

# Antioxidant and Anti-tyrosinase Activities from *Piper officinarum* C.DC (Piperaceae)

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## ABSTRACT

The present study was carried out to evaluate antioxidant and anti-tyrosinase activities from *Piper officinarum* stems, as well as investigation of its chemical constituents. In a series of *in vitro* assays, antioxidant (DPPH radical scavenging and total phenolic content) and anti-tyrosinase (mushroom tyrosinase) activities of various extracts of stem were evaluated. The isolation and purification of the constituents were carried out on the extracts using various chromatographic methods and identified by direct comparison of their spectroscopic data with respective published data. The results showed that the methanol extracts showed the highest DPPH (80.0%) at 1 mg/ml, as well as total phenolic content (50.5%). Phytochemical analysis of the stem extracts have isolated five compounds identified as 4-allyl resorcinol (**1**), aristolactam AII (**2**), aristolactam BII (**3**), stigmast-4-en-3-one (**4**) and 6-hydroxystigmast-4-en-3-one (**5**). Compound (**1**), (**2**) and (**3**) showed significant activity towards DPPH radical scavenging (I%=17.3-28.1%), while (**2**), (**3**) and (**4**) demonstrated potent inhibitory effects against tyrosinase mushroom (I%=11.1-24.4%). The results showed that the stem extracts has significant antioxidant activity that may help to discover new chemical classes of natural antioxidant substances.

## INTRODUCTION

*Piper officinarum* C.DC is an endemic species, climber and grows in Southeast Asia. It is locally known as 'lada panjang' or 'kechundai' in Malaysian region. The plant has been used to treat fever, jaundice, rheumatism, neuralgia, boils, and bronchitis (Wiert, 2006). Previous phytochemical studies on *P. officinarum* have resulted in the isolation of a number of amide and lignan compounds, while the extracts have showed antibacterial, interceptive activity and also anti-atherosclerotic and hypolipidaemic agents (Gupta *et al.*, 1972, 1976a, 1976b, 1977; Sharma *et al.*, 1998; Mrutyunjaya *et al.*, 2001; Rao *et al.*, 2011). The antioxidant and antimicrobial activities of the leaf and stem oils from *P. officinarum* collected from Sarawak, Malaysia have been reported by us (Salleh *et al.*, 2012). The stem oil was found to have moderate lipid peroxidation inhibition against  $\beta$ -carotene-linoleic acid bleaching. Both oils were weakly active against the

tested microbes. Herein, we report the antioxidant and anti-tyrosinase activities of various stem extracts and also their phytochemicals which has not been reported thus far.

## MATERIAL AND METHODS

### Plant materials

Sample of *P. officinarum* was collected from Sarawak, Malaysia, in January 2010. This species was identified by Mohizar Mohamad from the Forest Research Centre, Kuching, Sarawak and the voucher specimen (UiTMKS3004) was deposited at Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

### General

Melting points were determined using hot-stage mp apparatus equipped with a microscope, Leica Galen III and were uncorrected. VLC was carried out on silica gel 230-400 mesh and CC on silica gel 70-230 mesh as stationary-phase eluting with increasing polarity of solvents. TLC analysis was done on pre-coated silica gel F<sub>254</sub> plates (0.2-mm thickness).

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Spots were visualised by UV and anisaldehyde-sulphuric acid spraying reagent. NMR spectra were recorded in  $\text{CDCl}_3$  and  $\text{CD}_3\text{OD}$  using a Bruker Avance 400 MHz NMR spectrometer (400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$ ). The IR spectra were recorded on Perkin Elmer series 1600 spectrophotometer as KBr disc or thin film on NaCl discs. The Ultraviolet (UV) spectra were recorded on Shimadzu UV 1601PC spectrophotometer. High resolution mass spectral data were obtained from UCL Spectrometry Service, London, UK.

