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An ethanol extract of *Piper betle* Linn. mediates its anti-inflammatory activity via down-regulation of nitric oxide

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Abstract

The leaves of *Piper betle* (locally known as Paan) have long been in use in the Indian indigenous system of medicine for the relief of pain; however, the underlying molecular mechanisms of this effect have not been elucidated. The anti-inflammatory and immunomodulatory effects of an ethanolic extract of the leaves of *P. betle* (100 mg kg^{-1}; PB) were demonstrated in a complete Freund's adjuvant-induced model of arthritis in rats with dexamethasone (0.1 mg kg^{-1}) as the positive control. At non-toxic concentrations of PB (5–25 μg mL^{-1}), a dose-dependent decrease in extracellular production of nitric oxide in murine peritoneal macrophages was measured by the Griess assay and corroborated by flow cytometry using the nitric oxide specific probe, 4,5-diaminofluorescein-2 diacetate. This decreased generation of reactive nitrogen species was mediated by PB progressively down-regulating transcription of inducible nitric oxide synthase in macrophages, and concomitantly causing a dose-dependent decrease in the expression of interleukin-12 p40, indicating the ability of PB to down-regulate T-helper 1 pro-inflammatory responses. Taken together, the anti-inflammatory and anti-arthrotic activity of PB is attributable to its ability to down-regulate the generation of reactive nitrogen species, thus meriting further pharmacological investigation.

Introduction

Inflammation is the response of the vascular and supporting elements of a tissue to injury resulting in the formation of a protein-rich exudate, provided the injury has not been severe enough to cause tissue destruction (Majno & Joris 1996). The inflammatory response, characterized by movement of fluid and leukocytes from blood into extravascular tissues, is regulated by several mediators including cytokines, eicosanoids, nitric oxide (NO) and reactive oxygen species, which in turn are produced as part of a well-orchestrated immune response by lymphocytes, macrophages and other immunologically active cells (Korhonen et al 2005).

The management of inflammatory conditions includes the use of anti-inflammatory drugs, the chronic administration of which is often associated with several adverse effects. Natural plant products are emerging as important alternative therapeutic options, being cheap, abundantly available, and relatively less toxic. *Piper betle* Linn. (Piperaceae) is widely recognized as a traditional medicinal plant not only in India but in many countries across Southeast Asia. Locally known as "Paan", its leaves, and in a few cases leaf extracts, have been prescribed as an anti-stomatitis agent, antitussive, astringent, antiseptic, carminative, stimulant, tonic, expectorant, nerve stimulant, dental paste and inhibitor of nasal bleeding (Nadkarni 1976). Its ameliorative properties in elephantiasis and painful eye conditions have been documented (Nadkarni 1976). In more recent times, the pharmacological activities of *P. betle* with regard to its antimicrobial activity (Shitut et al 1999), antifertility properties (Sarkar et al 2000), anti-ulcer properties (Majumdar et al 2002), inhibition of platelet aggregation (Jeng et al 2002) and radiation-induced stress (Choudhary & Kale 2002; Bhattacharya et al 2005), and antidiabetic properties (Santhakumari et al 2003) have been demonstrated.

Studies elucidating the anti-inflammatory properties of *P. betle* are lacking. In the present study, the anti-inflammatory activity of an ethanol extract of the leaves of *P. betle*

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Linn. was examined in a rat model of chronic inflammation. The enzyme inducible nitric oxide synthase (iNOS), chief architect of a 1000-fold increase in NO production in response to diverse stimuli, is known to be involved in the pathogenesis of inflammatory disorders, including rheumatoid arthritis (MacMicking et al 1997). Interleukin-12 (IL-12), a heterodimeric cytokine produced by macrophages and dendritic cells upon induction by microbial products, has been implicated in the pathogenesis of T-helper 1 (Th1) mediated chronic inflammatory disorders such as arthritis and inflammatory bowel disease (Adorini 1999).

Materials and Methods

Reagents and chemicals

N-1 naphthyl ethylene diamine dihydrochloride was purchased from Loba Chemie Pvt Ltd (Mumbai, India). Sulfanilamide and phenazine methosulfate were from Sisco Research Laboratories (Mumbai, India). Complete Freund's adjuvant, RPMI-1640 phenol-red-free powdered medium, 4,5-diaminofluorescein diacetate (DAF-2 DA), lipopolysaccharide (LPS) and *N*-monomethyl arginine were from Sigma-Aldrich (St Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt or MTS was purchased from Promega (Madison, WI, USA). The RNAqueous kit was obtained from Ambion (Austin, TX, USA) and the OneStep RT-PCR kit was from Qiagen (Hilden, Germany). The primers were purchased from Sigma Genosys (St. Louis, MO, USA).

