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Hakimi Wan SALLEH, Wan Mohd Nuzul; AHMAD, Farediah; KHONG, Heng Yen

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Chemical composition of *Piper stylosum* Miq. and *Piper ribesioides* Wall. essential oils, and their antioxidant, antimicrobial and tyrosinase inhibition activities

[Composición química de los aceites esenciales de *Piper stylosum* Miq. y *Piper ribosoides* Wall. y sus actividades antioxidantes, antimicrobiana y de inhibición de la tirosinasa]

Wan Mohd Nuzul Hakimi Wan SALLEH[1], Farediah AHMAD[1] & Heng Yen KHONG[2]

[1]*Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia (UTM), 81310 Johor Bahru, Johor, Malaysia*
[2]*School of Chemistry and Environment Studies, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
Contactos | Contacts: **Farediah AHMAD** - ***E-mail address:*** **farediah@kimia.fs.utm.my**

Abstract: Chemical compositions of *P. stylosum* and *P. ribesioides* essential oils, and their antioxidant, antimicrobial and tyrosinase inhibition activities were determined. GC and GC–MS analysis of essential oils from leaves and stems of *P. stylosum* resulted in the identification of 50 (89.2%) and 45 (88.8%) components, respectively. The major components were aromadendrene (leaves 26.6%; stems 18.8%), sabinene (leaves 13.8%; stems 6.7%) and β-caryophyllene (leaves 11.5%; stems 17.9%). A total of 60 (87.0%) and 39 (82.9%) components were identified from leaves and stems of *P. ribesioides*, respectively. The most abundant components were β-caryophyllene (leaves 20.0%; stems 14.4%), camphene (leaves 16.3%; stems 12.3%) and δ-cadinene (leaves 4.4%; stems 7.8%). Antioxidant activity using DPPH and total phenolic content were tested for essential oils. However, the essential oils showed low antioxidant activity and phenolic content, compared to BHT. Studies of tyrosinase inhibition showed that the essential oils of *P. ribesioides* leaves had the highest inhibition (30.0%), although were lower than the control (kojic acid 81.8%). The evaluation of antimicrobial activities revealed that *P. ribesioides* essential oils showed strong activity against *Bacillus cereus* and *Staphylococcus aureus*, both with MIC value 62.5 µg/mL.

Keywords: Essential oil, *Piper stylosum*, *Piper ribesioides*, Antioxidant, Antimicrobial, Tyrosinase inhibition

Resumen: Se determinaron las composiciones químicas, las actividades antioxidante y antimicrobiana, y el contenido total de fenoles de los aceites esenciales de *P. stylosum* y *P. ribesioides*. El análisis GC y GC-MS de los aceites esenciaales de hojas y tallos de *P. stylosum* permitió la identificación de 50 (89.2%) y 45 (88.8%) de components, respectivamente. Los principales componentes fueron aromadendreno (hojas 26.6%; tallos 18.8%), sabineno (hojas 13.8%; tallos 6.7%) y β-cariofileno (hoja 11.5%; tallo 17.9%). Se identificaron 60 (87.0%) y 39 (82.9%) components en los aceites esenciales de hojas y tallos de P. ribesioides. Los componentes más abundantes fueron β-cariofileno (hojas 20.0%; tallos 14.4%), canfeno (hojas 16.3%; tallos 12.3%) y δ-cadineno (hojas 4.4%; tallos 7.8%). Los aceites esenciales se ensayaron para determinar sus actividades antioxidantes con DPPH y el contenido de fenoles totales. Para los aceites esenciales obtenidos se determinaron valores bajos en la actividad antioxidante con DPPH y el contenido total de fenoles, en comparación con BHT. Sin embargo, los ensayos de inhibición de tirosinasa most raron que el aceite esencial de las hojas de *P. ribesioides* presento la mayor inhibición (30.0%), aunque mas baja que el compuesto control (Àcido Kójico, 81.8%). Para el aceite esencial de P. ribesioides se determinó una MIC 62.5 mg/mL contra *Bacillus cereus* y Staphylococcus aureus.

