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Anti-inflammatory activity of constituents from *Glechoma hederacea* var. *longituba*

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ABSTRACT

Rosmarinic acid, its analogues, and a phenolic compound were obtained from *G. hederacea* var. *longituba*. There were two new compounds, methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate (**1**) and benzyl-4′-hydroxy-benzoyl-3′-*O*-β-D-glucopyranoside (**4**), and four known compounds (**2**, **3**, **5** and **6**). The structures of these compounds were determined on the basis of spectroscopic methods. Each compound was tested by NF-κB luciferase assay and three rosmarinic acid analogues inhibited NF-κB production and the induction of COX-2 and iNOS mRNA in HepG2 cells.

Inflammation is a cardinal host defence response to injury, autoimmune responses or infectious agents. It is a normal physiological response to injury or infection. In contrast, there are various inflammation-related diseases such as allergy, arthritis, and fibrosis. Inflammation can become chronic lasts weeks or months and causes tissue damage and makes a problem rather than the solution to infection and injury.[1,2] Chronic liver inflammation induces fibrosis, which is the excessive accumulation of extracellular matrix (ECM) proteins. Advanced hepatic fibrosis induced by chronic liver inflammation eventually progresses to liver cirrhosis.[3,4]

Glechoma hederacea var. *longituba* (Labiatae) is a perennial vine plant. It is distributed widely in Asia, Europe and America. *G. hederacea* is commonly known as 'ground ivy', 'creeping charlie' or 'grill-over-the-ground'.[5] *G. hederacea* var. *longituba* has been used for centuries in traditional oriental medicine for the treatment of cholelithiasis, urolithiasis, dropsy and various ailments, asthma, bronchitis, colds, and inflammation.[6,7] A previous study has reported on the anti-inflammatory activity of an extract of *G. hederacea* var. *longituba* on macrophage mediated inflammation.[5] In this study, anti-inflammatory compounds were isolated from *G. hederacea* and their structures were elucidated.

The whole plant of *G. hederecea* var. *longituba* (Labiatae) was collected in June 2009 in herbal garden of Chungnam National University and identified by Professor KiHwan Bae, one of the authors. The air-dried *G. hederecea* plant (7.4 kg) was extracted with ethanol (2 × 60 L), each for 3 days at room temperature. And filtrated ethanol soluble extract was concentrated in vacuo to obtain the extract (460 g) and then suspended in distilled water (2 L). The aqueous residue was partitioned and concentrated successively with hexane (2 × 2 L), ethyl acetate (2 × 2.5 L), and butanol (2 × 2 L) to obtain a hexane soluble fraction (GH, 35 g), an ethyl acetate soluble fraction (GE, 41 g), and a butanol soluble fraction (GB, 51 g). The fractions were tested NK-κB inhibitory activity. Among them GE showed inhibitory activity 35.2% at 1 mg/mL, and GH and GB inhibited 3.2% and 7.8% at 1 mg/mL, respectively (data not shown). GE was subjected to silica gel column chromatography (10 × 20 cm) and eluted with a $CHCl_3$/MeOH gradient (300:1 to 1:1). Thin layer chromatography (TLC) resulted in nine fractions (Fr. 1-9). Fr. 6 was chromatographed over a silica gel column (30 × 4 cm) eluting with gradient solvent system of $CHCl_3$/MeOH (250:1 to 1:1) to yield **1** (46 mg). Fr. 7 was chromatographed over a Sephadex LH-20 column (50 × 3 cm) using MeOH/H_2O (3:1) to yield six subfractions (fr. 71–76) and fr. 74 was further chromatographed over a YMC RP-18 column (45 × 3 cm) using 55% MeOH to yield **2** (45 mg) and **3** (6 mg). Fr. 8 was chromatographed over Sephadex LH-20 column (50 × 3 cm) using MeOH/H_2O (3:1) to afford nine subfractions (fr. 81-89) and fr. 82 was further chromatographed over a YMC RP-18 column (45 × 3 cm) using 50% MeOH and purified by prep-HPLC with MeOH/H_2O (45:55) to yield **4** (8 mg) and **5** (10 mg). Fr. 9 was chromatographed over a Sephadex LH-20 column (50 × 3 cm) using MeOH/H_2O (3:1) to afford five subfractions (fr. 91–95) and

