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Volatile Chemical Constituents and Bioactivity of Selected *Piper* Species in Sri Lanka

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Abstract The Genus *Piper* comprises with many economically and medicinally important species in which essential oil is one of the major secondary metabolites responsible for medicinal properties of these plants. The present study was aimed to investigate volatile chemical constituents and *in vitro* antibacterial, antioxidant and anti-inflammatory activities of essential oils extracted from leaves and fruits of eight *Piper* species found in Sri Lanka. Plant specimens were collected from natural habitats and cultivations. Essential oils were extracted using steam distillation method and subjected to gas chromatographic analysis. Identification of the volatile chemical constituents was performed by Gas chromatography mass spectrometry (GC/MS) analysis. Antioxidant activity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing power assay (FRAP). Human red blood cell (HRBC) membrane stabilizing method was used to evaluate anti-inflammatory activity. To evaluate the antibacterial activity, agar well diffusion assay was conducted for five *Piper* species and were tested against three pathogenic bacteria: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 121 and *Escherichia coli* ATCC 25922. As the major volatile constituents of *Piper nigrum*, β-caryophyllene (60.5-9.1%), caryophyllene oxide (8.49-1.3%), α-copaene (7.4-3.1%), cadina-1,(10)-4-diene (4.3-2.1%), (n)- *trans*-nerolidol (5.9-0.5%), 4-*epi* cubedol (11.0-0.5%) and β-linalool (5.7-0.7%) were identified. *P. betle* was dominated by safrole (39.7-33.0%) and eugenol (43.2-27.4%). *Piper longum*, *P. chuvya* and *P. sylvestre* contained (n)-*trans*-nerolidol (12.7-0.2%, 66.5% and 41.2% respectively) as the major compounds. *P. betle*, *P. chuvya* and *P. longum* leaves had high antioxidant activity when compared with the standard Butylated hydroxytoluene (BHT). Furthermore, *P. betle* exhibited high anti-inflammatory activity when compared to the standard (Aspirin). *P. nigrum* and *P. betle* showed significant antibacterial activity against *S. aureus*. Moreover, *P. betle* exhibited significant activity against *B. Subtilis*. The bioactivity test results revealed that some of the *Piper* species available in Sri Lanka are potential sources for developing new herbal drugs.

Keywords *Piper*, Essential oil, GC/MS, Antioxidant, Anti-inflammatory, Antibacterial

1. Introduction

The genus *Piper* belongs to the family Piperaceae which includes valuable economically and medicinally important species. Ten *Piper* species were recorded in Sri Lanka, among them *P. nigrum* L. and *P. betle* L. are mainly cultivated and *P. longum* L. to a lesser extent. *Piper zeylanicum* Miq., *P. trineuron* Miq. and *P. walkeri* Miq. are endemic to Sri Lanka, whereas *P. betle*, *P. longum*, *P. chuvya*, and *P. siriboa* are considered as introduced. *Piper walkeri* Miq. is a rare species and *P. sylvestre* Lam. is the most widespread wild *Piper* species in the country. Most of the *Piper* species are used in traditional medicine, which emphasizes the importance of the genus [1-4].

Black pepper (*P. nigrum*), the king of spices is the most widely used spice in the world and its main pungent principle is piperine. The aroma of pepper is determined by the composition of the essential oil usually obtained by steam distillation of black peppercorns [5]. The composition of black pepper oil was reported by several researches. It can be depended on various factors such as harvest year, cultivar, variation in the maturity of raw material, oil extraction method etc. [6]. According to Sruthi *et al*. [7] variability was observed in aroma quality of black pepper variety Panniyur 1, with respect to altitudes. A clear altitudinal variation was observed in β-caryophyllene and total phenol contents. α- and β-pinene, limonene, myrcene, linalool, α-phellandrene, sabinene, β-caryophyllene and germacrene-D are the main constituents of essential oil of black paper and these compounds contribute for the flavour of pepper [8,9].

A study of leaf volatile constituents of ten wild *Piper* species of Western Ghats using GC/MS, stated that β-caryophyllene, nerolidol and β-elemene as the most abundant compounds in *Piper* leaf oil [10]. GC/MS analysis of common betel leaf oil indicated safrole (48.69%) and

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chavibetol acetate (12.55%) as major compounds. It was reported that the composition of the essential oil varied with the maturity [11].

