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Chemical Constituents of the Leaves of *Glochidion obliquum* and Their Bioactivity

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A new flavonoid glycoside, globlin A (1), and eleven known compounds were isolated from methanolic extracts of the leaves of *Glochidion obliquum*. The structure of this new compound was established with a combination of 2D NMR techniques (COSY, NOESY, HMQC and HMBC) and HR-ESI-MS analyses. Chemical structures of the other known compounds were identified by comparison of their spectroscopic and physical data with those reported in the literature. Some of the isolates were examined for their bioactivities. Among the tested compounds, rotundic acid (4) displayed significant cytotoxicity and anti-inflammatory activities.

Key words: Flavonoid glycoside, Euphorbiaceae, Cytotoxicity, Anti-inflammatory, DPPH

INTRODUCTION

Glochidion is a relatively large genus of the Euphorbiaceae family, comprising approximately 300 species that are distributed from Madagascar to the Pacific Islands but mainly in tropical Asia. More than twenty species can be found in Vietnam, and most are commonly used as folk medicine in the treatment of influenza, dysentery, impaludism, rheumatoid arthritis, and dyspepsia (Delectis Florae Reipublicae Popularis Sinicae Edita, 1999; Nguyen, 2003). These species produce a wide variety of metabolites including sesquiterpenoids (Xiao et al., 2007, 2008), triterpenoids (Ganguly et al., 1966; Srivastava and Kulshreshtha, 1986, 1988; Puapairoj et al., 2005; Kiem et al., 2009), steroids (Hui et al., 1970), flavonoids (Otsuka et al., 2001), alkaloidal glycosides (Otsuka et al., 2004), and lignans (Takeda et al., 1998; Otsuka et al., 2000). In recent years, some lupane-type triterpenes from the *Glochidion* genus have been reported to inhibit tumor promotion and to be cytotoxic (Tanaka et al., 2004; Puapairoj et al., 2005). Therefore, in the continuing course of our integrated program aimed at new drug discovery, *G. obliquum* Decne. was selected as a target. In our investigation of the phytochemical diversity of this plant, the structure of a new flavonoid, globlin A (**1**), was established using a combination of spectroscopic methods including 1D- and 2D-NMR and HR-ESI-MS. We also identified eleven known compounds, including five triterpenoids, two phenolics, one flavonoid, two steroids, and one carbohydrate. We did this by comparison of their spectral and physical data with literature data. In the present study we report the structure of this new compound and the cytotoxicity and anti-inflammatory activities of all 12 isolates.

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MATERIALS AND METHODS

General

Melting points for the 12 isolates were determined using an Electrothermal IA-9200 melting point mea-

suring apparatus without correction. UV spectra were recorded on an Agilent UV-VIS recording spectrophotometer. The IR spectrum was obtained, as KBr discs, on a Hitachi 270-30 type spectrometer. Optical rotation was measured with a Jasco DIP-1000 KUY polarimeter. The electrospray ionization (ESI) mass spectrum was determined using an Agilent 1200 LC-MSD Trap spectrometer, and the HR-ESI-MS was completed with the aid of the Bruker APEX II mass spectrometer. ^{1}H- and ^{13}C-NMR, COSY, NOESY, HMQC, and HMBC spectra were recorded on the Bruker Avance-500 NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts are reported in parts per million (ppm, δ). Column chromatography (CC) was done on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, E. Merck). HPLC analysis of sugars was done on a Shimadzu LC-10ATVP series pumping system equipped with a Shimadzu SPD-6AV UV-Vis spectrophotometric detector at 210 nm, a Cosmosil packed column with 5C18-AR-II Waters type (4.6 × 250 mm, 5 mm), and a Rheodyne injector.

Plant materials

The leaves of *Glochidion obliquum* (Euphorbiaceae) were collected from Nghe An, Vietnam, during May 2008 and the plant materials were identified and authenticated by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnamese Academy of Science and Technology. A voucher specimen (Viet-TSWu-20080505) was deposited in the Herbarium of the Institute of Ecology and Biological Resources, Vietnamese Academy of Science and Technology, Hanoi, Vietnam.