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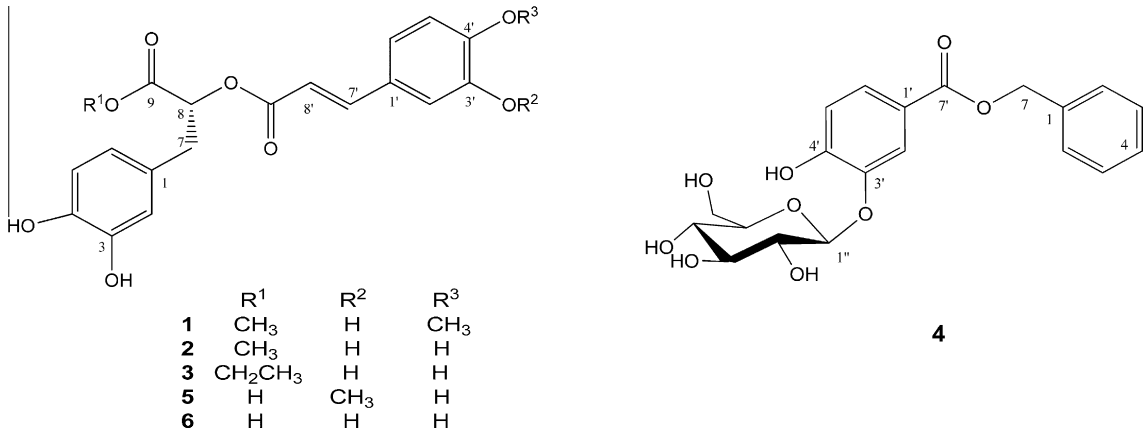

Figure 1. Chemical structures of compounds (**1**–**6**) isolated from *G. hederacea* var. *longituba*.

Table 1
^{1}H (300 MHz) and ^{13}C (75 MHz) NMR data of compounds **1** and **4** (in CD_3OD)

1			4		
Position	δ_H (J in Hz)	δ_C	Position	δ_H (J in Hz)	δ_C
1		128.9	1		137.1
2	6.71 (s)	117.7	2, 6	7.48 (dd, 1.8, 8.1)	129.5
3		146.4	3, 5	7.38 (br t)	129.8
4		145.6	4	7.36 (m)	129.6
5	6.70 (d, 8.1)	116.5	7	5.39 (d, 1.8)	68.3
6	6.57 (d, 8.1)	121.9	1′		113.5
7	3.06 (dd, 5.4, 14.4)	38.0	2′	7.62 (d, 3.0)	118.6
	3.02 (dd, 8.1, 14.4)		3′		151.8
8	5.20 (dd, 5.4, 8.1)	74.9	4′		158.5
9		172.3	5′	6.88 (d, 9.0)	119.3
1′		128.9	6′	7.32 (dd, 3.0, 9.0)	127.5
2′	7.08 (s)	115.0	7′		170.9
3′		148.2	1″	4.75 (d, 7.2)	103.8
4′		151.8	2″	3.31-3.32 (m)	75.0
5′	6.94 (d, 8.1)	112.6	3″	3.32-3.35 (m)	78.1
6′	7.06 (d, 8.1)	123.2	4″	3.39-3.41 (m)	71.3
7′	7.58 (d, 16.2)	147.7	5″	3.41-3.42 (m)	78.3
8′	6.32 (d, 16.2)	115.4	6″	3.68 (dd, 4.8, 12.3)	62.5
9′		168.3		3.81 (dd, 2.4, 12.3)	
4′-OCH_3	3.89 (s)	56.5			
CO-OCH_3	3.70 (s)	52.8			

fr. 92 was further chromatographed over a YMC RP-18 column (45 × 3 cm) using 50% MeOH to yield **6** (24 mg).

