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Free radical-scavenging activity of organic extracts and of pure flavonoids of *Blumea balsamifera* DC leaves

Fazilatun Nessa [a,1], Zhari Ismail [a,1], Nornisah Mohamed [a,1], Mas Rosemal Hakim Mas Haris [b,*]

[a] *School of Pharmaceutical Sciences Universiti Sains Malaysia, 11800 Penang, Malaysia*
[b] *School of Chemical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*

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Abstract

Phytochemical investigation on the leaves of *Blumea balsamifera* DC resulted in the isolation of 11 flavonoids. Their chemical structures were elucidated by means of elemental analyses and different spectroscopic methods, such as UV, IR, NMR and MS. The free radical scavenging activity of organic extracts of *B. balsamifera* DC leaves and that of pure flavonoids isolated from the leaves was evaluated using 1,1-diphenyl-2-picrylhydrazyl radical. A dose response curve was plotted for determining SC_{50} values (the concentrations required to inhibit radical formation by 50%). The antioxidant activities of crude extracts decreased in the order: methanol extract > chloroform extract > pet-ether extract. The antioxidant activities of all compounds tested decreased in the order: quercetin > rhamnetin > luteolin > luteolin-7-methylether > L-ascorbic acid > blumeatin > butylated hydroxyanisole > 5,7,3′,5′-tetrahydroxyflavanone > tamarixetin > butylated hydroxytoluene > α-tocopherol > dihydroquercetin-4′-methylether > dihydroquercetin-7,4′-dimethylether. This result indicates that flavonoid contents of different solvent extracts of *B. balsamifera* DC leaves were responsible for their antioxidant properties.

Keywords: *Blumea balsamifera* DC; Antioxidants; Flavonoids; 1,1-Diphenyl-2-picrylhydrazyl radical

1. Introduction

Free radicals are atoms or molecules with an unpaired electron (Diplock, 1994), which are highly reactive and can initiate harmful chain reactions by extracting an electron from a neighbouring molecule (Halliwell, 1997). There is considerable evidence that free radicals induce oxidative damage to biomolecules and play an important role in cardiovascular disease, aging, cancer, inflammatory diseases, and a variety of other disorders (Finkel & Holbrook, 2000; Halliwell, Gutteridge, & Cross, 1992; Petrone, English, Wong, & McCord, 1980). Antioxidants that scavenge free radicals are now known to possess preventive as well as therapeutic potential in free radical-mediated disease conditions (Halliwell, 1997; Noguchi & Nikki, 2000; Visioli, Borsani, & Galli, 2000). These observations have accelerated the search for potential pharmacological antioxidant principles from traditional medicinal plants.

Flavonoids are known to possess the ability to scavenge free radicals, including superoxide and hydroxyl radicals (Husain, Cillard, & Cillard, 1987; Torel, Cillard, & Cillard, 1986), in foods (Shahidi & Wanasundara, 1992). Furthermore, the redox properties of flavonoids allow them to act as reducing agents and, in some cases, as chelating agents for transition metals (Rice-Evans & Miller, 1996; Van Acker et al., 1996). The free radical-scavenging properties of flavonoids can protect the human body from free radical-mediated diseases and epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer and inflammation (Hertog, Feskens, Hollman,

* Corresponding author. Tel.: +60-465-335-63; fax: +60-465-748-54.
E-mail addresses: mas1@usm.my, rhmas@tm.net.my (M.R.H.M. Haris).
[1] Tel.: +60-465-778-88; fax: +60-457-700-17.

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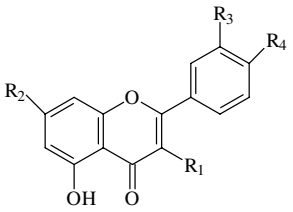
Katan, & Kromhout, 1993; Middleton, 1996; Middleton & Kandaswami, 1993; Verma, Johnson, Gould, & Tanner, 1998; Wei, Tye, Bresnick, & Birt, 1990). Moreover, in the food industry, addition of free radical scavenging antioxidants has been one of the effective ways of retarding fat oxidation. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are widely used. However, demands for natural food ingredients have continuously increased and there has been a general desire to replace synthetic food additives with natural alternatives (Branen, 1975). Unfortunately, known natural antioxidants, including tocopherols, are less effective than synthetic ones. This leads to interest and the need to identify new natural antioxidants for use as safe and effective additives in the food industry (Sherwin, 1990; Wanasundara & Shahidi, 1998).

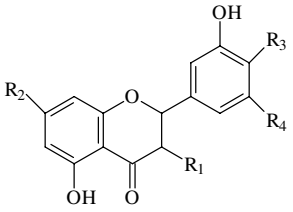
The study of known and new natural derivatives may support the development of new drugs and improve the treatment of various diseases. Use of *Blumea balsamifera* DC (Family: Compositae) leaves has been recommended as a folk medicine in the treatment of various diseases in south-east Asia (Zhari, Norhayati, & Jaafar, 1999). Several studies on the chemical constituents of *B. balsamifera* DC have been reported and a number of flavones, monoterpenes and triterpenes, have been isolated from this plant (Barua & Sharma, 1992; Fazilatun, Zhari, Nornisah, & Mas Rosemal, 2001; Lin, Long, & Deng, 1988; Ruangrungsi, Tappayuthpijaran, & Tantivatana, 1981).

Our present work was undertaken to further our study on the medicinal plant, *B. balsamifera* DC, found in Malaysia and to establish fairly comprehensive data on the chemical constituents of the leaves of this plant and their antioxidant properties. In our investigation we found a 3′,5′-substituted flavanone, similar to that found in the Chinese *B. balsamifera* DC, by Lin et al. (1988). In addition, we isolated velutin (I) and dihydroquercetin-7,4′-dimethylether (X) from pet-ether extracts, blumeatin (IX) and luteolin-7-methylether (VI) from chloroform extracts, and ombuine (II), tamarixetin (III), rhamnetin (IV), luteolin (V), luteolin-7-methylether (VI), quercetin (VII), 5,7,3′,5′-tetrahydroxyflavanone (VIII), blumeatin (IX) and dihydroquercetin-4′-methylether (XI) from methanol extracts. Earlier we reported the isolation of compounds I–VII (Fazilatun et al., 2001). Now, we report the isolation and structural determination, based on detailed analysis of UV, IR, 1D NMR, 2D NMR, EI-MS and ESI-MS spectral data, of four more flavonoids (VIII–XI). Also reported are the results of a study on the antioxidant activitis of different organic extracts and of pure flavonoids of *B. balsamifera* DC leaves in relation to known synthetic antioxidants. To date, this is the first report on free radical-scavenging effects of pure compounds obtained from the title plant (Fig. 1).



