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Full Length Research Paper

Anti-tyrosinase and anti-cancer activities of flavonoids from *Blumea balsamifera* DC

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Fractionation of an ethylacetate extract from leaves of *Blumea balsamifera* DC, led to isolation of nine flavonoids. The isolated compounds consisted of two dihydroflavonols, dihydroquercetin-4′-methyl ether (1) and dihydroquercetin-7,4′-dimethyl ether (2), two flavanones, 5,7,3′,5′-tetrahydroxyflavanone (3) and blumeatin (4), three flavonols, quercetin (5), rhamnetin (6) and tamarixetin (7), two flavones, luteolin (8) and luteolin-7-methyl ether (9). Their chemical structures were elucidated by spectroscopic methods including UV, NMR and MS analyses. Their inhibitory activities on mushroom tyrosinase using L-DOPA as substrate were evaluated. The anti-tyrosinase activities of dihydroflavonols (1 and 2) and flavonols (5-7) are stronger than arbutin, whereas flavanones (3 and 4) and flavones (8 and 9) are weaker than arbutin. The kinetic analysis showed that the dihydroflavonols (1 and 2), flavanones (3), and flavonols (5 and 6) are competitive inhibitors, whereas the flavones (8 and 9) are noncompetitive inhibitors. The inhibition constant (K_I) of compounds 1-3 were determined to be 0.10, 0.08, and 0.33 mM, respectively. Some compounds (1-5 and 9) were evaluated for cytotoxicity against KB, MCF-7 and NCI-H187 cancer cell lines. Compounds 2, 4, and 9 were active against the KB cells with the IC_{50} values of 17.09, 47.72 and 17.83 ug/ml, respectively. Compounds 2, 3 and 5 exhibited moderate activity against the NCI-H187 cells with the IC_{50} values of 16.29, 29.97 and 20.59 ug/ml. Luteolin-7-methyl ether (9) showed strong cytotoxicity against human lung cancer (NCI-H187) cell lines with IC_{50} of 1.29 µg/ml and moderate toxicity against oral cavity cancer (KB) cell lines with IC_{50} of 17.83 µg/ml.

Key words: *Blumea Balsamifera* DC, cytotoxicity, flavanoids, tyrosinase inhibitor.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase, widely distributed in nature. The enzyme catalyzes the first two reactions of melanin synthesis, (1) the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine, L-DOPA, and (2) the oxidation of L-DOPA to dopaquinone. This O-quinone is a highly reactive compound and can polymerize spontaneously to form melanin (Seo et al., 2003). Although the pigment melanin in human skin is a major defence mechanism against the ultraviolet light of the sun, the production of abnormal pigmentation, such as melasma, freckles, age-spots, liver spots, and other forms of melanin hyperpigmentation can be a serious aesthetic problem (Briganti et al., 2003). Hence, cosmetic agents that inhibit tyrosinase activity or that block melanogenic pathways and leading to skin lightening have been the subject of many researches (Kim et al., 2002; Shimizu et al., 2000; Son et al., 2000). In addition, natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low cost.

Blumea balsamifera is a medicinal plant that grows in Southeast Asia. The leaves are also used as a tea, and as a cure for certain disorders such as rheumatism and

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Table 1. The concentration of tyrosinase inhibition activity of extracts of *Blumea balsamifera* DC leaves (n = 3).

Extracts	IC_{50} (mg/ml)
Hexane extract	0.319 ± 0.015
Ethylacetate extract	0.206 ± 0.037
Water extract	0.345 ± 0.017
Paper mulberry extract	0.157 ± 0.023

hypertension. Its leaves have attracted attention as a part of the plant with various physiological activities, including plasmin-inhibitory, antifungal, and liver-protective effects (Norikura et al., 2008). In Thailand, the dried leaves are cut into small pieces and smoked as a cigarette to relieve sinusitis pain. An infusion of the leaves is used as a stomachic, carminative, diaphoretic, expectorant and emmenagogue. A decoction from fresh leaves alone or in combination with other plants is used as a bath for women after giving birth (Ruangrungsi et al., 1985). Several studies on the chemical constituents of *B. balsamifera* DC have been reported and a number of flavanoids, have been isolated from this plant (Ruangrungsi et al., 1981; Barua and Sharma, 1992; Fazilatun et al., 2001; Fazilatun et al., 2004; Ali et al., 2005). Furthermore, the flavonoids present in *B. balsamifera* DC leaves extracts are potent antioxidants (Fazilatun et al., 2004), but its anti-tyrosinase has not yet been reported. In addition, hydroxylated flavonoids are good target compounds for tyrosinase inhibitors because they share structural similarities with the natural substrate for tyrosinase (Jeong et al., 2009). Therefore, this study aimed to examine the flavanoids from *B. balsamifera* DC leaves on the inhibition of mushroom tyrosinase and also anti-cancer activity against three human KB, MCF-7 and NCI-H187 cell lines.

MATERIALS AND METHODS

General experimental methods

Melting points were determined on an electrothermal melting point apparatus and are uncorrected. UV-Vis spectra were measured with a Biochrom Libra S22 UV/Vis Spectrophotometer. ^{1}H and ^{13}C NMR were recorded using Bruker FTNMR UltraShield spectrometer. Chemical shifts were recorded in parts per million (δ) in acetone-d_6 and DMSO-d_6 with tetramethylsilane (TMS) as the internal reference. The ESITOFMS were obtained using a Micromass LCT mass spectrometer.

Chemicals

Column chromatography (CC) was carried out on silica gel 60 GF254 and Sephadex LH-20 (Merck). Silica gel 60 F254 precoated aluminum plates (0.2 mm, Merck) were used for thin layer chromatography (TLC) analysis. Mushroom tyrosinase was purchased from Sigma Chemical Co. The reagents for anti-tyrosinase assay were of AR grade.