Two new compounds were isolated (Fig. 1): a rosmarinic acid analogue, methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate (**1**) and a phenolic glucoside, benzyl-4′-hydroxy-benzoyl-3′-*O*-β-D-glucopyranoside (**4**). Additionally, four other known compounds were identified by spectroscopic evidence and their data were compared with those of literatures: methyl rosmarinate (**2**),[8–10] ethyl rosmarinate (**3**),[8–10] 3′-*O*-methyl-rosmarinic acid (**5**),[11] and rosmarinic acid (**6**).[9,12,13]

Compound **1** was obtained as a yellowish amorphous powder and its optical rotation was $[\alpha]_D^{25} = +1.9$ (c 0.1, MeOH). Its molecular formula was deduced to be $C_{20}H_{20}O_8$ by HRESIMS, which showed a molecular ion peak $[M+Na]^+$ at m/z 411.1045 (calcd for $C_{20}H_{20}O_8Na$, 411.1056). The UV spectrum exhibited signals at 327, 292, and 252 nm, and IR spectrum showed signals at 3406, 1709, 1611 and 1444 cm^{-1}. The ESIMS spectrum showed strong peak at m/z 193 corresponding to an isoferuloyl moiety.[14a] The ^{1}H, ^{13}C NMR spectra of **1** were similar to those of rosmarinic acid.[9,12,13] The ^{1}H NMR spectrum (Table 1) revealed the presence of two trisubstituted ABX system aromatic rings between δ 6.57 and 7.08, and two OCH_3 signals at δ 3.89 and 3.70. A strong correlation peak showed that the OCH_3 (δ 3.89) positioned C-4′ at δ 151.8 in HMBC data (Fig. 2). And long-range correlations appeared between H-7′ at δ 7.58 and C-1′ (δ 128.9), C-2′ (δ 115.0), C-3′ (δ 148.2), and C-6′ (δ 123.2); among them, the correlation from H-7′ to C-3′ was a 4-bond correlation and there was no significant correlation between H-7′ and C-4′ within the 4-bond. Also, the correlation between H-5′ and H-6′ as AB in the ABX system of the aromatic ring in ^{1}H-^{1}H COSY data supported to the location of OCH_3 (δ 3.89) at C-4′ in an isoferuloyl moiety. The structure of compound **1** was therefore determined to be a new rosmarinic acid analogue, methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate (Table 1).

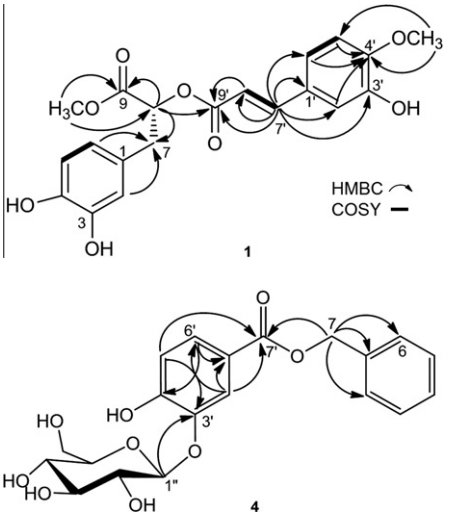

Figure 2. Key HMBC and COSY correlations of compound **1** and Key HMBC correlations of compound **4**.

Compound **4** was obtained as a yellowish powder and showed a negative optical rotation, $[\alpha]_D^{25} = -2.2$ (c 0.23, MeOH). Its molecular formula was deduced to be $C_{20}H_{22}O_9$ by HRESIMS, which revealed the $[M+Na]^+$ peak at m/z 429.1153 (calcd for $C_{20}H_{22}O_9Na$, 429.1162). [14b] The UV spectrum exhibited signal at 325 nm and IR spectrum showed signals at 3400, 1688, 1619 and 1075 cm^{-1}. The ^{1}H, ^{13}C NMR spectra were similar to those of 3,4-dihydroxy-7-(3′-*O*-β-D-glucopyranosyl-4′-hydroxy-benzoyl)-benzylalcohol.[15] The ^{1}H and ^{13}C NMR spectra revealed the presence of an anomeric proton at δ_H 4.75 (H-1″, J = 7.2) and δ_C 103.8 of a β-D-glucopyranosyl, an ester (C-7′, δ 170.9), an *O*-bearing methylene (H-7, δ 5.39) and monosubstituted benzene protons (H-2 to H-6, δ 7.36–7.48) of a benzyl formate moiety. Correlations between H-1″ and C-3′; H-2′, H-5′ and C-7′; H-7 and C-7′, C-1, C-2, and C-6 were observed

in the HMBC spectra (Fig. 2). Thus, the structure of this new phenolic compound was established as benzyl-4'-hydroxy-benzoyl-3′-*O*-β-D-glucopyranoside (Table 1).

