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Antimicrobial activity and Chemical composition of leaf oil in two varieties of *Piper betle* from northern plains of India

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Leaf essential oil of *Piper betle* variety *bangladesi* and *deswari*, obtained by steam distillation were assigned for their antimicrobial potential against a set of pathogenic microbes. Oil from the leaves of *Piper betle* variety *bangladesi* and *deswari* was found active against the selected strains of bacteria and fungi. GC-GC MS analysis of the *Piper betle* variety *bangladesi* and *deswari* oil resulted in the identification of twenty five and thirty five compounds representing 85.41% & 86.11% respectively. Major components identified (eugenol, α selinene, α farnesene, β selinene, methyl eugenol, germacerene D) were same in both the oils beside safrole which was not found in the leaf oil of *Piper betle* variety *bangladesi.* The two essential oils showed antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae* and antifungal against *Candida albicans, Candida rugosa, Saccharomyces cerevisiae, Aspergillus niger and Aspergillus flavus*.

Keywords: Antibacterial, Antifungal, Chemical composition, Essential oil, *Piper betle*

Introduction

Medicinal and aromatic plants, since times immemorial have been used in virtually all cultures as a source of alternative medicine, herbs and their essential oils have been known for their varying degrees of different biological activities [1]. *Piper betle* (Betelvine) leaves have strong pungent aromatic flavor and are widely used as masticatory in Asia. In India it is grown as an evergreen, perennial creeper which is dioecious and a glabrous climber (may climb as high as 10-15 ft). Leaves are glossy, heart shaped and highly variable among cultivars, 5-20 cm long. The plant has been extensively studied phytochemically and a large variety of compounds have been isolated[2-6]. Different varieties of betel leaves contain volatile oils in varying amounts[7] ranging from 0.15% to 0.2%. The liquid extract of the plant has been used traditionally in curing inflammation and infections of the respiratory tract, cough, dyspnoea, antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, an astringent, an antiseptic, pancreatic lipase stimulant and wound healer[8-13]. *Piper betle* var. *bangla desi* and *Piper betle* var. *deswari* grown in the northern plains of Uttar Pradesh differ in their morphological characteristics. *Bangla desi* variety is characterized by roundish to cordate leaf alumina with prominent basal lobes. *Deswari* variety has short, acuminate curved tip with cordate leaf base[14]. There are various reports[5,7] over chemical composition of *Piper betle* variety grown in different parts of India, however to the best of my knowledge chemical composition and antimicrobial activity of *Piper betle* var. *bangla desi* and *Piper betle* var. *deswari* against the selected strains of bacteria and fungi from the northern plains of Uttar Pradesh, India has not been studied so far. Therefore, aim of the present study was to identify chemical composition of the essential oil from the *Piper betle* varieties and to screened *in vitro* antibacterial and antifungal activity against selected strains.

Materials and methods

Experimental

Plant material

The leaves of *Piper betle* var. *bangladesi* and *Piper betle* var. *deswari* were collected from the local market of Luck now, Uttar Pradesh and verified by

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one of us TR. The plant materials were washed thoroughly with tap water and then with sterilized distilled water before isolation of the oil.

Oil isolation

Collective samples of fresh leaves of *Piper betle* var. *bangla desi* and *Piper betle* var. *deswari* (150 g each) were subjected to hydro distillation in a conventional Clevenger type apparatus for 3 h, which gave 0.12% and 0.15% (v/w) of oils, respectively, on a fresh weight basis. The oils were dried over anhydrous sodium sulfate and kept in sterile sample tubes in the refrigerator at 4°C until analysis.

