ANTIOXIDANT AND ANTITYROSINASE ACTIVITIES OF PIPER MAINGAYI HK. ESSENTIAL OILS

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Graphical abstract

Abstract

The study was aimed to investigate the chemical components of stem and fruit volatile oils of Piper maingayi HK. with their antioxidant and antityrosinase activities. GC and GC-MS analysis of the essential oils obtained from the fresh stem and fruit of P. maingayi HK. resulted in the identification of 34 and 18 components accounting for 83.6% and 78.7% of the total amount, respectively. Sesquiterpene hydrocarbons and oxygenated sesquiterpenes were the most highly represented classes as the former ranging from 64.7% to 70.7%, and the latter varying from 7.4% to 8.6%. The main constituents of the stem oil were: β-caryophyllene (26.2%), α-cedrene (8.4%), caryophyllene oxide (6.7%) and cis-calamenene (6.2%), while the fruit oil was dominated by δ-cadinene (22.6%), β-caryophyllene (18.8%), α-copaene (11.2%) and α-cadinol (7.1%). The stem and fruit of P. maingayi oils showed significant value for antioxidant and antityrosinase activities.

Keywords: Chemical composition, essential oil, piper maingayi, antioxidant, antityrosinase

Abstrak

Kajian ini bertujuan untuk mengkaji komponen kimia daripada minyak pati batang dan buah Piper. maingayi HK. serta aktiviti antioksidan dan antitirosinas. Analisis GC dan GC-MS minyak pati yang diperoleh daripada batang dan buah segar P. maingayi HK. menghasilkan pengecaman 34 dan 18 komponen menyumbang kepada masing-masing 83.6% dan 78.7% daripada jumlah keseluruhan. Hidrokarbon seskuiterpena dan seskuiterpena teroksidasi mewakili kelas tertinggi dengan yang pertama menyumbang diantara 64.7% hingga 70.7%, dan keduanya daripada 7.4% hingga 8.6%. Kandungan utama minyak pati dahan ialah: β-kariofilena (26.2%), α-sedrena (8.4%), kariofilena oksida (6.7%) dan cis-kalamenena (6.2%), manakala minyak buah dominasi oleh δ-kadinena (22.6%), β-kariofilena (18.8%), α-kopaena (11.2%) dan α-cadinol (7.1%). Minyak pati batang dan buah P. maingayi menunjukkan nilai yang signifikan untuk aktiviti antioksidan dan antitirosinas.

Kata kunci: Komposisi kimia, minyak pati, piper maingayi, antioksidan, antitirosinase

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1.0 INTRODUCTION

Oxidant and free radicals participate as both toxic and beneficial to the body [1]. However, excess of free radical production might play a role in immense diseases. Current life style and environment accumulate an over production of ROS and free radicals, such as super oxide anion radical (O2−•), hydrogen peroxide (H2O2) and hydroxyl radical (OH•) which introduced into the body as by products in normal metabolic functions. These molecules/radicals are immense factors in cellular injury such as cardiovascular diseases, cancer and others which accounted for a major portion of deaths today [2, 3]. The significant contribution as natural antioxidant by medicinal plants has prompted research interest. In distinction, Piper species is one of the medicinal plants used as folk’s medicine worldwide.

Previous phytochemical investigations of Piper maingayi Hk. by our group have led to the isolation of two alkenylamides [4]. Our first study in the leaf oil of this species found β-caryophyllene as the main constituent present in 39.6% of the total components [5]. To the best of our knowledge, there has been no report in the essential oils of stem and fruit of P. maingayi. Hence, in the present work, the detailed chemical compositions of stem and fruit oils of P. maingayi and their antioxidant and antityrosinase potentials are discussed.

2.0 EXPERIMENTAL

2.1 Plant Materials

Fresh stems and fruits of P. maingayi Hk. were collected in January 2014 from Hutan Simpan Fraser, Fraser Hill, Perak, and identified by Dr. Shamsul Khamis. The voucher specimen SK2329/14 was deposited at Herbarium of Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor.

