SUPPLEMENTARY MATERIAL

Anti-skin ageing effects of phenolic compounds from *Carpinus tschonoskii*

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Abstract: *Carpinus tschonoskii* (CT) is distributed through in southern regions of South Korea. We isolated six known compounds, including three ellagitannins, one gallotannin, and two flavonoids from CT. In conclusion, the tannins, especially ellagitannins, and CT extract showed potent anti-oxidative, anti-inflammatory and anti-skin ageing activities.

Keywords: *Carpinus tschonoskii*; Anti-skin ageing activities; Tannin, Flavonoid.

Experimental

Plant material

Fresh stems and leaves of CT (2.0 kg) were collected from the Jeolla Namdo Wando Arboretum (Korea) in October 2011. Their identity was certificated by Ph.D Kim Sung-Sik (Korea National Arboretum). A voucher specimen (CA2011) has been deposited at the herbarium of the College of Pharmacy, Chung-Ang University.

General experimental procedures

The stationary phases for column chromatography mediated isolation were performed on a Sephadex LH-20 column (10-25 m; GE Healthcare Bio-Science AB, Uppsala, Sweden), MCI-gel CHP 20P (75-150 m; Mitsubishi Chemical, Tokyo, Japan), and ODS-B gel column (40-60 m; Daiso, Osaka, Japan). The ODS-B gel was used as the stationary phase of a middle pressure liquid chromatography (MPLC) system equipped with a model 650E injector (Waters, Milford, MA, USA), 110UV/VIS detector (Gilson, Middleton, WI, USA), and TBP5002 pump (Tauto Biotech, Sanghai, China). Chemical structures were elucidated using several instrumental analyses. One-dimensional nuclear magnetic resonance (1D-NMR) including ^{1}H-(300 or 600MHz) nuclear magnetic resonance (NMR) experiments were recorded with Gemini 2000 and VNS devices (Varian, Palo Alto, CA, USA) at the Center for Research Facilities, Chung-Ang University.

Extraction, isolation and structure elucidation

Stems and leaves (2.0 kg) of CT were extracted for 72 h at room temperature with 80% acetone. After removing the acetone under vacuum, the aqueous solution was filtered through filter paper (Tokyo Roshi Kaisha Ltd, Japan) and re-filtered using Celite 545 (Duksan Pure Chemicals Co. Ltd, Seoul, Korea). Filtrate was concentrated and applied to a Sephadex LH-20 column (25-100 μm, 2000 g, 10 × 120 cm; Pharmacia, Uppsala, Sweden)

and eluted with water (H2O) containing increasing proportions of methanol (MeOH). Ten fractions were obtained. Fraction 5 (9.4 g) was separated on a MCI gel (50 μm, 400 g, 3× 50 cm, 0-100%, MeOH in H2O). Repeated column chromatography on a ODS-B gel (50 μm, 150 g, 1.5× 45 cm, 20-100%, MeOH in H2O) in a reverse phase MPLC system (5 mL/min, 280 nm) yielded **4** (570 mg). Repeated column chromatography of fraction 6 (4.57 g) on MCI gel CHP 20P (50 μm, 400 g, 3 × 50 cm, 0-100%, MeOH in H2O) yielded 3 (1.20 g). Repeated column chromatography on a ODS-B gel (50 μm, 250 g, 3× 50 cm, 20-100%, MeOH in H2O) in reverse phase MPLC system (5 mL/min, 280 nm) yielded **5** (90 mg). Fraction 9 (2.39 g) was separated by recrystallization to yield **6** (60 mg), and on Sephadex LH-20 (25-100 μm, 400 g, 3.5 × 60 cm, 0-100%, MeOH in H2O) to yield **1** (120 mg) and **2** (40mg).

