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Anti-inflammatory flavonoids from root bark of *Broussonetia papyrifera* in LPS-stimulated RAW264.7 cells

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ABSTRACT

Broussonetia papyrifera has been used as a diuretic, tonic and suppressor of edema. Bioactivity-guided fractionation and metabolite investigation of root bark extracts of this plant resulted in the isolation and identification of six 1,3-diphenylpropanes (**1**, **2**, **8**, **10**, **17**, **20**), flavanone (**3**), two chalcones (**4, 5**), five flavans (**6**, **11**, **14–16**), dihydroflavonol (**7**) and five flavonols (**9**, **12**, **13**, **18**, **19**), including five new compounds (**5**, **7**, **8**, **19**, **20**) that inhibit NO production in LPS-induced RAW264.7 cells. The structures of compounds **1–20** were elucidated on the basis of spectroscopic data (1D and 2D NMR, MS, MS/MS, and HRMS). In particular, compounds **3**, **5**, **7**, **12**, and **20** exhibited significant inhibitory effects on the NO, iNOS, and pro-inflammatory cytokine (TNF-α and IL-6) production. Therefore, this study suggests that the flavonoid-rich products of *B. papyrifera,* including the new compounds, could be valuable candidates for the development of pharmaceuticals or functional foods in the prevention and treatment of anti-inflammatory disease.

1. Introduction

Broussonetia papyrifera (L.) L'Her. ex Vent. (paper mulberry) is a fast-growing shade tree belonging to the Moraceae family that is widely distributed throughout Korea, China, and Japan [1]. The dried branches, leaves, and roots of *B. papyrifera* are used as a Korean traditional medicine for various therapeutic purposes, such as a diuretic, tonic and suppressor of edema [1,2]. In particular, isolated metabolites from the roots have characteristics including anti-inflammatory [3], antiasthmatic [4], antioxidant [5], anticancer [6], antinociceptive [7], antimicrobial [8], PTP1B [9], and aromatase inhibitors [10]. The extracts of this plant have also been described by the Korea Food and Drug Administration (KFDA) as a medicinal ingredient of Korean traditional medicine (http://www.koreantk.com/ktkp2014/medicine/medicine-view.view?medCd=M0003242&tempLang=en, accessed 15.10.18), and its effectiveness has been supported by our recent identification of bioactive metabolites, including chalcones, flavans, and flavonols with cancer [6], α-glucosidase [1], anticholinesterase [11], xanthine oxidase [5], and anti-SARS/MERS inhibitory activities [12]. Although previous studies of asthma and anti-inflammatory effects of extracts have been undertaken, no study has defined the relationship between the activities of the isolated compounds.

The discovery of new bioactive natural products as leads for pharmaceutical development are inspired by evidence-based traditional medicines and/or achieved by bioassay-guided fractionation using *in vitro* assays [13,14,15]. The bioassay-guided fractionation method is still used today for the discovery of therapeutic leads, and identification of bioactive substances from botanical RAW material (BRM) has increased as a part of a bioassay-guided fractionation strategy (chemistry, manufacturing and control, CMC) for the development of recent herbal- drugs [13,14].

Our ongoing project on respiratory diseases is aimed at discovering anti-inflammatory agents from natural products as prospective agents to protect cells from damage caused by an elicitor [14,16,17]. During the screening of Korean wild plant libraries, we found that the constituents of the roots of *B. papyrifera* had anti-inflammation properties based a bioactivity-guided isolation. Described herein are the isolation and spectroscopic analyses (1D, 2D NMR spectra and MS analysis) for structural elucidation, as well as their inhibitory effects on representative proinflammatory mediators (NO, IL-6, TNF-α, iNOS, and COX-2) in LPS-activated RAW264.7 macrophage. These results suggest that the compounds isolated from *B. papyrifera* can be therapeutic

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agents for the treatment of inflammatory diseases.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter (JASCO, Easton, Maryland, USA) with a 100 mm glass microcell, and ECD spectra were recorded on a JASCO J-815 CD spectrometer. 1D (^{1}H, ^{13}C, and DEPT) and 2D (COSY, HMQC, and HMBC) NMR spectra were obtained using a JEOL ECZ500R (^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz, JEOL Ltd., Akishima, Tokyo, Japan), Varian UNITY 400 NMR (^{1}H NMR at 400 MHz, ^{13}C NMR at 100 MHz, Varian, Palo Alto, California, USA) and Bruker AVANCE III HD 700 (^{1}H NMR at 700 MHz, ^{13}C NMR at 175 MHz, Bruker, Billerica, Massachusetts, USA) using methanol-d_4 (Merck Millipore, Darmstadt, Germany) and acetone-d_6 (Cambridge Isotope Laboratories, Andover, Massachusetts, USA) as an NMR solvent. Ultraviolet (UV) measurements were taken with a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, California, USA). Analysis and tentative identification of compounds was performed by ultra-performance liquid chromatography (ACQUITY UPLC™, Waters, Milford, Massachusetts, USA) equipped with a photodiode array (PDA, Waters) coupled to a quadrupole time of flight-mass spectrometer (Micromass QTof Premier™ mass spectrometry, Waters) to collect spectroscopic data, such as UV, MS, MS/MS, HRMS. An ACQUITY BEH C18 (1.7 μm, 2.1 × 100 mm, Waters) was used for UPLC-QTof-MS and UPLC-PDA. Middle performance liquid chromatography (MPLC) was performed using a YMC-Pack ODS AQ HG column (10 μm, 250 × 20 mm, YMC, Kyoto, Japan) with a Armen spot prep II System (Gilson, Middleton, Massachusetts, USA) and Multiple Preparative HPLC LC-Forte/R Series (YMC) for fractionation. Isolation of compounds was conducted on a preparative-HPLC (PLC 2020, Gilson) using Atlantis® T3 (5 μm, 250 × 19 mm, Waters), YMC-Actus Triart C18 ExRs (5 μm, 250 × 20 mm, YMC), YMC-J'sphere ODS-H80 (5 μm, 250 × 20 mm, YMC), YMC-Pack pro C8 (5 μm, 250 × 20 mm, YMC), YMC ODS aQ (10 μm, 250 × 20 mm, YMC), Hypersil GOLD (5 μm, 250 × 20 mm, Thermo Fisher, Massachusetts, USA), and Kinetex 5 μm Biphenyl (5 μm, 250 × 20 mm, Phenomenex, Torrance, California, USA) for column chromatography. Water was purified using a water purification system (Milli-Q Academic, Merck Millipore). Formic acid and leucine enkephalin (Sigma-Aldrich, St. Louis, Missouri, USA) were used for instrumental analysis. Methanol (MeOH, Honeywell, Morris Plains, New Jersey, USA) and acetonitrile (CH_3CN, Merck Millipore) of HPLC analytical grade were used for extraction and isolation. VLC open column (435 × 150 mm), Silica gel 60 (0.040–0.063 mm, Merck Millipore), glass TLC plate silica gel 60 F254 (Merck Millipore), and the LP grade solvents that hexane, chloroform, and ethyl acetate (SK Chemicals, Seoul, Korea) were re-distilled before use.

2.2. Plant material

The root bark of *Broussonetia papyrifera* was collected at Gonyang in Gyeongsangnam-do, South Korea, in June 2015 and identified by Dr. Joong Ku Lee. A voucher specimen (KRIBB 0059119) was deposited in the Korea Research Institute of Bioscience and Biotechnology (KRIBB) International Biological Material Center (IBMRC).

