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Benzyl Glycosides from the Aerial Parts of *Gynostemma laxum* and Their NF-κB Inhibitory Activity in HepG2 Cells

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Nuclear transcription factor-κB (NF-κB) represents a family of Rel domain-containing proteins including RelA, RelB, c-Rel, NF-κB1, and NF-κB2. The activation of NF-κB has been linked to multiple pathophysiological conditions such as cancer, arthritis, asthma, inflammatory bowel disease, and other inflammatory conditions.[1] NF-κB can be activated by various stimuli, such as microbial and viral products, cytokines, DNA damage and noxious chemicals. The induction of a number of pro-inflammatory mediators occurs as the results of increased inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) activities.[2] Therefore, suppression of iNOS and COX-2 activities can be an important approach to preventing inflammation in organs. NF-κB and the signaling pathways that regulate many physiological processes, including the innate- and adaptive-immune responses, cell death, and inflammation have become a focal point for intense drug discovery and development efforts.[3] Indeed, growing evidences has validated NF-κB as a target for anti-inflammatory and anticancer agents.

Gynostemma genus (Cucurbitaceae) is a climbing vine, attaching itself to supports using tendrils. Over 30 species of *Gynostemma* are known to grow throughout China, mostly in the South-west. In Vietnam, there are only two species of the *Gynostemma* genus recorded to date, *Gynostemma pentaphyllum* (Thunb.) Makino and *Gynostemma laxum* (Wall.) Cogn. Of these, *G. laxum* is distributed throughout Vietnam from the plains to mountainous areas. The aerial parts of *G. laxum* have been used as a bitter stomachic, a laxative, anti-inflammatory as well as anti-obesity. Up to date, no report has been reported about chemical components from this plant. In our screening project for anti-inflammatory agents from natural sources, we found *G. laxum* to possess anti-inflammatory effects. As a part of our continuing research to elucidate the anti-inflammatory compounds, we report herein the isolation, structural elucidation, and evaluation of the inhibitory effects on NF-κB activation and iNOS and COX-2 expression of a new benzyl glycoside, 2,4-dihydroxybenzyl-*O*-α-L-rhamnopyranoside (**1**), along with nine known compounds from *G. laxum*.

The dried aerial parts of *G. laxum* were extracted with MeOH and partitioned with *n*-hexane and ethyl acetate, and water. From these fractions and subsequently separation, one new and nine known compounds were isolated (see Figure 1).

Compound **1** was obtained as a white amorphous powder and its molecular formula, $C_{13}H_{18}O_7$, determined on the basis of ESI MS at *m/z* 287 $[M+H]^+$ (positive) and HR ESI MS at *m/z* 287.1147 $[M+H]^+$ (calcd $C_{13}H_{19}O_7$ for 287.1131). The ^{1}H-NMR spectrum of **1** (DMSO-d_6) (Table 1) showed the following signals: one secondary methyl group at δ_H 1.03 (d, J = 6.0 Hz), assigned to H-6 of the rhamnose; one anomeric proton at δ_H 4.72 (br s); one oxygenated methylene (δ_H 4.42 and 4.50); and one 1,2,4-trisubstituted aromatic ring with ABX coupling patterns [δ_H 7.20 (d, J = 8.0 Hz), 7.25 (d, J = 8.0 Hz), and 7.96 (s)]. The ^{13}C-NMR and DEPT spectra (Table 1) revealed 13 carbon signals, of which, 7 signals were assigned to a benzyl moiety and 6 signals belonged to rhamnose moiety. The ^{1}H- and ^{13}C-NMR data of **1** (Table 1) were similar to those of benzyl-*O*-α-L-rhamnopyranoside except for an appearance of two hydroxyl groups at aromatic ring.[4,5] In the HMBC spectrum (see Figure 2), two proton signals at δ_H 4.45 and 4.50 of oxygenated methylene group correlated with carbons C-1 (δ_C 126.3), C-2 (δ_C 148.1), and C-6 (δ_C 126.4); one proton signal at δ_H 7.25 (H-6) correlated with carbons C-2 (δ_C 148.1), C-4 (δ_C 154.0), and C-7 (δ_C

