

Evaluation of Antioxidant, Anticholinesterase and Antityrosinase Activities of Malaysian *Cinnamomum* Species

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ABSTRACT: The extracts of the leaves and bark of five *Cinnamomum* species (*C. altissimum*, *C. griffithii*, *C. javanicum*, *C. macrocarpum*, and *C. velutinum*) were screened to evaluate their antioxidant, anticholinesterase, and antityrosinase activities. The bark of *C. altissimum* showed the highest phenolic content (130.1 mg GA/g), free radical scavenging by DPPH (IC₅₀ 126.2 µg/mL) and ferric reducing antioxidant power (FRAP) assays (341.2 mg AA/g). All of the extracts inhibited linoleic acid peroxidation by greater than 70%, with the leaves of *C. altissimum* exhibiting the highest inhibition of 87.7%. The leaves of *C. javanicum* revealed the highest inhibition on anticholinesterase (AChE 30.8%) and butyrylcholinesterase (BChE 46.8%) enzymes. The leaves and bark of *C. altissimum* and *C. velutinum* exhibited greater than 20% tyrosinase inhibition, with the leaves of *C. altissimum* having the highest percentage of inhibition (34.6%). These bioactivities indicate that some *Cinnamomum* species have therapeutic potential in medicinal research and development of new drugs candidates.

Key words: Lauraceae, *Cinnamomum*, antioxidant, anticholinesterase, antityrosinase

INTRODUCTION

Cinnamomum, belonging to the family Lauraceae, is a genus of trees found in continental Asia, eastern and southeastern Asia, Australia, the Pacific region, and a few species in Central and South America. In Malaysia, twenty one species have been found in tropical rain forests where they grow at various altitudes from highland slopes to lowland forests and occur in both marshy places and on well-drained soils.¹ Cinnamon has been used as a spice for thousands of years. In Ayurvedic medicine, cinnamon bark has been used as an antiemetic, antidiarrheal, antifatulent, and general stimulant.² Systematic chemical studies with the essential oils of some of the Malaysian species have been carried out.

The oils were found to contain mainly safrole, eugenol, linalool, camphor, benzylbenzoate, or cinnamaldehyde as major components.³⁻⁵ The cinnamon oil is used as flavoring ingredient in foods and drinks, as a perfumery material, and in many pharmaceutical preparations for its carminative and astringent properties.⁶ Besides, the cinnamon extracts have been found to be impersonating the function of insulin, while the extracts also can potentiate insulin action in isolated adipocytes and also enhanced the insulin receptor function.^{7,8} Hence, considering the various medicinal benefits of *Cinnamomum*, an effort has been made to establish the scientific validity through screening for antioxidant, anticholinesterase, and antityrosinase inhibitory activities of methanolic leaves and bark extracts of five *Cinnamomum* species. Table 1 shows the traditional uses of the selected *Cinnamomum* species.⁹

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Table 1. List of five *Cinnamomum* species and their traditional uses.

Botanical name	Vernacular names	Traditional uses
<i>C. altissimum</i> Kosterm.	No report	The leaves, stem bark and stem wood have been used to treat wound infections.
<i>C. griffithii</i> Meisn.	Babau	A decoction of the roots is given to women after childbirth and also to treat fever.
<i>C. javanicum</i> Blume	Daun buluh	The leaves are used medicinally with <i>Kadsura scandens</i> to treat stomachache and to initiate abortion. The root decoction is drunk to treat fatigue and chest pain.
<i>C. macrocarpum</i> Hook.f.	Lavanga	The leaves are used for flavouring rice dishes. The bark is reported to be used as a substitute/adulterant of the bark of <i>C. zeylanicum</i> which is traded in large quantities as a spice as well as a raw plant drug. It is also used in Ayurveda, Folk and Sidha.
<i>C. velutinum</i> Ridl.	Medang teja	No report.

MATERIALS AND METHODS

Plant materials. Five *Cinnamomum* species were collected from Bau and Lundu, Sarawak and identified by Mohizar Mohamad. The voucher specimen (UiTMKS/C01-05) was deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

Solvents and chemicals. *Antioxidant:* β -carotene, linoleic acid, chloroform (HPLC grade), Tween 40, Folin-Ciocalteu reagent, gallic acid, anhydrous sodium carbonate 99%, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), and iron (III) chloride hexahydrate were purchased from Sigma-Aldrich, USA.

