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Selective inducible nitric oxide synthase suppression by new bracteanolides from *Murdannia bracteata*

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Abstract

Murdannia bracteata has been used as a Taiwanese folk medicine for its anti-inflammatory properties. However, neither its active ingredients nor its anti-inflammatory actions are well defined. Nitric oxide (NO), overproduced by activated macrophages via inducible NO synthase (iNOS), is suggested to be a significant pathogenic factor in various inflammatory tissue injuries. In order to elucidate the anti-inflammatory actions of *M. bracteata*, the present study was designed to isolate its active constituents and examine its effects on iNOS in lipopolysaccharide (LPS)-activated macrophages. Two new hydroxybutenolides, bracteanolide A (**1**) and B (**2**), together with (+)-(*R*)-*p*-hydroxyphenyllactic acid (**3**) and isovitexin (**4**), were isolated and identified from *M. bracteata* by the NO production assay. All of the compounds inhibited NO production except **3**. Their rank order of potency was **1** > **2** > **4**. Among these, **1** significantly inhibited NO production, which is associated with its suppression on iNOS induction in a concentration-dependent manner, with an IC_{50} of 33.27 ± 0.86 μM. Nevertheless, isometric tension recordings in isolated endothelium-intact rat aorta revealed that **1–4** did not affect acetylcholine-induced endothelial NO-dependent relaxation, an index of endothelial NOS (eNOS) activity. The selective inhibition on iNOS provides a possible explanation for the anti-inflammatory use of *M. bracteata*.

Keywords: *Murdannia bracteata*; Commelinaceae; Bracteanolide A; Bracteanolide B; Inducible nitric oxide synthase; RAW 264.7 cells

1. Introduction

Murdannia bracteata, a perennial herb, is widely distributed throughout the Indo-China Peninsula (Hong, 1997). It has long been used in folk medicine to treat hepatitis, stomatitis, pneumonia, nephritis and many other inflammatory diseases (Chiu and Chang, 1995). However, the pharmacological data is deficient in clearly establishing the scientific rationale for the anti-inflammatory medicinal use of this plant; the search for its active constituents is also limited.

Nitric oxide (NO) is one of the critical mediators released from a variety of cells, such as vascular endothelial cells and macrophages, that alters cardiovascular homeostasis, leading to changes in the physiological condition, even when large amounts of NO are released upon external stimulation (Wolf, 1997). Cellular NO, accompanied with L-citrulline, is synthesized from L-arginine by a family of NO synthase (NOS) (Hibbs et al., 1987). Among three identified isoforms of NOS, the constitutive endothelial NOS (eNOS) plays an important role in protection against the onset and progression of cardiovascular disorders under physiological conditions. The expression of inducible NOS (iNOS) is induced by pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS) or cytokines (Chartrain et al., 1994). NO, if produced in large quantities by activated macrophages overexpressing iNOS, has been implicated in the pathogenesis of a variety of inflammatory-mediated disorders including septic shock, stroke, DNA damage, and carcinogenesis caused by mutagenesis (Chen et al., 1998). Since cells cannot sequester and regulate the local concentration of NO, inhibition of NO synthesis is, therefore, a potential therapeutic approach for the treatment of these inflammatory diseases. Therefore, the

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase

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screening of bioactive compounds from natural resources, which modulates the activity and/or expression of iNOS, will prove invaluable.

NO production induced by LPS through iNOS expression in RAW 264.7 cells, a mouse macrophage cell line, may reflect the degree of inflammation and may provide a measure for assessing the effects of test drugs on the inflammatory process (Jiang et al., 2006). Through bioassay-guided fractionation, the ethyl acetate layer of the whole plant extracts of *M. bracteata* has the ability to inhibit NO production in cultured RAW 264.7 cells stimulated with bacterial LPS. *M. bracteata* may contain some bioactive components worthy of being investigated. In this study, a series of extraction, separation, purification, and structural elucidation was investigated, which has led to the isolation and identification of two new hydroxybutenolides as well as two known phenolics. In an attempt to evaluate their potential as anti-inflammatory agents, the isolated pure compounds were tested for their activities on iNOS in unstimulated- and LPS-stimulated RAW 264.7 cells. Their cytotoxicity was also assessed. The results indicated that constituents of *M. bracteata* significantly inhibited NO production in LPS-stimulated murine macrophages. Of all the compounds tested, the new extract bracteanolide A (**1**) is the most potent and selective for iNOS, which may have potential in the prevention and treatment of diseases caused by an increased expression of iNOS, although further study is still warranted. These results also serve as an additional rationale for the use of *M. bracteata* in inflammatory disorders.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured using a JASCO P-1020 digital spectropolarimeter. IR spectra were recorded on a Thermo IR 300 spectrometer. UV spectra were measured in MeOH on a Shimazu UV 1601 spectrophotometer. The NMR spectra were recorded in CD_3OD at room temperature on a Bruker DMX-500 SB spectrometer, and the solvent resonances of the residual undeuterated solvent were used as internal shift references. The 2D NMR spectra were recorded using standard pulse sequences. Positive ion FAB-MS and HR-FAB-MS data were obtained on a JOEL SX-102A mass spectrometer using *m*-nitrobenzyl alcohol (NBA) as the matrix. Sephadex LH-20 (Pharmacia Biotech) was used for gel permeation chromatography. HPLC was performed using a semi-preparative column (BDS Hypersil C18, 10 mm i.d. × 250 mm, Thermo Hypersil-Keystone, Runcorn, UK; detector, refractive index). TLC was performed using silica gel 60 F_{254} plates (200 μm, Merck, Germany).

