### Condensation of nuclear material

Condensation of nuclear material, a primary event in apoptosis-like cell death, was studied using PI. Cells with

condensed nuclei exhibit brighter red fluorescence than non-condensed nuclei. PB-BMM-treated promastigotes showed bright red fluorescent spots compared with a dull red generalized fluorescence in untreated cells (Fig. 1a).

### Externalization of phosphatidylserine in promastigotes and intracellular amastigotes after exposure to PB-BMM

During apoptosis in metazoan and unicellular cells, phosphatidylserine is translocated from the inner side to the outer layer of the plasma membrane, which can be measured by double staining with annexin V and PI. A significant number of promastigotes (60.29 %) treated with PB-BMM for 48 h stained positive for annexin V compared with 6.4 % in untreated cells (Fig. 1b, ii and i, respectively). The effect of PB-BMM was comparable to miltefosine (74.43 %; Fig. 1b, iii) (Paris *et al.*, 2004).

Harvested amastigotes from treated and untreated infected macrophages were stained with annexin V conjugated to fluorescein isothiocyanate (FITC). At 48 h, a significant number of amastigotes recovered from cells treated with PB-BMM or miltefosine stained positive for annexin V (64.89 and 70.32 %; Fig. 1b, v and vi, respectively) compared with controls at 24 h (8.02 %; Fig. 1b, iv).

### PB-BMM-induced generation of ROS in promastigotes and infected macrophages

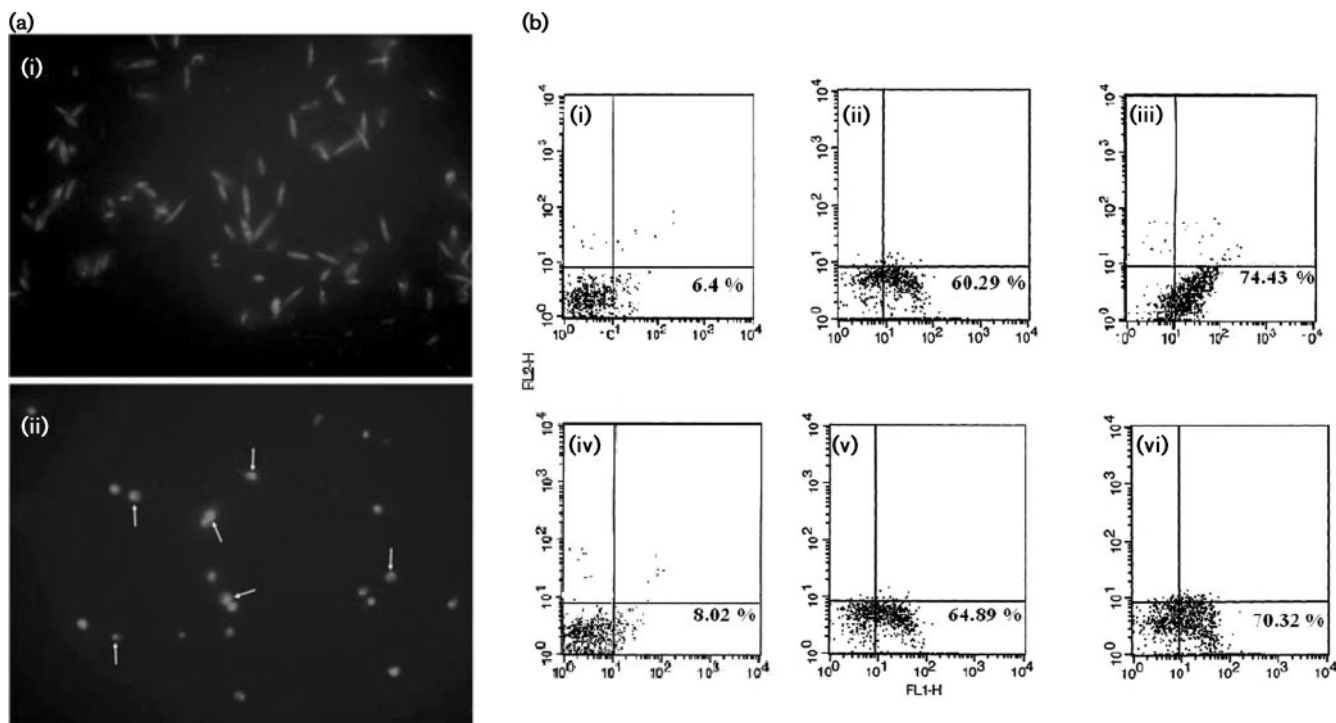
$\text{H}_2\text{DCFDA}$ , a non-polar compound, is converted on oxidation to the highly fluorescent 2',7'-dichlorofluorescein and this property has been utilized to monitor ROS generation. PB-BMM treatment of promastigotes led to a significant increase in ROS up to 24 h (fluorescence intensity units (FIU): 0 h,  $6.18 \pm 0.56$ ; 6 h,  $15.33 \pm 0.59$ ; 12 h,  $27.39 \pm 1.52$ ; 24 h,  $35.64 \pm 1.06$ ; Fig. 2a). An increase in ROS was also observed in infected macrophages following treatment with PB-BMM (FIU: 0 h,  $20.29 \pm 1.15$ ; 6 h,  $26.8 \pm 1.8$ ; 12 h,  $46.29 \pm 2.4$ ; 24 h,  $109.7 \pm 5.9$ ; Fig. 2b).