Extraction and isolation

The leaves of *G. obliquum* (6.3 kg) were powdered and soaked with methanol (20 L × 3) at room temperature, and the combined extracts were concentrated under reduced pressure to give a deep brown syrup (304 g). The crude extract was suspended in water and partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, successively to afford *n*-hexane (11 g), ethyl acetate (129 g), *n*-butanol (143 g), and water (21 g) fractions, respectively. Compounds soluble in ethyl acetate were chromatographed over a silica gel column by gradient elution with *n*-hexane and increasing concentrations of acetone to afford eight fractions (1-8). Fraction 2 was further subjected to silica gel column chromatography. It was eluted with *n*-hexane/acetone (19:1) to yield β-sitosterol (**2**) (783 mg; Kuo and Yeh, 1997). Silica gel column chromatography was used for fraction 3 as well. It was eluted with a step gradient elution of *n*-hexane/acetone (15:1, 10:1, 7:1, and 5:1) and led to the isolation of taraxerol (**3**) (132 mg; Sakurai et al., 1987). Purification of fraction 5 by column chromatography with silica gel (eluted by *n*-hexane/acetone (9:1)) afforded rotundic acid (**4**) (12.90 g; Saimaru et al., 2007) and pedunculoside (**5**) (6.80 g; Wu et al., 2007).

The *n*-butanol soluble residues (143 g) were subjected to silica gel column chromatography which was done using step gradient elution with chloroform and increasing concentrations of methanol (0%, 5%, 10%, 20%, 30%, 50%, 70%, 90%, and 100%) to afford nine fractions (1-9). Silica gel column chromatography of fraction 2 with the eluant mixtures of chloroform-methanol (19:1) and step gradient with methanol afforded euphorginol (**6**) (92 mg; Rasool et al., 1989), 2, 3, 24-trihydroxyurs-12-en-28-oic acid (**7**) (324 mg; Jung et al., 2004), and syringin (**8**) (71 mg; Kiem et al., 2003). Separation of fraction 3 by silica gel column chromatography (eluted with chloroform-methanol (15:1)) yielded globlin A (**1**) (987 mg), bergenin (**9**) (145 mg; Taneyama et al., 1983), and vitexin (**10**) (46 mg; Lin et al., 2000), successively. Fraction 6 was subjected to column chromatography with silica gel eluted with chloroform-methanol-water (9:1:0.05) and further purified by repeated silica gel column chromatography to yield *myo*-inositol (**11**) (57 mg; Breitmaier and Voelter, 1986) and β-sitosteryl-3-O-β-D-glucopyranoside (**12**) (268 mg; Kuo and Yeh, 1997), respectively.

Globlin A (1)

Colorless powder (MeOH); m.p. 244-246°C; $[\alpha]_D^{30}$ –69 (c 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 330 (2.50), 276 (3.20), 216 (3.50) nm; IR (KBr) ν_{max} 3406, 2927, 2360, 1687, 1660, 1605, 1556, 1511, 1497, 1366, 1304, 1248, 1209, 1186, 1067, 1025 cm^{-1}; ^{1}H-NMR (Pyridine-d_5, 500 MHz): δ 7.88 (2H, d, J = 9.0 Hz, H-2' and -6'), 7.06 (2H, d, J = 9.0 Hz, H-3' and -5'), 6.78 (1H, s, H-8), 6.76 (1H, s, H-3), 5.58 (1H, d, J = 7.5 Hz, H-1''), 5.23 (1H, br s, H-1'''), 4.37 (1H, br d, J = 11.0 Hz, H-6''), 4.24 (1H, dd, J = 3.3, 1.5 Hz, H-2'''), 4.22-4.15 (3H, m, H-2'', -4'', and -5''), 4.05-4.01 (2H, m, H-3''' and -5'''), 3.99 (3H, s, OCH_3-7), 3.97 (1H, br d, J = 11.0 Hz, H-6''), 3.93 (1H, m, H-4'''), 3.91 (1H, d, J = 9.5 Hz, H-3''), 3.78 (3H, s, OCH_3-4'), 1.45 (3H, d, J = 6.0 Hz, CH_3-6'''); ^{13}C-NMR (Pyridine-d_5, 125 MHz): δ 182.9 (C-4), 164.4 (C-2), 163.1 (C-4'), 159.7 (C-7), 153.9 (C-9), 152.9 (C-5), 129.8 (C-6), 128.6 (C-2' and -6'), 123.1 (C-1'), 115.0 (C-3' and -5'), 106.2 (C-10), 104.5 (C-3), 104.4 (C-1''), 101.8 (C-1'''), 92.1 (C-8), 77.9 (C-4''), 77.4 (C-4'''), 75.4 (C-2''), 73.6 (C-3'''), 72.2 (C-5''), 71.7 (C-2'''), 71.4 (C-3''), 69.3 (C-5'''), 67.8 (C-6''), 56.9 (OCH_3-7), 55.6 (OCH_3-4'),