Preparation of extract

Fresh *P. betle* leaves were collected locally and authenticated by the Botanical Survey of India (Shibpur, Howrah, India). After being air dried and powdered in a hand crusher, the dried, powdered leaves (300 g) were extracted twice with absolute ethanol (900 mL each time) and the resultant filtrate was solvent-recovered by distillation in a Soxhlet apparatus (Bhattacharya et al 2005). The concentrated extract, referred to as PB, was subsequently dried in a rotary evaporator and lyophilized, the yield being 3.63 g. For all experiments, propylene glycol was used as the solvent.

Identification of phytoconstituents in crude extract of PB

PB was dissolved in methanol (50 mL), treated with activated charcoal (0.2 g), and the mixture warmed at ~60°C. After filtration, the extract was concentrated in-vacuo and lyophilized to obtain a chlorophyll-free amorphous, yellowish brown solid (1.23% w/w yield).

The chemical constituents of PB were analysed by high-performance liquid chromatography (HPLC) with a Jasco model PU-2080 plus chromatogram using Hypersil GOLD (250×4.6 mm, particle size 5 μm; Thermo Electron Corporation, Waltham, MA, USA) column; the eluent was acetonitrile/water (1:1, flow rate 0.5 mL min^{-1}) and peaks were detected at 254 nm.

Animals

Male, Sprague-Dawley rats (120–150 g) were used in this study. All animals were housed in the departmental animal facility under standard temperature conditions (25 ± 5°C). A 12-h day/night cycle was maintained. Animals were fed a standard pellet diet and provided water *ad libitum*. All animals were acclimatized for a week before starting the experiment and experimental protocols received prior approval from the institutional animal ethical committee.

Test for chronic inflammation in a complete Freund's adjuvant-induced arthritis model in rats

Experimental animals were divided into three groups with eight rats per group. Arthritis was induced by injecting 0.1 mL of complete Freund's adjuvant intradermally into the sub-plantar region of the hind paw of each animal (Newbould 1963). From Day 1 to 13 the rats received PB (100 mg kg^{-1}, 0.5 mL orally) or dexamethasone (0.1 mg kg^{-1}, 0.5 mL orally), as a standard reference, while rats in the control group received propylene glycol (0.5 mL). Oedema formation in both hind paws was calculated on Days 3, 5, 9, 13 and 21 by plethysmometric measurement of paw volumes. The percent inhibition of oedema formation was calculated as follows (Tsai & Linn 1999): $(V_c - V_t)/V_c \times 100$, where V_c and V_t are the oedema volumes of the control and treated groups, respectively. On Day 21, the severity of the secondary lesions was graded using an arthritic index (Table 1) and the sum of the scores for each animal were calculated (Schorlemmer et al 1999).

Evaluation for primary lesions involved determining the percent inhibition of paw volume of the injected left paw versus vehicle control as measured on Day 5. For secondary lesions, the percentage inhibition of paw volume of the non-treated right paw versus control was measured on Day 21. Finally, the total percentage change was calculated by addition of percent inhibition of paw volume of the injected paw on Day 5 + percent inhibition of paw volume of the non-treated paw on Day 21 + percent change in the arthritic index (Schorlemmer et al 1999).

Preparation of mouse peritoneal macrophages

Swiss albino mice received thioglycollate (4% in 0.02 M sterile phosphate-buffered saline (PBS) pH 7.2; 1 mL per animal,

Table 1 Arthritic score

Site	Nature of lesion	Score
Ears	Absence of nodules and redness	0
	Presence of nodules and redness	1
Nose	No swelling of connective tissue	0
	Intensive swelling of connective tissue	1
Tail	Absence of nodules	0
	Presence of nodules	1
Forepaws	Absence of inflammation	0
	Presence of inflammation	1
Hind paws	Absence of inflammation	0
	Slight inflammation	1
	Moderate inflammation	2
	Marked inflammation	3

i.p.). After 96 h, macrophages were collected by peritoneal lavage using chilled RPMI-1640 phenol-red-free medium (10 mL). The peritoneal exudate was centrifuged at 300 *g* for 10 min and the resultant cell pellet was washed twice with medium and finally resuspended in RPMI-1640 phenol-red-free medium supplemented with 10% fetal calf serum, penicillin (50 units mL^{-1}) and streptomycin (50 $\mu g\,mL^{-1}$), referred to as Medium A. Viable macrophages (>95% as determined by Trypan blue dye exclusion) were maintained at 37°C in a 5% CO_2 humidified incubator.