2.3. Extraction and isolation

The dried root bark of *B. papyrifera* (13.36 kg) was extracted in 100% methanol (35 L × 3) at room temperature for 24 h. The filtered extracts were concentrated in vacuo at 42 °C to produce a dried extract (1.3 kg, yield 10.05%). The extracts were fractionated by MPLC (Armen spot prep II System) using a YMC-Pack ODS AQ HG column eluted with MeOH-H_2O [0–10 min 10% MeOH, 10–60 min 10–100% MeOH, 60–90 min 100% MeOH] and a flow rate of 30 mL/min to give 9 fractions (BP Frs. 1–9). The sub-fraction 6 (47.6 g) was sub-fractionated by VLC packed normal phase silica gel. Elution was performed with 12 L of hexane/ethyl acate (HE) mixture (3:1, v/v), 8 L of HE (1:1), 15 L of ethyl acetate, and 20 L of chloroform/methanol mixture (1:1) to give 4 fractions (BP Frs. 6-1 ~ 6-4). The fraction 6-1 (8.35 g) was sub-fractionated by Atlantis® T3 column using a gradient of MeOH-H_2O (60% → 90%) to give 5 fractions (BP Frs. 6-1-1 ~ 6-1-5). The fraction 6-1-2 (1.3 g) was separated on a YMC-Triart C18 ExRs column using a gradient of CH_3CN-H_2O (35% → 40%) to give compounds **1** (183.9 mg) and **2** (239.6 mg). The fraction 6-1-3 (1.2 g) was separated on a Kinetex 5 μm Biphenyl column using a gradient of MeOH/CH_3CN (1:1)-H_2O (40% → 55%) to give compound **7** (16.1 mg). The fraction 6-3 was sub-fractionated by Atlantis® T3 column using a gradient of CH_3CN-H_2O (45% → 75%) to give 11 fractions (BP Frs. 6-3-1 ~ 6-3-11). The fraction 6-3-2 (520.1 mg) was separated on a Kinetex 5 μm Biphenyl column using a gradient of CH_3CN-H_2O (30% → 45%) to give compound **3** (135.1 mg). The fraction 6-3-3 (763.8 mg) was separated on a YMC-Triart C18 ExRs column using a gradient of MeOH-H_2O (65% → 75%) to give compounds **4** (336.1 mg) and **5** (71.9 mg). The fraction 6-4 (10.0 g) was separated on a YMC Triart C18 ExRs column using a gradient of CH_3CN-H_2O (45% → 46%) to give compound **6** (26.4 mg). The sub-fraction 7 (50 g) was sub-fractionated by YMC ODS aQ column using a gradient of CH_3CN-H_2O (55% → 100%) at 20 mL/min to give 4 fractions (BP Frs. 7-1 ~ 7-4). The fraction 7-2 (5.7 g) was sub-fractionated by YMC ODS aQ column using a gradient of MeOH-H_2O (50% → 70%) to give 4 fractions (BP Frs. 7-2-1 ~ 7-2-4). The fraction 7-2-3 (1.4 g) was separated on a YMC Triart C18 ExRs column using a gradient of CH_3CN-H_2O (53% → 55%) to give compound **9** (43.5 mg). The fraction 7-2-2 (2.1 g) was separated on a YMC Triart C18 ExRs column using a gradient of MeOH-H_2O (50% → 70%) to give compound **11** (23.7 mg). The fraction 7-3 (12 g) was sub-fractionated by Hypersil GOLD column using a gradient of MeOH-H_2O (70% → 100%) to give 7 fractions (BP Frs. 7-3-1 ~ 7-3-7). The fraction 7-3-3 (333.5 mg) was separated on a Kinetex 5 μm Biphenyl column using a gradient of CH_3CN-H_2O (45% → 60%) to give compound **10** (28.0 mg). The fraction 7-3-5 (345.1 mg) was separated on a YMC-Pack pro C8 column using isocratic 55% MeOH to give compound **8** (39.4 mg), compounds **12** (44.9 mg) and **13** (29.4 mg). The fraction 7-3-4 (285.1 mg) was separated on a YMC Triart C18 ExRs column using a gradient of MeOH-H_2O (75% → 80%) to give compounds **14** (158.4 mg) and **16** (23.7 mg). The fraction 7-3-7 (337.5 mg) was separated on a YMC-Pack pro C8 column using a gradient of MeOH-H_2O (75% → 80%) to give compounds **15** (7.6 mg), **17** (48.7 mg), **18** (215.7 mg), and **19** (40.6 mg). The fraction 7-3-8 (457.1 mg) was separated on a YMC J'sphere ODS-H80 column using a gradient of MeOH-H_2O (75% → 80%) to give compound **20** (45.2 mg).

2.3.1. Broussochalcone C (5)

Yellowish powder; UV (MeOH) λ_{max} (log ε) 260 (1.04), 390 (1.36) ^{1}H NMR (400 MHz, methanol-d_4) δ 7.47 (1H, s, H-2), 7.40 (1H, s, H-6′), 7.19 (1H, d, J = 7.6 Hz, H-6), 6.81 (1H, d, J = 7.6 Hz, H-5), 6.66 (1H, s, H-3′), 6.62 (1H, s, H-β), 5.29 (1H, t, J = 7.1 Hz, H-8′), 3.25 (2H, d, J = 7.1 Hz, H-7′), 1.75 (3H, s, H-10′), 1.68 (3H, s, H-11′); ^{13}C NMR (100 MHz) δ 184.6 (C-β′), 168.4 (C-2′), 166.3 (C-4′), 149.3 (C-4), 147.8 (C-α), 146.7 (C-3), 134.3 (C-9′), 127.5 (C-5′), 126.3 (C-6), 125.6 (C-1), 125.2 (C-6′), 122.8 (C-8′), 118.9 (C-2), 116.6 (C-5), 114.5 (C- β), 114.3 (C-1′), 98.4 (C-3′), 28.8 (C-7′), 25.9 (C-10′), 17.8 (C-11′); HRESIMS (negative) m/z 337.1091 $[M-H_2O-H]^-$, calcd for $C_{20}H_{17}O_5$, 337.1076.

2.3.2. Broussoflavanonol A (7)

Yellowish powder; $[\alpha]_D^{25}$ − 114 (c 0.1, CH_3OH); ^{1}H NMR (400 MHz, acetone-d_6) δ 6.93 (1H, s, H-6′), 6.40 (1H, d, J = 7.5 Hz, C-12′), 6.00 (1H, s, H-6), 5.93 (1H, s, H-8), 5.72 (1H, d, J = 7.5 Hz, H-13′), 5.36 (1H, d, J = 10.6 Hz, H-2), 5.16 (1H, m, H-8′), 4.77 (1H, d, J = 10.6 Hz,

H-3), 3.51 (2H, d, J = 5.2 Hz, H-7′), 1.64 (3H, s, H-11′), 1.60 (3H, s, H-10′), 1.43 (6H, s, H-15′), 11.7 (5-OH); ^{13}C NMR (100 MHz) δ 198.5 (C-4), 168.3 (C-7), 165.1 (C-9), 164.4 (C-5), 143.8 (C-3′), 140.7 (C-4′), 131.4 (C-13′), 131.2 (C-9′), 129.6 (C-2′), 128.7 (C-1′), 124.3 (C-8′), 122.9 (C-12′), 119.9 (C-5′), 117.1 (C-6′), 101.5 (C-10), 97.3 (C-6), 96.2 (C-8), 80.7 (C-2), 77.7 (C-14′), 73.0 (C-3), 28.2 (C-15′), 28.1 (C-16′), 26.0 (C-10′), 25.4 (C-7′), 18.1 (C-11′)-; HRESIMS (negative) m/z 437.1598 $[M-H]^-$, calcd for $C_{25}H_{25}O_7$, 437.1600.

2.3.3. Kazinol V (8)

Yellowish powder; $[\alpha]_D^{25}$ −4.84 (c 0.05, CH_3OH); UV (MeOH) λ_{max} (log ε) 230 (2.53), 281 (2.25), 344 (2.07); ^{1}H NMR (700 MHz, acetone-d_6) δ 6.93 (1H, d, J = 8.1 Hz, H-6′), 6.50 (1H, br s, H-6″), 6.45 (1H, d, J = 2.3 Hz, H-3′), 6.36 (1H, dd, J = 2.3, 8.1 Hz, H-5′), 5.02 (1H, t, J = 6.7 Hz, H-8″), 4.57 (1H, dd, J = 8.4, 9.5 Hz, H-13″), 3.78 (3H, s, 2′-OCH_3), 3.16 (2H, d, J = 6.7 Hz, H-7″), 3.12 (1H, dd, J = 8.4, 15.6 Hz, H-12″), 3.05 (1H, dd, J = 9.5, 15.6 Hz, H-12″), 2.55 (2H, t, J = 7.4 Hz, H-1), 2.47 (2H, td, J = 2.2, 7.4 Hz, H-3), 1.74 (2H, m, H-2), 1.71 (3H, s, H-11″), 1.65 (3H, s, H-10″), 1.21 (3H, s, H-16″), 1.18 (3H, s, H-15″); ^{13}C NMR (175 MHz) δ159.3 (C-2′), 157.7 (C-4′), 145.9 (C-4″), 139.5 (C-5″), 133.6 (C-1″), 131.1 (C-9″), 130.8 (C-6′), 128.6 (C-3″), 127.3 (C-2″), 124.3 (C-8″), 122.1 (C-1′), 117.0 (C-6″), 107.3 (C-5′), 99.7 (C-3′), 90.4 (C-13″), 71.6 (C-14″), 55.5 (2′-OCH_3), 33.2 (C-2), 33.0 (C-3), 31.2 (C-12″), 30.4 (C-1), 29.3 (C-7″), 25.8 (C-10″, C-15″), 25.4 (C-16″), 18.0 (C-11″); HRESIMS (negative) m/z 425.2345 $[M-H]^-$, calcd for $C_{26}H_{34}O_5$, 425.2328.