### PB-BMM-stimulated NO production in infected macrophages

PB-BMM treatment caused a significant increase in diaminofluorescein-mediated fluorescence in infected macrophages (MFI: 0 h,  $93.36 \pm 2.12$ ; 12 h,  $198.6 \pm 3.35$ ; 24 h,  $267.7 \pm 14.35$ ; Fig. 2c), indicating the generation of NO.

### PB-BMM-induced time-dependent loss of $\Delta\Psi_m$ in promastigotes and isolated amastigotes

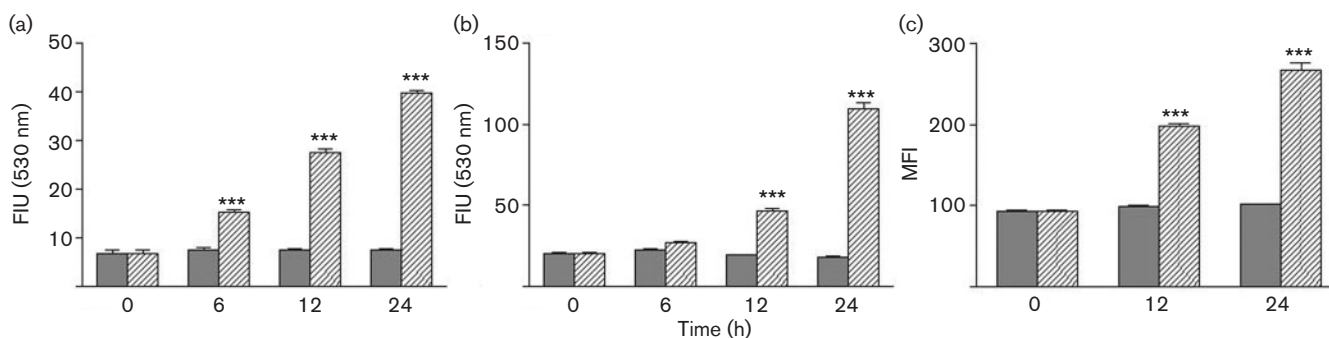
PB-BMM-induced time-dependent changes in  $\Delta\Psi_m$  were monitored at 6, 12 and 24 h post-treatment. A significant drop in  $\Delta\Psi_m$  of 44.9 % was observed 6 h post-treatment (590:530 nm ratio: control  $6.28 \pm 0.49$ ; drug-treated



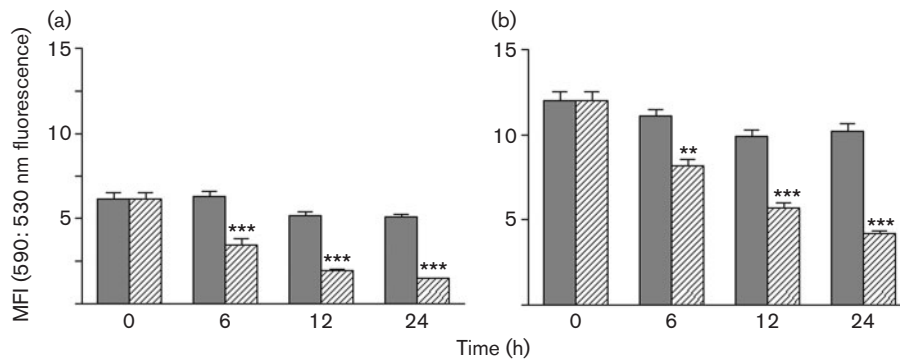
**Fig. 1.** Induction of apoptosis after PB-BMM treatment. (a) Analysis of DNA condensation in promastigotes treated with PB-BMM ( $10 \mu\text{g ml}^{-1}$ ) for 24 h by PI staining. (i) Untreated promastigotes; (ii) PB-BMM-treated promastigotes. Condensed nuclei are indicated by arrows. The results are representative of three independent experiments. (b) Externalization of phosphatidylserine following PB-BMM ( $\text{IC}_{50}$ ) treatment. The lower-left quadrant indicates the percentage of unstained cells, whilst the upper left shows PI-positive cells, the lower right shows annexin V-stained cells and the upper right shows PI- and annexin-V-positive cells. (i) Untreated promastigotes; (ii) PB-BMM-treated promastigotes; (iii) miltefosine-treated promastigotes; (iv) untreated amastigotes; (v) PB-BMM-treated amastigotes; (vi) miltefosine-treated amastigotes. The results shown are representative of at least three experiments.