Palabras clave: aceite esencial, *Piper stylosum*, *Piper ribesioides*, antioxidante, antimicrobiana, inhibición de la tirosinasa

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INTRODUCTION

The genus *Piper* has a large number of species and has been of worldwide interest due to their wide utilization as aromatic species and their use in the traditional medicine. Analysis of volatile constituents from Piperaceae species has revealed the presence of monoterpenes, sesquiterpenes and arylpropanoids that have shown interesting biological properties including cytotoxic, fungistatic, insecticide, molluscicidal, antioxidant and antimicrobial activities (Parmar *et al.,* 1997; Martins *et al.,* 1998; Santos *et al.,* 2001; Costantin *et al.,* 2001; Navickiene *et al.,* 2006). As part of an exhaustive research of the composition of the essential oils of the aromatic and medicinal plants from Malaysia, we report herein the results of the investigation of two *Piper* species widely used by the local traditional healers.

Piper stylosum also known as 'kaduk hutan' is a sprawling and upright herb with little height to 20 cm tall and creeping stem. The leaves use as a vegetable and seasoning, while the root is used as a poultice or decoction medicinally after confinement (Burkill, 1966). *Piper ribesioides* locally known as 'lada rimba', 'akar kalong ular', or 'sireh murai', is a vigorous climber, found in Indonesia and the Peninsular Malaysia. As a traditional medicine, the root is used to treat an illness caused from asthma, diarrhea, and abdominal pain. Stems of *P. ribesioides* have long been used to flavor food giving its spicy and peppery taste. The leaves treat body wind element abnormality, alleviate chest congestion and excrete phlegm while the flowers have been used to treat urticaria (Sudmoon *et al.*, 2012).

In the literature, there is only one study on the essential oil composition from the leaves of *P. ribesioides* (Aunphak, 1998), whereas the oil of *P. stylosum* seem not to have been reported before. In addition this is the first report on the antioxidant, antimicrobial and tyrosinase inhibition studies for these two essential oils.

MATERIAL AND METHODS

Plant material

Samples of *Piper stylosum* (SK1963/11) and *Piper ribesioides* (SK1962/11) were collected from Kuala Berang, Terengganu, Malaysia, in June 2011. This species was identified by Dr. Shamsul Khamis and the voucher specimens deposited at the Universiti Putra Malaysia, Serdang, Selangor.

Solvent and chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, butylated hydroxytoluene (BHT), kojic acid, 3,4-dihydroxy-L-phenylalanine (L-DOPA) and mushroom (EC 1.14.18.1; 25KU) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Methanol, ethanol and dimethylsulfoxide (DMSO) were analytical grade, HPLC grade chloroform, Folin-Ciocalteu's reagent, anhydrous sodium sulfate, sodium carbonate, were purchased from Merck (Darmstadt, Germany).

Extraction of essential oils

The fresh leaves and stems were subjected to hydrodistillation in an all glass Dean-stark apparatus for 8 hours. The oils were dried over anhydrous magnesium sulfate and stored at 4–6°C. The oils yields (w/w) were based on their fresh weight.

Gas chromatography (GC)

GC analysis were *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 a flow rate of 0.7 mL/min. The injector temperature was set at 250° C while the detector was kept at 280° C, respectively. The 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).

Gas chromatography-Mass spectrometry (GC-MS)

GC-MS chromatograms were recorded using a Hewlett Packard Model 5890A gas chromatograph and a Hewlett Packard Model 5989A 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. The injector temperature was set at 250° C. Oven temperature was programmed from 50° C (5 min hold) to 250° C at 10°C/min and finally held isothermally for 15 min. For GC-MS detection, an electron 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 (Wiley) and also by comparing their Kovats 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.

Antioxidant activity

DPPH Radical scavenging assay

The free radical scavenging activity was measured by the DPPH method with minor modification (Loo *et al.,* 2008). Stock solution of essential oil (1.0 mg/L) was diluted to final concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.63 and 7.81 µg/mL. Then, a total of 3.8 ml of 50 µM DPPH methanolic solution was added to 0.2 mL of each sample solution and allowed to react at room temperature for 30 min. The absorbance of the mixtures was measured at 517 nm. A control was prepared without sample or standard and measured immediately at 0 min. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. Inhibitions of DPPH radical in percent (I%) were calculated as follow:

$$[I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100]$$

where A_{blank} is the absorbance value of the control reaction (containing all reagents except the essential oils) and A_{sample} is the absorbance values of the essential oils. The sample concentration that provides 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicate.

