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ORIGINAL ARTICLE

Inhibition of Xanthine Oxidase by Phenolic Phytochemicals from *Broussonetia papyrifera*

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Abstract The roots of *Broussonetia papyrifera* were extracted into four different polar solvents: chloroform, 50% ethanol in water, ethanol, and water. The ethanol extract showed the most potent inhibition (72.3% at 20 g/mL) against xanthine oxidase (XOD). Chromatography of EE yielded nine phenolic phytochemicals, which were confirmed as broussochalcone A (**1**), broussochalcone B (**2**), 3,4-dihydroxyisolonchocarpin (**3**), 4-hydroxyisolonchocarpin (**4**), 3-'(3-methylbut-2-enyl)-3',4',7-trihydroxyflavane (**5**), kazinol A (**6**), kazinol B (**7**), kazinol E (**8**), and broussoflavan A (**9**). All isolated compounds (**19**) possessed potent antioxidant activities against 2,2-diphenyl-l-picrylhydrazyl and 2,2'-azino-bis-ethyl-benzthiazoline-6-sulfonic acid (ABTS) radicals with IC_{50} values ranging from 5.8 to 252.8 M. Although most compounds exhibited potent inhibition with IC_{50} values ranging 0.6–164 M against XOD, compounds **1** and **3** were found to be the principal contributors to the XOD inhibition in ethanol extract. The analysis of K_I and K_{IS} values proved that the two most promising compounds (**1** and **3**), present at high concentrations in the root barks as analyzed by using high-performance liquid chromatography analysis, were reversible mixed type I inhibitors.

Keywords antioxidant effect · *Broussonetia papyrifera* · phenolic phytochemicals · xanthine oxidase

Introduction

Active oxygen species has played a crucial role in development and progression of many human diseases (Halliwell, 1991). One of the most important sources of active oxygen species is xanthine oxidase (XOD), which is a key enzyme in the last two steps of purine metabolic pathway as well as has a capability of generating reactive oxygen species (ROS). Main function of XOD is to catalyze the oxidation of hypoxanthine into xanthine, and then to uric acid (Harris et al., 1999). Gout and hyperuricemia are metabolic disorders associated with the accumulation of uric acid in joints and kidneys, causing acute arthritis (Tomita et al., 2000; Kramer and Curhan, 2002). When antioxidant systems are inadequate or active oxygen species are overproduced, oxidative stress can damage lipids, proteins, and neucleic acids (Yu, 1994). Should a substance have dual functions of XOD inhibition and antioxidant activity, it could work not only as a treatment for gout but also as a protector against oxidative stress. Thus, there is a need to develop compounds having xanthine oxidase inhibitory activity together with quenching potential of ROS.

The *Broussonetia papyrifera* Vent., well known as a polyphenol-rich plant, has been used as a natural therapeutic agent (Ryu et al., 2010). This species belongs to Moraceae family and is distributed throughout China, Korea, and Japan. Especially, its roots have been used as a diuretic, tonic, and suppressant for edema in Chinese folk medicine (Zhang et al., 2001). Main phenolic phytochemicals constituents are chalcones, flavans, and flavonols, many of which have been proven to have anti-browning (Zheng et al., 2008), hyperglycemic (Chen et al., 2002), antimicrobial (Shon et al., 2004), and anti-inflammatory properties (Lin et al., 2008). Recently, we reported that polyphenols of *B. papyrifera* had significant inhibitory potential against α-glucosidase (Ryu et al., 2010) and acetylcholinesterase (Ryu et al., 2012).

However, to the best of our knowledge, there has been no report on the roots of *B. papyrifera* eliciting XOD inhibition; thus, with this in mind, we set about the present work. Nine phenolic phytochemicals were isolated from the roots of *B. papyrifera*, and their structures were identified by using spectroscopic methods. The isolated compounds were evaluated separately for their inhibitory activities against XOD and antioxidant capacity via 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis(ethylbenz-

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thiazoline-6-sulfonic acid) (ABTS). Their inhibition mechanisms were ascertained using Lineweaver-Burk and second plots. The relative abundance of these extracts within the native roots was also assessed using a HPLC chromatogram profile.

Materials and Methods

Chemicals and materials. CH_3CN (HPLC grade) and water were purchased from J. T. baker (USA). CD_3OD, acetone-d_6, and $CDCl_3$ were purchased from Cambridge Isotope Lab. Inc., (USA). Folin-Ciocalteu's phenol reagent, ABTS, DPPH, XOD, xanthine, allopurinol, and potassium persulfate were purchased from Sigma-Aldrich Co., (USA). All solvents were distilled before use. The root barks of *B. papyrifera* were collected at Gonyang, Korea on July, 2008 and identified by Prof. Myong Gi Chung. A voucher specimen (KHPark 210709) of this raw material is deposited at the Herbarium of Gyeongsang National University, Korea.

Instruments. NMR spectra were recorded with a Bruker AM500 instrument (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz, USA). Electron ionization (EI) and EI-high resolution (HR) mass spectra were obtained on a JEOL JMS-700 instrument (Japan). Optical rotations were measured on a Perkin-Elmer 343 polarimeter (USA). Melting points were measured on a Thomas Scientific Capillary Melting Point Apparatus (USA) and are uncorrected. Column chromatography was performed on silica gel (230–400 mesh, Merck, Germany), RP-18 (ODS-A, 12 nm, S-150 μM, YMC, Japan), and Sephadex LH-20 (GE Healthcare Bio-Science AB, Sweden). Thin layer chromatography was performed using precoated thin-layer chromatography (TLC) plates with silica gel 60 F_{254} (Merck, 0.25 mm, normal phase). The plates were visualized by UV illumination (254 nm, UK) or by spraying with 10% H_2SO_4 in ethanol followed by heating.