### Extraction and isolation of compounds

Soxhlet extraction of the powdered stems (500 g) of *P. officinarum*, sequentially with *n*-hexane, EtOAc and MeOH yielded the crude *n*-hexane (POH: 6.9 g, 2.7%), EtOAc (POE: 4.6 g, 1.8%) and MeOH (POM: 6.5 g, 2.6%) extracts, respectively. The POH extract (6.0 g) was fractionated by VLC on silica gel 60 (230–400 mesh) and eluted with *n*-hexane and *n*-hexane: $\text{CHCl}_3$  to afford 5 fractions (POH1 - POH5). The entire fraction does not yield compounds after recombined and separated by CC. The POE extract (4.0 g) was fractionated by VLC on silica gel 60 (230–400 mesh) and eluted with *n*-hexane:EtOAc to afford 8 fractions (POE1 – POE8).

Fraction POE2 (1.22 g) was subjected to silica gel column chromatography eluted with *n*-hexane: $\text{CHCl}_3$  as mobile phase to afford five fractions. Fractions 4 (11–32) were combined to yield **(1)** (20.1 mg, 2.5%) as a light brown viscous liquid. The POM extract (6.0 g) was subjected to VLC on silica and eluted with *n*-hexane: $\text{CHCl}_3$ :EtOAc and finally acetone. Fractions with similar TLC profiles were combined to form six fractions (POM1 - POM6). Fraction POM2 - POM4 was subjected to column chromatography on silica gel and eluted with gradient solvent system of  $\text{CHCl}_3$ :EtOAc to yield five new fractions. Fractions 4 (47–86) were combined followed by purification using preparative TLC to yield **(2)** (8.2 mg, 1.6%) and **(3)** (5.1 mg, 0.9%) as a pale yellow amorphous solid. Fraction POM5 - POM6 were further subjected to column chromatography on silica gel and eluted with the gradient solvent system of *n*-hexane:EtOAc to yield four new fractions.

Fractions 3 (26–75) and 4 (80-95) were combined, followed by purification using preparative TLC and recrystallized using cold *n*-hexane to yield **(4)** (6.2 mg, 2.1%) and **(5)** (5.4 mg, 1.8%) as white solids, respectively.

### Antioxidant activity

#### DPPH radical scavenging

The free radical scavenging activity of the extracts/compounds was measured by the DPPH method with minor modification (Loo *et al.*, 2008). Each sample of stock solution (1.0 mg/L) was diluted to final concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.63 and 7.81  $\mu\text{g/mL}$ . Then, a total of 3.8 ml of 50  $\mu\text{M}$  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 follows;

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

where  $A_{\text{blank}}$  is the absorbance value of the control reaction (containing all reagents except the test compound) and  $A_{\text{sample}}$  is the absorbance value of the test compounds. The sample concentration that provides 50% inhibition ( $\text{IC}_{50}$ ) was calculated by plotting inhibition percentages against concentrations of the sample. Analyses were run in triplicate and the result was expressed as average values with standard error mean (SEM).

#### Total phenolic content (TPC)

Total phenolic contents of the extracts were determined by Folin–Ciocalteu colorimetric method with minor modification (Loo *et al.*, 2008). Sample of stock solution (1.0 mg/mL) was diluted in methanol to final concentrations of 1000, 800, 600, 400, and 200  $\mu\text{g/mL}$ . 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%  $\text{Na}_2\text{CO}_3$  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 extracts was expressed as mg of gallic acid equivalent per gram of sample. Analyses were run in triplicate and the result was expressed as average values with standard error mean (SEM).