18.1 (C-6'''); ESI-MS *m/z* (%) 645 $[M+Na]^+$ (100); HR-ESI-MS *m/z* 645.1791 $[M+Na]^+$ (Calcd for $C_{29}H_{34}O_{15}Na$, 645.1795).

Acid hydrolysis of flavonoid glycosides

Compound **1** (10.0 mg) was refluxed at 100°C for 1 h with 2N HCl (5 mL). The acid hydrolysate was extracted with ethyl acetate and evaporated to dryness to yield a colorless amorphous solid, which, on recrystallization from methanol, afforded 2.5 mg of 5,6-dihydroxy-7,4'-dimethoxyflavone (Horie et al., 1997). This compound was identified by mp, UV, IR, ^{1}H-, ^{13}C-NMR and mass spectral analysis. The sugars in the aqueous layer were identified as D-glucose and D-rhamnose, respectively, using HPLC analysis, by comparison of their retention times and optical rotations with those of authentic samples as described in Shin et al. (2003).

In vitro cytotoxicity assay

All stock cultures of cells were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 1500-7500 cells per well with compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were fixed with cold 50% trichloroacetic acid and then stained with 0.4% sulforhodamine B (SRB). The absorbance at 562 nm was measured using a microplate reader after solubilizing the bound dye. The mean EC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant. Four human cancer cell lines, Daoy, Hep2, MCF-7, or HeLa, were used in the assay. Mitomycin C (5 μM, final concentration) and DMSO (0.3%, final concentration) were used as positive and vehicle controls. Results were expressed as percent of cell growth for the DMSO control (Scudiero et al., 1988).

Microglial cell culture and measurements of nitric oxide (NO)

The murine microglial cell lines (BV2) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (HyClone). Production of NO was measured by the Griess reagent as in a previous report (Wang et al., 2006). NO was measured by the accumulation of nitrite in the culture medium in the presence of test drug (0.1-50 μM) 24 h after stimulation with 0.5 μg/mL LPS (Sigma-Aldrich).

Measurement of NADPH oxidase (NOX) activity

NADPH oxidase activity was measured as described previously (Wang et al., 2006). BV2 cells (2×10^7 cells) were suspended in lysis buffer [20 mM potassium phosphate (pH 7.0), 1 mM ethylene-bis(oxyethylenenitrilo)-tetraacetic acid (EGTA), and a protease inhibitor cocktail (Roche Applied Science)]. The cell suspension was sonicated using five 10-s bursts at 30% power followed by cooling on ice for 30 sec between each sonication. The homogenate was centrifuged twice at 800 × g for 15 min to remove unbroken cells. Protein concentrations were determined using the Bradford reagent (Bio-Rad). Fifty micrograms of cell homogenate were added to the wells of a bioluminescence plate. NADPH oxidase activity was measured in a 50 mM phosphate buffer containing 1 mM EGTA, 150 mM sucrose, and 10 μM lucigenin. Various drugs were tested including diphenyleneiodonium (DPI, an NADPH oxidase inhibitor) and 0.1-50 μM of the test drugs. These drugs were added to the wells of a bioluminescence plate and incubated for 20 min at 37°C in the dark. O_2 production was stimulated with 200 μM NADPH (Sigma-Aldrich), and chemiluminescence was monitored for 30 min, after which the area under the curve (AUC) was calculated to represent ROS production by NADPH oxidase (NOX activity) and expressed as relative light units (RLU)/50 μg protein.

