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Chemical Constituents and Antioxidant Activity of *Cinnamomum microphyllum*

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ABSTRACT

The extract of *Cinnamomum microphyllum* showed strong antioxidant activity when it was tested against auto-oxidation of linoleic acid, superoxide, and DPPH radical scavenging activity. Further detailed investigations of the plant constituents and bioactivity studies led to the isolation and identification of known compounds consisting of three lignans, a coumarin, an ester and β-sitosterol. The structures of the compounds were determined using detailed spectroscopic analysis. The lignans were found to possess a significant antioxidant activity when tested against the three assay systems.

Keywords: *Cinnamomum microphyllum*, pinoresinol, syringaresinol, medioresinol, antioxidant

INTRODUCTION

Cinnamomum of the family *Lauraceae* is an evergreen or decidous shrub or small to large tree which can grow up to 50 meters in height. The genus grows widely in continental Asia, East and Southeast Asia, Australia, the Pacific and in Central and South America. About 21 species are found in Peninsular Malaysia (Kochummen, 1989) alone. Many ethnobotanical studies have reported the traditional uses of *Cinnamomum* species, especially in food, fragrances, fumigants, and traditional medicines (Burkill, 1966; Perry, 1980). Commercially known as cinnamon, it is considered as one of the oldest spices in the world (Wijesekera, 1978) and contains valuable chemicals such as eugenol, cinnamaldehyde, safrole, linalool, camphor and benzyl benzoate. Cinnamon oil and oleoresin are primarily used in the food processing, cosmetic, and pharmaceutical industries, while the cinnamon sticks or powdered form are used as flavourings and in confectionaries (Jayatilaka *et al.*, 1995).

Considerable experimental evidences have supported the view that reactive oxygen radicals play a vital role in an oxidation process which is recognized as the initial stage in the development of many chronic diseases such as cancer, atherosclerosis, diabetes, etc. (Abe and Berk, 1998; Lefer and Granger, 2000). These highly reactive free radicals, in the form of superoxide anion radicals, hydroxyl radicals, and non-free radical species, are unstable molecules and seek other electrons to pair, and thus lead to cell and DNA damages. The roles of antioxidant in food and herbal care products are very important, particularly functioning as free radical scavenger, reducing agents and quenchers for the formation of singlet oxygen (Youdim *et al.,* 1999). These antioxidants

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can neutralize these free radicals and help prevent any damages, as well as increase resistance against diseases and prolong life. Typical antioxidants, which are found in plants, consist mostly polyphenolics such as flavonoids, lignans, xanthones, etc. The objectives of the study were to identify the presence of chemical components in *C. microphyllum* and to evaluate the antioxidant activity of the extracts, essential oils, and pure compounds against linoleic acid autoxidation, superoxide and DPPH free radical scavenging assays. Earlier chemical studies on the plant revealed a rich source of essential oils and some have been shown to exhibit insecticidal, anticandidal, and antidermatophytic activities (Ibrahim *et al.,* 2005; Ibrahim *et al.,* 2008; Mastura *et al.,* 1999). Various types of secondary metabolites have been reported to occur in *Cinnamomum* species and some of the compounds were found to have cytotoxic, antioxidant, anti-platelet aggregation, and antimicrobial activities (Mukherjee *et al*., 1994; Kwon *et al.,* 1998; Su *et al.*, 1999; Zhu *et al*., 1994).

EXPERIMENTAL DESIGN

General Experimental Procedures

In this study, melting points were measured on a Kofler hot stage apparatus and uncorrected. The IR spectra were recorded using the KBr discs on a Perkin Elmer FTIR spectrophotometer model 1275X. The UV spectra were recorded in MeOH on a Shimadzu UV 160A spectrophotometer. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JOEL FTNMR 400 MHz spectrometer. Meanwhile, chemical shifts are shown in δ values (ppm) with tetramethylsilane as an internal standard.

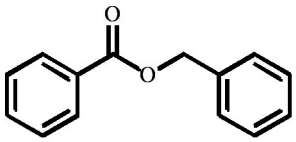
Plant Material

The leaf, stem bark, and stem of *Cinnamomum microphyllum* were collected from Gunung Berembun, Cameron Highlands in 1998, and a voucher specimen was deposited at the Herbarium, Forest Research Institute of Malaysia, in Kepong.

