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ScienceDirect
Food Chemistry 106 (2008) 529–535
Food Chemistry
www.elsevier.com/locate/foodchem

Tyrosinase inhibitors from paper mulberry (*Broussonetia papyrifera*)

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Received 10 March 2007; received in revised form 4 June 2007; accepted 4 June 2007

Abstract

Fractionation of a chloroform-soluble extract from twigs of *Broussonetia papyrifera*, led to the isolation of one new compound, 3,5,7,4′-tetrahydroxy-3′-(2-hydroxy-3-methylbut-3-enyl)flavone (**1**), and 10 known compounds, uralenol (**2**), quercetin (**3**), isolicoflavonol (**4**), papyriflavonol A (**5**), broussoflavonol F (**6**), 5,7,3′,5′-tetrahydroxyflavanone (**7**), luteolin (**8**), isoliquiritigenin (**9**), broussochalcone A (**10**) and 5,7,3′,4′-tetrahydroxy-3-methoxyflavone (**11**). Their structures were identified by interpretation of MS, ^{1}H NMR, ^{13}C NMR, HMQC and HMBC data. Their inhibitory activities on mushroom tyrosinase using L-tyrosine as substrate were investigated and the IC_{50} values of 3,5,7,4′-tetrahydroxy-3′-(2-hydroxy-3-methylbut-3-enyl)flavone, uralenol, quercetin and broussoflavonol F were found to be 96.6, 49.5, 57.8, and 82.3 μM, respectively, better than arbutin, a well-known tyrosinase inhibitor.

Keywords: *Broussonetia papyrifera*; Flavonol; Chalcone; Tyrosinase inhibition

1. Introduction

Appearance, flavor, texture and nutritional value are the four major attributes considered by consumers when they select food products. Color is a critical determinant for the appearance of many food products such as fruits, vegetables and crustaceans. The original colors of these products are determined by the pigments they contain. On the other hand, both enzymatic and non-enzymatic browning reactions can affect their final appearance. Enzymatic browning, which is catalyzed by polyphenol oxidases (tyrosinase, EC 1.14.18.1), is known to cause undesirable discolorization. The rate of enzymatic browning depends on the concentration of tyrosinase and phenolic substrates, oxygen availability, pH, temperature, etc. (Martinez & Whitaker, 1995). Various strategies have been developed to inhibit enzymatic browning. Among them, chemical treatment has been a popular approach and both synthetic and natural compounds have been extensively examined for their suitability of applications in food products (McEvily, Iyengar, & Otwell, 1992). Sulfites are representative chemicals of polyphenol oxidase (PPO) inhibitors. However, their use on fruits and vegetables has been banned by FDA due to safety reasons (Son, Moon, & Lee, 2000). Ascorbic acid and its derivatives were once suggested as promising alternatives, but their temporary action has limited their wide applications (Seo, Sharma, & Sharma, 2003; Soliva-Fortuny & Martin-Belloso, 2003). Recently, 4-hexylresorcinol has been recognized as one of the most effective polyphenol oxidase inhibitors for safe applications in the food industry (McEvily, Iyengar, & Otwell, 1991). It has been successfully used on shrimp (Frankos et al., 1991) and its effectiveness on some fruits and vegetables has also been verified (Soliva-Fortuny & Martin-Belloso, 2003). In some cases, 4-hexylresorcinol may require the combined action of reducing agents to achieve better results (Buta, Moline, Spaulding, & Wang, 1999).

Despite the large number of tyrosinase inhibitors reported, their identification and isolation from natural sources is currently one of the most important approaches (Son et al., 2000). Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low costs, especially when rich sources are identified. In addition, tyrosinase inhibitors

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.06.037

are becoming increasingly important in the cosmetic industry, due to their skin-whitening effects (Shimizu, Kondo, & Sakai, 2000). Furthermore, it has been reported that tyrosinase might contribute to the dopamine neurotoxicity and neurodegeneration associated with Parkinson's disease (Xu et al., 1997). These facts led us to focus our research work on the exploration of natural tyrosinase inhibitors.