Total phenolic content

Total phenolic contents of the essential oils were determined with minor modification (Loo *et al.,* 2008). Stock solution of essential oil (1.0 mg/mL) was diluted in methanol to final concentrations of 1000, 800, 600, 400, and 200 µg/mL. A 0.1 mL aliquot of sample was pipetted into a test tube containing 0.9 mL of methanol, then 0.05 mL Folin-Ciocalteu's reagent was added, and the flask was thoroughly shaken. After 3 min, 0.5 mL of 5% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Then, 2.5 mL of methanol was added and left to stand in the dark for 1 h. The absorbance measurements were recorded at 765 nm. The same procedure was repeated for the standard gallic acid solutions. The concentration of total phenolic compounds in the essential oils was expressed as mg of gallic acid equivalent per gram of sample. Test was carried out in triplicate and gallic acid equivalent value was reported as mean ± SD of triplicate.

Tyrosinase inhibition activity

Tyrosinase inhibition assay (EC 1.14.18.1) was performed according to previous study with minor modification (Liu *et al.,* 2009). The essential oils and kojic acid were dissolved in DMSO prepared as 0.1 mg/mL. The reaction was carried out using 96-well microplate and ELISA microplate reader (VersaMax Molecular Devices, USA) was used to measure the absorbance at 475 nm. 40 µL of essential oils dissolved in DMSO with 80 µL of phosphate buffer (pH 6.8), 40 µL of tyrosinase enzyme and 40 µL of L-Dopa were put in each well. Each sample was accompanied by a blank that had all the components except for L-Dopa. Kojic acid was used as reference standard inhibitors for comparison. Inhibitions of tyrosinase in percent (I%) were calculated as follow:

$$[I\% = [(A_{control} - A_{sample})/A_{control}] \times 100]$$

where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the essential oils.

Antimicrobial activity

Microbial strains

The test microorganisms, *Bacillus subtilis* (ATCC6633), *Bacillus cereus* (ATCC11778), *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC35218), *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumonia* (ATCC13883), *Candida albicans* (ATCC10231), *Candida neoformens* (ATCC90112) and *Saccharomyces cerevisiae* (ATCC7754) were used. The strains were grown on Nutrient broth (NB) for the bacteria and Potato dextrose broth (PDB) for yeasts and fungi (Oxoid, Italy).

Disc diffusion assay

Antimicrobial activity of the essential oils was carried out by the disc diffusion method (Murray *et al.,* 1999). The essential oils were dissolved in DMSO (4 mg/mL). Inocolumn of 400 µL suspension containing 10^8 CFU/mL of bacteria and 10^6 CFU/mL of fungi each was spread on the nutrient agar (NA)

and potato dextrose agar (PDA) medium. The discs (6 mm diameter) impregnated with 10 µL of the essential oils and DMSO (negative control) were placed on the inoculated agar, and were incubated for 24 h at 37 °C (bacterial), 48 h at 30 °C (yeast) and 72 h at 30 °C (fungi). Streptomysin sulfate (10 µg/mL) and nystatin (100 IU) were used as the positive controls for bacteria and fungi, respectively. Clear inhibition zones around the discs indicated the positive antimicrobial activity. All tests and analysis were carried out in triplicates.

Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) was determined by broth micro dilution method using 96-well microplates (Gulluce *et al.*, 2004). The inocula of the microbial strains were prepared from 24 h broth cultures and McFarland standard turbidity of suspensions was adjusted to 0.5. Essential oil (1 mg) was dissolved in DMSO (1 mL) to get 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. The mixture of samples and sterile broth (100 µL) 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 inoculated bacteria (100 µL) were added to each well. The final volume in each well was 200 µL. Strepyomysin sulfate and nystatin were used as positive controls for bacterial and fungal, respectively. Plates were incubated at 37 °C for 24 h. Microbial growth was indicated by the turbidity and the presence of pellet at the bottom of the well.

Statistical analysis

Data obtained from essential oil analysis, antioxidant and antimicrobial activity were expressed as mean values. The statistical analyses 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

The hydrodistillation of fresh leaves and stems parts of *P. stylosum* and *P. ribesioides* yielded yellowish, pleasant-smelling oils due to they are herbs. The highest yield was obtained from *P. stylosum* (leaves 0.08%; stems 0.07%), which was significantly different from the oils of *P. ribesioides* (leaves 0.03%; stems 0.04%). The volatile components identified in these oils are listed in Table 1.