Anti-oxidative activity

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

Anti-oxidant activity was determined on the basis of the scavenging capacity for the stable DPPH free radical (Sigma-Aldrich, St. Louis, MO, USA). Twenty microliter aliquots of the samples dissolved in absolute ethanol were added to 180 μl of a DPPH solution (0.1 mM, in absolute ethanol). After mixing gently and standing for 30 min, the optical density (O.D.) was measured at 518 nm using an ELISA reader (TECAN, Salzburg, Austria). The free radical scavenging activity was calculated as inhibition rate (%) = [1-(sample O.D. / control O.D.)] × 100. IC_{50} values were defined as the concentration that could scavenge 50% DPPH free radical. L-ascorbic acid was used as the positive control.

Measurement of nitroblue tetrazolium (NBT)/superoxide scavenging activity

A reaction mixture with a final volume of 632 µl in an Eppendorf tube was prepared with 50 mM phosphate buffer (pH 7.5) containing EDTA (0.05 mM), hypoxanthine (0.2 mM), 63 µl NBT (1mM) (Sigma-Aldrich), 63 µl of aqueous or ethanolic extract (distilled water used for the control), and 63 µl of xanthine oxidase (1.2 U/µL) (Sigma-Aldrich). Xanthine oxidase was added last. A blank was analyzed for each sample. The subsequent rate of NBT reduction was determined on the basis of sequential spectrophotometric determination of absorbance at 612 nm. The solutions were prepared daily and kept in the dark. The results are expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without sample (buffer only). Superoxide anion scavenging activities were calculated as [(1-(sample O.D.- blank O.D.) / (control O.D. - blank O.D.)) × 100]. IC_{50} was defined as the concentration at which 50% of NBT/superoxide anion was scavenged. Allopurinol (Sigma-Aldrich) was used as the positive control.

Viability and anti-inflammatory activities

Cell culture

RAW 264.7 macrophages and THP-1 human monocytic leukemia cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Each cell type was grown in a culture flask, then maintained at 37 °C in a humidified atmosphere (5% CO_2) in DMEM (Sigma-Aldrich) and RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and antibiotics, 100 IU/mL penicillin G and 100 mg/mL streptomycin (Gibco BRL, Grand Island, NY, USA) as previously described (Mosmann, 1983) and were used after cell counting with a hemocytometer.

Cell viability

Before the biological assay, cytotoxicity was measured by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Sigma-Aldrich) to formazan as previously described.(Mosmann, 1983) After cells were precultured in wells of a 96-well plate for 2 h, the cells were treated with the samples (12.5, 25. 50 and 100 μg/mL or μM). The cells were incubated for 24 h, the medium was replaced with fresh medium containing 0.5 mg/mL MTT, and incubation was continued for a further 4 h at 37 °C. The medium was removed and the produced MTT-formazan was dissolved in 200 μL dimethylsulfoxide. The reduction of MTT to formazan within the cells was quantified by measuring the absorbance at 540 nm using an ELISA reader (TECAN) as previously described.(Feelisch & Stamler, 1996; Park et al., 2005) Cytotoxicity was calculated as cell viability (%) = sample O.D. / blank O.D. × 100.

Inhibition of nitric oxide (NO) production

RAW 264.7 macrophages were cultured in wells of a 96-well plate and incubated for 2 h at 37 °C in a humidified atmosphere (5% CO_2). The cells were then incubated in a medium containing 0.1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich). After incubation for an additional 24 h, NO was analyzed using the Griess assay. Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4; Sigma-Aldrich) was added to each supernatant. L-NG-monomethyl Arginine citrate (L-NMMA) was used as the positive control. NO content was determined at 540 nm against a standard sodium nitrite curve as previously described.(Feelisch & Stamler, 1996; Park et al., 2005) NO production inhibitory activity was calculated as inhibition rate (%) = [1- (sample O.D- blank O.D.) / (control O.D. - blank O.D.)] × 100. IC_{50} was defined as the concentration inhibiting 50% of NO production.