2.2. Plant material

The whole plant of *Murdannia bracteata* (C. B. Clarke) J. K. Morton ex D. Y. Hong (Commelinaceae) were collected in the suburbs of Taipei, Taiwan on Oct. 11, 2005, and were identified by Dr. Ching-I Peng, a research fellow in the Research Center for Biodiversity, Academia Sinica, Taipei, Taiwan. The voucher specimens (no. 10112005) were deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan for future reference.

2.3. Extraction and isolation of **1–4**

The whole plant of *M. bracteata* (6 kg) was successively extracted three times with 12 l of MeOH at room temperature for 2 weeks. The methanolic extract (530 g) was adjusted to 85% in aqueous solution for an *n*-hexane partition, which generated two fractions soluble in aqueous methanol and *n*-hexane (12.5 g). Subsequently, the aqueous methanol-soluble fraction was then evaporated to dryness (103 g) and further partitioned between ethyl acetate (400 ml × 2) and water (400 ml). The remaining water solution was extracted two times with *n*-butanol (400 ml). Then, the subsequent separation was guided by bioassays used in this study. The ethyl acetate layer (15.5 g), with significant bioactivity, was evaporated to a brown residue and re-dissolved in methanol for chromatographic purification. The first separation step was carried out using gel permeation chromatography on a Sephadex LH-20 column (3 cm i.d. × 65 cm) and eluted by methanol with a flow rate of 2.5 ml/min. Each fraction (15 ml) collected from the ethyl acetate layer was checked for its compositions by TLC using ethyl acetate/acetic acid/H_2O (85:10:10) for development, and observation under UV 254 nm. Dipping in vanillin-sulfuric acid were used in the detection of compounds with similar chromophores. Subsequently, fractions containing similar compounds and exhibiting stronger bioactivity (#fr. 18–21, 0.65 g) were combined to give one major portion. HPLC of this portion on a reversed-phase column with acetonitrile/H_2O (1:4) as eluent, 2 ml/min, afforded 1 (25 mg; t_R, 8.96 min), 2 (8 mg; t_R, 14.90 min), 3 (11 mg; t_R, 10.13 min) and 4 (47 mg; t_R, 11.00 min).

Bracteanolide A (*1*). amorphous white powder; $[\alpha]^{25}{}_D = +21.4^\circ$ (*c* 0.1, MeOH); UV (MeOH): λ_{max} (log ε) = 214 (3.9), 245 (3.7), 303 (3.8), 329 (3.9) nm; IR (KBr): υ_{max} = 3324, 1726, 1603, 1299 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): see Table 1; ^{13}C NMR (CD_3OD, 125 MHz): see Table 1; FAB-MS (NBA): *m/z* = 209 $[M+H]^+$; HR-FAB-MS (NBA): *m/z* = 209.0453 $[M+H]^+$ calcd. for $C_{10}H_8O_5 + H^+$: 209.0450.

Bracteanolide B (*2*). amorphous white powder; $[\alpha]^{25}{}_D = -13.4^\circ$ (*c* 0.1, MeOH); UV (MeOH): λ_{max} (log ε) = 215 (3.9), 246 (3.8), 305 (3.8), 333 (3.9) nm; IR (KBr): υ_{max} = 3369, 1729, 1605, 1298 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): see Table 1; ^{13}C NMR (CD_3OD, 125 MHz): see Table 1; FAB-MS (NBA): *m/z* = 223 $[M+H]^+$; HR-FAB-MS (NBA): *m/z* = 223.0602 $[M+H]^+$ calcd. for $C_{11}H_{10}O_5 + H^+$: 223.0607.

2.4. Cell culture

RAW 264.7 cells (a transformed murine macrophage cell line) obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) were maintained by once-weekly passage in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin.