Measurement of NO production in macrophages

Macrophages obtained by peritoneal lavage as described above were resuspended in Medium A in 12-well tissue culture plates (2.5×10^6 macrophages mL^{-1}/well) and equilibrated at 37°C, 5% CO_2 for 3 h. Macrophages were incubated with LPS (10 $\mu g\,mL^{-1}$) in the absence or presence of PB (0–25 $\mu g\,mL^{-1}$) at 37°C, 5% CO_2 for an additional 48 h. The amount of NO generated was measured by the Griess assay (Sarkar et al 2005). Briefly, 0.5 mL of Griess reagent, a 1:1 mixture of naphthylethylenediamine dihydrochloride (0.1% in water) and sulfanilamide (1% in 5% phosphoric acid), was added to 0.5 mL of culture supernatant and incubated in the dark at 25–30°C for 10 min. Absorbances at 546 nm were measured spectrophotometrically and a standard curve was generated using sodium nitrite (0–100 μM) to calculate nitrite concentrations. To demonstrate specificity, macrophages were exposed to LPS (10 $\mu g\,mL^{-1}$) along with *N*-monomethyl arginine (L-NMMA, 100 μM), a proven inhibitor of NO production (Olken & Marletta 1993).

Assessment of macrophage viability

Macrophages (2×10^5 macrophages/200 μL/well) were incubated at 37°C, 5% CO_2 for 3 h, following which they were incubated with PB (0–100 $\mu g\,mL^{-1}$) or propylene glycol (up to 2 % v/v) and incubated at 37°C, 5% CO_2 for an additional 48 h. At the end of 48 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (2 $mg\,mL^{-1}$ in PBS; MTS) combined with phenazine methosulphate (0.92 $mg\,mL^{-1}$ in PBS) in a ratio of 10:1 was added (20 μL/well) and the plates were incubated at 37°C for 3 h (Ganguly et al 2006). Specific absorbances were measured at 490 nm by subtracting the background absorbance of the medium containing the drug/solvent from the total absorbance and percent viability was calculated as follows: $(\text{specific absorbance}_{\text{treatment}}/\text{specific absorbance}_{\text{control}})\times100$.

Flow cytometric determination of intracellular NO production

Murine peritoneal macrophages obtained as described above were seeded in 6-well tissue culture plates (1×10^6 macrophages mL^{-1}/well) and equilibrated at 37°C, 5% CO_2 for 1 h. To induce generation of NO, LPS (10 $\mu g\,mL^{-1}$) was added, followed by the addition of either PB (25 $\mu g\,mL^{-1}$) or L-NMMA (100 μM) for 24 h at 37°C, 5% CO_2. Following removal of the medium, the cells were incubated with DAF-2 DA (10 μM) for 30 min at 37°C, 5% CO_2. The reaction was terminated by placing the cells on ice; adherent cells were removed by gentle scraping and prior to acquisition, 1 $\mu g\,mL^{-1}$ propidium iodide (PI) was added and incubated for an additional 10 min at 20–25°C. The cells were then acquired on a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA, USA) using forward scatter versus side scatter to gate the macrophage population, a side scatter versus FL2 dot plot to distinguish viable macrophages (PI negative) from non-viable macrophages (PI positive), and a FL1 histogram to quantify fluorescence of viable macrophages. Acquisition and subsequent analyses were done using BD Cell Quest Pro software.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Macrophages (1×10^7/5 mL) were seeded in 90-mm tissue culture Petri dishes and incubated with LPS alone or in combination with PB (2.5–25 $\mu g\,mL^{-1}$) or PG (0.5 % v/v). At the end of 24 h, total RNA was isolated using the RNAqueous Kit (Ambion, Inc., Austin, TX, USA) as per the manufacturer's instructions. Subsequently, RT-PCR was carried out with the OneStep RT-PCR kit using 100 ng of isolated RNA. The RNA was reverse-transcribed into cDNA, which was further amplified, using gene-specific primers for β-actin (sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, anti-sense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), iNOS (sense: 5′-CATGGCTTGCCCCTGGAAGTTTCTCT-TCAAAG-3′, anti-sense: 5′-GCAGCATCCCCTCTGATGGT-GCCATCG-3′) and IL-12 p40 (sense: 5′ CAGAAGCTAACCATCTCCTGGTTTG-3′, anti-sense: 5′-TCCGGAGTAATTTGGTGCTTCACAC-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 manufacturer's 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 90 s) (Portugal et al 2004). With regard to β-actin and IL-12 p40, samples underwent 35 cycles of denaturing (94°C for 30 s), annealing (58°C for 45 s) and extension (72°C for 30 s) (Kim et al 2003). After a terminal extension step at 72°C for 10 min, RT-PCR products were resolved by electrophoresis on agarose gels (1.5%) containing ethidium bromide (0.5 $\mu g\,mL^{-1}$) and visualized with the Molecular Imager ChemiDoc XRS System (Bio Rad, CA, USA). The extent of iNOS and IL-12 p40 expression was quantified by densitometric analysis using TotalLab Nonlinear Dynamic Image Analysis software (Nonlinear USA Inc., Durham, NC, USA).