Bhuiyan *et al* [12] has investigated essential oil composition of the inflorescences and leaf of *P*. *longum* Linn. grown in Bangladesh by GC/MS. The study revealed the presence of few monoterpene hydrocarbons, a moderate content of sesquiterpenes and high content of aliphatic hydrocarbons. The inflorescences oil was rich in eugenol (33.11%), caryophyllene (9.29%), cinnamyl acetate (5.91%) and β-pinene (4.74%).

Sugumaran *et al* [13] has identified sixty five components in vellaikodi variety of *P*. *betle* leaf oil extracted by hydro-distillation method in India. They reported 5-(2-propenyl)-1,3-benzodioxole (25.67%) as the first major constituent in the oil, the second was eugenol, (18.27%) and third, 2-methoxy-4-(2-propenyl) acetate-phenol (8.00%).

Bioactivity of all the essential oils revealed both antibacterial and antifungal activities. The effectiveness of the essential oils against different bioactivities varied from each other as chemical composition of the volatiles was different. Terpenes are the main group of compounds present in the essential oils of *Piper* species which are responsible for their antimicrobial activity [14].

Early investigations have been stated that different extractions and essential oils of *P*. *betle*, *P*. *nigrum* leaves and *P*. *longum* fruits contain large number of bioactive compounds including polyphenols, alkaloids, steroids, saponins and tannins hence, possess different bioactivities such as antioxidant, antibacterial, digestive, anti-inflammatory, stimulant, antifungal and nematicidal properties [15-22].

Essential oils of black pepper and white pepper showed considerable antioxidant activity against DPPH radicals scavenging activity assay and FRAP assay [22]. *P*. *betle* leaves have exhibited activity against *Streptococcus pyogens*, *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli*. Further, Piperine extracted from *P*. *nigrum* fruits and the crude extract has showed the antibacterial activity against *S*. *aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E*. *coli* [23].

Present study was aimed to evaluate volatile chemical constituents of selected *Piper* species in Sri Lanka and their antioxidant, anti-inflammatory and antibacterial properties.

2. Material and Methods

2.1. Collection of Plant Material

Healthy plant materials (leaves and fruits) were collected from the cultivations of growers and the field collections of the Intercropping and Betel Research Station (Table 1). Plant specimens were identified by referring to the morphological features provided in "A Revised Handbook to the Flora of Ceylon" [1], by using the authenticated specimens available in the National Herbarium and by using available field collections at the Intercropping and Betel Research Station.

Herbarium specimens were prepared and deposited as voucher specimens in National Herbarium, Peradeniya, Sri Lanka (Herbarium numbers are included in the Table 1).

Table 1. Piper species and varieties used for the study

Herbarium number (PDA)	Collected species	Variety name
NJ 01	*P*. *sylvestre* Lam.	
NJ 02	*P*. *zeylanicum* Miq.	
NJ 03	*P*. *longum* L.	Thippili, Gaja Thippili
NJ 05	*P*. *walkeri Miq*.	
NJ 06	*P*. *nigrum* L.	MB 12, MW 21, IW 05, MW 18, Panniyur, Kuching, KW 30, KW 31, KW 33, GK 49
NJ 07	*P*. *betle* L.	Maneru, Ratadalu, Nagawalli
NJ 08	*P*. *chuvya* (Miq.) C. DC.	
NJ 09	*P*. *siriboa L*.	

2.2. Isolation of Essential Oils

To collect the essential oil, air dried leaves and fruits (20 g) of *Piper* spp. (fruits and leaves of *P*. *nigrum* and *P*. *longum*, and leaves of the other *Piper* species) were crushed into small pieces and steam distilled by passing steam into the crushed leaves for 3 hours. The condensed solution (400 mL) was saturated with NaCl and subsequently extracted with CH_2Cl_2 (Analar grade 3, 100 mL) to obtain essential oil. The CH_2Cl_2 phase was dried with anhydrous Na_2SO_4 and concentrated using a flash evaporator. A stream of nitrogen gas was passed through the concentrated sample to remove any remaining CH_2Cl_2 before use in the bioassays and GC analysis.