Broussonetia papyrifera, known as paper mulberry, is a deciduous tree or shrub, which grows naturally in Asia and Pacific countries such as China, Thailand and USA. The roots, barks and fruits are all used in traditional Chinese medicines and the fruits have been used for treatment of impotence and ophthalmic disorders in China (Lee et al., 2001). The chemical components of paper mulberry have been extensively studied with various compounds identified. The constituents of this plant have been shown to inhibit lipid peroxidation (Ko, Yu, Ko, Teng, & Lin, 1997), exhibit antiplatelet effects (Lin et al., 1996), inhibit PTP1B enzyme (Chen, Hu, An, Li, & Shen, 2002) and inhibit aromatase (Lee et al., 2001). Besides, paper mulberry extract was found to show depigmenting effects with Kazinol F [5-(3′-(2,4-dihydroxyphenyl) propyl-3,4-bis(3-methyl-2-butenyl)-1,2-benzendiol)] from the root bark extract showing strong tyrosinase inhibitory activity (Jang et al., 1997). As part of our continuing search for tyrosinase inhibitory agents of natural origin, the chloroform-soluble extract of *B. papyrifera* twigs was found to have potent inhibitory activity against mushroom tyrosinase. Bioassay-guided fractionation of the chloroform-soluble extract of *B. papyrifera* led to the isolation of one new and ten known compounds with moderate tyrosinase inhibitory activities. The isolation and structural elucidation of these active components are described in the following sections.

2. Materials and methods

2.1. Plant material

Twigs of *B. papyrifera* were collected from Arboretum of Zhongshan, Nanjing, Jiangsu Province, PR China in May 2006. A voucher specimen (accession number 20060501) has been deposited at Department of Botany, The University of Hong Kong.

2.2. Chemicals and instruments

Mushroom tyrosinase (3900 units/mg), L-tyrosine, 2,4-dihydroxyacetophenone, boric acid, ethylene glycol and 4-hydroxybenzaldehyde were purchased from Sigma Chemical Co (St. Louis, USA). HPLC grade solvents were purchased from BDH (Poole, UK). Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences AB (Uppsala Sweden). Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Company (Qingdao, PR China). TLC plates (25 DC-platten Kieselgel 60 F_{254}) were obtained from Merck (Darmstadt, Germany). Semi-preparative HPLC was carried out on a Waters Delta 600 system equipped with a 2487 dual-wavelength detector, a Masslynx V4.0 software and a Phenomenex Luna C18 (2) column (250 × 21.2 mm, 5 μm). ^{1}H NMR, ^{13}C NMR, and 2D NMR (^{1}H–^{1}H COSY, HMBC and HMQC) spectra were obtained on a Bruker 500 DRX NMR Spectrometer. LC–MS was run on an Agilent LC–MSD system equipped with an electrospray ionization source, Bruker Daltonics 4.0 and Data analysis 4.0 software. The tyrosinase inhibitory activity was monitored on a UV-1206 Spectrophotometer (Shimadzu Corporation, Japan).

2.3. Extraction and isolation

Dried plant material (3.3 kg) was ground by a mini-grinder (Model: DF-15, Shenzhen Laitong Company, Shenzhen, PR China) and packed into a paper extraction bag. Extraction was performed with 95% ethanol (3 × 10 L) at room temperature using a traditional Chinese medicine extractor (Model: YFX20T, Donghuayuan Medical Company, Beijing, PR China). The extracts were combined and concentrated in vacuo at 45 °C. The concentrated extract was suspended in water; the resultant suspension was partitioned with *n*-hexane (3 × 1.0 L) to give a *n*-hexane-soluble syrup (22.2 g). Next, the aqueous layer was partitioned with chloroform (3 × 1.0 L) to give a chloroform-soluble extract (42.2 g). The aqueous layer was then partitioned successively with ethyl acetate (3 × 1.0 L) and *n*-butanol (3 × 500 mL) to give 12.2 g of ethyl acetate extract and 8.9 g of *n*-butanol extract, respectively. Then the four extracts were dissolved in DMSO and subjected to mushroom tyrosinase inhibition assay. The chloroform-soluble extract showed moderate tyrosinase inhibitory activity. This extract was fractionated on a silica gel column, with *n*-hexane–chloroform (2:1), chloroform, chloroform–ethyl acetate (5:1), chloroform–ethyl acetate (1:1), ethyl acetate, ethyl acetate–methanol (5:1), and methanol, as the mobile phases (2000 mL each). This gave 18 pooled fractions (Fr1–Fr18). Among them, Fr12–Fr14 showed the strongest tyrosinase inhibitory activity, and these fractions were purified separately. Separation was performed individually on Sephadex LH-20 with MeOH–$CHCl_3$ (1:1) as the mobile phase. This resulted in six subfractions for Fr12 (Fr12-Sub1-6), six subfractions for Fr13 (Fr13-Sub1-6), and eight subfractions for Fr14 (Fr14-Sub1-8). Fr12-Sub6, Fr13-Sub6, and Fr14-Sub6-8 were found to exhibit the strongest tyrosinase inhibitory activity among the different corresponding subfractions, and were further purified. Fr12-Sub6 was purified by silica gel column chromatography with $CHCl_3$ as the mobile phase to obtain compound **4** (56.5 mg), **5** (14.8 mg), and **6** (8.2 mg). Fr13-Sub6 was purified by silica gel column chromatography eluted with *n*-hexane–ethyl acetate (3:1) to give compound **1** (6.0 mg), **9** (5.1 mg), and **10** (10.4 mg). Fr14-Sub6 was purified by semi-preparative RP-HPLC using H_2O–MeOH gradient mobile phase