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity

Drugs were diluted with MeOH to yield a range of concentrations (0.1-50 μM). DPPH (Sigma-Aldrich) solution (200 μL, final concentration: 200 μM in MeOH) was added to 10 μL of each diluted sample in a 96-well microplate, and the resulting solution was allowed to react for 30 min in the dark at ambient temperature. The absorbance at 517 nm was determined by a microplate-spectrophotometer. Trolox (OXIS) was included as a reference (Lin et al., 2006).

RESULTS AND DISCUSSION

Air-dried, powdered leaves of *G. obliquum* were extracted with methanol at room temperature and concentrated to give a dark brown syrup. The methanol extract was suspended in water and partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, successively to afford *n*-hexane, ethyl acetate, *n*-butanol, and water soluble fractions, respectively. Further purification by a combination of conventional chromatographic techniques yielded a new compound, globlin A (**1**). Eleven known compounds were also identified from the ethyl acetate and *n*-butanol extracts including β-sitosterol (**2**), taraxerol (**3**), rotundic acid (**4**), pedunculoside (**5**), euphorginol (**6**), 2, 3, 24-trihydroxyurs-12-en-28-oic acid

(**7**), syringin (**8**), bergenin (**9**), vitexin (**10**), *myo*-inositol (**11**), and β-sitosteryl-3-O-β-D-glucopyranoside (**12**). The structure of this new flavonoid glycoside, globlin A (**1**), was established on the basis of 1D and 2D NMR and mass spectroscopic analyses.

Compound **1** was isolated as an optically active colorless powder with an m.p. 244-246°C. The HR-ESI-MS of **1** displayed a sodium adduct ion peak at *m/z* 645.1791 $[M+Na]^+$, corresponding to the pseudomolecular formula of $C_{29}H_{34}O_{15}Na$. The UV absorption maxima at 330, 276, and 216 nm were characteristic of a flavone skeleton (Mabry et al., 1970). The IR absorption bands at 3406, 1687, and 1660 cm^{-1} showed the presence of a hydroxyl moiety, a carbon-carbon double bond, and hydrogen-bonded carbonyl groups, respectively. In the ^{1}H-NMR spectrum, a typical set of A_2B_2 signals at δ 7.88 (2H, d, J = 9.0 Hz) and 7.06 (2H, d, J = 9.0 Hz) were attributed to H-2', -6' and H-3', -5' of the *para*-substituted B-ring. Two singlets at δ 6.78 (1H, s) and 6.76 (1H, s) were assumed to be H-8 and H-3 since they displayed ^2J,^3J-HMBC correlations with the carbon signals at δ 159.7 (C-7), 153.9 (C-9), 129.8 (C-6), 106.2 (C-10); and δ 182.9 (C-4), 164.4 (C-2), 123.1 (C-1'), respectively. Two anomeric proton signals at d 5.58 (d, J=7.5 Hz) and 5.24 (br s) suggested the presence of two sugar units. In addition, there were oxygenated methine and methylene protons at δ 4.37 (1H, br d, J=11.0 Hz), 4.24 (1H, dd, J=3.3, 1.5 Hz), 4.22-4.15 (3H, m), 4.05-4.01 (2H, m), 3.97 (1H, br d, J =11.0 Hz), 3.93 (1H, m), and 3.91 (1H, d, J=9.5 Hz), which were identified as the proton signals of the sugar moieties. Another upfield methyl doublet at δ 1.45 (3H, d, J=6.0 Hz) was the characteristic signal for the rhamnose fragment. Moreover, two methoxy groups at d 3.99 (s, 3H) and 3.78 (s, 3H) were assigned to be located at positions C-7 and C-4', respectively, since they exhibited NOE crosspeaks with the H-8 (d 3.99) and H-3' (δ 7.06) in the NOESY experimental measurement. With the aid of ^{13}C-NMR and DEPT spectral analytical techniques, twelve carbon signals were identified, including one methyl (δ 18.1), one oxymethylene (δ 67.8), eight oxymethine (δ 77.9, 77.4, 75.4, 73.6, 72.2, 71.7, 71.4, 69.3), and two anomeric carbons (δ 104.4 and 101.8). They were identified as components of D-glucose (δ 104.4, 77.9, 75.4, 72.2, 71.4, and 67.8) (Watanabe et al., 2003) and D-rhamnose (δ 101.8, 77.4, 73.6, 71.7, 69.3, and 18.1) (Burger et al., 1998) by comparison with literature values and the assistance of comprehensive COSY, NOESY, HMQC, and HMBC spectroscopic examinations. The sugar fraction of acid hydrolysates of compound **1** was further analyzed with HPLC by comparing their retention times and optical rotations with those of authentic samples as described in Shin et al. (2003); the sugar moiety was confirmed as mentioned. In the HMBC spectrum, long range correlations from H-1" (δ 5.58) to C-6 (δ 129.8); and H-1'" (δ 5.13) to C-2'" (δ 71.7), C-5'" (δ 69.3), and C-6" (δ 67.8), established that the sugar moiety, D-rhamnosyl-(1 → 6)-D-glucose, was attached at C-6 through the C–O linkage. The stereochemistry of the sugar moiety was established with reference to the relative coupling constants of protons, and the configurations of glucose and rhamnose were determined as β and α, respectively, according to the coupling constants of the corresponding anomeric protons H-1" (7.5 Hz) and H-1'" (~0 Hz). In addition, the methoxy proton signals at δ 3.99 (OCH_3-7) and 3.78 (OCH_3-4') displayed ^3J-HMBC correlations with the carbon signals at δ 159.7 (C-7) and 163.1 (C-4'), respectively, which indicated the substitution of two methoxy