Table 1
^{1}H and ^{13}C NMR spectroscopic data (CD_3OD, 500 MHz) for compounds **1** and **2** [δ in ppm, mult. (J in Hz)]

Position	1			2		
	^{13}C[a]	^{1}H	HMBC (H→C)	^{13}C[a]	^{1}H	HMBC (H→C)
2	174.1 s			173.5 s		
3	111.8 d	6.23 s	2, 4, 5, 1′	112.6 d	6.30 s	2, 4, 5, 1′
4	165.9 s			163.9 s		
5	99.9 d	6.45 s	2, 3	104.8 d	6.30 s	2, 3
1′	122.6 s			122.3 s		
2′	116.0 d	7.20 s	4, 3′, 4′, 6′	115.7 d	7.14 s	4, 3′, 4′, 6′
3′	146.7 s			146.9 s		
4′	150.5 s			150.8 s		
5′	116.5 d	6.82 d (8.2)	1′, 3′, 4′	116.6 d	6.82 d (8.1)	1′, 3′, 4′
6′	122.3 d	7.18 d (8.2)	4, 2′, 4′	122.1 d	7.13 d (8.1)	4, 2′, 4′
$-OCH_3$				55.6 q	3.52 s	5

[a] Multiplicities were obtained from DEPT experiments.

2.5. NO measurement

Cell aliquots (5×10^5 cells/ml) were grown to confluence on 24-well plates for 24 h. The medium was changed to serum-free DMEM for another 4 h to render the attached cells quiescent. To assess the effects on LPS-induced NO production, compounds **1–4**, two positive control N^{ω}-nitro-L-arginine (L-NNA, a non-selective NOS inhibitor) and aminoguanidine (a selective iNOS inhibitor; 100 μM) or vehicle (dimethyl sulfoxide; 0.1%) were added in the absence or presence of LPS (50 ng/ml) to the cells for another 24 h. The culture supernatant was subsequently collected for the nitrite assay as a reflection of NO production (Green et al., 1982). Briefly, an aliquot of supernatant was mixed with an equal volume of Griess reagent (prepared by adding 1 part 0.1% napthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured by a microplate spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT, USA). Fresh medium was used as the blank. The nitrite concentration was determined by reference to a standard curve by using sodium nitrite diluted in the stock culture medium. Results are expressed as percentage of inhibition calculated versus vehicle plus LPS-treated cells.

2.6. Cytotoxicity assay

A redox indicator, alamarBlue, was used to measure the cytotoxicity as shown previously (Kwack and Lynch, 2000). After the culture supernatant was removed for NO measurement described above, a solution of 10% al amarBlue in DMEM was added to each well containing RAW 264.7 cells. The plates were incubated at 37 °C in humidified 5% CO_2 for 3 h. Following incubation, the absorbance of the alamarBlue was read spectrophotometrically at dual wavelengths of 570 and 600 nm.

2.7. Western blot assay

The expressions of iNOS in LPS-stimulated RAW 264.7 cells was analyzed. RAW 264.7 cells (1.5×10^7 cells/ml) were grown to confluence on culture dishes for 24 h. After starvation in a serum-free medium for 4 h, the cells were simultaneously exposed to LPS (50 ng/ml) and compounds **1–4** for another 24 h. At the end of the experiments, the cells were washed and lysed in ice-cold lysis buffer. The lysates were centrifuged at 3000 × g for 20 min at 4 °C. The cytoplasmic protein concentration in the supernatants was determined by the protein-dye method of Bradford (1976), using bovine serum albumin as a standard. Total protein (50 μg per lane) from each sample was run on 8% SDS-polyacrylamide gels and transferred to PVD membranes (Bio-Rad Laboratories Inc., Hercules, CA). Membranes were then serially incubated, first with blocking buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.2% (vol/vol) Tween 20, and 5% (wt/vol) non-fat milk for 1 h. The next incubation was performed with a mouse monoclonal antibody against mouse iNOS (1:1000, Transduction Laboratories, San Diego, CA). A final incubation was carried out with anti-rabbit IgG horseradish peroxidase (1:5000). Immunoreactive bands were visualized with a chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

2.8. Animals

Adult male Sprague-Dawley rats, weighing 250–280 g (National Laboratory Animal Center, Taipei, Taiwan), were tested. The rats were allowed to acclimate to environmentally controlled quarters with a constant temperature of 20–22 °C, relative humidity 55% and a light cycle of 12:12 h. Standard laboratory chow (Purina Mills, Richmond, IN, USA) and drinking water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committees of National Research Institute of Chinese Medicine and were conducted in accordance with the National Institutes of Health Animal Care standards.