Total phenolic and flavonoid contents. Total phenolic content in each extract were measured according to the modified Folin-Denis method (Lee et al., 2008). Briefly, Folin-Ciocalteu reagent (0.5 mL) and 10% Na_2CO_3 solution (0.5 mL) was added to each extract (1 mL) and mixed vigorously. After 5 min, the absorbance was measured at 724 nm. Total phenolic content of each extract was calculated in mg of gallic acid equivalent (mg GAE/g). The flavonoid content of each extract was measured using the Choi method against a dietary flavonoid such as catechin (Choi et al., 2006). Subsequently, 0.5 mL of extract was mixed with 0.1 mL of 10% $Al(NO_3)_3$ and 0.1 mL of 1 M CH_3CO_2K, followed by measurement of absorbance at 420 nm. The standard calibration curve was made using catechin equivalents (mg CE/g).

DPPH and ABTS radicals scavenging activities. The DPPH radical scavenging activity of the samples was measured by the method of Blois (1958) with a slight modification. Briefly, various concentrations of sample extract (0.1 mL) were respectively added to 0.49 mL of methanol and 0.39 mL of DPPH ethanolic solution (1 mM). The mixture was vortexed vigorously and was allowed to stand for 30 min at room temperature in the dark. The absorbance of the mixture was determined using a spectrophotometer (Beckman Coulter DU650, USA) at 517 nm. Trolox was used as the reference. The scavenging activity was expressed as a percentage using the following formula: DPPH radical scavenging activity (%) = (1 – absorbance of sample/absorbance control) × 100. The ABTS radical scavenging activity was based on the ability of different substances to scavenge the $ABTS^{\bullet+}$ radical cation in comparison to a standard (Trolox). $ABTS^{\bullet+}$ was dissolved in EtOH to achieve a concentration of 7 mM. This radical cation was produced by reacting the $ABTS^{\bullet+}$ stock solution with 2.45 mM potassium persulfate, and the mixture was left standing for 10–14 h until the reaction was complete and the absorbance was stable. For measurement, the $ABTS^{\bullet+}$ stock solution was diluted in ethanol to an absorbance of 0.70 at 734 nm (Beckman Coulter DU650, USA). After the addition of 0.9 mL of diluted $ABTS^{\bullet+}$ to 0.1 mL of sample, the absorbance was taken 1 min after the initial mixing (Fellegrin et al., 1999). This scavenging activity (%) was expressed as a percentage using the following formula: $ABTS^{\bullet+}$ radical scavenging activity (%) = [(absorbance of control – absorbance of sample)/absorbance of control] × 100.

Xanthin oxidase inhibitory activity. Measurement of xanthin oxidase inhibitory activity was carried out according to the method of Kong et al. (2000) with slight modifications. First, 15 μL of 1 unit XOD in buffer (200 mM sodium pyrophosphate/HCl, pH 7.5) and stock solution (5 μL) of the test extracts or compounds in DMSO were mixed at 37°C. The reaction was started by adding 100 μL of 20 mM xanthine in double-distilled water to the mixture. The reaction mixture was incubated at ambient temperature, and the absorbance at 295 nm was determined every 1 min up to 5 min. Allopurinol was used as a positive control. Three replicates were made for each test sample. The percent inhibition ratio (percent) was calculated according to the following equation: % inhibition = [(rate of control reaction – rate of sample reaction)/rate of control reaction] × 100.

Enzyme kinetic assay and progress linear determination. The inhibition kinetics of the enzyme by the isolated compound was analyzed by the Lineweaver-Burk plots compared to data obtained in the absence of inhibitor (Ryu et al., 2012). Kinetic assays and progress linear were carried out using 1 U/mL units xanthine oxidase and xanthine as a substrates in double-distilled water at 37°C. Enzyme activities were measured continuously for 5 min using a spectrophotometer. To determine the kinetic parameters associated with inhibition mechanism of xanthine oxidase, linear progressions for steady state were obtained at several inhibitor concentrations using changed substrate concentrations. The inhibition constants for inhibitor binding with free or enzyme-substrate complex, K_I or K_{IS}, respectively with xanthine as substrate, were obtained from the second plots of the slopes of the straight lines or vertical intercept ($1/V^{app}_{max}$), respectively, versus the concentration of inhibitors. K_I and K_{IS} are represented by the Equations (1), (2), and (3) (Zhang et al., 2006; Chiari et al., 2011):

$$1/V = K_m/V_{max} (1 + [I]/K_I) 1/S + 1/V_{max} \quad (1)$$

$$\text{Slope} = K_m/K_I V_{max}\ [I] + K_m/V_{max} \quad (2)$$

$$\text{Intercept} = 1/K_{IS} V_{max}\ [I] + 1/V_{max} \quad (3)$$

Extraction and isolation. The root barks of *B. papyrifera* were extracted in separate flasks (10 g dry bark each) with 200 mL of chloroform, 50% ethanol in water, ethanol or distilled water at room temperature for 3 days to examine inhibitory activities against antioxidant and XOD as a function of solvent used (Table 2). The ethanol extract was determined as the target extract for isolation of XOD inhibitors as it gave the strongest XOD inhibition. The air-dried root barks (1.0 kg) of *B. papyrifera* were chopped and extracted with ethanol (10 L × 4) at room temperature for 3 days. The combined filtrate was concentrated in vacuo to yield a dark brown gum (143 g, 14.3%). This crude extract (55 g) was fractionated by silica gel flash column chromatography (CC) employing a gradient of chloroform to acetone to afford five fractions (fractions A-E). The pure compounds **1**–**9** have been isolated by different chromatographic methods and characterized as described previously (Filho et al., 1975; Fukai and Nomura, 1985; Dagne et al., 1989; Ryu et al., 2010). Fraction B (6.5 g) was fractionated by silica gel flash CC (4.5 × 70 cm, 300 g) employing a gradient of *n*-hexane-EtOAc (30:1 → 1:1) to afford 12 subfractions (B1-B12). Subfractions B7-B10, enriched with **5**–**8**, were combined (1683 mg) and further purified by silica gel flash CC (3.0 cm × 50 cm, 150 g) to yield compounds **5** (61 mg), **6** (115 mg), and a mixture of compounds **7** and **8**. Further purification by Sephadex LH-20 (Pharmacia Biotech AB, Sweden) (1.5 × 50 cm, 60 g) with CH_3OH as an eluent yielded compounds **7** (88 mg) and **8** (54 mg). Fraction C (5.3 g) was fractionated by silica gel flash CC (4.5 × 70 cm, 250 g) employing a gradient of *n*-hexane- EtOAc (15:1 → 1:1) resulting in 18 subfractions (C1-C18). Subfractions C10-C14 (375 mg) were rechromatographed on a silica gel column (2.5 × 30 cm, 80 g) eluting with *n*-hexane-EtOAc (10:1 → 1:1) to yield compound **9** (23 mg). Fraction D (11.5 g) was subjected to flash CC (4.5 × 70 cm, 300 g) employing a gradient $CHCl_3$-acetone (50:1 → 1:1) giving 15 Subfractions (D1–D15). Subfraction D3–D6, enriched with **1**–**4**, were combined (981 mg) and further purified by reversed-phase CC (ODS-A, 12 nm, S-150 μM) eluting with CH_3OH:H_2O (4:1) to afford compounds **1** (231 mg), **2** (187 mg), **3** (8 mg), and **4** (5 mg).