Statistical analysis

Results were expressed as the mean ± s.d. Statistical analysis was performed using the non-parametric Kruskal–Wallis analysis of variance and the Mann–Whitney *U*-test. To analyse the effect of time on oedema volume, we used Friedman's analysis of variance followed by Wilcoxon's matched pairs signed rank test. Concentration effects on cell viability were analysed using one-way analysis of variance and Tukey's multiple comparison test. Differences were considered significant at $P<0.05$.

Results

Four major phytoconstitutents of PB were identified by HPLC analysis. The HPLC chromatogram (Figure 1) identified the four major constituents as chevibetol (0.35%), allylpyrocatechol (0.41%), and their respective glycosides (0.021% and 0.047%) by comparing their HPLC retention times with those of standard compounds. The HPLC retention times for allylpyrocatechol glycosides, chevibetol glycosides, allylpyrocatechol and chevibetol were 6.7 min, 9.5 min, 19.1 min and 23.7 min, respectively.

PB exhibited anti-inflammatory and moderate anti-arthrotic activity in the chronic adjuvant-induced inflammation model. PB decreased oedema formation in the adjuvant-treated left paw on Day 5 ($P<0.05$), as compared with the control (Table 2). On Day 5, the critical day for measurement of activity against primary adjuvant-induced inflammation in the injected paw, PB (100 mg kg^{-1}) caused a significant decrease (24.73%, $P<0.05$) in oedema volume; a 62.37% decrease ($P<0.05$) was demonstrated by dexamethasone (0.1 mg kg^{-1}), the reference drug. With regard to the composite arthritic scores (Table 2) from Days 13–21, the median arthritic score in PB-treated animals was 1.5-fold less than the controls ($P<0.05$), being 2.0 versus 3.0, respectively; dexamethasone-treated animals showed a 6.0-fold decrease ($P<0.05$) in the arthritic score, being 0.5. Accordingly, the total % change in the PB group was 58.06% (24.73% + 0 + 33.33%); in the dexamethasone group the % change was 145.7% (62.37% + 0 + 83.33%).

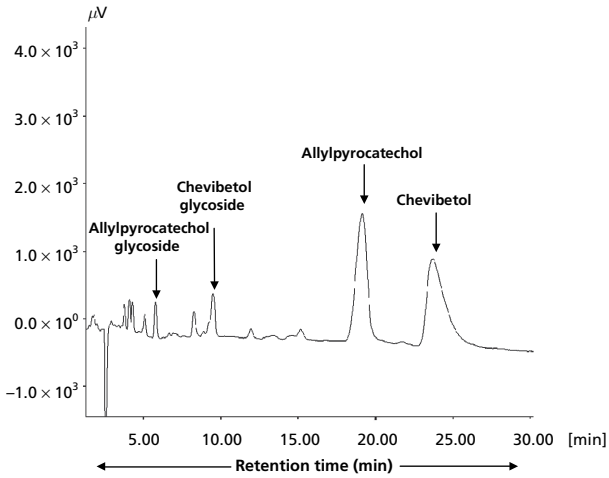

Figure 1 HPLC profile of an ethanol extract of *Piper betle* (PB). The chemical constituents of PB were analysed by HPLC, detecting several peaks at 254 nm. Allylpyrocatechol glycoside (0.047%), chevibetol glycoside (0.021%), allylpyrocatechol (0.41%) and chevibetol (0.35%) were identified using standards, their HPLC retention times being 6.7, 9.5, 19.1 and 23.7 min, respectively.

