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STUDIES ON *PIPER BETLE* OF SRI LANKA

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Abstract: More than twelve cultivars of *Piper betle* are reported in Sri Lanka but no chemical studies have been carried out on them. The present study describes the morphological, physico - chemical, chemical and antimicrobial activities of six main cultivars of *P. betle* Linn namely *Galdalu, Mahamaneru, Kudamaneru, Ratadalu, Nagawalli* and *Malabulath*. The chemical constituents identified in the essential oil of *Malabulath* by Gas Chromatography-Mass Spectrometry (GC-MS) were different to those from the other cultivars. The major compound in *Malabulath* was allylpyrocatechol diacetate while that in all other cultivars categorized as common betel was safrole. Chemical compositions of the essential oil of the leaf, stalk, stem, fruit and root were different. The major compounds in the leaf, stem, stalk and root oil was safrole but in the fruit it was β-phellandrene. The composition of the oil also varied with maturity. The essential oil from common betel was active against *Escherichia coli* (NCTC 10148), *Pseudomonas aeruginosa* (NCTC 10662), *Staphylococcus epidermidis* (NCTC 4276), *Staphylococcus aureus* (NCTC 8532), and *Streptococcus pyrogens*. The essential oil and the ethanol extract were also active against *Cladosporium sp.*

Key words: antimicrobial activities, GC-MS studies, *Piper betle*, Sri Lanka.

INTRODUCTION

Piper betle Linn. (Sinhala: *Bulath*; English: Betel vine) belongs to the genus *Piper* of the family Piperaceae.[1] Over 700 species of plants belonging to the genus *Piper* are found distributed in both hemispheres.[2] Of these about 30 species have been recorded from India.[1] In Sri Lanka, 18 species are found and three are endemic.[3]

P. betle Linn. is cultivated in Sri Lanka, India, Malaysia, Indonesia, Philippine Islands and East Africa. In Sri Lanka, betel is commonly used as a cultural symbolism.[4] The leaves of this plant are economically and medicinally important. Betel leaves have been traditionally used for chewing purposes along with other condiments. This chewing combination is known as betel quid. Sri Lankan betel industry has a long-standing history dating back to 340 AD.[4] Colombo, Gampaha, Kalutara, Kurunegala, Kegalle, Ratnapura, Matale and Galle are the main betel cultivating districts in the country. In Sri Lanka, more than twelve cultivars of betel have been recognized by the villagers but no systematic nomenclature and scientific classification of these cultivars are recorded. The objectives of the present study are to identify the different cultivars of betel found in Sri Lanka using morphological characters and chemical properties of the oil.(to assist a scientific classification of the different *P. betle* Linn. cultivars found in Sri Lanka). In most of the plants the constituents depend on the maturity and the part analysed. Hence in the present study, the chemical constituents of the essential oils from different parts of the plant at different maturity levels were also investigated.

Antimicrobial properties of *P. betle* Linn. found in India are reported[1] but no studies on the plants found in Sri Lanka are available. Hence a study on the antimicrobial properties too was carried out.

METHODS AND MATERIALS

Plant material : *P. betle* plant materials were collected from Narammala, Panduwasnuwara, Thissawa, Thabbomulla, Walpitagama, Udagama (Kurunegala district), Wadinapaha, Divulapitiya, Dewalapola (Gampaha district), Karagoda, Bibullawella, Uyangoda (Matara district) and Hiyara east, Akmeemana, Yakkalamulla, Paradaradeniya (Galle district). Six cultivars of *P. betle* namely *Galdalu, Mahamaneru, Kudamaneru, Ratadalu, Nagawalli* and *Malabulath* were used in the present study. From 5-6 plants of each type 6-8 wk old leaves (250 g) from the sixth to eight position from the apex of the vine were collected along with some plagiotrophic branches. The roots and the fruits

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from the same plant were also collected. The samples were identified by the third author.

Morphological studies: Morphological observations regarding colour and size were recorded for each of the leaf samples collected. Internodal distances of the plagiotrophic branches of each sample were measured.

Study of the anatomy: Cross sections of the stem and leaf of each of the samples were obtained and stained. These cross sections were mounted and observed under the microscope. The microscopic photographs were taken and used for comparative study. Similarly microscopic cross sections of the leaves were photographed and studied.

Five betel leaves were selected from each sample. From each leaf the lower epidermal peels were trimmed from the leaf lamina between the tip and the base and from half-way between the margin and the mid rib. The lamina peels of each were examined under the microscope using grid eye piece. The stomatal indices were calculated using the Salisburgs formula.

Determination of the moisture content: The leaves (5.11 g) were dried in an oven at 105 ^{0}C for 2 h, cooled and weighed. The procedure was repeated till a constant weight was obtained. The determination was done in triplicate. Similarly the moisture contents in the stem, the stalk, the fruit and the root of common betel were determined.