Anticholinesterase: L-3,4-dihydroxyphenylalanine (L-dopa), mushroom tyrosinase and kojic acid were obtained from Sigma-Aldrich, USA.

Antityrosinase: Electric eel AChE (Type-VI-S, EC 3.1.1.7), horse serum BChE (EC 3.1.1.8), acetylcholine iodide, S-butrylthiocholine chloride, 5,5'-dithiobis (2-nitrobenzoic acid) 99% (DTNB), and galantamine were purchased from Sigma-Aldrich, USA.

Plant extraction. The dried and powdered leaves and bark of the above-mentioned *Cinnamomum* species (about 100 g, each) were subjected to cold extraction method by using methanol as solvent. The extracts were filtered and

the solvent was removed under vacuum using a rotary evaporator (Eyela, Japan). The extracts were obtained and kept in freeze until the experimental practices. Percentage yields (w/w) of the crude extracts are given in Table 2.

Antioxidant activity

β -Carotene/linoleic acid bleaching: The β -carotene-linoleic acid bleaching assay as described previously was used with minor modifications.¹⁰ A mixture of β -carotene and linoleic acid was prepared by adding together of 0.5 mg β -carotene in 1 mL CHCl_3 , 25 μL linoleic acid and 200 mg Tween 40. The CHCl_3 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 extracts and BHT were individually dissolved in methanol (2 g/L) and 350 μL volumes of each of them 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 50°C for 2 h, together with a negative control (blank) contained the same volume of methanol. The absorbance values were measured at 470 nm on UV-vis spectrophotometer. Percentage inhibitions (I%) of the extracts were calculated using the following equation:

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

where $A_{\beta\text{-carotene after 2 h}}$ assay is the absorbance values of β -carotene after 2h 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 \pm SD of triplicates.

DPPH radical scavenging: The free radical scavenging activity was measured by the DPPH method with minor modifications.¹⁰ Each sample of stock solution (1 mg/mL in MeOH) was diluted in various concentrations (1000-7.8 μ g/mL). Then, a total of 3.8 mL of 50 μ M 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 the samples) and A_{sample} is the absorbance values of the test samples. The sample concentration providing 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 \pm SD triplicates.

Total phenolic content (TPC): TPC of extracts were determined by gallic acid equivalent with minor modifications.¹⁰ A sample of stock solution (1 mg/mL in MeOH) was diluted to final concentrations of 1000 μ g/mL. A 0.1 mL aliquot of sample was pipetted into a test tube containing 0.9 mL of MeOH, 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 2h with intermittent shaking. Then, 2.5 mL of MeOH was added and left to stand in the dark for 1h. The absorbance measurements were recorded at 765 nm. The same procedure was repeated for the standard gallic acid solutions. The concentration of total phenolic contents in the extracts was expressed as mg of gallic acid equivalent per gram of sample. Tests

were carried out in triplicate and the gallic acid equivalent value was reported as mean \pm SD of triplicate.

Ferric reducing antioxidant power (FRAP): The total reducing capacity was determined using FRAP assay.¹¹ The stock solutions included 300mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 $^{\circ}C$ prior to use. The extracts (150 μ L) was allowed to react with 2850 μ L of the FRAP solution for 30 min in the dark condition. After incubation, the absorbance was read at 593 nm using a UV-Vis spectrophotometer. The results were calculated by standard curves prepared with known concentrations of ascorbic acid (AA) and were expressed as mg AA/g.

Anticholinesterase activity. AChE and BChE inhibitory activities were measured slightly modified by Ellman spectrophotometric method.¹² Briefly, 140 μ L sodium phosphate buffer (pH 8.0), 20 μ L DTNB, 20 μ L extracts (concentration of 1 mg/mL), and 20 μ L AChE/BChE solution were added by a multichannel automatic pipette in a 96-well microplate and incubated for 15 min at 25 $^{\circ}C$. The reaction was then initiated with the addition of 10 μ L AChI/BuChI. The hydrolysis of AChI/BuChI chloride was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion from the reaction of DTNB with thiocholine, catalyzed by enzymes at 412 nm, and utilizing a 96-well microplate reader (Epoch Micro-Volume Spectrophotometer). Galantamine was used as a reference. The percentage inhibition (I%) of AChE/BChE was determined by comparing the rates of reaction of samples relative to the blank sample (EtOH in phosphate buffer pH 8) using the following formula:

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

where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. Analyses were expressed as mean \pm SD of triplicates.