2.3. Gas Chromatography and Gas Chromatography and Mass Spectrometry

The essential oils of *Piper* species (fruit oil of *P*. *nigrum* and *P*. *longum* and leaf oil of other *Piper* spp.) were subjected to gas chromatography (GC) to identify the components in the oil. A Shimadzu Gas Chromatograph (GC-2025) with a splitless injector (220°C), flame ionization detector (FID) (270°C), and a fused silica carbowax 20M capillary column (30m 0.25mm) was used under the following conditions: 60°C for 5min followed by 60 to 110°C at 5°C min^{-1}, 110 to 200°C at 3°C min^{-1} and 200 to 220°C at 5°C min^{-1} and held for 5 min. Carrier gas was Nitrogen. Gas Chromatography-Mass Spectrometry (GC/MS) was performed using a Thermo Scientific TRACE 1300 Chromatograph fitted with a split-split-less injector, coupled with an ISQ mass spectrophotometer. GC conditions were same as above. The constituents of the volatile oils were identified by comparing retention time with standards.

The below mentioned bioassays were tested against leaf oil of *P*. *chuvya*, *P*. *sylvestre*, *P*. *betle*, *P*. *longum* and both

fruit oil and leaf oil of *P. nigrum* species.

2.4. Scavenging Ability on 2,2-diphenyl-1-picrylhydrazyl Radicals (DPPH)

The assay was conducted in a flat bottom 96-well microtiter plate according to the method described by Chatatikun & Chiabchalard [24]. Methanolic DPPH solutions (0.16mM, 40µL) were added to various concentrations of BHT (1.0, 0.25, 0.5, 0.125, 0.0625, 0.03125mg mL^{-1}) in the 96-well plate. All reagents were mixed and incubated for 30 min at room temperature under dark conditions. The absorbance of each well was measured at 517nm with a Microplate Reader (Biotek, USA). Different concentrations of DPPH in methanol solutions were used to plot the standard curve and then the BHT concentration (µg mL^{-1}) in the reaction medium was calculated from the calibration curve. To determine the IC_{50} (Inhibitory Concentrations), the inhibition rate was calculated and plotted *versus* test concentrations [25].

2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

The assay was conducted in a 96-well microtitre plate. Test samples (10 µl), (1.0, 0.25, 0.5, 0.125, 0.0625, 0.03125 mg mL^{-1}), sodium phosphate buffer (0.2 M, pH 6.6, and 25µl) and potassium ferricyanide (1% w/v, 25µl) were added one after the other into wells of the microtitre plate. The similar procedure was performed for the standard (BHT) solution. The control contained all the reagents except the sample. The mixture was incubated at 45°C for 20min, and was terminated by the addition of (10% w/v, 25µl) trichloroacetic acid. This mixture was diluted with 85µl of deionized water and ally freshly prepared ferric chloride (0.1% w/v, 17µl) was added and kept for 10min. The absorbance of the mixture was measured at 700nm, using a microplate reader [26].

2.6. Anti-inflammatory Activity Using HRBC Membrane Stability Assay

In vitro anti-inflammatory activity of essential oils of different *Piper* species was assessed by human red blood cell (HRBC) membrane stabilizing method [27] with slight modifications. Fresh blood sample was collected from healthy human volunteers and transferred to heparinised centrifuge tubes. The blood samples were then centrifuged at 3000rpm and washed three times with an equal volume of normal saline. Amount of red blood cells (RBC) were measured and reconstituted as 10% (v/v) suspension with normal saline. The reaction mixture (5.5mL) contained 5mL of the test solution and 0.5mL of 10% RBCs suspension. Saline was added to the control test tube instead of the sample and aspirin was used as the standard drug. All the centrifuge tubes with the reaction mixtures were incubated in a water bath at 56°C for 30min and cooled under running tap water. The reaction mixtures were then centrifuged at 3000rpm for 10 min and the absorbance of the supernatants were measured at 560nm. The assay was performed in triplicate.