Inhibition of pro-inflammatory cytokine production

THP-1 cell culture. The mature macrophage-like state was induced by treating THP-1 monocytes (10^5 cells/mL) for 48 h with 10 nmol 12-O-tetradecanoyl- phorbol-13-acetate (TPA; Sigma-Aldrich) in wells of 24-well plates with 1 mL cell suspension in each well. Differentiated plastic-adherent cells were washed once with phosphate buffered saline (PBS) and fresh RPMI 1640 (Sigma-Aldrich) containing 10% FBS, 100 IU/ml penicillin G. (Gibco BRL) was added. Differentiated THP-1 cells were incubated for 1 h at 37 °C in a humidified atmosphere (5% CO_2) prior to incubation for 24 h in a medium containing 0.1 μg/mL LPS (Sigma-Aldrich). Each supernatant was stored in an Eppendorf tube at 37℃

Inhibitory activity of pro-inflammatory cytokine production by ELISA. Cytokine concentration of interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) were determined in the supernatants recovered from cultured THP-1 cells by enzyme-linked immunosorbent assay (ELISA). After THP-1 cells were exposed to LPS, the cytokine levels from these cells were measured for the inhibitory effect of those compounds (**1**-**6**) (50 μM). LPS and TPA treated cells comprised the control. Cytokine contents were quantified by measuring the absorbance at 405 nm using an ELISA reader (TECAN). The amount of produced cytokines was calculated using a standard calibration curve as described previously.(Lee et al., 2011)

Anti-skin ageing activity

Material

Anti-skin ageing activities were experimented with five samples (compounds **1**-**4** and extract). Stock solution was prepared with solvent and stock concentration and then stored at -20°C.

Measurement of cell viability

Cell viability was determined using an MTT reduction assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases.(Mosmann, 1983) For experiments, HaCaT human keratinocyte cells (10^5 cell/well) were cultured in 24-well plates and the cells were treated with the samples (nontoxic 0.5, 1 and 2.5 μg/mL or μM). The cells were incubated for an additional 24 h, and the medium was replaced with fresh medium contained 5 mg/mL MTT and the incubation continued for a further 4 h at 37 °C. The medium was then removed and the MTT-formazan produced was dissolved in 200 uL dimethylsulfoxide. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance at 540 nm using a spectrophotometer (Thermo Fischer Scientific, Vantaa, Finland) as previously describe.(Mosmann, 1983; Feelisch & Stamler, 1996; Park et al., 2005) Cytotoxicity was calculated as cell viability (%) = sample O.D. / blank O.D.× 100.

Cell culture

CCD986sk human skin fibroblast cell line was purchased from American Type Culture Collection (ATCC, Manassas, Va, USA). Cells were grown as a monolayer culture in 75 cm2 plastic tissue culture flasks (Nunc, Rochester, NY, USA) in Dulbecco's modified eagle's medium (DMEM; Gibco BRL) with 10% FBS (Gibco BRL) and 1% antibiotic-antimycotic solution 100X (WelGENE Inc., Daegu, Korea). Cells were maintained at 37 °C in a 5% CO2 incubator. The cells (1 × 104 cell/well) were cultured in a 96-well plate and were treated with the samples (nontoxic 0.5, 1 and 2.5 μg/mL or μM) for 24 h.

RNA isolation and cDNA synthesis

Protein concentrations were determined using the 2X QuantiTect SYBR Green RT-PCR Master mix method (Qiagen) according to the manufacturer's instructions. This study

used to PCR primer from the basis of QuantiTect® primer assay. Real-time PCR conditions are summarized in Table.

Table: Real–time cycler conditions

Step	Time	Temperature
Reverse transcription	30 min	50 °C
PCR initial activation step	15 min	95 °C
Three-step cycling		
Denaturation	15 s	94 °C
Annealing	30 s	50-60 °C
Extension	30 s	72 °C
Number of cycles	40 cycles	

