Original article:

CHEMICAL COMPOSITIONS AND ANTIBACTERIAL ACTIVITY OF THE LEAF AND STEM OILS OF *PIPER PORPHYROPHYLLUM* (LINDL.) N.E. BR.

Wan Mohd Nuzul Hakimi Wan Salleh[1], Farediah Ahmad*[1], Hasnah Mohd Sirat[1], Khong Heng Yen[2]

[1] Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia (UTM), 81310 Skudai, Johor, Malaysia
[2] School of Chemistry and Environment Studies, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Sarawak, Jalan Meranek, 94300 Kota Samarahan, Sarawak, Malaysia

* corresponding author: E-mail: farediah@kimia.fs.utm.my; Tel: +607-5534317; Fax: +6-075566162

ABSTRACT

The essential oils obtained by hydrodistillation from the fresh leaf and stem of *Piper porphyrophyllum* N.E. Br. were analyzed by GC and GC/MS. Thirty four constituents were identified in the leaf oil, while thirty eight constituents were identified in the stems oil. The most abundant components in the leaf oil included bicyclogermacrene (14.7 %), α-copaene (13.2 %) and β-phellandrene (9.5 %) while sabinene (15.5 %), bicyclogermacrene (12.3 %) and α-copaene (8.1 %) were the main constituents in the stem oil. The evaluation of antibacterial activity by using micro-dilution method revealed that both oils were moderately active against all the *Gram*-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and *Gram*-negative bacteria (*Pseudomonas aeruginosa, Pseudomonas putida* and *Escherichia coli*) with minimum inhibitory concentration (MIC) values in the range 125-1000 µg/ml.

Keywords: Chemical compositions, essential oils, *Piper porphyrophyllum*, antimicrobial activity

INTRODUCTION

The genus *Piper* belongs to Piperaceae family, comprises five genera and approximately 1400 species distributed in the tropical and subtropical regions. *Piper* and *Peperomia* are the most representative of the Piperaceae family (Parmar et al., 1997; Dos Santos et al., 2001). *Piper* species are used all over the world in traditional remedies in the Indian Ayurvedic system of medicine and in folklore medicine of Latin America and West Indies (Jussara et al., 2006). The leaves of *P. betle* (Sireh) were used for masticatory and also for relieving constipa-tion in children and poulticing ulcerated noses. The leaves were often heated and applied to the chest to relieve cough and asthma (Ghosh and Bhattacharya, 2005). The decoction of *P. sarmentosum* Roxb. (Kadok) leaves was used as an embrocation to cure pains in bones and applied to the forehead of children suffering from headaches (Thitima et al., 2004). The leaves of *P. umbellatum* (Segumbar urat) were used for stomachaches and its fruits were chewed with *P. betel* for the treatment of coughs. The leaves were also eaten raw or cooked as a seasoning (Turibio et al., 2008). *Piper* species have been investigated as a source

of new natural products with potential anti-oxidant (Yamaguchi et al., 2006), antimicrobial (Toshiya et al., 1991), antifungal (Terreaux et al., 1998; Lago et al., 2004), anti-inflammatory (Lie et al., 2006; Rajudin et al., 2008; Zakaria et al., 2010), antileishmanial (Hardik et al., 2007) and insecticidal activities (Siddiqui et al., 2004). Various compounds including amides, alkaloids, lignans, neolignans, propenylphenols, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones, flavanones, terpenes and steroids have been isolated from *Piper* species (Sengupta and Ryan, 1987; Jensen et al., 1993; Wu et al., 1997).