PB suppressed nitric oxide release in murine peritoneal macrophages. The effect of PB on NO production was examined in murine peritoneal macrophages using the nitrite accumulation assay. The NO production triggered by LPS was attenuated in a dose-dependent manner by PB (0–25 μg mL^{-1}) (Figure 2). In macrophages exposed to PB (15 and 25 μg mL^{-1}), the enhanced NO levels observed in LPS-treated macrophages was decreased by 28.57% ($P<0.05$) and 40% ($P<0.05$), respectively. To demonstrate specificity of the reaction, LPS-treated macrophages were incubated with L-NMMA, an inhibitor of iNOS. Compared with LPS-treated macrophages, L-NMMA caused a 74.29% decrease ($P<0.05$) in NO production (Figure 2).

PB decreased intracellular NO levels in peritoneal macrophages. To further corroborate that PB causes a reduction in NO production, levels of intracellular NO in peritoneal macrophages were determined by flow cytometry using DAF-2 DA, a non-fluorescent freely cell permeant probe that measures NO in viable cells (Tarpey et al 2004). Within the cell, the diacetate groups are hydrolysed by cytosolic esterases releasing DAF-2 and the reagent is sequestered inside the cell. Nitric-oxide-derived nitrosating agents such as dinitrogen trioxide or N_2O_3 then nitrosate the non-fluorescent dye DAF-2 DA to its highly fluorescent triazole derivative, DAF-2T.

The mean fluorescence channel values (Figure 3) indicated that the LPS-induced increase in intracellular NO was attenuated by addition of PB (25 μg mL^{-1}) as the mean fluorescence channel value decreased from 71.83 to 12.9, translating into a 82.04% decrease in fluorescence. L-NMMA, a potent blocker of iNOS activity also decreased LPS-induced fluorescence, the mean fluorescence channel value being 23.3.

Table 2 Effect of an ethanol extract of *Piper betle* (PB) in a complete Freund's adjuvant-induced arthritis model of chronic inflammation

Group	Median arthritic score and range (% change)	Oedema volume (mL) (% inhibition)				
		Day 3	Day 5	Day 9	Day 13	Day 21
Control	3.0; 3.0–4.0	0.8 ± 0.11	0.93 ± 0.02	0.53 ± 0.09	0.63 ± 0.09	0.5 ± 0.12
PB (100 mg kg^{-1})	2.0*; 1.0–3.0 (33.33%)	0.71 ± 0.18 (11.25%)	0.7 ± 0.18* (24.73%)	0.65 ± 0.15 –	0.61 ± 0.18 (3.17%)	0.56 ± 0.20 –
Dexamethasone (0.1 mg kg^{-1})	0.5*; 0.0–1.0 (83.33%)	0.35 ± 0.04 (68.75%)	0.35 ± 0.05* (62.37%)	0.23 ± 0.14 (56.60%)	0.18 ± 0.18 (71.43%)	0.25 ± 0.05 (50.00%)

The % change in the arthritic index was calculated by the method of Schorlemmer et al (1999). For oedema volume, the results are mean ± s.d., n = 8. *$P<0.05$, significantly different compared with control.

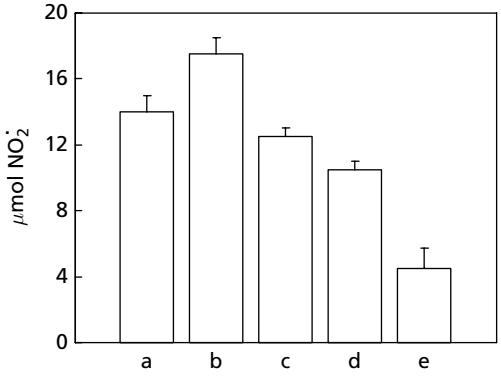

Figure 2 An ethanol extract of *Piper betle* (PB) decreased extracellular nitric oxide (NO) release in murine peritoneal macrophages. Murine peritoneal macrophages (2.5×10^6 mL^{-1}/well; (a) were incubated with 10 μg mL^{-1} lipopolysaccharide (b) in the presence of 15 μg mL^{-1} PB (c), 25 μg mL^{-1} PB (d) or 100 μM L-NMMA (e) at 37°C, 5% CO_2 for 48 h and assayed for levels of NO. Data represent the mean ± s.d.