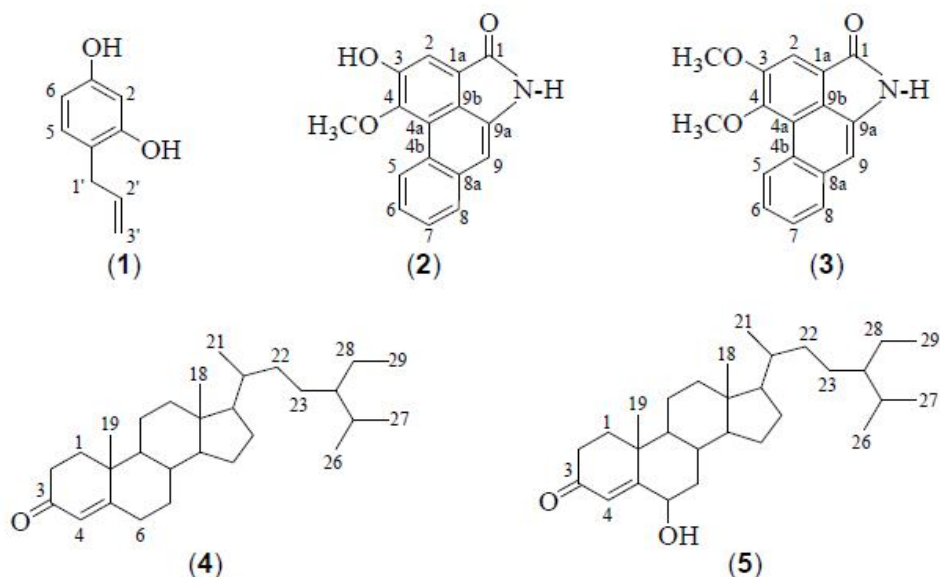
#### Anti-tyrosinase activity

Tyrosinase inhibition assay (EC1.14.18.1) was performed by using the dopachrome method with L-DOPA as substrate with slight modifications (Liu *et al.*, 2009). Briefly, the extracts/compounds and kojic acid (Sigma) 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  $\mu\text{L}$  of extracts dissolved in DMSO with 80  $\mu\text{L}$  of phosphate buffer (pH 6.8), 40  $\mu\text{L}$  of tyrosinase enzyme and 40  $\mu\text{L}$  of L-Dopa (Sigma) 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. The percentage of tyrosinase inhibition (I%) was calculated as follows;

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

where  $A_{\text{control}}$  is the absorbance of the control reaction and  $A_{\text{sample}}$  is the absorbance of the extracts/reference. Analyses were run in triplicate and the result was expressed as average values with standard error mean (SEM).



**Fig. 1:** Chemical constituents from stem extracts of *P. officinarum*.

### Statistical analysis

Data obtained from antioxidant and tyrosinase inhibition results were stated in mean  $\pm$ SD. Analysis of variance was performed by ANOVA procedures (SPSS 14.0 for Windows). Data were considered statistically different at  $p < 0.01$ .

### RESULTS

The current investigation was to evaluate the antioxidant and anti-tyrosinase activity as shown in **Table 1**. In addition, studies on the phytochemicals of the stem extracts of *P. officinarum* revealed that the presents of one phenylpropanids, two alkaloids, and two triterpenes.

They were identified as 4-allyl resorcinol (**1**), aristolactam AII (**2**), aristolactam BII (**3**), stigmast-4-en-3-one (**4**), and 6-hydroxystigmast-4-en-3-one (**5**). The identification of all compounds was achieved by physical properties: UV, IR,  $^1\text{H}$  and  $^{13}\text{C}$  NMR and 2D NMR and MS. These data were also confirmed by comparison with previously reported spectral data (Junior *et al.*, 1999; Zhao *et al.*, 2005; Ghosh and Bhattacharya, 2005; Zhao *et al.*, 2011). The chemical structures of the isolated compounds as in **Figure 1**.