(90:10 to 0:100, 0–45 min) at a flow rate of 8 mL/min and at a wavelength of 254 nm. One active compound, **11** (17.4 mg) was obtained. Fr14-Sub7 was separated by silica gel column chromatography with *n*-hexane–ethyl acetate (2:3) as the mobile phase to give compounds **7** (29.3 mg) and **8** (5.1 mg). Fr14-Sub8 was repeatedly separated by semi-preparative HPLC, using H_2O–MeOH (60:40) as the mobile phase to give compound **2** (77.9 mg), H_2O–MeOH gradient system (90:10 to 0:100, 0 min to 45 min) to give compound **3** (63.7 mg), with mobile phase flowing at 8 mL/min and a detection wavelength at 254 nm.

2.4. Spectral data

Uralenol (**2**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.59 (1H, d, $J = 2.1$ Hz, H-2′), 7.52 (1H, d, $J = 2.1$ Hz, H-6′), 6.34 (1H, d, $J = 2.1$ Hz, H-8), 6.16 (1H, d, $J = 2.1$ Hz, H-6), 5.34 (1H, m, H-2″), 3.30 (2H, m, H-1″), 1.75 (6H, s, CH_3-4″, 5″). ^{13}C NMR (CD_3OD): δ 177.4 (C-4, s, C=O), 165.7 (C-7, s), 162.6 (C-9, s), 158.3 (C-5, s), 148.5 (C-4′, s), 146.9 (C-2, s), 145.9 (C-3′, s), 137.3 (C-3, s), 133.5 (C-3″, s), 129.6 (C-1′, s), 123.9 (C-6′, d), 123.4 (C-5′, s), 122.2 (C-2″, d), 113.5 (C-2′, d), 104.7 (C-10, s), 99.4 (C-6, d), 94.5 (C-8, d), 29.4 (C-1″, t), 26.1 (C-4″, q), 18.1 (C-5″, q). Positive ESI-MS m/z 371 $[M + H]^+$.

Quercetin (**3**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.73 (1H, s, H-2′), 7.62 (1H, d, $J = 8.0$ Hz, H-6′), 6.87 (1H, d, $J = 8.0$ Hz, H-5′), 6.38 (1H, s, H-8), 6.16 (1H, s, H-6). ^{13}C NMR (CD_3OD): δ 177.5 (C-4, s, C=O), 165.7 (C-7, s), 162.6 (C-9, s), 158.4 (C-5, s), 148.9 (C-4′, s), 148.2 (C-2, s), 146.3 (C-3′, s), 137.4 (C-3, s), 124.3 (C-1′, s), 121.8 (C-6′, d), 116.5 (C-5′, d), 116.4 (C-2′, d), 104.7 (C-10, s), 99.4 (C-6, d), 94.6 (C-8, d). Positive ESI-MS m/z 303 $[M + H]^+$.

Isolicoflavonol (**4**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.93 (1H, d, $J = 2.2$ Hz, H-2′), 7.87 (1H, dd, $J = 8.6$, 2.2 Hz, H-6′), 6.83 (1H, d, $J = 8.6$ Hz, H-5′), 6.33 (1H, d, $J = 2.0$ Hz, H-8), 6.15 (1H, d, $J = 2.0$ Hz, H-6), 5.35 (1H, m, H-2″), 3.30 (2H, m, H-1″), 1.76 (3H, s, CH_3-4″), 1.74 (3H, s, CH_3-5″). ^{13}C NMR (CD_3OD): δ 177.4 (C-4, s, C=O), 165.6 (C-7, s), 162.6 (C-5, s), 158.5 (C-4′, s), 158.4 (C-9, s), 148.5 (C-2, s), 137.2 (C-3, s), 133.7 (C-3″, s), 130.6 (C-2′, d), 129.6 (C-3′, s), 128.3 (C-6′, d), 123.7 (C-1′, s, C-2″, d), 115.8 (C-5′, d), 104.7 (C-10, s), 99.4 (C-6, d), 94.6 (C-8, d), 29.4 (C-1″, t), 26.1 (C-4″, q), 18.1 (C-5″, q). Positive ESI-MS m/z 355 $[M + H]^+$.