2.3.4. Broussonol G (19)

Yellowish powder; $[\alpha]_D^{25}$ −9.62 (c 0.1, CH_3OH); UV (MeOH) λ_{max} (log ε) 230 (2.55), 260 (2.51), 302 (2.30), 353 (2.25); ^{1}H NMR (400 MHz, acetone-d_6) δ 6.27 (1H, s, H-6′), 6.19 (1H, dd, J = 10.6, 17.2 Hz, H-14), 6.09 (1H, s, H-6), 5.20 (1H, m, H-8′), 4.84 (1H, d, J = 17.2 Hz, H-15a), 4.69 (1H, d, J = 10.6 Hz, H-15b), 3.34 (1H, m, H-13′), 3.32 (2H, m, H-7′), 3.07 (1H, m, H-12′), 2.95 (1H, m, H-12′), 1.76 (3H, s, H-11′), 1.66 (3H, s, H-10′), 1.51 (3H, s, H-16′), 1.47 (3H, s, H-13), 1.44 (3H, s, H-12), 1.43 (3H, s, H-15′); ^{13}C NMR (100 MHz) δ 192.1 (C-4), 167.7 (C-7), 163.9 (C-5), 163.6 (C-9), 151.5 (C-14), 150.7 (C-3), 145.0 (C-1′), 144.0 (C-2), 136.3 (C-2′), 131.5 (C-9′), 129.0 (C-5′), 124.7 (C-4′), 123.4 (C-8′), 114.4 (C-8), 108.1 (C-15), 107.2 (C-6′), 105.7 (C-3′), 100.8 (C-10), 98.1 (C-6), 84.6 (C-14′), 63.1 (C-13′), 41.4 (C-11), 32.1 (C-15′), 30.8 (C-12′), 30.5 (C-13), 30.0 (C-12), 27.9 (C-16′), 26.7 (C-7′), 25.9 (C-10′), 18.0 (C-11′); HRESIMS (negative) m/z 521.2193 $[M-H]^-$, calcd for $C_{30}H_{34}O_8$, 521.2175.

2.3.5. Kazinol W (20)

Yellowish powder; UV (MeOH) λ_{max} (log ε) 230 (2.52), 281 (2.18); ^{1}H NMR (500 MHz, acetone-d_6) δ 6.92 (1H, d, J = 10.0 Hz, H-6′), 6.50 (1H, br s, H-6″), 6.42 (1H, d, J = 2.9 Hz, H-3′), 6.34 (1H, dd, J = 2.9, 10.0 Hz, H-5′), 4.90 (1H, t, J = 7.9 Hz, H-8″), 3.76 (3H, s, 2′-OCH_3), 3.18 (2H, d, J = 7.9 Hz, H-7″), 2.66 (2H, t, J = 8.5 Hz, H-12″), 2.55 (2H, t, J = 9.4 Hz, H-1), 2.50 (2H, t, J = 10.0 Hz, H-3), 1.79 (2H, t, J = 8.5 Hz, H-13″), 1.72 (3H, s, H-11″), 1.72 (2H, m, H-2), 1.65 (3H, s, H-10″), 1.27 (6H, s, H-15″, H-16″); ^{13}C NMR (125 MHz) δ 159.3 (C-2′), 157.6 (C-4′), 144.8 (C-5″), 140.4 (C-4″), 132.5 (C-1″), 131.2 (C-9″), 130.8 (C-6′), 128.8 (C-2″), 124.8 (C-8″), 122.1 (C-1′), 120.6 (C-3″), 114.2 (C-6″), 107.3 (C-5′), 99.6 (C-3′), 74.0 (C-14″), 55.5 (2′-OCH_3), 33.8 (C-13″), 33.6 (C-3), 33.1 (C-2), 30.5 (C-1), 27.7 (C-7″), 26.7 (C-15″), 26.7 (C-16″), 25.8 (C-10″), 21.0 (C-12″), 18.0 (C-11″); HRESIMS (negative) m/z 427.2473 $[M-H]^-$, calcd for $C_{26}H_{35}O_5$, 427.2484.

2.4. Cell culture

Murine macrophage RAW264.7 cells were purchased from ATCC (Rockville, MD, USA). The cells were cultured at 37 °C and 5% CO_2 in DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Welgen) and 1% penicillin-streptomycin (GibcoTM, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.5. Measurement of secreted NO level and cell viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded into a 96 well plate at a concentration of 1×10^4 cells/well. After incubation for 4 h, cells were treated with various concentrations of samples (2.5–30.0 μg/mL) for 20 h. Then, 5 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h for the formation of formazan. After removing the supernatant, dimethyl sulfoxide (DMSO) was added to dissolve the formazan, followed by agitation for 10 min at room temperature. The absorbance at 570 nm was measured to determine the number of viable cells using a microplate reader (Spark10M, TECAN, Switzerland). Cells were seeded into a 96 well plate at a concentration of 5×10^4 cells/well and allowed to adhere for 4 h. Then, cells were pretreated with various concentrations of samples (2.5–30.0 μg/mL) for 1 h and stimulated with lipopolysaccharide (LPS) at 0.5 μg/mL for 24 h. To measure NO secretion, the cell supernatant was harvested and reacted with the Griess reagent (Griess reagent I, 1% sulfanilamide; Griess reagent II, 0.1% N-(1-naphathyl)-ethylenediamine dihydrochloride and 5% phosphoric acid) for 10 min at room temperature in the dark. The absorbance at 540 nm was detected and the concentration was calculated using a nitrite standard solution.

2.6. Enzyme-linked immunosorbent assay

Pro-inflammatory cytokines in the cell supernatants were determined using ELISA kits (Mouse IL-6 Set, Cat. No. 558534; Mouse TNF-α Set, Cat. No. 555240, BD Biosciences, Santa Clara, CA, USA) and analyzed according to the manufacturer's instructions.

2.7. RT-PCR

Total RNA was isolated from the harvested cells using the TRIzol® reagent. The synthesis of complementary DNA was performed using a QuantiTech reverse transcription kit (Cat. No. 205310, Qiagen, Hilden, Germany). IL-6, TNF-α, iNOS, COX-2, and β -actin encoded cDNAs were amplified by PCR using the primer sequences as follows: IL-6 (forward; 5′-GAGGATACCACTCCCAACAGACC-3′, reverse; 5′-AAGTGCATCATCG TTGTTCATACA-3′), TNF-α (forward; 5′-CATCTTCTCAAAATTCGAGTG ACAA-3′, reverse; 5′-TGGGAGTAGACAAGGTACAACCC-3′), iNOS (forward; 5′-CAAGAGTTTGACCAGAGGACC-3′, reverse; 5′-TGGAACCACT CGTACTTGGGA-3′), COX-2 (forward; 5′-GAATGCTTTGGTCTGGTGC CTG-3′, reverse; 5′-GTCTGCTGGTTTGGAATAGTTGC-3′), and β-actin (forward; 5′-TGTTTGAGACCTTCAACACC-3′, reverse; 5′-CGCTCATTGC CGATAGTGAT-3′). Reaction products were electrophoresed on 1.5% agarose gels stained with the RedSafe™ kit (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea). Images of the gel were captured with an Olympus C4000 zoom camera system (Olympus, Tokyo, Japan).

2.8. Western blotting

Protein was extracted using a protein lysis buffer (NP40; Cat. No. EBA-1049, ELPIS-Biotech, Inc., Daejeon, Korea). The lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4 °C. Total protein was quantified using the Pierce™ BCA protein assay kit (Cat. No. 23225, Thermo Fisher Scientific, Inc.). An equal amount of protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature to prevent non-specific binding. The membranes were incubated at 4 °C overnight with primary antibody. The following primary antibodies and dilutions were used: anti-iNOS (1:1000 dilution; Cat. No. ADI-905-431, Enzo Life Science, NY, USA) and anti-β-actin (1:2000 dilution; Cat. No. MA5-15379,

Invitrogen). The following day, the membranes with the primary antibodies were washed 3 times with Tris-buffered saline containing 0.1% Tween20 (TBS-T) buffer for 10 min and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution; anti-mouse, Cat. No. sc-2005; anti-rabbit, Cat. No. sc-2030, Santa Cruz Biotechnology, Inc.). The membranes were washed 3 times with TBS-T buffer for 10 min and then visualized using a chemiluminescence (ECL; Cat. No. 32106, Thermo Fisher Scientific, Inc., MA, USA) system.

