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Anti-inflammatory and PPAR Transactivational Effects of Components from *Ginkgo biloba* Seeds

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Ginkgo biloba, one of the oldest species of trees, has existed on earth for 200 million years. The *G. biloba* leaves and seeds have been used in traditional medicine and food for thousands of years. Although numerous pharmacological effects and chemical components of *G. biloba* leaves extracts have been reported, few studies have been conducted on *G. biloba* seeds. In traditional food and medicine, *G. biloba* seeds have been used as supplementary materials for desserts, glazed fruits and beverages.[1] Some studies on the proteins of *G. biloba* seeds showed that the protein content of dry and defatted *G. biloba* seed powder was about 7%, and a number of proteins with antifungal and antioxidant activities were isolated.[2-5] As part of our search for bioactive components from this valuable medicinal plant,[6] together with the methanol extract of *G. biloba* seeds was found to be active on NF-κB and PPARs transactivational effects during the preliminary screening process, the present study describes the isolation and structural elucidation of 13 compounds from the methanol extract of *G. biloba* seeds. The effects of compounds **1-13** on TNFα-induced NF-κB transcriptional activity in human hepatocarcinoma (HepG2) cells were evaluated using an NF-κB-luciferase assay. To confirm the inhibitory effects of the compounds on NF-κB transcriptional activity, the effects of the compounds on the upregulation of the pro-inflammatory proteins iNOS and COX-2 were assessed in TNFα-stimulated HepG2 cells by RT-PCR. Futhermore, the effects of compounds **1-13** on the transcriptional activity of PPARs and individual PPAR subtypes in HepG2 cells were also evaluated using a PPRE-luciferase and GAL4-PPAR chimera assays.

The MeOH extract of *G. biloba* seeds (200 g) was suspended in H_2O and successively partitioned with *n*-hexane, CH_2Cl_2, and EtOAc to give four fractions. The aqueous fraction was subjected to multiple chromatographic steps over Diaion HP-20, silica gel, and reversed-phase C_{18}, yielding compounds **1-13**. By comparing the NMR and MS data with those reported in the literature, the structures of the isolated compounds were identified as: (2*E*,4*E*)-dihydrophaseic acid (**1**);[7] *rel*-5-(1*R*,5*S*-dimethyl-3*R*,4*R*,8*S*-trihydroxy-7-oxabicyclo-[3,2,1]-oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid (**2**);[8] 2-phenylethyl-β-D-glucoside (**3**);[9] (2*R*)-3-(4-hydroxy-3-methoxyphenyl)propan-1,2-diol (**4**);[10] coniferin (**5**);[11] *trans*-isoconiferin (**6**);[12] *trans*-ferulic acid 4-β-D-glucoside (**7**);[13] adenosine (**8**);[14] thymidine (**9**);[15] cedrusin (**10**);[16] uroligneoside (**11**);[17] ceplignan-4-*O*-β-D-glucoside (**12**);[18] and (+)-8-hydroxy-pinoresinol 4,4'-di-*O*-β-D-glucopyranoside (**13**).[19]

Compound **1** was isolated as colorless, viscous liquid. The molecular formula of **1** was determined to be $C_{15}H_{22}O_5$ based on its NMR data and ion peaks on high HRESIQTOFMS: $[M-H]^-$ at *m/z* 281.1387 (calcd. for $C_{15}H_{21}O_5$, 281.1389) and $[M+Cl]^-$ at 317.1157 (calcd. for $C_{15}H_{22}O_5Cl$, 317.1156). The ^{1}H NMR spectrum of **1** contained signals for three tertiary methyls at δ_H 1.93 (s, H_3-6), 1.11 (s, H_3-9'), and 0.89 (s, H_3-10'), and three signals of olefinic protons at δ_H 5.81 (1H, s, H-2), 7.69 (1H, d, J = 15.6 Hz, H-4), and 6.22 (1H, d, J = 15.6 Hz, H-5). The large coupling constant (J = 15.6 Hz) of the two olefinic protons indicated *trans*-geometry of the double bond. The ^{13}C NMR and DEPT spectra indicated 15 carbons, including three methyl, three methylene, four methine, and five quaternary carbons. The ^{13}C NMR spectrum of **1** showed the presence of a carboxyl carbon (δ_C 174.6, C-1), two double bonds [(δ_C 127.2, C-2), (δ_C 139.8, C-3), (δ_C132.1, C-4), and (δ_C 129.2, C-5)], two oxygenated quaternary carbons [(δ_C 86.8, C-5') and (δ_C 82.2, C-8')], one oxymethylene (δ_C 76.3, C-7'), one oxymethine (δ_C 65.1, C-3'), and two methylenes [(δ_C 43.5, C-2') and (δ_C 45.0, C-4')], implying that **1** is a megastigmane. The position of the oxymethylene group at C-7' was assigned from the HMBC correlation of H_2-7' to C-1, C-2', C-5', C-8', and C-1'. The positions of the two double bonds at C-2 and C-4 were assigned by HMBC correlations of H-2 to C-1, and C-6, H_3-6 to C-2, C-3, and C-4, and H-5 to C-3, C-4, and C-8'. In the NOESY spectrum, the NOE correlations between H-2/H-4 and H-6/H-5 indicated *E* geometry of the two double bonds at C-2 and C-4. The NOE correlations between H_3-10'/H-5 and H_3-9'/H-5 suggested α-orientation of the hydroxyl group at C-8' and β-orientation of H_3-10'. Moreover, the NOE correlations between H_3-10'/H-2'β, H-5/H-2'β, H-5/H-4'β, H-2'α/H-3', H-3'/H-4'α, and H-3'/H-7'β clearly indicated the α-orientation of H-3. Based on the above analysis and comparison of the NMR data of **1** with those of a similar compound isolated from the stem bark of *G. biloba*, (2*E*,4*E*,1'*R*,3'*S*,5'*R*,8'*S*)-dihydrophaseic acid 3'-*O*-β-D-glucopyranoside,[6] the structure of **1** was established as (2*E*,4*E*)-dihydrophaseic acid. Notably, this is