GC and GCMS analysis

The essential oil was analyzed using a Perkin-Elmer GC 8500, equipped with flame ionization detector, using BP-1 (polydimethyl siloxane, 50 m × 0.25 mm). Nitrogen was used as carrier gas at 10 psi inlet pressure. Temperature programming, 5 min at 60°C heated to 240°C at 5°C/min and then held isothermal at 240° for 9 min, then heated at 3°C/min to 245°C and held isothermal at 245° for 5 min. The injector and detector temperatures were 250°C and 300°C, respectively. The oils were injected neat with a split ratio of 1:80. Duplicate analysis was performed. Quantitative results are mean data derived from GC analysis. GC/MS data were obtained on a Perkin-Elmer turbo mass spectrometer instrument using a PE-Wax column (60 m × 0.32 mm i.d., film thickness 0.25 m). The carrier gas was helium; temperature programming, 5 min at 70°C, rising at 2°C/min to 120°C and 5°C/min to 240°C. Duplicate analysis was performed. Quantitative results are mean data derived from GC analysis. The individual peaks were identified by comparison of their retention indices on the BP-1 column with literature values[15-18]. The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the Wiley and NIST libraries mass spectral database and from literature information[19]. Chirality analysis was not performed. Relative amounts of individual components are based on GC peak areas obtained without FID response factor correction. The retention indices were obtained from gas chromatograms by logarithmic interpolation between bracketing *n*-alkanes. A homologous series of *n*-alkanes (C8-C22, Poly Science, Niles, USA) was used as standards.

Microbial strains used

The antimicrobial activity of essential oils was analyzed using the following strains procured as microbial type culture collections (MTCC) from the Institute of Microbial Technology, Chandigarh. The bacterial strains used were *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis*, (MTCC 435), *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella pneumoniae* (MTCC 39) and *Escherichia coli* (MTCC 443), while the fungal strains used in the assays were *Candida albicans* (MTCC 227), *Candida rugosa* (NCIM 3467), *Saccharomyces cerevisiae* (MTCC 36), *Aspergillus flavus* (MTCC 227), *Aspergillus niger* (MTCC 1344).

Antimicrobial activity

Disk diffusion assay

The antimicrobial activity testing was done according to Bauer *et al.*[20] and Wannisorn *et al*.[21]. All strains were sub-cultured from -80°C stock culture into 5 mL Mueller-Hinton broth for bacteria, and Sabouraud dextrose broth (SDB) for fungus (Hi-Media), and incubated for 24 h at the desired temperatures. The inoculums of the test microbes were prepared equivalent to the 0.5 McFarland Standards, as described in NCCLS protocols. A uniform lawn of each of the selected microbes was made using 100 µL inoculums on nutrient agar for bacteria and SDA for fungi. Then filter paper (Whatman) discs (5 mm) containing essential oil (5 µL) were placed over the seeded plates. The plates were incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for fungi. The activity was measured in terms of zone of microbial growth inhibition. First, the diameter of the total zone was measured (including disc), then the diameter of the disc was subtracted from the total zone to obtain the net zone of growth inhibition. The tests were performed in triplicate to confirm the findings. The net zone of growth inhibition above 10 mm was considered as highly active, 4-10 mm moderately active and less than 4 mm was considered to be either least active or inactive.

Broth dilution assay

The minimum inhibitory dilution (MID) was determined according to Petersdorf *et al.*[22] using the two fold serial micro broth dilution technique using a 96 well micro plate. The tested oils were added to sterile Mueller Hinton broth for bacteria in micro titer

plates before the diluted microbial suspension (final inoculum of 10^4 cfu/mL) was added. Oil was assayed in triplicate. The MID values were taken as the lowest concentration of the test oils in the wells of the micro titer plate that showed no turbidity after 24 h of incubation at 37°C for bacteria. The turbidity of the wells in the micro titer plate was interpreted as visible growth of the microorganism.