Broussochalcone A (**1**): yellow needles; mp 182–184°C; EIMS *m/z* 340 $[M]^+$; HREIMS *m/z* 340.1313, (calcd for $C_{20}H_{20}O_5$, 340.1311); ^{1}H-NMR (500 MHz, acetone-d_6) δ 13.5 (1H, s, 2-OH), 7.96 (1H, s, H-6'), 7.72 (1H, d, *J*=15.3 Hz, H-α), 7.72 (1H, d, *J*=15.3 Hz, H-β), 7.32 (1H, s, H-2),7.20 (1H, d, *J*=12.0 Hz, H-6),6.92 (1H, d, *J*=13.5 Hz, H-5),6.41 (1H, s, H-3'), 5.35 (1H, br t, *J*=14.3 Hz. *J*=7.2 Hz, H-2"), 3.31 (2H, d, *J*=11.5 Hz, H-1"), 1.61 (3H, s, H-5"), 1.58 (3H, s, H-4"); ^{13}C-NMR (125 MHz) δ 193.1 (C=O), 166.3 (C-2'), 163.9 (C-4'), 149.6 (C-4), 146.8 (C-β), 145.7 (C-3), 132.8 (C-3"), 132.7 (C-6'), 128.7 (C-1), 124.4 (C-2"), 123.8 (C-6), 121.7 (C-5'), 119.0 (C-α), 116.9 (C-5), 116.3 (C-2), 114.8 (C-1'), 103.9 (C-3'), 29.2 (C-1"), 26.3 (C-4"), 18.4 (C-5") (Matsumoto et al., 1985).

Broussochalcone B (**2**): yellow needles; mp 168–169°C; EIMS *m/z* 324 $[M]^+$; HREIMS *m/z* 324.1363, (calcd for $C_{20}H_{20}O_4$, 324.1362); ^{1}H-NMR (500 MHz, acetone-d_6) δ 7.76 (1H, d, *J*=15.5 Hz. H-β), 7.73 (1H, s, H-6'), 7.59 (2H, d, *J*=8.3 Hz, H-2, 6),7.54 (1H, d, *J*=15.5 Hz, H-α), 6.85 (2H, d, *J*=8.1 Hz, H-3, 5),6.31 (1H, s, H-3'), 5.34 (1H, s, H-2"), 3.27 (2H, d, *J*=7.0 Hz, H-1"), 1.72 (6H, m, H-4", 5"); ^{13}C-NMR (125 MHz) δ 192.0 (C=O), 164.4 (C-4'), 163.0 (C-2'), 160.1 (C-4), 143.9 (C-β), 131.8 (C-6'), 130.7 (C-3"), 130.7 (C-2, 6), 126.4 (C-1), 122.6 (C-2"), 120.6 (C-5'), 117.0 (C-α), 115.5 (C-3, 5), 112.0 (C-1'), 101.9 (C-3'), 29.4 (C-1"), 27.5 (C-4"), 16.5 (C-5") (Matsumoto et al., 1985).

3,4-Dihydroxyisolonchocarpin (**3**): amorphous yellow powder; mp 242–243°C; EIMS *m/z* 338 $[M]^+$; HREIMS *m/z* 338.1153, (calcd for $C_{20}H_{18}O_5$, 328.1154); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.69 (1H, d, *J*=15.2 Hz, H-β), 7.41 (1H, s, H-6'),7.32 (1H, d, *J*=15.3 Hz, H-α), 7.15 (1H, s, H-2), 7.07 (1H, d, *J*=6.9 Hz, H-6),6.84 (1H, s, H-5), 6.31 (1H, s, H-3'), 6.25 (1H, d, *J*=9.9 Hz, H-1"), 5.32 (1H, d, *J*=9.9 Hz, H-2"), 1.39 (6H, s, H-4", 5"); ^{13}C NMR (125 MHz) δ 192.0 (C=O), 166.9 (C-2'), 160.9 (C-4'), 146.5 (C-3), 144.6 (C-β),144.0 (C-4), 129.3 (C-2"), 128.8 (C-1'), 127.8 (C-6'), 123.6 (C-α), 121.5 (C-6), 118.7 (C-1"), 116.1 (C-5), 115.2 (C-2), 114.7 (C-1), 113.9 (C-5'), 105.2 (C-3'), 78.4 (C-3"), 29.0 (C-4", 5") (Filho et al., 1975).

4-Hydroxyisolonchocarpin (**4**): amorphous yellow powder; mp 196–197°C; EIMS *m/z* 322 $[M]^+$; HREIMS *m/z* 322.1203, (calcd for $C_{20}H_{20}O_4$, 322.1205); ^{1}H-NMR (500 MHz, CD_3OD) δ 7.74 (1H, d, *J*=14.8 Hz, H-β), 7.48 (1H, s, H-2),7.42 (1H, s, H-6'), 7.34 (1H, d, *J*=14.7 Hz, H-α), 6.82 (2H, s, H-3, 5), 6.30 (1H, s, H-3'), 6.24 (1H, d, *J*=9.6 Hz, H-1"), 5.51 (1H, d, *J*=10.0 Hz, H-2"), 1.38 (6H, s, H-4", 5"); ^{13}C-NMR (125 MHz) δ 192.2 (C=O), 166.9 (C-2', 4'), 160.9 (C-4), 144.7 (C-β), 131.0 (C-2"), 129.2 (C-2, 6), 127.8 (C-6'), 126.1 (C-1), 121.5 (C-1"), 118.1 (C-α), 116.5 (C-3, 5), 114.7 (C-1'), 113.9 (C-5'), 105.2 (C-3'), 78.4 (C-3"), 29.0 (C-4", 5") (Dagne et al., 1989).