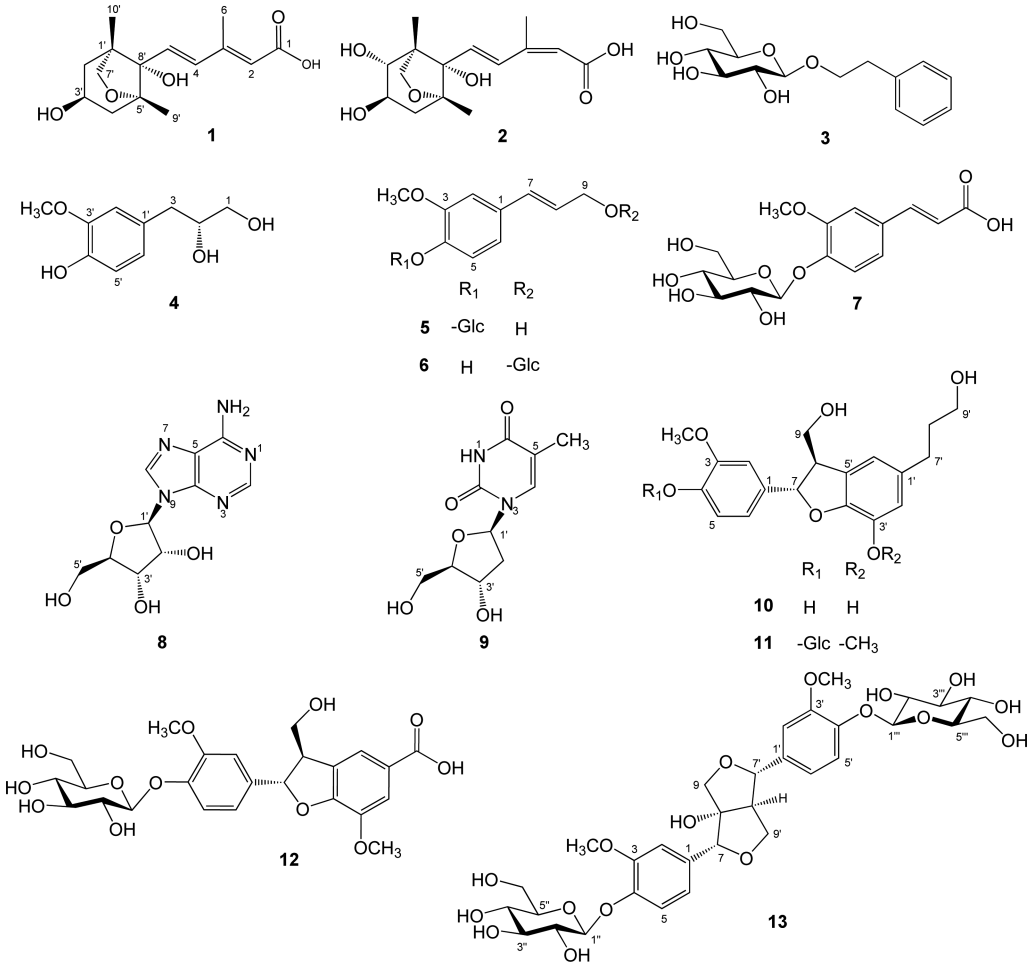

Figure 1. Structures of compounds **1-13**.

the first report of NMR data for (2*E*,4*E*)-dihydrophaseic acid.[7]

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay (Promega Celltiter 96-Aqueous One Solution Assay) as described in Supporting information. The result showed that compounds **1-13** had no significant cytotoxicity toward HepG2 cells at the concentration of 10 μM (data not shown).