Extraction and separation

Leaves of *B. balsamifera* DC (5.2 kg) were collected at Thumbon Thasud, Chiang Rai, Thailand, in April, 2008. They were air dried and grinded and subsequently extracted with ethanol at room temperature for 5 days. The extract was evaporated to dryness under reduced pressure to give the ethanolic extract (102.9 g) which was suspended in water (2000 ml) and re-extracted with hexane, and ethylacetate respectively. Each soluble fraction was evaporated under reduced pressure to give the hexane (41.1 g), ethylacetate (36.9 g), and water (22.7 g) extracts. Each extract was dissolved in DMSO and subjected to mushroom tyrosinase assay. The ethylacetate extract showed the highest tyrosinase inhibitory activity among those extracts (Table 1). The ethylacetate extract was then separated by silica gel CC and eluted initially with hexane enriched with dichloromethane, then with ethylacetate followed by increasing amounts of methanol in ethylacetate and finally with methanol-ethylacetate (3:7). Each fraction was monitored by TLC, fractions that appeared similar on TLC were combined to yield 9 major fractions, F1-F9. Fraction F2 (30.1 g) was subjected to flash CC with hexane-ethylacetate (4:1) to afford 5 fractions, which were further purified by preparative TLC with hexane-ethylacetate (1:1) to give dihydroquercetin-7,4'-dimethyl ether (7) (1.5 g) and blumeatin (4) (53.7 mg). Fraction F4 (8.3 g) was further purified by flash CC with hexane-ethylacetate (9:1) and subsequently by flash CC with dichloromethane-ethylacetate-acetone (18:1:1) to give rhamnetin (6) (9.2 mg), luteolin (8) (20.3 mg), luteolin-7-methyl ether (9) (15.1 mg), dihydroquercetin-4′-methyl ether (1) (30.1 mg), and 5,7,3′,5′-tetrahydroxyflavanone (3) (302.9 mg). Fraction F5 (4.65 g) was applied to a silica gel flash CC with hexane-ethylacetate (1:1) to afford tamarixetin (7) (8.5 mg). Fraction F7 (5.5 g) was also further purified by Sephadex LH-20 with ethanol and subsequently by preparative TLC with dichloromethane-ethylacetate-acetone (7:2:1) to afford quercetin (5) (22.7 mg).

Spectral data

Dihydroquercetin-7-methylether (1): colorless crystals. ^{1}H NMR (Acetone-d_6): δ 11.72 (1H, s, 5-OH), 7.09 (1H, d, J = 2.1 Hz, H-2′), 7.01 (1H, dd, J = 8.4, 2.1 Hz, H-6′), 6.99 (1H, d, J = 8.4 Hz, H-5′), 5.99 (1H, d, J = 1.8 Hz, H-8), 5.95 (1H, d, J = 1.8 Hz, H-6), 5.06 (1H, d, J = 11.2 Hz, H-2), 4.63 (1H, d, J = 11.6 Hz, H-3), 3.87 (3H, s, 4′-OCH_3). ^{13}C NMR (Acetone-d_6): 197.2 (C-4), 167.3 (C-7), 164.1 (C-9′), 163.2 (C-5), 148.0 (C-4′), 146.4 (C-3′), 130.2 (C-1′), 119.6 (C-6′), 114.6 (C-2′), 111.1 (C-5′), 100.5 (C-10), 96.2 (C-8), 95.2 (C-6), 83.5 (C-2), 72.2 (C-3), 55.4 (4′-OCH_3). ESITOFMS m/z 319 $[M + H]^+$. Dihydroquercetin-7,4′-dimethylether (2): colorless crystals. ^{1}H NMR (Acetone-d_6): δ 11.69 (1H, s, 5-OH), 7.74 (1H, s, 3′-OH), 7.09 (1H, d, J = 2.1 Hz, H-2′), 7.02 (1H, dd, J = 8.8, 2.1 Hz, H-6′), 6.98 (1H, d, J = 8.8 Hz, H-5′), 6.08 (1H, d, J = 2.1 Hz, H-6), 6.06 (1H, d, J = 2.0 Hz, H-8), 5.10 (1H, d, J = 11.6 Hz, H-2), 4.79 (1H, d, J = 4.4 Hz, 3-OH), 4.67 (1H, dd, J = 11.6, 4.4 Hz, H-3), 3.88 (3H, s, 4′-OCH_3), 3.86 (s, 7-OCH_3). ^{13}C NMR (Acetone-d_6): 195.8 (C-4), 168.4 (C-7), 163.8 (C-5), 163.1 (C-9), 148.0 (C-4′), 146.5 (C-3′), 130.1 (C-1′), 119.6 (C-6′), 114.6 (C-2′), 111.1 (C-5′), 100.2 (C-10), 94.9 (C-6), 93.8 (C-8), 83.6 (C-2), 72.3 (C-3), 55.5 (7-OCH_3), 55.4 (4′-OCH_3). ESITOFMS m/z 333 $[M + H]^+$.

5, 6, 3′, 5′-tetrahydroxyflavanone (3): colorless crystals. ^{1}H NMR (Acetone-d_6): δ 12.20 (1H, s, 5-OH), 7.03 (1H, s, H-2′), 6.89 (1H, s, H-6′), 6.89 (1H, s, H-4′), 5.95 (1H, d, J = 2.1 Hz, H-8), 5.93 (1H, d, J = 2.1 Hz, H-6), 5.39 (1H, dd, J = 12.4, 2.8 Hz, H-2), 3.14 (1H, dd, J = 17.2, 12.8 Hz, H-3), 2.72 (1H, dd, J = 17.2, 2.8 Hz, H-3). ^{13}C NMR (Acetone-d_6): 197.3 (C-4), 167.5 (C-5), 165.4 (C-9), 164.4 (C-7),

146.5 (C-3′), 146.1 (C-5′), 131.6 (C-1′), 119.3 (C-6′), 116.1 (C-4′), 114.8 (C-2′), 103.3 (C-10), 96.9 (C-6), 80.0 (C-2), 95.9 (C-8), 43.6 (C-3). ESITOFMS *m/z* 289 $[M + H]^+$.