To evaluate and compare the anti-inflammatory activity of potential compounds from *G. hederacea,* the nuclear transcription NF-κB cell-reporter system was used.[16] First, to determine non-toxic concentrations, HepG2 cells were treated with various concentrations (0.1, 1, 10 and 20 μM) of the compounds and cell viability was measured by MTS assay.[17] All six compounds displayed no significant cytotoxicity up to 20 μM, indicating that the inhibitory effects of these compounds on NF-κB activation were not due to cytotoxicity (Fig. 3). Next, the anti-inflammatory activity of the compounds was measured by luciferase assay in HepG2 cells. One of the rosmarinic acid analogues, compound **1**, reduced NF-κB activation by 45.4% at 20 μM and 28.7% at 10 μM respectively, and rosmarinic acid analogues **2** and **3** showed similar inhibitory activity (44.3% and 57.0% at 20 μM, respectively). On the other hand, compound **6** reduced NF-κB activation by 22.7% at 20 μM, and compounds **4** and **5** showed very weak inhibitory activities (Fig. 4). These results suggest that compounds **1**, **2**, **3** and **6** have an anti-inflammatory effect through the reduction of NF-κB in HepG2 cells.

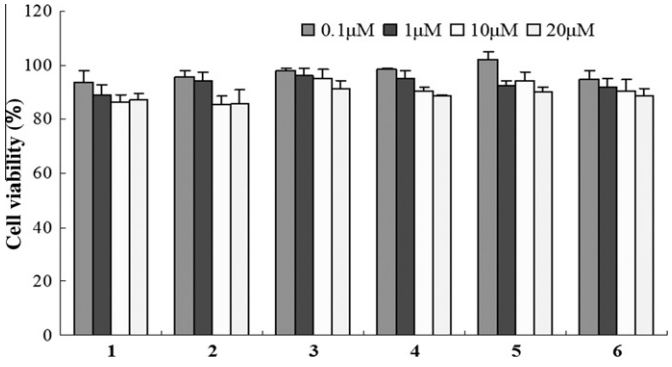

Figure 3. HepG2 cells were incubated with the compounds at various concentrations for 24 h. Cell viability was determined by MTS assay. Non-treated control is at 100%. Methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate (**1**), methyl rosmarinate (**2**), ethyl rosmarinate (**3**), benzyl-4′-hydroxy-benzoyl-3′-*O*-β-D-glucopyranoside (**4**), 3′-*O*-methyl-rosmarinic acid (**5**) and rosmarinic acid (**6**). Data are expressed as the mean ± SD ($n = 3$).

To confirm the anti-inflammatory activity of the compounds that inhibited NF-κB activation in the luciferase assay, compounds **1**, **2** and **3** were selected to examine their effects on the expression of NF-κB target genes by RT-PCR.[18] HepG2 cells treated with TNF-α (10 ng/mL) significantly upregulated the mRNA expression of NF-κB target genes iNOS and COX-2, respectively. All three compounds significantly inhibited the induction of iNOS and COX-2 mRNA in a dose-dependent manner, indicating that these compounds attenuated the synthesis of these transcripts at the transcriptional level (Fig. 5). HepG2 cells activated by TNF-α increase the expression of factors related to inflammation, including NF-κB, iNOS and COX-2. Therefore, compounds **1, 2** and **3** showed potent anti-inflammatory activity by the inhibition of NF-κB activation, iNOS and COX-2 expression.

It has been reported that *G. hederacea* var. *longituba* shows an inhibitory effect on IFN-γ/LPS-induced NO in a dose-dependent manner, and the pro-inflammatory cytokine, IL-12p70, and TNF-α production on stimulated mouse peritoneal macrophages.[5] Nevertheless, there has been no report of the active constituents. In this study, it was newly revealed that the rosmarinic acid analogues, new compound **1**, and other analogues **2** and **3** possess anti-inflammatory activity. These compounds displayed better activity than that of rosmarinic acid in HepG2 cells. Rosmarinic acid is known for its anti-inflammatory activity via the inhibition of IKK-β activity in NF-κB signaling which upregulates the expression of *CCL11* and *CCR3*.[19] A literature reported that anti-inflammatory activity of isoferulic acid is better than that of ferulic acid which is partial structures of **1** and **5**, respectively.[20] In this study, anti-inflammatory activity of **1** was significantly better than **5** as the report of isoferulic acid and ferulic acid. Further study is required to establish relationship between chemical structure of the compounds and the anti-inflammatory activity. Based on these results, it is suggested that not only rosmarinic acid but also some of its analogues from *G. hederacea* var. *longituba* could be useful resources for the prevention and treatment of diseases related to inflammation.