Papyriflavonol A (**5**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.76 (1H, d, $J = 2.0$ Hz, H-2′), 7.51 (1H, d, $J = 2.0$ Hz, H-6′), 6.21(1H, s, H-8), 5.35 (1H, m, H-2″), 5.33 (1H, m, H-2‴), 3.33 (2H, m, H-1″), 3.29 (2H, m, H-1‴), 1.77, 1.74, 1.72, 1.65 (12H, s, CH_3-4″, 5″, 4‴, 5‴). ^{13}C NMR (CD_3OD): δ 177.7 (C-4, s, C=O), 162.9 (C-7, s), 160.1 (C-5, s), 155.4 (C-9, s), 148.4 (C-3′, s), 146.9 (C-4′, s), 145.8 (C-2, s), 137.3 (C-3, s), 133.7 (C-3″, s), 132.7 (C-3‴, s), 129.6 (C-5′, s), 123.8 (C-2″, d), 123.7 (C-2‴, d), 121.5 (C-6′, d), 114.3 (C-2′, d), 107.8 (C-1′, s), 104.7 (C-10, s), 99.4 (C-6, s), 94.5 (C-8, d), 29.4 (C-1″, t), 26.1 (C-4″, q; C-4‴, q), 22.6 (C-1‴, t), 18.3 (C-5‴, q), 18.1 (C-5″, q). Positive ESI-MS m/z 439 $[M + H]^+$.

Broussoflavonol F (**6**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.99 (1H, dd, $J = 8.4$, 1.7 Hz, H-6′), 7.96 (1H, d, $J = 1.7$ Hz, H-2′), 6.84 (1H, d, $J = 8.4$ Hz, H-5′), 6.22 (1H, s, H-6), 5.35 (1H, m, H-2″), 5.33 (1H, m, H-2‴), 3.33 (2H, m, H-1″), 3.29 (2H, m, H-1‴), 1.78, 1.75, 1.73, 1.66 (12H, s, CH_3-4″, 5″, 4‴, 5‴). ^{13}C NMR (CD_3OD): δ 177.6 (C-4, s, C=O), 162.8 (C-7, s), 160.1 (C-5, s), 158.4 (C-4′, s), 155.5 (C-9, s), 148.4 (C-2, s), 137.0 (C-3, s), 133.9 (C-3″, s), 132.6 (C-3‴, s), 130.1 (C-2′, d), 129.6 (C-3′, s), 128.6 (C-6′, d), 124.1 (C-1′, s), 123.9 (C-2‴, d), 123.5 (C-2″, d), 115.7 (C-5′, d), 107.8 (C-8, s), 104.7 (C-10, s), 99.4 (C-6, d), 29.3 (C-1″, t), 26.1 (C-4″, q; C-4‴, q), 22.6 (C-1‴, t), 18.3 (C-5‴, q), 18.0 (C-5″, q). Positive ESI-MS m/z 423 $[M + H]^+$.

5,7,3′,5′-Tetrahydroxyflavanone (**7**): yellow powder. ^{1}H NMR (CD_3OD): δ 6.91 (1H, s, H-4′), 6.77 (2H, s, H-2′, 6′), 5.88 (1H, d, $J = 2.0$ Hz, H-8), 5.86 (1H, d, $J = 2.0$ Hz, H-6), 5.27 (1H, dd, $J = 12.7$; 3.1 Hz, H-2), 3.05 (1H, dd, $J = 17.1$; 12.7 Hz, H-3$_\alpha$), 2.68 (1H, dd, $J = 17.1$; 3.1 Hz, H-3$_\beta$). ^{13}C NMR (CD_3OD): δ 197.9 (C-4, s, C=O), 168.5 (C-7, s), 165.6 (C-5, s), 165.0 (C-9, s), 147.0 (C-3′, s), 146.6 (C-5′, s), 131.9 (C-4′, d), 119.4 (C-1′, s), 116.4 (C-2′, d), 114.8 (C-6′, d), 103.5 (C-10, s), 97.2 (C-6, d), 96.3 (C-8, d), 80.6 (C-2, d), 44.2 (C-3, t). Positive ESI-MS m/z 289 $[M + H]^+$.