2.2 Extraction of Essential Oils

Approximately, each of the fresh samples of P. maingayi Hk. was cleaned and chopped into small pieces. Samples were loaded into a round-bottomed flask (5 L) containing 2 L distilled water which covered the entire samples. The flask was connected to a Clevenger’s distillation unit. The sample was then hydrodistilled for 6 hrs. The essential oil, which was collected in the side arm, was extracted with diethyl ether (Et2O) (3 x 10 mL) and dried over anhydrous magnesium sulfate (MgSO4). The extracted essential oil was placed in a glass amber bottle and refrigerated 4°C until further analysis. The composition of the essential oils were analysed using GC and GC-MS in Ultra 1 column. Identification of the constituents was carried out by comparison of the mass spectra from the Wiley Library in the GC-MS and Kovat indices [6]. The KI was calculated by using the equation below:

\[ KI = 100 \left( \frac{\log t_{sample}}{\log t_{x+1}} - \frac{\log t_{s}}{\log t_{x+1}} \right) + 100x \]

where, \( t_{sample} \) = retention time of the sample component; \( t_{s} \) = retention time of the saturated hydrocarbons elute before the sample component; \( t_{x+1} \) = retention time of the saturated hydrocarbon which contains x+1 carbon and that just elutes after the sample component; \( x \) = number of carbon atoms in saturated hydrocarbons

2.3 Gas Chromatography (GC)

GC analysis was carried out using an Agilent 6890 series II. A gas chromatograph analysis was equipped with flame ionization detector (25.0 m long x 200 μm i.d. film thickness x 0.11 μm) with non-polar Ultra-1 fused equipped silica capillary column (25 m long, 0.33 mm thickness and 0.20 μm inner diameter). The samples were injected by splitter using Helium as the carrier gas at a flow rate of 1.5 mL/min and 30 psi inlet pressure; split ratio 1:20. The column oven was programmed from 40°C for 5 min to 300°C at a rate of 4°C/min and the final temperature was kept isothermal for 10 min. Injector and detector temperatures were set at 300°C and 310°C. Samples were diluted using (1/100, diethyl ether v/v) of 1.0 μL were injected manually (split ratio 1:50). Calculation of peak area percentage was carried out using GC HP Chemstation software (Agilent Technologies).

2.4 Gas Chromatography-Mass Spectrometry (GCMS)

The qualitative GC-MS analysis was carried out an Agilent Technologies Model 6890N gas chromatography and an Agilent Technologies 5973i mass spectrometer. The GC equipped with Ultra-1 column (25 m long, 0.33 μm thickness and 0.20 mm inner diameter), Helium was used as the carrier gas at flow rate 1 mL/min. Injector temperature was set at 250°C. Oven temperature was programmed from 50°C (5 min hold) to 300°C at 5°C/ min and finally held isothermally for 10 min. As 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.

2.5 Identification of Components

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 (KI) with reference to homologues series of C9-C24 n-alkanes, as well as matching with those of authentic compounds or data in the literature [6]. The quantitative data were
obtained electronically from FID area percentage without the use of correction factor.

2.6 β-Carotene/Linoleic Acid Discoloration Assay

The β-carotene/linoleic acid discoloration assay described by Miraliabakri and Shahidi [7] was used, with minor modifications. A mixture of β-carotene and linoleic acid was prepared by adding together of 0.5 mg β-carotene in 1 mL CHCl₃ (HPLC grade), 25 µL linoleic acid and 200 mg Tween 40. The CHCl₃ was then completely evaporated under vacuum and 100 mL of oxygenated distilled water was subsequently added to the residue and mixed gently to form a clear yellowish emulsion. The samples (2 g/L) were prepared in MeOH and 350 µL of each sample solution were added to 2.5 mL of the above mixture in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50°C for 2 h together with two blanks, one contained positive control (BHT and BHA) and the other contained the same volume of MeOH. The absorbance was measured at 470 nm on an ultraviolet-visible (UV-Vis) spectrometer. Antioxidant activities (inhibition percentages, %) of the samples were calculated using the following equation:

\[ I\% = \left( \frac{A_{\beta\text{-carotene after 2 h}}}{A_{\text{initial } \beta\text{-carotene}}} \right) \times 100 \]

Where \( A_{\beta\text{-carotene after 2 h}} \) is the absorbance value of β-carotene after 2 h assay remaining in the samples and \( A_{\text{initial } \beta\text{-carotene}} \) is the absorbance value of β-carotene at the beginning of the experiment. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.6 Total Phenolic Content Assay