Determination of the essential oil content : Fresh leaves (251.9 g), roots (160.7 g), stems (175.8 g), stalks (245.9 g), and fruits (176.7 g) obtained from each cultivar except from the cultivar identified as *Malabulath* were cut in to small pieces and the essential oil extracted separately as follows. In the case of *Malabulath* only the leaves were used for the extraction. The cut plant materials were separately water distilled for 6 h using a Clevenger oil arm fitted with condensers through which cooled water was circulated to prevent low volatiles from escaping. The volatile oil was collected in hexane-pentane mixture. The yield was calculated on a dry weight basis. Determinations were carried out in triplicate.

Determination of the physical properties of the essential oil: Refractive index of the oil was measured using an Abbe Atogo IT refractometer. The temperature was maintained at 23 ^{0}C using a thermostat controlled water circulator. Specific gravity of the oil was measured using a specific gravity bottle.

Preparation of the ethanol extract: Fresh leaf samples (20.8 g) were cut into small pieces and refluxed with ethanol (400 ml) using a Soxhlet extractor for 8 h. The extract was evaporated under reduced pressure and a dark brown mass (0.96 g) was obtained.

Determination of the chemical constituents of the essential oil : The volatile oil extracted from each of the betel cultivars was subjected to Gas Liquid Chromatography (GLC) analysis using Shimadzu GC – 14 B equipped with FI detector and Supelcowax TM-10 fused silica capillary column. Retention data and peak enhancement techniques were used for the identification of compounds. The GLCs of cultivars *Galdalu, Mahamaneru, Kudamaneru, Ratadalu* and *Nagawalli* were similar and they were referred to as common betel. The volatile oil from the leaves, stalks, stems, fruits and roots of common betel and from the leaf of *Malabulath* were subjected to the GC-MS using Hewlett-Packard 5890 Series II equipped with FI detector and DB-5 MS capillary column. The volatile oils of young and mature *P.betle* leaves of common betel were also analysed .

Antibacterial screening: In the antibacterial studies cultivars *Galdalu, Mahamaneru, Kudamaneru, Ratadalu* and *Nagawalli* were classified as common betel. The leaf oil and the ethanol extract obtained from the common cultivars of betel were used for the antibacterial studies. The antibacterial activity was studied by the 'Disk diffusion method'[5] using *Escherichia coli* (NCTC 10148), *Pseudomonas aeruginosa* (NCTC 10662), *Staphylococcus epidermidis* (NCTC 4276), *Staphylococcus aureus* (NCTC 8532), *Streptococcus pyogens*. The experiment was conducted in triplicate. This method has diffusion and solubility limitations. The MIC values were determined using the serial dilution technique.[6]

RESULTS AND DISCUSSION

Table 1: Morphological features of the different cultivars of betel

Character	Betel Cultivars					
	Malabulath	*Galdalu*	*Mahamaneru*	*Kudamaneru*	*Ratadalu*	*Nagawalli*
Leaf colour	Yellowish Green	Yellowish Green	Yellowish Green	Yellowish Green	Green	Green with yellow patch
Internodal Distances(cm)	10.10 - 15.70	3.80 - 12.50	4.00 - 13.60	6.20 - 13.20	6.8 - 13.40	7.60 - 13.50
L /W	1.22 ± 0.01	1.53 ± 0.17	1.55 ± 0.18	1.70 ± 0.07	1.48 ± 0.06	1.86 ± 0.01
Stomatal index	7.23 ± 0.12	9.57 ±0.64	9.32 ± 0.45	9.34 ± 0.40	9.98 ± 0.88	10.03 ± 0.92

Table 2 : Morphological characters and stomatal index of common betel and *Malabulath*

Betel cultivar	Leaf length / width ratio	Stomatal index	Leaf colour
Common betel	1.62 ± 0.16	9.65 ± 0.69	Green - yellowish green
Malabulath	1.22 ± 0.01	7.27 ± 0.12	Green - yellowish green

Table 3: Comparison of different cultivars of betel

Character	Betel cultivars					
	Malabulath	*Galdalu*	*Mahamaneru*	*Kudamaneru*	*Ratadalu*	*Nagawalli*
Moisture content in leaves	83.75 ± 0.23	83.22 ± 1.19	83.21 ± 2.57	79.49 ± 4.18	85.13 ± 0.52	85.28 ± 0.03
Oil g/100 g	1.03 ± 0.01	1.11 ± 0.34	1.12 ± 0.33	1.02 ± 0.21	0.89	0.84 ± 0.09
Specific gravity	1.03	1.05	1.05	1.05	1.05	1.05
Refractive index	1.4926	1.5231	1.5162	1.5152	1.5155	1.5158
EtOH extract g/100 g	32.75 ± 0.06	40.50 ± 8.14	42.91 ± 4.74	33.27 ± 1.26	40.91 ± 2.37	41.88 ± 0.55