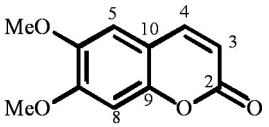
Extraction and Isolation

The ground dried bark (2.1 kg) and stem (1.4 kg) were separately and sequentially extracted by cold percolation with hexane, chloroform, and methanol, over a period of two weeks. The bark extracts were concentrated under reduced pressure to give 116.1 g, 82.8 g, and 392.0 g of dark viscous extracts, respectively. A similar treatment of the stem gave 13.4 g, 19.4 g, and 55.0 g of dark viscous extracts, respectively. A portion of the hexane extract of the bark (30 g) was subjected to VLC and eluted with solvent gradient of petroleum ether, ethyl acetate and methanol to afford 22 major fractions of 200 ml each. Further chromatographic separation of fractions 7 and 10-20 gave benzyl benzoate (**1**) as a colourless oil (54 mg) and β-sitosterol (42 mg), respectively. In particular, β-Sitosterol was recrystallised from $CHCl_3$ as white needles with m.p. 131-133°C (Hill *et al*., 1991; m.p. 136-137°C).

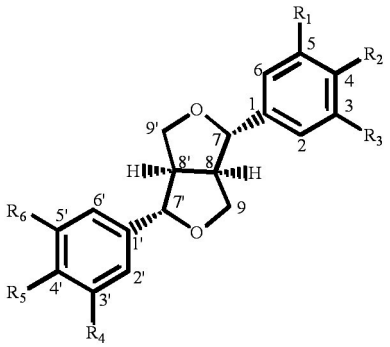
A portion of the chloroform bark extract (30.0 g) was subjected to flash column chromatography using chloroform and ethyl acetate as eluents to afford 83 fractions. Fractions 21-26 were combined and rechromatographed with silica gel to give scoparone (**2,** 75 mg) and recrystallised from chloroform as white plates, m.p. 147-148°C (Valenciennes *et al*., 1999; m.p. 143-145°C). Fractions 51-74 were further separated by a series of silica gel column chromatography and preparative TLC to give pinoresinol (**3,** 38 mg**)** as brownish oil, syringaresinol (**4**, 52 mg**)** as white needle-shaped crystals, while m.p. 172-173°C (Abe and Yamauchi, 1988; m.p. 173-174°C) and medioresinol (**5,** 29 mg**)** as white needles, m.p. 170-171°C (Abe and Yamauchi, 1988; m.p. 170-172°C).



Benzyl benzoate (**1**)



Scoparone (**2**)



Pinoresinol (**3**) $R_1 = R_6 = H$; $R_2 = R_5 = OH$; $R_3 = R_4 = OMe$
Syringaresinol (**4**) $R_1 = R_3 = R_4 = R_6 = OMe$; $R_2 = R_5 = OH$
Medioresinol (**5**) $R_1 = H$; $R_2 = R_5 = OH$; $R_3 = R_4 = R_6 = OMe$

Benzyl benzoate (1): IR ν_{max} (KBr) cm^{-1}: 1717, 1448, 1324, 1273, 1241, 1199, 978; UV λ_{max} (MeOH) nm (log ε): 208 (2.43); ^{1}H-NMR (400 MHz) δ: 8.07 (1H, *d*, *J*=7.5 Hz, H-4), 7.55 (2H, *t*, *J*=7.5 Hz, H-3, H-5), 7.33-7.45 (7H, *m*, H-3', H-7', H-4', H-5', H-6', H-2, H-6), 5.36 (2H, *s*, H-1');. ^{13}C-NMR (100 MHz) ppm: 166.3 (C=O), 135.9 (C-1), 132.8 (C-4), 129.9 (C-2'), 129.5 (C-2/C-6), 128.2 (C-3/C-5), 128.4 (C-4'/6'), 128.0 (C-3'/C-7'), 127.9 (C-5'), 66.5 (-CH_2); MS m/z (%): 212 (M^+, 22), 194 (6), 105 (100), 91 (51), 77 (36), 65 (12).