Blumeatin (4): colorless crystals. ^{1}H NMR δ (400 MHz, Acetone-d_6): δ 12.16 (1H, s, 5-OH), 7.12 (1H, s, H-4′), 6.95 (2H, s, H-2′ and H-6′), 6.12 (1H, d, *J* = 2.1 Hz, H-8), 6.13 (1H, d, *J* = 2.1 Hz, H-6), 5.50 (1H, dd, *J* = 12.6, 2.8 Hz, H-2), 3.95 (3H, s, 7-OCH_3), 3.20 (1H, dd, *J* = 17.1, 12.6 Hz, H-3b), 2.81 (1H, dd, *J* = 17.1, 2.8 Hz, H-3a), and ^{13}C NMR (Acetone-d_6): 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). ESITOFMS *m/z* 303 $[M + H]^+$.

Quercetin (5): yellow powder. ^{1}H NMR (Acetone-d_6 + DMSO-d_6): δ 12.39 (1H, s, 5′-OH), 7.83 (1H, d, *J* = 2.1 Hz, H-2′), 7.68 (1H, dd, *J* = 8.4, 2.1 Hz, H-6′), 6.98 (1H, d, *J* = 8.4 Hz, H-5′), 6.51 (1H, d, *J* = 2.1 Hz, H-8), 6.26 (1H, d, *J* = 2.1 Hz, H-6). ^{13}C NMR (Acetone-d_6 + DMSO-d_6): δ 175.6 (C-4), 164.1 (C-7), 160.9 (C-9), 156.3 (C-5), 147.5 (C-4′), 146.3 (C-2), 144.9 (C-3′), 135.6 (C-3), 122.1 (C-10), 119.8 (C-6′), 115.1 (C-5′), 114.7 (C-2′), 102.7 (C-1′), 97.9 (C-6), 93.1 (C-8). ESITOFMS *m/z* 303 $[M + H]^+$.

Rhamnetin (6): yellow powder. ^{1}H NMR (Acetone-d_6 + DMSO-d_6): δ 12.08 (1H, s, 5′-OH), 7.76 (1H, d, *J* = 2.1 Hz, H-2′), 7.74 (1H, dd, *J* = 8.4, 2.1 Hz, H-6′), 7.07 (1H, d, *J* = 8.4 Hz, H-5′), 6.52 (1H, d, *J* = 2.1 Hz, H-8), 6.22 (1H, d, *J* = 2.1 Hz, H-6), 3.89 (3H, s, 7-OCH_3). ^{13}C NMR (Acetone-d_6 + DMSO-d_6): δ 177.5 (C-4), 165.7 (C-7), 162.6 (C-9), 158.4 (C-5), 148.9 (C-4′), 148.2 (C-2), 146.3 (C-3′), 137.4 (C-3), 124.3 (C-10), 121.8 (C-6′), 116.5 (C-5′), 116.4 (C-2′), 104.7 (C-10), 99.4 (C-6), 94.6 (C-8), 55.8 (7-OCH_3). ESITOFMS *m/z* 317 $[M + H]^+$.

Tamarixetin (7): yellow powder. ^{1}H NMR (Acetone-d_6 + DMSO-d_6): δ 12.43 (1H, s, 5′-OH), 7.80 (1H, d, *J* = 2.1 Hz, H-2′), 7.77 (1H, dd, *J* = 8.7, 2.1 Hz, H-6′), 7.12 (1H, d, *J* = 8.7 Hz, H-5′), 6.51 (1H, d, *J* = 1.8 Hz, H-8), 6.26 (1H, d, *J* = 1.8 Hz, H-6), 3.93 (3H, s, 4′-OCH_3). ^{13}C NMR (Acetone-d_6 + DMSO-d_6): δ 175.2 (C-4), 164. 2 (C-7), 156.3 (C-9), 160.8 (C-5), 149.2 (C-4′), 145.9 (C-2), 146.3 (C-3′), 136. 3 (C-3), 123.6 (C-10), 119.6 (C-6′), 114.4 (C-5′), 111.3 (C-2′), 102.8 (C-1′), 98.0 (C-6), 93.1 (C-8), 55.1 (4′-OCH_3). ESITOFMS *m/z* 317 $[M + H]^+$.