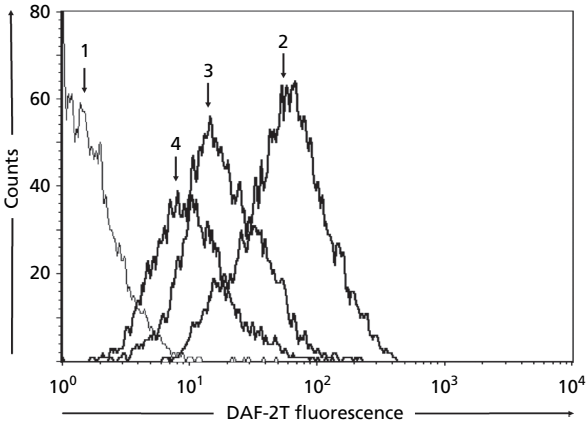

Figure 3 An ethanol extract of *Piper betle* (PB) reduces intracellular nitric oxide (NO) production in murine peritoneal macrophages. Representative histogram profile of murine peritoneal macrophages (1×10^6 mL^{-1}/well; 1) that were treated with 10 μg mL^{-1} lipopolysaccharide (2) in conjunction with 100 μM L-NMMA (3) or 25 μg mL^{-1} PB (4). Cells were incubated at 37°C, 5% CO_2 for 24 h and assayed for intracellular NO using 4,5-diaminofluorescein diacetate. DAF-2T, triazole derivative of 4,5-diaminofluorescein-2 diacetate.

PB (25 μg mL^{-1}) was non-toxic to peritoneal macrophages. To confirm that the observed suppression of NO production (Figures 2 and 3) was not due to any cytotoxic effect of PB or the vehicle, propylene glycol (PG), macrophages were incubated with PB (0–100 μg mL^{-1}) or PG (0–2% v/v, in medium) and cell viability was evaluated. No cytotoxicity was observed with PB at a concentration of 25 μg mL^{-1}, with higher concentrations being toxic (Figure 4). Macrophages incubated with PG showed no loss in viability up to a concentration of 0.5% v/v (data not shown), while viability was reduced to 80% with higher concentrations (1–2% v/v), confirming that the PB-mediated decrease in NO production is not due to PB- or PG-mediated cytotoxicity.

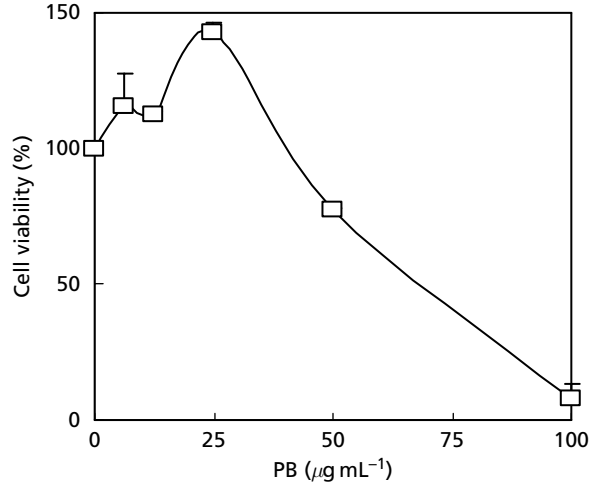

Figure 4 Cytotoxicity of an ethanol extract of *Piper betle* (PB) on murine peritoneal macrophages. Murine peritoneal macrophages (2×10^5/200 μL/well) were incubated with PB (0–100 μg mL^{-1}) for 48 h at 37°C, 5% CO_2 and cell viability was measured by the MTS assay. Each point corresponds to the mean ± s.d. of three experiments in duplicate.

PB down-regulated transcription of iNOS and IL-12 p40. Since PB down-regulated NO synthesis, we next examined the iNOS mRNA expression by RT-PCR. As evident in Figure 5, PB (0–25 μg mL^{-1}) co-incubated with LPS (10 μg mL^{-1}) for 24 h caused a dose-dependent reduction in iNOS expression ($P < 0.01$).

IL-12, a heterodimeric cytokine, has been implicated in the pathogenesis of several Th1-mediated chronic inflammatory disorders. The effect of PB on the expression of IL-12 p40 was therefore studied. As evident from Figure 4, PB co-incubated with LPS (10 μg mL^{-1}) decreased the IL-12 p40 expression in a dose-dependent manner ($P < 0.05$).

Discussion

The immunologically mediated chronic inflammatory model of complete Freund's adjuvant-induced arthritis is an established model of rheumatoid arthritis (Williams 1998). This method involves the injection of complete Freund's adjuvant into the hind paw of the rat to induce inflammation (Newbould 1963), the immunogenicity of which has been attributed to bacterial peptidoglycan and muramyl dipeptide, constituents of the complete Freund's adjuvant. Antigen presenting cells, primarily macrophages, perform a central role in the model of immunologically mediated chronic synovial inflammation and consequent arthritis. Once activated, macrophages synthesize inflammatory mediators such as prostaglandin E_2 and pro-inflammatory cytokines such as tumour necrosis factor α and interleukin-1, which in turn produce a variety of enzymes that are ultimately responsible for initiation of cartilage and bone destruction (Brennan et al 2006).