3'-(3-Methylbut-2-enyl)-3',4',7-trihydroxyflavane (**5**): yellow sticky oil; $[\alpha]_D$ −5.8° ($CHCl_3$, *c* 0.35); EIMS *m/z* 326 $[M]^+$; HREIMS *m/z* 326.1516, (calcd for $C_{20}H_{22}O_4$, 326.1518); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 6.87 (2H, m, H-2', 6'), 6.68 (1H, m, H-5), 6.36 (2H, m, H-6, 8), 5.13 (1H, m, H-2"), 5.06 (1H, m, H-2), 3.41 (2H, d, *J*=6.4 Hz, H-1"), 2.73 (2H, m, H-4), 2.04 (2H, m, H-3b), 1.98 (1H, m, H-3a), 1.70 (3H, s, H-5"), 1.65 (3H, s, H-4"); ^{13}C-NMR (125 MHz) δ 156.5 (C-8a), 155.3 (C-7), 143.8 (C-5'), 142.8 (C-4'), 133.5 (C-3"), 132.5 (C-1'), 130.6 (C-5), 126.6 (C-3'), 122.9 (C-2"), 118.8 (C-2'), 114.6 (C-4a), 113.7 (C-6'), 108.7 (C-6), 104.1 (C-8), 75.6 (C-2), 29.9 (C-3), 25.6 (C-1"), 25.5 (C-4), 18.4 (C-4", 5") (Chen et al., 2002).

Kazinol A (**6**): yellowish powder; mp 129–130°C; $[\alpha]_D$ −10.7° ($CHCl_3$, *c* 0.13); EIMS *m/z* 394 $[M]^+$; HREIMS *m/z* 394.2141, (calcd for $C_{25}H_{30}O_4$, 394.2144); ^{1}H-NMR (500 MHz, acetone-d_6) δ 6.90 (1H, d, *J*=6.2 Hz, H-5), 6.88 (1H, s, H-6'), 6.37 (1H, d, *J*=2.4 Hz, *J*=8.2, H-6), 6.27 (1H, d, *J*=2.4 Hz, H-8), 5.12 (1H, t, *J*=10.9 Hz, *J*=2.2 Hz, 2"), 5.07 (1H, t, *J*=17.4 Hz, *J*=3.2 Hz, H-2'"), 5.04 (1H, d, *J*=1.8 Hz, H-2), 3.42 (2H, d, *J*=10.5 Hz, H-1"),

3.34 (2H, m, H-1'''), 2.82 (1H, m, H-4), 2.71 (1H, m, H-4), 2.07 (1H, m, H-3), 1.88 (1H, m, H-3), 1.76 (3H, s, H-5''), 1.71 (3H, s, H-4''), 1.67 (6H, s, H-4''', 5'''); ^{13}C-NMR (125 MHz) δ 56.6 (C-8a), 156.5 (C-7), 142.9 (C-3'), 142.4 (C-4'), 131.1 (C-3''), 130.2 (C-3'''), 130.1 (C-1'), 129.0 (C-5), 126.7 (C-2'), 124.6 (C-5'), 123.7 (C-2'''), 112.9 (C-2''), 110.8 (C-6''), 110.2 (C-4a), 107.9 (C-6), 103.0 (C-8), 74.7 (C-2), 26.8 (C-1''), 26.8 (C-4), 25.2 (C-1'''), 25.2 (C-3), 24.9 (C-5''), 24.8 (C-5'''), 17.2 (C-4''), 17.1 (C-4''') (Ikut et al., 1985).

Kazinol B (**7**): amorphous yellow powder; mp 86−88°C; $[\alpha]_D$ −19.0° ($CHCl_3$, *c* 0.38); EIMS *m/z* 392 $[M]^+$; HREIMS *m/z* 392.1989, (calcd for $C_{25}H_{28}O_4$, 392.1988); ^{1}H-NMR (500 MHz, acetone-d_6) δ 6.82 (1H, d, *J*=8.4 Hz, H-5), 6.65 (1H, s, H-2'), 6.29 (1H, d, *J*=2.6 Hz, H-6), 6.27 (1H, s, H-8), 6.20 (1H, d, *J*=9.9 Hz, H-1'''), 5.46 (1H, d, *J*=9.8 Hz, H-2'''), 5.06 (2H, t, *J*=12.1 Hz, *J*=6.3 Hz, H-2''), 4.99 (2H, dd, J_1=10.7 Hz, J_2=1.9 Hz, H-2), 3.33 (2H, t, *J*=14.8 Hz, *J*=7.7 Hz, H-1''), 2.78 (1H, m, H-4), 2.64 (1H, m, H-4), 1.99 (1H, m, H-3), 1.89 (1H, m, H-3), 1.62 (3H, s, H-4''), 1.58 (3H, s, H-5''), 1.37 (3H, s, H-4'''), 1.34 (3H, s, H-5'''); ^{13}C-NMR (125 MHz) δ 156.8 (C-8a), 155.4 (C-7), 142.6 (C-3'), 139.0 (C-4'), 132.7 (C-3''), 132.0 (C-1'), 130.5 (C-2'''), 130.3 (C-5), 126.2 (C-2'), 123.3 (C-2''), 122.7 (C-6'), 119.4 (C-5'), 115.5 (C-1'''), 114.5 (C-4a), 108.4 (C-6), 104.1 (C-8), 77.7 (C-3'''), 74.5 (C-2), 30.1 (C-4), 28.5 (C-4'''), 28.2 (C-5'''), 26.1 (C-5''), 19.5 (C-1''), 18.3 (C-3), 14.5 (C-4'') (Ikut et al., 1985).