The anti-inflammatory activity of compounds **1-13** was evaluated through the inhibition of a TNFα-induced NF-κB luciferase reporter and by the attenuation of TNFα-induced pro-inflammatory gene (iNOS and COX-2) expression in HepG2 cells. The result showed that compounds **9** and **12** significantly inhibited TNFα-induced NF-κB transcriptional activity, with IC_{50} values of 8.7 and 7.1 μM, respectively (Table 1). Consistent with their inhibitory effects on NF-κB activation, compounds **9** and **12** significantly inhibited the induction of COX-2 and iNOS mRNA in a dose-dependent manner (Figure 2), suggesting that the compounds reduced the transcription of these genes. Moreover, the housekeeping protein β-actin was unchanged by the presence of compounds **9** and **12** at the same concentrations.

The effects of compounds **1-13** on PPARs activation were evaluated using a PPRE-luciferase reporter assay. The result showed that compounds **4** and **13** significantly activated PPARs transcriptional activity, with EC_{50} values of 8.5 and

Table 1. Inhibitory effects of compounds **9** and **12** on the TNFα-induced NF-κB transcriptional activity

Compound	IC_{50} (μM)
9	8.7 ± 2.5
12	7.1 ± 1.8
Sufasalazine	0.9 ± 0.1

The values are mean ± SD (n = 3). Compounds **1-8**, **10**, **11** and **13** were inactive at tested concentrations.

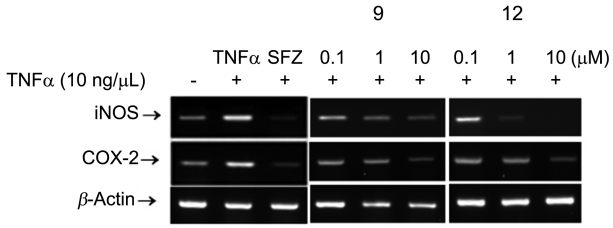

Figure 2. Effects of compounds **9** and **12** on iNOS and COX-2 mRNA expression in HepG2 cells.

Table 2. PPARs, α, γ, and $\beta(\delta)$ transactivational activities of compounds **1-13**

Compound	EC_{50} (μM)			
		Gal4-PPRAα-LBD	Gal4-PPRAγ-LBD	Gal4-PPRAβ(δ)-LBD
1	> 60*	> 60	n.d.	
2	22.5 ± 3.1	> 60	n.d.	n.d.
3	21.7 ± 2.5	> 60	> 60	> 60
4	8.5 ± 1.3	47.7 ± 2.1	> 60	n.d.
5	41.6 ± 4.1	> 60	n.d.	n.d.
6	22.9 ± 3.2	> 60	53.4 ± 2.6	> 60
7	26.8 ± 2.5	> 60	> 60	> 60
8	26.9 ± 3.6	n.d.	50.4 ± 1.9	> 60
9	28.3 ± 1.8	> 60	n.d.	52.8 ± 1.9
10	25.3 ± 3.9	> 60	> 60	> 60
11	n.d.	> 60	> 60	> 60
12	n.d.	53.3 ± 3.3	> 60	> 60
13	17.0 ± 2.7	n.d.	> 60	> 60
Bezafibrate	1.1 ± 0.3			
Ciprofibrate		0.9 ± 0.2		
Troglitazone			0.8 ± 0.1	
L-165.041				0.6 ± 0.07

EC_{50}: the concentration of tested compound that gave 50% of the maximal reporter activity. *A compound is considered inactive with EC_{50} > 60 μM. n.d: not determined

17.0 μM, respectively (Table 2). Compounds **2**, **3**, and **6-10** displayed moderate activities, with EC_{50} values ranging from 21.7 to 28.3 μM. Compound **5** weakly activated PPARs transcription, with an EC_{50} value of 41.6 μM, whereas compounds **1**, **11**, and **12** were inactive at the experimental concentrations. Given these primary data, to determine specifically how the compounds modulate PPAR transcriptional activity, we further examined the PPAR transactivational effects of the isolated compounds on individual PPAR subtypes, including PPARα, γ, and $\beta(\delta)$. The results indicated that compounds **4** and **12** increased PPARα transcriptional activity, with EC_{50} values of 47.7 and 53.3 μM, respectively. Compounds **6** and **8** activated PPARγ trascriptional activity, with EC_{50} values of 53.4 and 50.4 μM, respectively, whereas compound **9** increased PPAR$\beta(\delta)$ transcriptional activity, with an EC_{50} value of 52.8 μM (Table 2).

