Landrace/gender-based differences in phenol and thiocyanate contents and biological activity in *Piper betle* L.

*Piper betle* L. leaves are used in many countries as masticatory with areca nut, lime and spices such as cardamom, clove and cinnamon, which act as ‘breath fresheners’ and help in the prevention of halitosis. It is called ‘paan’ in Hindi and ‘tambula’ in Sanskrit. Frequent references of *P. betle* can be found in ancient Sanskrit texts, including *Charaka*, *Susruta* Sambha and Astonga Hridayam. Since this crop is under obligate vegetative propagation and cultivated widely, it is claimed to have hundreds of landraces, which can be broadly grouped into five to six types such as Banglia, Desavari, Kapoori, Sanchi, Meetha and Khaesi. Its utility as an anti-inflammatory and antimicrobial is emphasized at several places


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poor, causing significant mortality and morbidity.

VL, caused by Leishmania donovani and lymphatic filariasis transmitted by Wuchereria bancrofti or Brugia malayi, are considered as major tropical and neglected diseases of the developing world by DNDi and TDR/WHO. Annually, 500,000 new cases of VL occur worldwide\(^5\), of which nearly 40–50% is in India. The situation has turned more serious with the recent emergence of VL as an opportunistic infection in the HIV-infected population. On the other hand, more than a billion global population is at risk of filarial infection, ~128 million people are already infected and ~40 million seriously incapacitated by the disease, with one-third of the infected people living in India\(^5\).

The current arsenal of therapeutic agents against VL and filariasis is limited. Antimonials, the mainstay of treatment for VL, can no longer be used in highly endemic northeastern India because of drug resistance. Traditional second-line drugs (pentamidine and amphotericin B) possess serious side effects, are difficult to administer and newer formulations of amphotericin B are not affordable in less developed countries. The first effective oral drug, miltefosine, has been licensed in India in 2002, but the development of other drugs in clinical phases (paromomycin and sitamaquine) is slow. The two available antifilarial drugs, viz. diethylcarbamazine (DEC) and ivermectin, are principally microfilaricidal with limited action on adult filarial parasites. A drug which may either kill the adult parasite or adversely affect the reproductive potential of adult worms, is therefore needed. Given the limitations of the current treatments, there is an urgent need for the development of new therapeutics. India being rich in traditional medicinal plant species, provides an opportunity for exploiting them for various diseases and metabolic disorders. Leaves of *P. betle* are widely used by both the rural and urban population in India in various forms; however, there have been no reports in the literature to the best of our knowledge on its antiparasitic activity. We have attempted to explore the antileishmanial and antifilarial potentials of the leaves of *P. betle* in vitro.

*P. betle* is known to have explicit diuretic and about hundred landraces are reported. Differences between landraces in terms of leaf shape, size (Figure 1) and chlorophyll content have been reported\(^6\)–\(^11\).

In the present study, Bangla Mahoba (BM\(^5\)) and Kapoori Velialkodi (KV\(^5\)) were used. The water decoction and methanol extracts of leaves of both the plants and their fractions have been evaluated *in vitro* using different stages of experimental human parasite, *L. donovani* and a sub-periodic strain of human lymphatic filarial, *B. malayi*.

*P. betle* landraces were grown in the botanical garden at the National Botanical Research Institute, Lucknow under fully protected cultivation. Fully grown mature leaves of BM\(^5\) and KV\(^5\) were harvested, washed, weighed and loaded in elevengear apparatus for preparation of decoction. One kilogram of leaves (200 g dry weight) was boiled in 1 l of water for total 18 h, the decoction was first filtered through a sieve followed by Whatman No. 1 filter paper and further clarified by centrifugation at 10,000 g for 10 min in cold and stored in a refrigerator until further use. Methanol extract was prepared using shake-dried leaf powder. The extract was concentrated by Rota vapour and further fractionated into hexane, chloroform, butanol and water fractions. Methanol extract and its fractions were vacuum-dried and stored in vacuo under cold condition.

The total phenolic content was measured in the decoction, methanol extract and its fractions according to the Folin–Ciocalteu assay\(^12\). Thiocyanate in *P. betle* leaves was determined according to the method described by Betts and Dainton\(^13\). Results were expressed as milligrams of phenol per gram of dry sample.

The antileishmanial efficacy of the plant was assessed *in vitro* against GFP-transfected *L. donovani* promastigotes and intracellular amastigotes by flow cytometry\(^14,15\). Briefly, log-phase GFP-transfected promastigotes (1 × 10⁶ cells/ml) were incubated with twofold dilutions of water decoction starting from 500 μl/ml. With regard to crude methanolic extract or its fractions, the concentrations used were 100, 50 and 25 μg/ml, followed by FACS analysis. Growth of promastigotes was also monitored after 24, 48 and 96 h by counting the number of motile promastigotes microscopically in a Neubauer chamber slide. For assessing activity against intracellular amastigotes, J774 A.1 macrophages (10⁴ cells/well) infected with GFP-transfected promastigotes (10:1) were used. The level of infection in infected macrophages before and after drug treatment was quantitated by flow cytometry. Percentage of inhibition and 50% inhibitory concentrations (IC\(_{50}\)) were calculated by linear regression analysis. Tests were performed at least in triplicate on three different days in order to verify the results. Miltefosine was used as positive control (IC\(_{50}\) 5.0 μg/ml). The efficacy of samples was also assessed by Giemsa staining\(^16\).