2.9. Vascular tension experiment

The methods employed were essentially the same as our previously published methods (Wang et al., 1996; Ko et al., 2006). In brief, aortic rings from Sprague-Dawley rats were fixed in organ chambers isometrically under passive tension of

1.8 g for 60 min. Functional integrity of the endothelium was confirmed by an observation of more than 95% relaxation in response to acetylcholine (1 μM) in tissues pre-contracted with phenylephrine (0.3 μM). To investigate the effects of compounds **1–4** on eNOS function, cumulative concentrations of acetylcholine (10 nM–10 μM) were applied during the sustained phase (considered as 100%) of phenylephrine (0.3 μM)-induced contraction in endothelium-intact aortic rings. Following washing and recovery for 30 min, similar experiments were carried out in the presence of **1–4**, L-NNA or aminoguanidine at 10 μM or vehicle (dimethyl sulfoxide; 0.1%) for 20 min. The construction of concentration–response curves for acetylcholine was based on the percentage of relaxation of the agonist-induced contraction. A complete relaxation was considered attained when the pre-contracted rings returned to the base line position.

2.10. Reagents

The following drugs were used: acetylcholine, aminoguanidine, bovine serum albumin, dimethyl sulfoxide, DMEM, L-NNA, LPS (*Escherichia coli* Serotype 055:B5), napthylethylenediamine dihydrochloride, phenylephrine and sulfanilamide, from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA); alamarBlue, from Biosource International Co. (Camarillo, CA, USA); FCS, from PAA Laboratories GmbH (Pasching, Austria); penicillin–streptomycin from Invitrogen Co. (Carlsbad, CA, USA); mouse monoclonal antibody against mouse iNOS from Transduction Laboratories (San Diego, CA, USA). Compounds **1–4** were dissolved in dimethyl sulfoxide to make stock solutions, respectively, and stored at −30 °C. The final concentration of the vehicle in the solutions never exceeded 0.1% and had no effects on NO production, iNOS expression, vascular tension or cell viability assay.

2.11. Statistic analysis

For each experimental series, data are presented as mean ± S.E. and n represents the number of independently performed experiments. All data were analyzed by an IBM-compatible statistical software package (SPSS for Windows, Ver. 10.0). The significance of the concentrations and sample treatments was determined by two-way analysis of variance (ANOVA) with repeated measures. If there were significant interactions, the simple main effect of each factor was assessed using the Kruskal-Wallis nonparametric post hoc analysis for ANOVA. Post hoc comparisons were carried out between means, according to suitability. P-values less than 0.05 indicated a statistically significant difference.

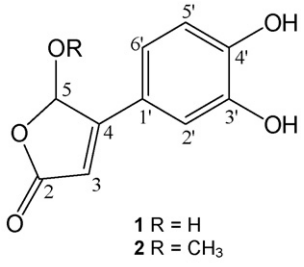
3. Results and discussion

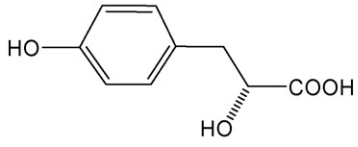
A methanolic extract of the whole plant of *M. bracteata* was fractionated by liquid–liquid partitioning into fractions soluble in *n*-hexane, ethyl acetate, *n*-butanol, and H_2O, successively. Among them, the ethyl acetate-soluble fraction has the most significant inhibitory activity on iNOS with an overall yield of 0.26%. This fraction was further chromatographed sequentially by Sephadex LH-20 column chromatography and HPLC, and each step was guided by a bioassay to give compounds **1–4**. Of these, **1** and **2** were identified to be new based on their spectral analysis.

The molecular formula for **1**, $C_{10}H_8O_5$, was determined by ^{13}C NMR and HR-FAB-MS data. The IR spectrum of 1 indicated the presence of a hydroxyl (3324 cm^{-1}), a carbonyl (1726 cm^{-1}) and aromatic (1603 cm^{-1}) groups. The ^1H NMR spectrum of 1 (Table 1) displayed an ABX coupled aromatic system at δ_H 7.20 (1H, s), 7.18 (1H, d, J = 8.2 Hz) and 6.82 (1H, d, J = 8.2 Hz), ascribable to H-2′, -6′ and -5′, respectively, as well as one olefinic proton at δ_H 6.23 (1H, s), and one hemiacetal proton at δ_H 6.45 (1H, s) in association with its ^{13}C NMR data, **1** was characteristic of an aromatic ring annexed to a conjugated γ-butyrolactone skeleton (Rasmussen et al., 1995; Li et al., 2003). Thus, the structure of **1** (Fig. 1) was established to be 2,5-dihydro-5-hydroxy-4-[3′,4′-dihydroxyphenyl]-furan-2-one, and named bracteanolide A.

Compound **2** possessed spectroscopic data closely comparable to that of **1** except that its OH-5 functionality in **1** was replaced with a methoxy group in **2**. Its ^1H NMR (Table 1) exhibited one signal for an additional methoxy singlet at δ_H 3.52 when compared with that of **1**. Therefore, **2** was assigned as 2,5-dihydro-5-methoxy-4-[3′,4′-dihydroxyphenyl]-furan-2-one, and named bracteanolide B.

Spectroscopic data of (+)-(*R*)-*p*-hydroxyphenyllactic acid (3) having been isolated from the metabolites of *Ceratocystis* spp.