Structure A

Structure A	R_1	R_2	R_3	R_4
(I) Velutin	H	OCH_3	OCH_3	OH
(II) Ombuin	OH	OCH_3	OH	OCH_3
(III) Tamarixetin	OH	OH	OH	OCH_3
(IV) Rhamnetin	OH	OCH_3	OH	OH
(V) Luteolin	H	OH	OH	OH
(VI) Luteolin 7-methyl ether	H	OCH_3	OH	OH
(VII) Quercetin	OH	OH	OH	OH



Structure B

Structure B	R_1	R_2	R_3	R_4
(VIII) 5,7,3',5'-Tetrahydroxyflavanone	H	OH	H	OH
(IX) Blumeatin	H	OCH_3	H	OH
(X) Dihydroquercetin-7,4'-dimethylether	OH	OCH_3	OCH_3	H
(XI) Dihydroquercetin-4'-methylether	OH	OH	OCH_3	H

Fig. 1. Structures of flavonoids isolated from the leaves of *Blumea balsamifera* DC.

2. Materials and methods

2.1. General experimental methods

Melting points were determined on a Gallenkamp instrument and are uncorrected. FTIR spectra were recorded on a Bomem Hartmann and Braun, MB-Series instrument; UV spectra were recorded on a Hitachi U-2000 spectrophotometer. EI and ESI MS spectra were obtained from a Finnigan LC-Q Classic, Ion Trap Spectrometer and a Hewlett–Packard Mass Spectrometer (Model No. 5989A). ^{1}H and ^{13}C NMR spectra were acquired using Bruker Avance 300 and 400 MHz spectrometers equipped with 5 mm bore gradient-pulse inverse probeheads. Samples were dissolved in DMSO-d_6 and chemical shifts were recorded in δ (ppm) relative to that of TMS ($\delta = 0.00$ ppm).

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, BHT, BHA, (+)-α-tocopherol and methanol (spectroscopic grade) were purchased from Sigma Chemical Co.

(USA). Chromatography of samples was carried out on Merck silica gel and Sephadex LH-20. AR grade solvents were used for the extraction and chromatographic analysis.

2.3. Extraction and separation

The leaves of *B. balsamifera* DC (herbarium voucher specimen, FRI 57083 Botany unit of The Forest Research Institute of Malaysia) (6.5 kg) were oven dried at 40 °C for 6 days. They were then crushed into powder and subsequently extracted with pet-ether (60–80 °C), chloroform and methanol. After removal of the solvent by evaporation under reduced pressure, the yield of residue from pet-ether, chloroform and methanol extracts were about 4%, 2% and 5%, respectively. The crude pet-ether extracts (PEB) (15 g) were subjected to vacuum liquid chromatography (VLC) [silica gel 60 GF_{254}, E. Merck, 100 gm] with petroleum ether–ethyl acetate as the consecutive mobile phases to give I (107.7 mg) and X (108 mg). The crude chloroform extracts (CEB) (10 g) were subjected to VLC [silica gel 60 GF254, E. Merck, 100] with petroleum ether–ethyl acetate–methanol as the consecutive mobile phases to give VI (8 mg) and IX (215 mg). The crude methanol extracts (MEB) (35) were suspended in water (500 ml) and filtered. The resulting residues (15) were repeatedly subjected to VCL [silica gel 60 GF_{254}, 100] with petroleum ether–ethyl acetate–methanol as the consecutive mobile phases, on Sephadex LH-20 with chloroform–methanol as eluents and followed by preparative TLC to give compounds II (2.7 mg), III (8 mg), IV (10 mg), V (50.4), VI (94.6 mg), VII (20 mg), VIII (155 mg), IX (58 mg), and XI (233 mg).

2.4. Spectral data

5,7,3′,5′-Tetrahydroxyflavanone (**VIII**). M.p. 264–266 °C; $FeCl_3$ test: (+); UV max (MeOH): 330 (sh), 288, 208; NaOMe 322, 244, 212; $AlCl_3$ 322, 244, 212; $AlCl_3$/HCl 374, 307, 222, 206; NaOAc 323, 242 (sh), 229; NaOAc/H_3BO_3 328 (sh), 290, 211 nm; IR bands (KBr disc): 3259, 1617, 1461, 1308, 1262, 1182, 1084, 829, 729 cm^{-1}; ESI-MS Negative ion m/z (%): $M-1^-$ 287 (36), 151 (56), 135.1 (22); Positive ion m/z (%): $M+1^+$ 289 (27). Elemental analysis: found, C 62.03, H 4.42; calculated for $C_{15}H_{14}O_6$, C 62.51, H 4.19. ^{1}H NMR δ (400 MHz, DMSO): 2.67 (1H, dd, $J = 17.1$ Hz, 3.0 Hz, H-3a), 3.19 (1H, dd, $J = 17.1$, 12.5 Hz, H-3b); 5.37 (1H, dd, $J = 12.5$ Hz, 3.0 Hz, H-2), 5.88 (2H, s, H-6 and H-8), 6.75 (2H, s, H-2′ and H-6′), 6.88 (1H, s, H-4′), 9.04 (1H, s, 5′-OH), 9.09 (1H, s, 3′-OH), 10.81 (1H, s, 7-OH) and 12.97 (1H, s, 5-OH); ^{13}C NMR δ (100 MHz, DMSO): 196.5 (C4), 166.7 (C7), 163.6 (C9), 163 (C5), 145.8 (C3′ or C5′), 145.3 (C5′ or C3′), 129.6 (C1′), 118.1 (C4′), 115.5 (C6′), 114.5 (C2′), 101.9 (C10), 95.9 (C6), 95.1 (C8), 78.5 (C2) and 42.1 (C3). The assignments of ^{13}C chemical shifts were confirmed by HMQC experiment.