Table 1
Constituents identified in the leaves and stems oil of P. stylosum and P. ribesioides

	Constituents	RI [a]	Area (%)			
			PSLO	PSSO	PRLO	PRSO
1	α-Thujene	924	0.1	-	0.1	-
2	α-Pinene	926	0.9	0.7	5.4	3.3
3	**Camphene**	945	0.2	0.1	**16.3**	**12.3**
4	**Sabinene**	967	**13.8**	**6.7**	0.1	-
5	β-Pinene	974	0.2	0.6	0.2	-
6	Myrcene	984	0.3	0.1	1.1	0.7
7	α-Phellandrene	1002	0.5	0.4	0.1	-
8	δ-3-Carene	1008	-	-	0.2	0.9
9	α-Terpinene	1014	1.0	0.7	0.1	-
10	Limonene	1019	-	0.5	2.5	2.5
11	*p*-Cymene	1020	-	0.2	-	-
12	*o*-Cymene	1022	0.2	-	0.1	-
13	1,8-Cineole	1026	-	-	1.9	0.9
14	(*Z*)-β-Ocimene	1032	0.4	0.1	-	-
15	γ-Terpinene	1048	1.9	1.1	0.1	-
16	*cis*-Sabinene hydrate	1065	-	-	0.1	-
17	Terpinolene	1080	0.5	0.3	0.2	0.2
18	Linalool	1082	1.4	1.4	0.8	0.9
19	*trans*-Linalool oxide	1085	-	-	0.1	-

20	Fenchol	1115	-	-	0.1	-
21	Camphor	1141	0.2	0.1	0.1	-
22	Terpinen-4-ol	1149	5.1	2.7	0.1	0.3
23	Borneol	1165	-	-	0.8	1.5
24	α-Terpineol	1171	0.3	0.4	0.7	0.8
25	n-Decanal	1201	0.2	-	-	-
26	Bornyl acetate	1287	0.1	-	0.1	-
27	Bicycloelemene	1313	0.1	-	0.1	0.5
28	δ-Elemene	1332	0.2	-	0.1	-
29	α-Cubebene	1344	2.2	0.9	2.6	2.3
30	Cyclosativene	1369	0.4	0.7	-	-
31	α-Copaene	1372	0.8	3.2	1.8	1.2
32	α-Ylangene	1373	-	-	0.4	0.4
33	Isoledene	1374	0.3	-	-	-
34	Calarene	1384	0.1	0.3	-	-
35	β-Bourbonene	1387	-	-	0.1	-
36	β-Elemene	1389	2.0	1.6	0.2	0.4
37	α-Gurjunene	1401	0.3	0.2	0.2	-
38	β-Cubebene	1402	-	-	2.2	2.4
39	β-Gurjunene	1431	-	-	0.4	-
40	**β-Caryophyllene**	1432	**11.5**	**17.9**	**20.0**	**14.4**
41	γ-Elemene	1434	1.9	2.9	0.4	0.6
42	α-Guaiene	1437	-	0.2	-	-
43	**Aromadendrene**	1441	**26.6**	**18.8**	1.1	0.8
44	*epi*-Bicyclosesquiphellandrene	1453	-	-	0.5	0.6
45	α-Humulene	1455	2.4	3.8	2.1	1.5
46	Dehydroaromadendrene	1460	-	0.1	0.1	-
47	Germacrene D	1470	1.6	2.0	1.4	2.1
48	γ-Gurjunene	1475	0.1	-	-	-
49	β-Chamigrene	1476	1.6	-	-	-
50	γ-Muurolene	1478	-	-	0.9	-
51	α-Amorphene	1480	0.6	0.7	3.1	4.4
52	β-Selinene	1490	0.5	1.3	0.4	-
53	Valencene	1496	0.5	0.8	0.4	-
54	α-Selinene	1498	-	0.8	0.2	-
55	α-Muurolene	1502	1.0	1.6	1.2	1.9
56	β-Bisabolene	1506	0.5	-	-	-
57	γ-Cadinene	1514	0.5	0.2	1.5	0.5
58	δ-Cadinene	1515	1.9	2.8	4.4	7.8
59	Cis-Calamenene	1528	0.5	-	1.6	2.6
60	(*Z*)-Nerolidol	1531	0.2	-	0.6	0.4
61	Cadina-1,4-diene	1532	0.3	-	0.5	0.7
62	α-Cadinene	1537	-	0.4	0.4	0.5
63	α-Calacorene	1544	-	-	0.8	1.6
64	Selina-3,7(11)-diene	1545	-	0.3	-	-
65	Elemicin	1555	0.4	6.3	-	-
66	Germacrene B	1559	1.0	1.0	1.8	2.0
67	Caryophyllene alcohol	1570	-	0.2	-	-
68	Viridiflorol	1572	-	-	0.2	0.4
69	Spathulenol	1574	-	-	0.7	0.7
70	Caryophyllene oxide	1582	0.2	0.4	1.0	0.5