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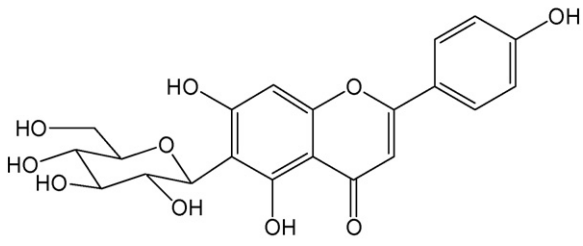

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Fig. 1. Chemical structure of compounds **1–4** from *Murdannia bracteata*.

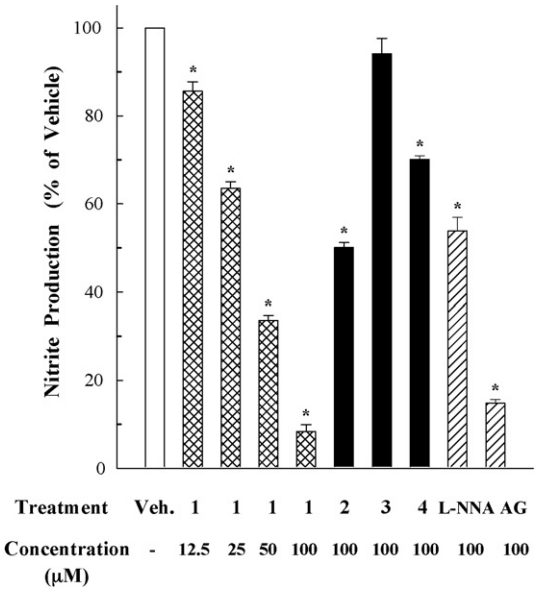

Fig. 2. Inhibitory effect of compounds **1–4** on LPS-induced NO production. RAW 264.7 cells were co-incubated with LPS (50 ng/ml) and **1** (12.5–100 μM), **2–4** (100 μM), N^{ω}-nitro-L-arginine (L-NNA, 100 μM), aminoguanidine (AG, 100 μM) or vehicle for 24 h. NO production was determined by measuring the amount of NO metabolites in the medium. $n = 6–8$ in each group. $^{*}P < 0.05$ when compared with vehicle-treated cells. Vehicle group represented as 100% is equal to 30.60 ± 0.05 μM of NO produced in the medium per well of cells.

were interpreted by comparison with those reported in the literature (Ayer et al., 1986). Compound **4**, a major component, was obtained as a yellow powder whose spectral data was consistent with that of isovitexin, having been isolated from *Terminalia catappa* (Lin et al., 2000).

To access the possible cytotoxicity of **1–4** against RAW 264.7 cells, a mouse macrophage cell line, we conducted a cell viability test determined in the absence of LPS using the alamaBlue assay. Up to a concentration of 100 μM, all compounds revealed no significant cytotoxicity (data not shown). Thus, concentrations of 100 μM were chosen for subsequent experiments. NO production induced by LPS through iNOS expression in RAW264.7 cells may reflect the degree of inflammation and may provide a measure for assessing the effects of drugs on the inflammatory process. In this experiment, aminoguanidine, a selective iNOS inhibitor, and L-NNA, a non-selective NOS inhibitor, were used as positive inhibitors. To investigate the effect of **1–4** on NO production, RAW 264.7 cells were stimulated with 100 μM of **1–4** in the absence or presence of LPS (50 ng/ml). Without LPS, RAW 264.7 cells released undetectable levels of NO after 24-h incubation (data not shown). From these results, all the compounds failed to stimulate RAW 264.7 cells to produce detectable amounts of NO (data not shown), but inhibited NO production by LPS-activated RAW 264.7 cells to varying extents. As shown in Fig. 2, LPS significantly elicited the accumulation of 30.60 ± 0.05 μM of nitrite, which is a stable metabolite in the medium. Among the tested compounds, **1**, at 100 μM, is the most potent in the assay, with 91.60 ± 1.51% inhibition, even better than the positive inhibitors aminoguanidine and L-NNA. Under the same conditions, the inhibitory effects of aminoguanidine and L-NNA were 85.21 ± 0.87% and 46.16 ± 3.07%, respectively. Fig. 2 shows that the new bracteanolide A (**1**, 12.5–100 μM) strongly inhibited LPS-induced NO production in a concentration-dependent manner, with an IC_{50} value of 33.27 ± 0.86 μM. The observed effect seems not to be related to the cytotoxicity of **1**, since it showed no impairment of cell viability (Fig. 3). In order to determine if the observed inhibitory effect of **1** on the inflammatory mediators was directly related to the modulation of iNOS induction, we examined its protein expression levels by Western blot analysis. As shown in Fig. 4A, 130 kDa of iNOS protein expression was undetectable in unstimulated RAW 264.7 cells. In response to LPS, the amount of iNOS was markedly up-regulated. At the indicated concentrations, **1** had an apparent suppressive effect on iNOS protein induction without affecting housekeeping protein expression. This effect was also concentration-dependent (Fig. 4B). The inhibitory effect of **1** on iNOS protein expression in activated RAW 264.7 cells could account, at least in part, for the suppression of NO production. Similar experiments were conducted for aminoguanidine and L-NNA as illustrated in Fig. 4A. Clearly, **1** is more potent than either of these two positive controls.

