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Antioxidant and Anticholinesterase Activities of Essential Oils of *Cinnamomum griffithii* and *C. macrocarpum*

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The essential oils of *Cinnamomum griffithii* and *C. macrocarpum* were analyzed by GC and GC–MS and evaluated for their antioxidant and anticholinesterase activities. The essential oils of leaf and bark of *C. griffithii* were characterized by the presence of 30 components, with methyl eugenol (38.5-43.8%) as the major component. A total of 11 components were characterized in the leaf and bark of *C. macrocarpum* essential oil with the most abundant component was safrole (54.5-59.5%). The bark oil of *C. griffithii* demonstrated significant activity on DPPH (IC_{50} 73.4 µg/mL) and a high phenolic content (192.0%), while the leaf oil inhibited oxidation of β-carotene/linoleic acid with an inhibition value of 65.5 µg/mL. Acetylcholinesterase and butyrylcholinesterase inhibition were assessed and the results showed that *C. macrocarpum* bark oil exhibited significant activity with inhibition values of 55.8% and 66.1%, respectively at a concentration of 1 mg/mL.

Keywords: Antioxidant, Anticholinesterase, Essential oil, *Cinnamomum griffithi*, *Cinnamomum macrocarpum*.

The genus *Cinnamomum* contains about 350 species distributed in tropical and subtropical regions of America, South East Asia and Australia. Approximately 21 species of this genus have been recognized in Peninsular Malaysia. A number of these species are used in traditional medicine and as spices, especially in food, fragrances, fumigants, and traditional medicines [1]. The genus *Cinnamomum* commercially is known as cinnamon, and is considered as one of the oldest spices in the world. Cinnamon oil is widely used in the food processing, cosmetic, flavoring, confectionary and pharmaceutical industries, to treat inflammatory diseases and antifungal diseases [2]. The antioxidant and antimicrobial activities of some C*innamomum* species have been extensively investigated and reported [3-9]. Hence, in the present study, work has been carried out to explore the possible antioxidant properties by different methods, together with anticholinesterase activities of the essential oils of *C. griffithii* Meisn. and *C. macrocarpum* Hook.f..

The essential oils of *C. griffithii* and *C. macrocarpum* were obtained by hydrodistillation and their compositions were identified by GC and GC-MS analyses. Table 1 shows the chemical compositions identified in the leaf and bark oils of both species. Thirty components, constituting 91.8% (leaf) and 78.9% (bark) of the essential oil composition of *C. griffithii*, and eleven components, constituting 81.3% (leaf) and 80.0% (bark) of the essential oil composition of *C. macrocarpum* were determined. The main constituents of the essential oil of both species were identified as methyl eugenol (leaf 38.5%; bark 43.8%) and safrole (leaf 59.5%; bark 54.5%), respectively. Safrole, the major component of *C. macrocarpum* oil in this study was in accordance with the previous report by Sandigawad and Patil [10]. They had succesfully found safrole (43%) and camphor (41%) as their major components from the leaf oil of *C. macrocarpum*, collected from India. The chemical compositions of the essential oil of various *Cinnamomum* species have been widely investigated. Methyl eugenol, popularly used as an anti-allergic and anti-nociceptive agent [11], was also indicated as the major component of *C. subavenium* (Taiwan; leaf 75.9%) [12], *C. cordatum* (Malaysia; bark 92.1%) [13] and *C. riparium* (India; leaf 62.7%) [14]. Meanwhile, safrole known as a mutagenic and carcinogenic agent [15] has been found previously in the essential oils of *C. rhyncophyllum* (Malaysia; bark 41.5%) [2], *C. longepaniculatum* (China; leaf 90.0%) [16] and *C. petrophilum* (China; leaf 92.9-94.3%) [17]. Chemical differences in the oil composition of plant species in relation to their geographical origins and harvesting season have been reported. Chemical and biological diversity of aromatic and medicinal plants depend on such factors as cultivation area, climatic conditions, vegetation phase and genetic modifications. In fact, these factors influence the plant's biosynthetic pathways and consequently, the relative proportion of the main characteristic compounds [18].

The results of antioxidant activity are given in Table 2. Based on the results, the bark oil from *C. griffithii* displayed the highest activity for DPPH radical scavenging and phenolic content with IC_{50} of 73.4 µg/mL and 192.0 mg GA/g. In the β-carotene/linoleic acid bleaching test, the highest inhibition was produced by the leaf oil of *C. griffithii* (65.5%), followed by the leaf oil of *C. macrocarpum* (60.6%). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities were investigated by using Ellman's method and the results are given in Table 3. The bark oils of *C. macrocarpum* exhibited the highest activity for AChE and BChE inhibition with values of 55.8% and 66.1%, respectively at a concentration of 1 mg/mL. In addition, the bark oil of *C. griffithii* also showed significant activity against AChE (52.2%) and BChE (63.2%). Galanthamine was used as a standard, which exhibited 95.9% inhibition of AChE and 88.7% inhibition of BChE at the same concentration of 1 mg/mL. The level of antioxidant and anticholinesterase activities of these essential oils were probably related to their richness in phenylpropanoid components, which are methyl eugenol and safrole. However, the synergistic effect of more than one individual oil compound could be responsible for their activity [19-20].