Luteolin (**8**): yellow powder. ^{1}H NMR (DMSO-d_6): δ 12.97 (1H, OH-5), 7.40 (1H, d, $J = 2.1$ Hz, H-2′), 7.39 (1H, dd, $J = 8.1$; 2.1 Hz, H-6′), 6.87 (1H, d, $J = 8.1$ Hz, H-5′), 6.65 (1H, s, H-3), 6.42 (1H, d, $J = 1.2$ Hz, H-8), 6.16 (1H, d, $J = 1.2$ Hz, H-6). ^{13}C NMR (DMSO-d_6): δ 181.6 (C-4, s, C=O), 164.4 (C-7, s), 163.8 (C-2, s), 161.5 (C-9, s), 157.3 (C-5, s), 149.8 (C-4′, s), 145.8 (C-3′, s), 121.4 (C-1′, s), 118.9 (C-6′, d), 116.0 (C-5′, d), 113.3 (C-2′, d), 103.6 (C-10, s), 102.8 (C-3, d), 98.9 (C-6, d), 93.8 (C-8, d). Positive ESI-MS m/z 287 $[M + H]^+$.

Isoliquiritigenin (**9**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.96 (1H, d, $J = 8.9$ Hz, H-6′), 7.78 (1H, d, $J = 15.3$ Hz, H-β), 7.61 (2H, d, $J = 8.6$ Hz, H-2, 6), 7.60 (1H, d, $J = 15.3$ Hz, H-α), 6.83 (2H, d, $J =$ 8.6 Hz, H-3, 5), 6.40 (1H, dd, $J = 8.9$, 2.4 Hz, H-5′), 6.28 (1H, d, $J = 2.4$ Hz, H-3′). ^{13}C NMR (CD_3OD): δ 193.7 (C=O), 167.6 (C-4′, s), 166.5 (C-2′, s), 161.7 (C-4, s), 145.8 (C-β, d), 133.5 (C-6′, d), 131.9 (C-2, 6, d), 128.0 (C-1, s), 118.5 (C-α, d), 117.0 (C-3 and C-5, d), 114.8 (C-1′, s), 109.3 (C-5′, d), 103.9 (C-3′, d). Positive ESI-MS m/z 257 $[M + H]^+$.

Broussochalcone A (**10**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.67 (1H, s, H-6′), 7.66 (1H, d, $J = 15.3$ Hz, H-β), 7.47 (1H, d, $J = 15.3$ Hz, H-α), 7.14 (1H, d, $J = 1.8$ Hz, H-2), 7.06 (1H, dd, $J = 8.2$, 1.8 Hz, H-6), 6.80 (1H, d, $J = 8.2$ Hz, H-5), 6.28 (1H, s, H-3′), 5.30 (1H, m, H-1″), 3.29 (2H, m, H-2″), 1.75, 1.73 (6H, s, CH_3-4″, 5″). ^{13}C NMR (CD_3OD): δ 193.5 (C=O), 165.8 (C-4′, s), 164.5 (C-2′, s), 150.0 (C-4, s), 147.0 (C-3, s), 145.9 (C-β, d), 133.4 (C-4″, s), 132.1 (C-6′, d), 128.6 (C-1,

s), 124.0 (C-6, d), 123.6 (C-2″, d), 122.1 (C-5′, s), 118.5 (C-α, d), 116.8 (C-5, d), 115.9 (C-2, d), 114.5 (C-1′, d), 103.5 (C-3′, d), 28.9 (C-1″, t), 26.1 (C-4″, q), 18.0 (C-5″, q). Positive ESI-MS m/z 341 $[M + H]^+$.

5,7,3′,4′-Tetrahydroxy-3-methoxyflavone (**11**): yellow powder. ^{1}H NMR (CD_3OD): δ 7.61 (1H, s, H-2′), 7.62 (1H, d, $J = 8.2$ Hz, H-6′), 6.87 (1H, d, $J = 8.2$ Hz, H-5′), 6.37 (1H, s, H-8), 6.18 (1H, s, H-6), 3.77 (OCH_3). ^{13}C NMR (CD_3OD): δ 180.1 (C-4, s, C=O), 166.0 (C-7, s), 163.2 (C-5, s), 158.5 (C-9, s), 157.7 (C-2, s), 150.0 (C-4′, s), 146.6 (C-3′, s), 139.7 (C-3, s), 123.1 (C-1′, s), 122.5 (C-6′, d), 116.6 (C-5′, d), 116.6 (C-2′, d), 104.2 (C-10, s), 99.9 (C-6, d), 94.8 (C-8, d), 60.6 (OCH_3, q). Positive ESI-MS m/z 317 $[M + H]^+$.