Luteolin (8): yellow powder. ^{1}H NMR (Acetone-d_6 +DMSO-d_6): δ 13.00 (1H, s, 5-OH), 7.47 (1H, d, *J* = 2.1 Hz, H-2′), 7.43 (1H, dd, *J* = 8.4, 2.1 Hz, H-6′), 6.97 (1H, d, *J* = 8.4 Hz, H-5′), 6.61 (1H, s, H-3), 6.50 (1H, d, *J* = 2.1 Hz, H-8), 6.23 (1H, d, *J* = 2.1 Hz, H-6). ^{13}C NMR (Acetone-d_6 + DMSO-d_6): δ 181.6 (C-4), 164.4 (C-7), 163.8 (C-2), 161.7 (C-9), 157.3 (C-5), 149.5 (C-4′), 145.7 (C-3′), 121.7 (C-1′), 118.4 (C-6′), 115.5 (C-5′), 112.9 (C-2′), 103.6 (C-10), 102.5 (C-3), 98.5 (C-6), 93.4 (C-8). ESITOFMS *m/z* 287 $[M + H]^+$. Luteolin-7-methyl ether (9): yellow powder. ^{1}H NMR (Acetone-d_6+DMSO-d_6): δ 13.00 (1H, s, 5-OH), 7.52 (1H, d, *J* = 2.1 Hz, H-2′), 7.49 (1H, dd, *J* = 8.1, 2.1 Hz, H-6′), 6.99 (1H, d, *J* = 8.1 Hz, H-5′), 6.68 (1H, d, *J* = 1.2 Hz, H-8), 6.62 (1H, s, H-3), 6.32 (1H, d, *J* = 1.2 Hz, H-6), 3.93 (3H, s, 7-OCH_3). ^{13}C NMR (Acetone-d_6 +DMSO-d_6): δ 181.8 (C-4), 165.2 (C-7), 164.13 (C-2), 161.5 (C-9), 157.3 (C-5), 151.2 (C-4′), 148.1 (C-3′), 121.3 (C-1′), 120.2 (C-6′), 115.6 (C-5′), 109.8 (C-2′), 104.6 (C-10), 103.0 (C-3), 97.5 (C-6), 92.1 (C-8), 55.5 (7-OCH_3). ESITOFMS *m/z* 301 $[M + H]^+$.

Measurement of anti-tyrosinase activity

Tyrosinase-inhibition activity of the extract of *B. balsamifera* DC leaves and isolated compounds were performed by using L-DOPA as a substrate according to Kubo et al. (2000) with slight modification. All extracts or compounds were first dissolved in DMSO at 1.0 mg/ml and then diluted to different concentration using DMSO. Each sample (100 µl) was diluted with 1800 µl of 0.1 M sodium phosphate (pH 6.8) and 1000 µl of L-DOPA solution (with 0.1 M sodium phosphate, pH 6.8). Then, 100 µl of mushroom tyrosinase solution (138 units) was added in the reaction. The dopachrome formation was measured using UV-Vis spectrophotometer at 475 nm, for 6 min. The percentage of tyrosinase-inhibition activity was calculated follows:

% Tyrosinase inhibition = [A- (B-C)]/A * 100

Where, A = absorbance of control treatment; without test sample, B = absorbance of test sample treatment; with tyrosinase, C = absorbance of test sample blank treatment; without tyrosinase.

The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (IC_{50}).

Determination of mode of tyrosinase inhibition

The mode of inhibition on the enzyme was assayed by Lineweaver-Burk plot. The assay was varied the concentration of L-DOPA (0.5, 1.0, 1.5 and 2.5 mM). The kinetic constants (Km and Vmax) were determined by the plot of the reciprocal of the reaction rate $(\mu mol/min)^{-1}$ versus the reciprocal of substrate concentration $(mM)^{-1}$.

Determination of inhibition constant

The assay varied the concentration of L-DOPA (0.5, 1.0, 1.5 and 2.5 mM) and inhibitor (0, 20, 40, 60 and 80 µg/ml). The inhibition constants were obtained by the second plots of the apparent Michaelis-Menten constant (Km_{app}) versus the concentration of the inhibitor as described by Chen et al. (2005) and calculated follow:

$Km_{app} = (Km/Ki)[I] + Km$

Determination of copper chelation

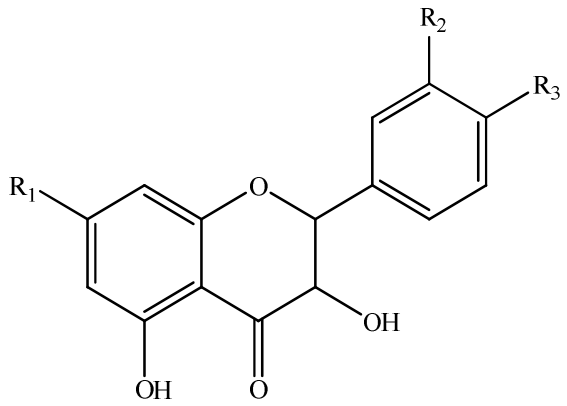
The copper chelation of the inhibitors was determined by the UV-Vis spectra according to Kubo et al. (2000). The reaction mixture contained 1800 µl of 0.1 M phosphate buffer (pH 6.8), 1000 µl of DI water, 100 µl of 0.14 mM $CuSO_4$ solution and 100 µl of 0.050 mM inhibitor solution. The reaction mixture was incubated at 25°C for 30 min and UV-Vis absorption spectra were measured at 240 to 540 nm.

Determination of ability to chelate copper in the enzyme

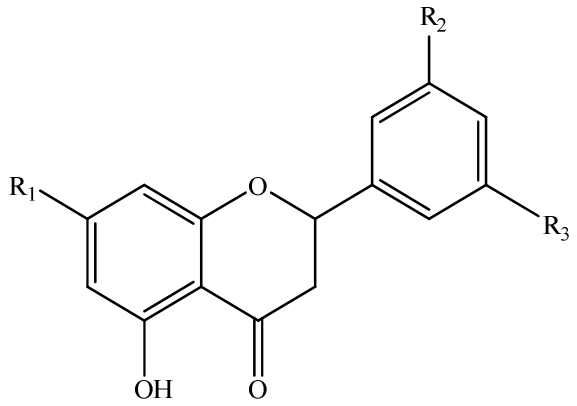
For the determination of ability to chelate copper in the enzyme of isolated compounds, the mixture consisting of 1800 µl of 0.1 M phosphate buffer (pH 6.8), 1000 µl of water, 100 µl of 0.05 mM sample solution and 100 µl of the aqueous solution of the mushroom tyrosinase (138 units) was incubated at 25°C for 30 min, and then the spectra were recorded.