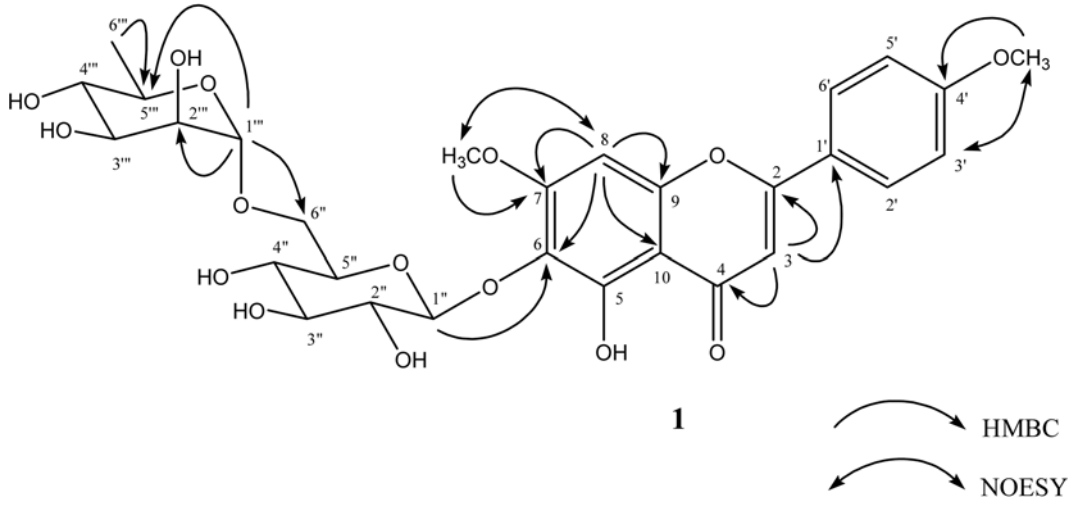

Fig. 1. Structure and significant NOESY and HMBC correlations of globlin (**1**).