Bracteanolide B (**2**), an analogue of bracteanolide A, has no influence on cell viability at concentrations up to 100 μM in the presence of LPS (Fig. 3). Different from bracteanolide A, **2** exerts a moderate inhibitory activity on NO production at the maximal test concentration (Fig. 2). The nitrite level of **2** was 50.11 ± 1.12% of cells treated with LPS plus vehicle group. Only 4, at the concentration used for NO inhibition, has weakly reduced cell viability by 9.25 ± 1.78% in the presence of LPS (Fig. 3). This compound appeared to inhibit NO production in LPS-activated RAW 264.7 cells (Fig. 2), but this effect was comparable with its cytotoxicity upon cells during culture in

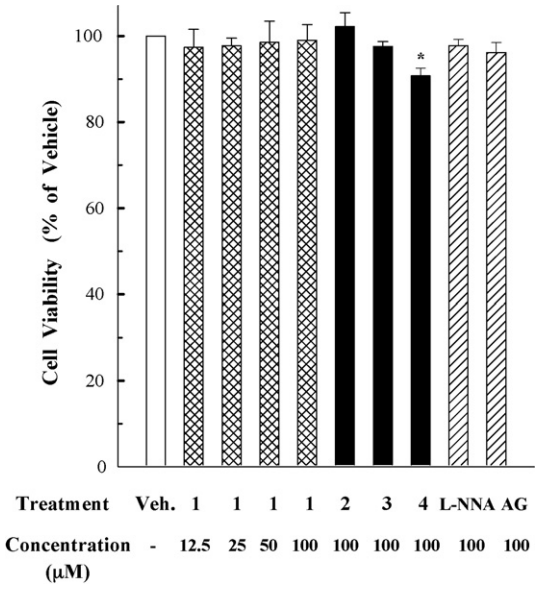

Fig. 3. Effect of compounds **1–4** on cell viability in LPS-activated RAW 264.7 cells. RAW 264.7 cells were co-incubated with LPS (50 ng/ml) and **1** (12.5–100 μM), **2–4** (100 μM), N^{ω}-nitro-L-arginine (L-NNA, 100 μM), aminoguanidine (AG, 100 μM) or vehicle for 24 h. Cell viability was detected by alamarBlue assay. $n = 6–8$ in each group. $^{*}P < 0.05$ when compared with vehicle-treated cells.

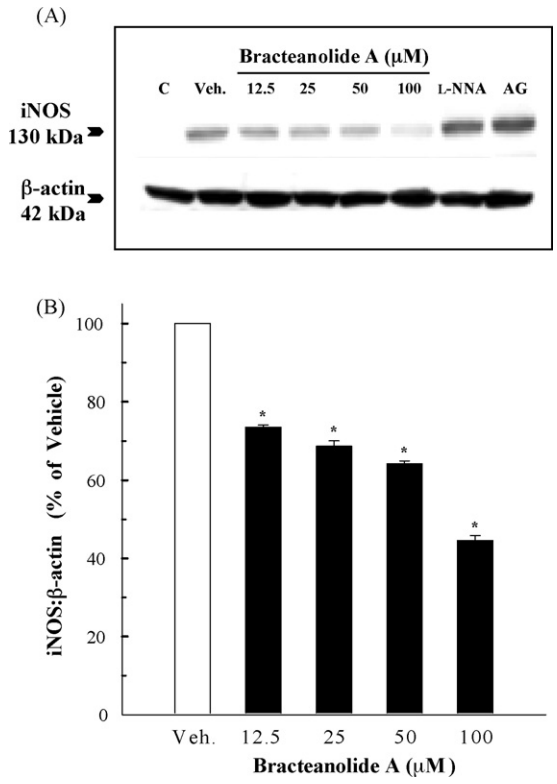

Fig. 4. The inhibitory effect of bracteanolide A (**1**) on LPS-induced iNOS protein expression in RAW 264.7 cells. (A) A representative immunoblot from three separate experiments is shown. Lysates were prepared from 24 h-LPS (50 ng/ml)-stimulated cells in combination with bracteanolide A (12.5–100 μM), N^{ω}-nitro-L-arginine (L-NNA, 100 μM), aminoguanidine (AG, 100 μM) or vehicle. (B) Results were generated as integrated intensity units by densitometry and expressed as percentage of vehicle from three separate experiments. $^{*}P < 0.05$ when compared with vehicle-treated cells.

the presence of LPS. In contrast to the other three compounds, the effect of **3** seems not to be linked to NO production at the tested concentrations, although it did not affect cell viability (Figs. 2 and 3).