2.9. Statistics

All data are expressed as the means ± S.E.M. for triplicate independent experiments. Statistical comparisons between the different treatments were performed using one-way ANOVA with Student's *t*-test. $^{*}P$ values < 0.05 were considered to be statistically significant.

3. Result and discussion

3.1. Bioassay-guided fractionation of extracts from B. Papyrifera using LPS-stimulated RAW264.7 cells and isolation of compounds

To isolate and identify specific secondary metabolites of *B. papyrifera* for anti-inflammatory activity, a preliminary literature review and bioassay-guided fractionation were undertaken. MPLC separation for the bioassay-guided fractionation was carried out using a C18 column with a linear gradient of MeOH/water, resulting in nine fractions (BP Frs.1–9). The NO screening method is most widely used as a first step for evaluating anti-inflammatory effects because NO is variously associated with the mechanism of inflammation [18,19,20]. Therefore, we further investigated the effects of the extracts with sub-fractions in LPS-induced RAW264.7 cells. Based on the result that no significant cell death was observed at concentrations of up to 10 μg/mL, fractions 6 and 7 of *B. papyrifera* root bark exhibited potent NO inhibition as 32.81% and 36.84%, respectively (Fig. 1), and further isolation of anti-inflammatory constituents from them were carried out.

3.2. Structural elucidation of isolated compounds

The methanolic extract of the dried root bark of *B. papyrifera* led to the isolation of twenty compounds (**1–20**) including five new compounds (**5**, **7**, **8**, **19**, and **20**) and 15 previously reported diphenylpropanes (**1**, **2**, **10**, and **17**), flavanone (**3**), chalcone (**4**), flavans (**6**, **11**, and **14–16**), and flavonols (**9**, **12**, **13**, and **18**) (Fig. 2). The known compounds (**1–4**, **6**, and **9–18**) were identified as broussonin B (**1**) [21], broussonin A (**2**) [21,22], (2*R*)-7, 3′, 4′-trihydroxy-6-prenylflavanone (**3**) [23,24], broussochalcone A (**4**) [12,25], broussoflavan A (**6**) [12], 8-(1,1-Dimethylallyl)-5′-(3-methylbut-2-enyl)-3′,4′,5,7- tetrahydroxyflanvonol (**9**) [9], kazinol F (**10**) [12], kazinol A (**11**) [12], broussoflavonol B (**12**) [6], broussonol D (**13**) [2], kazinol B (**14**) [12], (−)-(2*S*)-kazinol I (**15**) [22], daphnegiravan H (**16**) [26], kazinol J (**17**) [12], and broussoflavonol C (**18**) [27] by carefully comparison of their spectroscopic data (^{1}H and ^{13}C NMR, *m/z* values, MS/MS, HRMS) with those in the previous literature values. The discussion of structural identification will focus on new compounds **5**, **7**, **8**, **19**, and **20** (vide infra).

Compound **5** was isolated as a yellowish powder. The molecular formula was determined to be $C_{20}H_{20}O_6$ from the ion peak $[M-H-H_2O]^-$ at *m/z* 337.1091 (calcd for $C_{20}H_{17}O_5$, 337.1076) in the HRESIMS, which was 2 amu lower than that of **4** $[M-H]^-$ and showed the same fragment patterns at *m/z* 203 [^{1,3}A] and *m/z* 159 [^{1,3}A-CO_2]. Thus **5** was tentatively identified as a broussochalcone A (**4**) derivative. The ^{13}C NMR spectrum (100 MHz, methanol-d_4) of **5** showed the resonance of all twenty carbon atoms and the ^{13}C NMR resonance at δ 147.8 were identified for C-α [28]. In the HMBC spectrum, The assignment of H- β was confirmed by its heteronuclear long-range correlations to C-2 at δ 118.9, C-6 at δ 126.3, C-α at δ 147.8 and C-β′ at δ 184.6 and a proton signal at δ 7.40 (H-6′) showed a correlation with a ketone carbon signal at δ 184.6 (C-β′) [28]. The ^{1}H NMR (400 MHz, methanol-d_4) spectrum showed the three coupled singlets of the ABX system at δ 7.47 (1H, s), δ 7.19 (1H, d, J = 7.6 Hz), and δ 6.81 (1H, d, J = 7.6 Hz) were identified for H-2, H-6, and H-5, respectively, of the B-ring and two singlet proton signals in the aromatic region of the A-ring at δ 7.40 (1H, s) and δ 6.66 (1H, s) were identified for H-3′ and H-6′ [12,28]. Additionally, a singlet typical of olefinic proton resonating at δ 6.62 (1H, s) was ascribed to H-β to the carbonyl [28]. These data compared to compound **4** suggested that **5** consisted of a α-hydroxychalcone backbone [12,28]. In the ^{1}H NMR spectrum, a prenyl group appeared as a triplet at δ 5.29 (1H), a doublet at δ 3.25 (2H), and two singlets at δ 1.75 (3H) and δ 1.68 (3H) and the proton signals of the 5′-prenyl group at δ 5.29 (H-8′) and δ 3.25 (H-7′) showed a correlation with each other in the COSY spectrum. In addition, the 5′-prenyl proton signal at δ 3.25 (H-7′) showed a correlation with two quaternary carbons at δ 166.3 (C-4′) and δ 127.5 (C-5′) and the proton signal of 6′ at δ 7.40 showed a correlation with carbon at δ 28.8 (C-7′) on the A-ring in the HMBC spectrum. Thus, compound **5** was identified as 5′-prenyl-α-hydroxybutein and named broussochalcone C.

Compound **7** was isolated as a yellowish powder. The molecular formula was determined to be $C_{25}H_{26}O_7$ from the ion peak $[M-H]^-$ at *m/z* 437.1598 (calcd for $C_{25}H_{25}O_7$, 437.1600) in the HRESIMS. The ^{13}C NMR spectrum (100 MHz, acetone-d_6) of **7** showed the resonance of all twenty-five carbon atoms. The chemical shift values of C-2 to C-4 on the C-ring were similar to those of the corresponding data for flavanonol [29]. In the ^{1}H NMR (400 MHz, acetone-d_6) spectrum, a weak signal at δ 11.70 was assigned to the phenolic 5-OH, which was hydrogen-bonded to the C-4-carbonyl group. Two meta-coupled broad singlets at δ 6.00 (1H, s) and δ 5.93 (1H, s) were assignable to H-6 and H-8 of the A-ring. A prenyl group appeared as a multiplet at δ 5.16 (1H), a multiplet at δ 3.51 (2H), and two singlets at δ 1.60 (3H) and δ 1.64 (3H). Two doublets at δ 6.40 (1H, d, J = 7.5 Hz) and δ 5.72 (1H, d, J = 7.5 Hz) and a singlet at δ 1.43 (6H) showed the 2,2-dimethy-2H-chromene moiety. In the COSY spectrum, the proton signals of the 2,3-dihydroflavonol moiety at δ 5.36 (H-2) and δ 4.77 (H-3), of the 2′-prenyl group at δ 5.16 (H-8′) and δ 3.51 (H-7′), and of the 2,2-dimethy-2H-chromene moiety at δ 6.40 (H-12′) and δ 5.72 (H-13′) showed a correlation with each other. From the HMBC spectrum, the 2′-prenyl proton signal at δ 3.51 (H-7′) showed a correlation with a carbon signal at δ 128.7 (C-1′) and the oxygenated quaternary carbon at δ 143.8 (C-3′) on the B-ring. A proton signal at δ 6.40 (H-12′) showed correlations with a carbon signal at δ 140.7 (C-4′) and a carbon signal at δ 117.1 (C-6′) on the B-ring. The presence of a flavanonol skeleton was deduced between the two oxygenated methine proton signals at δ 5.36 (1H, d, J = 10.6 Hz) and δ 4.77 (1H, d, J = 10.6 Hz), which indicated a *trans*-diaxial relationship, and therefore, the absolute configuration had to be (2*R*, 3*R*) or (2*S*, 3*S*) [30]. Based on the negative CD Cotton effect at 280–310 nm and the positive CD Cotton effect at 320–360 nm, **7** was assigned with the absolute configuration (2*R*, 3*R*) [30,31]. The $[\alpha]_D^{25}$ value was −114. Thus, compound **7** was identified as (2*R*,3*R*)-3,5,7-trihydroxy-2-(8-hydroxy-2,2-dimethyl-7-(3-methylbut-2-en-1-yl)-2H-chromen-6-yl)chroman-4-one and named as broussoflavanonol A.