Taken together, this study provides scientific rationale for the use of *G. biloba* seeds in the prevention and treatment of inflammatory and metabolic diseases and warrants further studies of the potential of *G. biloba* seeds in functional foods and medicine.

Experimental

Plant Materials. The seeds of *G. biloba* were purchased from herbal market at Kumsan, Chungnam, Korea, in August, 2010. The plant material was identified by one of us (Y. H. Kim). A voucher specimen (CNU10110) was deposited at herbarium, College of Pharmacy, Chungnam National University.

Extraction and Isolation. The dried seeds (10.5 kg) were extracted three times with hot MeOH under reflux. After concentration, the MeOH extract (200 g) was suspended in H_2O and then partitioned successively with *n*-hexane, CH_2Cl_2, and EtOAc to give *n*-hexane (sA, 110 g), CH_2Cl_2 (sB, 1.1 g), EtOAc (sC, 1.3 g) and aqueous (sD, 80 g) fractions, respectively. The aqueous fraction sD was chromatographed on a column of highly porous polymer (Diaion HP-20) and eluted with a step-wise gradient of 0, 30, 60 and 100% (v/v) MeOH in H_2O to give four fractions (sD1-sD4). Fraction sD2 (27 g) was separated by YMC RP column chromatography (CC), using MeOH-H_2O (1:2) as eluents to give six subfractions (sD2.1-sD2.6). Compound **9** (7 mg) was isolated by silica gel CC, eluting with CH_2Cl_2-MeOH (9:1) from subfraction sD2.1 (200 mg). Subfraction sD2.2 was separated by CC over silica gel, eluting with CH_2Cl_2-MeOH (10:1), and further purified by silica gel CC, using CH_2Cl_2-acetone (2:1) as eluents, to obtain compounds **4** (9 mg) and **2** (6 mg). Compound **13** (11 mg) was isolated by preparative TLC using CH_2Cl_2-MeOH-H_2O (4:1:0.1) from subfraction sD2.4 (50 mg). Subfraction sD2.3 (200 mg) was separated by silica gel CC, eluting with CH_2Cl_2-MeOH-H_2O (5:1:0.1) to give two subfractions sD2.31-sD2.32. Compounds **6** (10 mg) and **7** (5 mg) were isolated by preparative TLC using CH_2Cl_2-MeOH-H_2O (3:1:0.1) from subfractions sD2.31 (35 mg) and sD2.32 (42 mg), respectively. Compound **10** (8 mg) was isolated by CC over silica gel, eluting with CH_2Cl_2-MeOH (10:1) from subfraction sD2.6 (65 mg). Subfraction sD2.5 (0.3 g) was separated by silica gel CC, eluting with CH_2Cl_2-MeOH-H_2O (6:1:0.1) to give two subfractions sD2.51-sD2.52. Compounds **1** (3 mg) and **3** (9 mg) were isolated by preparative TLC using CH_2Cl_2-MeOH-H_2O (4:1:0.1) from subfractions sD2.51 (80 mg) and sD2.52 (66 mg), respectively. Fraction sD3 (8 g) was separated by silica gel CC, eluting with CH_2Cl_2-MeOH (10:1) to provide four subfractions (sD3.1-sD3.4). Compounds **8** (13 mg) and **5** (11 mg)

were isolated by precipitation and filtered with MeOH from subfractions sD3.1 (100 mg) and sD3.2 (97 mg), respectively. Compounds **11** (14 mg) and **12** (10 mg) were isolated by preparative TLC using CH_2Cl_2-MeOH-H_2O (4:1:0.1) from subfractions sD3.3 (170 mg) and sD3.4 (106 mg), respectively.