Piper porphyrophyllum (Lindl.) N.E. Br. is an endemic plant of Malaysia, known locally as '*lada hutan, sireh rimau, kerakap rimau* and *akar bugu*'. This wild *Piper* with purple and speckled leaves was purportedly effective against leprosy, stomachaches in children and a variety of skin diseases (Ahmad et al., 2002; Rajudin et al., 2010). It is also used in traditional medicine for postpartum treatment as well as bone pain (Burkill, 1966). The leaves oil of *P. porphyrophyllum* was reported for the first time (Chieng et al., 2005) with β-guaiene, seychellene and α-copaene as the major constituents in the oil. The ethanolic leaves extract was found to exhibit antibacterial activity (Wiart et al., 2004), while the petroleum ether leaves extract was shown to exhibit high inhibition of anti-inflammatory activity (Rajudin et al., 2008). In this study, we would like to report the chemical composition of the oils from leaves and stems of *P. porphyrophyllum,* collected from Sarawak, Malaysia with their antibacterial activity.

MATERIALS AND METHODS

Plant material

Samples of *Piper porphyrophyllum* (Lindl.) N.E. Br. were collected from Sarawak, Malaysia, in July 2010. This species was identified by Mrs. Mohizar Mohamad from the Forest Research Centre, Kuching, Sarawak and the voucher specimen (UiTMKS 3002) was deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

Extraction of essential oil

The fresh leaves and stems were subjected to hydrodistillation in on all glass *Dean-stark* apparatus for 8 hours. The oils obtained were dried over anhydrous magnesium sulfate and stored at 4-6 °C. The oil yields (w/w) were 0.20 % and 0.18 % for leaves and stems, respectively based on fresh weight-basis.

GC and GC-MS analysis

GC analysis was performed on a *Hewlett Packard 6890 series II A* gas chromatograph equipped with an *Ultra-1* column (25 m long, 0.33 μm thickness and 0.20 mm inner diameter). Helium was used as a carrier gas at flow rate of 0.7 ml/min. Injector and detector temperature were set at 250 and 280 °C, respectively. Oven temperature was kept at 50 °C, then gradually raised to 280 °C at 5 °C/min and finally held isothermally for 15 min. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 μl were injected manually (split ratio 50:1). The injection was repeated three times and the peak area percents were reported as means ± SD of triplicates. Calculation of peak area percentage was carried out by using the GC *HP Chemstation software (Agilent Technologies)*.

GC/MS chromatograms were recorded using a *Hewlett Packard Model 5890 A* gas chromatography and a *Hewlett Packard Model 5989 A* mass spectrometer. The GC was equipped with *Ultra-1* column (25 m long, 0.33 μm thickness and 0.20 mm inner diameter). Helium was used as carrier gas at flow rate of 1 ml/min. Injector temperature was 250 °C. Oven temperature was programmed from 50 °C (5 min hold) at 10 °C/min to 250 °C and finally held isothermally for 15 min. For GC/MS detection, an election ionization system, with ionization energy of 70 eV was used. A scan rate of 0.5 s (cycle time: 0.2 s) was

applied, covering a mass range from 50-400 amu.

Identification of constituents

The constituents of the oils were identified by comparison of their mass spectra with reference spectra in the computer library (*Wiley275.L* and *NIST05.L*) and also by comparing their retention indices, with those of authentic compounds or data in the literature (Adams, 2001). The quantitative data were obtained electronically from FID area percentage without the use of correction factor.

Antimicrobial activity

The tested microorganism, *Staphylococcus aureus* (ATCC29737), *Bacillus subtilis* (ATCC6633) (*Gram*-positive bacteria), *Pseudomonas aeruginosa* (ATCC9027), *Pseudomonas putida* (ATCC49128) and *Escherichia coli* (ATCC10536) (*Gram*-negative bacteria) were used. The bacteria were cultured in an appropriate nutrient broth (NB) at 37 °C overnight. The concentrations of the cultures were adjusted to obtain turbidity comparable to that of McFarland standard.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC was determined by broth micro dilution method using 96-well microplates (Gill et al., 2002). Each test sample (1 mg) was dissolved in DMSO (1 mL) to get 1000 μg/mL stock solution. A number of wells was reserved in each plate for positive and negative controls. Sterile broth (100 μL) was added to well from row B to H. The stock solutions of samples (100 μL) were added to wells at row A and B. Then, the mixture of samples and sterile broth (100 μL) at row B were transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.63 and 7.81 μg/mL). The inoculate (100 μL) was added to each well. Plates were incubated at 37 °C for 16-20 hours. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well. Samples from the MIC study which did not show any growth of bacteria were removed from each well (10 μL) and then subcultured on the surface of the freshly prepared nutrient agar in 50 mm x 15 mm disposable petri dishes. Then, the petri dishes were inverted and incubated for 16-20 hours at 37 °C. After 16-20 hours, the number of surviving organisms was determined.