Blumeatin (**IX**). M.p. 218–220 °C. $FeCl_3$ test: (+); UV max (MeOH): 331 (sh), 287, 228 (sh), 207; NaOMe 331 (sh), 287, 243 (sh), 213; $AlCl_3$ 377, 309, 211; $AlCl_3$/HCl 375, 308, 223, 206; NaOAc 331 (sh), 287, 229; NaOAc/$H_3BO_3$331 (sh), 287, 231 nm; IR bands (KBr disc): 3215, 1620, 1451, 1388, 1302, 1205, 1088, 826, 728, 562 cm^{-1}; EI-MS m/z (%): M^+ 302 (57), 285 (6), 243 (3), 193 (30), 180 (57), 167 (100), 136 (29), 95 (14), 67 (4). Elemental analysis: found, C 63.41, H 4.69; calculated for $C_{16}H_{14}O_6$, C 63.57, H 4.67. ^{1}H NMR δ (400 MHz, DMSO): 2.72 (1H, dd, $J = 2.9$, 17.1 Hz, H-3a), 3.25 (1H, dd, $J = 12.5$, 17.1 Hz, H-3b), 3.787 (3H, s, 7-OCH_3), 5.42 (1H, dd, $J = 2.9$, 12.5 Hz, H-2), 6.07 (1H, d, $J = 2$ Hz, H-6), 6.09 (1H, d, $J = 2$ Hz, H-8), 6.75 (2H, s, H-2′ and H-6′), 6.88 (1H, s, H-4′); 9.05 (1H, s, 5′-OH), 9.10 (1H, s, 3′-OH) and 12.11 (1H, s, 5-OH); ^{13}C NMR δ (100 MHz, DMSO): 197.1 (C4), 167.5 (C7), 163.3 (C5), 162.9 (C9), 145.9 (C3′ or C5′), 145.3 (C5′ or C3′), 129.4 (C1′), 118.1 (C4′), 115.4 (C6′), 114.5 (C2′), 102.7 (C10), 94.7 (C6), 93.9 (C8), 78.8 (C2), 42.2 (C3) and 56.0 (7-OCH_3). The assignments of ^{13}C chemical shifts were confirmed by XHCORR experiment.

Dihydroquercetin-7,4′-dimethylether (**X**). M.p.165–167 °C; $FeCl_3$ test: (+); UV max (MeOH): 335 (sh), 288, 229 (sh), 206; NaOMe 354, 289, 245 (sh), 212; $AlCl_3$ 382, 314, 223, 206; $AlCl_3$/HCl 380 (sh), 309, 285 (sh), 223, 206; NaOAc 357 (sh), 288, 228; NaOAc/H_3BO_3 335 (sh), 288, 230 nm; IR bands (KBr disc): 3427, 1637, 1512, 1451, 1282, 1142, 1087, 1014, 815 cm^{-1}; ESI-MS Positive ion m/z (%): $M+Na^+$ 355 (27), $M+H^+$ 333 (23), 315 (23), 287 (64), 259 (28), 209 (23), 167 (35), 163 (14), 137 (16). Elemental analysis: found, C 61.24, H 4.89, calculated for $C_{17}H_{16}O_7$, C 61.44, H 4.85. ^{1}H NMR δ (400 MHz, DMSO): 3.783 (3H, s, 4′-OCH_3); 3.788 (3H, s, 7-OCH_3), 4.57 (1H, dd, $J = 6.3$ and 11.3 Hz, H-3), δ 5.08 (1H, d, $J = 11.3$ Hz, H-2), 5.86 (1H, d, $J = 6.3$ Hz, 3-OH), 6.08 (1H, d, $J = 2.1$, H-6), 6.11 (1H, d, $J = 2.1$ Hz, H-8), 6.90 (1H, d, $J = 1.5$ Hz, H-6′), 6.92 (1H, s, H-2′), 6.94 (1H, d, $J = 1.5$ Hz, H-5′), 9.08 (IH, s, 3′-OH) and 11.86 (1H, s, 5-OH). ^{13}C NMR δ (100 MHz, DMSO): 198.3 (C4), 167.7 (C7), 163.1 (C5), 162.5 (C9), 148.1 (C4′), 146.3 (C3′), 129.6 (C1′), 119.4 (C6′), 115.2 (C2′), 111.9 (C5′), 101.5 (C10), 95.0 (C6), 93.9 (C8), 83.1 (C2), 71.7 (C3), 56.1 (7-OCH_3) and 55.8 (4′-OCH_3). The assignments of ^{13}C chemical shifts and the stereo dynamic positions of the methoxyl groups were confirmed by HMQC and NOESY experiments, respectively.

Dihydroquercetin-4′-methylether (**XI**). M.p. 172–174 °C; $FeCl_3$ test: (+); UV max (MeOH): 331 (sh), 289, 229 (sh), 209; NaOMe 324, 245 (sh), 213; $AlCl_3$ 378 (sh), 314, 285 (sh), 223, 207; $AlCl_3$/HCl 374 (sh), 311, 282 (sh), 223, 207; NaOAc 325, 285 (sh), 223, 207; NaOAc/H_3BO_3 331 (sh), 293, 231 nm; IR bands: 3249, 1622, 1460, 1263, 1153, 1081, 1023, 800 cm^{-1}; ESI-MS

Positive ion m/z (%): $M+1^+$ 319 (11), 301 (100), 273 (40), 256 (47), 240 (6), 212 (19), 195 (21), 153 (76) 137 (8), 124 (7). Elemental analysis: found, C 60.10, H 4.47; calculated for $C_{17}H_{16}O_7$, C 60.37, H 4.43. ^{1}H NMR δ (400 MHz, DMSO): 3.78 (3H, s, 4′-OCH_3), 4.52 (1H, dd, $J = 6.1$ and 11.2 Hz, H-3), 5.03 (1H, d, $J = 11.2$ Hz, H-2), 5.80 (1H, d, $J = 6.1$ Hz, 3-OH); 5.86 (1H, d, $J = 2.1$ Hz, H-6), 5.91 (1H, d, $J = 2.1$ Hz, H-8), 6.86 (1H, d, $J = 1.5$ Hz, H-6′), 6.89 (1H, s, H-2′), 6.94 (1H, d, $J = 1.5$ Hz, H-5′), 9.07 (1H, s, 3′-OH), 10.86 (IH, s, 7-OH) and 11.89 (1H, s, 5-OH); ^{13}C NMR δ (100 MHz, DMSO): 199.9 (C4), 169.1 (C7), 165.6 (C5), 164.8 (C9), 150.3 (C4′), 148.5 (C3′), 132.1 (C1′), 121.5 (C6′), 117.4 (C2′), 114.1 (C5′), 102.8 (C10), 98.3 (C6), 97.3 (C8), 85.2 (C2), 73.9 (C3) and 58.1 (4′-OCH_3). The assignments of ^{13}C chemical shifts were confirmed by HMQC experiment.