The total phenolic constituents of the plant extracts were determined by literature methods involving gallic acid (mg GA/g) equivalents per gram, through the calibration curve with gallic acid [8]. Sample of stock solution of the extracts (1000 µg/mL) was diluted in MeOH to get the final concentration of 100, 800, 600, 400 and 200 µg/mL. Then, 1.0 mL aliquot of sample containing 1000 µg of extract was pipetted into a test tube containing 0.9 mL of MeOH, followed by 0.05 mL Folin-Ciocalteu reagent and the flask was vortexed for 15 sec. After 3 min, 0.5 mL of 5% Na₂CO₃ solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Then, 2.5 mL of MeOH 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 (1000, 800, 600, 400, and 200 µg/mL) and a standard curve obtained with the following equation:

\[ \text{Absorbance} = 0.0021x - 0.0023, \quad r^2 = 0.9840 \]

The concentration of total phenolic compounds in the extracts was expressed as µg of gallic acid equivalent per milligram of sample. The test was carried out in triplicate and gallic acid equivalent value was reported as mean ± SD of triplicate.

2.7 ABTS Assay

The antioxidant potential of essential oils of P. maingayi species was also evaluated using 2, 2'-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) [9]. The assay is based on the capacity of antioxidants to scavenge ABTS radical cation causing a reduction in absorbance at 734 nm. Briefly, ABTS 7 mM and potassium persulphate (K₂S₂O₄) 2.45 mM solutions were prepared and mixed. The resultant mixture was stored in dark at room temperature for 12-16 h to get dark colored solution containing ABTS radical cation.

Prior to use, ABTS radical cation solution was diluted with phosphate buffer (0.01 M) pH 7.4. The stock solution of sample was prepared at 1000 µg/mL. The stock solutions were diluted to give a series of concentration at 200, 150, 100, 50 and 25 µg/mL of sample. Radical scavenging ability of the fractions was analyzed by mixing the test sample with ABTS solution and measured at absorbance of 734 nm. The reduction in absorbance was measured spectrophotometrically after mixing the solutions for 30 min. Ascorbic acid was used as positive control. The assay was repeated in triplicate and percentage inhibition was calculated using formula:

% Inhibition = \( \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100 \)

Where \( A_{\text{blank}} \) absorbance of the blank sample, and \( A_{\text{sample}} \) absorbance of the plant extract. The antioxidant effect was expressed in terms of percent inhibition and as IC₅₀ (sample concentration required for 50% reduction of ABTS radicals). The sample concentration that provides 50% inhibition (IC₅₀) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC₅₀ values were reported as logarithmic values of triplicate.

2.8 DPPH Free Radical Scavenging Assay:

The free radical scavenging activity was measured by the DPPH method as described by Miller et al., [10] with minor modifications. Each sample of stock solution (1.0 mg/L) was diluted to final concentration of 1000, 500, 250, 125, 62.5, 31.3 and 15.63 µg/mL. Then, a total of 3.8 mL of 50 µM DPPH in MeOH solution was added to 0.2 mL of each sample solution and allowed to react at room temperature for 30 min in dark. 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 were calculated as follow:

\[
\% \text{ Inhibition} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100
\]

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 values of the test compounds. 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 logarithmic values of triplicate.

2.9 Antilysofase Assay

Tyrosinase inhibitory activity was assayed using the modified dopachrome method with slight modifications [11]. The concentration of sample used was 1000 \(\mu\)g/mL diluted in 20mM phosphate buffer solution with pH 6.8 (PBS). L-DOPA was used as the substrate and mushroom tyrosinase as the enzyme. The assays were conducted in 96-well microtiter plate, and Epoch Biotek reader was used to measure the absorbance at 475 nm. Mushroom tyrosinase enzyme (MTE) was prepared from stock solution to 480 units/mL in PBS. For each concentration of the sample solution, four wells designated A, B, C and D each contained a reaction mixture (180 \(\mu\)L) as follows: (A) 20 \(\mu\)L of MTE; (B) 140 \(\mu\)L of PBS, and 20 \(\mu\)L of methanol; (C) 20 \(\mu\)L of MTE, 140 \(\mu\)L of 20 mM PBS and 20 \(\mu\)L of sample solution; (D) 160 \(\mu\)L of PBS and 20 \(\mu\)L of sample solution.