$3.46 \pm 0.57$ ; Fig. 3a), which increased to 62% after 12 h of treatment (control  $5.19 \pm 0.32$ ; drug-treated  $1.95 \pm 0.2$ ; Fig. 3a). A further drop in  $\Delta\Psi_m$  of 71% at 24 h (control

$5.12 \pm 0.24$ ; drug-treated  $1.46 \pm 0.14$ ; Fig. 3a) was observed, indicating that PB-BMM caused sustained hypopolarization of the mitochondrial membrane in promastigotes.



**Fig. 2.** PB-BMM induces the generation of ROS (a, b) and NO (c). Increase in ROS generation in promastigotes (a) and infected macrophages (b) after treatment with PB-BMM for 6, 12 and 24 h. (c) NO generation in infected macrophages after treatment with PB-BMM for 0, 12 and 24 h. Each bar represents  $\text{MFI} \pm \text{SD}$ . Grey bars, untreated; hatched bars, PB-BMM treated. Asterisks indicate significant differences between treated and untreated groups: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**Fig. 3.** Decrease in  $\Delta\Psi_m$  in promastigotes (a) and isolated amastigotes (b) following treatment with PB-BMM for the indicated times and staining with the potentiometric probe JC-1 (10  $\mu$ M).  $\Delta\Psi_m$  values are expressed as the ratio of 590:530 nm fluorescence. Each bar represents the MFI  $\pm$  SD. Grey bars, untreated; hatched bars, PB-BMM treated. Asterisks indicate significant differences between treated and untreated groups: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Whilst monitoring  $\Delta\Psi_m$  in intracellular amastigotes, the macrophage mitochondria might interfere and lead to erroneous data. Isolation of the amastigotes from macrophages after drug treatment and measurement of  $\Delta\Psi_m$  could have been performed afterwards, but in this case the preparation time would have contributed to alterations in the  $\Delta\Psi_m$  (Sudhandiran & Shaha, 2003). Therefore, isolated and purified amastigotes were treated with PB-BMM for various time periods, and changes in  $\Delta\Psi_m$  were monitored. The decrease in  $\Delta\Psi_m$  due to PB-BMM treatment after 6 h was 26% (590:530 nm ratio: control  $11.09 \pm 0.7$ ; drug-treated  $8.16 \pm 0.61$ ; Fig. 3b) followed by a further decrease to 43.2% at 12 h post-treatment (control  $9.86 \pm 0.65$ ; drug-treated  $5.6 \pm 0.56$ ; Fig. 3b). PB-BMM treatment extended the decrease in  $\Delta\Psi_m$  to 59% after 24 h (control  $10.20 \pm 1.02$ ; drug-treated  $4.18 \pm 0.36$ ; Fig. 3b).

### Nuclear DNA fragmentation in promastigotes and intracellular amastigotes following PB-BMM treatment by *in situ* labelling of DNA fragments by a TUNEL assay

DNA fragmentation, a hallmark of apoptosis in metazoan and unicellular cells, was assessed by *in situ* labelling of DNA fragments by a TUNEL assay. Flow cytometric analysis of promastigotes and intracellular amastigotes isolated from macrophages treated or not with PB-BMM and probed with a TUNEL assay showed an increase in the number of cells staining positive for TUNEL. TUNEL-positive cells are represented by increased forward scatter. This indicated a greater degree of staining for fragmented DNA (nearly 60% in promastigotes, Fig. 4b, c; 48% in amastigotes, Fig. 4d, e).