71	Globulol	1590	-	-	0.7	-
72	Ledol	1602	-	-	-	0.9
73	τ-Muurolol	1635	-	1.2	-	3.3
74	α-Cadinol	1645	-	1.5	1.5	3.2
75	Eudesma-7(11)-en-4-ol	1700	0.2	0.6	-	-
76	Phytol	1942	0.4	-	0.1	-
77	Hexadecanoic acid	1959	1.1	-	-	-
Group components						
	Phenylpropanoids		**0.4**	**6.3**	**-**	**-**
	Monoterpene Hydrocarbons		**20.0**	**11.5**	**26.5**	**22.0**
	Oxygenated Monoterpenes		**7.1**	**4.6**	**4.8**	**4.4**
	Sesquiterpene Hydrocarbons		**59.4**	**62.5**	**50.9**	**47.1**
	Oxygenated Sesquiterpenes		**0.6**	**3.9**	**4.7**	**9.4**
	Others		**1.7**	**-**	**0.1**	**-**
Identified Components (%)			**89.2**	**88.8**	**87.0**	**82.9**

[a]Retention indices on Ultra-1 capillary column
PSLO - *P. stylosum* leaf oil; PSSO - *P. stylosum* stem oil
PRLO - *P. ribesioides* leaf oil; PRSO - *P. ribesioides* stem oil

Sixty components were identified in the *P. stylosum* essential oil, representing approximately 89.2% (50 components) and 88.8% (45 components) of the leaves and stems oil, respectively. Sesquiterpene hydrocarbons were the most dominant components which constituted 59.4 - 62.5% of the oils. The most abundant components were aromadendrene (leaves 26.6%; stems 18.8%), sabinene (leaves 13.8%; stems 6.7%) and β-caryophyllene (leaves 11.5%; stems 17.9%). In addition, monoterpene hydrocarbons (11.5 - 20.0%) were present in appreciable amounts. Elimicin (leaves 0.4%; stems 6.3%) was the only phenylpropanoids found in this oil but at low percentage. Meanwhile, 15 components were not detected in the stem oil; these were α-thujene, o-cymene, n-decanal, bornyl acetate, bicycloelemene, δ-elemene, isoledene, γ-gurjunene, β-chamigrene, β-bisabolene, cis-calamenene, (*Z*)-nerolidol, cadina-1,4-diene, phytol and hexadecanoic acid. A total of 62 components were identified in the *P. ribesioides* essential oil, corresponding to 87.0% (60 components) and 82.9% (39 components) of leaves and stems essential oils, respectively. Both essential oils were characterized by large amounts of sesquiterpene hydrocarbons (47.1 - 50.9%), followed by monoterpene hydrocarbons (22.0 – 26.5%). The major components were β-caryophyllene (leaves 20.0%; stems 14.4%), camphene (leaves 16.3%; stems 12.3%), α-pinene (leaves 5.4%; stems 3.3%) and δ-cadinene (leaves 4.4%; stems 7.8%). All components identified in the leaves essential oil were found in the stems essential oil. The principal difference between the two essential oils was the absence of ledol and t-muurolene from both parts of the plants. Previous study on the leaves oil of this plant has been reported, which gave β-caryophyllene (35.2%), (*Z*)-nerolidol (35.9%) and bicyclogermacrene (5.1%) as the major components (Aunphak, 1998). This result was similar with ours which gave β-caryophyllene (20.0%) as one of the major components. However, Aunphak (1998) reports (*Z*)-nerolidol and bicyclogermacrene in appreciable amounts. The *P. ribesioides* essential oil revealed a chemical profile that is more characteristic of the genus *Piper*. The presence of β-caryophyllene in significant amounts in the leaves (20.0%) and stems (14.4%) essential oils is in agreement with the previous study from *P. obliquum* (27.6%) (Mundina et al., 1998), *P. lancaefolium* (20.6%) (Mundina *et al.*, 2001), *P. guineense* (57.5%) (Jirovetz *et al.*, 2002), *P. tuberculatum* (40.2%) (Navickiene *et al.*, 2006), *P. amapense* (25.0%) and *P. duckei* (23.5%) (Santos *et al.*, 1998) indicating that the occurrence of β-caryophyllene as the major component may be a characteristic of *Piper* essential oils. These results confirm that the variability in the composition of essential oils depends essentially upon the origin of the samples as well as the influence of geographic circumstances and climate (Santos *et al.*, 1998).