2.5. Measurement of antioxidant activity

The antioxidant activity of different organic solvent extracts (PEB, CEB and MEB) of *B. balsamifera* DC leaves and the isolated pure flavonoids, tamarixetin (III), rhamnetin (IV), luteolin (V), luteolin-7-methylether (VI), quercetin (VII), 5,7,3′,5′-tetrahydroxyflavanone (VIII), blumeatin (IX), dihydroquercetin-7,4′-dimethylether (X) and dihydroquercetin-4′-methylether (XI) were measured in terms of radical-scavenging ability, according to the method of von Gadow, Joubert, and Hansmann (1997). BHT, BHA, α-(+)-tocopherol and L-ascorbic acid were used as reference compounds. A methanolic solution of the antioxidant (50 μl) at different concentrations was placed in a cuvette and 2 ml of 23.7-μg/ml methanolic solution of DPPH radical was added. Absorbance measurement commenced immediately. The decrease in absorbance at 517 nm was determined continuously with data capturing at 30 second intervals with a Hitachi-2000 spectrophotometer and Data Capture software, until absorbance stabilized (±30 min). A blank sample (without antioxidant), containing the same amount of methanol and DPPH radical, was prepared and measured daily. DPPH radical standard solution was freshly prepared daily and kept in a flask protected from light with aluminium foil and stored at 4 °C between the measurements. All determinations were performed in three replicates. The percent inhibition of DPPH radical by the samples was calculated according to the following formula of Yen and Duh (1994):

$$\text{Percent inhibition} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100,$$

where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance of the antioxidant at $t = 30$ min. A dose response curve was plotted for determining the SC_{50} values. SC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity (Choi et al., 2002).

2.6. Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Tukey's test ($p < 0.05$) for multiple comparisons using Jandel SigmaStat for windows, Statistical Software (Version 2.0, USA).

3. Results and discussion

3.1. Structure determination of isolated compounds

5,7,3′,5′-Tetrahydroxyflavanone (**VIII**) is a white crystalline powder, m.p. 264–266 °C. Its molecular formula $C_{15}H_{14}O_6$ is confirmed by ESI-MS negative ions (287.1, 151, 135.1) spectral, ESI-MS positive ion (289.1) spectral and elemental (found, C 62.03%, H 4.42 %; calculated, C 62.51%, H 4.19%) analyses. Its UV (288 nm, band II) and ^{1}H NMR [δ 2.67 (1H, dd, $J = 17.1$ Hz, 3.0 Hz, H-3a), δ 3.19 (1H, dd, $J = 17.1$ Hz, 12.5 Hz, H-3b) and δ 5.37 (1H, dd, $J = 12.5$ Hz, 3.0 Hz, H-2)] spectral data (an AMX coupling system) show characteristics of a flavanone. The presence of cross peaks in its COSY 45 (^{1}H–^{1}H) spectrum confirms the distinctive couplings between H-2, which is attached to the chiral centre, and the adjacent diastereotopic protons, H-3a ($J^0_{60} = 3.0$ Hz) and H-3b ($J^0_{180} = 12.5$ Hz). Its IR spectrum shows a strong absorption band at 1617 cm^{-1} due to a chelated carbonyl group. Its UV spectrum exhibits characteristics of 5,7-dihydroxyflavanone (Mabry, Markham, & Thomas, 1970). The bathochromic shift of band II (36 nm) with sodium acetate suggests the presence a free hydroxyl group at the C-7 position (Mabry et al., 1970). The presence of a free hydroxyl group at the C-5 position is indicated by the presence of bathochromic shifts of band I (47 nm) and of band II (34 nm) and no hypsochromic shift of bands upon addition of hydrochloric acid (Mabry et al., 1970). Its ^{1}H NMR spectrum exhibits five aromatic protons: two are for H-6 and H-8 of the A ring, which overlapped and appeared as a singlet integrating for two protons at δ 5.88, and the remaining three protons are for H-2′, H-6′ and H-4′ of the B ring that appeared at δ 6.75 (overlapped, s, 2H) and δ 6.88 (s, 1H), respectively, suggesting VIII as 5,7,3′,5′-tetrahydroxyflavanone. Its negative ion ESI-MS spectrum shows peaks at m/z 287.1, 151 and 135, which are ascribed to $[M–H]^+$, $[A_1–H]^+$ and $[B_3–H]^+$ species (ions), respectively, suggesting the presence of two hydroxyls in both A and B rings. The heteronuclear multiple quantum correlation (HMQC) spectrum reveals the presence of cross peaks for H-6, H-8, H-2′, H-6′ and H-4′ with their corresponding carbons. Assignments of ^{13}C chemical shifts were made with reference to its XHCORR spectrum. Thus, based on

elemental analysis and spectral (UV, IR, NMR and MS) information, VIII is characterized as 5,7,3′,5′-tetrahydroxyflavanone. We note here that the isolation and characterization of VIII from the leaves of *B. balsamifera* DC, to date, has never been reported.