Result and discussion

The volatile oils were obtained by conventional hydro distillation of *P. betle* var. *bangla desi* and *P. betle* var. *deswari* leaves in a Clevenger-type apparatus were evaluated for their antimicrobial activity against a set of pathogenic microbes including bacterial and fungal strains. Antibacterial results of both essential oils are given in table 1 which showed that both the essential oils have antibacterial activity. Although the oil from the leaves of *P. betle variety bangladesi* was found comparatively more active than that of the oil from the leaves of *P. betle variety deswari* but the observed zone of inhibition of both the oils were not considerably high comparative to streptomycin. Zone of growth inhibition of both oil, given in the table 2, showed that oil from the leaves of *P. betle variety bangladesi* was found highly active

Table 1—Components of the leaf essential oils of *P. betle variety bangladesi and P.betle variety deswari* leaf oils from the northern plains of India

Compounds	RI[a]	*P. betle var. bangla desi Leaf oil (%)*[$]	*P. betle var. deswari Leaf oil (%)*[$]
α-Pinene	941	-	0.06
Camphene	948	-	0.09
Sabinene	976	-	1.21
Myrcene	980	-	0.40
A-Terpinene	1014	-	0.04
β-Phellandrene	1024	1.0	0.58
β-Ocimene	1039	0.22	0.14
γ-Terpinene	1053	-	0.74
Terpinolene	1081	0.14	0.18
Cis-sabinene hydrate	1084	-	0.06
Terpineol-4	1176	0.08	0.10
Safrole	1279	-	27.48
Eugenol	1347	50.29	28.44
Iso-safrole	1374	-	1.62
β-Bourbonene	1384	0.53	0.29
β-Elemene	1389	0.16	0.17
Methyl Eugenol	1394	1.17	1.46
Caryophyllene	1434	0.26	1.14
Aromadendrene	1447	0.33	0.17
β-Farnesene	1451	0.04	0.06
A-humulene	1456	-	0.13
Methyl isoeugenol	1463	1.20	2.60
Germacerene-D	1479	2.82	0.91
β-Selinene	1487	10.14	1.72
α-Selinene	1493	11.39	7.32
α-Farnesene	1504	2.48	4.70
Cubebol	1517	0.26	0.03
Hydroxy chavicol	1522	1.20	0.53
Eugenyl acetate	1534	0.41	1.72
α-Cadinene	1538	0.08	0.40
Germacerene-B	1545	0.12	0.42
E-Nerolidol	1561	0.08	0.21
Spathulenol	1577	0.31	0.02
β -Caryophyllene oxide	1582	0.18	0.02
Globulol	1586	0.52	0.06
Total		85.41%	86.11%

Compounds are listed in their order of elution from a BP-1 column; [a]RI indicates the retention indices on a BP-1 column; [$]indicates relative area (peak area relative to the total peak area)

Table 2—Minimum inhibitory dilution (MID) and zone of inhibition of *P. betle variety deswari and P. betle variety bangladesi essential* oils against pathogenic bacterial strains

Bacterial strains	*Piper betle variety deswari*		*Piper betle variety bangla desi*		Penicillin	Strepto-mycin
	MID (mg/ml)	Zone of Inhibition	Zone of Inhibition	MID (mg/ml)	Zone of Inhibition	Zone of Inhibition
Bacilius subtilis	>1.00	0	00	>1.00	20	-
S. aureus	>1.00	09	12	>1.00	18	-
S. epidermidis	1.00	10	14	0.50	23	-
E. coli	>1.00	0	00	>1.00	-	29
P. aeroginosa	>1.00	0	00	>1.00	-	34
K. peumoniae	>1.00	07	09	>1.00	-	30

Table 3—Zone of Inhibition of *P. betle variety deswari and P. betle variety bangladesi essential* oils against pathogenic fungal strains

Fungal strains	*Piper betle variety deswari*	*Piper betle variety bangla desi*	Amphotericin-B (50 µg)
	Zone of Inhibition	Zone of Inhibition	Zone of Inhibition
Candida albicans	09	12	23.5
Candida rugosa	13	12	21
Staphylococcus cerevisiae	0	17	22
A. niger	0	10	25
A. flavus	12	17	25

against *Staphylococcus aureus & Staphylococcus epidermidis*, while moderately active against *Klebsiella pneumoniae*, similarly oil from the leaves of *P. betle variety deswari* was found moderately active against *S. aureus, S. epidermidis & K. pneumoniae.*