2.5. Synthesis of 2′,4,4′-trihydroxychalcone

The synthesis was achieved by following the procedures obtained from the literature (Guan, Yin, Quan, & Quan, 2004). 2,4-Dihydroxyacetophenone (1.00 g) and 4-hydroxybenzaldehyde (0.46 g) were dissolved in ethylene glycol (5 mL), then boric acid (0.70 g) was added under constant stirring. The resulting mixture was stirred at 120 °C for 6 h. The reaction mixture was first separated on Sephadex LH-20 column with MeOH as mobile phase. Then it was purified by semi-preparative RP-HPLC using MeOH–H_2O (65:35) as mobile phase at a flow rate of 8 mL/min and at wavelength of 254 nm to give 2′,4,4′-trihydroxychalcone (65.0 mg, yield 3.85%). The structure was confirmed by ^{1}H NMR and was found to be identical with Isoliquiritigenin.

2.6. Mushroom tyrosinase inhibitory assay

Tyrosinase inhibitory activity of plant extracts and isolated compounds was determined by spectrophotometric method. The procedure followed that described by Vanni, Gastaldi, and Giunata (1990). All fractions or compounds were first dissolved in DMSO at 1.0 mg/mL and then diluted to different concentrations using DMSO. Each sample (30 μL) was diluted with 970 μL 0.05 mM sodium phosphate buffer (pH 6.8) in a test tube. This was followed by addition of 1 mL L-tyrosine solution and finally 1.0 mL mushroom tyrosinase (200 units/mL) was added. The test mixture (3.0 mL) was mixed by Vortex and incubated for 20 min at 37 °C and absorbance at 490 nm was measured. The absorbance of the same mixture without the test sample was used as the control. The same experiment was repeated at least twice and they gave similar results. The IC_{50}, the concentration of plant extract at which half the original tyrosinase activity is inhibited, was determined for each compound. The percent inhibition of tyrosinase activity was calculated as follows:

$$\% \text{ inhibition} = (A - B)/A \times 100$$

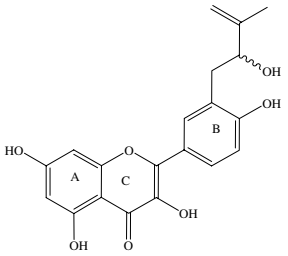
where A = absorbance at 490 nm without test sample, and B = absorbance at 490 nm with test sample.

3. Results and discussion

Bioassay-guided separation of the chloroform-soluble extract of paper mulberry twigs led to the purification of 11 compounds. Separation was achieved by classic column chromatography on silica gel, Sephadex LH-20 and with the aid of semi-preparative reverse-phase HPLC. Structures for 10 compounds were identified by comparing MS, ^{1}H NMR and ^{13}C NMR data with the literature, as uralenol (**2**) (Jia, Ma, & Wang, 1991), quercetin (**3**) (Batterham & Highet, 1964), isolicoflavonol (**4**) (Hatano, Yasuhara, Miyamoto, & Okuda, 1988), papyriflavonol A (**5**) (Son, Kwon, Chang, Kim, & Kang, 2001), broussoflavonol F (**6**) (Fang, Shieh, Wu, & Lin, 1995), 5,7,3′,5′-tetrahydroxyflavanone (**7**) (Nessa, Ismail, Mohamed, & Haris, 2004), luteolin (**8**) (Ternai & Markham, 1976), isoliquiritigenin (**9**) (Vaya, Belinky, & Aviram, 1997), broussochalcone A (**10**) (Matsumoto et al., 1985), and 5,7,3′,4′-tetrahydroxy-3-methoxyflavone (**11**) (Wang, Hamburger, Gueho, & Hostettmann, 1989). Several of them, 3,5,7,4′-tetrahydroxy-3′-(2-hydroxy-3-methylbut-3-enyl)flavone, 5,7,3′,5′-tetrahydroxyflavanone, luteolin and 5,7,3′,4′-tetrahydroxy-3-methoxyflavone, were isolated for the first time from paper mulberry (Fig. 1).