The antifilarial efficacy of the plant and its fractions was assessed *in vitro* on

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**Figure 1.** Differences in shape and pigment content in leaf of Kapoori Vellaikodi (a) and Bangla Mahoba (b) landraces of *P. betle.*
adult female *B. malayi*, infective larvae and microfilariae as described earlier. Adult *B. malayi* were isolated from the peritoneal cavity of gerbils, infective larvae from the mosquitoes (*Aedes aegypti*) and microfilariae from the peritoneal washing of infected gerbils. The parasites were exposed to different concentrations of water decoction or methanol extract/fraction (twofold dilutions of 500 μl/ml or 500 μg/ml respectively) at 37°C for 24 h. The efficacy of the sample was assessed by microscopic observation of worm motility and viability of parasites was checked by MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyltetrazolium bromide) reduction assay. Treated parasites were also observed for reversal of immotility in the fresh medium, which was expressed as high (5±4/4); moderate (3±); low (2±) and dead (D). Lethal concentration (LC₁₀₀) has been evaluated as μg/ml on the basis of either total immobility of the worm/larvae/mf or more than 50% inhibition in MTT reduction by the treated worm/larvae/mf compared to untreated controls.

In *P. betle*, landrace/gender-based differences were reported on the basis of leaf shape and chlorophyll content and in essential-oil composition. The present study further reports the landrace/gender-based differences in total phenol and thiocyanate content (Table 1). Threefold higher total phenol content and twofold higher thiocyanate content were observed in the female plant (BM). Similar trends were also observed in methanol extract and its n-hexane, chloroform and n-butanol fractions, but not in the case of the aqueous fraction. Thus, at least for these two groups of compounds, there are distinct landrace/gender-based differences in *P. betle* which opens an interesting field of investigation. Phenols and thiocyanates are known to have biological activity, viz. antimalarial and antioxidant activity, as shown in several studies. Polyphenols are reported to possess anthelmintic, anti-inflammatory, antidiarrheal, antiulcer, antiviral, antiallergic and vasodilatory actions. Thiocyanates are also known to have antibacterial and antifilarial (both microfilaricidal and macrofilaricidal) activities. These compounds are known to exert antileishmanial and immunomodulatory activity. *P. betle* contains both these chemical constituents and hence tested for its antiparasitic properties.

Both promastigote and amastigote stages were found to be sensitive to water decoction as well as methanol extract assayed by flow cytometry (Table 2). The activity (IC₅₀) of the two preparations against both promastigotes and intracellular amastigotes was found to be 10 μg/ml (methanol extract) or 10 μg/ml (water decoction) without any observable cytotoxicity to macrophages. Antileishmanial activity against promastigotes was confined to chloroform fraction, where IC₅₀ was 5 μg/ml, which was comparable to the standard drug miltefosine, followed by n-hexane fraction (IC₅₀= 25 μg/ml). These fractions also inhibited 50% growth of intracellular amastigotes at 10 and 25 μg/ml concentrations respectively. The activity of n-butanol was moderate, but the aqueous fraction was inactive against both promastigotes as
well as intracellular amastigotes. With regard to B. malayi, both water decoction and crude methanol extract of BM in general appeared to possess good antifilarial efficacy against all the life-stages of B. malayi. viz. adult worms, vector derived infective larvae and microfilariae. The LC_{100} for all the three life-stages for water decoction was between 1.5 and 6.25 μg/ml. Regarding crude methanolic extract, LC_{100} values ranged between 7.8 and 31.25 μg/ml. Interestingly, among the different fractions, the chloroform fraction was the most active on adult B. malayi, showing LC_{100} of 3.9 μg/ml, followed by hexane fraction which was effective at 31.2 μg/ml; n-butanol and aqueous fractions were ineffective (Table 2).

On the other hand, the water decoction of KV was found totally ineffective against both the parasites (data not shown) even up to a concentration of 500 μg/ml. This result indicates that possibly other constituents apart from polyphenols and thiocyanates, could also be responsible for antileishmanial and antifilarial activity. Furthermore, we have observed that P. betle BM also possesses immunomodulatory activity by stimulating nitric oxide production in the peritoneal macrophages of mice (unpublished).

The present findings thus indicate that pan leaves possess bioactive principles whose identification, characterization, purification and further biological evaluation are warranted. Studies are in progress to evaluate its efficacy in vivo. Here we have reported the antileishmanial and antifilarial efficacies of betel vine or pan. Further, in view of the above study, field surveillance on the incidence of parasitic infections amongst betel users and non-users may provide useful information.


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