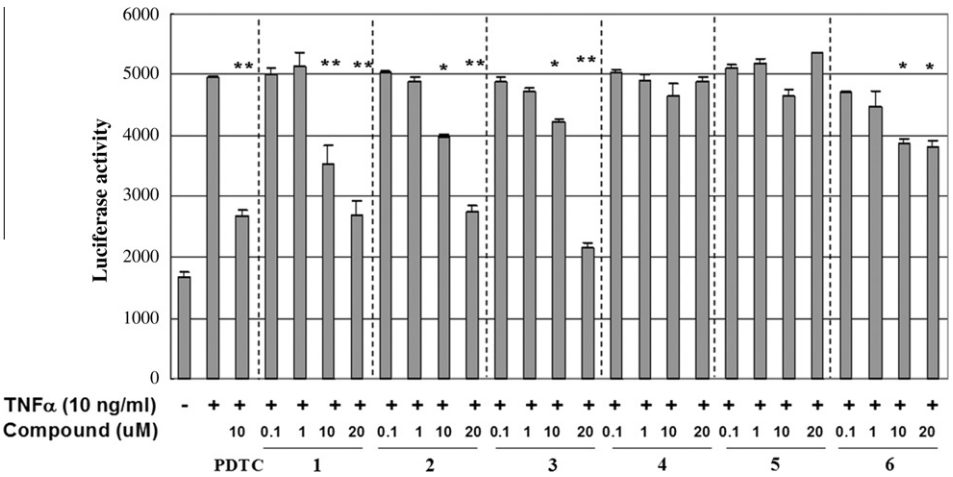

Figure 4. Effect of the compounds on TNF-α induced NF-κB luciferase reporter activity in HepG2 cell lines. HepG2 cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with either vehicle (DMSO) and compounds, prior to 1 h of treatment with TNF-α (10 ng/mL). Unstimulated HepG2 cells acted as a negative control. Cells were then harvested and luciferase activities were assessed. Results are expressed as relative luciferase activity. Methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate (**1**), methyl rosmarinate (**2**), ethyl rosmarinate (**3**), benzyl-4′-hydroxy-benzoyl-3′-*O*-β-D-glucopyranoside (**4**), 3′-*O*-methyl-rosmarinic acid (**5**), and rosmarinic acid (**6**). PDTC (pyrrolidine dithiocarbamate) is a positive control. Data are expressed as the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