Scoparone (2): IR ν_{max} (KBr) cm^{-1}: 2924, 1623, 1519, 1457, 1385, 1281; UV λ_{max} (MeOH) nm (log ε): 340 (0.43), 291 (0.24); ^{1}H-NMR (400 MHz) δ: 7.64 (1H, *d, J*=9.5 Hz, H-4), 6.87 (2H, *s*, H-8, H-5), 6.31 (1H, *d, J*=9.5 Hz , H-3), 3.97 (3H, *s*, 6-OMe), 3.94 (3H, *s,* 7-OMe); ^{13}C-NMR (100 MHz) ppm: 161.7 (C-2), 153.2 (C-7), 150.4 (C-9), 146.7 (C-6), 143.5 (C-4), 111.8 (C-10), 113.9 (C-3), 108.5 (C-5), 100.4 (C-8), 56.7 (2xOMe). MS m/z (%):206 (M^+, 100), 191 (51), 178 (22), 163 (44), 135 (29), 120 (20), 107 (36), 92 (24), 79 (50).

Pinoresinol (3): IR ν_{max} (KBr) cm^{-1}: 3458, 2997, 1613, 1519, 1462, 1427, 1366, 1343, 1325, 1278, 1245; UV λ_{max} (MeOH) nm (log ε): 289 (1.10), 230 (1.25); MS m/z (%): 358 (M^+, 48), 205 (10), 191 (7), 163 (32), 151 (100), 137 (69), 124 (23), 103 (11), 77 (13). ^{1}H-NMR (400 MHz: $CDCl_3$) and ^{13}C-NMR (100 MHz: $CDCl_3$) – *see* Table 1.

Syringaresinol (4); IR ν_{max} (KBr) cm^{-1}: 3472, 3097, 2996, 2947, 2866, 1612, 1521, 1459, 1426, 1368, 1341, 1327; UV λ_{max} (MeOH) nm (log ε): 272 (0.05), 258 (0.04), 208 (1.10); MS m/z (%): 418 (M^+, 38), 388 (59), 182 (44), 181 (100), 167 (73). ^{1}H-NMR (400 MHz: $CDCl_3$) and ^{13}C-NMR (100 MHz: $CDCl_3$) – *see* Table 1.

TABLE 1
^{1}H-NMR and ^{13}C-NMR data for pinoresinol (**3**), syringaresinol (**4**) and medioresinol (**5**)

No.	Pinoresinol (**3**)		Syringaresinol (**4**)		Medioresinol (**5**)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	-	133.2	-	132.3	-	132.4
2	6.92, (*d*, 0.5 Hz)	119.2	6.61 (*s*)	102.9	6.92 (*d*, 2.4 Hz)	102.9
3	-	146.9	-	147.4	-	146.9
4	5.63 (*s*, OH)	145.5	5.94 (*s*, OH)	134.5	5.50 (*s*, OH)	146.5
5	6.91 (*d*, 7.5 Hz)	114.5	-	147.4	6.91 (*d*, 8.5 Hz)	114.5
6	6.84 (*dd*, 0.5, 7.5 Hz)	108.8	6.61 (s)	102.9	6.84 (*dd*, 8.5, 2.4 Hz)	102.9
7/7’	4.76 (*brd*, 4.0 Hz)	86.1	4.75 (*d*, 4.0 Hz)	86.3	4.76 (*dd*, 5.0, 3.2 Hz)	86.4/86.0
8/8’	3.12 (*brd*)	54.6	3.12 (*brd*, 1.5 Hz)	54.6	3.13 (*m*)	54.7/54.3
9a/9’a	4.27 (*dd*, 9.0, 7.0 Hz)	72.2	4.30 (*dd*, 7.0, 9.0 Hz)	72.0	4.30 (*m*)	72.1/71.8
9b/9’b	3.90 (*dd*, 9.0, 3.2 Hz)	72.2	3.91 (*dd*, 7.0, 5.5 Hz)	72.0	3.91 (*m*, 2H)	72.1/71.8
1’	-	133.2	-	132.3	-	133.1
2’	6.92 (*d*, 0.5 Hz)	119.2	6.61 (*s*)	102.9	6.60 (*s*, 1H)	108.8
3’	-	146.9	-	147.4	-	147.4
4’	5.63 (*s*, OH)	145.5	5.51 (*s*, OH)	134.5	5.61 (*s*, OH)	134.5
5’	6.91 (*d*, 7.5 Hz)	114.5	-	147.4	-	147.4
6’	6.84 (*dd*, 0.5, 7.5 Hz)	108.8	6.61 (*s*)	102.9	6.60 (*s*, 1H)	119.2
OMe	3.93 (*s*, 6H, 2xOMe)	56.4	3.95 (*s*, 12, 4xOMe)	56.6	3.93 (OMe)	56.6
					3.95 (2xOMe)	56.2