Kazinol E (**8**): yellow powder; mp 146−147°C; $[\alpha]_D$ +0.33° ($CHCl_3$, *c* 0.41); EIMS *m/z* 462 $[M]^+$; HREIMS *m/z* 462.2761, (calcd for $C_{30}H_{38}O_4$, 462.2770); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 6.92 (1H, s, H-5), 6.37 (1H, s, H-2'), 6.18 (1H, q, *J*=17.7 Hz, *J*=10.5 Hz, H-4''), 5.66 (1H, s, H-8), 5.32 (1H, d, *J*=17.7 Hz, H-5''), 5.26 (1H, d, *J*=10.6 Hz, H-5''), 5.12 (1H, t, *J*=5.5 Hz, *J*=12.1 Hz, H-2'''), 5.04 (1H, dd, *J*=1.3 Hz, *J*=5.6 Hz, H-2), 4.99 (1H, t, *J*=4.3 Hz, *J*=6.4 Hz, H-2''''), 3.38 (2H, d, *J*=6.4 Hz, H-1''''), 3.31 (2H, m, H-1'''), 2.90 (1H, m, H-4), 2.74 (1H, m, H-4), 2.10 (1H, m, H-3), 1.99 (1H, m, H-3), 1.79 (3H, s, H-5''''), 1.72 (3H, s, H-4''''), 1.68 (3H, s, H-5'''), 1.66 (3H, s, H-4'''), 1.42 (6H, s, H-2'', 3''); ^{13}C-NMR (125 MHz)δ 155.3 (C-8a), 153.8 (C-7), 148.3 (C-4''), 142.1 (C-3', 4'), 133.7 (C-3''''), 132.1 (C-3'''), 131.2 (C-1'), 129.8 (C-5'), 126.8 (C-5), 126.6 (C-6'), 124.8 (C-6), 123.8 (C-2'''), 122.3 (C-2''''), 113.7 (C-4a), 113.1 (C-5''), 105.6 (C-8), 74.9 (C-2), 39.8 (C-1''), 30.0 (C-4), 27.3 (C-1''''), 27.3 (C-2'', 3''), 25.9 (C-3), 25.7 (C-5'''), 25.6 (C-1'''), 25.6 (C-5''''), 18.1 (C-4''''), 18.0 (C-4'''), 11.1 (C-2') (Ikauta et al., 1985).

Broussoflavan A (**9**): yellowish sticky oil; $[\alpha]_D$ −17.65° (acetone, *c* 0.092); EIMS *m/z* 426 $[M]^+$; HREIMS *m/z* 426.2047, (calcd for $C_{25}H_{30}O_6$, 462.2047); ^{1}H-NMR (500 MHz, acetone-d_6) δ 7.35 (1H, s, H-6'), 6.91 (1H, d, *J*=8.5 Hz, H-5), 6.39 (1H, d, *J*=8.5 Hz, H-6), 6.31 (1H, s, H-8), 5.18 (1H, brt, H-2''), 5.10 (1H, d, *J*=9.0 Hz, H-2), 4.54 (1H, d, *J*=8.5 Hz, H-2'''), 3.59 (1H, d, *J*=8.5 Hz, H-1'''), 3.46 (1H, m, H-1''), 2.91 (1H, m ,H-4), 2.73 (1H, m ,H-4), 2.10 (1H, m H-3), 1.96 (1H, m, H-3), 1.72 (3H, s, H-5''), 1.65 (3H, s, H-4''), 1.48 (3H, s, H-5'''), 1.23 (3H, s,H-4''); ^{13}C-NMR (125 MHz) δ 157.9 (C-8a), 157.7 (C-7), 143.9 (C-3'), 140.4 (C-4'), 133.4 (C-1'), 131.4 (C-3''), 131.3 (C-5), 125.6 (C-2'), 125.0 (C-2''), 123.8 (C-5'), 117.0 (C-6'), 114.2 (C-4a), 109.3 (C-6), 104.4 (C-8), 80.4 (C-3'''), 77.3 (C-1'''), 76.2 (C-2), 70.3 (C-2'''), 31.1 (C-3), 27.4 (C-5'''), 26.4 (C-4), 26.3 (C-5''), 25.6 (C-1''), 20.2 (C-4'''), 18.5 (C-4'') (Fang et al., 1994).

Analytical HPLC conditions. Quantification of the relative abundance of the isolated phenolic phytochemicals within the ethanol extract of *B. papyrifera* root barks was carried out by analytical HPLC. To characterize phenolic compounds, the ground root barks (1 g) were extracted with 20 mL of ethanol for 3 days at room temperature. The supernatant was centrifuged at 3000 g for 3 min, and then filtered through a 0.45-μm syringe filter (Whatman Inc., UK) prior to HPLC analysis. A sample (20 μL) of the crude ethanol extract was injected into a Capcell Pak C 18 column (4.6×250 mm, UG 120Å, 5 μM). The mobile phase was composed of 1% acetic acid in water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 0–0.5 min, 0% B; 10 min, 50% B; 20 min, 55% B; 30 min, 60% B; 50 min, 85% B; 70 min, 100% B, and then held for 10 min before returning to the initial conditions. The total running time was 70 min with a flow rate of 1.0 mL/min. The column temperature was maintained at 30°C, and peaks of phenolic phytochemicals were monitored at 260 nm.

Statistical analysis. All experiments were repeated at least three times. Results are expressed as mean ± SD (standard deviation). The statistical significance of differences between groups was evaluated for two parallel experiments using the student's t-test.