Table 1: Constituents identified in the essential oils of *C. griffthii* and *C. macrocarpum*.

Components	KI[a]	Percentage (%)			
		CGL	CGB	CML	CMB
β-Pinene	974	1.2	-	-	-
α-Terpinene	1014	1.4	-	-	-
Limonene	1019	1.6	-	-	-
p-Cymene	1020	4.9	-	-	-
Camphor	1142	-	-	1.6	-
Borneol	1165	2.6	-	-	-
α-Terpineol	1171	1.1	-	-	-
Safrole	1285	6.4	7.0	59.5	54.5
α-Cubebene	1345	3.9	1.0	-	-
α-Ylangene	1372	0.6	-	-	-
α-Copaene	1374	-	-	-	1.1
β-Patchoulene	1379	0.6	2.7	-	-
Methyl eugenol	1403	38.5	43.8	11.1	12.0
Longifolene	1407	-	-	-	2.1
γ-Selinene	1438	1.1	1.7	-	-
Aromadendrene	1440	-	4.5	-	5.2
Dehydroaromadendrene	1460	-	1.2	-	-
β-Cadinene	1473	-	2.1	-	-
γ-Gurjunene	1475	0.7	1.5	3.9	-
Germacrene D	1485	2.6	-	-	-
β-Selinene	1490	2.9	-	-	-
δ -Selinene	1492	1.8	1.1	-	-
Valencene	1496	3.6	-	-	-
α-Selinene	1498	2.1	-	-	-
Epizonarene	1501	-	3.8	-	-
δ-Cadinene	1520	1.7	2.8	-	-
cis-Calamenene	1528	2.8	1.2	1.6	-
α-Cadinene	1537	1.4	-	-	-
α-Calacorene	1545	-	-	-	2.0
Selina-3,7(11)-diene	1545	-	3.2	-	3.1
Elemicin	1555	-	1.3	-	-
Germacrene B	1560	1.4	-	3.6	-
Spathulenol	1578	2.1	-	-	-
Caryophyllene oxide	1582	4.8	-	-	-
Group components					
Phenylpropanoids		44.9	52.1	70.6	66.5
Monoterpene hydrocarbons		9.1	-	-	-
Oxygenated monoterpenes		3.7	-	1.6	-
Sesquiterpene hydrocarbons		27.2	26.8	9.1	13.5
Oxygenated sesquiterpenes		6.9	-	-	-
Identified Components (%)		91.8	78.9	81.3	80.0

[a]Kovat's indices (KI) experimental; CGLO - *C. griffthii* leaf oil; CGBO - *C. griffthii* bark oil; CMLO - *C. macrocarpum* leaf oil; CMBO - *C. macrocarpum* bark oil

Table 2: Antioxidant activity of the essential oils of *C. griffthii* and *C. macrocarpum*.

Essential oils	Part	β-Carotene (I%)[a]	DPPH IC_{50} (μg/mL)[b]	TPC (mg GA/g)[c]
C. griffithii	Leaf	65.5 ± 0.2	82.4	181.7 ± 0.2
	Bark	55.9 ± 0.3	73.4	192.0 ± 0.2
C. macrocarpum	Leaf	60.6 ± 0.3	99.3	141.5 ± 0.2
	Bark	52.4 ± 0.4	109.3	149.5 ± 0.3
BHT		125.5 ± 0.3	42.5	-

[a]Percentage inhibition at a concentration of 2 g/L; [b]IC_{50} value at the concentrations of 1000-7.8μg/mL; [c]TPC at a concentration of 1 mg/mL

Table 3: Anticholinesterase activities of the essential oils of *C. griffthii* and *C.macrocarpum*.

Essential oils	Part	Cholinesterase inhibition (I%)[a]	
		AChE	BChE
C. griffithii	Leaf	42.5 ± 0.2	50.4 ± 0.3
	Bark	52.2 ± 0.3	63.2 ± 0.3
C. macrocarpum	Leaf	25.8 ± 0.1	36.3 ± 0.2
	Bark	55.8 ± 0.3	66.1 ± 0.3
Galantamine		95.9 ± 0.2	88.7 ± 0.2

[a]Percentage inhibition at a concentration of 1 mg/mL

This study evidenced the richness in methyl eugenol and safrole of *C. griffithii* and *C. macrocarpum*, respectively, which exhibited considerable antioxidant and anticholinesterase activities. These two species might be good natural products for further investigation into the development of new antioxidants and anticholinesterase agents, so they could be used as natural additives in food, cosmetic and pharmaceutical industries. Thus, further phytochemical and biological studies could be carried out to identify their active constituents.