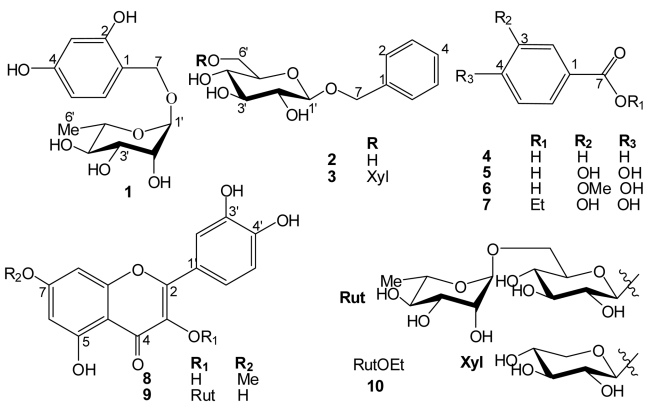

Figure 1. Structures of isolated compounds **1-10** from *Gynostemma laxum*.

Table 1. ^{1}H and ^{13}C NMR data of compound **1**

Pos.	δ_C[a,c]	δ_H[a,d] (*J* in Hz)
Aglycone		
1	126.3	-
2	148.1	-
3	137.7	7.92 (1H, s)
4	154.0	-
5	126.9	7.20 (1H, d, 8.0)
6	126.4	7.25 (1H, d, 8.0)
7	70.8	4.42 (1H, d, 12.5) 4.50 (1H, d, 12.5)
Rha		
1'	101.6	4.72 (1H, br s)
2'	71.7	3.78 (1H, d, 3.0)
3'	71.8	3.56 (1H, dd, 3.0, 9.5)
4'	73.5	2.24 (1H, t, 9.5)
5'	70.3	3.43 (1H, m)
6'	18.2	1.03 (3H, d, 6.0)

[a]Measured in DMSO-d_6. [b]125 MHz. [c]500 MHz, Assignments were done by HMQC and HMBC experiments; Rha, L-rhamnopyranosyl.

70.8) confirmed that one oxygenated methylene and two hydroxyl groups were at C-1, C-2, and C-4, respectively. On the other hand, acid hydrolysis of **1** (see Experimental) proved the presence of L-rhamnose (identified as TMS derivative). Furthermore, the L-rhamnose moiety at C-7 of benzyl was confirmed by the HMBC cross peaks from the H-1 (δ_H 4.72) to C-7 (δ_C 70.8) and contrary from H-7 (δ_H 4.42 and 4.50) to C-1 (δ_C 101.6). Consequently, the structure of **1** was determined as 2,4-dihydroxybenzyl-*O*-α-L-rhamnopyranoside.

The remain compounds were identified as benzyl-*O*-β-D-glucopyranoside (**2**),[6] benzyl β-D-xylopyranosyl-(1→6)-*O*-β-D-glucopyranoside (**3**),[7] benzoic acid (**4**), 3,4-dihydroxybenzoic acid (**5**),[8] vanillic acid (**6**),[9] 3,4-dihydroxybenzoate ethyl (**7**),[10] quercetin (**8**),[11] rhamnetin 3-*O*-rutinoside, (**9**),[12] and ethyl-β-rutinoside (**10**)[13] (see Figure 1). Their structures were established on the basis of spectral and chemical evidence, which were in agreement with those of reported in literature. All these compounds were initially isolated from *G. laxum.*

The pharmacological properties of local food plants are an attractive area for investigation. Accumulating evidence suggests that many locally consumed foods have functional properties, notably anti-inflammatory activity.[14] To investigate cellular toxicity of the isolated compounds **1-10**, they

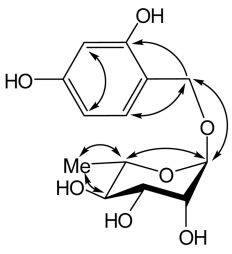

Figure 2. Important HMBC correlations of compound **1**.