RESULTS AND DISCUSSION

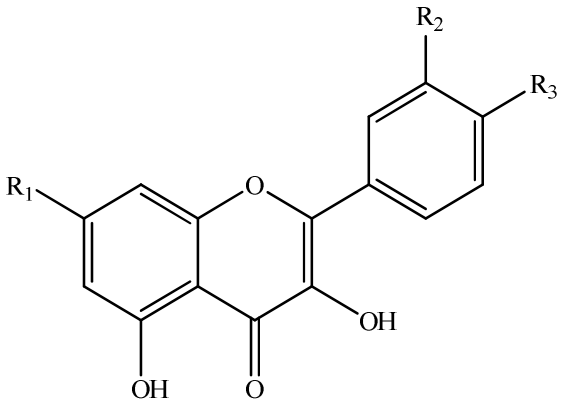
Table 1 summarizes results of mushroom tyrosinase inhibition of the crude extracts from *B. balsamifera* DC leaves. In this table, values are presented as IC_{50} of tested compounds for tyrosinase inhibition. Paper mulberry extract, a natural skin lightening cosmetics, was chosen as a positive control. The ethylacetate-soluble



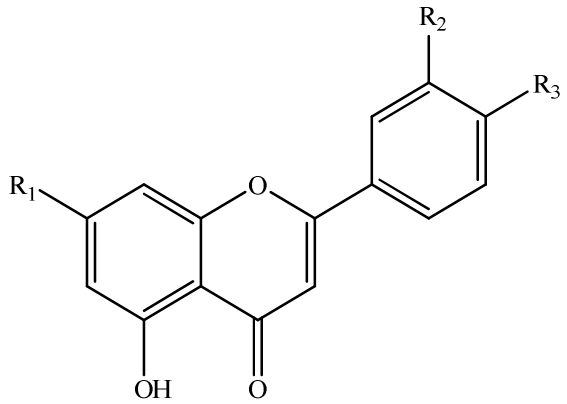
	R_1	R_2	R_3
(**1**) Dihydroquercetin-4'-methylether	OH	OH	OCH_3
(**2**) Dihydroquercetin-7,4'-dimethylether	OCH_3	OH	OCH_3



	R_1	R_2	R_3
(**3**) 5,7,3',5'-Tetrahydroxyflavanone	OH	OH	OH
(**4**) Blumeatin	OCH_3	OH	OH



	R_1	R_2	R_3
(**5**) Quercetin	OH	OH	OH
(**6**) Rhamnetin	OCH_3	OH	OH
(**7**) Tamarixetin	OH	OH	OCH_3



	R_1	R_2	R_3
(**8**) Luteolin	OH	OH	OH
(**9**) Luteolin-7-methyl ether	OCH_3	OH	OH

Figure 1. Structures of the isolated compounds.

extract showed potent anti-tyrosinase activity compared to the hexane and water extract, overall, the activity decreased in the following order: ethylacetate extract > hexane extract > water extract (Table 1). The separation of the ethylacetate extract of *B. balsamifera* DC leaves afforded 9 flavonoids including dihydroflavonols (1 and 2), flavanones (3 and 4), flavonols (5-7) and flavones (8 and 9). Separation of ethylacetate extract was achieved by column chromatography on silica gel and sephadex LH-20. Structures of the isolated compounds were identified by spectroscopic method including UV, ^{1}H NMR, ^{13}C NMR, HMQC, HMBC and MS data and also comparing with the reported literatures, as dihydroquercetin-4'-methyl ether (1), dihydroquercetin-7,4'-dimethyl ether (2), 5,7,3',5'-tetrahydroxyflavanone (3), and blumeatin (4) (Fazilatun et al., 2004), quercetin (5), rhamnetin (6), tamarixetin (7) (Lee et al., 2008), luteolin (8) (Zheng et al., 2008), and luteolin-7-methyl ether (9) (Fazilatun et al., 2001) (Figure 1).

Tyrosinase inhibitory activity of isolated compounds

Table 2. The concentration of tyrosinase inhibition activity and mode of inhibition of isolated compounds (n = 3).

Compounds	IC_{50} (mM) ±SD	Mode of inhibition
Dihydroquercetin-4′-methyl ether (1)	0.115 ± 0.013	Competitive
Dihydroquercetin-7,4′-dimethyl ether (2)	0.162 ± 0.042	Competitive
5,7,3′,5′-Tetrahydroxyflavanone (3)	0.423 ± 0.049	Competitive
Blumatin (4)	0.624 ± 0.029	*
Quercetin (5)	0.096 ± 0.004	Competitive
Rhamnetin (6)	0.107 ± 0.017	Competitive
Tamarixetin (7)	0.144 ± 0.004	*
Luteolin (8)	0.258 ± 0.015	Non-competitive
Luteolin-7-methyl ether (9)	0.350 ± 0.002	Non-competitive
Kojic acid	0.044 ± 0.005	-
Arbutin	0.233 ± 0.025	-

* unable to establish.

(1-9), using L-DOPA as substrate, was examined. Each compound was assayed at different concentrations and their relative activities were expressed as IC_{50} values (Table 2). Two well known tyrosinase inhibitors, arbutin and kojic acid, were used as the reference compounds. The tyrosinase inhibitory activity of dihydroflavonols (1 and 2) and flavonols (5-7) are stronger than arbutin but flavanones (3 and 4) and flavones (8 and 9) are weaker than arbutin. However, all compounds exhibited lower anti-tyrosinase activity than kojic acid.