2.7. Agar Well Diffusion Method

Antibacterial activity was evaluated against *E. coli* ATCC 25922, *B. subtilus* MTCC 121 and *S. aureus* ATCC 25923 bacteria as the method described by Jeyaseelan *et al*. [28]. Bacterial suspension (100µl) was poured on the nutrient agar medium by using a sterile micro pipette and spread evenly. Four wells were developed by using 7 mm diameter sterile cork borer in each Petri dish and two wells were filled with 100µl of tetracycline (0.5mg mL^{-1}) and sterile dimethyl sulfoxide (DMSO) (100µl) as the standard and control. Different concentrations (5mg mL^{-1}, 10mg mL^{-1} and 25mg mL^{-1}) of isolated essential oils of leaves and fruits of *Piper* species were prepared. Culture plates were incubated at 37°C for 12 to 18 hours. The zone of inhibition was recorded (in cm) by measuring the diameter of the inhibition zone around the well, including the well diameter. The readings were taken in three different fixed directions and the average values were tabulated. Two replicates were carried out for one concentration of each essential oil. The obtained data were subjected to one way ANOVA ($p<0.05$) and Turkey's multiple comparison using MINITAB 17 statistical package.

3. Results and Discussion

3.1. Gas Chromatography Mass Spectroscopy

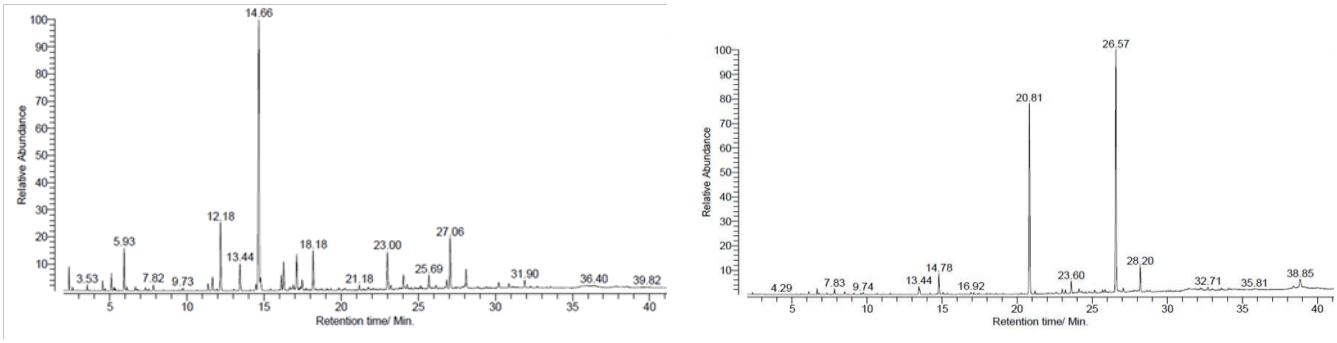

Figure 1. Gas chromatogram of P. nigrum (MW18) fruit oil and P. betle (Ratadalu) leaf oil

Table 2. Percentage composition of the essential oils of P. nigrum varieties

Retention time	Compound	MW18	KW33	MB12	KW31	MW21	IW05	Panniyur	Kuching	KW30	GK49
3.53	α-Pinene	0.4	0.2	<0.2	-	<0.2	-	<0.1	-	0.4	-
5.10	δ-3-Carene	1.4	1.0	0.6	-	-	0.2	0.6	-	1.3	-
5.27	β-Pinene	0.3	0.2	0.3	-	-	<0.1	0.2	-	0.6	-
5.93	Limonene	3.7	3.1	2.0	-	<0.2	1.4	3.5	<0.2	3.7	<0.3
12.18	α-Copaene	7.4	5.2	3.3	-	3.8	6.0	6.3	3.9	4.3	3.1
13.44	β-Linalool	3.0	2.7	5.7	-	1.7	0.7	0.8	1.6	2.8	3.5
14.66	**β-caryophyllene**	**38.1**	**60.5**	**38.7**	**9.1**	**49.7**	**47.3**	**46.1**	**44.4**	**51.5**	**45.5**
14.77	Terpinen-4-ol	1.3	-	3.0	-	1.0	-	-	1.2	-	1.6
16.12	Cubedol	1.7	0.6	0.3	-	-	0.2	2.4	<0.2	-	<0.3
16.27	Humulene	3.3	3.0	2.2	0.96	2.8	2.7	0.6	2.7	2.8	2.8
16.67	β-Ylangene	0.5	0.7	0.3	0.48	0.3	0.6	<0.1	1.0	0.3	0.9
16.91	α–Terpineol	0.5	0.6	0.3	0.51	0.5	0.9	0.7	1.0	0.6	1.0
17.11	4-*epi*-Cubedol	4.1	0.6	1.4	0.48	1.0	11.0	9.4	1.1	1.2	0.8
17.47	α-Murrolene	1.2	<0.1	0.5	-	0.7	<0.1	-	<0.2	1.0	-
18.18	Cadina 1,(10), 4 diene	4.3	2.2	2.1	3.06	2.4	3.8	2.9	3.4	2.4	3.0
21.18	Heptadecane (geranyl iso valerate)	0.5	0.3	0.8	1.43	0.6	0.5	0.5	1.0	0.6	0.8
23.00	Caryophyllene oxide	4.5	1.3	4.0	8.49	3.6	3.5	2.4	4.6	3.1	4.3
24.04	*trans*-Nerolidol	5.9	1.4	3.6	4.86	2.4	1.0	0.5	3.2	1.6	2.9
26.85	α-Cadinol	1.0	<0.1	1.3	4.90	1.1	1.0	0.7	1.5	0.9	1.5
27.07	1-Naphthalenol	0.8	0.3	0.5	0.75	<0.2	<0.1	0.3	0.4	0.2	0.8
28.10	Spathulenol	2.16	0.8	2.8	6.94	2.0	0.8	0.5	2.1	0.9	2.8