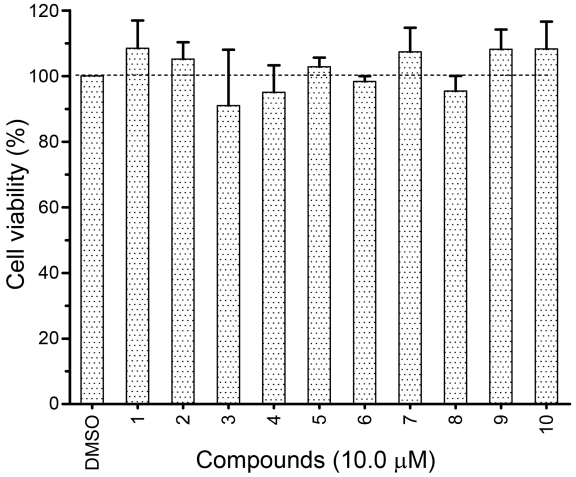

Figure 3. Effects of compounds **1-10** on cell viability using MTS assay in HepG2 cells. All values are means ± S.D ($n = 3$) *vs.* control.

were applied at various concentrations to HepG2 cells for 24 h after which cell viability was measured in an MTS assay as described in Material and Methods. None of the compounds displayed cytotoxicity at the concentration of 10.0 μM (see Figure 3). They were therefore used in subsequent experiments at below concentration of 10.0 μM. Compounds were examined inhibitory effects on NF-κB transcriptional activation in HepG2 cells. Cells were treated with compounds at various concentrations (0.1, 1.0, and 10.0 μM) prior to stimulation with TNFα (10 ng/mL). The data showed that treatment with compounds **1**, **9**, and **10** reduced TNFα-mediated NF-κB activation (see Figure 4). The remains of compounds showed the inactivity. Activation of NF-κB and

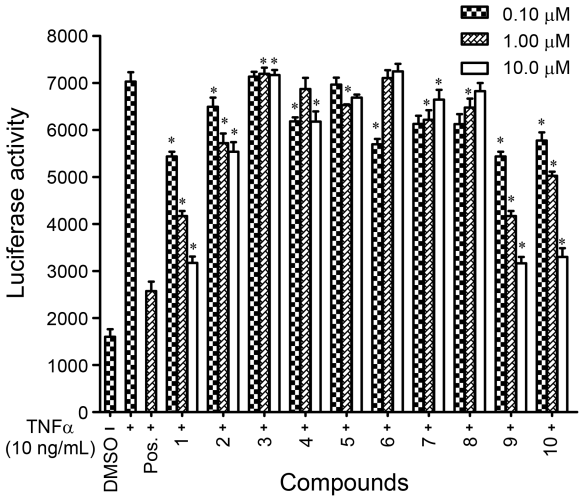

Figure 4. Effects of compounds **1-10** on the TNFα-induced NF-κB activation in HepG2 cells. HepG2 cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with vehicle (DMSO) or one of the compounds, prior to 1 h of treatment with TNFα (10 ng/mL). Unstimulated HepG2 cells acted as a negative control. Cells were then harvested and luciferase activities were assessed. Results are expressed as relative luciferase activity. Sulfasalazine was used as a positive control. All values represent the mean ± S.D ($n = 3$). $P < 0.05$ *vs.* control.

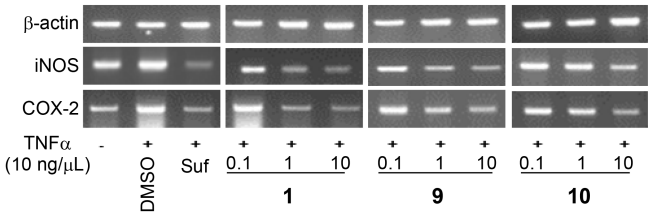

Figure 5. Inhibitory effects of compounds **1**, **9**, and **10** on the TNFα-induced expression of iNOS and COX-2 mRNAs in HepG2 cells. HepG2 cells were pretreated with one of the listed compounds for 1 h and then treated with TNFα (10 ng/mL) for 6 h. Total mRNAs were prepared from the cell pellets using TRIzol. Relative levels of mRNAs were measured by RT-PCR.

subsequent transcriptional induction of pro-inflammatory mediators play a critical role in inflammation.[15] Therefore, we assessed the ability of three compounds (**1**, **9**, and **10**) that significantly inhibited TNFα-mediated NF-κB activation to modulate iNOS and COX-2 gene expressions. The results indicated that compound **1** dramatically and compounds **9** and **10** slightly reduced TNFα-induced iNOS and COX-2 gene expression in a dose dependent manner (see Figure 5).

