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Superoxide Radical Scavenging Properties of Extracts and Flavonoids Isolated from the Leaves of *Blumea balsamifera*

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Abstract

In view of the pharmacological interest in flavonoids, the superoxide radical scavenging capacity of pet-ether, chloroform, and methanol extracts and flavonoids of *Blumea balsamifera* DC leaves on nonenzymatically (phenazine methosulfate/NADH) generated superoxide radicals were evaluated. The methanol extract (93.91 ± 1.37%) exhibited higher radical scavenging activity than the chloroform extract (84.58 ± 1.51%). The pet-ether extract was inactive toward nonenzymatically generated superoxide radicals. The superoxide radical scavenging capacity of flavonoids (100 μM) as determined was decreased in the order quercetin > luteolin > 5,7,3′,5′-tetrahydroxyflavanone > blumeatin > rhamnetin > tamarixetin > luteolin-7-methyl ether > dihydroquercetin-4′-methyl ether > dihydroquercetin-4′,7-dimethyl ether. It was concluded that flavonoids with a free hydroxyl group were more active than methylated compounds, and the flavonoid content of extracts contributed to their superoxide radical scavenging activity.

Keywords: *Blumea balsamifera*, flavonoids, leaf extracts, superoxide radical scavenging activity.

Introduction

The superoxide radical is ubiquitous in aerobic cells (Cerutti, 1994). Although only mildly reactive toward biological molecules, the superoxide radical may be transformed to the more reactive and damaging hydroxyl radical in the Haber-Weiss and Fenton reactions (Aust et al., 1985; Babbs, 1985; Deby & Goutier, 1990). The healthy cell is capable of maintaining the balance of production and inactivation of the superoxide radical (Sies, 1993). However, the superoxide radical is overproduced in inflammation. If not inactivated by chemical or biochemical defenses, this excess superoxide may damage cells (Aust & Svingen, 1982; Tien et al., 1982; Deby & Goutier, 1990).

In view of the crucial role of superoxide anion ($O_2^{\bullet -}$) in the development of inflammation and age-related disease and the suggested role of superoxide generation inhibition as a contributing factor in the process (Murakami et al., 2000; Perry et al., 2000) we evaluated the superoxide scavenging capacities of crude extracts and flavonoids isolated from the leaves of *Blumea balsamifera* DC (Compositae). The leaves of this plant are locally used in folk medicine as stomachic, expectorant, antispasmodic, diaphoretic, after-birth medicine, and are also believed to be useful as a treatment for fever, lumbago, to increase appetite, skin diseases, wounds, liver cirrhosis, and in urolithiasis (Zhari et al., 1999). Phytochemical studies on the chemical constituents of *Blumea balsamifera* resulted in the isolation of flavonoids, that is, dihydroquercetin-7,4′-dimethyl ether (**1**), blumeatin (**2**), tamarixetin (**3**), rhamnetin (**4**), luteolin-7-methyl ether (**5**), luteolin (**6**), quercetin (**7**), 5,7,3′,5′-tetrahydroxyflavanone (**8**), and dihydroquercetin-4′-methyl ether (**9**) (Fig. 1). The beneficial role of herbal extracts in inflammation has long been studied, and it has been suggested that excess superoxide was eliminated by the flavonoids (Sichel et al., 1991). Because flavonoids are superoxide scavengers (Robak & Gryglewski, 1988; Cos et al., 1998; Nagao et al., 1999), this work describes the superoxide scavenging capacity of different solvent extracts of *Blumea balsamifera* leaves and their major flavonoids against nonenzymatically generated superoxide radicals. To date, no literature has reported on the superoxide scavenging properties of *Blumea balsamifera* leaves and their major flavonoids (Fig. 1).