P. betle or Paan is a well known medicinal plant in India (Nadkarni 1976), its leaf extracts finding use in clinical situations where inflammation is an important feature. We tested an ethanolic extract of *P. betle* leaves for anti-inflammatory

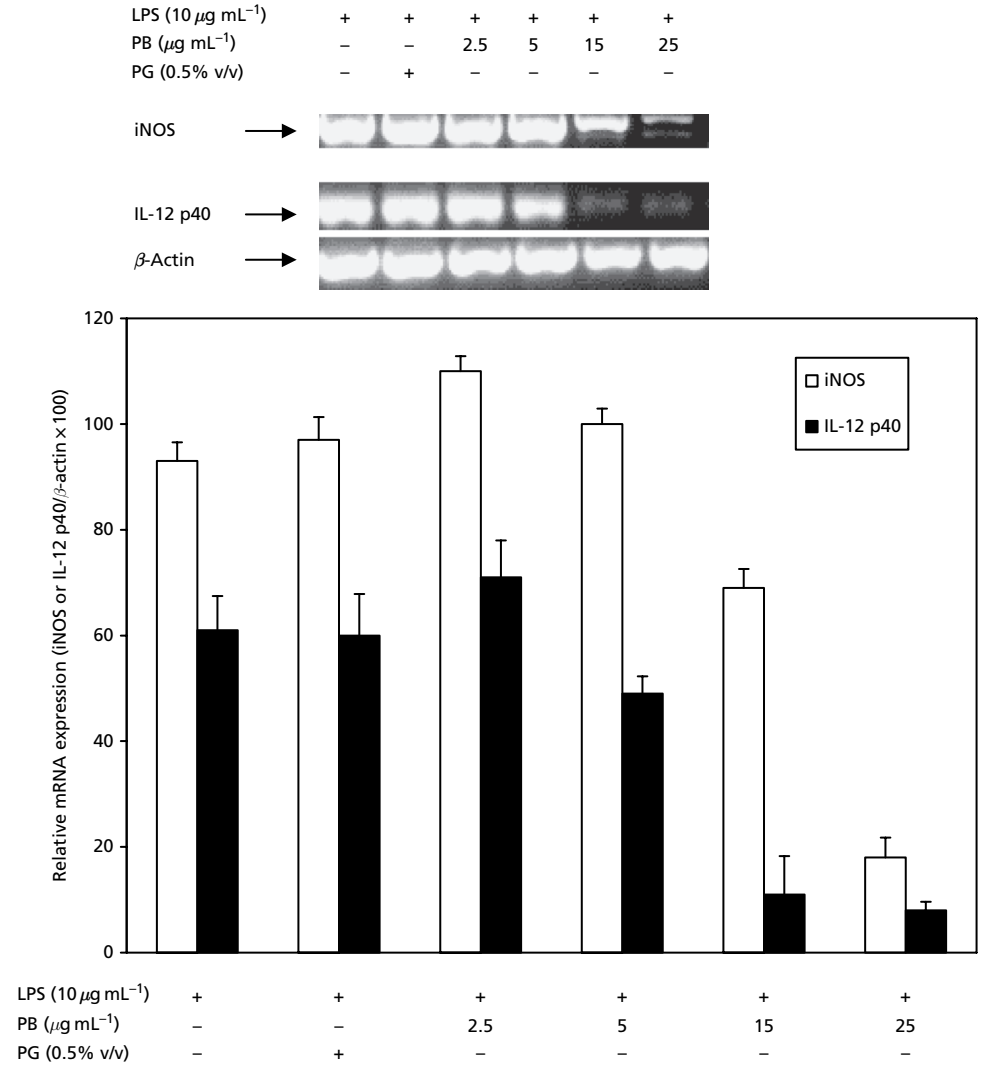

Figure 5 An ethanol extract of *Piper betle* (PB) down-regulates enzyme inducible nitric oxide synthase (iNOS) and interleukin-12 p40 (IL-12 p40) expression in murine peritoneal macrophages. Murine peritoneal macrophages were treated with lipopolysaccharide (LPS) in conjunction with PB or propylene glycol (PG) for 24 h. RNA was isolated and subjected to RT-PCR. RT-PCR products of β-actin, iNOS and IL-12 p40 mRNA were resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of iNOS and IL-12 p40 was quantified densitometrically with TotalLab software. The results are representative of three experiments.

activity and wanted to establish some of the underlying mechanisms contributing towards its anti-inflammatory activity. Investigation of the phytoconstituents of PB revealed the presence of phenolic compounds, chevibetol and allylpyrocatechol, and their respective glycosides in significant proportions (Figure 1). Since phenolics are reputed to be potent anti-inflammatory compounds (Surh et al 2001), it was expected that the phenolics identified in PB would contribute towards its anti-inflammatory activity.