Compound **8** was isolated as a yellowish powder. The molecular formula was determined to be $C_{26}H_{34}O_5$ from the ion peak $[M-H]^-$ at *m/z* 425.2345 (calcd for $C_{26}H_{33}O_5$, 425.2328) in the HRESIMS, which was 16 amu higher than **17**, indicating that **8** has a dihydrobenzofuran group instead of the prenyl and hydroxyl group in **17**. The NMR data of compound **8** were similar to that of **17**, with the exception of the 2-(1-hydroxy-1-methylethyl)-dihydrofurano group. The ^{13}C NMR spectrum (175 MHz, acetone-d_6) of **8** showed the resonance of all twenty-six carbon atoms, and the DEPT 135 spectrum revealed the presence of five methylenes, at δ 33.2 (C-2), δ 33.0 (C-3), δ 31.2 (C-12″), δ 30.4 (C-1), and δ 29.3 (C-7″) carbon atoms. In the ^{1}H NMR (700 MHz, acetone-d_6) spectrum, the chemical shift values at δ 2.55 (2H, t, J = 7.4 Hz), δ 2.47

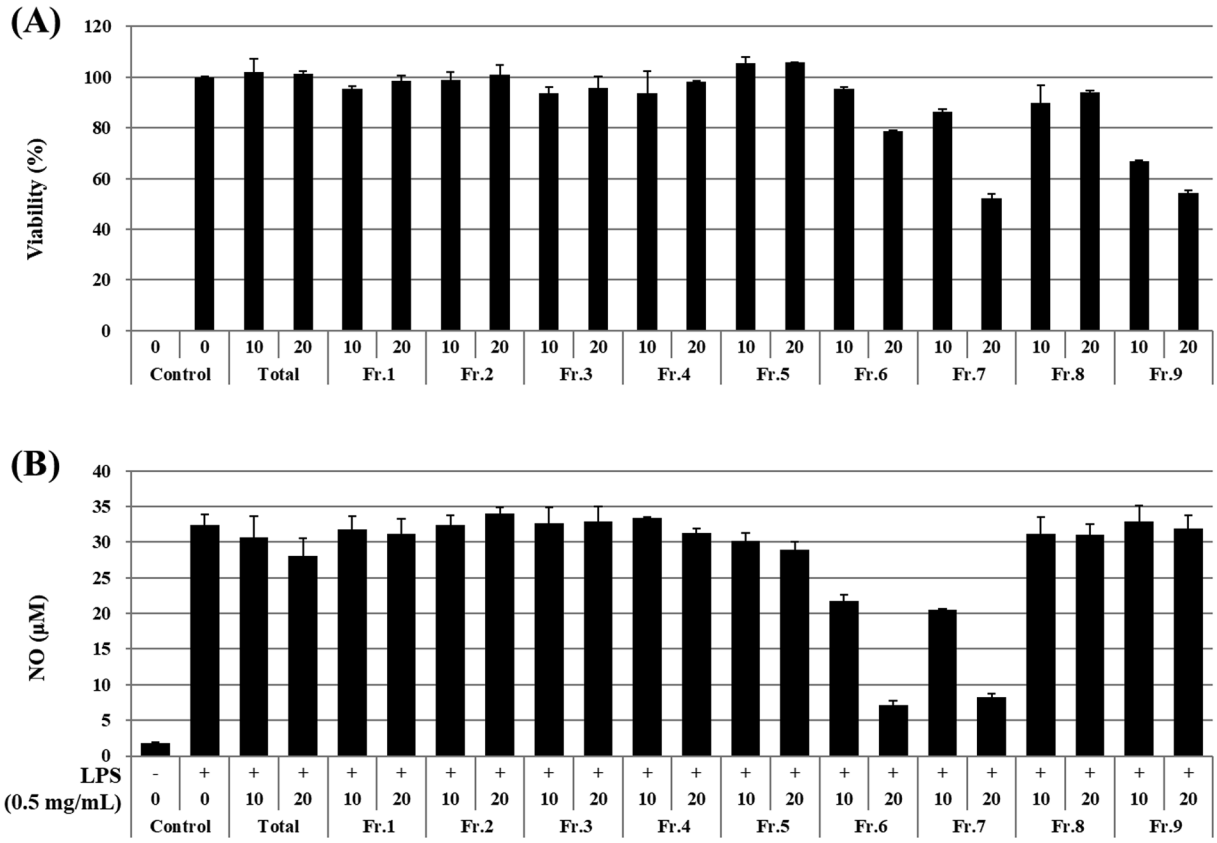

Fig. 1. Effects of the MeOH extract and the actively-guided fractions (10 and 20 μg/mL) from *B. papyrifera* root bark on cell viability (A) and NO production (B) in LPS-stimulated RAW264.7 cells. The error bars represent the mean ± SD for three independent experiments.

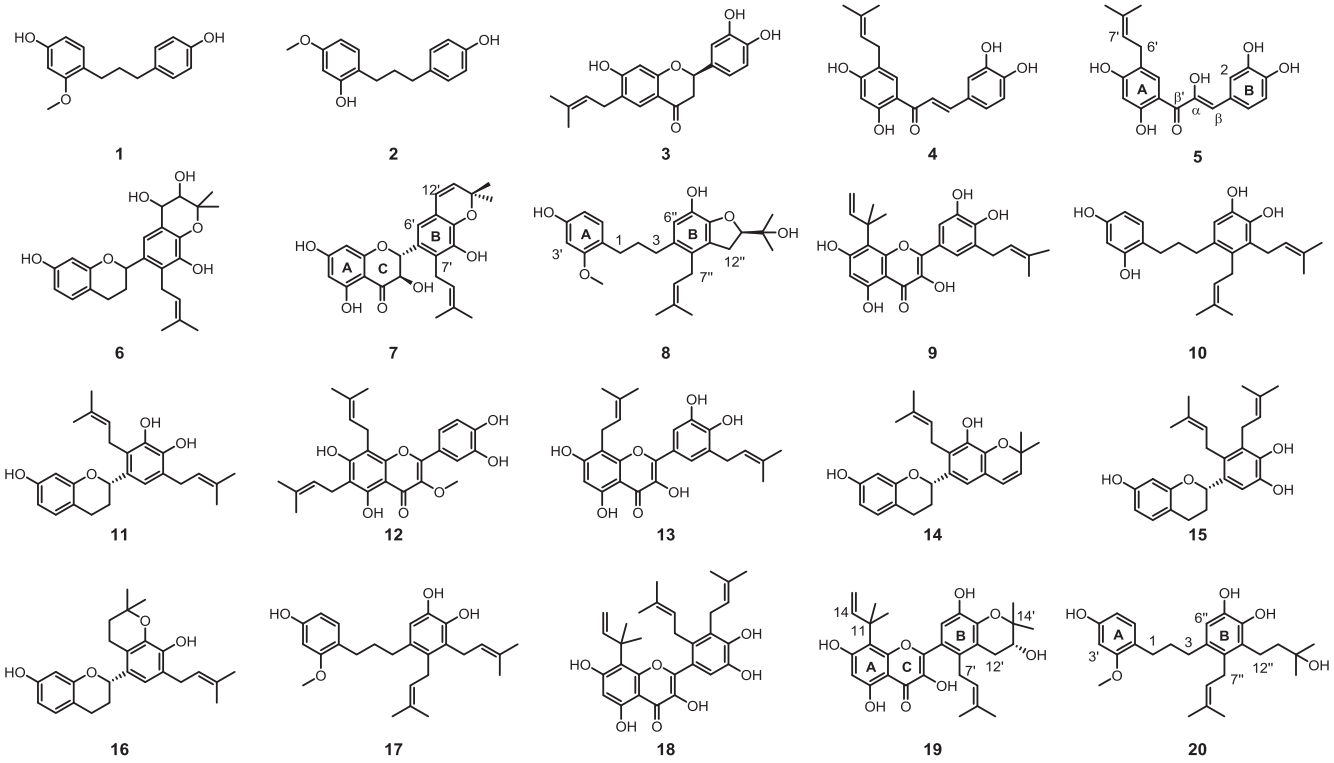

Fig. 2. Structures of compounds **1–20** isolated from root bark of *Broussonetia papyrifera*.