Reference

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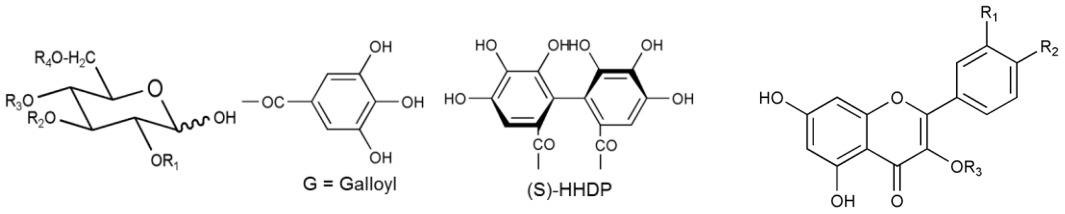
Park S, Hong S, Han X, et al. 2005. Inhibitory constituents of LPS-induced nitric oxide production from Arctium lappa., 11: 85-8.

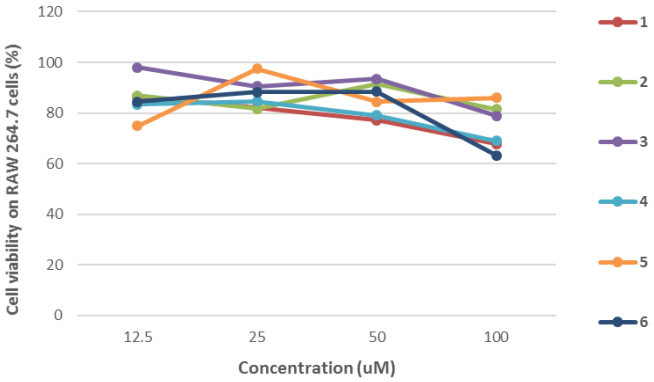
Figure S1. Chemical structures of the isolated compounds



	R_1	R_2	R_3	R_4
1	G	G	(S)-HHDP	
2	(S)-HHDP		(S)-HHDP	
3	H	G	(S)-HHDP	
4	G	G	H	H

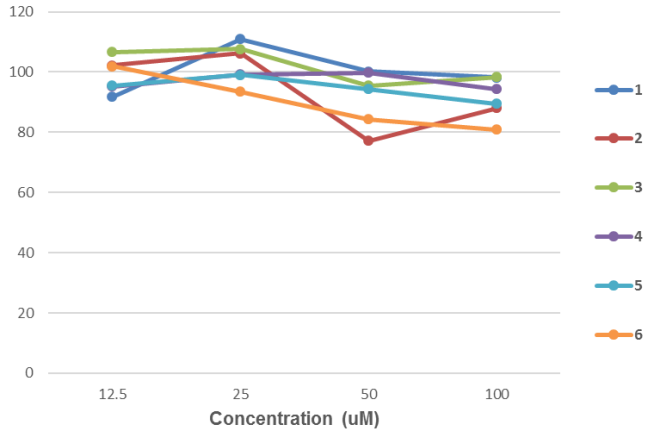
	R_1	R_2	R_3
5	H	OH	α-L-rhamnose
6	OH	OH	H

Figure S2. Viability effects of the compounds (**1**-**6**) on RAW 264.7 macrophage cells.



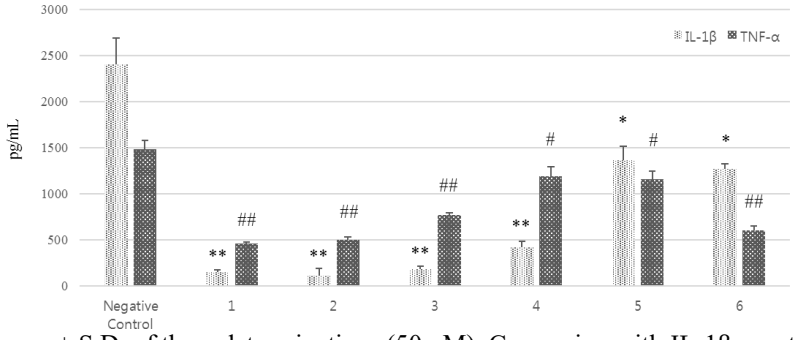
Results are expressed as percent of control absorbance. Values represent mean ± S.D. of three determinations.