In conclusion, from the aerial parts of *G. laxum*, a new compound, 2,4-dihydroxybenzyl-*O*-α-L-rhamnopyranoside (**1**), and nine known compounds were isolated. Compounds **1**, **9**, and **10** showed significant NF-κB inhibitory activation stimulated by TNFα in a dose-dependent manner with IC_{50} values of 7.6 ± 0.4, 9.2 ± 0.3, and 9.3 ± 0.3 μM, respectively. Furthermore, compound **1** dramatically and compounds **9** and **10** slightly reduced TNFα-induced iNOS and COX-2 gene expression in a dose dependent manner. These results suggested compound **1** could be an anti-inflammatory agent.

Experimental

General Procedures. Optical rotations were determined on a Jasco DIP-370 automatic polarimeter. Preparative HPLC was carried out using a Waters HPLC system (600 pump, 600 controller, and a 996 photodiode array detector). The NMR spectra were recorded using a Bruker DRX 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz). The ESI-MS were obtained from an AGILENT 1200 SERIES LC-MSD Trap spectrometer. GC spectra were recorded on a Shimadzu-2010 spectrometer. Column chromatography was performed using a silica gel 60 (70-230 mesh or 230-400 mesh, Merck KGaA, 64271 Darmstadt, Germany) or YMC RP-18 resins (30-50 μm, Fujisilisa Chemical Ltd. Kasugai, Aichi, Japan), and thin layer chromatography (TLC) using a pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

Plant Material. The aerial parts of *G. laxum* were collected in Da Bac, Hoa Binh province, Vietnam in June, 2010, and identified by one of the authors, Prof. Dr. Pham Thanh Ky. A voucher specimen (HUP001) was deposited at the Herbarium of Hanoi University of Pharmacy.

Extraction and Isolation. The dried aerial parts of *G. laxum* (1 kg) were extracted with MeOH three times at room temperature to yield 90 g of a dark solid extract, which was then suspended in water and successively partitioned with *n*-hexane and ethyl acetate (EtOAc) to obtain *n*-hexane (GL1, 20.0 g), EtOAc (GL2, 18.0 g), and water (GL3, 50.0 g) extracts after removal solvent *in vacuo*. GL2 (18.0 g) was chromatographed on a silica gel column and eluted with $CHCl_3$-MeOH gradient (50:1 → 1:1, v/v) to obtain four sub-fractions, GL2A (4.0 g), GL2B (3.5 g), GL2C (7.4 g), and GL2D (3.1 g). Sub-fraction GL2A was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (15:1, v/v) to yield **8** (8.0 mg). Sub-fraction GL2C was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (6:1, v/v) to give three smaller fractions, GL2C1 (1.8 g), GL2C2 (2.5 g), and GL2C3 (3.1 g). Fraction GL2C1 was chromatographed on an YMC RP-18 column eluting with MeOH-water (5:1, v/v) to yield **4** (12.0 mg) and **7** (8.5 mg). Fraction GL2C2 was chromatoghraphed on a silica gel column and eluted with $CHCl_3$-MeOH-water (5:1:0.1, v/v/v) to yield **2** (30.0 mg) and **9** (15.0 mg). The water soluble fraction (GL3, 50.0 g) was chromatographed on a Diaion HP-20P column eluting with water containing increasing concentrations of MeOH (0%, 25%, 50%, 75%, and 100%) to obtain five sub-fractions GL3A (10.0 g), GL3B (8.1 g), GL3C (15.0 g), GL3D (6.6 g), and GL3E (5.3 g). The fraction GL3B (8.1 g) was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH-water (3:1:0.15, v/v/v) to yield **3** (5.0 mg) and **10** (8.3 mg). Sub-fraction GL3C was chromatographed on a silica gel column eluting with CH_2Cl_2-MeOH-water (4:1:0.1, v/v/v) to give three smaller fractions, GL3C1~GL3C3. Fraction GL3C1 was chromatographed on an YMC RP-18 column eluting with acetone-water (1:3, v/v) to yield **5** (4.3 mg) and **6** (6.0 mg). Fraction GL3C3 was chromatographed on a silica gel column using CH_2Cl_2-MeOH-water (3:1:0.17, v/v/v) as an eluent to obtain **1** (6.5 mg).