### Oligonucleosomal DNA fragmentation in isolated amastigotes following treatment with PB-BMM

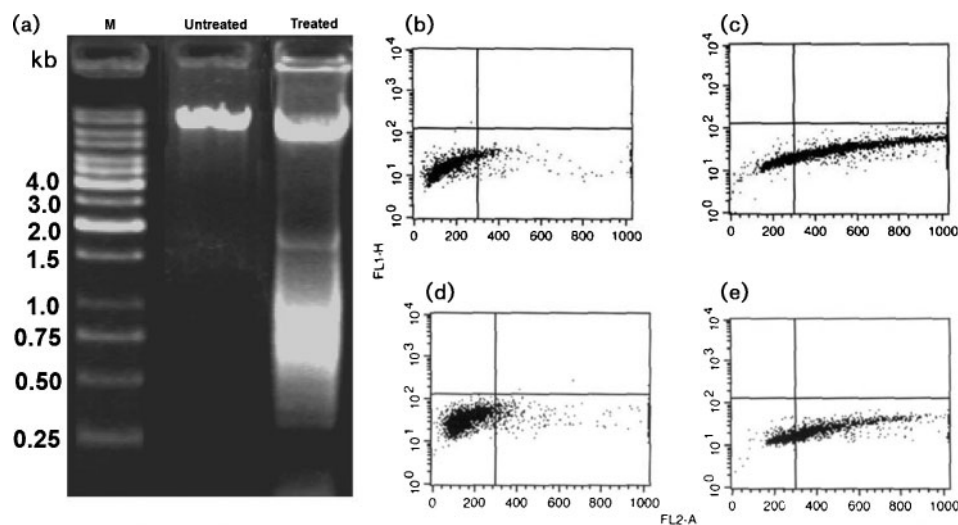
DNA laddering is a classical sign of apoptosis. Oligonucleosomal DNA fragmentation analysis of treated

isolated amastigotes showed clear fragmentation of the genomic DNA into oligonucleosomal fragments in the characteristic ladder form seen during apoptosis, compared with untreated cells (Fig. 4a).

## DISCUSSION

Several plants have been shown to possess interesting anti-leishmanial activities, validating their use in folk medicine (Rocha *et al.*, 2005). In our earlier studies, we reported gender/landrace-based differences in the anti-leishmanial activity of the methanolic extract of *P. betle*, a dioecious plant (Tripathi *et al.*, 2006). It was observed that PB-BMM showed potent anti-leishmanial activity, whereas no activity was observed with PB-KVM. Moreover, selective elimination of the parasite without affecting host macrophage cells led us to evaluate the mode of death induced in both forms of the parasite. As apoptosis can cause the selective killing of parasites without affecting the entire population (Debrabant *et al.*, 2003), this mode of cell death was studied in PB-BMM-treated *L. donovani* parasites. Interesting information regarding the phenomenon of apoptosis in *Leishmania* has been obtained in promastigotes (Paris *et al.*, 2004), but has been less studied in intracellular amastigotes, the form responsible for disease pathogenesis, due to its intracellular localization in splenic macrophages and the tedious methods required for their isolation, along with low yield. Studies carried out by Sarkar *et al.* (2008) were based on the anti-leishmanial activity of *P. betle* leaves (without assigning any particular landrace) and its mode of action against the non-pathogenic (promastigote) form. Therefore, observations of mechanisms by which effective drugs induce their anti-leishmanial effect against intracellular amastigotes would help to develop rational strategies for evaluation of the efficacy of preparations from plants.

To clarify the PB-BMM mode of action against *L. donovani*, we demonstrated that PB-BMM-induced cell



**Fig. 4.** Detection of PB-BMM-induced DNA fragmentation. (a) DNA fragmentation in isolated amastigotes after treatment. (b–e) Flow cytometric analysis after TUNEL staining of: (b) untreated promastigotes, (c) PB-BMM-treated promastigotes at 24 h post-treatment, (d) amastigotes isolated from untreated macrophages and (e) amastigotes isolated from PB-BMM-treated macrophages at 24 h post-treatment.