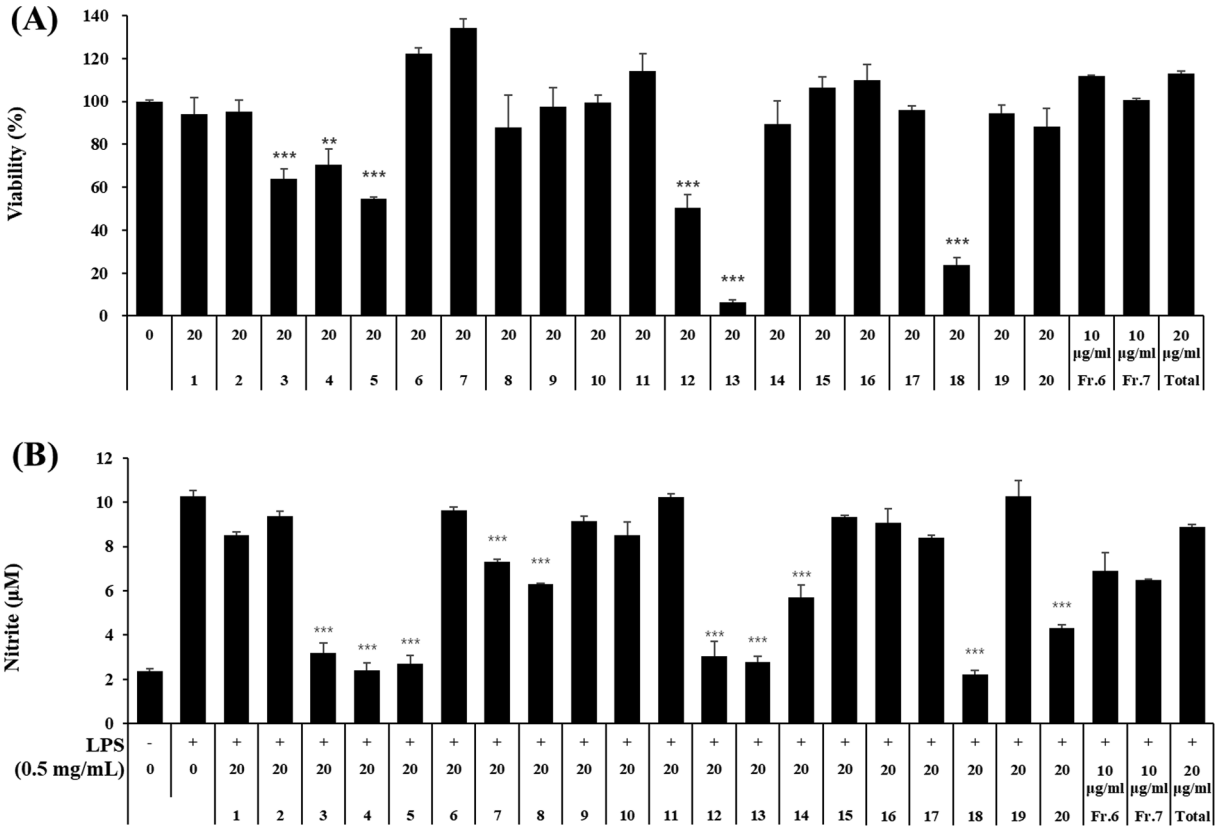

Fig. 3. Effects of isolated compounds **1**–**20** (20 μM) on cell viability (A) and NO production (B) in LPS-stimulated RAW264.7 cells. The error bars represent the mean ± SD of three independent experiments.

(2H, t, J = 7.4 Hz), and 1.74 (2H, m) were similar to those of the corresponding data for 1,3-diphenylpropane [21]. The three coupled protons of the ABX system at δ 6.93 (1H, d, J = 8.1 Hz), δ 6.45 (1H, d, J = 2.3 Hz), and δ 6.36 (1H, dd, J = 2.3, 8.1 Hz) were identified for H-6′, H-3′, and H-5′, respectively, of the A-ring. A singlet at δ 3.78 (3H) showed a methoxy group. The prenyl group appeared as a triplet at δ 5.02 (1H), a doublet at δ 3.16 (2H), and two singlets at δ 1.71 (3H) and δ 1.65 (3H). In addition, the 2-(1-hydroxy-1-methylethyl)-dihydrofurano group appeared as three doublet of doublet at δ 3.12 (1H), δ 3.05 (1H), and δ 4.57 (1H) and two singlets at δ 1.21 (3H) and δ 1.18 (3H) [32]. In the ^{1}H-^{1}H COSY spectrum, the proton signals of the 1,3-diphenylpropane moiety at δ 2.55 (H-1), δ 2.47 (H-3) and δ 1.74 (H-2) and the proton signals of the 2′-prenyl group at δ 5.02 (H-8″), and δ 3.16 (H-7″) and of the dihydrobenzofuran group at δ 3.12 (H-12″), δ 3.05 (H-12″), and δ 4.57 (H-13″) were correlated with each other. From the HMBC spectrum, the 1,3-diphenylpropane moiety proton signals at δ 2.55 (H-1) and δ 2.47 (H-3) showed a correlation with a tertiary carbon signal at δ 130.8 (C-6′) and δ 117.0 (C-6″) and two quaternary carbons at δ 159.3 (C-2′) and δ 127.3 (C-2″). The 2′-prenyl proton signal at δ 3.18 (H-7″) showed a correlation with two quaternary carbons at δ 133.6 (C-1″) and δ 128.6 (C-3″) on the B-ring. In addition, the dihydrobenzofuran proton signals at δ 3.12 and 3.05 (H-12″) showed a correlation with a carbon signal at δ 145.9 (C-4″) and carbon signal at δ 127.3 (C-2″), and a proton signal at δ 4.57 (C-13″) showed a correlation with a carbon at δ 145.85 (C-4″) on the B-ring. The methoxy group proton signal at δ 3.78 showed a correlation with the carbon signal at δ 159.3 (C-2′). On the basis of precedent research, the coupling constants of three signals at δ 4.57 (1H, dd, J = 8.4, 9.5 Hz; H-13″), 3.12 (1H, dd, J = 8.4, 15.6 Hz; H-12″), and 3.05 (1H, dd, J = 9.5, 15.6 Hz; H-12″) were compared [29]. The cyclization patterns of the 2-(1-hydroxy-1-methylethyl)-dihydrofurano group was established as 13″ α and the $[\alpha]_D^{25}$ value was −4.84 [33]. Thus, compound **8** was identified as 13″α-2″-(3-methylbut-2-enyl)-3″,4″-[2-(1-hydroxy-1-methylethyl)]-dihydrofuranyl)-2′,5″-dihydroxyl-4′-methoxydiphenylpropane and named kazinol V.

Compound **19** was isolated as a yellowish powder. The molecular formula was determined as $C_{30}H_{34}O_8$ from the ion peak $[M-H]^-$ at m/z 521.2193 (calcd for $C_{30}H_{33}O_8$, 521.2175) in the HRESIMS. The NMR spectrum of **19** was similar to that of **18**, with the exception of 2,2-dimethyl-chroman-3-ol. The ^{13}C NMR spectrum (125 MHz, acetone-d_6) of **19** showed the resonance of all thirty carbon atoms. The chemical shift values of C-2 to C-10 were similar to those of the corresponding data for 8-prenyl-5,7-dihydroxylflavonol [27]. In the ^{1}H NMR (500 MHz, acetone-d_6) spectrum, δ 12.82 (1H, s) was assigned to the phenolic 5-OH, which was strongly hydrogen-bonded to the C-4-carbonyl group in each case. The prenyl groups appeared at at δ 5.20 (1H, m), δ 3.32 (2H, m), and two singlets at δ 1.51 (3H, s) and δ 1.43 (3H, s). The 1,1-dimethylallyl group ascribed at δ 6.19 (1H, dd, J = 10.6, 17.2 Hz), δ 4.84 (1H, d, J = 17.2 Hz) and δ 4.69 (1H, d, J = 10.6 Hz), and a two singlets at δ 1.47 (3H, s), and δ 1.44 (3H, s). In addition, the 2,2-dimethyl-chroman-3-ol groups appeared as three multiplet protons at δ 3.34 (1H), δ 3.07 (1H), and δ 2.95 (1H) and two singlets at δ 1.51 (3H, s) and δ 1.43 (3H, s). In the COSY spectrum, the proton signals of the 1,1-dimethylallyl group at δ 6.19 (H-14), δ 4.84 (H-15a), and δ 4.69 (H-15b), the signals of the 2′-prenyl group at δ 1.76 (H-11′) and δ 1.66 (H-10′), and the signals of the 2,2-dimethyl-chroman-3-ol groups at δ 3.34 (H-13′), δ 3.07 (H-12′), and δ 2.95 (H-12′) showed a correlation to each other. From the HMBC spectrum, the proton signals of the 1,1-dimethylallyl group at δ 6.19 (H-14) showed a correlation with the quaternary carbon signal at δ 114.4 (C-8) on the A-ring. In addition, the 2′-prenyl proton signal at δ 3.36 (H-7′) showed a correlation with two quaternary carbons at δ 145.0 (C-1′) and δ 105.7 (C-3′) on the B-ring. In addition, the 2,2-dimethyl-chroman-3-ol proton signals H-12′ at δ 3.07 and δ 2.95 showed a correlation with two quaternary carbons at δ 136.3