Each well was mixed and incubated at 25°C for 10 min. Then, 20 \(\mu\)L of 0.85 mM L-DOPA (Sigma) in PBS was added. After incubation at 25°C for 20 min, the amount of dopachrome in each reaction mixture was measured as the difference of the optical density before and after incubation. The percent inhibition of tyrosinase activity was calculated using the equation below:

\[
\% \text{ Inhibition} = \frac{(A-B)-(C-D)}{(A-B)} 
\]

Where: A represents the difference of optical density before and after incubation without test sample; B represents the difference of optical density before and after incubation without test sample and enzyme; C represents the difference of optical density before and after incubation with test sample; D represents the difference of optical density before and after incubation with test sample but without enzyme. Kojic acid (Sigma) was used as positive standard. Test was carried out in triplicate and reported as mean ± SD of triplicate.

2.10 Statistical Analysis

Data obtained from essential oil analysis and antioxidants are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA (p > 0.05). A statistical package (SPSS version 11.0) was used for the data analysis.

3.0 RESULTS AND DISCUSSION

Hydrodistillation of the fresh stem and fruit of \(P.\) maingayi Hk. has yielded 0.09% and 0.17% (w/w) as colourless oils with light pungent odour. The chemical compositions of these oils are listed in Table 1. The GC and GC-MS analysis revealed 34 and 18 components respectively accounting for 83.6% and 78.7% of the total oils. Sesquiterpene hydrocarbons were the major components in the stem (64.7%) and fruit (70.7%) oils, with \(\beta\)-caryophyllene (26.2%) and \(\gamma\)-erythronene (18.8%) being the most substantial component in both oils. \(\beta\)-Caryophyllene has also been reported to be the significant constituent in aerials part of \(P.\) dilatatum L.C. Rich (15.5%) [12] and in fruits of \(P.\) tuberculatum Jacq. (17.7%) [13].

Other major constituents from the stem were \(\alpha\)-cedrene (8.4%), \(\alpha\)-copaene (6.2%) and of \(\delta\)-cadinene (5.2%) while the fruit oil composed of \(\alpha\)-cadinene (22.6%) and \(\beta\)-copaene (11.2%). \(\delta\)-Cadinene was found to be the major constituent in the aerials part of \(P.\) capense L.f. (16.8%) [14], in rhizomes of \(P.\) betel L. (11.7%) [15] and \(P.\) auctifolium Ruiz. and Pav., (6.8%) [16]. All oils displayed similar occurrence of constituents such as linalool, \(\alpha\)-cubebene, \(\alpha\)-copaene, \(\alpha\)-humulene, \(\beta\)-selinene, \(\alpha\)-selinene, germacrene D, \(\alpha\)-calamene, \(\delta\)-cadinene and \(\alpha\)-cadinol. However, few monoterpane hydrocarbons such as \(\alpha\)-pinene, sabinene, \(\beta\)-pinene, \(\alpha\)-phellandrene, \(\alpha\)-cyrene, camphor, terpinen-4-ol, \(\gamma\)-muurolene, and \(E\)-nerolidol were absent in the fruit oil but present in the stem oil.

The antioxidant activity of each essential oil was evaluated by \(\beta\)-carotene/linoleic acid discoloration and total phenolic content (TPC) assays, together with the DPPH free radical scavenging activity and ABTS radical assays. The results are given in Table 2. The \(\beta\)-carotene/ linoleic acid discoloration assay activity of the antioxidant activity at 1000 \(\mu\)g/mL, revealed a significant activity for both stem (91.8 ± 2.1%) and fruit (83.6 ± 1.7%) oils. The TPC which was measured as percentage of mg gallic acid (GAE) equivalents was expressed using the standard curve equation; \(y = 0.0021x + 0.0023\). The stem and fruit oils displayed a significant activity at 279.6 and 176.8 mg of GAE/g extract.