(2*E*,4*E*)-Dihydrophaseic Acid (1): Colorless, viscous liquid; $[\alpha]_D^{25}$ +16.7 (*c* 0.1, MeOH). HRESIQTOFMS: *m/z* 281.1387 $[M-H]^-$ (calcd for $C_{15}H_{21}O_5$, 281.1389), *m/z* 317.1157 $[M+Cl]^-$ (calcd for $C_{15}H_{22}O_5Cl$, 317.1156). ^{1}H NMR (600 MHz, CD_3OD) δ 7.69 (d, J = 15.6 Hz, H-4), 6.22 (d, J = 15.6 Hz, H-5), 5.81 (s, H-2), 4.06 (m, H-3'), 3.77 (dd, J = 6.6, 1.8 Hz, H-7'α), 3.66 (d, J = 6.6 Hz, H-7'β), 1.98 (m, H-4'α), 1.93 (s, H_3-6), 1.80 (m, H-2'α), 1.72 (dd, J = 13.8, 10.2 Hz, H-4'β), 1.64 (m, H-2'β), 1.11 (s, H_3-9'), 0.89 (s, H_3-10'); ^{13}C NMR (150 MHz, CD_3OD) δ 174.6 (C-1), 139.8 (C-3), 132.1 (C-4), 129.2 (C-5), 127.2 (C-2), 86.8 (C-5'), 82.2 (C-8'), 76.3 (C-7'), 65.1 (C-3'), 48.8 (C-1'), 45.0 (C-4'), 43.5 (C-2'), 19.5 (C-6), 18.7 (C-9'), 15.4 (C-10').

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Supporting Information. The HRESIQTOFMS and NMR spectra of compound **1**, physicochemical properties of compounds **2-13**, and experimental information.

References

1. Deng, Q.; Wang, L.; Wei, F.; Xie, B.; Huang, F.; Huang, W.; Shi, J.; Huang, Q.; Tian, B.; Xue, S. *Food Chem.* **2011**, *124*, 1458.
2. Huang, W.; Deng, Q.; Xie, B.; Shi, J.; Huang, F. H.; Tian, B.; Huang, Q.; Xue, S. *Food Res. Int.* **2010**, *43*, 86.
3. Zhou, H.; Chen, X.; Wang, C.; Ye, J.; Chen, H. *Molecules* **2012**, *17*, 14778.
4. Huang, W.; Xie, B.; Wang, Y.; Yang, E. N.; Luo, R. *Sci. Agric. Sinica* **2004**, *37*, 1537.
5. Wang, H.; Ng, T. B. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 407.
6. Ngan, N. T.; Quang, T. H.; Tai, B. H.; Song, S. B.; Lee, D.; Kim, Y. H. *J. Agric. Food Chem.* **2012**, *60*, 2815.
7. Martin, G. C.; Dennis, F. G., Jr.; MacMillan, J.; Gaskin, P. *J. Am. Soc. Hortic. Sci.* **1977**, *102*, 16.
8. Kikuzaki, H.; Kayano, S.; Fukutsuka, N.; Aoki, A.; Kasamatsu, K.; Yamasaki, Y.; Mitani, T.; Nakatani, N. *J. Agric. Food Chem.* **2004**, *52*, 344.
9. Ali, F.; Iqbal, M.; Naz, R.; Malik, A.; Ali, I. *J. Chem. Soc. Pak.* **2011**, *33*, 90.
10. Ouyang, M. A.; Chen, P. Q.; Wang, S. B. *Nat. Prod. Res.* **2007**, *21*, 769.
11. Guvenalp, Z.; Ozbek, H.; Kuruzum-Uz, A.; Kazaz, C.; Demirezer, L. O. *Turk. J. Chem.* **2009**, *33*, 667.
12. Nakanishi, T.; Iida, N.; Inatomi, Y.; Murata, H.; Inada, A.; Murata, J.; Lang, F. A.; Iinuma, M.; Tanaka, T. *Heterocycles* **2004**, *63*, 2573.
13. Park, B.-J.; Tomohiko, M. *Chem. Nat. Compd.* **2011**, *47*, 363.
14. Ciuffreda, P.; Casati, S.; Manzocchi, A. *Magn. Reson. Chem.* **2007**, *45*, 781.
15. Larsen, E.; Aleem, A. A. H. A.; Pedersen, E. B. *J. Heterocycl. Chem.* **1995**, *32*, 1645.
16. Agrawal, P. K.; Agarwal, S. K.; Rastogi, R. P. *Phytochemistry* **1980**, *19*, 1260.
17. Shen, Y.-C.; Hsieh, P.-W.; Kuo, Y.-H. *Phytochemistry* **1998**, *48*, 719.
18. Jia, L.; Bi, Y. F.; Jing, L. L.; Zhou, S. A.; Kong, D. Y. *J. Asian Nat. Prod. Res.* **2010**, *12*, 962.
19. Deyama, T.; Nishibe, S.; Kitagawa, S.; Ogihara, Y.; Takeda, T.; Omoto, T.; Nikaido, T.; Sankawa, U. *Chem. Pharm. Bull.* **1988**, *36*, 435.