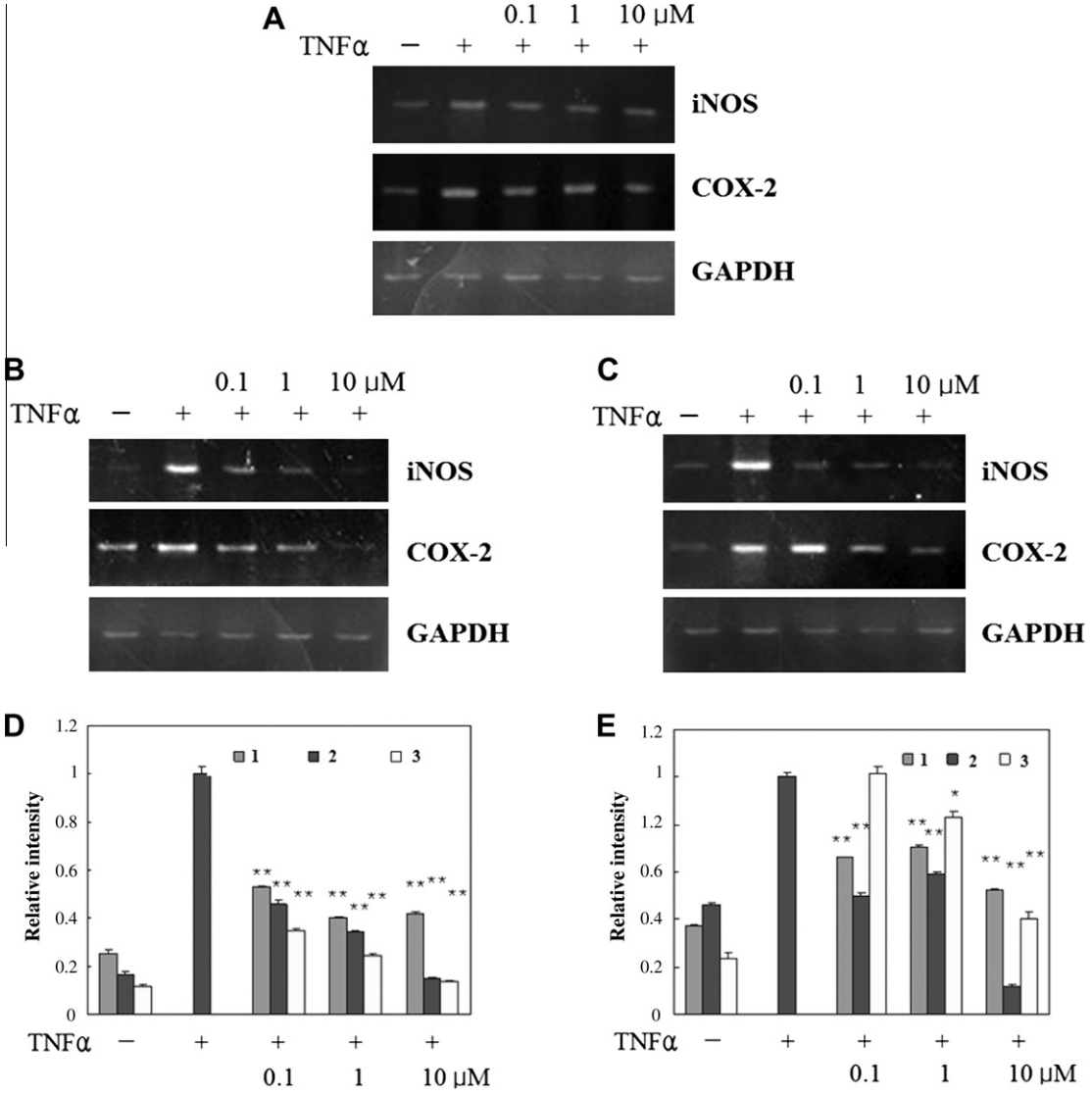

Figure 5. Effect of compounds **1**, **2**, and **3** on iNOS and COX-2 mRNA expression in HepG2 cell line. HepG2 cells were pretreated in the absence and presence of **1**, **2**, and **3** for 1 h before TNF-α treatment (10 ng/mL), then exposed to TNF-α for 6 h. Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNAs were assessed by RT-PCR. (A) Treated **1**, (B) treated **2**, (C) treated **3**, (D) iNOS, and (E) COX-2 in HepG2 cells. Data are expressed as the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