Compound **1** was found to be a novel compound and its structure was elucidated by interpretation of MS and NMR data. Compound **1** gave a molecular ion peak $[M + H]^+$ at m/z 371 in positive ESI-MS, consistent with a molecular formula of $C_{20}H_{18}O_7$. In its ^{1}H NMR spectrum (Table 1), characteristic proton signals for a five-carbon hydroxylated alkenyl group at δ 1.81 (3H, singlet), 2.86 (1 H, $J = 13.8$, 7.5 Hz), 2.96 (1 H, $J = 13.8$, 5.3 Hz), 4.40 (1H, dd, $J = 7.5$, 5.3 Hz, H-2″), 4.77 (1H, broad singlet) and 4.88 (1H, broad singlet), and five aromatic proton signals at δ 6.17 (1H, broad singlet), 6.39 (1H, broad singlet), 6.87 (1H, $J = 8.4$ Hz), 7.85 (1H, $J = 8.4$ Hz) and 7.96 (1H, broad singlet) were observed, suggesting that **1** has a flavonoid skeleton with four substituted hydroxyl groups and one hydroxylated five-carbon alkenyl group (Wandji, Fomum, Tillequin, Seguin, & Koch, 1994). This was supported by ^{13}C NMR data (Table 1), which totally showed 20 carbon signals with five signals [δ 18.2 (q), 38.5 (t), 76.7 (d), 111.5 (d), and 148.7 (s)] assignable to a 2-hydroxy-3-methylbut-3-enyl group and other carbon signals assignable to a flavonol skeleton. The full assignment of ^{1}H NMR and ^{13}C NMR (Table 1) of compound **1** was achieved with the aid of HMQC and HMBC spectra. The positions of the substituents were deduced as occurring at C-5, C-7, C-3, and C-4′ (four hydroxyls) and C-3′ (2-hydroxyl-3-methylbut-3-enyl) using the HMBC and HMQC NMR technique (the key HMBC correlations are shown in Fig. 2). Thus, the structure of the new compound **1** was elucidated as 3,5,7,4′-tetrahydroxy-3′-(2-hydroxy-3-methylbut-3-enyl)flavone.

Tyrosinase inhibitory activity of compounds **1**–**11** using L-tyrosine as substrate was examined. Each compound was assayed at different concentrations and their relative activ-



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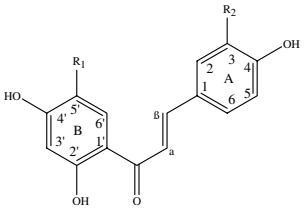
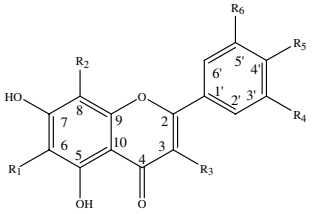
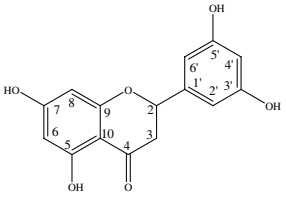


9 R_1= R_2= H

10 R_1= prenyl, R_2= OH





2 R_1= H, R_2= H, R_3= OH, R_4= prenyl, R_5= OH, R_6= OH **7**

3 R_1= H, R_2= H, R_3= OH, R_4= OH, R_5= OH, R_6= H

4 R_1= H, R_2= H, R_3= OH, R_4= prenyl, R_5= OH, R_6= H

5 R_1= prenyl, R_2= H, R_3= OH, R_4= prenyl, R_5= OH, R_6= OH

6 R_1= H, R_2= prenyl, R_3= OH, R_4= prenyl, R_5= OH, R_6= H

8 R_1= R_2= R_3= H, R_4= R_5= OH, R_6= H

11 R_1=R_2=R_6=H, R_4=R_5=OH, R_3=OCH_3

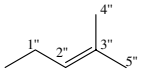
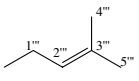
Prenyl:  (attached at B ring)  (attached at A ring)

Fig. 1. Structures of compounds isolated from *Broussonetia papyrifera*.