Experimental

Plant materials: *C. griffithii* Meisn. and *C. macrocarpum* Hook.f. were collected from Bau and Lundu, Sarawak and identified by Mrs Mohizar Mohamad. The voucher specimens (UiTMKS/C02&C04) were deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

Solvents and chemicals**:** *Antioxidant:* β-Carotene, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Germany). Folin-Ciocalteu's reagent, anhydrous sodium sulfate, sodium carbonate and polyoxyethylenesorbitanmonopalmitate (Tween-40) were purchased from Merck (Germany). *Anticholinesterases*: Acetylcholinesterase (Type-VI-S, EC3.1.1.7), butyrylcholinesterase (EC3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithio-bis(2-nitrobenzoic)acid (DTNB) and galantamine were obtained from Sigma-Aldrich (Germany).

Extraction of essential oils: The fresh leaves and bark from each species were subjected to hydrodistillation in an all glass Dean-Stark apparatus for 8 h. The oils obtained were dried over anhydrous magnesium sulfate and stored at 4-6°C. The oil yields (w/w) were calculated based on a fresh weight-basis.

Gas chromatography (GC): GC analysis was performed on a Hewlett Packard 6890 series II A gas chromatograph equipped with an Ultra-1 column (100% polymethylsiloxanes) (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 temperatures were set at 250°C 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 3 times and the peak area percentages were reported as means ±SD of triplicates. Calculation of peak area percentage was carried out by using 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 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 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 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 components: The chemical components of the essential oils were identified by comparing their MS with the reference spectra in the computer library (Wiley) and also by comparing their retention indices and Kovat's index with the literature data [21]. The quantitative data were obtained electronically from FID area percentage without the use of correction factor.

Antioxidant activity - β-Carotene/linoleic acid bleaching: The β-carotene-linoleic acid bleaching assay was used, as described previously, with minor modifications [22]. A mixture of β-carotene and linoleic acid was prepared by adding together 0.5 mg

β-carotene in 1 mL chloroform, 25 µL linoleic acid and 200 mg Tween 40. The chloroform 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 essential oils and BHT were individually dissolved in methanol (2 g/L) and 350 µL volumes of each were added to 2.5 mL of the above emulsion in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50ºC for 2 h, together with a negative control (blank) containing the same volume of methanol. The absorbance values were measured at 470 nm using a UV-vis spectrophotometer. Percentage inhibitions (I%) of the samples were calculated using the following equation:

$$I\% = [\ A_{\beta\text{-carotene after 2 h}} / A_{\text{initial }\beta\text{-carotene}}\ ] \times 100$$

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 experiments. All tests were carried out in triplicate and percentage inhibitions were reported as means ± SD of triplicates.

DPPH radical scavenging: The free radical scavenging activity was measured by the DPPH method with minor modifications [23]. Each sample of stock solution (1 mg/mL in MeOH) was diluted to a final concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL. Then, a total of 3.8 mL of 50 µM DPPH methanolic solution (1 mg/50 mL) 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. The percentage inhibitions (I%) of DPPH radical were calculated as follow:

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

where A_{blank} is the absorbance value of the control reaction (containing all reagents except samples) and A_{sample} is the absorbance values of the essential oils. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. The IC_{50} values were reported as means ±SD of triplicates.

Total phenolic content (TPC): Total phenolic content of the essential oils was determined as in a previous study [24], with minor modifications. A sample of stock solution (1 mg/mL in MeOH) was diluted to a final concentration of 1000 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 thoroughly shaken. After 3 min, 0.5 mL of 5% Na_2CO_3 solution was added and the mixture 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 phenolics in the oils was expressed as mg of gallic acid equivalent per g of sample. Tests were carried out in triplicate and the gallic acid equivalent value was reported as mean ±SD of triplicate.

Anticholinesterase activity (AChE): AChE and BChE inhibitory activities were measured by the Ellman method, with slight modification of the spectrophotometric method [25-26]. Electric eel AChE and horse serum BChE were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic) acid (DTNB) was used for the measurement of the anticholinesterase activity. Briefly, in this method, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of the essential oils and 20 µL of AChE/BChE solution were added by multichannel automatic pipette to a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated by the addition of 10 µL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer, USA). Percentage of inhibition (I%) of AChE/BChE was determined by comparison of the rate of reaction of each sample relative to a blank sample (ethanol in phosphate buffer pH = 8) using the following formula:

$$I\% = [\ E - S / E] \times 100$$

where E is the activity of enzyme without the test sample and S is the activity of enzyme with test sample. Galantamine was used as the standard. The experiments are reported as mean ±SD of triplicates.

Statistical analysis: Data obtained from essential oil analysis and biological activities 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.

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