Statistical analysis

Data obtained from essential oil analysis and antibacterial activity were expressed as mean values. The statistical analysis were carried out employing one way *ANOVA* ($p<0.05$). A statistical package (*SPSS version 11.0*) was used for the data analysis.

RESULTS AND DISCUSSION

Chemical composition of essential oil

The essential oils obtained from the hydrodistillation of the leaf and stem of *P. porphyrophyllum* were pale yellow oils with perfumery odour. The results showed that the leaf yielded slightly higher oil (0.20 %) than the stem (0.18 %). The chemical compositions of the leaf and stem oils studied are presented in Table 1.

Table 1: Constituents identified in the leaf and stem oils of *Piper porphyrophyllum*

Constituents	RI [a]	Area (%) [b]	
		Leaf	Stem
α-Pinene	926	2.9 ± 0.03	**7.8 ± 0.02**
Camphene	945	-	0.6 ± 0.06
Sabinene	967	3.8 ± 0.09	**15.5 ± 0.12**
Myrcene	984	-	0.7 ± 0.14
δ-3-Carene	1008	0.4 ± 0.06	0.4 ± 0.04
Limonene	1019	0.3 ± 0.05	1.5 ± 0.02
β-Phellandrene	1032	**9.5 ± 0.10**	2.2 ± 0.11
γ-Terpinene	1048	0.3 ± 0.09	1.1 ± 0.08
Terpinolene	1080	-	0.3 ± 0.15
Linalool	1082	1.2 ± 0.04	**5.4 ± 0.02**
Camphor	1112	-	0.2 ± 0.16
Terpinen-4-ol	1149	0.2 ± 0.09	1.1 ± 0.18
α-Terpineol	1171	-	0.2 ± 0.02
Bornyl acetate	1278	1.0 ± 0.01	-
Bicycloelemene	1313	-	1.3 ± 0.15
δ-Elemene	1332	**4.3 ± 0.12**	-
α-Cubebene	1344	7.4 ± 0.14	3.6 ± 0.05
α-Copaene	1372	**13.2 ± 0.08**	**8.1 ± 0.04**
β-Bourbonene	1375	3.3 ± 0.12	0.3 ± 0.01
α-Gurjunene	1401	0.8 ± 0.01	0.4 ± 0.01
β-Cubebene	1402	-	2.6 ± 0.10
α-Cedrene	1409	1.0 ± 0.14	-
β-Caryophyllene	1432	**6.4 ± 0.07**	**7.1 ± 0.12**
Aromadendrene	1441	2.2 ± 0.08	0.8 ± 0.05
Dehydroaromadendrene	1442	0.6 ± 0.05	-
Epi-Bicyclosesquiphellandrene	1453	2.2 ± 0.03	-
α-Humulene	1455	0.9 ± 0.02	1.0 ± 0.08
Germacrene D	1470	0.7 ± 0.04	4.2 ± 0.12
α-Amorphene	1480	2.4 ± 0.02	1.2 ± 0.05
α-Selinene	1482	-	0.4 ± 0.02
α-Muurolene	1489	0.3 ± 0.11	0.7 ± 0.01
β-Selinene	1490	1.1 ± 0.19	-
Bicyclogermacrene	1494	**14.7 ± 0.18**	**12.3 ± 0.14**
(*E*,*E*)-α-Farnesene	1498	0.4 ± 0.15	-
cis-Calamenene	1505	0.6 ± 0.03	0.9 ± 0.08
γ-Cadinene	1514	-	0.5 ± 0.02
δ-Cadinene	1515	**4.7 ± 0.17**	**5.0 ± 0.06**
Cadina-1,4-diene	1532	0.5 ± 0.01	0.6 ± 0.10
α-Cadinene	1535	0.2 ± 0.19	0.2 ± 0.05
Globulol	1570	-	1.3 ± 0.04
Viridiflorol	1572	-	2.5 ± 0.02
Spathulenol	1574	1.5 ± 0.10	0.8 ± 0.01
Calarene	1602	0.3 ± 0.14	0.4 ± 0.02
t-Muurolol	1635	1.9 ± 0.08	1.3 ± 0.06
α-Cadinol	1645	**6.1 ± 0.16**	1.0 ± 0.04
Monoterpene Hydrocarbons		17.2 ± 0.05	30.1 ± 0.07
Oxygenated Monoterpenes		1.4 ± 0.02	6.9 ± 0.04
Sesquiterpene Hydrocarbons		68.9 ± 0.11	51.2 ± 0.12
Oxygenated Sesquiterpenes		9.8 ± 0.08	7.3 ± 0.05
Oil Yield, % (w/w)		0.20	0.18
Identified Components (%)		97.3 ± 0.12	95.5 ± 0.10