Major compounds present in essential oils of *Piper* species are in bold font in each table. β-caryophyllene was the major compound in black pepper varieties and the highest amount was detected in KW33 (60.5%) whereas KW31 showed some alterations by giving an unidentified compound (27.9%) as the major constituent.

Figure 1 indicates the gas chromatographs of *P*. *nigrum* (MW 18) fruit oil and *P*. *betle* (Ratadalu) leaf oil.

Table 2 provides the essential oil composition of the fruit oil of ten black pepper varieties which revealed 21 compounds.

The results revealed that, other than β-caryophyllene, caryophyllene oxide, α-copaene, cadina 1,(10), 4 diene, (n)-*trans*-nerolidol, 4-epi cubedol and β-linalool were also present in high amounts in *P*. *nigrum* though the percentage compositions varied with the variety (Table 2).

Highest β-caryophyllene content was detected in *P*. *nigrum* variety KW33 compared to all tested *P*. *nigrum* varieties and the highest (n)-*trans*-nerolidol content was found in variety MW18. However, some compounds were not detected in KW31 compared to the other *P*. *nigrum* varieties. In many previous studies β-caryophyllene was recorded as the major sesquiterpene of black pepper [6,7] which is supported by the findings of the present study. Table 3 summarised the percentage compositions of leaf oil of the three betel varieties. The variety Ratadalu contained eugenol as the major compound while Maneru and Nagawalli contained safrole.

Table 3. Percentage composition of the essential oils of P. betle varieties

Retention time	Compound	Ratadalu	Maneru	Nagawalli
6.12	Eucalyptol	0.3	0.4	0.3
11.54	cis- β-Terpineol	0.1	0.2	0.2
13.44	β-linalool	1.7	0.9	1.2
14.78	Terpinen-4-ol	3.2	3.3	3.8
16.92	α-terpineol	0.2	0.2	0.1
20.18	**Safrole**	33.0	**39.7**	**39.5**
23.00	Caryophyllene oxide	0.7	0.2	0.2
23.60	Methyl eugenol	2.0	1.3	1.5
26.57	**Eugenol**	**43.2**	38.8	27.4
28.20	Eugenyl acetate	3.9	5.0	12.1

As reported in Arambewela et al [11] safrole and chavibetol acetate were the major components of common betel. Eugenol content was also detected in high amount during the young stage and harvesting stage. Safrole, eugenol and eugenyl acetate were detected as the main constituents of the betel leaf oil in this study.

The major compound and the percentage compositions of the two thippili varieties varied (Table 4).

Percentage compositions of the leaf oil of other *Piper* species are indicated in Table 5. As depicted in Table 5, 13 compounds in *P*. *zeylanicum*, 7 compounds in *P*. *walker*i, 6 compounds in *P*. *sylvestre*, 9 compounds in *P*. *siriboa* and 7

compounds in *P*. *chuvya* could be identified. *P*. *longum*, *P*. *chuvya* and *P*. *sylvestre* contained *trans*-nerolidol as the major compound.

