Anti-oxidative and Anti-inflammatory Effects of Flavonoids from the Silk of *Zea mays* Linn

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Activity-guided isolation of the silk of *Zea mays* Linn (ZMS) yielded three flavonoids, chrysoeriol-6-*C*-β-boivinopyranosyl-7-*O*-β-glucopyranoside (**1**), chrysoeriol (**2**) and luteolin (**3**). Anti-oxidative activity was measured using the 2,2-diphenyl-1-picryl-hydrazyl and superoxide dismutase-like superoxide anion (generated by xanthine oxidase/hypoxanthine) scavenging assays. Anti-inflammatory activity was confined by quantifying the protein and mRNA levels of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated HaCaT cells using western blotting and real-time polymerase chain reaction. The results showed that luteolin **3** exhibited potent 2,2-diphenyl-1-picryl-hydrazyl ($IC_{50} = 18.31 \pm 1.29$ μM) and superoxide dismutase-like superoxide anion scavenging activity ($IC_{50} = 1.47 \pm 0.14$ μM) and dose-dependently inhibited the protein expression of inducible nitric oxide synthase and cyclooxygenase-2 as well as their mRNA levels.

Key Words: *Zea mays* Linn, Flavonoid, Anti-oxidative, Anti-inflammatory, Inducible nitric oxide synthase, Cyclooxygenase-2.

INTRODUCTION

Reactive oxygen species such as superoxide anion, hydroxyl radical and peroxynitrite have been reported to play a critical role in the process of inflammation in various tissues. In skin, reactive oxygen species can be produced by chemical ionization, UV radiation and enzymatically by polymorpho-nuclear leukocytes that infiltrate the sites of infection[1]. Excessive reactive oxygen species cause extensive damage to DNA, proteins and lipids and have been reported to be pro-inflammatory mediators[2].

Inducible nitric oxide synthase and cyclooxygenase-2 are known to be key enzymes in inflammation and oxidative stress[3]. The inducible nitric oxide synthase produces a highly reactive nitrogen radical[4] (nitric oxide) and cyclooxygenase-2 produces prostaglandins that contribute to the pain of inflammation[5]. These two enzymes, which are related to pathological conditions of inflammation, are highly induced by pro-inflammatory cytokines, such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β)[6]. Thus, the inhibitory activities of these proteins and their mRNAs have proved useful in a variety cell culture studies and animal inflammatory models[7].

Zea mays Linn (ZMS) has been used as oriental traditional medicine for the treatment of dysuria, uricosuria, edema, cystitis, gout, kidney stones and nephritis[8,9]. Several flavonoids such as chrysoeriol 6-*C*-β-fucopyranoside, 2"-*O*-β-L-rhamnosyl-6-*C*-3"-deoxyglucosyl-3'-methoxyluteolin and maysin have been isolated from *Zea mays* Linn[10,11]. Anti-glycation activity and insecticide effects of *Zea mays* Linn, have been reported[12]. Furthermore, anti-oxidant activity and anti-inflammatory effects of *Zea mays* Linn extract have also been reported[13,14].

As part of our on-going screening program to search for new anti-oxidative and anti-inflammatory natural products[15], we examined the silk of *Zea mays* Linn (ZMS) for DPPH and SOD-like superoxide anion (generated by xanthine oxidase/hypoxanthine) scavenging activities and inhibition of the expression of inducible nitric oxide synthase and cyclooxygenase-2.

EXPERIMENTAL

Thin layer chromatography (TLC) was carried out using pre-coated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) and chloroform-methanol-water (80:20:2, 70:30:4 volume ratio). The spots were detected using UV (254 nm) and by spraying with $FeCl_3$ and 10 % H_2SO_4, followed by heating. The stationary phases for the column chromatographic isolation were performed on a Sephadex LH-20 column (25-10 μm, Pharmacia), a MCI-gel CHP 20P column (75-150 μm, Mitsubishi) and an YMC-gel ODS-A column (230-70 and 500-400 mesh, YMC Co.). Compounds were identified by [1]H- and

^{13}C NMR (Varian Gemini 2000, USA or Brucker Amx-500) and by mass spectrometry (MS) (API 3000 triple quadrupole mass spectrometry, Canada) and were characterized by comparisons with literature values.

In this study, we used Lot #2008042500210001 of *Zea mays* Linn (hot water extract), which was supplied by Kwang Dong Pharmaceutical Co. Ltd., in September 2008. The hot water extracts were the "tea of the silk of *Zea mays* Linn" (beverage goods of Kwang Dong Pharmaceutical Co. Ltd).