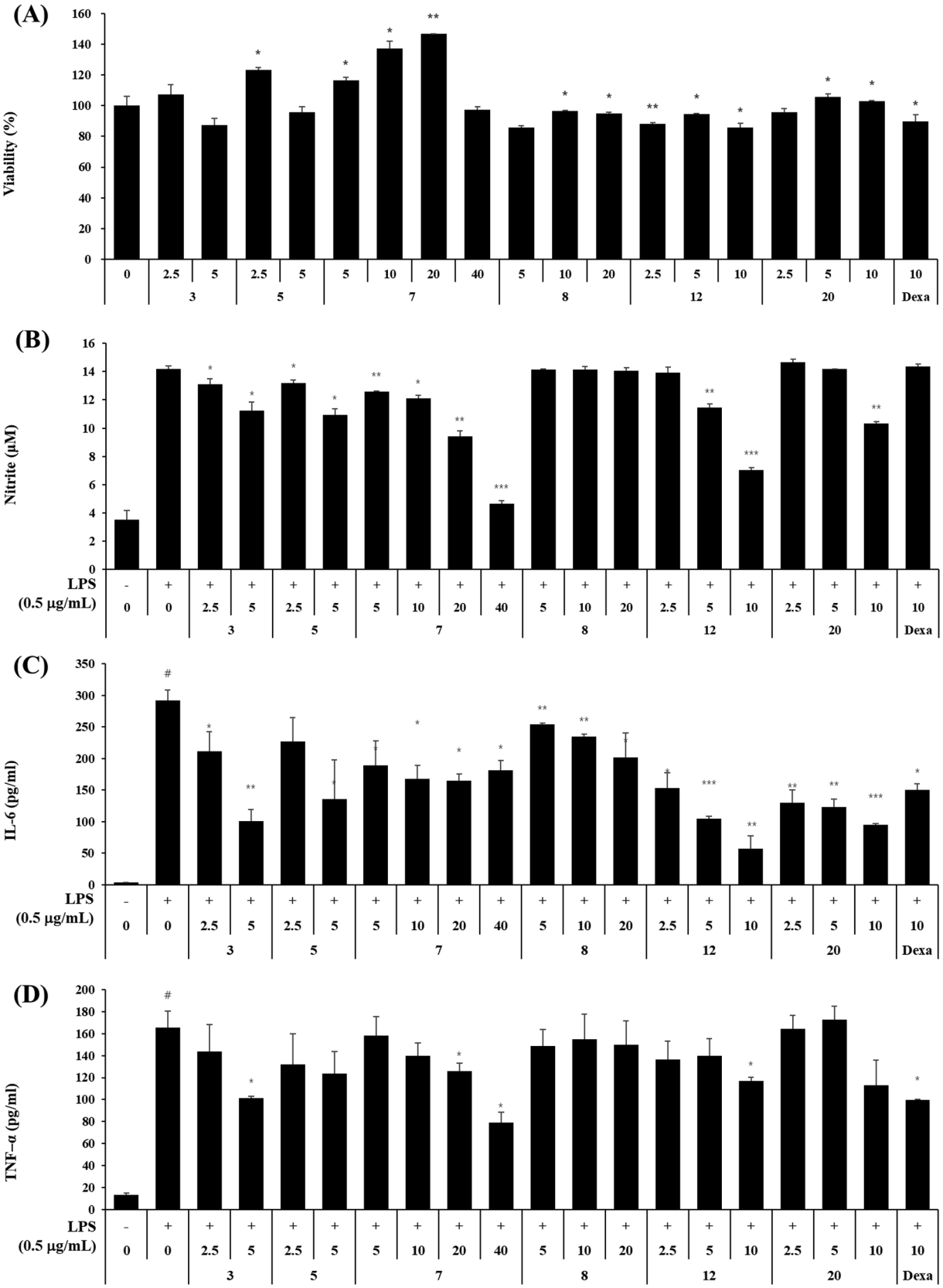

Fig. 4. Effects of active compounds **3**, **5**, **7**, **8**, **12**, and **20** (2.5–40 μM) on cell viability (A), NO production (B), TNF-α (C), and IL-6 (D) production in LPS-stimulated RAW264.7 cells. The error bars represent the mean ± SD of two independent experiments. *p < 0.05 vs. LPS, **p < 0.005 vs. LPS, ***p < 0.0005 vs. LPS. Positive control: Dexamethasone (Dexa).

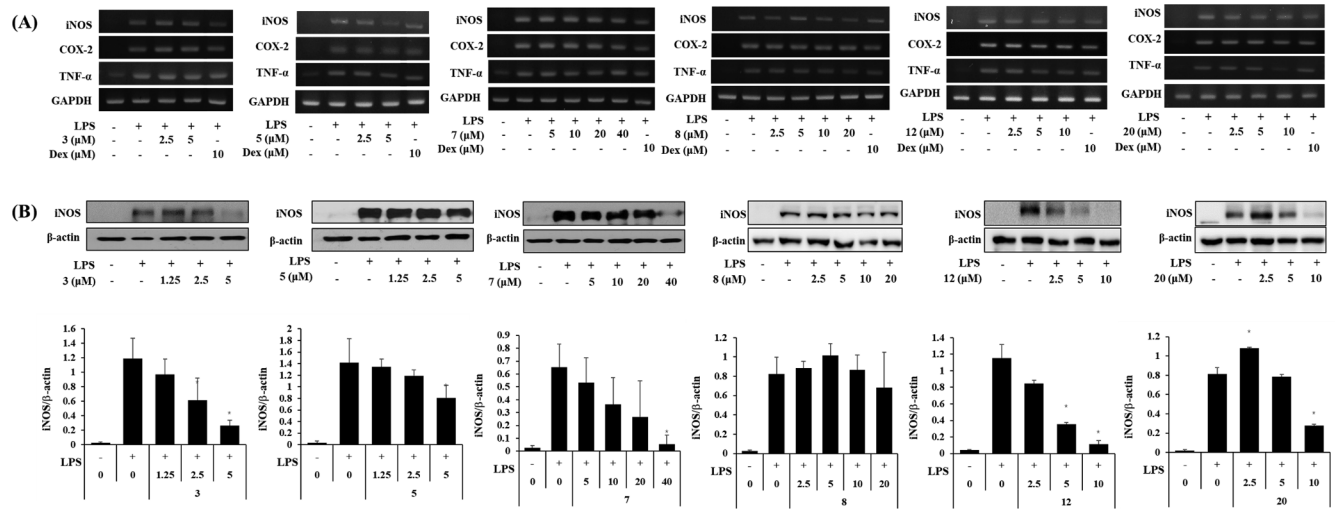

Fig. 5. Effects of active compounds, **3**, **5**, **7**, **8**, **12**, and **20** (1.25–40 μM) on iNOS, COX-2, and TNF-α DNA expression (A) and iNOS protein expression (B) in LPS-stimulated RAW264.7 cells. The error bars represent the mean ± SD of two independent experiments. *p < 0.05 vs. LPS.

(C-2′) and δ 124.7 (C-4′). The $[\alpha]_D^{25}$ was measured as −9.62, in reasonable agreement with the literature value ($[\alpha]_D$ −8.0). On the basis of a comparison of its $[\alpha]_D$ data with those of other 2,2-dimethylchroman-3-ol, Suciati et al. proposed that compound **19** has an *R* configuration [34]. Thus, compound **19** was identified as 2-[3,8-dihydroxy-2,2-dimethyl-5-(3-methylbut-2-enyl)-3,4-dihydrochromen-6-yl]-3,5,7-trihydroxy-8-(2-methylbut-3-en-2-yl)chromen-4-one and named broussonol G.