Results and Discussion

In the preliminary screening, total phenolic (TP) and total flavonoid (TF) contents were determined in various tissues of *B. papyrifera* (Table 1). In the presence of the 10 g test sample, the TP and TF of *B. papyrifera* decreased in the following order: root barks extract (22.6 mg GAE/g, 12.45 mg CE/g) > stem barks extract (16.97 mg GAE/g, 7.46 mg CE/g) > leaves extract (8.15 mg GAE/g, 9.92 mg CE/g) > heartwoods extract (4.38 mg GAE/g, 3.79 mg CE/g). Furthermore, among extracts from different plant tissues of *B. papyrifera*, root barks extract exhibited the highest

Table 1 Total phenolic and total flavonoid contents of ethanol extract of *B. papyrifera*

Specimen	TPC[a] (mg of GAE[b]/g)	TFC[a] (mg of CE[c]/g)
Root barks	22.6±0.5	12.4±0.4
Stem barks	16.9±1.1	7.5±0.3
Leaves	8.2±0.8	6.9±1.1
Heartwoods	4.4±0.4	3.8±0.6

[a]All extracts were examined in a set of experiments repeated three times.
[b]Gallic acid was used as a standard for measuring of the total phenolic content.
[c]Catechin was used as a standard for measuring of the total flavonoid content.

Table 2 Comparison of extraction yield, XOD inhibitory activity, DPPH radical scavenging activity, and $ABTS^{\bullet+}$ scavenging activity of *B. papyrifera* root barks extract

Extraction solvent	Extraction yield (%)[a]	XOD		DPPH	ABTS
		IC_{50} (μg/mL)	Inhibition (%)[b]	Inhibition (%)[b]	Inhibition (%)[b]
$CHCl_3$	8.7±2.2	20.2±0.1	70.3±4.1	66.5±4.5	79.4±3.8
50% EtOH	14.4±1.5	25.8±0.4	58.8±3.3	> 200	> 200
EtOH	14.3±1.0	13.4±0.3	72.3±2.7	79.3±2.3	88.5±1.7
Water	10.9±2.3	NT	>200	>200	>200

All extracts were examined in a set of experiments repeated three times.
[a]Extraction yields are given as g/100 g dry weight.
[b]DPPH and ABTS, sample concentration was 20 μg/mL; XOD, sample concentration was 30 μg/mL.
[c]NT is not test.

TP and TF contents. These results indicated that *B. papyrifera* root barks contain significantly high phenolic and flavonoid contents.

The $CHCl_3$, 50% EtOH in water, EtOH, and H_2O extracts of the above mentioned *B. papyrifera* root barks were also screened for their antioxidant capacity by both chemicals and enzymatic methods. All the four extracts showed a significant quenching effects on DPPH and ABTS free radicals. DPPH and ABTS radicals were quenching most effectively by EtOH extract with around 80% inhibition at 20 μg/mL (Table 2).

Activity guided fractionation of the ethanol extract of *B. papyrifera* gave nine phenolic phytochemicals which were purified over silica gel, Sephadex LH-20 and octadecyl-functionalized silica gel as delineated above. The structure identification of these compounds was carried out by spectroscopic analyses and by comparison with published data. The isolated compounds (**19**) were identified as broussochalcone A (**1**), broussochalcone B (**2**), 3,4-dihydroxy-isolonchocarpin (**3**), 4-hydroxyisolonchocarpin (**4**), 3-(3-methylbut-2-enyl)-3,4,7-trihydroxyflavane (**5**), kazinol A (**6**), kazinol B (**7**), kazinol E (**8**), broussoflavan A (**9**) (Fig. 1). The discussion of structural identification will focus on compound **1,** which emerged to be the most potent XOD inhibitor. The structural elucidation of compound **1** is detailed as follows: the molecular formula $C_{20}H_{20}O_5$ and eleven degrees of unsaturation, as deduced from HREIMS ($[M]^+$ at *m/z* 340.1313). The ^{13}C-NMR

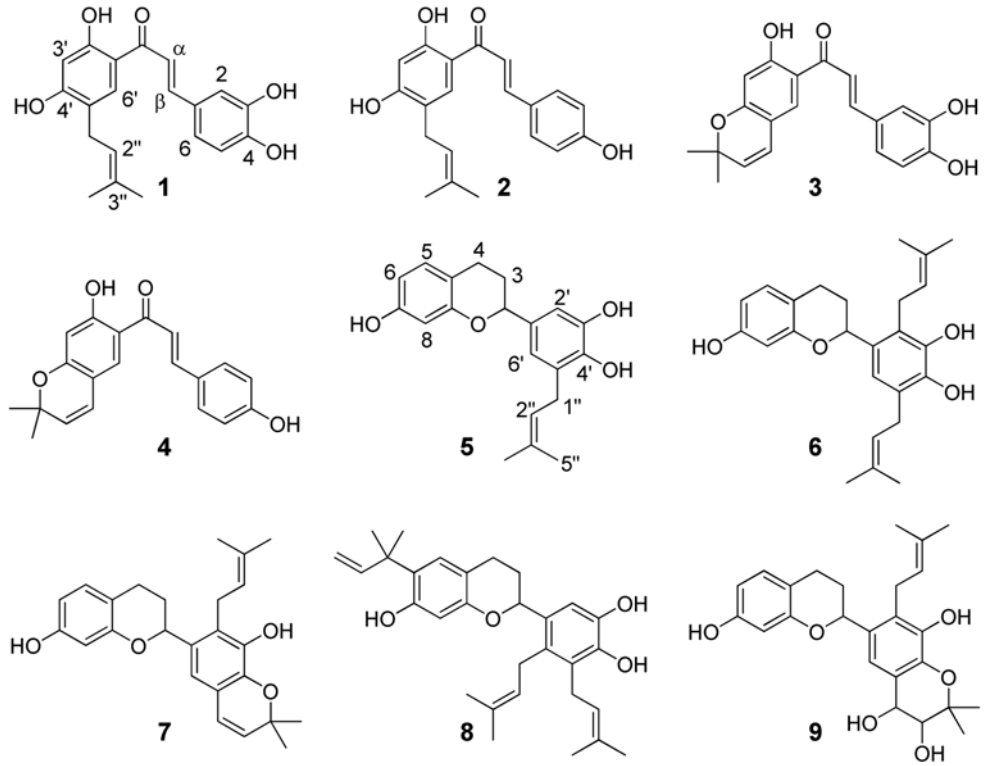

Fig. 1 Phenolic phytochemicals isolated from ethanol extractions of *Broussonetia papyrifera* root barks; **1**, broussochalcone A; **2**, broussochalcone B; **3**, 3,4-dihydroxyisolonchocarpin; **4**, 4-hydroxyisolonchocarpin; **5**, 3'-(3-methylbut-2-enyl)-3',4',7-trihydroxyflavane; **6**, kazinol A; **7**, kazinol B; **8**, kazinol E; **9**, broussoflavan A.