Figure S3. Viability effects of the compounds (**1**-**6**) on human THP-1 cells.



Results are expressed as percent of control absorbance. Values represent mean ± S.D. of three determinations.

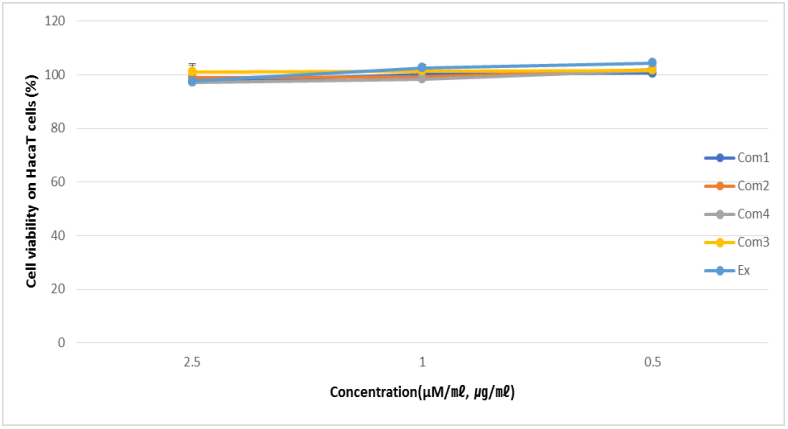
Figure S4. Effect of compounds (**1**-**6**) on IL-1β and TNF-α production.



Values represent mean ± S.D. of three determinations (50 μM). Comparing with IL-1β negative control group.

*: p<0.05, **: p<0.01. Comparing with TNF-α negative control group #: p<0.05, ##: p<0.01

Figure S5. Viability of human HaCat keratinocytes treated with compounds **1**-**4** and Extract from CT.



Cell viability was measured by the MTT assay.
Results are expressed as percent of control absorbance (0.5, 1.0, 2.5 μM).

Table S1. The anti-oxidative and anti-inflammatory activities on compounds (**1**-**6**)

Samples	IC_{50} (μM)		
	DPPH free radical scavenging activity	NBT/superoxide scavenging activity	NO production inhibitory activity
1	5.18±0.05[b)]	5.54±1.57[b)]	20.13±1.07 [b)]
2	4.39±0.17[b)]	2.36±0.66[a)]	24.82±3.41 [b)]
3	3.29±0.15[a)]	2.42±0.81[a)]	32.33±12.30 [a)]
4	8.28±0.59[c)]	2.64±0.07[a)]	73.79±1.58 [c)]
5	>100 [f)]	>100[e)]	>100 [f)]
6	30.06±1.13[e)]	12.42±2.17[d)]	22.38±1.15 [e)]
Ascorbic acid	19.43±0.67[d)]	-	-
Allopurinol	-	9.90±0.05[c)]	-
L-NMMA	-	-	44.36±1.18 [d)]

Values represent mean ± S.D. of three determinations. Different superscript letters indicate a significant difference (p value < 0.05).

Table S2. The anti-skin ageing activities on tannins (**1**-**4**) and extract.

Samples	μM (compound) or μg/mL (extract)				
	EC_{50}				IC_{50}
	PCOLCE	Elastin	TIMP-1	TIMP-2	MMP-1
1	2.28±0.40[a)]	2.29±0.38[a)]	-	0.57±2.12[a)]	2.38±0.28[b)]
2	2.23±2.18[a)]	2.74±1.13[a)]	4.15±1.89[a)]	0.89±0.20[a)]	2.51±0.36[b)]
3	2.66±0.51[a)]	1.98±0.49[a)]	6.61±0.65[b)]	1.49±0.97[a)]	1.92±0.55[b)]
4	-	1.75±1.65[a)]	7.42±6.49[b)]	-	1.14±0.27[a)]
Ex	3.80±3.85	-	4.21±0.90	3.15±1.73	1.73±0.25

Values represent mean ± S.D. of three determinations. Different superscript letters indicate a significant difference (p value < 0.05). -, Not Increased.