TABLE 2
Antioxidant activity of *C. microphyllum* extracts and isolated compounds

Extracts/isolated compounds	Inhibition of linoleic acid (%)	Superoxide scavenging activity (%)	DPPH scavenging activity (%)
Hexane (stem)	5.1	95.2	12.5
Chloroform (stem)	34.7	97.0	52.0
Methanol (stem)	38.7	94.6	98.7
Hexane (bark)	16.8	16.7	12.7
Chloroform (bark)	49.2	76.5	76.0
Methanol (bark)	5.4	96.3	86.7
Essential oil (leaf)	-	6.4	-
Essential oil (bark)	-	-	-
Scoparone (**2**)	-	-	-
Pinoresinol (**3**)	84.9	76.8	95.8
Medioresinol (**5**)	79.6	73.4	94.6
Control (5% linoleic acid)	0	-	-
BHT	100		

Medioresinol (5); IR ν_{max} (KBr) cm^{-1}: 3774, 3457, 3139, 3097, 2996, 1612, 1520, 1459, 1368, 1327, 1282, 1204; UV λ_{max} (MeOH) nm (log ε): 279 (0,10), 258 (0.03), 206 (1.12), ; MS m/z (%): 388 (M^+, 29), 205 (15), 181 (23), 163 (37), 151 (100), 137 (66), 124 (23), 55 (31). ^{1}H-NMR (400 MHz: $CDCl_3$) and ^{13}C-NMR (100 MHz: $CDCl_3$) – *see* Table 1.

Essential Oil Extraction

The leaf and bark of the plant were subjected to hydrodistillation using Clevenger-type apparatus for 4 hours. The oily layers obtained were separated and dried over anhydrous sodium sulphate. The percentage of essential oils obtained from the leaves and bark were 1.9% and 2.3%, respectively. These were the averaged yield over two experiments and calculated based on the dry weight of the plant materials.

Antioxidant Assays

Autoxidation of linoleic acid in a water-alcohol system. The autoxidation assay was carried out using the method described by Osawa and Namiki (1981), but with a slight modification. 2,6-ditert-Butylphenol (BHT) at 4.0 mg was used as a positive control.

Xanthine/Xanthine oxidase (X/XOD) superoxide scavenging assay. The assay was carried out according to the method described by Chang *et al.* (1996), with a slight modification.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The effect of *C. microphyllum* extracts on DPPH radical was estimated according to the method proposed by Blois (1958). The plant extracts or compounds (0.5 mg/ml) were added to a solution of DPPH (0.5 mg/ml) in methanol. The mixture was shaken and left to stand at room temperature for 10 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm.

RESULTS AND DISCUSSION

A positive preliminary screening of crude extracts of *C. microphyllum* against two of the assay systems prompted the researchers to investigate the stem bark and stem extracts of the plant further. The hexane, chloroform and methanol extracts of both parts of the plant were chosen for a detail study.

The chromatographic separation of the hexane extract gave the simple benzyl benzoate (**1**) as colourless oil and the ubiquitous β-sitosterol as white powder with m.p. 131-133ºC. The first compound identified from the chloroform extract of the bark was scoparone (**2**), and this was followed by the isolation of pinoresinol (**3**) as brownish oily solid with UV spectrum maximum absorptions at 289 and 230 nm, and molecular ion peak at m/z 358 in the mass spectrum which corresponded to the molecular formula $C_{20}H_{22}O_6$. The mass spectral analysis was consistent with the fragmentation patterns reported for 2,6-diaryltetrahydrofuran lignans (Latip *et al.,* 1999). This was further supported by the ^{1}H-NMR spectrum which showed the typical characteristic of the symmetrical tetrahydrofuran lignan. A total of six aromatic protons were observed in the range of δ 6.84-6.92 for the two aryl groups, as well as eight protons for the bifuran ring, represented by the resonances at δ 3.12 (H-8, H-8'), 4.76 (H-7, H-7'), 4.27 (H-9a, H-9'a), and 3.90 (H-9b, H-9'b). The chemical shifts observed for the benzylic protons, at H-7 and H-7' appeared at a higher field (δ 4.76), were consistent with the equatorial orientation of the aryl substituents (Pelter *et al*., 1976).