Antifungal activity of both the oils are given in the table 3, which showed that the oil from the leaves of *P.betle variety bangladesi* was highly active against *Candida albicans, Candida rugosa, Staphylococcus cervisiae & Aspergillus flavus* whereas the oil from the leaves of *P. betle variety deswari* was found highly active against *C. rugosa & A. flavus* and moderately active against *C. albicans.* Furthermore, it was also observed that *P. betle variety bangladesi* oil had higher activity against the test fungi in comparison with *P. betle variety deswari* oil. Components of leaf essential oil *P. betle variety bangladesi & P. betle variety deswari* were analyzed by using GC and GC-MS, which resulted in the identification of twenty five and thirty five components respectively. Eugenol (50.29%), α selinene (11.39%), β selinene (10.14%), germacerene D (2.82%), α farnesene (2.48%), hydroxyl chavicol (1.20%), methyl eugenol (1.17%) are the major components identified, accounted for 79.4% of the total oil from the leaf of *P. betle variety bangladesi.* Similarly, major component identified in the leaf oil of *P. betle variety deswari* were eugenol (28.44%), safrole (27.48%), α selinene (7.32%), α farnesene (4.70%), β selinene (1.72%), methyl eugenol (1.46%), germacerene D (0.91%), eugenyl acetate (1.72%), isosafrole (1.62%) and caryophyllene (1.14) Thus, we see that, major components in two oils are almost same, apart from safrole which was present in the *P. betle variety deswari* but absent in the leaf oil of *P. betle variety bangladesi*. It is interesting to note that safrole; a hepatotoxic and carcinogenic component was present in the *Piper betle variety deswari* grown in the plains of northern India whereas it is not present in the leaf essential oil of *P. betle variety bangladesi*. There have been reports that chewing betel quid causes oral cancer[23-25]. Therefore, keeping in view of health aspects, it is recommended to grow the variety like *bangladesi* with no safrole content than variety like *deswari*.

Conclusion

The GC-GC MS analysis of the *Piper betle* oils of variety *bangladesi* and *deswari* oil resulted in the identification of eugenol, α selinene, α farnesene, β selinene, methyl eugenol, germacerene D as major components in the oils. Safrole and isosafrole were absent in the leaf essential oil of *Piper betle* variety *bangladesi,* therefore, use of *deswari* variety for chewing purpose is not recommended due to presence of carcinogenic compound safrole. Further,

antimicrobial potential of both the oils was evaluated against a set of pathogenic microbes and both the oils were found active against the selected strains of bacteria and fungi, therefore, these oils could be used in the development of antimicrobial agents as a herbal remedy, an alternative to synthetic antimicrobial agents.

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References

1 Sharma M L, Rawat S K S, Balasubrahmanyam V R & Singh A, Studies on essential oil of betlevine leaf (*Piper betle* Part II. Vars; Desi Bangla, Ramtek Bangla and Calcutta Bangla, *Indian Perfumer*, **27** (1983) 91-93.

2 Evans P H, Bowers W S & Vunk E J X, Identification of fungicidal and nematocidal components in the leaves of *Piper betle* (Piperaceae), *J Agr Food Chem,* **32** (1983) 1254-1256.

3 Balasubramanyam V R & Rawat A K S, Studies on morphology and chemistry of *Piper betle* L, *J Plantation Crops*, **18** (1990) 78-87.

4 Saeed S A, Farnaz S, Simjee R U & Malik A, Triterpenes and b-sitosterol from *Piper betle*: isolation, antiplatelet and anti-inflammatory effects, *Biochem Soc T*, **21** (1993) 462S.

5 Sharma M L, Rawat A K S & Balasubramanyam V R, Studies on essential oil of betle leaf (*Piper betle* L.) Part III vars. Desi Desawari and Mahoba Desawari, *Indian Perfumer*, **31** (1987) 319-324.

6 Jantan I B, Ahmad A R, Ahmad A S & Ali N A M, A comparative study of the essential oils of Piper species from Peninsular Malaysia, *Flavour Frag J*, **9** (1994) 339-342.