Table 4: Major compounds identified by GLC from the volatile oil of the leaf of six betel cultivars

Cultivar	Compounds					
	β -Phellandrene	4 –Terpineol	Safrole	Eugenol	Chavibitol acetate	Allylpyrocatechol diacetate
Galdalu	3.8	2.03	37.5	17.2	1.1	1.1
Mahamaneru	6.1	3.8	33.1	9.26	6.74	6.82
Kudamaneru	1.62	2.5	35.7	19.9	3.8	1.74
Ratadalu	1.76	11.0	37.5	10.5	2.96	3.54
Nagawalli	5.5	2.5	36.6	8.5	6.7	3.5
Malabulath	6.93	-	-	-	-	34

Table 5: Composition of the essential oil of leaf of *Malabulath* and leaf, stalk, stem, fruit and root of common betel detected by GC – MS

R_t min	Compound	Leaf oil g/100 g (Common betel)	Stem oil g/100 g (Common betel)	Stalk oil g/100 g (Common betel)	Fruit oil g/100 g (Common betel)	Root oil g/100 g (Common betel)	Leaf oil g/100 g (*Malabulath*)	MW
12.37	α- Pinene	-	0.82	-	2.31	12.86	-	136
12.77	Camphene	-	4.06	-	0.89	13.48		136
13.78	β - Phellandrene	2.58	-	-	25.04	12.72	0.93	136
14.15	*p* –Cymene	0.75	-	-	3.01	-	-	134
14.33	Limonene	-	-	-	5.29	2.23	-	136
16.28	1, 8- Cineole	-	-	-	-	3.19	-	154
17.11	α -Terpineol	-	-	-	-	1.51	-	154
17.12	4 –Terpineol	3.61	1.51	1.85	0.98	-	-	154
19.05	Safrole	48.69	41.74	40.47	22.16	31.81	-	162
20.00	Eugenol	11.93	13.19	11.48	6.11	4.50	-	164
20.46	γ - Muurolene	1.71	2.15	4.76	0.98	-	3.76	204
21.18	α- Elemene	-	4.34	3.41	-	1.80	-	204
21.27	β-Caryophellen	0.93	5.03	1.57	1.83	1.57	-	204
21.77	α-Humulene	0.97	2.97	4.48	0.88	-	3.97	204
22.24	Chavibitol acetate	12.55	10.30	11.63	7.24	4.74	-	206
22.33	γ - Selinene	-	4.54	9.64	2.53	2.89	3.19	204
22.49	β - Cadinene	1.00	0.59	1.12	0.45	0.38	5.60	204
23.81	Allylpyrocatechol diacetate	11.34	5.14	-	11.25	4.25	34.01	234

Table 6: Effect of stage of maturity on the composition of the major compounds in common betel leaf oil

Compound	Young stage %	Harvesting stage %	Mature stage %
Safrole	27.98	48.69	38.71
Eugenol	13.41	11.73	9.72
Allylpyro catechol diacetate	1.17	11.34	11.38
Chavibitol acetate	2.12	12.55	6.70
β -Phellandrene	10.66	2.58	2.55

Antifungal study: The antifungal activity was studied by the Bioautographic TLC Assay[7] using *Cladosporium* sp. The experiment was conducted in triplicate.

According to morphological and anatomical studies the parameters such as stomatal index and leaf length to width ratio were similar in *Kudamaneru, Mahamaneru, Galdalu Ratadalu* and *Nagawalli* but different in *Malabulath*.(Table 1&2). The physical parameters of the essential oil (Table 3) and constituents of the essential oils of *Kudamaneru, Mahamaneru, Galdalu, Ratadalu* and *Nagawalli* too were similar but were different to those of *Malabulath*. Safrole was detected as the major compound in the leaf oil of common betel but Allyl pyrocatechol diacetate was the major compound in the leaf oil of *Malabulath*. Hence the results show that the cultivars of betel analysed in the present study fall into two chemically and morphologically different groups namely *Malabulath* and common betel. Common betel includes *Kudamaneru, Mahamaneru, Galdalu, Ratadalu* and *Nagawalli*. The appearance of *Nagawalli* leaves differed from the other cultivars. Therefore it can be concluded that chemotaxanomically there are two different groups of betel in Sri Lanka namely *Malabulath* and the other referred to as common betel.