#### 4-Allyl resorcinol (1)

Light brown viscous liquid (20.12 mg, 2.52%); m.p 67-68°C; IR (film)  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3517, 1637, 1611, 1463, 1267;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  3.29 (2H, d,  $J = 6.8$  Hz, H-1'), 5.07 (2H, m, H-3'), 5.15 (1H, s, 3-OH), 5.24 (1H, s, 1-OH), 5.90 (1H, m, H-2'), 6.62 (1H, dd,  $J = 8.0$  and 2.0 Hz, H-6), 6.72 (1H, d,  $J = 2.0$  Hz, H-2), 6.81 (1H, d,  $J = 8.0$  Hz, H-5);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  39.4 (C-1'), 115.3 (C-5), 115.5 (C-3'), 115.7 (C-6), 120.9 (C-2), 133.1 (C-4), 137.1 (C-2'), 141.7 (C-1), 143.5 (C-3); EIMS  $m/z$  (rel. int.): 150 [ $\text{M}^+$ ,  $\text{C}_9\text{H}_{10}\text{O}_2$ ] (100), 131 (26), 123 (38), 103 (36).

#### Aristolactam AII (2)

Pale yellow amorphous solid (8.2 mg, 1.6%); m.p 258-260°C; IR (KBr)  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3335, 2918, 1717, 1597, 1498;  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{COCD}_3$ ):  $\delta$  4.04 (3H, s, 4-OCH<sub>3</sub>), 7.20 (1H, s, H-9), 7.56 (2H, m, H-6, H-7), 7.77 (1H, s, H-2), 7.91 (1H, m, H-8), 9.35 (1H, m, H-5), 9.52 (1H, s, O-H), 9.65 (1H, s, N-H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{COCD}_3$ ):  $\delta$  56.8 (4-OCH<sub>3</sub>), 104.3 (C-9), 107.9 (C-2), 124.9 (C-1a, C-4a), 126.7 (C-7, C-8), 127.9 (C-5, C-6), 128.5 (C-4b, C-9b), 134.7 (C-8a), 135.3 (C-9a), 148.1 (C-4), 149.1 (C-3),  $\delta$  168.6 (C-1); EIMS  $m/z$  (rel. int.): 265 [ $\text{M}^+$ ,  $\text{C}_{16}\text{H}_{11}\text{NO}_3$ ] (100), 250 (32), 222 (17), 166 (34).

#### Aristolactam BII (3)

Pale yellow amorphous solid (5.1 mg, 0.9%); m.p 254-256°C; IR (KBr)  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3163, 3014, 2920, 1715, 1599, 1499;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  4.09 (3H, s, 4-OCH<sub>3</sub>), 4.14 (3H, s, 3-OCH<sub>3</sub>), 7.08 (1H, s, H-9), 7.55 (1H, m, H-6), 7.60 (1H, m, H-7), 7.72 (1H, m, H-8), 7.83 (1H, s, H-2), 9.25 (1H, m, H-5);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  60.3 (4-OCH<sub>3</sub>), 60.6 (3-OCH<sub>3</sub>), 105.7 (C-9), 114.3 (C-2), 121.0 (C-4a), 124.3 (C-1a), 126.1 (C-7, C-8), 127.7 (C-9b), 129.2 (C-5, C-6), 131.0 (C-4b), 132.3 (C-8a), 134.4 (C-9a), 146.5 (C-4), 157.9 (C-3),  $\delta$  173.9 (C-1); EIMS  $m/z$  (rel. int.): 279 [ $\text{M}^+$ ,  $\text{C}_{17}\text{H}_{13}\text{NO}_3$ ] (42), 151 (100), 149 (18), 127 (35), 113 (68), 111 (23), 107 (20), 93 (24), 88 (27).

