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Isolation of Hepatoprotective Phenylpropanoid from *Lactuca indica*

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Abstract – In continuing our search for biologically active compounds from Korean Compositae medicinal plants, we investigated the constituents of the aerial parts of *Lactuca indica* L. and isolated a phenylpropanoid derivative from its MeOH extract. The chemical structure was characterized by spectroscopic methods, including 1D and 2D NMR to be di-*E*-caffeoyl-*meso*-tartaric acid (**1**). Compound **1** was isolated for the first time from this plant. In this paper, we suggest that the NMR assignment at C-2 of (+)-taraxafolin-B should be corrected. In the human HBV-transfected liver cell line HepG2.2.15, the compound **1** effectively reduced HBV DNA level in the release of mature HBV particles from HepG2.2.15 cultivation.
Keywords – *Lactuca indica* L. Compositae, Di-*E*-caffeoyl-*meso*-tartaric acid, Hepatoprotective activity

Introduction

Lactuca indica L. (Compositae) is widely distributed throughout Korea. This indigenous herb is an edible wild vegetable that is traditionally used as a folk medicine for inflammatory, bacterial infection, and intestinal disorders (Kan, 1986). Various compounds including terpenoids (Hui *et al*., 1971; Fan *et al*., 2004), sterols (Fan *et al*., 2004) and flavonoids (Makoto *et al*., 1978) were isolated from this plant. Several biological activities of this plant have been reported, including antimutagenic activity against indirect-acting mutagens (AFB_1 and B(a)P) (Kusamran *et al*., 1998) and stimulation of differentiation of the mouse melanoma cell line, B16 2F2 (Hata *et al*., 2003). In continuing our search for biologically active compounds from Korean Compositae medicinal plants, we investigated the constituents of the aerial parts of *L. indica* and reported terpenoids, phenolic constituents and their hepatoprotective activity (Kim *et al*., 2007; Kim *et al*., 2008). We conducted a further chemical investigation of the aerial parts of *L. indica*, which led to isolation of a phenylpropanoid derivative, di-*E*-caffeoyl-*meso*-tartaric acid (**1**). The structure of **1** was elucidated by spectroscopic methods, including 1D and 2D NMR. Compound **1** was isolated for the first time from this plant. Compound **1** was evaluated for hepatoprotective activity by the HBV assay *in vitro*. This paper describes the isolation, structural elucidation, and hepatoprotective activity of **1**.

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Experimental

General – Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 Polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. UV spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer. LC-ESI/MS data on an Agilent 1100LC/ MSD trap SL LC/MS. Preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector and Econosil® RP-18 10 μ column (250 × 10 mm). Silica gel 60 (Merck Co., Germany, 70~230 mesh, and 230~400 mesh) and RP-C_{18} silica gel (Merck Co., Germany, 230 - 400 mesh) were used for column chromatography. TLC was performed using Merck precoated Silica gel F_{254} plates and RP-18 F_{254s} plates. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.).

Plant materials – The aerial parts of *L. indica* were collected in Suwon, Korea, in May 2005, and the plant was identified by one of the authors (Y.H.K.). A voucher specimen (SKKU-2005-05) of the plant was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation – The aerial parts of *L. indica* (5 kg) were extracted at room temperature with 80% MeOH and evaporated under reduced pressure to give a residue (200 g), which was dissolved in water (800 mL) and solvent-partitioned (*n*-hexane, $CHCl_3$ and *n*-BuOH) to give *n*-hexane (20 g), $CHCl_3$ (12 g), and *n*-BuOH (75 g) soluble extracts. The *n*-BuOH fraction (45 g) was separated over a silica gel column with a solvent system of $CHCl_3$: MeOH : H_2O (30 : 10 : 1 - 13 : 7 : 1) as the eluent to give six fractions (B1-B6). Fraction B6 (3.0 g) was separated over an RP-C_{18} silica gel column with 30% MeOH as the eluent to give six subfractions (B61-B66). Subfraction B62 (350 mg) was applied to column chromatography over Sephadex LH-20 (Pharmacia Co.), eluting with a solvent system of MeOH : H_2O (4 : 1) and purified further by semi-preparative HPLC, using aqueous 28% MeOH over 30 min at a flow rate of 2.0 mL/min (Econosil® RP-18 10 μ column; Shodex refractive index detector) to afford **1** (25 mg, R_t = 13.5 min).