death in *L. donovani* shares several phenotypic features with other cases of metazoan apoptosis (Debrabant *et al.*, 2003), including phosphatidylserine exposure, PI staining, *in situ* TUNEL staining of nicked DNA and oligonucleosomal DNA fragmentation (Das *et al.*, 2001). During programmed cell death in metazoan and unicellular cells, phosphatidylserine is transferred from the inner side to the outer layer of the plasma membrane. The apoptotic nature of death induced by PB-BMM in both promastigotes and intracellular amastigotes was therefore confirmed by double staining with annexin V–FITC and PI, as annexin V, a Ca<sup>2+</sup>-dependent phospholipid-binding protein with affinity for phosphatidylserine, is routinely used to demonstrate externalization of phosphatidylserine. Annexin V–FITC labelling experiments were also performed for cells treated with PB-KVM to further confirm our finding that this landrace/gender of plant showed no activity against the parasite studied. The results of this experiment, similar to those of the untreated control, further confirmed that there are gender/landrace-based differences in the activity of methanolic extracts from *P. betle* landraces (data not shown).

*P. betle* has been shown to possess antioxidant activity, but observation that metabolic activation of some plant extracts can lead to the production of toxic pro-oxidants and cause cell injury under different conditions (Cao *et al.*, 1997) led us to evaluate whether PB-BMM could induce oxidative stress in promastigotes as well as in infected macrophages. It is well established that ROS generation in cells following drug treatment can direct the cells towards apoptosis (Chipuk & Green, 2005). The increase in ROS after exposure to PB-BMM followed by death of the parasite suggested that PB-BMM-mediated generation of

ROS by promastigotes and amastigote-infected macrophages was responsible for their apoptotic death. Generation of NO after drug treatment in infected macrophages further indicated the involvement of ROS in amastigote death. NO generated *in vitro* by NO-donating compounds has been shown to induce DNA fragmentation (Holzmuller *et al.*, 2002).

Further studies were conducted to determine the changes occurring after oxidative stress that were responsible for apoptotic cell death. Earlier studies have established that the mitochondrion is a possible target of ROS-induced apoptosis in promastigotes and intracellular amastigotes, occurring via the loss of  $\Delta\Psi_m$  (Sudhandiran & Shaha, 2003). In this study, a sharp decrease in  $\Delta\Psi_m$  indicated that mitochondrial dysfunction occurred, which initiated the changes required for the cell to enter the apoptosis-like pathway following treatment with PB-BMM. Apart from the mitochondrial dysfunction studies, all other studies related to PB-BMM inducement of apoptosis-like death were performed in intracellular amastigotes. Therefore, it was necessary to confirm whether death in isolated amastigotes occurred by the same method or not. The observation of a DNA ladder after treatment showed that isolated amastigotes also showed apoptosis-like cell death.

The observation of an apoptotic mode of cell death induced in *Leishmania* parasites by *P. betle*, and the fact that this effect was 'landrace specific' as PB-KVM was non-effective against *Leishmania*, led us to evaluate the differences in essential oil composition of both landraces to identify possible active components responsible for the anti-leishmanial activity. GC and GC-MS of the essential oil confirmed a significantly higher content of eugenol

along with eugenyl acetate in PB-BME compared with PB-KVE. Other active phenols such as anethol, chavicol, chavicol acetate and hexadecanoic acid/methyl benzoate were present in PB-BME but absent in the ineffective PB-KVE. The effectiveness of eugenol in stimulating apoptosis is known in human melanomas (Kim *et al.*, 2006). Moreover, it is known that eugenol at lower concentrations acts as an anti-oxidant and anti-inflammatory agent, whereas at higher concentrations it can work as a pro-oxidant, resulting in enhanced production of tissue-damaging free radicals (Suzuki *et al.*, 1985; Wright *et al.*, 1995). PB-BME was found to have a significantly higher eugenol content (approx. threefold higher than PB-KVE), and apoptosis induced by PB-BMM was found to be mediated via ROS generation. This finding, along with the fact that eugenol at a higher concentration enhances the generation of ROS and induces apoptosis, supports the theory that the anti-leishmanial efficacy of PB-BMM is likely to be due to the higher eugenol content. Ueda-Nakamura *et al.* (2006) demonstrated the leishmanicidal activity of the eugenol-rich essential oil derived from *Ocimum gratissimum*, which further supports our observation. The presence of other active phenols in PB-BME may also contribute to the higher efficacy of PB-BMM.

The present findings indicate that *P. betle* leaves possess bioactive components, and further identification, characterization, purification and biological evaluation of these are in progress. As the raw leaves are consumed by many people who inhabit areas with a high incidence of leishmaniasis, the use this folk medicine may have therapeutic merit and deserves to be explored further. This study has provided a novel rationale for extending the search for bioactive plants below the species level to ensure better efficacy and reproducibility.

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