Table 4. Percentage composition of the essential oils of P. longum varieties

Retention time	Compound	Thippili	Gaja thippili
13.44	β-linalool	8.4	0.7
14.26	Bornyl acetate	1.9	<0.1
14.78	Terpinen-4-ol	0.9	0.3
15.33	Cis-linalool oxide	1.6	-
15.51	**Unidentified**	10.1	**0.6**
16.92	α-terpineol	3.4	0.1
17.84	Linalool oxide	4.2	0.1
23.01	Caryophyllene oxide	1.0	0.3
24.05	**(n)-trans-nerolidol**	**12.7**	0.2
26.73	Cholestan-3-ol 2-methylene-(3α,5β)	1.2	0.1

Terpinen-4-ol (11.95%) and eugenol (10.04%) were found to be the major compounds in *P*. *siriboa* while β linalool (4.92%) and terpeneol-cis-β (4.1%) were also present in high amount. *P*. *walkeri* leaf oil was mainly comprised with 6, isopropenyl-4,8a-dimethyl-3,5,6,7,8,8a hexa hydro-2 (1H)-naphthalenone and with an unidentified compound.

The major compound of *P*. *zeylanicum* was detected as *epi*globulol (39.6%). The study by Utpala *et al*. [10] about wild *Piper* species of Western Ghats for leaf volatile oil revealed the presence of β-caryophyllene, nerolidol and β-elemene. The present study also indicated *trans*-nerolidol and β-caryophyllene in many wild *Piper* species (Table 5).

Table 5. Percentage composition of the essential oils of other Piper species

Retention time	Compound	*P. zeylanicum*	*P. walkeri*	*P. sylvestre*	*P. siriboa*	*P. chuvya*
3.53	α–pinene	0.5	-	0.6	-	0.3
6.12	Eucalyptol	-	-	-	0.5	-
11.54	cis-β-Terpineol	-	-	-	4.1	-
13.45	β–linalool	-	-	0.4	4.9	0.6
14.66	β–caryophyllene	-	-	4.1	-	-
14.79	**Terpinen-4-ol**	0.2	-	-	**12.**	0.3
15.74	α–Guaiene	-	0.5	-	-	-
16.13	Cubedol	4.8	-	-	1.7	-
16.27	Humulene	-	-	8.4	-	-
17.10	4-epi-Cubedol	14.3	-	-	-	-
18.19	Cadina 1,(10), 4 diene	1.5	-	-	-	-
20.81	Safrole	-	-	-	2.6	-
20.88	Benzyl alcohol	-	4.7	-	-	-
21.19	Nona decane	0.7	-	-	-	-
23.01	Caryophyllene oxide	0.6	-	7.0	3.5	1.2
23.23	2.6.10.tri methyl tetra decane	-	0.4	-	-	-
23.60	Methyl eugenol	-	-	-	2.2	-
24.05	**trans-Nerolidol**	1.6	0.8	**41.2**	-	**66.5**
24.95	**Epiglobulol**	**39.6**	-	-	-	-
26.57	Eugenol	-	-	-	10.0	9.0
26.70	Cubenol	1.9	-	-	-	-
27.50	α-Eudesimol	3.8	-	-	-	-
27.67	β-Eudesimol	5.2	-	-	-	-
28.11	Spathulenol	2.9	-	-	-	-
28.20	Eugenyl acetate	-	-	-	-	2.0
28.24	Globulol	-	7.0	-	-	-
28.97	**6, isopropenyl – 4,8a – dimethyl – 3,5,6,7,8,8a hexa hydro – 2 (1H)- naphthalenone**	-	**54.6**	-	-	-
29.56	Unidentified	-	14.9	-	-	-

3.2. Scavenging Ability on DPPH Radicals

Total antioxidant activity of the essential oils of different *Piper* species was determined by DPPH and FRAP assays and compared with the standard (BHT). The positive control, BHT displayed the highest percentage inhibition (74.48%) at 1mg mL^{-1} concentration and the results are presented in Figure 2.