Di-*E*-caffeoyl-*meso*-tartaric acid (1) Yellowish powder, mp: 120 - 125 °C; $[\alpha]_D^{25}$: +50.6° (c 0.51, MeOH); IR ν_{max} cm^{-1}: 3392, 2713, 1701, 1601, 1525, 1385, and 1157; UV λ_{max} (MeOH) nm (log ε): 217 (2.8), 235 (2.3), 243 (2.3), 299 (2.8), and 325 (3.0); ESI-MS *m/z*: 497 $[M + Na]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.62 (2H, d, *J* = 16.0 Hz, H-7', H-7"), 7.08 (2H, d, *J* = 1.7 Hz, H-2', H-2"), 6.92 (2H, dd, *J* = 8.1, 1.7 Hz, H-6', H-6"), 6.79 (2H, d, *J* = 8.1 Hz, H-5', H-5"), 6.43 (2H, d, *J* = 16.0 Hz, H-8', H-8"), 5.74 (2H, s, H-2, H-3); ^{13}C-NMR (CD_3OD, 125 MHz): δ 173.3 (C-1, C-4), 167.9 (C-9', C-9"), 148.1 (C-4', C-4"), 145.7 (C-7', C-7"), 145.5 (C-3', C-3"), 126.9 (C-1', C-1"), 121.7 (C-6', C-6"), 115.3 (C-5', C-5"), 114.6 (C-2', C-2"), 114.0 (C-8', C-8"), 75.3(C-2, C-3); ^{1}H (D_2O, 500 MHz) and ^{13}C (D_2O, 125 MHz) NMR data, see Table 1.

The HBV assay *in vitro* – The hepatoprotective activity of the compound **1** was investigated using the HBV assay *in vitro* (Kim *et al*., 2007). Cultures of HepG2.2.15 cells grown in DMEM media were supplemented with purified compound **1** from plant extracts. On days 4 of treatment the presence of released HBV particles in the culture media (10 μL) was determined by PCR techniques and compared to untreated controls, positive control with 1-deoxynojirimycin (You *et al*., 2003), and the compound **1**. Intracellular HBV-specific RNAs were extracted from the harvested HepG2.2.15 cells and could be also determined by RT-PCR techniques (Yang *et al*., 2005). To monitor the inhibition of HBV secretion from HepG2.2.15 cells, the target region of the HBV surface antigen was amplified in a PCR or RT-PCR reaction. The following set of primers was employed to amplify the HBsAg sequences: forward primer, 5'-TGC CTC ATC TTC TTG TTG GTT CT-3'; backward primer, 5'- CCC CAA TAC CAC ATC ATC CAT ATA-3' amplifies a 336 nt length of DNA products. The amplified DNA fragments at the HBsAg sequences were then revealed by conventional agarose gel electrophoresis. In order to identify intracellular HBV-specific RNA expression in the HepG2.2.15 cells during treatment, the same target region of HBV surface antigen was amplified in a RT-PCR reaction with the primer sets used above.

Results and Discussion

Compound **1** was obtained as a yellowish powder. The ESI-MS (*m/z* 497 $[M + Na]^+$) and ^{1}H- and ^{13}C-NMR spectral data of **1** gave a molecular formula of $C_{22}H_{18}O_{12}$. The UV spectrum exhibited absorption maxima at 243 and 325 nm, suggesting the presence of aromatic ring in the molecule. The IR spectrum showed absorption bands for hydroxyls (3392 cm^{-1}), α,β-unsaturated carbonyl (1701 cm^{-1}), and aromatic (1601 and 1525 cm^{-1}) functionalities. The ^{1}H-NMR spectrum showed three aromatic protons signals at δ 7.08 (d, *J* = 1.7 Hz), 6.92 (dd, *J* = 8.1, 1.7 Hz), and 6.79 (d, *J* = 8.1 Hz), two olefinic proton signals at δ 7.62 (d, *J* = 16.0 Hz) and 6.43 (d, *J* = 16.0 Hz). The ^{13}C-NMR spectrum demonstrated the presence of six aromatic carbon signals at δ 148.1, 145.5, 126.9, 121.7, 115.3, and 114.6, two olefinic carbon signals at δ 145.7 and 114.0 and a carbonyl carbon signal at δ 167.9, which implied the presence of a *trans*-caffeoyl moiety from the characteristic shifts and coupling constant of signals at δ 7.62 (*J* = 16.0 Hz) and 6.43 (*J* = 16.0 Hz). In addition, there was an oxygenated proton signal at δ 5.74 (s) in the ^{1}H-NMR spectrum. The HMQC spectrum revealed that the proton at δ 5.74 is attached to the carbon signal at C-2/C-3 (δ 75.3). The carbon signal at δ 75.3 and a carboxyl carbon signal at δ 173.3 observed in the ^{13}C-NMR spectrum were characteristic shifts of the tartaric acid (Bergmana *et al*., 2001). The downfield shift of H-2/H-3 compared to tartaric acid showed that the caffeoyl moiety was attached at the hydroxyl function of the tartaric acid (Bergmana *et al*., 2001). This was also supported by the HMBC spectrum, which showed that H-2/H-3 were correlated to C-1/C-4 (δ 173.3) and C-9'/C-9" (δ 167.9). Although a caffeoyl moiety was assigned in the ^{1}H- and ^{13}C-NMR signals, the molecular ion peak $[M + Na]^+$ at *m/z* 497 indicated the presence of a symmetrical dicaffeoyltartaric acid. The relative configuration of tartarate residue of **1** was established as *meso*-tartaric acid, based