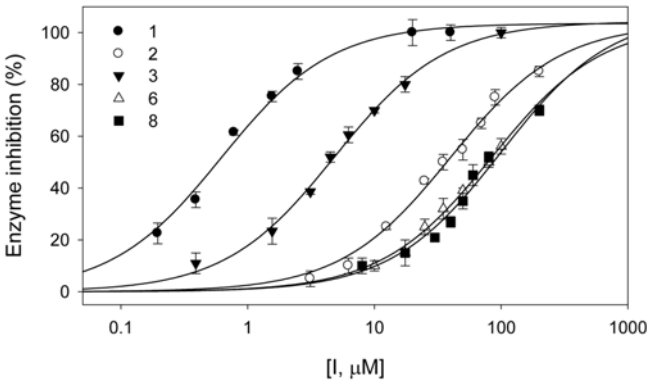

Fig. 2 Effect of compounds (**1**–**3**, **6,** and **8**) on the activity of xanthine oxidase for the catalysis of xanthine.

Table 3 XOD inhibitory activity, DPPH radical scavenging activity, and $ABTS^{\bullet+}$ scavenging activity of *B. papyrifera*

Compound	XOD		DPPH	ABTS
	IC_{50} (μM)[a]	Inhibition type (K_I, K_{IS} μM)	IC_{50} (μM)[a]	IC_{50} (μM)[a]
1	0.6±0.04	Mixed type I (0.27, 0.71)	27.6±0.3	5.8±0.1
2	58.7±0.2	NT[b]	81.2±0.7	24.1±1.2
3	1.8±0.1	Mixed type I (0.83, 1.07)	21.8±0.2	7.7±0.4
4	>200	NT	129.4±11.3	31.1±1.0
5	164.0±0.2	NT	64.1±2.1	9.1±0.2
6	80.9±0.3	NT	30.9±1.3	8.3±0.3
7	>200	NT	252.8±5.4	8.8±0.5
8	62.0±0.4	NT	108.8±7.6	10.0±0.3
9	>200	NT	150.2±4.5	9.3±0.4
Trolox[c]	-	-	5.6±0.1	4.6±0.2
Allopurinol[c]	2.0±0.3	NT	-	-

All extracts were examined in a set of experiments repeated three times.
[a]Sample concentration was 50% enzyme activity loss.
[b]NT is no tested.
[c]Trolox and allopurinol were used as positive controls.

data enable carbons corresponding to the carbonyl and eight C-C double bonds to be identified, and thus accounted for nine of the eleven degrees of unsaturation. The extra two degrees of unsaturation were ascribed to a two-cyclic system. The chalcone skeleton was deduced from α,β-unsaturated C-C bond in δ_H 7.72 (1H, d, J=15.3 Hz) and δ_H 7.72 (1H, d, J=15.3 Hz). The presence of isoprenyl group was deduced from successive connectivities from H-1 (δ_H 3.31) to H-5 (δ_H 1.61) in the ^1H-^1H COSY spectrum. HMBC correlation H-1 (δ_H 3.31) and C-4 (δ_C 163.9) proved the location of the isoprenyl group. Thus, compound **1** was identified as 2,4,3,4-tetrahydroxy-5-(3,3-dimethylallyl)-chalcone, called as broussochalcone A.

The isolated compounds (**1**–**9**) were evaluated separately for their antioxidant capacity by using DPPH and ABTS radicals. Most compounds were more effective on ABTS radical than on that of DPPH. The most potent ABTS scavengers **1** (IC_{50}=5.8 μM) and **3** (IC_{50}=7.7 μM) have a 3,4-diol motif (catechol) in B-ring.