Antioxidant activities of the investigated essential oils were evaluated by measuring the scavenging activity on DPPH-radicals and total phenolic content by Folin-Ciocalteu's method. In the

DPPH-test, the ability of the essential oil to act as the donor of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H was measured spectrophotometrically. Nevertheless, essential oils did not display a noteworthy DPPH-radical scavenging activity. Table 2 shows the IC_{50} of the essential oils in the range 605.8 – 831.5 ug/mL, lower than that of BHT. This result is expected because the chemical composition of *P. stylosum* and *P. ribesioides* essential oils shows low oxygenated compounds and a dominance of sesquiterpene hydrocarbons components. Essential oils that rich in oxygenated monoterpenes have relatively important DPPH scavenging properties (Zouari *et al.,* 2011). The total phenolic contents (TPC) of the essential oils were expressed as equivalents of gallic acid (mg GA/g) of essential oil. As shown in Table 2, the essential oils were found to have various phenolic levels, ranging from 15.4 to 24.8 mg GA/g essential oil. *P. ribesioides* stems oil having the highest contents of total phenolic (24.8 ± 0.2 mg GA/g). Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. The highest level of inhibitory activity was from the *P. ribesioides* essential oil (leaves 30.0 ± 0.5%; stems 19.2 ± 0.2%) but at low percentage compared to kojic acid, 81.8 ± 0.5%. However, the *P. stylosum* essential oil showed weak activity against tyrosinase.

Table 2
Antioxidant and tyrosinase inhibition activities of the leaves and stems oil of P. stylosum and P. ribesioides[a]

Samples	DPPH IC_{50} (µg/ml)	Total phenolic content Gallic acid equivalent (mg GA/g)	Tyrosinase Inhibitory activity at 0.1 mg/mL
PSLO	605.8	15.4 ± 0.2	NA
PSSO	623.2	18.2 ± 0.4	3.87 ± 0.4
PRLO	831.5	20.5 ± 0.5	30.0 ± 0.5
PRSO	692.4	24.8 ± 0.2	19.2 ± 0.2
BHT	43.5	ND	ND
Kojic acid	ND	ND	81.8 ± 0.5

[a]Data represent mean ± standard deviation of three independent experiments
PSLO - *P. stylosum* leaf oil; PSSO - *P. stylosum* stem oil
PRLO - *P. ribesioides* leaf oil; PRSO - *P. ribesioides* stem oil
NA - not active; ND - not determined

The inhibition zone of the disc diameters and minimum inhibitory concentrations (MIC) of the essential oils against the microorganisms tested are shown in Table 3. The antimicrobial activity revealed that the essential oils have moderate to strong, and in a few cases very weak activity against the tested microorganisms. The inhibition zones for most of the bacterial strains were in the range of 6.4 ± 0.1 to 14.7 ± 0.2 mm. The essential oil of *P. stylosum* leaves exhibited the highest zones of inhibition against *S. aureus* (14.7 ± 0.2 mm), while the essential oil of *P. ribesioides* leaves was found to be almost equally effective against *B. cereus* and *S. aureus* (13.3 ± 0.2 and 13.2 ± 0.1 mm, respectively). The highest activity was shown by the *P. ribesioides* essential oils against *B. cereus* and *S. aureus* with MIC values 62.5 µg/mL. *P. stylosum* essential oils were found to be equally effective against *B. cereus* and *S. aureus* with MIC values 125 µg/mL. Moderate activities were shown by all the essential oils towards *B. subtilis* which gave MIC value 500 µg/mL. In addition, essential oil of *P. stylosum* leaves showed strong antifungal activity towards *C. albicans* and *C. neoformens* with MIC value 125 µg/mL. Moreover, the MIC values of the other strains tested were all higher than 1000 µg/mL and were considered weak, except essential oil of *P. stylosum* stems and *P. ribesioides* leaves exhibited MIC value 500 µg/mL towards *E. coli*. This activity is assumed to be related with the high percentage of aromadendrene and β-caryophyllene, which were the major components of *P. stylosum* and *P. ribesioides*, respectively.