Dihydroflavonols (1 and 2) showed higher diphenolase inhibitory activity than flavanones derivatives (dihydroquercetin-4'-methyl ether (1) > dihydroquercetin-7,4'-dimethyl ether (2) > 5,7,3',5'-tetrahydroxyflavanone (3) > blumeatin (4). In addition, flavones showed less activity than corresponding flavonols (quercetin (5) > luteolin (8); rhamnetin (6) > luteolin-7-methyl ether (9) suggesting that the presence of the 3-hydroxyl group was essential for high anti-tyrosinase activity. Rhamnetin (6) showed high anti-tyrosinase activity than tamarixetin (7). It seems that the presence of 3',4'-hydroxyls group was essential for high anti-tyrosinase activity and the presence of a methoxyl group at the C-4' position reduced its activity. The presence of methoxyl at the C-7 position tended to reduce the anti-tyrosinase activity, as indicated by the experimental results: dihydroquercetin-4'-methyl ether (1) > dihydroquercetin-7,4'-dimethyl ether (2) and 5,7,3',5'-tetrahydroxyflavanone (3) > blumeatin (4); quercetin (5) > rhamnetin (6); luteolin (8) > luteolin-7-methyl ether (9). Flavones (8-9) showed higher anti-tyrosinase activities than flavanones (luteolin (8) > 5,7,3′,5′-tetrahydroxyflavanone (3); tamarixetin (7) > blumeatin (4)). In addition, dihydroquercetin-4'-methyl ether (1) exhibited comparatively weaker anti-tyrosinase activity than tamarixetin (7). It suggests that the presence of the C2–C3 double bond is also essential for tyrosinase inhibitor ability.

The mode of tyrosinase inhibition of the compounds was performed using Lineweaver-Burk plot. Most of the isolated compounds are competitive inhibitor for L-DOPA oxidation, whereas, flavones (8 and 9) are noncompetitive tyrosinase inhibition. The tyrosinase inhibitory activity of the isolated flavonoids might be due to chelating with copper in the active center of tyrosinase. The binding ability with copper in mushroom tyrosinase enzyme of the isolated compounds was investigated. The UV-Vis spectrophotometric analyses were conducted to establish the binding copper ability of flavonoids by adding Cu^{2+} and by incubation with the enzyme. The UV-Vis spectra of dihydroflavonols (1 and 2) and flavanones (3 and 4) showed no significant shift by adding Cu^{2+} and by incubation with the enzyme. The results suggest that the isolated dihydroflavonols and flavanones compete with substrate to combine with free enzyme by not chelate copper in enzyme pathway. The equilibrium constant for inhibitor binding with free enzyme, Ki, of compounds 1-3 were evaluated. The inhibition constants (Ki) were determined by the second plot of apparent Michaelis-Menten constant (Km_{app}) versus the concentration of inhibitor and calculated from slope or X axis intercept (Figures 2 to 4). The inhibition constant of compounds 1-3 was obtained 0.10, 0.08 and 0.33 mM, respectively.

The UV-Vis spectra of flavonols (5-7) exhibited hypsochromic shift after incubated with tyrosinase for 30 min (Table 3). The results suggested that the isolated flavonols chelated with copper in tyrosinase. This result confirmed that the tyrosinase inhibition of flavonols come from their ability to chelate copper in the enzyme. The spectra of the isolated flavones, luteolin (8) and luteolin-7-methyl ether (9), showed slightly characteristic bathochromic shifts by adding Cu^{2+} and slightly shifted to the short wavelength by incubation with the enzyme, but not significantly compared to those observed with flavonols (5-7). The mode of inhibition of flavones (8 and 9) showed noncompetitive type, apart from they indicated the 3-hydroxy group in the structure of flavonols played important role for inhibition mechanism of tyrosinase. For

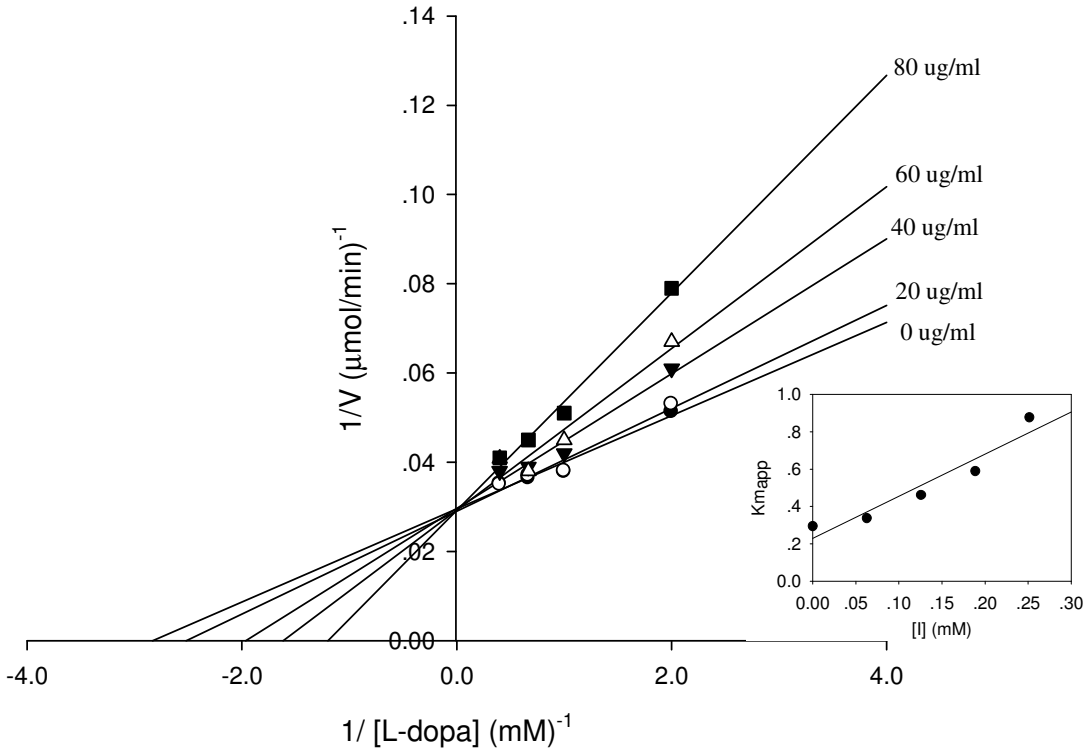


Figure 2. Lineweaver-Burk plot for inhibition of dihydroxyquercetin-4′-methylether (1) on the oxidation of L-DOPA by tyrosinase. The inhibitor concentrations were 0, 20, 40, 60 and 80 ug/ml, respectively.