Blumeatin (**IX**) is an off-white crystalline powder, m.p. 218–220 °C (MeOH). Its molecular formula $C_{16}H_{14}O_6$ is confirmed by EI-MS ($M^+ = 302$) spectral and elemental (found, C 63.41%, H 4.69 %; calculated, C 63.57%, H 4.67%.) analyses. Its UV spectrum in methanol shows one major absorption peak at 287 nm (band II), suggesting that compound IX was either a flavanone or a dihydroflavonol derivative (Mabry et al., 1970). Its ^{1}H NMR spectrum distinguishes it as the former derivative, based on the characteristic resonances of the diastereotopic protons at δ 2.72 ppm (1H, dd, $J = 3.0$ and 17.1 Hz) and at δ 3.25 ppm (1H, dd, $J = 12.5$ and 17.1 Hz) for H-3a and H-3b, respectively, which were coupled to the resonance at δ 5.42 ppm (1H, dd, $J = 3.0$ and 12.5 Hz) for H-2. The homonuclear coupling between H-2 and H-3 is confirmed by the presence of cross peaks in its COSY 45 (^{1}H–^{1}H) spectrum. From its XHCORR spectrum, the resonances at δ 6.07 ppm (1H, d, $J = 2.1$ Hz) and δ 6.09 (1H, d, $J = 2.1$ Hz) are assignable to protons attached to carbons C-6 and C-8, respectively, of the A ring, and that at δ 6.75 ppm (2H, overlapped s) is assignable to protons attached to carbons C-2′ and C-6′, while that at δ 6.88 ppm (1H, s) is assignable to a proton attached to carbon C-4′ of the B ring. The resonance at δ 3.78 ppm (3H, s) is ascribed to the methoxyl protons located at C-7, which is confirmed by the presence of cross peaks due to the through-space interactions between the methoxyl protons and the aromatic protons at C-6 and C-8 as observed in its NOESY spectrum. The EI-MS spectrum shows peaks at 302 $[M]^+$, 167 $[A_1 + H]^+$ and 136 $[B_3]^+$ which affirmed the presence of one methoxyl on the A ring and two hydroxyls on the B ring. The pronounced bathochromic shift of band I (46 nm) and band II (22 nm) with aluminium chloride and no hypsochromic shift of absorption bands upon addition of hydrochloric acid in the presence of aluminium chloride suggests the presence of a free hydroxyl at C-5 position (Mabry et al., 1970). The absence of bathochromic shift of band II upon addition of sodium acetate and sodium methoxide indicates the absence of a free hydroxyl at the C-7 position (Mabry et al., 1970). Hence, and with comparison to the ^{1}H NMR spectra of VIII and X, the proton resonances at δ 9.10 and δ 9.05 ppm are assignable to the hydroxyls attached to C-3′ and C-5′ of the B ring, respectively, and the proton resonance at δ 12.11 ppm is assignable to the hydroxyl attached to C-5 of the A ring. Assignments to the corresponding carbons were made with the aid of its XHCORR spectrum. Thus, based on elemental analysis and spectral (UV, IR, NMR and MS) information, the structure of compound IX is deduced to be 5,3′,5′-trihydroxy-7-methoxyflavanone, namely Blumeatin (Lin et al., 1988).

Dihydroquercetin-7,4′-dimethylether (**X**) is a white crystalline powder, m.p.165–167 °C (MeOH). Its molecular formula $C_{17}H_{16}O_7$ is confirmed by positive ion ESI-MS ($MH^+ = 333$) spectral and elemental (found, C 61.24%, H 4.89%; calculated, C 61.44%, H 4.85%) analyses. Its UV spectrum (285 nm, band II) in methanol suggests it is either a flavanone or dihydroflavonol derivative (Mabry et al., 1970). However, based on of the splitting pattern and relative abundance of the resonances at δ 4.58 ppm (1H, dd, $J = 6.3$ and 11.3 Hz) for H-3, δ 5.08 ppm (1H, d, $J = 11.3$ Hz) for H-2 and δ 5.85 ppm (1H, d, $J = 6.3$ Hz) for the hydroxyl proton (3-OH) as observed in its ^{1}H NMR spectrum, it is likely the latter derivative. The bathochromic shift of band II with aluminium chloride (26 nm) and with hydrochloric acid (23 nm) suggests the presence of a free hydroxyl group at C-5 of the A ring (Mabry et al., 1970). The absence of bathochromic shift of band II with sodium methoxide and sodium acetate indicates the absence of a free hydroxyl at C-7 position (Mabry et al., 1970). In its ^{1}H NMR spectrum, the A ring protons that appeared at 6.90 (1H, d, $J = 1.5$ Hz), 6.92 (1H, s) and 6.94 (1H, d, $J = 1.5$ Hz) are assigned to H-6′, H-2′ and H-5′, respectively (confirmed by from its HMQC spectrum), and the B ring protons that appeared at δ 6.09 (d, $J = 2.2$ Hz) and δ 6.12 (1H, d, $J = 2.2$ Hz) are assigned to H-6 and H-8, respectively (confirmed from its HMQC spectrum). Assignment of the hydroxyl resonance at δ 5.85 ppm (d, $J = 6.3$ Hz) is based on the analysis of the COSY 45 (^{1}H–^{1}H), D_2O-experiment and HMQC spectra. In the COSY 45 (^{1}H–^{1}H) spectrum, cross peaks are discernible between H-2 and H-3 and between H-3 and the hydroxyl (3-OH). Upon addition of D_2O, the signal at δ 5.85 ppm disappeared confirming that rapid exchange with deuterium had taken place and that the signal was due to a hydroxyl proton. Finally, the HMQC spectrum shows that the proton signal at δ 5.85 ppm does not correlate with any of the carbons in the molecule. The overlapped signals (two singlets) at δ 3.783 and δ 3.788 ppm (integrating both for 6H) are assigned to the two methoxyl groups located at C-4′ and C-7, respectively. This assignment was confirmed by its NOESY spectrum. The methoxyl protons exhibit NOE with the protons on C-2′, 5′ and 6′ and with protons on C-6 and 8. Its positive ion ESI-MS spectrum shows peaks at m/z 333, 167 and 137 that correspond to $[M + H]^+$, $[A_1 + H]^+$, B_4^+ species, respectively. Based on all the above information and ^{13}C chemical shift assignments (noted in the experimental section), compound X is characterized as 3,5,3′-trihydroxy-7,4′-dimethoxyflavanone, namely dihydroquercetin-7,4′-dimethylether (Ruangrungsi et al., 1981).