Table I. EC_{50} values of tested compounds against human tumor cell lines

Tested compounds	ED_{50} (μM)			
	Daoy	Hep-2	MCF-7	HeLa
1	(–)	(–)	(–)	(–)
3	(–)	(–)	(–)	(–)
4	24.67 ± 0.43	29.22 ± 1.00	29.43 ± 1.13	(–)
5	(–)	(–)	(–)	(–)
6	(–)	(–)	(–)	(–)
8	(–)	(–)	(–)	(–)
9	(–)	(–)	(–)	(–)
10	(–)	(–)	(–)	(–)
Mitomycin *C*	0.30 ± 0.03	0.30 ± 0.003	0.21 ± 0.03	0.45 ± 0.03

(–): ED_{50} > 40, μM

Table II. IC_{50} values (μM) of tested compounds on nitric oxide synthase (NOS) and NADPH oxidase (NOX) activities in murine microglial cells

Compounds	NOS	NOX
1	80.1± 1.6	N.A.
3	60.6±12.7	52.2
4	39.5± 9.3	80.9
5	78.7± 1.5	N.A.
6	60.5± 7.1	N.A.
8	78.9±18.1	N.A.
9	98.4±10.0	N.A.
10	65.7± 2.9	N.A.
Reference	18.9± 3.2 (L-NAME)	0.4±0.2 (DPI)

N.A.: not effective.

groups attached at C-7 and -4'. The successive NOESY and HMBC experiments completed the assignments of all the proton and carbon signals of **1** and therefore its chemical structure was established as 5,6-dihydroxy-7,4'-dimethoxyflavone 6-O-α-D-rhamnosyl-(1 → 6)-β-D-glucose (Fig. 1) and given the trivial name of globlin A.

Among these isolated compounds, **1**, **3-6**, and **8-10** were examined for cytotoxicity against tumor cell lines Daoy (human medulloblastoma), Hep-2 (human laryngeal carcinoma), MCF-7 (human breast adenocarcinoma), and HeLa (human cervical epitheloid carcinoma), as described previously (Scudiero et al., 1988). The EC_{50} values are summarized in Table I. Only compound **4** exhibited moderate cytotoxicity with EC_{50} values of 24.67±0.43, 29.22±1.00, and 29.43±1.13 μM against the Daoy, Hep-2, and MCF-7 tumor cell lines, respectively. The anti-inflammatory potentials of compounds **1**, **3-6**, and **8-10** were evaluated by examining their effects on LPS-induced *i*NOS-dependent NO production and NOX-dependent ROS production in microglial cells. Inhibition of NOX and NOS were measured by ROS and NO production, respectively, in the presence of 1-100 μM of drugs. DPI and L-NAME were included as positive controls. Data were calculated as 50% inhibitory concentrations (IC_{50}s) and expressed as the mean ±S.E.M. from 3~6 experiments performed on different days using cells from different passages. All tested compounds had IC_{50} values in the range of 39.5-98.4 μM, which were not quite as potent as L-NAME (IC_{50} 18.9 μM), a non specific NOS inhibitor that inhibits LPS-induced NO production. These data are shown in Table II. NOX is the major ROS-producing enzyme in activated inflammatory cells (Van den Worm et al., 2001). Previous reports indicated that compounds with anti-inflammatory activity are also potent inhibitors of NOX activity (Liou et al., 2003; Lin et al., 2006). Therefore, the effects of these isolates on NOX activity in lysates of microglial cells were determined. Compounds **3** and **4** showed weaker inhibition of NOX activity (IC_{50} values of 52.2 and 80.9 μM, respectively) than the specific NOX inhibitor DPI (IC_{50} 0.4 μM) (Table II). This suggests that NOX might not be the direct target of these compounds. In addition, the free radical-scavenging capacities of these com pounds were examined in a cell-free DPPH solution. Trolox, a vitamin E analogue included as a positive control, displayed a stronger free radical-scavenging effect than any of the compounds examined (data not shown). Thus, none of these compounds showed considerable free radical-scavenging activity.

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