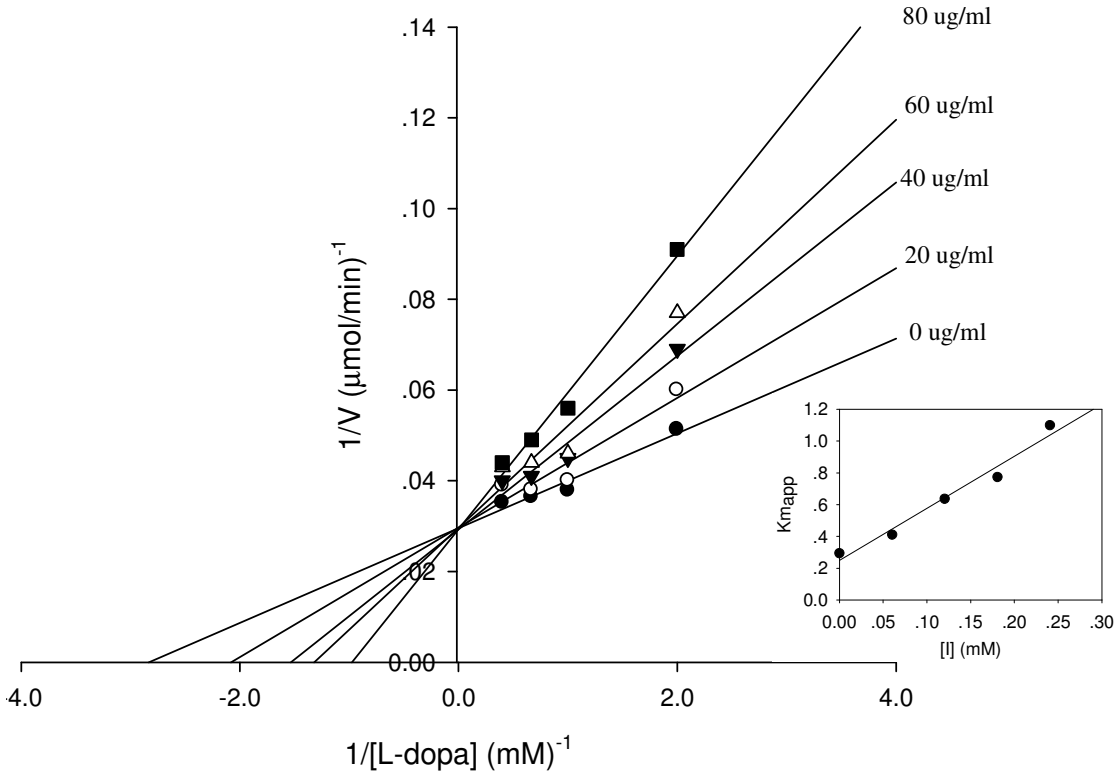


Figure 3. Lineweaver-Burk plot for inhibition of dihydroxyquercetin-7,4′-methylether (2) on the oxidation of L-DOPA by tyrosinase. The inhibitor concentrations were 0, 20, 40, 60 and 80 ug/ml, respectively.