In addition to the inflammatory iNOS pathway, basal release of NO chiefly derived from constitutively activated eNOS contributes to vasorelaxation. Endothelium-dependent relaxations are achieved by a combination of nitric oxide (NO), endothelium-derived prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) (Rubanyi, 1993; Fleming et al., 1996). The tonical contribution of NO, derived from activation of eNOS, is most prominent in the aorta, whereas that of EDHF is most prominent in the distal resistance arteries (Shimokawa et al., 1996). According to previous reports (Ku et al., 1996; Chataigneau et al., 1999), endothelium-dependent relaxation induced by acetylcholine in the aorta from wild-type eNOS(+/+) mice was completely abolished by acute inhibition of NOS indicating that, in large-conduit vessels, NO is the major endogenous endothelium-derived vasodilator. In order to explore whether **1–4** has an effect on modification of eNOS function, besides iNOS, we examined the effects of **1–4** on acetylcholine-evoked relaxation in isolated endothelium intact aortic rings. In the vasorelaxation bioassay, each tested compound, given individually did not alter the baseline tension of the aortic rings (data not shown). Fig. 5 shows the vasorelaxation effects of acetylcholine (10 nM 10 μM) in the presence of **1**, reference compounds or vehicle in aortic rings pre-contracted with phenylephrine (0.3 μM). When the concentration of acetylcholine reached 1.0 μM, a marked relaxation occurred (97.46 ± 0.81%) in the vehicle-treated group. The non-selective NOS inhibitor L-NNA (10 μM) completely inhibited acetylcholine-evoked vasorelaxation whereas aminoguanidie, a selective iNOS inhibitor, did not change the effect induced by acetylcholine. In the presence of **1** (10 μM) for 20 min, the vasorelaxation induced by acetylcholine was not obviously affected. The maximal relaxation of this compound was 100%, which was similar to aminoguanidine (10 μM) yielding maximal relaxation of 98.08 ± 1.92%. These findings suggest that **1**, unlike L-NNA, did not modify the activity of eNOS. This compound exhibits significant and comparable selectivity for the inhibition of iNOS and may be developed as a potential therapeutic strategy for anti-inflammation, without changes in vascular tension and systemic blood pressure. Similar to **1**, compounds **2–4** did not change the endothelial NO-dependent relaxation induced by acetylcholine, an index of eNOS acitivity (data not shown).

The overall experimental results of the present study suggest that the constituents, two hydroxybutenolides as well as isovitexin, isolated from *M. bracteata*, substantially inhibit NO production in LPS-stimulated RAW 264.7 murine macrophages. The new compound bracteanolide A (**1**) is the most potent. Its dramatic inhibitory effect on NO synthesis is associated with its decreased expression of iNOS protein. The modulating effect on iNOS is selective, since it did not effect acetylcholine-evoked endothelial NO-dependent vasorelaxation an index of eNOS activity. All of these findings seem to provide a rationale for the

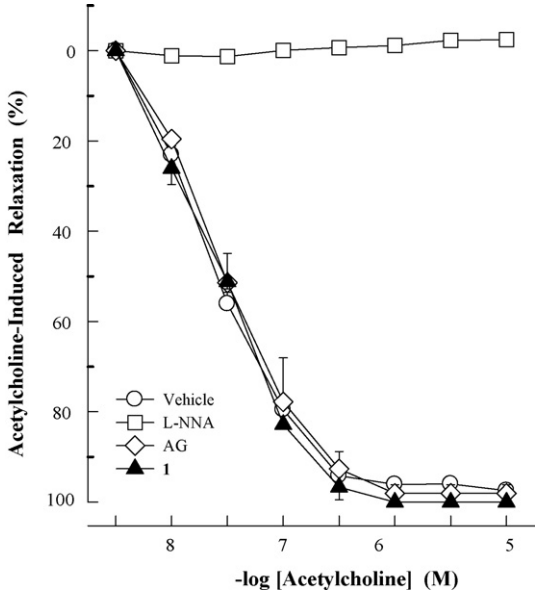

Fig. 5. The vasorelaxation effects of acetylcholine (10 nM–10 μM) in the presence of compound **1** (10 μM), N^{ω}-nitro-L-arginine (L-NNA, 10 μM), aminoguanidine (AG, 10 μM) or vehicle for 20 min in rat aortic rings with intact endothelium precontracted with phenylephrine (0.3 μM). $n = 6–8$ in each group. $^{*}P < 0.05$ when compared with vehicle-treated cells.

anti-inflammatory use of *M. bracteata*, which might be linked with its ability to reduce NO production and immunoregulatory function.