Dihydroquercetin-4′-methylether (**XI**) forms white crystals, m.p.172–174 °C (MeOH). Its molecular

formula $C_{16}H_{14}O_7$ is confirmed by positive ion ESI-MS data ($MH^+ = 319$) and elemental analysis (found, C 60.10, H 4.47; calculated for $C_{17}H_{16}O_7$, C 60.37, H 4.43). In its UV spectrum, band II appeared as a major absorption peak at 285 nm. The pronounced bathochromic shift of band II upon addition of sodium acetate and sodium methoxide indicates the presence of a free hydroxyl at the C-7 position (Mabry et al., 1970). Addition of aluminium chloride and hydrochloric acid resulted in bathochromic shift of band II and band I by about 25 and 21 nm, respectively, characteristic of a second free hydroxyl at the C-5 position (Mabry et al., 1970). In its ^{1}H NMR spectrum, the appearance of H-3 at δ 4.52 ppm (1H, dd, $J = 6.1$, 11.2 Hz) and H-2 at δ 5.02 ppm (1H, d, $J = 11.2$ Hz) suggests that XI is a dihydroflavonol derivative. The doublet at δ 5.80 ppm with coupling constant of 6.1 Hz is attributable to the hydroxyl attached to C-3. This assignment is supported by the fact that the proton signal vanished upon addition of D_2O and did not show distinctive correlation with any carbon in the HMQC spectrum. Further, the homonuclear couplings of H-3 to both H-2 ($J = 11.2$ Hz) and 3-OH ($J = 6.1$ Hz) are confirmed by the presence of the distinctive cross peaks in the COSY 45 (^{1}H–^{1}H) spectrum. The AB system doublets at δ 5.86 and δ 5.91 ppm characteristic of meta couplings ($J \approx 2.1$ Hz) are assignable to protons at C-6 and C-8, respectively, in a dihydroflavonol oxygenated at C-5 and C-7. The assignment of the resonances at 6.86 (1H, d, $J = 1.5$ Hz), 6.89 (1H, s), 6.94 (1H, d, $J = 1.5$ Hz) to H6′, H-2′ and H-5′, respectively, is confirmed by analyzing its HMQC spectrum. The singlet at δ 3.78 ppm, integrated for three protons, is assigned to the methoxyl protons located at C-4′ as confirmed by the presence of cross peaks (in its NOESY spectrum) between the methoxyl protons and the aromatic protons at C-2′, C-5′ and C-6′. The positive ion ESI-MS spectrum shows fragmental peaks at m/z 153 and 137 assignable to $[A_1 + H]^+$ and $[B_4 + H]^+$ species, respectively, suggesting that the methoxyl group is on the B ring. In the ^{13}C NMR spectrum, the well-distinguished signals at δ 85.1 and δ 73.9 ppm are characteristic of a dihydroflavonol derivative, and they are assignable to C-2 and C-3, respectively. Thus, compound XI is characterized as 3,5,7,3′-tetrahydroxy-4′-methoxyflavanone, namely dihydroquercetin-4′-methylether (Ruangrungsi et al., 1981).

3.2. Antioxidant activity of solvent extracts of B. balsamifera DC leaves according to the DPPH radical-scavenging method

The DPPH radical has been widely used to test the free radical-scavenging ability of various natural products (Brand Williams, Cuvelier, & Berset, 1995; Chen, Wang, Rosen, & Ho, 1999; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999) and has been accepted as a model compound for scavenging free radicals in lipids. In the DPPH radical-scavenging method, a compound with high antioxidant potential effectively traps the radical, thereby preventing its propagation and the resultant chain reaction (Brand Williams et al., 1995; Yen & Duh, 1994).

The free radical-scavenging activities of different solvent extracts of *B. balsamifera* DC leaves were determined using the DPPH assay as shown in Fig. 2. The results, expressed as SC_{50} values, were calculated by regression analysis as presented in Table 1 (the slopes and correlation coefficients of the regression lines are also presented in the table). The methanol extract (MEB) and chloroform extract (CEB) showed potent free radical-scavenging activity on the DPPH radical compared to pet-extract (PEB) and, overall, the activity decreased in the following order: α-tocopherol > MEB > CEB > PEB. α-Tocopherol was used as reference compound. The high SC_{50} value of PEB indicates that it is a poor free radical scavenger. There was a statistically significant difference ($p < 0.05$) in SC_{50} values in antioxidant activity between extracts and α-tocopherol, but there was no significant difference ($p < 0.05$) in SC_{50} values between CEB and MEB. The high polyphenolic contents of MEB and CEB could be responsible for the high antioxidant activity.

The free radical-scavenging activity of the extracts is attributed to their hydrogen donating ability (Shimada, Fujikawa, & Nakamura, 1992). It is well known that free radicals cause auto-oxidation of unsaturated lipids in food (Kaur & Perkins, 1991). On the other hand, antioxidants are believed to intercept the free radical chain during oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of the lipid (Sherwin, 1978). Our data reveal that the methanol and chloroform extracts of *B. balsamifera* leaves are free radical inhibitors and primary antioxidants that react with free radicals.

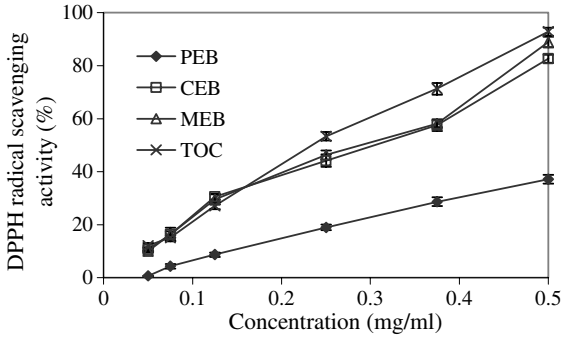


Fig. 2. Free radical-scavenging activities of different solvent extracts [PEB (pet-ether), CEB (chloroform) and MEB (methanol)] of *B. balsamifera* leaves measured using the DPPH-assay. Results are means ± SD ($n = 3$). (+)-α-Tocopherol (TOC) was used as reference compound.

Table 1
SC_{50} values of different solvent extracts of *Blumea balsamifera* leaves for scavenging of free radicals, as assessed by DPPH radical-scavenging method

Solvent extracts of *Blumea balsamifera* DC leaves	SC_{50} (mg/ml) ± SD[a]	*m*	*r*
MEB (methanol extract)	b*0.282 (3)[b] ± 0.002	162	0.97
CEB (chloroform extract)	b0.294 (2) ± 0.001	149	0.98
PEB (Pet-ether extract)	c1.13 (1) ± 0.013	21.4	0.99
TOC [(+)-α-tocopherol]	a0.255 (4) ± 0.001	182	0.99

Means ± SD ($n = 3$).
[a] The concentration sufficient to obtain 50% of a maximum scavenging capacity (procedure described in materials and methods). SC_{50} values were calculated from regression lines, where *m* is slope of the regression line; *r* is correlation coefficient.
[b] Figures in parentheses denote rank in ascending order of antioxidant activity.
* Means with different small letters within the column are significantly different ($p < 0.05$).