[a] Retention indices on *Ultra-1* capillary column
[b] Each value is expressed as means ± SD of three injections

Thirty-four components (97.3 %) and thirty-eight components (95.5 %) from the leaf and stem oils, respectively were successfully identified. The main fraction in the leaf and stem oils were sesquiterpene hydrocarbons which consists of 68.9 % and 51.2 %, respectively. The most abundant components in the leaf oil were bicyclogermacrene (14.7 %), α-copaene (13.2 %), β-phellandrene (9.5 %), α-cubebene (7.4 %) and β-caryophyllene (6.4 %), while sabinene (15.5 %), bicyclogermacrene (12.3 %), α-copaene (8.1 %), α-pinene (7.8 %) and β-caryophyllene (7.1 %) were the main components in the stem oil. Most of the components were similar in both oils. However, bornyl acetate (1.0 %), δ-elemene (4.3 %), α-cedrene (1.0 %), dehydroaromadendrene (0.6 %), *epi*-bicyclosesquiphellandrene (2.2 %), β-selinene (1.1 %) and (*E,E*)-α-farnesene (0.4 %) were absent in the stem oil. In addition, camphene (0.6 %), myrcene (0.7 %), terpinolene (0.3 %), camphor (0.2 %), α-terpineol (0.2 %), β-cubebene (2.6 %), α-selinene (0.4 %), bicycloelemene (1.3 %), γ-cadinene (0.5 %), globulol (1.3 %) and viridiflorol (2.5 %) were found to be absent in the leaf oil.

In comparison to previous study (Chieng et al., 2005), the leaf oil of *P. porphyrophyllum* was reported to show high amounts of β-guaiene (8.4 %), seychellene (8.3 %) and α-cadinene (6.9 %). However, these two constituents, β-guaiene and seychellene were not detected in both oils studied, while α-cadinene was present in lower percentage which only gave 0.2 % from both oils. The differences of the leaf oil composition of this study compared to the previous report was probably due to the different environmental and genetic factors, chemotypes and nutritional status of the plants, which may influence the oil composition (Marjo et al., 2001).

Antimicrobial activity

The antibacterial activity of the investigated oils was evaluated by determining the MIC and MBC values against two *Gram*-positive bacteria (*B. subtilis*, *S. aureus*) and three *Gram*-negative bacteria (*P. aeruginosa*, *P. putida*, *E. coli*). The results of the antibacterial activity are shown in Table 2.

