margin incurved towards the attachment of the lobules; bract lobules oblong, 0.95 mm long, 0.35 mm wide, apex acute with one marginal 7–8-celled long tooth at base; bracteoles free or slightly adnate on one side with bract margin at extreme base, oblong, 0.70 mm long, 0.25 mm wide, bilobed, sinus deep, two-third of the lobes, very narrow, acute, lobes acute, margin entire–subundulate with one tooth on each side of the margin at base. Perianth 1.75 mm long, 0.95 mm wide, two-thirds emergent, pyriform–obcuneate, smooth, four-keeled (two lateral, two ventral), apex beaked. Sporophyte not developed.

Distribution and ecology: Eastern Himalaya – Meghalaya: East Khasi Hills (BSI campus, Shillong) and Ri Bhoi district (Barapani).

Plants grow epiphytically on the bark of trees in association with *Chiloscyphus* sp., *Cheilolejeunea serpentina* (Mitt.) Mizut. and *Plagiochila phalangea* Tayl., between 765 and 1411 m altitude.

Range: Endemic to India.

Specimens examined: *Frullania udarii* sp. nov., HOLOTYPE: 208067-A (LWG): BSI campus, Barapani (Ri Bhoi district) Meghalaya, altitude ca. 765 m, growing on tree trunks up to 5 ft height in loose population of 5 cm^2 area, 12.11.1998, leg., V. Nath & party, det. V. Nath & A. P. Singh. PARATYPE: 208008-A, 208070-A (LWG): BSI campus, Barapani (Ri Bhoi district) Meghalaya, altitude ca. 765 m, growing on basal region of tree trunks in loose population of 3–5 cm^2 area, 12.11.1998, leg., V. Nath & party; 208724-C, 208726-C, 208727-A (LWG); BSI campus, Shillong (East Khasi Hills) Meghalaya, altitude ca. 1411 m, growing on tree trunks up to 5 ft height in loose population of 3–4 cm^2 area, 20.09.2000, leg., V. Nath & party, det. V. Nath & A. P. Singh.

Amongst Indian taxa, *F. udarii* approaches *F. muscicola* Steph., which is a more plastic and variable species occasionally having explanate lobules. The former being monoecious clearly differs from the latter in sexuality. *F. muscicola* is a dioecious taxon with saccate to rarely explanate–cucullate lobules and usually five-keeled (two lateral, two ventral and one dorsal) to occasionally three-keeled (two lateral and one ventral) perianth. *F. udarii* also resembles *F. neurota* Tayl. in sexuality (monoecious), perianth keels (four-keeled), plant length, incurved apices of leaf lobes and subrotund basal appendages. However, *F. neurota* differs from *F. udarii* in (reddish-brown) plants, which have galeate lobules without or with weakly developed beak and rounded apex, laminal portion triangular, free margin entire, slightly sinuose, keels more wider and long.

On the basis of the critical examination of the populations, type/authentic specimens of other species loaned from various herbaria and relevant literature[2–9], *F. udarii* has been determined as a new species under the genus.

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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[1]. 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 Samhita* and *Astanga Hradayam*[2]. 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 Bangla, Desavari, Kapoori, Sanchi, Meetha and Khasi[3]. Its utility as an anti-inflammatory and antimicrobial is emphasized at several places[2,3]. *P. betle* leaves have been reported to possess antioxidant[4], antibacterial, digestive, stimulant[5], antifungal and nematocidal[6] properties. Despite this, there is lack of information regarding its activity against various tropical diseases such visceral leishmaniasis (VL) or kala-azar and filariasis, which are prevalent in India and are considered to be diseases of the

poor, causing significant mortality and morbidity.

VL caused by *Leishmania donovani* and lymphatic filariasis transmitted by *Wucchereria 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[7], 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[8].

The current arsenal of therapeutic agents against VL and filariasis is limited. Antimonial drugs, 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 dioecy and about hundred landraces are reported. Differences between landraces in terms of leaf shape, size (Figure 1) and chlorophyll content have been reported[9–11]. In the present study, Bangla Mahoba (BM♀) and Kapoori Vellaikodi (KV♂) 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 filariid, *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♀ and KV♂ were harvested, washed, weighed and loaded in clevenger 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 by using shade-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 (1×10^6 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, 25 and 10 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 (10^5 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

Figure 1. Differences in shape and pigment content in leaf of Kapoori Vellaikodi (***a***) and Bangla Mahoba (***b***) landraces of *P. betle*.