PB by effecting a decrease in primary oedema in test rodents following injection of complete Freund's adjuvant (Table 2) clearly demonstrated anti-inflammatory and anti-arthrotic activity. The anti-arthrotic effect of PB, evident in the downgrading of the arthritic score, suggested an influence on the contributory role of macrophages, key immune cells involved in the pathogenesis of arthritis.

NO is considered as a primary biological signalling and effector molecule in inflammation and immune responses. Macrophages generate NO as a free radical via iNOS, which catalyses the oxidation of guanidino nitrogen of L-arginine, thereby releasing L-citrulline and NO (MacMicking et al 1997). NO downstream can activate cyclooxygenases that catalyse production of prostaglandins and leukotrienes, proven mediators of inflammation (Korhonen et al 2005). Quantification of NO, in actuality the amount of nitrites, one of two primary stable and non-volatile breakdown products of NO, is measured using Griess reagent (Sarkar et al 2005). Enhanced NO production by macrophages upon exposure to

substances of microbial origin such as LPS is facilitated by a characteristic increase in iNOS activity. This has been documented in both peritoneal macrophages (Son et al 2006) and macrophage cell lines (Padwad et al 2006). Peritoneal macrophages co-incubated with LPS and PB exhibited a dose-dependent reduction in nitrite accumulation vis-à-vis LPS treated macrophages (Figure 2) and also intracellular NO levels (Figure 3), suggesting that PB, by decreasing the release of NO, inhibited release of inflammatory inhibitors such as prostaglandins, culminating in suppression of inflammation. This decrease in NO production in macrophages was not due to PB- or PG-induced cytotoxicity (Figure 4) but specifically by inactivation of macrophages.

NO production in physiological systems is attributed to three isoforms of the enzyme NOS, namely, the constitutively expressed eNOS and nNOS and the inducible iNOS, which play critical roles in the maintenance of homeostasis in circulatory, nervous and immune systems, respectively. Distinct from the constitutively expressed isoforms, iNOS, when triggered by diverse immune stimuli, is linked to a >1000-fold increase in NO production over normal physiological rates of NO synthesis (MacMicking et al 1997). In resting cells, iNOS is not expressed normally but is induced upon stimulation with agents such as pro-inflammatory cytokines and LPS (MacMicking et al 1997). Inducible enzymes, iNOS and COX-2, have been implicated in the overproduction of prostaglandins and NO, key players in the pathophysiology of arthritis and other inflammatory conditions (MacMicking et al 1997). The effect of PB on iNOS expression, determined by quantitating RT-PCR products of iNOS mRNA, indicated that PB had a dose-dependent inhibitory effect even when co-stimulated with LPS (Figure 5), thus accounting for the PB-induced reduction of NO (both intracellular and extracellular) in peritoneal macrophages (Figures 2 and 3).

The key functions of IL-12, a heterodimeric cytokine produced by macrophages and dendritic cells when challenged by microbes or microbial products, include induction and maintenance of Th1 responses (Trinchieri 1995). IL-12 has also been implicated in the pathogenesis of Th1-mediated chronic inflammatory disorders such as multiple sclerosis, arthritis and inflammatory bowel disease (Adorini 1999). The biologically active IL-12 is a heterodimer composed of two subunits, the inducible p40 and the constitutively expressed p35, which are encoded by two separate genes. The p40 gene is only detected in cells that produce bioactive IL-12 (Gubler et al 1991) and is strongly induced by intracellular bacteria and bacterial products that stimulate Th1 responses in-vivo (D'Andrea et al 1992). Up-regulated IL-12 p40 levels have also been associated with increased NO production by iNOS in pathological states (Pahan et al 2001). RT-PCR analysis for IL-12 p40 indicated that PB caused a dose-dependent down-regulation of its expression (Figure 5), indicating that PB is possibly acting further upstream on the interlinked IL-12 p40–iNOS expression cascade.

In conclusion, the present study demonstrated the anti-inflammatory and anti-arthrotic activity of the ethanolic extract of leaves of *P. betle* Linn., which was partly mediated by suppression of NO production, resulting in inhibition of the release of inflammatory mediators.

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