The essential oil displayed a broad antimicrobial spectrum and exerted a much stronger antimicrobial effect against *Gram*-positive bacteria than *Gram*-negative bacteria. The leaves oil showed good MIC value, 125 µg/mL towards *B. subtilis, S. aureus* and *E. coli.* The results were confirmed by the MBC values which showed 125 µg/mL, 250 µg/mL and 250 µg/mL inhibition of leaf oil against *B. subtilis, S. aureus* and *E. coli*, respectively. The stem oil exhibited MIC value of 125 µg/mL against *P. putida* and supported by MBC value at 250 µg/mL. Based on our findings, the sensitivity of the bacterial strains to the leaf oil decreases in the order: *B. subtilis > S. aureus = E. coli > P. putida > P. aeruginosa*, while for the stem oil, the order is: *P. putida > P. aeruginosa = E. coli = B. subtilis = S. aureus.*

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of leaf and stem oils of *Piper porphyrophyllum*

Bacteria	MIC (µg/mL)		MBC (µg/mL)		Streptomycin sulphate
	Leaf	Stem	Leaf	Stem	
***Gram*-positive bacteria**					
Bacillus subtilis (ATCC 6633)	125	1000	125	1000	7.81
Staphylococcus aureus (ATCC29737)	125	1000	250	1000	7.81
***Gram*-negative Bacteria**					
Pseudomonas aeruginosa (ATCC 9027)	1000	250	1000	500	7.81
Pseudomonas putida (ATCC49128)	250	125	500	250	7.81
Escherichia coli (ATCC10536)	125	1000	250	1000	7.81

The variation of the antimicrobial activity could be correlated to chemical variation in the oil composition (Burt, 2004). Some studies have concluded that the whole essential oils have greater antibacterial activity than the major components, indicating that minor components are critical for the activity due to their synergistic effect (Vandepitte et al., 1991).

Previous studies on the antimicrobial activity of the ethanolic extract of the leaf of *P. porphyrophyllum* showed that the activity was found to increase after fractionation (hexane, dichloromethane and aqueous), particularly in the aqueous fraction. However, no activity was shown against the tested *Candida* (Wiart et al., 2004). To the best of our knowledge, this is the first report on the antibacterial activity of the essential oil of *P. porphyrophyllum.*

CONCLUSION

Our results demonstrated that bicyclegermacrene and sabinene were the main components in the leaf and stem oils of *P. porphyrophyllum*. Since these components are used as flavouring agents in food industry, the essential oil of *P. porphyrophyllum* can be used as a source of these components. The essential oils were found to possess moderate antibacterial inhibition against *Gram*-positive rather than *Gram*-negative bacteria. The present study indicates the prospect of using essential oils as possible therapeutic agents against microbial infections.

ACKNOWLEDGMENTS

REFERENCES

Adams RP. Identification of essential oil by gas chromatography-quadrupole/mass spectroscopy. Carol Stream. IL: Allured Publ. Corp., 2001.

Ahmad FB, Tawan CS, Ipor IB, Sani HA. Brief account on the wild *Piper* (Piperaceae) of the Crocker Range, Sabah. ASEAN Rev Biodiv Environ Conserv 2002:1–11.

Burkill HI. A dictionary of economic products of the Malay Peninsula (pp 1766-84). Kuala Lumpur: Ministry of Agriculture and Co-operative, 1966.

Burt S. Essential oils: their antibacterial properties and potential applications in foods: a review. Int J Food Microbiol 2004; 94:223–53.

Chieng CT, Assim ZB, Ahmad FB. The essential oil composition of the leaves of five *Piper* species for taxonomic purposes. ACGC Chem Res Com 2005;19:6–12.

Dos Santos PRD, Moreira DL, Guimaraes EF, Kaplan MAC. Essential oil analysis of 10 Piperaceae species from the Brazilian Atlantic Forest. Phytochemistry 2001;58: 547–51.

Ghosh K, Bhattacharya TK. Chemical constituents of *Piper betle* Linn. (Piperaceae) Roots. Molecules 2005;10:798–802.

Gill AO, Delaquis P, Russoa P, Holley RA, Evaluation of antilisterial action of cilantro oil on vacuum packed ham. Int J Food Microbiol 2002;73:83–92.

Hardik SB, Gaganmeet S, Ranvir S, Chinmoy SD, Shyam SS, Kamlesh KB et al. Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum*. J Nat Med 2007;61:418–21.