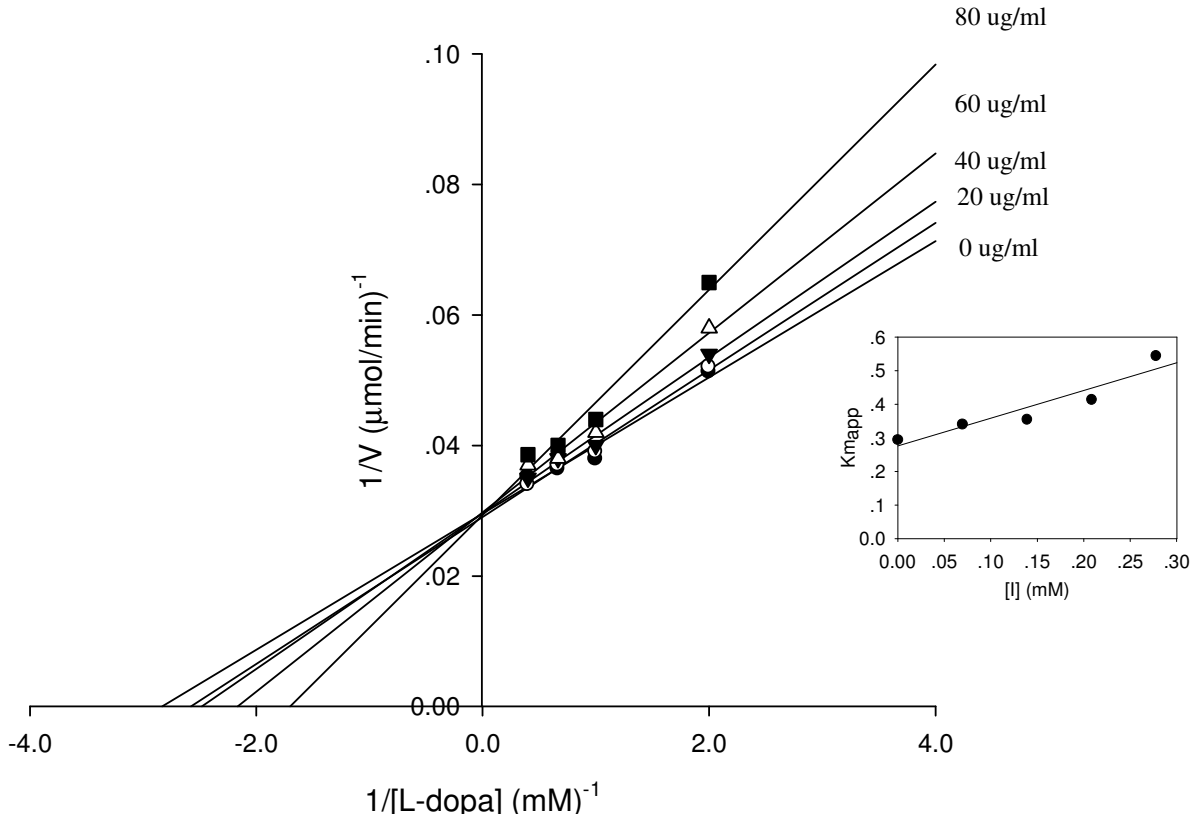


Figure 4. Lineweaver-Burk plot for inhibition of 5,7,3′,5′-tetrahydroxyflavanone (3) on the oxidation of L-DOPA by tyrosinase. The inhibitor concentrations were 0, 20, 40, 60 and 80 ug/ml, respectively.

Table 3. The shift UV-Vis spectra of isolated compounds by adding Cu^{2+} and tyrosinase.

Compounds	Shift by Cu^{2+} (nm)	Shift by tyrosinase (nm)
Dihydroquercetin-4′-methyl ether (1)	325 → 325	325 → 315
Dihydroquercetin-7,4′-dimethyl ether (2)	326 → 327	326 → 313
5,7,3′,5-Tetrahydroxyflavanone (3)	322 → 318	322 → 321
Blumatin (4)	324 → 321	324 → 320
Quercetin (5)	371→ 440	371 → 367
Rhamnetin (6)	373 → 435	373 → 325
Tamarixetin (7)	375 → 359	375 → 353
Luteolin (8)	350 → 399	350 → 316
Luteolin 7-methyl ether (9)	348 → 396	358 → 315

luteolin (8), the results were in good agreement with previously report (Kubo et al., 2000). The sufficient quantity compounds 1-5 and 9 were evaluated for cytotoxicity against KB, BC and NCI-H187 cells (Table 4). Compounds 2 and 9 were moderate toxicity against the KB cells with the IC_{50} values of 17.09 and 17.83 ug/ml, respectively, whereas, compound 4 exhibited weak activity with the IC_{50} values of 47.72 ug/ml. All compounds were inactive against the MCF7 cells. Compounds 2, 3 and 5 exhibited moderate activity against the NCI-H187 cells with the IC_{50} values of 16.29, 29.97 and 20.59 ug/ml, respectively, whereas, compound 9 exhibited strong activity against the NCI-H187 cells with the IC_{50} values of 5.21 ug/ml.

Conclusion

Nine flavanoids isolated from the leaves of *B. balsamifera* DC showed moderate of anti-tyrosinase activity.

Table 4. Biological activity of flavonoids isolated from the leaves of *B. balsamifera*

Compounds	Cytotoxicity (IC_{50}, ug/ml)		
	KB[a]	MCF7[b]	NCI-H187[c]
Dihydroquercetin-4′-methyl ether (1)	Inactive	Inactive	Inactive
Dihydroquercetin-7,4′-dimethyl ether (2)	17.09	Inactive	16.29
5,7,3′,5′Tetrahydroxyflavanone (3)	Inactive	Inactive	29.97
Blumeatin (4)	47.72	Inactive	Inactive
Quercetin (5)	Inactive	Inactive	20.59
Luteolin-7-methyl ether (9)	17.83	Inactive	5.21
Tamoxifen	-	4.03	-
Doxorubicine	0.222	9.65	0.073
Ellipticine	0.553	-	1.39

[a]KB = Oral cavity cancer; [b] MCF7 = Breast cancer; [c] NCI-H187 = Small cell lung cancer.

Dihydroflavonols (1 and 2) and flavanones (3 and 4) exhibited competitive inhibitor on tyrosinase activity but did not chelate copper in enzyme. The activity of flavonols (5-7) was found to come from their ability to chelate copper in the enzyme. The corresponding flavones (8 and 9) were indicated as noncompetitive inhibitor. The anti-tyrosinase activities of all compounds tested decreased in the order: dihydroquercetin-4'-methyl ether (1) > dihydroquercetin-7,4'-dimethyl ether (2); 5,7,3',5'-tetrahydroxyflavanone (3) > blumeatin (4); quercetin (5) > rhamnetin (6) > tamarixetin (7); luteolin (8) > luteolin-7-methyl ether (9). The anti-tyrosinase activities of dihydroflavonols (1 and 2) and flavonols (5-7) are stronger than arbutin, whereas flavanones (3 and 4) and flavones (8 and 9) are weaker than arbutin. Compounds 2, 4, and 9 were active against the KB cells with the IC_{50} values of 17.09, 47.72 and 17.83 ug/ml, respectively. All compounds were inactive against the MCF7 cells. Compounds 2, 3, 5 and 9 exhibited activity against the NCI-H187 cells with the IC_{50} values of 16.29, 29.97, 20.59 and 5.21 ug/ml, respectively. These findings have ecological and economic significance for application of *B. balsamifera* DC leaves extract as skin-lightening agent in cosmetic industry.

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