Table 1. Landrace/gender-based differences in total phenol and thiocyanate contents in *Piper betle* landraces Bangla Mahoba (BM♀) and Kapoori Vellaikodi (KV♂)

Extract/fraction	Thiocyanate content mg g^{-1}		Phenol content mg g^{-1}	
	BM♀	KV♂	BM♀	KV♂
Water decoction	3.64 ± 0.15	1.17 ± 0.11	78.46 ± 5.22	40.62 ± 2.72
Crude methanol extract	4.52 ± 0.10	2.67 ± 0.11	421.81 ± 40.84	276.90 ± 33.74
n-Hexane fraction	8.71 ± 0.19	6.30 ± 0.91	432.96 ± 31.65	63.89 ± 9.82
Chloroform fraction	22.64 ± 0.50	10.66 ± 0.50	766.50 ± 50.63	368.67 ± 3.39
n-Butanol fraction	6.04 ± 0.18	3.22 ± 0.19	156.25 ± 22.44	109.23 ± 6.39
Water fraction	0.92 ± 0.04	1.59 ± 0.08	53.92 ± 18.06	32.74 ± 0.29

Table 2. *In vitro* activity of *P. betle* (BM♀) decoction, crude methanolic extract and its fractions against different stages of *Leishmania donovani** and *Brugia malayi***

Extract/fraction	Antileishmanial activity$		Antifilarial activity#		
	Promastigotes	Intracellular amastigotes	Infective larva	Microfilaria	Adult worm
Water decoction***	10	10	<1.5	<3.1	6.25
Crude methanol extract	10	10	31.25	<7.8	15.6
n-Hexane fraction	25	25	15.6	< 7.8	31.2
Chloroform fraction	5	10	<7.8	>7.8	3.9
n-Butanol fraction	50	50	> 125	125	125
Aqueous fraction	>100	>100	< 125	>125	<1000

#Concentrations tested were twofold dilutions starting from 500 µg/ml.

$Concentrations tested were 100, 50, 25 and 10 µg/ml.

*IC_{50} has been evaluated as µg/ml on the basis of FACS reading and the dose at which 50% inhibition of parasite growth compared to untreated controls was observed.

**LC_{100} has been evaluated as µg/ml on the basis of either total immobility of the worm or more than 50% inhibition in the reduction of MTT by the treated worm compared to untreated controls.

***In water decoction IC_{50}/LC_{100} has been given as µl/ml.

adult female *B. malayi*, infective larvae and microfilariae as described earlier[17,18]. Adult *B. malayi* were isolated from the peritoneal cavity of gerbils, infective larvae from the mosquitoes (*Aedes aegypti*)[19] and microfilariae from the peritoneal washing of infected gerbil[20]. 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+); moderate (3+); low (2+); sluggish (1+) and dead (D). Lethal concentration (LC_{100}) 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[18].

In *P. betle*, landrace/gender-based differences were reported on the basis of leaf shape and chlorophyll content and in essential-oil composition[3,9,11]. 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. anticancer[21] and antioxidant activity[22], as shown in several studies. Polyphenols are reported to possess anthelmintic, anti-inflammatory, antidiarrhoeal, antiulcer, antiviral, antiallergic and vasodilatory actions[23]. Thiocyanates are also known to have antibacterial[24] and antifilarial[25] (both microfilaricidal and macrofilaricidal) activities[26]. These compounds are known to exert antileishmanial and immunomodulatory activity[26]. *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 as assessed by flow cytometry (Table 2). The activity (IC_{50}) of the two preparations against both promastigotes and intracellular amastigotes was found to be 10 µg/ml (methanol extract) or 10 µl/ml (water decoction) without any observable cytotoxicity to macrophages. Antileishmanial activity against promastigotes was confined to chloroform fraction, where IC_{50} was 5 µg/ml, which was comparable to the standard drug miltefosine, followed by *n*-hexane fraction (IC_{50}–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 µl/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 µl/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 paan 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 paan. 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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