Antityrosinase activity. Tyrosinase inhibition assay was performed according to the previous method with slight modifications.¹³ Briefly, the extracts and kojic acid were dissolved in DMSO prepared in the amount of 1 mg/mL. The extracts (40 μ L) dissolved in DMSO with 80 μ L phosphate buffer (pH 6.8), 40 μ L tyrosinase enzyme, and 40 μ L L-DOPA was placed in each well. Each sample was accompanied by a blank that contained all the components except L-DOPA. Kojic acid was used as a reference standard inhibitor for comparison. The reaction was carried out using a 96-well microplate and a microplate reader (Epoch Micro-Volume Spectrophotometer) were used to measure the absorbance at 475 nm. The percentage inhibition (I%) was calculated as follows:

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

where, A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the extracts/reference. Analyses were expressed as mean \pm SD of triplicates.

Statistical analysis. Data obtained from biological activities were expressed as means \pm SD and were compared using student's t-test. 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 antioxidant activity of the leaves and bark extracts was evaluated by four tests: DPPH radical scavenging, β -carotene-linoleic acid, total phenolic content, and FRAP assays. The results for antioxidant activities are presented in table 2.

Table 2. Percentage yield and antioxidant activities of five *Cinnamomum* species.

Sample	Yield (%)	β -carotene (I%)	DPPH IC ₅₀ (μ g/mL)	TPC (mg GA/g)	FRAP (mg AA/g)
CAL	2.13	87.7 \pm 0.4	242.4	110.6 \pm 0.4	340.6 \pm 0.8
CAB	2.22	85.2 \pm 0.3	126.2	130.1 \pm 0.2	341.2 \pm 0.4
CGL	2.96	82.0 \pm 0.2	226.4	69.6 \pm 0.2	149.2 \pm 0.4
CGB	2.45	81.5 \pm 0.3	197.4	87.2 \pm 0.3	95.2 \pm 0.2
CJL	2.80	84.0 \pm 0.3	223.5	76.8 \pm 0.3	207.6 \pm 0.3
CJB	2.68	78.6 \pm 0.2	197.4	72.0 \pm 0.3	125.0 \pm 0.2
CML	2.12	70.8 \pm 0.3	200.6	45.8 \pm 0.2	109.8 \pm 0.5
CMB	2.24	70.3 \pm 0.4	159.4	54.4 \pm 0.2	240.8 \pm 1.3
CVL	1.92	74.2 \pm 0.2	196.6	79.6 \pm 0.2	176.2 \pm 0.2
CVB	1.96	72.0 \pm 0.3	178.5	72.0 \pm 0.3	243.0 \pm 0.4
BHT		125.5	32.4	ND	ND

Data represent mean \pm SD of three independent experiments; ND – not determined; CAL - *C. altissimum* leaf; CAB - *C. altissimum* bark; CGL - *C. griffithii* leaf; CGB - *C. griffithii* bark; CJL - *C. javanicum* leaf; CJB - *C. javanicum* bark; CML - *C. macrocarpum* leaf; CMB - *C. macrocarpum* bark; CVL - *C. velutinum* leaf; CVB - *C. velutinum* bark.

In DPPH assays, the test intends to measure the hydrogen atom or electron donor capacity of the extracts to the stable radical DPPH formed in solution. It measures the capacity of the extract to scavenge free radicals in solution.¹⁴ The bark extract

of *C. altissimum* and *C. macrocarpum* showed the lowest IC₅₀ value in DPPH which exhibited strong antioxidant activity, with IC₅₀ value of 126.2 and 159.4 μ g/mL, respectively. However, this value was four times lower than those found with the