The correlations in the COSY spectrum strongly supported the connectivity of the 2,6-diaryltetrahydrofuran ring even further. The compound displayed only ten carbon signals in the ^{13}C-NMR spectrum (Yang *et al*., 1999), while the assignments of the individual protons and carbons were supported by DEPT, HSQC, and HMBC spectra. Three pairs quaternary carbons, observed at 146.9, 145.5 and 133.2 ppm, were assigned to C-3/C-3', C-4/C-4' and C-1/C-1', respectively. The two methylene carbons at C-9/C-9' occurred at 72.2 ppm (Table 1). Based on these data and the comparison made with the reported values, the compound was concluded to be pinoresinol (Abe and Yamauchi, 1988; Cowan *et al*., 2001).

Further separation of the extract gave syringaresinol (**4**) as white needles with m.p. 171-172ºC (Abe and Yamauchi, 1988; m.p. 173-174 ºC) and molecular ion peak at m/z 418 which corresponded to the molecular formula $C_{22}H_{26}O_8$. A broad band observed at 3472 cm^{-1} in the IR spectrum indicated the presence of hydroxyl groups. The presence of 2,6-diaryl-3,7-dioxobicyclo[3.3.0]octane system is clearly indicated in the ^{1}H-NMR spectrum. A sharp singlet integrated for four protons occurred at δ 6.61 and it was assigned to the four equivalent aromatic protons at H-2, H-2', H-6 and H-6', while the other sharp singlet at δ 3.95 was due to the four methoxyl groups. The ^{13}C-NMR and DEPT spectra exhibited only 8 carbon resonances indicating the presence of a symmetrical lignan with equivalent aryl carbon signals of each aromatic ring. All the assignments of individual protons and carbons were further confirmed by the HSQC and HMBC experiments.

Subsequently, medioresinol (**5**) was also obtained as white prisms with m.p. 170-171ºC (Abe and Yamauchi, 1988; m.p. 170-172ºC). The ^{1}H-NMR spectrum of the compound also showed the characteristics of a pinoresinol-type lignan, which are very similar to the previous two, except the spectrum now showed resonances for five aromatic protons and three methoxyl groups. This suggested a non-symmetrical structure with the two aryl groups having different substitution patterns. The protons in one of the aromatic rings displayed an ABX system, with the occurrence of two doublets at δ 6.91 (*d*, 8.5 Hz) and 6.92 (*d*, 2.4 Hz) and a doublet of doublets at δ 6.84 (*dd*, 8.5, 2.4 Hz). In addition, the presence of 18 carbon resonances in the ^{13}C-NMR spectrum, instead of 10 resonances as previously shown by pinoresinol, also supported this deduction. The positions of the hydroxyl and methoxyl substituents were confirmed by ^1J correlation in HSQC and ^2J and ^3J correlations in the HMBC spectra. These data are in complete agreement with those reported in the literature for medioresinol (Abe and Yamauchi, 1988).

All the extracts of the bark and stem showed a strong superoxides cavenging activity, except the hexane extract of the bark. The antioxidant activity for the hexane, chloroform, and methanol extracts of the stem were found to be 95.2, 97.0, and 94.6%, respectively (*see* Table 2). Meanwhile, the inhibition values for the bark were 16.9, 76.5, and 96.3%. Similarly, all the extracts of the stem and bark indicated a strong activity towards the DPPH scavenging test system, except the two hexane extracts. The strong activity showed by these extracts might be due to the presence of phenolic compounds. When tested against inhibition of linoleic acid, the extracts only displayed a medium to weak activity. Nevertheless, the essential oils extracted from the leaves and bark failed to give any positive results. However, the two pure lignans were found to produce strong antioxidant properties and this finding supports the suggestion that the presence of phenolic compounds is essential for its activity. Lignans such as pinoresinol have been shown to possess strong antioxidant properties (Owen *et al.,* 2000). Hence, the presence of such compounds in *C. microphyllum* could be responsible for the antioxidant activity of the plant.

In summary, the separation of the chemical constituent present in the extracts of *C. microphyllum* had resulted in the isolation of six compounds consisting of three lignans, a coumarin, an ester, and β-sitosterol. Most of the stem and bark extracts were strongly antioxidant against superoxide and DPPH scavenging tests. Two of the lignans, namely pinoresinol (**3**) and medioresinol (**5**), were excellent candidates as antioxidant agents with their strong activities.

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