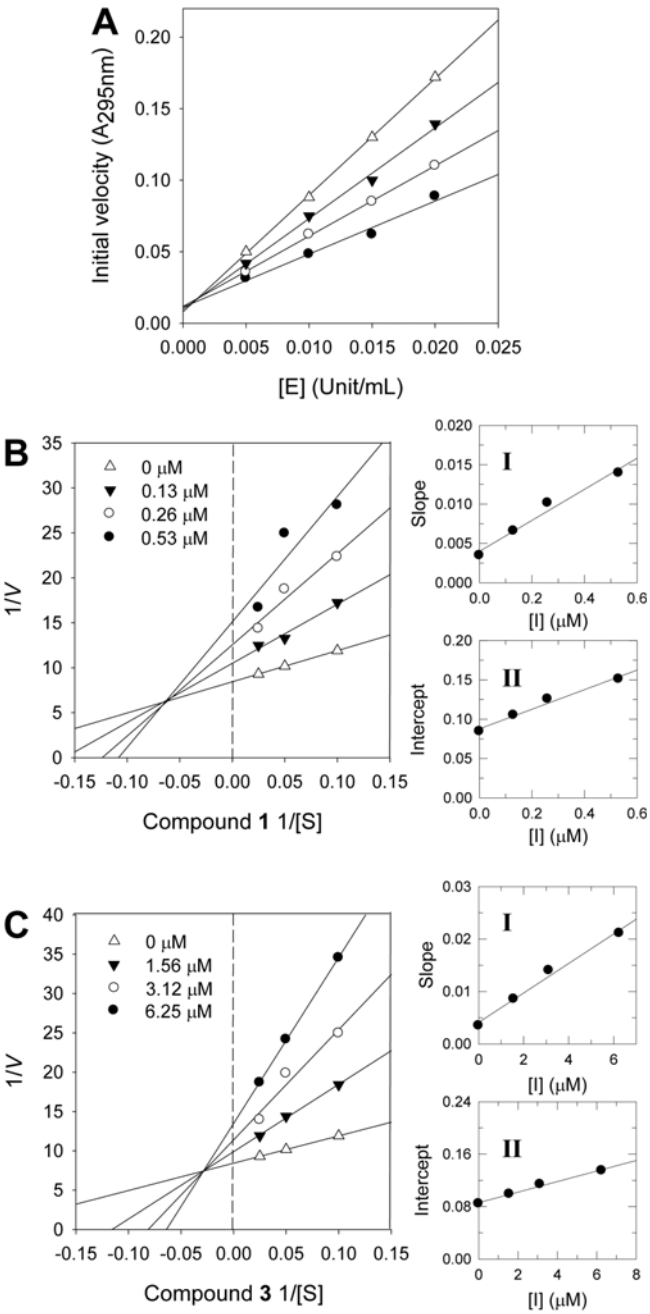

Fig. 3 (A) Catalytic activity of xanthine oxidase as a function of enzyme concentration at different concentrations of compound **1** (△, 0 μM; ▼, 0.13 μM; ○, 0.26 μM; ●, 0.53 μM). (B-C) Kinetic mechanism assays of xanthine oxidase inhibition by broussochalcone A (**1**), and 3,4-dihydroxyisolonchocarpin (**3**). Lineweaver-Burk plots were constructed for evaluation of the inhibition of xanthine oxidase by compounds **1** and **3**. The plots are expressed as 1/velocity versus 1/xanthine (mM^{-1}) without or with an inhibitor in the reaction solution. Insets (I) and (II) represent the secondary plot of the slope and the intercept of the straight lines versus concentration of compounds **1** and **3**, respectively.

All isolated phenolic phytochemicals (**1**–**9**) were also screened for inhibitory activities against XOD. Although most compounds (**1**, **2**, **3**, **5**, **6**, and **8**) showed dose-dependent inhibitory effects on XOD, they had different degree of inhibition with IC_{50} values ranging from 0.6 to 164.0 μM (Fig. 2 and Table 3). The highest inhibitory compounds were compounds **1** (IC_{50}=0.6 μM) and **3** (IC_{50}=1.8 μM), which can be favorably compared with the current commercially available inhibitor allopurinol (IC_{50}=2.0 μM). The activity was affected by subtle changes in structure. Chalcone series (**1**–**4**) was more effective than flavan series (**5**–**9**). It appears that better inhibition is observed when there is 3,4-dihydroxyl motif in B-ring of chalcone.

As depicted in Fig. 3, kinetic analysis of the inhibition elucidated a typical progress curves for reversible mixed-type inhibitor. All compounds manifested the same relationship between enzyme activity and concentration. The inhibition of XOD by compound **1** (the most effective species) is illustrated in Fig. 3A. Plots of the initial velocity versus enzyme concentration in the presence of different concentrations of compound **1** gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration resulted in lowering of the slope of the line, indicating that these compounds were reversible inhibitors. The enzyme inhibition properties of these derivatives were modeled using Lineweaver-Burk double-reciprocal plots. As depicted in Fig. 3B-C, the inhibition kinetics analyzed by Lineweaver-Burk plots show that compounds **1** and **3** are mixed-type inhibitors, because increasing the concentration resulted in a family of lines with a common intercept on the y-axis but with different gradients. More detailed parameters can be obtained by changing the inhibitor concentrations (Equations 2 and 3), which allows differentiation of the mechanism between mixed types I and II. The parameters K_I and K_{IS} can be fitted to Equations (2 and 3). The equilibrium constants for its binding with free enzyme (K_I) and with enzyme-substrate complex (K_{IS}) were obtained from the second plots of the K_m/V_{max} and $1/V_{max}$ versus concentration of compounds **1** and **2**, respectively. We established that the progress linear yielded the following: compound **1**, K_I =0.27 μM, and K_{IS} = 0.71 μM; compound **2**, K_I = 0.83 μM, and K_{IS}=1.07 μM (Fig. 3 Insets). The affinity of inhibitor for free enzyme is stronger than that of inhibitor for the enzyme-substrate complex, because K_{IS} is about two times greater than K_I. Thus, compounds **1** and **2** are a mixed type I inhibitor of the enzyme. All compounds examined in this study were detected in the HPLC chromatogram (Table 4). The most active XOD inhibitor **1** was present in very high concentration, because it appeared as principal peak in the chromatogram with 27.9 min of retention time (Supplementary material).

The present study demonstrated that the EtOH extract of *B. papyrifera* roots had the most potent inhibitory activity towards xanthine oxidase. Purification of this fraction gave nine phenolic phytochemicals that displayed xanthine oxidase inhibitory activities. Interestingly, the two compounds, broussochalcone A (**1**), and 3,4-dihydroxyisolonchocarpin (**3**), were the principal contributors to the xanthine oxidase inhibition. The analysis of K_I nad K_{IS} values proved that the two most promising compounds (**1** and **3**) had a reversible mixed type I behavior. Moreover, the XOD inhibitor 1, present at very high concentration in the *B. papyrifera* root barks appeared as the principal peak in the HPLC chromatogram at 27.9 min.

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Table 4 HPLC chromatogrphic and UV-Vis and Mass spectral data of phenolic phytochemicals identified in *B. papyrifera*

Peak	HPLC t_R (min)	Molecular weight	UV-vis maxima (nm)	Fragments (*m/z*)	Identification
1	27.9	340	203, 262, 376	340(100), 205(69), 149(49), 123(27)	broussochalcone A
2	30.4	324	195, 242, 369	324(100), 205(64), 187(31), 149(59)	broussochalcone B
3	28.8	338	201, 234, 241, 393	338(40), 323(100), 187(97)	3,4-dihydroxyisolonchocarpin
4	30.8	322	232, 238, 271, 360	322(29), 307(88), 187(100)	4-hydroxyisolonchocarpin
5	28.2	326	226, 276, 284, 289	326(36), 270(19), 143(25), 123(100)	3-(3-methylbut-2-enyl) -3,4,7-trihydroxyflavane
6	39.6	394	233, 268, 276, 285, 312	394(22), 215(32), 173(24), 123(100)	kazinol A
7	35.2	392	239, 274, 283	392(76), 377(100), 255(51), 123(62)	kazinol B
8	37.9	462	203, 232, 283	462(16), 215(36), 191(100)	kazinol E
9	37.3	426	220, 285, 398	426(8), 408(41), 352(22), 123(95)	broussoflavan A

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