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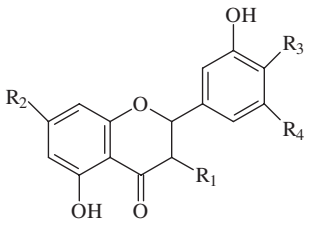
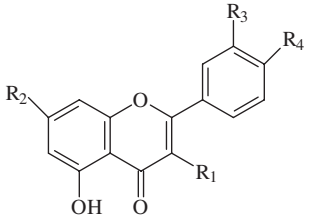


Structure A

Structure A	R_1	R_2	R_3	R_4
(**1**) Dihydroquercetin-7,4′-dimethyl ether	OH	OCH_3	OCH_3	H
(**2**) Blumeatin	H	OCH_3	H	OH
(**8**) 5,7,3′,5′-Tetrahydroxyflavanone	H	OH	H	OH
(**9**) Dihydroquercetin-4′-methyl ether	OH	OH	OCH_3	H



Structure B

Structure B	R_1	R_2	R_3	R_4
(**3**) Tamarixetin	OH	OH	OH	OCH_3
(**4**) Rhamnetin	OH	OCH_3	OH	OH
(**5**) Luteolin-7-methyl ether	H	OCH_3	OH	OH
(**6**) Luteolin	H	OH	OH	OH
(**7**) Quercetin	OH	OH	OH	OH

Figure 1. Structure of flavonoids isolated from the leaves of *Blumea balsamifera* studied for their scavenging capacity on nonenzymatically (phenazine methosulfate/NADH) generated superoxide radicals.

Materials and Methods

General experimental methods

Melting points were determined on a Gallenkamp and are uncorrected. Fourier-transform intrared (FTIR) spectra were recorded on a Bomem Hartmann and Braun, MB-Series; UV spectra were recorded on a Hitachi U-2000 spectrophotometer. Mass spectra (MS) (ESI/EI) were measured on a Finnigan LC-Q Classic, Ion Trap Spectrometer and Hewlett Packard Mass Spectrometer (model no. 5989A). ^{1}H NMR (DMSO-D_6) was measured on Bruker Avance 300 MHz and 400 MHz Spectrometers. Chemical shifts were recorded in δ (ppm) relative to that of tetramethylsilane (TMS) (δ = 0.00 ppm).

Chemicals

Sodium phosphate dibasic 12 hydrate (99%) and potassium phosphate monobasic (anhydrous, 99%), β-nicotinamide-adenine dinucleotide (β-NADH$^+$, disodium salt, ~98%), nitroblue tetrazolium (NBT, ~98%), and phenazine methosulfate (PMS) were all purchased from Sigma (St. Louis, MO, USA). Chromatography of samples was carried out on Merck silica gel or Sephadex LH-20. Analytical reagent (AR) grade (Merck, Dermstedt, Germany) solvents were used in the extraction and chromatographic analysis.

Plant material

The leaves of the plants were collected from the Kedah State, Malaysia, in 1999; a herbarium voucher specimen (FRI 57083) has been deposited in the botany unit of The Forest Research Institute of Malaysia.

Extraction and isolation

The leaves of *Blumea balsamifera* (6.5 kg) were oven-dried at 40 °C for 6 days. They were then crushed into powder and extracted with pet-ether (60–80 °C), chloroform, and methanol, subsequently. After removal of the solvent by evaporation under reduced pressure, the yield of residue from pet-ether, chloroform, and methanol extracts was 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 g] with petroleum ether-ethyl acetate (2 : 8) as the eluent to give **1** (107.7 mg). The crude chloroform extracts (CEB) (10 g) were subjected to VLC [silica gel 60 GF254, E. Merck, 100 g] with petroleum ether-ethyl acetate (1 : 1) as the eluent to give **2** (207.7 mg). The crude methanol extracts (MEB) (35 g) were suspended in water (500 ml) and filtered. The resulting residues (15 g) were repeatedly subjected to VLC [silica gel 60 GF_{254}, 100 g] with petroleum ether-ethyl acetate-methanol (8 : 1 : 1) as the eluent, and repeated chromatograph on Sephadex LH-20 with chloroform-methanol (19 : 1; 9 : 1, and 8 : 2) as eluents, to give **3** (38.9 mg), **4** (12 mg), **5** (50.8 mg), **6** (102.8 mg), **7** (19.5 mg), **8** (155 mg), and **9** (233 mg).