Over 100 cultivars of betel have been identified by farmers and traders in India.[8] The chemical studies on *P. betle* of India have revealed that composition of the volatile oils in the leaves can be used as markers for the identification of different cultivars[9]. The chemical composition of common betel oil of Sri Lanka appears to be closer to that of cultivar, *Deshawari* in India.[9]

GC-MS analysis of oil of *Malabulath* and common betel leaves indicated that the major constituents of common betel oil are safrole (48.69 %) and chavibitol acetate (12.55%) while *Malabulath* does not contain these two compounds (Table 5). The major compound in *Malabulath* oil is allylpyrocatechol diacetate (34.01%) which is the third major compound in common betel oil (11.34%). Further *p*-cymene , 4-terpineol, safrole, eugenol, β-caryophellene and chavibitol acetate detected in common betel leaves were not detected in *Malabulath*.

Hence in the present study GC-MS and microbiological studies were carried out considering the cultivars *Kudamaneru, Mahamaneru, Ratadalu, Galdalu* and *Nagawalli* as one group and referred to as common betel.

The GC-MS analysis of the essential oil of different parts of common betel (Table 5) indicated that the composition of the stalk was different to that of the other parts. The stalk did not contain detectable amounts of allylpyrocatechol diacetate. The major compound detected in the oil from the leaf, the stem, the stalk and the root was safrole but in the fruit, it was β-phellandrene.

Table 6 indicates that the composition of the oil also differs according to the stage of maturity of the leaf. It was observed that the major compounds safrole (48.69%) and chavibitol acetate (12.55%) content in the leaf was maximum at harvesting stage. Further it is seen from table 6 that eugenol and β- phellandrene content decreased with maturity. It was observed that β-phellandrene content remained constant after maturity. Allyl pyrocatechol diacetate content

increased up to harvesting stage and remained constant thereafter.

The change in the composition of the oil with maturity and the part of the plant explains why the ayurvedic physicians specify the maturity of the plant and part of the plant in drug preparations.

The antimicrobial activity of local *P. betel* leaf oil and the ethanol extract has not been studied previously. In the antibacterial study the essential oil from the common betel leaves showed activity against *Escherichia coli, Streptococcus pyogenes* and *Staphylococcus aureus.* The MIC values were 3.12 x 10^2, 2.50 x 10^3, and 5.00 x 10^3 respectively. The ethanol extracts showed high activity against *Streptococcus pyogenes, Escherichia coli* and *Staphylococcus aureus.* The MIC values were 1.25x10^3, 5.00x10^3 and 5.00x10^3 respectively (Table 8). This antibiotic activity can be related to the

Table 7: Diameter of the inhibition zones of test bacterial colonies exposed to essential oil and ethanol extract of common betel leaf

Sample		Diameter of the inhibition zones[a]				
	conc. µg/ disc	*Escherichia coli*	*Staphylococcus aureus* (NCTC 10148)	*Staphylococcus epidermidis* (NCTC 8532)	*Pseudomonas aeruginosa* (NCTC 4276)	*Streptococcus* Pyogenes* (NCTC 10662)
Essential oil	2000	21.00 ± 1.00	12.67 ± 1.15	15.67 ± 0.58	14.00 ± 2.00	14.67 ± 0.57
Ethanol extract	100	17.33 ± 2.08	10.67 ± 0.58	12.33 ± 1.28	14.33 ± 0.58	12.00 ± 2.00
Gentamycin	10	31.67 ± 0.58	20.33 ± 2.08	26.33 ± 0.58	21.67 ± 1.53	17.83 ± 0.29

[a] Values are means of three readings

Table 8: Minimum Inhibitory Concentration (MIC) of common betel leaf oil and ethanol extract of common betel leaf

Sample		MIC values [a] (µg / ml)				
	Staphylococcus aureus	*Staphylococcus epidermidis* (NCTC 8532)	*Pseudopmonas aeruginosa* (NCTC 4276)	*Escherichia coli* (NCTC 10662)	*Streptococcus Pyogenes** (NCTC 10148)
Essential oil	5.00 x 10^3	1.00 x 10^4	1.00 x 10^4	3.12 x 10^2	2.50 x 10^3
Ethanol extract	5.00 x 10^3	1.00 x 10^4	1.00 x 10^4	5.00 x 10^3	1.25 x 10^3
Gentamycin	2	2	2	0.5	16

[a] carried out in duplicate

use of this plant as an antiseptic in Ayurvedic medicine.

Antifungal activity of *P. betle* on *Cladosporium* sp. has not been previously reported. The antifungal activity against *Cladosporium* sp. indicates that the essential oil of *P. betle* possess at least three fungicidal compounds having R_f 0.54, 0.40, 0.11. The ethanol extract contained at least one fungicidal compound having R_f 0.61 on silica gel TLC plates. These studies will be useful in the development of value added products from betel.

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