Compound **20** was isolated as a yellowish powder. The molecular formula was determined to be $C_{26}H_{36}O_5$ from the ion peak $[M-H]^-$ at *m/z* 427.2473 (calcd for $C_{26}H_{35}O_5$, 427.2484) in the HRESIMS, which was 18 amu higher than **17**, indicating that **20** has a 3-hydroxy-3-methylbutyl group instead of the 3″-prenyl group in **17**. The NMR data for compound **20** were similar to that of **17**, with the exception of the 3-hydroxy-3-methylbutyl group [12]. The ^{13}C NMR spectrum (125 MHz, acetone-d_6) of **20** showed the resonance of all twenty-six carbon atoms and the DEPT spectrum revealed the presence of five methyls at δ 55.5 (2′-OCH_3), δ 26.7 (C-15″, 16″), δ 25.8 (C-10″), and δ 18.0 (C-11″), six methylenes at δ 33.8 (C-13″), δ 33.6 (C-3), δ 33.1 (C-2), δ 30.5 (C-1), δ 27.8 (C-7″), and δ 21.0 (C-12″), and five methines at δ 130.8 (C-6′), δ 124.8 (C-8″), δ 114.2 (C-6″), δ 107.3 (C-5′), and δ 99.6 (C-3′) carbon atoms. From the ^1H NMR (500 MHz, acetone-d_6) spectrum, the chemical shift values at δ 2.55 (2H, t, *J* = 9.4 Hz), δ 2.50 (2H, t, *J* = 10.0 Hz), and 1.72 (2H, m) were similar to those of the corresponding data for 1,3-diphenylpropane [21]. These data suggested that **20** consisted of a 1,3-diphenylpropane backbone. The three coupled singlets of the ABX system at δ 6.92 (1H, d, *J* = 10.0 Hz), 6.42 (1H, d, *J* = 2.9 Hz), and δ 6.34 (1H, dd, *J* = 2.9, 10.0 Hz) were identified for H-6′, H-3′, and H-5′, respectively, of the A-ring. A singlet at δ 3.76 (3H) showed a methoxy group. The prenyl group appeared as triplet at δ 4.90 (1H), a doublet at δ 3.18 (2H), and two singlets at δ 1.72 (3H) and δ 1.65 (3H). In addition, the 3-hydroxy-3-methylbutyl group appeared as two triplets at δ 2.66 (2H) and δ 1.79 (2H) and a singlet at δ 1.27 (6H). In the COSY spectrum, the proton signals of the 1,3-diphenylpropane moiety at δ 2.55 (H-1), δ 2.50 (H-3) and δ 1.71 (H-2) and the proton signals of the 2′-prenyl group at δ 4.95 (H-8″), and δ 3.21 (H-7″) and the proton signals of the 3-hydroxy-3-methylbutyl group at δ 2.66 (H-12″), and δ 1.79 (H-13″) showed a correlation with each other. From the HMBC spectrum, the 1,3-diphenylpropane moiety proton signals at δ 2.55 (H-1) and δ 2.50 (H-3) showed a correlation with a tertiary carbon signal at δ 130.8 (C-6′) and δ 114.2 (C-6″) and two quaternary carbons at δ 159.3 (C-2′) and δ 128.9 (C-2″). The 2′-prenyl proton signal at δ 3.18 (H-7″) showed a correlation with two quaternary carbons at δ 132.5 (C-1″) and δ 120.5 (C-3″) on the B-ring. Moreover, the 3-hydroxy-3-methylbutyl proton signal at δ 3.35 (H-12″) showed a correlation with the oxygenated quaternary carbon signal at δ 140.4 (C-4″) and the quaternary carbon at δ 128.8 (C-2″) on the B-ring. The methoxy group at δ 3.76 showed a correlation with the carbon signal at δ 159.3 (C-2′). Thus, compound **20** was identified as 2″-(3-methylbut-2-enyl)-3″-(3-hydroxy-3-methylbutyl)-2′,4″,5″-trihydroxyl-4′-methoxydiphenylpropane and named kazinol W.

3.3. Cell cytotoxicity and inhibitory effects of isolated compounds on NO, TNF-α, and IL-6 production in RAW264.7 cells

The isolated compounds **1**–**20** from active fractions of *B. papyrifera* root bark were evaluated for their anti-inflammatory effects on NO production in LPS-stimulated RAW264.7 cells because all compounds were isolated from BP Frs. 6 and 7 that significantly inhibit NO. The isolated compounds **3**–**5**, **12**, **13**, and **18** showed cell cytotoxicity affecting the viability of the RAW264.7 cells at 20 μM by MTT assay (Fig. 3). The most cytotoxic compounds, broussonol D (**13**) and broussoflavonol C (**18**), were excluded and the active compounds **4** and **14** were also excluded, as these were reported for NO production inhibition with an IC_{50} of 11.3 and 21.6 μM, respectively [35,36]. Although cell cytotoxicity of RAW264.7 cells was seen after treatment with 20 μM of compounds **3**, **5**, **7**, **8**, **12**, and **20,** treating cells with various concentrations (2.5–40.0 μM) of those did not affect cell viability (Fig. 4A-B). To further investigate the inflammatory activity, the pro-inflammatory cytokine (TNF-α and IL-6) expression in RAW264.7 cells induced by these inhibitors was probed. The target compounds especially showed a significant NO, TNF-α, and IL-6 inhibitory effect. Additionally, the most active compounds (**3**, **5**, **7**, **12**, and **20**) inhibited TNF-α and IL-6 production in a dose-dependent manner (2.5–40.0 μM) in RAW264.7 cells (Fig. 4C-D). They were more potent than the positive control, dexamethasone, a synthetic glucocorticoid and potent immunosuppressant that inhibits the cytokine production induced by lipopolysaccharides (LPS). These results indicate that compounds **3**, **5**, **7**, **8**, **12**, and **20** are non-toxic, potent inhibitors of inflammation in the concentration range tested.

3.4. Effects of compounds *3*, *5*, *7*, *8*, *12*, and *20* on iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells

The inhibition of iNOS and COX-2 is important because they mediate inflammatory events to produce pro-inflammatory cytokines such as TNF-α and IL-6 [18,19,20]. Moreover, TNF-α mediates the actions of cytokines such as IL-6 [18,19,20]. TNF-α and IL-6 are principal

mediators in LPS-stimulated tissue injury. As shown in Fig. 5, to evaluate the effect of the active compounds **3**, **5**, **7**, **8**, **12**, and **20** on iNOS, COX-2, and TNF-α, LPS-induced RAW264.7 cells were pretreated with various concentrations of samples (2.5–10 μM) for 24 h. The iNOS protein expression induced by LPS was decreased by 42.8–91.4% by compounds **3**, **5**, **7**, **12**, and **20** at 5–40 μM with a minor decrease by compound **8** of 17.2% at 20 μM compared to the LPS only group. This *in vitro* study indicates that compounds **3**, **5**, **7**, **8**, **12**, and **20** may have anti-inflammatory effects by inhibiting the production of NO through downregulation of iNOS and COX-2 expression.

4. Conclusion

In summary, a bioactivity-guided fractionation and metabolite investigation from the methanolic extracts of root bark of *Broussonetia papyrifera* (L.) L Her. ex Vent. led to the isolation of twenty compounds; six 1,3-diphenylpropanes (**1**, **2**, **8**, **10**, **17**, and **20**), flavanone (**3**), two chalcones (**4** and **5**), five flavans (**6**, **11**, and **14–16**), dihydroflavonol (**7**) and five flavonols (**9**, **12**, **13**, **18**, and **19**), including five new compounds (**5**, **7**, **8**, **19**, and **20**). From the screening for inhibition of NO and pro-inflammatory cytokines (TNF-α and IL-6) in LPS-stimulated RAW264.7 cells, compounds **3**, **5**, **7**, **8**, **12**, and **20** exhibited potent anti-inflammatory effects by reducing NO production through downregulating iNOS, COX-2, and TNF-α expression and iNOS protein expression. This study therefore underscores that *B. papyrifera* is a valuable source of natural BRM for pharmaceuticals and functional foods for anti-inflammatory diseases such as asthma, COPD, and atopy.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103233.

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