Determination of superoxide radical scavenging activity

The scavenging activity against chemically generated superoxide radicals of the crude extracts and flavonoids was measured by means of spectrophotometric measurement of the product on reduction of nitro blue tetrazolium (NBT) (Robak & Gryglewski, 1988). Test samples were dissolved in DMSO and diluted in water to give a final concentration of 12% (v/v) for DMSO (Hatano et al., 1991). Superoxide anions were generated in a nonenzymatic [phenazine methosulfate (PMS)/NADH] system. The reaction mixture contained 1 ml of test solution, 1.9 ml 0.1 M phosphate buffer, pH 7.4, 1 ml of 20 µM PMS, 156 µM NADH, and 25 µM of NBT in phosphate buffer, pH 7.4. After 2 min of incubation at 25 °C, the color was read on a Hitachi U-2000 spectrophotometer at 560 nm against blank samples, which contained no PMS. The percentage of scavenging activities (%) was calculated as follows:

Scavenging activities % (capacity to scavenge the superoxide radical) = [1 − (absorbance of sample at 560 nm)/(absorbance of control at 560 nm)] × 100.

Results and Discussion

The chemical structures of the compounds **1–9** were elucidated by means of different analytical methods such as UV, IR, NMR, MS, elemental analyses, and comparison with literature value as dihydroquercetin-7,4′-dimethyl ether (**1**) (Ruangrungsi et al., 1981), blumeatin (**2**) (Lin et al., 1988), tamarixetin (**3**) (Fazilatun et al., 2001), rhamnetin (**4**) (Fazilatun et al., 2001), luteolin-7-methyl ether (**5**) (Fazilatun et al., 2001), luteolin (**6**) (Fazilatun et al., 2001), quercetin (**7**) (Fazilatun et al., 2001), 5,7,3′,5′-tetrahydroxyflavanone (**8**) (Anthoni et al., 1998; Fazilatun et al., 2000), and dihydroquercetin-4′-methyl ether (**9**) (Ruangrungsi et al., 1981).

In determining the scavenging effects of extracts from the leaves of *Blumea balsamifera* against chemically generated superoxide radicals, three different solvent extracts, that is, pet-ether (PEB), chloroform (CEB), and methanol (MEB), were used. Three different concentrations (100, 250, and 500 µg/ml) were prepared for each extract as shown in Table 1. The superoxide radical scavenging activity of different organic extracts was measured by reduction of nitro blue tetrazolium (Robak & Gryglewski, 1988). Methanol extract (93.91 ± 1.37%) exhibited a higher radical scavenging activity than chloroform extract (84.58 ± 1.51%). Pet-ether extract was inactive toward chemically generated superoxide radicals. The scavenging activities of different solvent extracts of the leaves of *Blumea balsamifera* are presented in Figure 2.

It can be seen that various extracts of *Blumea balsamifera* leaves prepared by different solvents exhibited various degrees of antioxidant activity. The phytochemical studies on the leaves of *Blumea balsamifera* revealed that extracts CEB and MEB were richer in flavonoids than PEB. Therefore, the difference in the scavenging of superoxide radicals of the different organic extracts may be ascribed to their different polyphenolic compositions.