ities were expressed as IC_{50}values. As shown in Table 2, the IC_{50} values of 3,5,7,4′-tetrahydroxy-3′-(2-hydroxy-3-methylbut-3-enyl)flavone (**1**), uralenol (**2**), quercetin (**3**) and broussoflavonol F (**6**) were 96.6, 49.5, 57.8, and 82.3 μM, respectively. Their activities are better than arbutin, a well-known tyrosinase inhibitor. However, other compounds (Compound **4**, **5**, **7**–**11**) showed weaker activities than arbutin. Based on the literature, quercetin, papyriflavonol A, broussochalcone A (Lee et al., 2004) and isoliquiritigenin (Ohad, Jacob, Ramadan, Saritizael, & Snait, 2003) were previously evaluated for their inhibition of monophenolase activity using L-tyrosine as substrate and the activities of quercetin, papyriflavonol A and broussochalcone A were in good agreement with our results. However for isoliquritigenin, the IC_{50} value found in this study was much higher than that reported by Ohad et al. (2003), which reported that isoliquritigenin showed stronger inhibition on the monophenolase activity of tyrosinase than kojic acid and arbutin, while our data showed it is a much weaker tyrosinase inhibitor when compared with arbution and kojic acid. In order to confirm our structure and activity for isoliquritigenin, a commercial reference sample of isoliquritigenin was obtained from Indofine Chemical Company, Inc. (NJ, USA) and isoliquiritigenin was also synthesized. The synthesized and the purchased compounds were both identical to isoliquiritigenin purified from paper mulberry and they showed very weak tyrosinase inhibition. Recently, Khatib et al. (2005) studied the structure/activity relationship of a few chalcones, and they found that a 2,4-substituted resorcinol subunit on ring A of chalcone contributed predominantly to tyrosinase inhibitory potency while a 2,4-substituted resorcinol subunit on ring B of chalcone did not contribute significantly to chalcones's tyrosinase inhibitory activities. Such discrepancy might be due to impurities in the sample or misidentification of the active compound.

Due to the limited amounts of compounds from paper mulberry, the application of these compounds in prevent-

Table 1
NMR data of compound **1** in CD_3OD

Carbon	δ_H	δ_C
2		159.0 (s)
3		137.3 (s)
4		177.5 (s)
5		162.6 (s)
6	6.17 (1H, s)	99.4 (d)
7		158.4 (s)
8	6.39 (1H, s)	94.6 (d)
9		158.4 (s)
10		104.7 (s)
1′		123.8 (s)
2′	7.96 (1H, s)	132.4 (d)
3′		127.1 (s)
4′		159.0 (s)
5′	6.87 (1H, d, $J = 8.4$ Hz)	116.3 (d)
6′	7.85 (1H, d, $J = 8.4$ Hz)	129.0 (d)
1″	2.96 (1H, dd, $J = 13.8$; 5.3 Hz)	38.5 (t)
	2.86 (1H, dd, $J = 13.8$; 7.5 Hz)	
2″	4.40 (1H, dd, $J = 7.5$; 5.3 Hz)	76.7 (d)
3″		148.7 (s)
4″	4.88 (1H, s), 4.77 (1H, s)	111.5 (t)
5″	1.81 (3H, s)	18.2 (q)

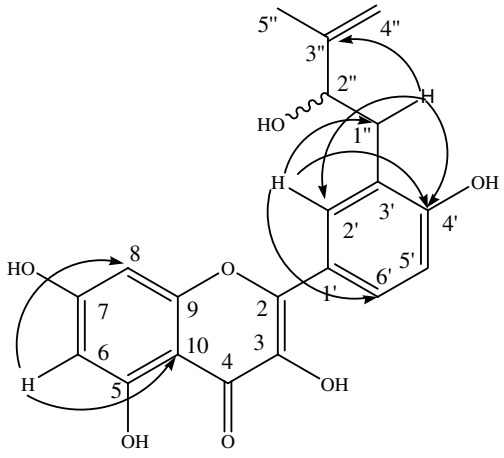


Fig. 2. The key HMBC correlations observed for compound **1**.

Table 2
Tyrosinase inhibitory activity of compounds **1**–**11** purified from the twigs of paper mulberry

Compound	IC_{50} (μM) mean ± SD
1	96.6 ± 1.3
2	49.5 ± 0.7
3	57.8 ± 2.7
4	>1000
5	82.3 ± 3.0
6	388.6 ± 12.0
7	>1000
8	>1000
9	480.8 ± 15.9
10	421.5 ± 6.8
11	>1000
Arbutin	146.0 ± 2.6

ing browning in food systems was not evaluated. However, based on their mushroom tyrosinase inhibitory activities, some of these compounds including the new compound and paper mulberry twig extract do have the potential to be used as natural anti-browning agents in foods.

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