Extraction and isolation: The hot water extracts (Lot #2008042500210001) of *Zea mays* Linn were prepared with decompression filtration. The filtrate was then concentrated (627 g) and solvent fractionation was performed with ethyl acetate, butyl alcohol and water. The ethyl acetate fraction (62 g), which showed anti-oxidative activity with an IC_{50} value of 310.88 ± 1.39 μg/mL compared with the positive control, L-ascorbic acid ($IC_{50} = 14.75 \pm 3.87$ μg/mL) (data not shown), was applied to a Sephadex LH-20 column (25-100 μm, 2000 g, 10×90 cm) (Pharmacia, Uppsala, Sweden) and eluted with an H_2O-MeOH gradient to yield seven sub-fractions. Sub-fractions 3 and 4 showed anti-oxidative activity with IC_{50} values of 51.22 ± 2.36 μg/mL and 54.82 ± 3.47 μg/mL, respectively, compared with a positive control, L-ascorbic acid ($IC_{50} = 13.92 \pm 2.56$ μg/mL) (data not shown). Sub-fraction 3 was further separated on a Sephadex LH-20 column (25-100 μm, 400 g, 5×50 cm) (Pharmacia, Uppsala, Sweden) eluted with 60 % MeOH and a MCI-gel CHP 20P column (75-150 μm, 200 g, 3×25 cm) (Mitsubishi, Tokyo, Japan) eluted with 50 % MeOH. Prep-thin layer chromatography (PTLC) was carried out for final purification using pre-coated silica gel 60 F_{254} glass plates (Merck, Darmstadt, Germany) and chloroform-methanol-water (70:30:4, volume ratio) to yield chrysoeriol 6-*C*-*β*-boivinopyranosyl-7-*O*-*β*-glucopyranoside (**1**, 75 mg). Sub-fraction 4 was further separated on YMC-gel ODS-A (230/70 mesh, 300 g, 5×60 cm) (YMC, Japan) and eluted using a H_2O-MeOH gradient to yield two sub-fractions (4-1 and 4-2). PTLC was carried out for sub-fraction 4-1 using pre-coated silica gel 60 F_{254} glass plates (Merck, Darmstadt, Germany) and chloroform-methanol (10:1, volume ratio) to yield chrysoeriol (**2**, 30 mg). Sub-fraction 4-2 was further applied to Sephadex LH-20 column (150 μm, 100 g, 3×30 cm) (Pharmacia, Uppsala, Sweden) and eluted with EtOH-H_2O gradient to yield luteolin (**3**, 105mg).

Chrysoeriol 6-*C*-*β*-boivinopyranosyl-7-*O*-*β*-glucopyranoside (1): Yellow amorphous powder; negative LC-MS: m/z 594 [M-H]$^-$, ^{1}H NMR (600 MHz, DMSO-d_6+D_2O): δ 6.87 (1H, s, H-8), 6.46 (1H, s, H-3), 7.39 (1H, dd, J = 8.4, 1.8 Hz, H-6'), 7.16 (1H, d, J = 1.8 Hz, H-2'), 6.32 (1H, d, J = 8.4 Hz, H-5'), 5.24 (1H, dd, J = 13.2, 2.4 Hz, H-1"), 4.77 (1H, d, J = 7.2 Hz, H-1'''), 3.87 (1H, dd, J = 13.2, 6.6 Hz, H-5"), 3.83 (1H, d, J = 3.0 Hz, H-3"), 3.78 (1H, m, H-6a'''), 3.69 (3H, s, -OCH$_3$), 3.44 (2H in total, m, H-6b''' and H-5'''), 3.35 (1H, t, J = 8.4 Hz, H-2'''), 3.30 (1H, t, J = 9.0 Hz, H-3'''), 3.16 (1H, d, J = 3.6 Hz, H-4"), 3.12 (1H, t, J = 7.8 Hz, H-4"), 2.83 (1H, m, H-2a"), 1.21 (1H, d, J = 13.2 Hz, H-2b"), 1.02 (3H, m, -CH$_3$). ^{13}C NMR (150 MHz, DMSO-d_6+D_2O) : (Table-1).

Chrysoeriol (2): Yellow amorphous powder; negative LC-MS: *m/z* 302 [M-H]$^-$, ^{1}H NMR (300 MHz, DMSO-d_6) : δ

12.0 (1H, m, OH-5), 5.94 (1H, d, J = 1.8, H-8), 5.70 (1H, d, J = 1.8, H-6), 6.37 (1H, s, H-3), 7.35 (1H, dd, J = 8.4, 1.8 Hz, H-6'), 7.28 (1H, J = 1.8, H-2'), 6.32 (1H, d, J = 8.4 Hz, H-5'), 3.79 (3H, s, -OCH$_3$). ^{13}C NMR (75 MHz, DMSO-d_6): (Table-1).