Table 1 ^{1}H- and ^{13}C-NMR spectral data for compound **1** from *L. indica* and (+)-taraxafolin-B from *T. formosanum* in D_2O

Position	**1**[a]		(+)-taraxafolin-B[b]	
	δ_H	δ_C	δ_H	δ_C
1,4		173.7		173.9
2,3	5.50 (s)	74.9	5.49 (s)	75.1
1',1"		127.2		127.4
2',2"	6.97 (d, 1.6)	115.3	7.11 (d, 1.6)	115.6
3',3"		144.3		144.6
4',4"		147.2		147.5
5',5"	6.76 (d, 8.0)	116.3	6.84 (d, 8.0)	116.6
6',6"	6.91 (dd, 8.0, 1.6)	123.1	7.04 (dd, 8.0, 1.6)	123.3
7',7"	7.50 (d, 16.0)	146.9	7.60 (d, 16.0)	147.1
8',8"	6.28 (d, 16.0)	114.1	6.37 (d, 16.0)	114.5
9',9"		168.8		169.0

[a] ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) in D_2O (δ in ppm)
[b] ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) in D_2O (δ in ppm)
Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

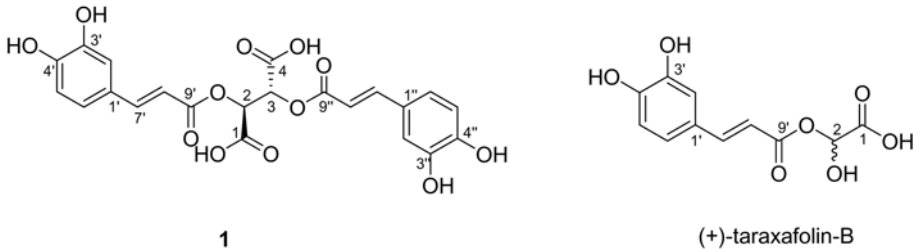

Fig. 1. The structures of **1** from *L. indica* and (+)-taraxafolin-B.

on the value of $^{3}J_{2,3}$ (Bergmana *et al*., 2001) and comparison with published ^{13}C-NMR data (Veit *et al*., 1991). Therefore, based on all the above evidence, the structure of **1** was assigned as di-*E*-caffeoyl-*meso*-tartaric acid.

According to the survey of literature, the ^{1}H- and ^{13}C-NMR data of (+)-taraxafolin-B, which was reported by Yann *et al*. were very similar to those of the compound **1** (Yann *et al*., 2005). The spectral data of **1**, particularly ^{13}C-NMR data, were completely matched with those of (+)-taraxafolin-B isolated from *T. formosanum* (Table 1). We suggest that the assignment at C-2 of (+)-taraxafolin-B should be corrected since its carbon signal at C-2 (δ 75.1) implies the presence of only one functional group responsible for such a deshielding effect. In the structure of (+)-taraxafolin-B, the carbon signal at C-2 should appear at δ 95~105, similar to the anomeric carbon of sugar (Stephen *et al*., 1977) or the carbon of methylenedioxy group (Kim *et al*., 2010).

The hepatoprotective activity of the isolated compound **1** was assessed using an *in vitro* HBV assay. The HepG2.2.15 cell line used in this experiment constitutively expresses HBV via an integrated HBV genome and is used extensively for drug evaluation (Korba *et al*., 1992). After compound **1** was added to the culture media and incubated for 4 days, 10 μL media was used for HBV DNA amplification by PCR. The release of virus particles or the expression of HBV-specific RNAs was positively inhibited by compound **1** at micromolar concentrations after 4 days of treatment. No amplified DNA or a very weak DNA band at the position of 336 bp after agarose gel electrophoresis indicate a positive result, that is, inhibition of virus production. No inhibitory effect on virus production or viral gene expression may result in a unique DNA band at the 336 bp position of the agarose gel. The lack of amplification of viral DNA means that a specific inhibitory molecule had interrupted the processes of viral DNA replication or transcription. From analysis of this PCR products on the agarose gel electrophoresis, we found that compound **1** showed stronger antiviral activity than that of the control molecule of 1-deoxynojirimycin (You *et al*., 2003), that is, significant

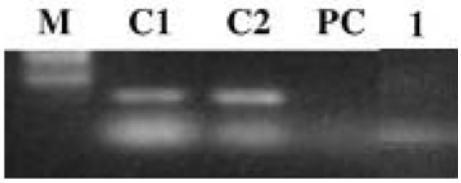

Fig. 2. Compound **1** isolated from the *n*-BuOH soluble fraction was assayed for amplification of 336 bp length of HBsAg DNA fragment. 0.39 mM of **1** was added and tested by PCR. **M** lane: 1 Kb size marker; **C1** and **C2** lanes: untreated mock and butanol treated mock as control, respectively. **PC** lane is treated with 1-deoxynojirimycin as a positive control for HBV inhibition.

hepatoprotective activity against hepatitis B virus replication, as shown in Fig. 2.

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