7 Sharma M L, Rawat S K S, Balasubrahmanyam V R & Singh A, Studies on essential oil of betlevine leaf (*Piper betle* Part II. Vars; Desi Bangla, Ramtek Bangla and Calcutta Bangla, *Indian Perfumer,* **27** (1983) 91-93.

8 Singh M, Shakya S, Soni V K, Dangi A, Kumar N & Bhattacharya S M, The n-hexane and chloroform fractions of *Piper betle* L., trigger different arms of immune responses in BALB/c mice and exhibit antifilarial activity against human lymphatic filarid Brugia malayi, *Int Immunopharmacol*, **9** (2009) 716-728.

9 Anonymous: The Wealth of India, The Dictionary of Indian Raw Materials and Industrial Products. Raw Material, revised edition Publication and Information directorate, CSIR, New Delhi, **5** (1992) 84-94.

10 Norton S A, Betel: consumption and consequences, *J Am Acad Dermatol,* **38** (1998) 81-88.

11 Prabhu M S, Patel K, Saraswathi G & Srinivasan K, Effect of orally administered betel leaf (*Piper betle* leaf Linn.) on digestive enzymes of pancreas and intestinal mucosa and on bile production in rats, *Indian J Exp Biol*, **33** (1995) 752-756.

12 Bhattacharya S, Banerjee D, Bauri A K, Chattopadhyay S & Bandyopadhyay S K, Healing properties of the *Piper betle* phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action, *World J Gastroentero,* **21** (2007) 3705-3713.

13 Garg S C & Jain R, Biological activity of essential oil of Piper betlle L, *J Essent oil Res*, **23** (1992) 601-606.

14 Rawat A K S, Tripathi R D, Khan A J & Balasubrahmanyam V R, Essential oil components as marker for identification of *Piper betle* L. cultivars. L, *Biochem Syst Ecol,* **17** (1988) 35-38.

15 Brophy J J, Goldsack R J, Forster P I, Carven L A & Lepschii J, The leaf essential oils of the Australian members of the genus *Callistemon* (Myrtaceae), *J Essent Oil Res,* **10** (1998) 595-606.

16 Riaz M & Chaudhury F M, The chemical composition of Pakistani *Callistemon citrinus* oils, *J Essent Oil Res,* **2** (1990) 327-328.

17 Raina V K, Srivastava S K, Aggarwal K K, Syamasundar K V & Khanuja S P S, Essential oil composition of *Cymbopogan martini* from different places of India, *Flavour Frag J,* **8** (2003) 312-315.

18 Srivastava S K, Ahmad A, Jain N & Aggarwal K K, Essential oil composition of *Callistemon citrinus* leaves from the lower region of Himalayas, *J Essent Oil Res,* **13** (2001) 359-361.

19 Adams R P, Identification of Essential Oils by Ion Trap Mass Spectroscopy. Academic (1990), San Diego, C.A.

20 Baeur A, Kirby W, Sherries J C & Turck M, Antibiotic susceptibility testing by standardized single disk method, *Am J Clin Pathol,* **45** (1966) 493-496.

21 Wannisorn B, Joarikasem S & Soontorntanasart T, Antifungal activity of lemon grass oil and lemon grass cream, *Phytother Res,* **10** (1996) 551-554.

22 Petersdorf R G & Sherris J C, Methods and significance of in vitro testing of bacteria sensitivity to drugs, *Am J Med,* **39** (1995) 766-767.

23 IARC. *Tobacco Habits Other than Smoking; Betel Quid and Areca-nut Chewing; and Some related Nitrosamines*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 37.IARC Press: Lyon, 1985.

24 Moore S R, Johnson N W, Pierce A M & Wilson D E, The epidemiology of mouth cancer: a review of global incidence, *Oral Dis,* **6** (2000) 65-74.

25 Sankaranarayanan R, Oral cancer in India: An epidemiological and clinical review, *Oral Surg Oral Med O* **69** (1990) 325-330.