antioxidant standard, BHT with IC_{50} 32.4 $\mu\text{g/mL}$. The other extracts that showed IC_{50} value $< 200 \mu\text{g/mL}$, were *C. griffithii* (bark 197.4 $\mu\text{g/mL}$), *C. javanicum* (leaves 197.4 $\mu\text{g/mL}$) and *C. velutinum* (leaves 196.6 $\mu\text{g/mL}$; bark 178.5 $\mu\text{g/mL}$). In β -carotene-linoleic acid, the activity measured as the inhibition of oxidation of linoleic acid can simulate the oxidation of the membrane lipid components and also measures the capacity of inhibition of conjugated diene hydroperoxide arising from the linoleic acid oxidation. This test measures the antioxidant activity toward linoleic acid relatively to β -carotene.¹⁴ In β -carotene-linoleic acid, the results showed that the *Cinnamomum* extracts were in the range of 70.3–87.7%. Moderate inhibitory activity was exhibited by the leaves and bark extracts of *C. altissimum* extracts which revealed 87.7% and bark 85.2%, respectively, followed by extracts of *C. griffithii* (leaves 82.0%; bark 81.5), and *C. javanicum* (leaves 84.0%; bark 78.6%). In phenolic content assay, the phenolic compounds have been shown to be responsible for the antioxidant activity of plant materials and hence many of the natural polyphenols possess therapeutic potential.¹⁵ The total phenolic content (TPC) values were quantified based on the linear equation obtained from gallic acid standard calibration curve ($y = 0.0021x - 0.0223$; $r^2 = 0.9928$). Thus, TPC values were expressed as gallic acid equivalent (mg GAE/g extracts). From the table, leaves and bark extract of *C. altissimum* had the highest content of phenolic which gave 110.6 ± 0.4 and 130.1 ± 0.2 mg GA/g extract, respectively. FRAP assay is presented as an accurate method for assessing antioxidant power. Ferric to ferrous ion reduction at low pH causes a colored ferroustripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with that containing ferrous ions in known concentration. In FRAP assay, the highest values showed by the bark extracts of *C. altissimum* (341.2 mg AA/g). FRAP assay is widely used in the evaluation of the antioxidant component in dietary polyphenols due to inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is

straightforward and speedy.¹⁶ Previous work carried out on *Cinnamomum* species had obtained higher antioxidant activity in plant extracts such as *C. camphora*, *C. cassia*, *C. tamala*, *C. zeylanicum*, *C. osmophloeum*, *C. triplinerve*, *C. iners*, and *C. verum*.¹⁷⁻²⁰ This suggests that flavonoid glycosides, proanthocyanidins, cinnamic acid, coumarin, and lignans known to have antioxidant activity may be responsible for the activity.²¹

According to the results at table 3 we obtained, the leaves extract from *C. javanicum* (AChE 30.8%; BChE 46.8%) and *C. velutinum* (AChE 30.5%; BChE 46.5%) exerted the highest AChE and BChE inhibition. However the results of the extracts for AChE was three times lower than galantamine which gave 95.9%, while for BChE was two times lower than that standard which gave 88.7%. In addition, the leaves extract of *C. macrocerpum* (AChE 29.5%; BChE 45.7%) and *C. griffithii* (AChE 27.1%; BChE 43.8%) were also found to have significant inhibition. However, no activity were recorded on AChE from the bark of *C. griffithii* and *C. javanicum* as well as leaves and bark of *C. altissimum*. Inhibition of AChE, the key enzyme in the breakdown of acetylcholine, is considered as one of the treatment strategies against several neurological disorders such as Alzheimer disease.²² Anticholinesterase inhibitory activity of the extracts was tested against acetylcholinesterase and butyrylcholinesterase at different concentrations. Our literature survey revealed the presence of few report on cholinesterase inhibitory activity of *Cinnamomum* species. Kumar *et al.*²³ reported that aqueous extract of *C. zeylanicum* gave inhibitory activity of 46.8% for AChE and 51.7% for BChE enzyme. The ethanol extract showed percentage inhibition of 40.8% and 51.5% for AChE and BChE, respectively. *Cinnamomum* extract has been found to have an inhibitory effect on tau aggregation related to AD. The extract can also promote complete disassembly of recombinant tau filaments and cause substantial alteration of the morphology of paired-helical filaments isolated from AD brain. Recently, orally administered *Cinnamomum* extract has been found to reduce β -amyloid oligomerization and correct cognitive impairment in AD animal models.²⁴

Table 3. Anticholinesterase and antityrosinase activities of five *Cinnamomum* species.