The antioxidant activities of polyphenols were attributed to their redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers as well as to their metal chelating abilities [17]. Therefore, the preliminary screening of \(\beta\)-carotene and TPC of \(P.\) maingayi Hk. were augmented with ABTS and DPPH assays.
The stem and fruit oils were found to be effective scavengers of the ABTS radical with an IC₅₀ values at 12.6 and 13.9 μg/mL compared to the standard BHT (IC₅₀ 9.4 μg/mL). Both stem and fruit oils displayed significant radical scavengers in DPPH assay with IC₅₀ 14.9 and 20.8 μg/mL respectively. The tyrosinase inhibition activity was found to be significant for stem oil (65.5 ± 0.2%), but lower than the positive control, kojic acid (91.3 ± 0.1%). Kojic acid was used as a standard in this study as it is an effective tyrosinase inhibitor. Previous studies mentioned that aromatic or aliphatic aldehydes such as anisaldehyde, cuminaldehyde and (2E)-alkenal were reported to possess as tyrosinase inhibitors [18]. P. maingayi essential oils showed moderate anti-tyrosinase activity due to lack of phenolic compounds which can cause chelating with copper metal of the enzyme. The major constituents from stem and fruits oils may contribute to both antioxidant and anti-tyrosinase activities. However, it is complex to ascribe the antioxidant and anti-tyrosinase capacities of whole P. maingayi essential oils to only some principal constituents, due to the fact that essential oils were composed with variety of constituents. Thus, the results of antioxidant activities may suggest the presence of synergistic effect among various compositions in the essential oils contributed mainly by the dominant constituents.

### 4.0 CONCLUSION

In conclusion, the GC and GC-MS analysis of the essential oils obtained from fresh stems and fruits of P. maingayi Hk resulted in the identification of 34 and 18 components accounting for 83.6% and 78.7% of the total amount, respectively. Sesquiterpenes hydrocarbons and oxygenated sesquiterpenes were the most highly represented classes with the former ranging from 64.7% to 70.7%, and the latter varying from 7.4% to 8.6%. The main constituents of stems essential oil were: β-caryophyllene (26.2%), α-cedrene (8.4%), caryophyllene oxide (6.7%) and cis-calamenene (6.2%), while the fruits essential oil was dominated by δ-cadinene (22.6%), β-caryophyllene (18.8%), α-copaene (11.2%) and α-cadinol (7.1%). The antioxidant activity of each essential oil was evaluated by β-carotene/linoleic acid discoloration and total phenolic content (TPC) assays, together with the DPPH free radical scavenging activity and ABTS radical assays. The stem and fruit oils were found to be effective scavengers of the ABTS radical with an IC₅₀ values at 12.6 and 13.9 μg/mL and DPPH assay with IC₅₀ 14.9 and 20.8 μg/mL respectively. The tyrosinase inhibition activity was found to be significant for stem oil (65.5 ± 0.2%), but lower than the positive control, kojic acid (91.3 ± 0.1%).
Table 2 Antioxidant activity by β-carotene/linoleic acid discoloration, TPC, DPPH and ABTS and antityrosinase assays

<table>
<thead>
<tr>
<th>Samples</th>
<th>β-carotene/linoleic acid discoloration (%)</th>
<th>TPC (mg GAE/g)</th>
<th>DPPH IC₅₀ (µg/mL)</th>
<th>ABTS IC₅₀ (µg/mL)</th>
<th>Mushroom tyrosinase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EO Stem</td>
<td>91.8 ± 2.1</td>
<td>176.8 ± 0.3</td>
<td>14.9</td>
<td>12.6</td>
<td>65.5 ± 0.2</td>
</tr>
<tr>
<td>Fruit</td>
<td>83.6 ± 1.7</td>
<td>279.6 ± 0.8</td>
<td>20.8</td>
<td>13.9</td>
<td>57.5 ± 0.4</td>
</tr>
<tr>
<td>BHT</td>
<td>95.2 ± 0.6</td>
<td>ND</td>
<td>18.5</td>
<td>9.4</td>
<td>ND</td>
</tr>
<tr>
<td>BHA</td>
<td>90.2 ± 0.3</td>
<td>ND</td>
<td>20.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AA</td>
<td>ND</td>
<td>ND</td>
<td>15.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kojic Acid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>91.3 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean ± SD of three independent experiments performed in triplicate; AA=Ascorbic acid; BHT=Butylatedhydroxytoluene; BHA=Butylatedhydroxyanisole; EO=Essential Oils

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