Figure 3 illustrates the superoxide anion scavenging activities of flavonoids isolated from the leaves of *Blumea balsamifera*. The scavenging capacity of flavonoids (100 µM) was decreased in the order quercetin (**7**) > luteolin (**6**) > 5,7,3′,5′-tetrahydroxyflavanone (**8**) > blumeatin (**2**) > rhamnetin (**4**) > tamarixetin (**3**) > luteolin-7-methyl ether (**5**) > dihydroquercetin-4′-methyl ether (**9**) > dihydroquercetin-4′,7-dimethyl ether (**1**) as shown in Table 2. It has been observed that flavonoids with a free hydroxyl group were more active than methylated compounds.

The presence of methyl group at C-7 position markedly reduces the scavenging activity as it is observed in luteolin-7-methyl ether, blumeatin and dihydroquercetin-7,4′-dimethyl ether (luteolin > luteolin-7-methyl ether; 5,7,3′,5′-tetrahydroxyflavanone > blumeatin; quercetin > rhamnetin and dihydroquercetin-4′-methyl ether > dihydroquercetin-7,4′-dimethyl ether). The results agreed with Cos et al. (1998).

Flavonoids with free hydroxyl groups act as free-radical scavengers, and multiple hydroxyl groups, especially on the B-ring, enhance their antioxidant activity (Jovanovic et al., 1994). This was observed in our experiment, as quercetin was more active than luteolin.

Flavanone and dihydroflavonol derivatives showed lower activity than the flavone and flavonol derivatives. It appears that the hydroxyl groups at C-5 and C-7 and the double-bond between C-2 and C-3 are important for radical scavenging activity. The structure of flavonone and dihydroflavonol differ from flavones and flavonols by the presence of a single bond between C-2 and C-3 in the former and a double-bond in the latter. Apparently, this structural difference influences the superoxide scavenging activity. With a double-bond between C-2 and C-3, ring B will be coplanar with rings A and C due to the conjugation. Saturation of this double-bond will destroy the conjugation and coplanarity. This suggests that a planar flavonoid structure is important for scavenging activity (Cos et al., 1998). In contrast, a number of publications suggest there is no effect of the presence of a double-bond between C-2 and C-3 on the antioxidant activity of the flavonoids (Hudson & Lewis, 1983; Husain et al., 1987; Pratt & Hudson, 1990).

However, though 5,7,3′,5′-tetrahydroxyflavanone and blumeatin are flavonone derivatives, they exerted higher scavenging activity on nonenzymatically generated superox-

Table 1. Scavenging capacity of different solvent extracts of *Blumea balsamifera* on nonenzymatically (phenazine methosulfate/NADH) generated superoxide anions.

	Scavenging of superoxide radicals (%)[a] Concentrations		
Samples	100 µg/ml	250 µg/ml	500 µg/ml
Methanol extract (MEB)	65.80 ± 2.06	86.40 ± 2.50	93.91 ± 1.37
Chloroform extract (CEB)	46.52 ± 0.66	69.41 ± 2.00	84.58 ± 1.51
Pet-ether extract (PEB)	1.22 ± 0.12	3.44 ± 0.25	7.26 ± 0.11

[a] Each value is expressed as mean ± SD (n = 3).