Samples	Anticholinesterase (%)		Antityrosinase (%)
	AChE	BChE	
CAL	NA	19.9 ± 0.2	34.6 ± 0.3
CAB	NA	12.8 ± 0.2	32.2 ± 0.3
CGL	27.1 ± 0.2	43.8 ± 0.3	NA
CGB	NA	18.1 ± 0.2	NA
CJL	30.8 ± 0.2	46.8 ± 0.3	18.4 ± 0.2
CJB	NA	20.6 ± 0.2	6.4 ± 0.1
CML	29.5 ± 0.3	45.7 ± 0.2	21.3 ± 0.2
CMB	6.4 ± 0.2	28.0 ± 0.1	28.2 ± 0.3
CVL	30.5 ± 0.2	46.5 ± 0.3	13.9 ± 0.2
CVB	NA	22.3 ± 0.2	11.4 ± 0.3
Galantamine	95.9 ± 0.2	88.7 ± 0.2	ND
Kojic acid	ND	ND	81.8 ± 0.5

Data represent mean ±SD of three independent experiments; NA - not active; ND – not determined; CAL - *C. altissimum* leaf; CAB - *C. altissimum* bark; CGL - *C. griffithii* leaf; CGB - *C. griffithii* bark; CJL - *C. javanicum* leaf; CJB - *C. javanicum* bark; CML - *C. macrocarpum* leaf; CMB - *C. macrocarpum* bark; CVL - *C. velutinum* leaf; CVB - *C. velutinum* bark

Table 3 showed that leaves and bark extracts of *C. altissimum* revealed the highest antityrosinase activity with 34.6% and 32.2%, respectively. However it is three times lower than that of kojic acid, 81.8% inhibition. Meanwhile, the extracts that showed inhibition more than 20% were leaves (21.3%) and bark (28.2%) of *C. macrocarpum*. Tyrosinase, also known as monophenol monooxygenase, catechol oxidase or diphenol oxidase, is a coppercontaining enzyme present in plant, animal, and fungal species that catalyzes the production of melanin and other pigments from tyrosine by oxidation. Tyrosinase inhibitors are used in treatment of various skin diseases such as hyperpigmentation and melasma. Many inhibitors of this enzyme have been reported to contain phenolic structure such as kojic acid and arbutin.²⁵ Antityrosinase activity of the extracts was tested using L-dopa as a substrate. A few studies on *Cinnamomum* species have been tested on their extracts for antityrosinase activity. Methanolic extracts of *C. zeylanicum* leaves showed inhibition 72.7% at 100 µg/mL, while the methanol extract of *C. cassia* twigs was found to possess tyrosinase inhibitory activity with percentage inhibition >85% at the same concentration.^{26,27} The present investigation was delineated to evaluate the preliminary screening

of enzyme inhibitory activities of the plant extracts. The expected bioactive components could be flavonoids, aromatic acids, polyphenols, or aromatic aldehydes as these compounds consist of hydrophobic parts which could probably act as competitive inhibitors in the synthesis of melanin.²⁸ Previous studies showed that proanthocyanidins have been isolated from *C. zeylanicum* and *C. cassia*.^{29,30} Meanwhile, little is known of the lightening effect of proanthocyanidin on UV-induced pigmentation of the skin but they showed that grape seed extract, rich in proanthocyanidins, inhibited the activity of mushroom tyrosinase and inhibited melanogenesis without inhibiting the growth of cultured B16 mouse melanoma cells.³¹ 'Quebracho', an extract rich in proanthocyanidins, isolated from the heartwood of *Schinopsis lorentzii* with 70% aqueous acetone, showed 47% tyrosinase inhibition.³²

CONCLUSIONS

The present findings support the use of the five *Cinnamomum* species in traditional medicine and indicate their potential in providing biologically active compounds. Since the activities exhibited by these species were from leaves and stems and not the bark that is generally used, substitution of the bark with these plant parts will impact on conservation.

This will add more value on the sustainable uses of these species, especially in areas where they are protected due to overexploitation. The data will also add to the knowledge base needed to advance the local management of diseases.

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