4. Conclusion

To our knowledge, this is the first report on the scientific rationale of *M. bracteata* for anti-inflammatory medicinal use. The specific iNOS inhibitory effect of **1** could be potentially developed as a selective inhibitor of iNOS for future therapeutic use.

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References

Ayer, W.A., Browne, L.M., Feng, M.C., Orszanska, H., Saeedi-Ghomi, H., 1986. The chemistry of the blue stain fungi. Part 1. Some metabolites of *Ceratocystis* species associated with mountain pine beetle infected lodgepole pine. Canadian Journal of Chemistry 64, 904–909.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Chartrain, N.A., Geller, D.A., Koty, P.P., Sitrin, N.F., Nussler, A.K., Hoffman, E.P., Billiar, T.R., Hutchinson, N.I., Mudgett, J.S., 1994. Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. Journal of Biological Chemistry 269, 6765–6772.

Chataigneau, T., Feletou, M., Huang, P.L., Fishman, M.C., Duhault, J., Vanhoutte, P.M., 1999. Acetylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice. British Journal of Pharmacology 126, 219–226.

Chen, Y.C., Lin-Shiau, S.Y., Lin, J.K., 1998. Involvement of reactive oxygen species and caspase 3 activation in arsenite induce apoptosis. Journal of Cellular Physiology 177, 323–324.

Chiu, N.Y., Chang, K.H. (Eds.), 1995. The Illustrated Medicinal Plants of Taiwan (VI). SMC Publishing Inc., Taipei, p. 256.

Fleming, I., Bauersachs, J., Busse, R., 1996. Paracrine functions of the coronary vascular endothelium. Molecular and Cellular Biochemistry 157, 137–145.

Green, L.C., Wagner, D.A., Glogowski, J., Wishnik, I.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite and [^{15}N]nitrate in biologic fluids. Analytical Biochemistry 126, 131–138.

Hibbs Jr, J.B., Taintor, R.R., Vavrin, Z., 1987. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science 235, 473–476.

Hong, D.Y., 1997. Angiospermae: monocotyledoneae. In: Wu, K.F. (Ed.), Flora, Reipublicae Popularis Sinicae. Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita, Tomus, 13. Science Press, Beijing, pp. 109–110.

Jiang, J.S., Shih, C.M., Wang, S.H., Chen, T.T., Lin, C.N., Ko, W.C., 2006. Mechanisms of suppression of nitric oxide production by 3-*O*-methylquercetin in RAW 264.7 cells. Journal of Ethnopharmacology 103, 281–287.

Ko, H.C., Chen, K.T., Chen, C.F., Su, J.P., Chen, C.M., Wang, G.J., 2006. Chemical and biological comparisons on *Evodia* with two related species of different locations and conditions. Journal of Ethnopharmacology 108, 257–263.

Ku, D., Guo, L., Dai, J., Acuff, C.G., Steinhelper, M.E., 1996. Coronary vascular and endothelial reactivity changes in trans-genic mice overexpressing atrial natriuretic factor. American Journal of Physiology 271, H2368–H2376.

Kwack, K., Lynch, R.G., 2000. A new non-radioactive method for IL-2 bioassay. Molecular Cell 10, 575–578.

Li, L.Y., Deng, Z.W., Fu, H.Z., Li, J., Proksch, P., Lin, W.H., 2003. Chemical constituents from the marine sponge *Iotrochoto birotulata*. Pharmazie 58, 680–681.

Lin, Y.L., Kuo, Y.H., Shiao, M.S., Chen, C.C., Ou, J.C., 2000. Flavonoid glycoside from *Terminalia catappa* L. Journal of the Chinese Chemical Society 47, 253–256.

Rasmussen, S., Wolff, C., Rudolph, H., 1995. Compartmentalization of phenolic constituents in *Sphagnum*. Phytochemistry 38, 35–39.

Rubanyi, G.M., 1993. The role of endothelium in cardiovascular homeostasis and diseases. Journal of Cardiovascular Pharmacology 22 (Suppl 4), S1–S14.

Shimokawa, H., Yasutake, H., Fujii, K., Owada, M.K., Nakaike, R., Fukumoto, Y., Takayanagi, T., Nagao, T., Egashira, K., Fujishima, M., Takeshita, A., 1996. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. Journal of Cardiovascular Pharmacology 28, 703–711.

Wang, G.J., Shan, J., Pang, P.K., Yang, M.C., Chou, C.J., Chen, C.F., 1996. The vasorelaxing action of rutaecarpine: direct paradoxical effects on intracellular calcium concentration of vascular smooth muscle and endothelial cells. The Journal of Pharmacology and Experimental Therapeutics 276, 1016–1021.

Wolf, G., 1997. Nitric oxide and nitric oxide synthase: biology, pathology, localization. Histology and Histopathology 12, 251–261.