2,4-Dihydroxybenzyl-O-α-L-rhamnopyranoside (1): A white amorphous powder, $[\alpha]_D^{25}$: -58^0 (c = 0.5, MeOH), positive ESI-MS: *m/z* 287 $[M + H]^+$, $C_{13}H_{18}O_7$, HR ESI MS found *m/z* 287.1147 $[M+H]^+$ (Calcd $C_{13}H_{19}O_7$ for 287.1131), ^{1}H- and ^{13}C-NMR: see Table 1.

Acid Hydrolysis of 1. Compound **1** (2.0 mg) was dissolved in 1.0 N HCl (dioxane/H_2O, 1:1, v/v, 1.0 mL) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 mL of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with n-hexane and H_2O (0.1 mL, each), and the organic layer was analyzed by gas liquid chromatography (GC): Column: column SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (2.0 mL/min). The

retention time of persilylated rhamnose was found to be 4.50 min, when compared with the standard solution prepared by the same reaction from the standard rhamnose. The retention times of persilylated L-rhamnose was 4.50 min.

Cell Culture and Reagents. Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 10 μg/mL streptomycin at 37 °C and 5% CO_2. Human TNFα was purchased from ATgen (Seoul, Korea). Cells were counted with a hemocytometer and the number of viable cells was determined through trypan blue dye exclusion.

Cytotoxicity Assay. An MTS assay (Celltiter 96-Aqueous One Solution Assay; Promega, Madison, WI) was performed to analyze the effect of the different compounds on cell viability. Cells were cultured overnight in 96-well plates (1×10^4 cells/well). Cell viability was assessed after the incubation with the compounds at a concentration of 10.0 μM for 24 h. Number of viable cells was determined by measuring the A_{490nm} of the dissolved formazan product after addition of MTS for 30 min as described by the manufacturer. All values are means ± S.D (n-3) *vs*. control.

Luciferase Assay. Cells were seeded at 1.5×10^5 cells/well in a 12-well plate and grown for 24 h. All cells were transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Luciferase activity was assayed using an LB 953 Autolumat (EG&G Berthold, Nashua, NH) as described previously.[16] NF-κB-Luc and PPRE-Luc plasmids were kindly provided by Dr. Kyoon E. Kim (Chungnam National University, Daejeon, Korea). All experiments were performed in triplicate. HepG2 cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with vehicle (DMSO) or one of the compounds, prior to 1 h of treatment with TNFα (10 ng/mL). Unstimulated HepG2 cells acted as a negative control. Cells were then harvested and luciferase activities were assessed. Results are expressed as relative luciferase activity. Sulfasalazine was used as a positive control. All values represent the mean ± S.D ($n = 3$). $P < 0.05$ *vs.* control.

Reverse Transcription-polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cells using easy-BLUE (Intron biotechnology, Seoul, Korea). Approximately 2 μg of total RNA were reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega) for 1 h at 42 °C. The resulting cDNA was PCR-amplified using Taq polymerase pre-mixture (TaKaRa, Japan). PCR products were subjected to electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5'-TCATCCGCTATGCTGGCTAC-3', iNOS antisense 5'-CTCAGGGTCACGGCCATTG-3', COX-2 sense 5'-GCCCAGCACTTCACGCATCAG-3', COX-2 antisense 5'-GACCAGGCACCAGACCAAAGACC-3', GAPDH sense 5'-TGTTGCCATCAATGACCCCTT-3', and GAPDH antisense 5'-CTCCACGACGTACTCAGCG-3'. The specificity of the products generated using each set of primers was examined using gel electrophoresis and further confirmed using a melting curve analysis.

Effects of compounds **1**, **9**, and **10** on COX-2 and iNOS mRNA expression in HepG2 cells were assessed. HepG2 cells were pretreated with one of the listed compounds for 1 h and then treated with TNFα (10 ng/mL) for 6 h. Total mRNAs were prepared from the cell pellets using TRIzol. Relative levels of mRNAs were measured by RT-PCR.

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