#### Stigmast-4-en-3-one (4)

White solids (6.24 mg, 2.08%); m.p 75-77°C (lit. [8] 80-82°C); IR (KBr)  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 2960, 1687, 1460, 1414;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.72 (3H, s, H-18), 0.82 (3H, d,  $J = 6.4$  Hz, H-27), 0.84 (3H, d,  $J = 6.4$  Hz, H-26), 0.88 (3H, t,  $J = 7.6$  Hz, H-29), 0.92 (3H, d,  $J = 6.8$  Hz, H-21), 1.16 (3H, s, H-19), 1.22-2.46 (29H, m, CH<sub>2</sub> and CH overlapping), 5.73 (1H, s, H-4);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.27 (C-29), 14.62 (C-18), 15.98 (C-19), 17.06 (C-21), 18.42 (C-27), 20.85 (C-26), 23.02 (C-11), 24.38 (C-28),

26.27 (C-15), 28.17 (C-23), 29.52 (C-16), 31.96 (C-25), 33.86 (C-7), 35.21 (C-6), 36.29 (C-22), 38.73 (C-2), 39.82 (C-8), 42.52 (C-1), 43.61 (C-20), 44.96 (C-10), 46.05 (C-12), 48.21 (C-13), 49.30 (C-24), 52.01 (C-9), 53.90 (C-17), 56.07 (C-14), 123.73 (C-4), 171.47 (C-5), 198.83 (C-3); CIMS *m/z* (rel. int.): 413 [(M+1), C<sub>29</sub>H<sub>48</sub>O] (24), 411 (12), 399 (4), 152 (6), 151 (100).

### 6-Hydroxystigmast-4-en-3-one (5)

White solids (5.4 mg, 1.8%); m.p 201-204°C; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3337, 2938, 1668, 1464, 1384; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  0.79 (3H, s, H-18), 0.82 (3H, d, *J* = 6.0 Hz, H-27), 0.84 (3H, d, *J* = 6.0 Hz, H-26), 0.89 (3H, t, *J* = 7.6 Hz, H-29), 0.92 (3H, d, *J* = 8.8 Hz, H-21), 1.39 (3H, s, H-19), 1.52-2.54 (28H, m, CH<sub>2</sub> and CH overlapping), 4.36 (1H, s, H-6), 5.83 (1H, s, H-4); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  11.9 (C-29), 11.9 (C-18), 18.7 (C-21), 19.0 (C-27), 19.4 (C-19), 19.7 (C-26), 20.9 (C-11), 23.0 (C-28), 24.1 (C-15), 26.1 (C-23), 28.1 (C-16), 29.1 (C-25), 29.7 (C-8), 33.8 (C-22), 34.2 (C-2), 36.1 (C-20), 37.0 (C-1), 37.9 (C-10), 38.5 (C-7), 39.5 (C-12), 42.5 (C-13), 45.8 (C-24), 53.6 (C-9), 55.8 (C-14), 56.0 (C-17), 73.3 (C-6), 126.3 (C-4), 168.4 (C-5), 200.8 (C-3); CIMS *m/z* (rel. int.): 429 [(M-1), C<sub>29</sub>H<sub>48</sub>O<sub>2</sub>] (100), 411 (65), 391 (42), 375 (11), 151 (24).

## DISCUSSION

Many of these constituents were previously isolated from *Piper* genus. Compound (1), has been isolated previously from *P. betle*, compound (2) from *P. boehimeriaefolium*, *P. attenuatum*, *P. hamiltonii* and *P. longum*, while compound (3) from *P. argyrophyllum*, *P. attenuatum*, *P. boehimerzyolium*, *P. chiadoense*, *P. longum* and *P. wightii* (Desai *et al.*, 1988; Desai *et al.*, 1989; Parmar *et al.*, 1997; Ghosh and Bhattacharya, 2005). All these constituents were also reported from plants of the various families such as Menispermaceae, Papaveraceae and Aristolochiaceae. To the best of our knowledge, this is the first report on the chemical constituents from stem of *P. officinarum*.

The antioxidant activities (DPPH radical scavenging and phenolic content) and tyrosinase inhibition of various extracts (*n*-hexane, ethyl acetate and methanol) from the stems of *P. officinarum* are shown in **Table 1**.