References and notes

1. Lucas, S. M.; Rothwell, N. J.; Gibson, R. M. *Br. J. Pharmacol.* **2006**, *147*, S232.
2. Ferrero-Miliani, L.; Nielsen, O. H.; Andersen, P. S.; SE, G. *Clin. Exp. Immunol.* **2007**, *147*, 227.
3. Han, K. H.; Yoon, K. T. *Intervirology* **2008**, *51*, 11.
4. Elsharkawy, A. M.; Mann, D. A. *Hepatology* **2007**, *46*, 590.
5. An, H. J.; Jeong, H. J.; Um, J. Y.; Kim, H. M.; Hong, S. H. *J. Ethnopharmacol.* **2006**, *106*, 418.
6. Jo, D.; Lee, J.; Noh, J.; Kim, O.; Kwon, J. *J. Food Sci. Nutr.* **2001**, *6*, 142.
7. Yamauchi, H.; Kakuda, R.; Yaoita, Y.; Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **2007**, *55*, 346.
8. Park, S. H.; Oh, H. S.; Kang, M. A.; Cho, H. J.; Prasad, J. B.; Won, J. H.; Lee, K. H. *Bioorg. Med. Chem.* **2007**, *15*, 3938.
9. Kiran, I.; Sarfraz, N., et al *Chem. Biodivers.* **2005**, *2*, 104.
10. Huang, H.; Sun, H. D.; Wang, M. S.; Zhao, S. X. *J. Nat. Prod.* **1996**, *59*, 1079.
11. Baba, S.; Osakabe, N.; Natsume, M.; Terao, J. *Life Sci.* **2004**, *75*, 165.
12. Woo, E. R.; Piao, M. *Arch. Pharm. Res.* **2004**, *27*, 173.
13. Wang, Z. J.; Zhao, Y. Y.; Wang, B.; Ai, T. M.; Chen, Y. Y. *Chin. Chem. Lett.* **2000**, *11*, 997.
14. (a) *Methyl isoferuloyl-7-(3,4-dihydroxyphenyl) lactate* (**1**): yellowish amorphous powder; $[\alpha]_D^{25} = +1.9$ (*c* 0.1, MeOH); IR (KBr) ν_{max}: 3406 (OH), 1709 (C=O), 1611, 1444 (C=C), 1267, 1158 cm^{-1}; ESIMS *m/z* 388 $[M]^+$; HRESIMS *m/z* 411.1045 $[M+Na]^+$ (calcd for $C_{20}H_{20}O_8Na$, 411.1056); UV (MeOH) λ_{max} (log ε) 327 (8.4), 292 (7.1), 252 (3.2) nm; ^1H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) in Table 1. (B) *Benzyl-4′-hydroxy-benzoyl-3′-O-β-D-glucopyranoside* (**4**): yellowish powder; $[\alpha]_D^{25} = -2.2$ (*c* 0.23, MeOH); IR (KBr) ν_{max}: 3400 (OH), 1688 (C=O), 1619 (aromatic C=C), 1217, 1075 cm^{-1}; ESIMS *m/z* 406 $[M]^+$, 405 $[M-H]^+$, 242 $[M-Glc]^+$, 151 $[M-Glc-PhCH_2]^+$, 135 $[M-Glc-PhCH_2O]^+$, 107 $[M-Glc-PhCH_2COO]^+$, 79; HRESIMS *m/z* 429.1153 $[M+Na]^+$ (calcd for $C_{20}H_{22}O_9Na$, 429.1162); UV (MeOH) λ_{max} (log ε) 325 (2.7) nm; ^1H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) in Table 1.
15. Liao, L. P.; Bi, Z. M.; Li, P.; Zhang, T. D. *Linchan Huaxue Yu Gongye* **2005**, *25*, 13.
16. The NF-κB luciferase assay is designed to monitor the activity of NF-κB-regulated signal transduction pathways in cultured cells. The NF-κB responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the NF-κB transcriptional response element. Cells were seeded at 5×10^5 cells/mL (1.5 mL) in 6-well and grown for 24 h. All cells were transfected by the lipofectamine LTX (Invitrogen, Carlsbad, CA) as described by the manufacturer. Luciferase activity was assayed using an LB 953 Autolumat (EG&G Berthold, Nashua, NH) and was normalized based on the expression of RSV-β-galactosidase. β-Galactosidase activity was assayed colorimetrically. NF-κB-Luc plasmid was provided by Dr. Kyoon E. Kim (Department of Biochemistry, Chungnam National University).
17. *Cytotoxicity assay on HepG2 cells*: A MTS assay (Promega Celltiter 96-Aqueous One Solution Assay) was used to analyze affects of the compounds on cell viability. Cells were cultured overnight in 96-well plate (1×10^4 cells/well). Cell viability was assessed after addition of the compounds at various concentrations for 24 h. The number of viable cells was assessed by determination of the $A_{490\ nm}$ of the dissolved formazan product after addition of MTS for 30 min as described by the manufacturer (Promega, Madison, WI).

18. Total RNA extraction was isolated with Easy-blue reagent (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, Madison, WI) for 1 h at 42 °C. PCR for synthetic cDNA was performed 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′-CTCCACGACGTACT CAGCG-3′. The specificity of products generated by each set of primers was examined using gel electrophoresis.
19. Lee, J. S.; Jung, E. S.; Kim, Y. J.; Lee, J. Y.; Park, J. H.; Hong, S. T.; Hyun, C. G.; Park, D. H.; Kim, Y. S. *Br. J. Pharmacol.* **2006**, *148*, 366.
20. Sakai, S.; Kawamata, H.; Kogure, T.; Mantani, N.; Terasawa, K.; Umatake, M.; Ochiai, H. *Mediat. Inflammun.* **1999**, *8*, 173.