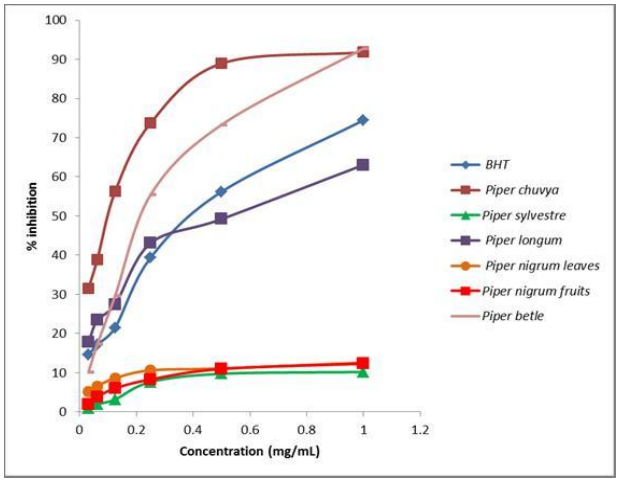

Figure 2. DPPH radical scavenging activity of the essential oils of P. chuvya leaves, P. betle leaves, P. nigrum fruits, P. nigrum leaves, P. sylvestre leaves and P. longum leaves. The results were compared with the positive control

Radical scavenging activity of the essential oils increased in a concentration dependent manner. At the concentration of 0.5mg mL^{-1} the inhibitory effect of the *P. chuvya* was found to be 89% for DPPH (Figure 2). *Piper chuvya* has exhibited significant activity with low IC_{50} value (0.22 mg mL^{-1}) in comparison to the standard (0.54 mg mL^{-1}).

P. betle exhibited significant free radical scavenging activity with low IC_{50} value (0.39mg mL^{-1}) compared with the standard (0.54mg mL^{-1}). The study of Prakash *et al*., [29] also revealed that the highest free radical scavenging activity (72% at 0.01mg mL^{-1}) of essential oil of *P. betle*. *Piper chuvya* showed greater radical scavenging activity than *P. betle*. Essential oils of *P. nigrum* fruits and laves have exhibited lower inhibition percentages (less than 50%) and higher IC_{50} values (4.18mg mL^{-1} and 4.93mg mL^{-1}) compared to the standard.

3.3. Ferric Reducing Antioxidant Power (FRAP) Assay

Activity of the essential oils increased in a concentration dependent manner (Figure 3). Essential oil of *P. betle* showed remarkable ferric reducing antioxidant power (mean absorbance, 0.982±0.28) comparatively higher than the standard (0.956±0.27). Further, it can be observed that the reducing power increased with the increment of the concentration of essential oil (0.03125-1mg mL^{-1}). These results are in agreement with the findings of Chakraborty and Shah [18].

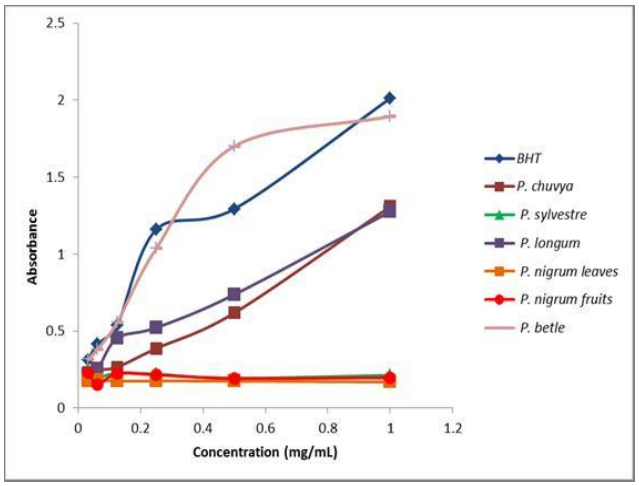

Figure 3. Absorbance values of essential oils of P. chuvya leaves, P. betle leaves, P. nigrum fruits, P. nigrum leaves, P. sylvestre leaves, P. longum leaves. The results were compared with the positive control during FRAP assay

3.4. Anti-inflammatory Activity

Total anti-inflammatory activity of the essential oils of *Piper spp*. was evaluated by the Human Red Blood Cell Membrane (HRBCM) stability assay and compared with the positive control aspirin.

Aspirin showed 91% inhibition at the concentration of 1.00mg mL^{-1} and anti-inflammatory activity of all the essential oils were less than the positive control (Figure 4). Comparatively higher mean inhibition percentage was demonstrated by essential oil of *P. betle* leaves (51.4%) and it was dose dependent and significantly increased the activity at higher concentration (76% at 0.5mg mL^{-1} and 79% at 1mg mL^{-1}). It was reported that methanol extracts of *P. betle* leaves had anti–inflammatory activity by Rintu *et al*. [22] which is also supported by the present study.