Table 3
Antimicrobial activities of the leaves and stems oil of *P. stylosum* and *P. ribesioides* [a]

Microorganisms		PSLO	PSSO	PRLO	PRSO	SS	NY
Gram positive							
Bacillus cereus	**DD** [b]	11.2 ± 0.2	9.0 ± 0.2	13.3 ± 0.2	11.7 ± 0.2	18.8 ± 0.2	ND
	MIC[c]	125	125	62.5	62.5	7.8	ND
Bacillus subtilis	**DD**	7.5 ± 0.2	7.4 ± 0.1	7.2 ± 0.2	6.8 ± 0.1	18.5 ± 0.1	ND
	MIC	500	500	500	500	7.8	ND
Staphylococcus aureus	**DD**	14.7 ± 0.2	11.7 ± 0.2	13.2 ± 0.1	12.0 ± 0.2	17.3 ± 0.2	ND
	MIC	125	125	62.5	62.5	7.8	ND
Gram negative							
Escherichia coli	**DD**	6.5 ± 0.2	6.4 ± 0.1	9.0 ± 0.3	6.5 ± 0.2	17.4 ± 0.1	ND
	MIC	>1000	500	500	1000	7.8	ND
Pseudomonas aeruginosa	**DD**	6.7 ± 0.2	6.8 ± 0.2	6.8 ± 0.2	6.7 ± 0.2	18.0 ± 0.2	ND
	MIC	>1000	>1000	1000	>1000	7.8	ND
Klebsiella pneumoniae	**DD**	6.5 ± 0.2	6.5 ± 0.2	6.8 ± 0.2	6.8 ± 0.2	17.2 ± 0.2	ND
	MIC	>1000	1000	>1000	1000	7.8	ND
Fungus							
Candida albicans	**DD**	10.8 ± 0.1	6.6 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	ND	15.8 ± 0.2
	MIC	125	1000	1000	1000	ND	7.8
Candida neoformens	**DD**	9.2 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	6.8 ± 0.2	ND	16.5 ± 0.1
	MIC	125	1000	>1000	>1000	ND	7.8
Saccharomyces cerevisiae	**DD**	7.0 ± 0.2	6.5 ± 0.2	6.8 ± 0.1	6.8 ± 0.1	ND	16.8 ± 0.1
	MIC	1000	1000	1000	1000	ND	7.8

[a]Data represent mean±standard deviation of three independent experiments
[b]DD – disc diffusion (zone of inhibition including the diameter of disc - 6 mm)
[c]MIC - Mininum inhibitory concentration (µg/ml)
PSLO - *P. stylosum* leaves oil; PSSO - *P. stylosum* stems oil
PRLO - *P. ribesioides* leaves oil; PRSO - *P. ribesioides* stems oil
SS - streptomysin sulphate; NY - nystatin; ND - not determined

Studies on the antimicrobial activity of single aroma compounds found that aromadendrene inhibited Gram-positive bacteria, *S. aureus*, *S. epidermidis*, *S. agalactiae* and *S. pyogenes* (Mulyaningsih *et al.*, 2010), while β-caryophyllene was found active against the Gram-positive bacteria, *S. aureus* and *E. faecalis* (Schmidt *et al.*, 2010). Furthermore, the results of the bioassays showed the essential oils exhibited good antibacterial activity against all Gram-positive bacteria (Farag *et al*., 1989). According to previous studies, Gram-positive bacteria are more sensitive to essential oil than Gram-negative bacteria due to their outer membrane barriers (Tassou & Nychas, 1995). Gram-positive bacteria are more susceptible due to outer peptidoglycan layer which is not an effective permeability barrier while Gram-negative bacteria have outer phospholipids membranes (Burt, 2004).

CONCLUSION

The result demonstrated that aromadendrene, sabinene and β-caryophyllene were the most abundant components in *P. stylosum* oils, while *P. ribesioides* oils gave β-caryophyllene, camphene, and δ-cadinene as the mojor components. The essential oils showed strong antimicrobial activity; therefore it might well be used as an antimicrobial agent, as well as in food preservatives. However, further investigation should be carried out on their activities against other foodborne pathogens.

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