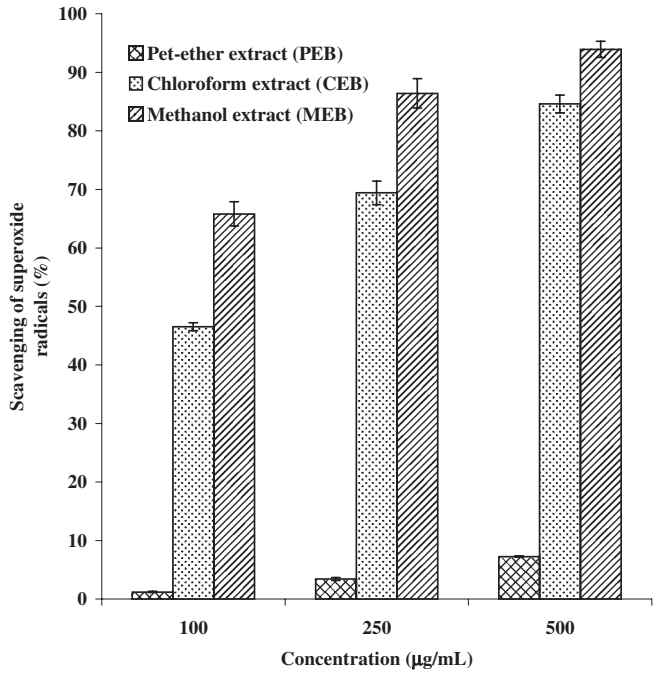


Figure 2. The superoxide radical scavenging activities of different solvent extracts of *Blumea balsamifera* leaves. Values represent mean of triplicate analysis.

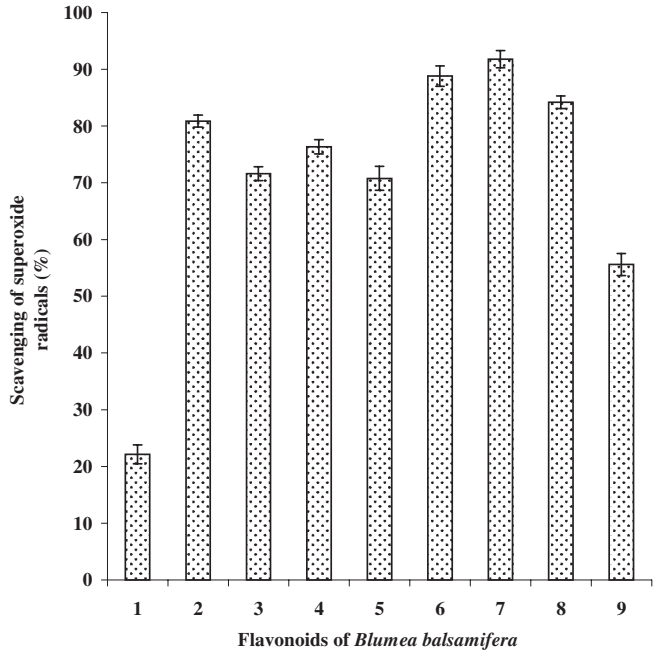


Figure 3. The superoxide radical scavenging activities of the flavonoids (100 µM) of *Blumea balsamifera* leaves. Values present mean of triplicate analysis.

ide radicals than rhamnetin and tamarixetin. The dihyroflavonol derivatives exerted lower scavenging activity. It seems saturation of the C-2 and C-3 bond with 3-OH group markedly reduces the superoxide scavenging activity.

Table 2. Scavenging capacity of flavonoids of *Blumea balsamifera* on nonenzymatically (phenazine methosulfate/NADH) generated superoxide anions.

Flavonoids	*Scavenging of superoxide radicals (%)[a]
Dihydroquercetin-4′,7-dimethyl ether (**1**)	22.14 ± 1.65
Blumeatin (**2**)	80.88 ± 1.06
Tamarixetin (**3**)	71.59 ± 1.23
Rhamnetin (**4**)	76.33 ± 1.24
Luteolin-7-methyl ether (**5**)	70.77 ± 2.11
Luteolin (**6**)	88.79 ± 1.78
Quercetin (**7**)	91.77 ± 1.52
5,7,3′,5′-Tetrahydroxyflavanone (**8**)	84.18 ± 1.11
Dihydroquercetin-4′-methyl ether (**9**)	55.59 ± 1.94

[a] Each value is expressed as mean ± SD (n = 3).

Conclusions

The results of this study provide evidence that extract and flavonoids of *Blumea balsamifera* leaves exhibit interesting antioxidant properties, expressed by the capacity to scavenge superoxide radicals. Therefore, this plant could be a promising remedy for radical-mediated diseases by decreasing superoxide concentrations. In addition, interesting antioxidant properties of *Blumea balsamifera* justify further investigation of its other beneficial biological properties.

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