The hydrogen atoms or electron donation ability of the corresponding extracts and pure compounds were measured from the bleaching of the purple colored DPPH. Results indicate that the methanol extracts showed the highest activity which gave inhibition of 80.0%, comparable to that of synthetic antioxidant, BHT (87.4%) at concentration, 1 mg/mL. The highest phenolic content was recorded from the methanol extracts (50.5%). The content of phenolic compounds could be used as an important indicator of antioxidant activity. Several reports have convincingly shown a close relationship between antioxidant activity and phenolic content (Yoon *et al.*, 2011). However, the results for the low value of total phenolic content for the current finding indicate the low levels of phenolic compound in the extracts.

**Table 1.** Antioxidant and anti-tyrosinase activities of *P. officinarum*<sup>a</sup>

Extracts	DPPH (I%) <sup>b</sup>	TPC (%) <sup>c</sup>	Tyrosinase (I%) <sup>d</sup>
<i>n</i> -Hex	18.9 ± 0.2	36.0 ± 0.2	2.2 ± 0.2
EtOAc	49.8 ± 0.2	33.2 ± 0.4	2.3 ± 0.4
MeOH	80.0 ± 0.4	50.5 ± 0.2	4.5 ± 0.4
Compounds			
(1)	17.3 ± 0.2	ND	NA
(2)	28.1 ± 0.3	ND	14.4 ± 0.2
(3)	25.4 ± 0.2	ND	24.4 ± 0.4
(4)	ND	ND	11.1 ± 0.2
(5)	ND	ND	NA
Standards			
BHT	87.4 ± 0.2	ND	ND
Kojic acid	ND	ND	81.8 ± 0.2

<sup>a</sup>Data represent mean ±SEM of three independent experiments. <sup>b</sup>Percentage inhibition at 1 mg/mL. <sup>c</sup>Gallic acid equivalent (mg GA/g). <sup>d</sup>Percentage inhibition at 0.1 mg/mL. NA-not active; ND-not determined; BHT-butylated hydroxytoluene.

Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. Therefore, these agents have good commercial potential in both food processing and cosmetic industries. Kojic acid was used as a positive control, as it has been reported to display the highest tyrosinase inhibition activity. At 0.1 mg/ml the tyrosinase inhibition of extracts ranges from 2.2 - 4.5%, which indicate that the extracts have weak activity compared to kojic acid.

The inhibition of tyrosinase ability might depend on the hydroxyl groups of the phenolic compounds of the mushroom extracts that could form a hydrogen bond to active site of the enzyme, leading to a lower enzymatic activity. Some tyrosinase inhibitors act through hydroxyl groups that bind to the active site on tyrosinase, resulting in steric hindrance or change conformation. Phenolic compounds proved to be effective inhibitors of tyrosinase activity as reported before (Baek *et al.*, 2008). However, compound (2), (3) and (4) showed potent inhibitory activity but still lower than that of kojic acid, while compound (1) and (5) were inactive against mushroom tyrosinase.

Previous studies on the other *Piper* species; *P. nigrum* and *P. longum* showed that both species have high tyrosinase inhibition activity (Khanom *et al.*, 2000; Mukherjee *et al.*, 2001). Piperlonguminine from *P. longum* was discovered to inhibit melanin production in melanoma B16 cells stimulated with  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH), 3-isobutyl-1-methylxanthine or protoporphyrin IX, with stronger depigmenting efficacy than kojic acid (Kyeong-Soo *et al.*, 2005). In addition, 2',6',4-trihydroxy-4'-methoxydihydrochalcone (asebogenin), a dihydrochalcone from *P. elongatum* displayed the strongest tyrosinase activity among the test compounds, and had almost the same activity to that of kojic acid (Masuoka *et al.*, 2003).

## CONCLUSIONS

Based on our findings, it can be concluded that *P. officinarum* stems methanol extract possess a significant antioxidant and anti-tyrosinase activity. These results also suggest

that the extracts could serve as potential source of bioactive compounds. Further research is needed in which the extract could possibly be exploited for pharmaceutical use.

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