3.3. Antioxidant activity of flavonoids of B. balsamifera DC leaves according to the DPPH radical-scavenging method

The DPPH scavenging activity-directed fractionation and isolation experiment confirmed the presence of flavonoids velutin (I) and dihydroquercetin-7,4′-dimethylether (X) in pet-ether extracts, and blumeatin (IX) and luteolin-7-methylether (VI) in chloroform extracts, ombuine (II), tamarixetin (III), rhamnetin (IV), luteolin (V), luteolin-7-methylether (VI), quercetin (VII), 5,7,3′,5′-tetrahydroxyflavanone (VIII), blumeatin (IX) and dihydroquercetin-4′-methylether (XI) in methanol extracts. This is the first report of the presence of these six (III–VIII) antioxidant principles in *B. balsamifera* leaves. The structures of the compounds were confirmed by spectroscopic evidence and by comparing the data with the literature values (Fazilatun et al., 2001).

In Figs. 3–5, the scavenging of DPPH radicals, due to their reduction by different concentrations of antioxidants, is illustrated. In this assay, absorbance at 517 nm decreases as a result of colour changes from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH–H. The more rapidly the absorbance decreases, the more potent is the antioxidant activity of the compound in terms of hydrogen donating ability (Yen & Duh, 1994). Almost no decrease in absorbance occurred with the addition of dihydroquercetin-7,4′-dimethyl ether or dihydroquercetin-4′-methyl ether.

The dose–response studies of the DPPH scavenging reaction of tested compounds and extract, at various concentrations in 30 min reaction, showed that the steady state of the reaction was mostly related with concentration and period of reaction time. L-Ascorbic acid reacted with DPPH immediately and reached a

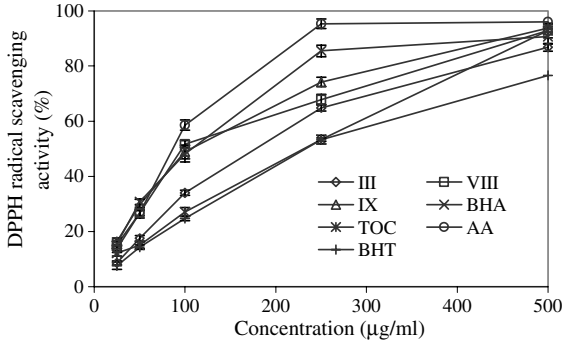


Fig. 3. Free-radical-scavenging activity of tamarixetin (III), 5,7,3′,5′-tetrahydroxyflavanone (VIII) and blumeatin (IX) found in *B. balsamifera* leaves measured using the DPPH assay. Results are means ± SD ($n = 3$). (+)-α-Tocopherol (TOC), BHT, BHA and L-Ascorbic acid (AA) were used as reference compounds.

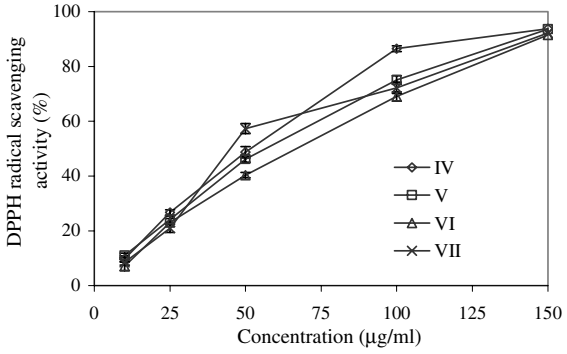


Fig. 4. Free radical-scavenging activities of rhamnetin (IV), luteolin (V), luteolin-7-methylether (VI) and quercetin (VII) found in *B. balsamifera* leaves measured using the DPPH assay. Results are means ± SD ($n = 3$).

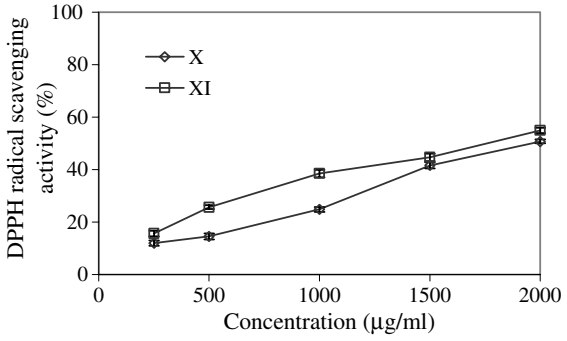


Fig. 5. Free radical-scavenging activities of dihydroquercetin-7,4′-dimethylether (X) and dihydroquercetin-4′-methylether (XI) found in *B. balsamifera* leaves measured using the DPPH assay. Results are means ± SD ($n = 3$).

steady state within 1 min (Brand Williams et al., 1995). Quercetin, rhamnetin, luteolin and luteolin-7-methyl ether reacted rapidly with DPPH radical at concentration ranges from 100–150 μg/ml. Blumeatin and 5,7,3′,5′-tetrahydroxyflavanone at 250–500 μg/ml reached a steady state within 1 min.

The SC_{50} value of each flavonoid was determined on the basis of a 30 min reaction time by regression analysis from its corresponding graph, as depicted in Figs. 3–5. The results are presented in Table 2. There was no significant difference ($p < 0.05$) in SC_{50} values in antioxidant activity between blumeatin, BHA, and 5,7,3′,5′-tetrahydroxyflavanone; ascorbic acid and blumeatin or luteolin and luteolin-7-methyl ether. Overall, the antioxidant activity decreased in the order: quercetin > rhamnetin > luteolin > luteolin-7-methyl ether > L-ascorbic acid > blumeatin > BHA > 5,7,3′,5′-tetrahydroxyflavanone > tamarixetin > BHT > α-tocopherol > dihydroquercetin-4′-methyl ether > dihydroquercetin-7,4′-dimethyl ether. BHT, BHA, L-ascorbic acid and α-tocopherol were used as reference compounds.