Jensen S, Hansen J, Boll PM. Lignans and neolignans from *Piperaceae*. Phytochemistry 1993;33:523–30.

Jussara MOM, Alaide BO, Fernao CB, Julio AL, Antonio PC, Ligia S et al. Essential oil constituents of *Piper vicosanum* Yunker from the Brazillian Atlantic Forest. J Essent Oil Res 2006;18:392–5.

Lago JHG, Ramos CS, Casanova DCC, Moradin AA, Bergamo DCB, Furlan M et al. Benzoic acid derivatives from *Piper* species and their fungitoxic activity against *Cladosporium cladosporioides* and *C. Sphaerospermum*. J Nat Prod 2004;67: 1783–8.

Lie CL, Chien CS, Yuh CS, Tung HT. Anti-inflammatory neolignans from *Piper kadsura*. J Nat Prod 2006;69:842–4.

Marjo K, Eija P, James ES. Variation in volatile compounds from tansy (*Tanacetum vulgare* L.) related to genetic and morphological differences of genotypes. Biochem Syst Ecol 2001;29:267–85.

Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A et al. Phytochemistry of the genus *Piper*. Phytochemistry 1997;46:597–673.

Rajudin E, Ahmad F, Sirat HM, Jamaluddin F, Mustapha NM, Ali NM et al. Anti-inflammatory activity of *Piper magnibaccum* (Piperaceae). Nat Prod Com 2008;3: 1719–21.

Rajudin E, Ahmad F, Sirat HM, Arbain D, Aboul-Enein HY. Chemical constituents from tiger's betel, *Piper porphyrophyllum* N.E.Br. (Fam. Piperaceae). Nat Prod Res 2010;24:387–90.

Sengupta S, Ryan AB. The chemistry of *Piper* species: a review. Fitoterapia 1987; 58:147–65.

Siddiqui BS, Gulzar T, Mahmood A, Begum S, Khan B, Afshan F. New insecticidal amides from petroleum ether extract of dried *Piper nigrum* L. whole fruits. Planta Med 2004;52:1349–52.

Terreaux C, Gupta M, Hostettmann K. Antifungal benzoic acid derivatives from *Piper dilatatum*. Phytochemistry 1998;49: 461–4.

Thitima R, Puttan S, Kanchanawadee S, Chanika W, Phongpan R, Phaopong W et al. Chemical constituents and bioactivity of *Piper sarmentosum*. J Ethnopharmacol 2004;93:173–6.

Toshiya M, Inazumi Y, Yasumasa Y, William GP, Hiroe K, Nobuji N. Antimicrobial phenylpropanoids from *Piper sarmentosum*. Phytochemistry 1991;30:3227–8.

Turibio KT, Joseph N, Jiawei L, Anne CMO, Sheraz AKT, Shamsun NK et al. Bioactive Aristolactams from *Piper umbellatum*. Phytochemistry 2008;69: 1726–31.

Vandepitte J, Engback K, Piot P, Heuck CC. Basic microbiology procedures in clinical bacteriology, Vol. 85 (pp 502-16). Geneva: World Health Organization; 1991.

Wiart C, Hannah NA, Yassim M, Hamimah H, Sulaiman M. Antimicrobial activity of tiger's betel (*Piper porphyrophyllum* N. E. Br, Piperaceae). Phythother Res 2004;18: 783–4.

Wu QL, Wang SP, Tu GZ, Feng YX, Yang JS. Alkaloids from *Piper puberullum*. Phytochemistry 1997;44:727–30.

Yamaguchi L, Lago JHG, Tanizaki TM, Mascio PD, Kato MJ. Antioxidant activity of prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* Kunth. Phytochemistry 2006;67:1838–43.

Zakaria ZA, Patahuddin H, Mohamad AS, Israf DA, Sulaiman MR. *In vivo* anti-nociceptive and anti-inflammatory activities of the aqueous extract of the leaves of *Piper sarmentosum.* J Ethnopharmacol 2010;128: 42–8.