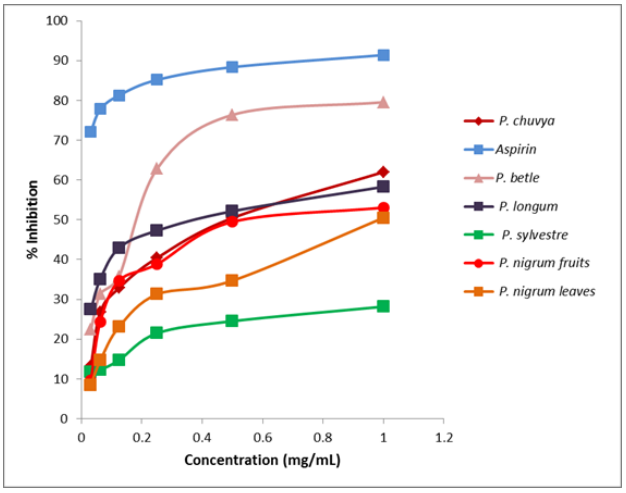

Figure 4. Anti-inflammatory activity of the essential oils of P. chuvya leaves, P. betle leaves, P. nigrum fruits, P. nigrum leaves, P. sylvestre leaves and P. longum leaves. The results were compared with the positive control, aspirin

3.5. Antibacterial Assay

The present study was focused on evaluating the antibacterial properties of essential oils of five different *Piper* species by using agar well diffusion assay. The antibacterial assays of the essential oils of *P. longum*, *P. sylvestre*, *P. chuvya* and *P. nigrum* leaves did not display antibacterial properties at 5mg mL^{-1} concentration. Inhibition zones were developed against *S. aureus* when treated with two different concentrations (15 and 25mg mL^{-1}) of essential oils of *P. betle* leaves and *P. nigrum* fruit oil separately (Table 6).

Essential oil of *P. betel* leaves showed antibacterial activity against *B. subtilis* at 25mg mL^{-1} concentration. However, the zone of inhibition on different bacterial strains was significantly increased with increment of concentration of essential oils in the present study as Chakraborty and Shah [18] and Arambewela *et al*., [15] stated.

Most of the compounds found in the essential oils of *Piper* species are known to have different bioactivities. The sesquiterpene compound, such as β-caryophyllene contain anti-inflammatory activity and antibacterial activity [30] while nerolidol is used as a natural pesticide against mites [31].

Hence the *Piper* species including wild species and cultivated varieties studied in this research demonstrated the potential of developing new herbal drugs as these species were rich in different bioactive compounds.

Table 6. Mean diameters of the zone of inhibition against S. aureus and B. subtilis by different concentrations of essential oils of P. nigrum fruits and P. betle leaves, after 18 hours of incubation

Plant species	Bacterial strain	Concentration (mg mL^{-1})				
		Control (DMSO)	Standard (Tetracycline) 5	5	15	25
P. nigrum	*S. aureus*	0.7^{d}±0.0	2.5^{a}±0.15	0.7^{d}±0.0	1.3^{b}±0.05	1.6^{c}±0.025
P. betle	*S. aureus*	0.7^{d}±0.0	3.5^{a}±0.01	0.7^{d}±0.0	1.2^{c}±0.05	1.8^{b}±0.05
	B. subtilis	0.7^{c}±0.0	3.3^{b}±0.05	0.7^{c}±0.0	0.7^{c}±0.0	3.6^{a}±0.05

Well diameter is 0.7 cm, and 0.7 cm value indicates absence of inhibition zone.
Each data point represents the mean of two trials (n) ± standard error. $^{a-d}$ Column wise values with different superscripts of this type indicate significant difference (P < 0.05) Indicate which statistical have you used.

4. Conclusions

Major compounds and percentage compositions of volatile chemical constituents in the *Piper* species were identified. Variations in the percentage compositions of the volatile constituents of varieties belong to the same species can be observed. Essential oils of *P. betle*, *P. chuvya* and *P. longum* leaves had high DPPH free radical scavenging activity compared to the positive control and the high anti-inflammatory properties were observed in essential oil of *P. betle* leaves. Essential oils of *P. nigrum* fruits and *P. betle* leaves showed significant antibacterial activity against *S. aureus* whereas essential oil of *P. betle* exhibited significant activity on *B. subtilis*. The results of this study suggested that some of the *Piper* spp. available in Sri Lanka are potential sources for developing new herbal drugs.

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