Flavonoids with free hydroxyl groups act as free radical-scavengers, and multiple hydroxyl groups, especially on the B-ring, enhance their antioxidant activity (Jovanovic, Steenken, Tosic, Marjanovie, & Simic, 1994). Flavonols showed higher antioxidant activities than the corresponding flavones (quercetin > luteolin) and, in contrast, flavanone derivatives showed comparatively higher antioxidant activity than dihydroflavonol derivatives (blumeatin > 5,7,3′,5′-tetrahydroxyflavanone > dihydroquercetin-4′-methyl ether > dihydroquercetin-7,4′-dimethyl ether), but showed lower activity than flavones and flavonols in our assay procedures.

Quercetin and rhamnetin showed high antioxidant activity than tamarixetin. It seems that the presence of 3′,4′-hydroxyls group was essential for high antioxidant activity and the presence of a methoxyl group at the C-4′ position greatly reduced its activity as observed in our experiment (quercetin > rhamnetin > tamarixetin). Luteolin, bearing four hydroxyls group, showed less activity than quercetin, suggesting that the presence of the 3-hydroxyl group was essential for high antioxidant activity (quercetin > luteolin). The presence of methoxyl at the C-7 position tended to reduce the free radical-scavenging activity, as indicated by the experimental results: quercetin > rhamnetin; luteolin > luteolin-7-methyl ether and dihydroquercetin-4′-methyl ether > dihydroquercetin-7,4′-dimethyl ether. However, we found no statistically significant difference ($p < 0.05$) in SC_{50} values in antioxidant activity between 5,7,3′,5′-tetrahydroxyflavanone and blumeatin, or luteolin and luteolin-7-methyl ether (Table 2).

5,7,3′,5′-Tetrahydroxyflavanone and blumeatin, bearing hydroxyl group at C-3′ and 5′ and having no C2–C3 double bond, exhibited comparatively higher antioxidant activity than tamarixetin, a flavonol. It appears that the presence of the C2–C3 double bond is not essential for DPPH radical-scavenging ability. Also, dihydroflavonol derivatives reacted with DPPH radical poorly compared with their corresponding flavanone derivatives. This finding suggests that the absence of the unsaturated bond at C2–C3, and the presence of the 3-OH group, greatly reduced the DPPH radical-scavenging ability (blumeatin > 5,7,3′,5′-tetrahydroxyflavanone > dihydroquercetin-4′-methyl ether > dihydroquercetin-7,4′-dimethyl ether). However, in light of conflicting reports by other researchers (Hudson & Lewis, 1983; Husain et al., 1987; Pratt & Hudson, 1990), we believe more data are required to better understand the roles of the double bond at C2–C3 on the antioxidant and free radical-scavenging activities.

The weaker radical-quenching abilities observed for the compounds in this test may be ascribed to their molecular structures. Compounds having higher num-

Table 2
SC_{50} values of flavonoids of *Blumea balsamifera* leaves as assessed by DPPH radical-scavenging method

Compounds	SC_{50} (μg/ml) ± SD[a]	*m*	*r*
Tamarixetin (III)	g*145 (5)[b] ± 0.52	27.0	0.97
Rhamnetin (IV)	b55.3 (12) ± 0.51	0.86	0.99
Luteolin (V)	c67.0 (11) ± 0.83	0.58	0.97
Luteolin-7-methyl ether (VI)	c73.4 (10) ± 0.74	0.58	0.98
Quercetin (VII)	a44.8 (13) ± 0.53	31.6	0.96
5,7,3′,5′-Tetrahydroxyflavanone (VIII)	f97.3 (6) ±1.87	22.2	0.96
Blumeatin (IX)	def90.8 (8) ±1.82	22.9	0.97
Dihydroquercetin-7,4′-dimethyl ether (X)	k1992 (1) ±8.74	0.02	0.98
Dihydroquercetin-4′-methyl ether (XI)	j1795 (2) ±5.82	18.2	0.98
BHA	e93.9 (7) ±1.48	27.1	0.97
BHT	h205 (4) ±2.73	23.5	0.95
(+)-α-Tocopherol (TOC)	i254 (3) ± 0.40	0.17	0.99
L-Ascorbic acid (AA)	d84.1 (9) ± 0.628	30.8	0.95

Means ± SD ($n = 3$).

[a] SC_{50} values were calculated from regression lines, where *m* is slope of the regression line; *r* is correlation coefficient.

[b] Figures in parentheses denote rank in ascending order of antioxidant activity.

* Means with different small letters within the column are significantly different ($p < 0.05$).

bers of phenolic hydroxyl groups exhibited stronger free radical-scavenging activities, which is in keeping with the notion that phenolic hydroxyl groups are able to donate hydrogens and that phenoxyl radicals, once formed, are stablilized by delocalization of electrons (Kovatcheva et al., 2001).

Based on the present results, it can be seen that the flavonoids of *B. balsamifera* DC except dihydroflavonol derivatives, exhibited more potent hydrogen-donating abilities than did BHA, BHT or α-tocopherol. Quercetin, rhamnetin, luteolin and luteolin-7 methyl ether showed higher antioxidant activity than ascorbic acid, a well-known antioxidant. It can be concluded that the flavonoid contents of different solvent extracts of *B. balsamifera* DC leaves contributed to their different antioxidant properties.

4. Conclusion

To date, we have isolated and characterized eleven flavonoids (I–XI, see Fig. 1) from the leaves of *Blumea balsamifera* DC. Details of the analysis of the UV, IR, NMR, EI-MS and ESI-MS spectral data of VIII–XI are presented in this paper. To the best of our knowledge, this is the first report of the isolation of VIII, namely 5,7,3′,5′-tetrahydroxyflavanone, from *B. balsamifera* DC leaves. The antioxidant activity results indicate that flavonoids present in *B. balsamifera* DC leaves extracts are potent antioxidants, comparable in activity with α-tocopherol and the widely used synthetic antioxidants BHT and BHA. Therefore, the leaves extracts can be used for a variety of beneficial chemo-preventive effects. Furthermore, this report suggests that *B. balsamifera* DC leaves extracts may provide a new source of natural food antioxidants. Investigations on the use of *B. balsamifera* DC leave extracts, as natural antioxidants, are in progress.

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