Luteolin (3): Yellow amorphous powder; negative LC-MS: *m/z* 286 [M-H]$^-$, ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.0 (1H, m, OH-5), 6.54 (1H, d, J = 1.8, H-8), 6.26 (1H, d, J = 1.8, H-6), 6.59 (1H, s, H-3), 7.45 (1H, dd, J = 8.4, 2.4 Hz, H-6'), 7.50 (1H, d, J = 2.4, H-2'), 7.01 (1H, d, J = 8.4 Hz, H-5'). ^{13}C NMR (75 MHz, DMSO-d_6): (Table-1).

TABLE-1			
^{13}C-NMR DATA OF COMPOUNDS **1-3**			
Carbon number	[a]**1** δ (ppm)	[b]**2** δ (ppm)	[b]**3** δ (ppm)
C-2	166.1	162.2	165.1
C-3	99.0	100.7	104.1
C-4	181.1	179.8	183.0
C-5	162.8	161.0	162.8
C-6	112.6	99.6	99.6
C-7	162.8	162.2	165.2
C-8	94.4	95.9	94.7
C-9	156.4	158.2	158.8
C-10	105.1	100.7	105.1
C-1'	123.5	120.8	123.4
C-2'	108.7	109.0	113.9
C-3'	151.0	149.2	146.5
C-4'	151.0	149.2	150.2
C-5'	118.4	116.7	116.5
C-6'	123.5	120.8	119.9
-OCH$_3$	55.6	55.7	
Sugar	(Boivinose)		
C-1"	65.2		
C-2"	30.5		
C-3"	67.6		
C-4"	69.5		
C-5"	70.4		
6"-CH$_3$	17.6		
Sugar	(Glucose)		
C-1'''	102.6		
C-2'''	74.0		
C-3'''	77.5		
C-4'''	70.4		
C-5'''	77.9		
C-6'''	62.1		

[a]^{13}C NMR (150 MHz, DMSO-d_6) [b]^{13}C NMR (75 MHz, DMSO-d_6)

DPPH radical scavenging activity: Anti-oxidative activities were measured using 1,1-diphenyl-2-picrylhydrzyl (DPPH) (Sigma, St. Louis, MO) scavenging activities[16]. Briefly, 20 μL of the compounds in absolute EtOH was added to 180 μL of DPPH solution (0.1 mM, in absolute EtOH). After gentle mixing, the reactions were allowed to stand for 0.5 h and the optical densities were measured at 492 nm using an ELISA reader (TECAN, Salzburg, Austria). Free radical scavenging activities were calculated as [(1-(sample O.D./control O.D.) × 100] and were expressed as IC_{50} values, which were defined as the concentrations at which 50 % of DPPH free radicals were scavenged. L-ascorbic acid was used as a positive control.

Superoxide anion scavenging activity: A reaction mixture with a final volume of 632 μL was prepared with 50 mM phosphate buffer (pH 7.5) containing EDTA (0.05 mM), hypoxanthine (0.2 mM), 63 μL nitro-blue tetrazolium (1 mM)

(Sigma, St. Louis, MO), 63 µL of aqueous or ethanolic extract (distilled water for the control) and 63 µL of xanthine oxidase (1.2 U/µL) (Sigma, St. Louis, MO) in an eppendorf tube. The xanthine oxidase was added last. For each sample, a blank was carried out. The subsequent rate of nitro-blue tetrazolium reduction was determined on the basis of sequential spectro-photometric determinations of absorbance at 560 nm. The solutions were prepared daily and kept from light. The results are expressed as the percentage inhibition of the nitro-blue tetrazolium 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] and were expressed as IC_{50} values, which were defined as the concentrations at which 50 % of nitro-blue tetrazolium/Superoxide anions were scavenged[17]. Allopurinol (Sigma, St. Louis, MO) was used as a positive control.

Cell culture: HaCat human keratinocyte cells were grown in a 75 cm^2 flask in a 5 % CO_2 atmosphere at 37 ℃ in DMEM medium (Sigma, St. Louis, MO) containing 10 % fetal bovine serum, 100 IU/mL penicillin G and 100 mg/mL streptomycin (Gibco BRL, Grand Island, NY).

Western blotting: HaCaT human keratinocyte cells were cultured in 6-well plates and then treated with or without 10 µg/mL of LPS and samples (10 or 100 µM) for 24 h. Cells were then lyzed in a buffer containing 50 mM *tris*-Cl (pH 8.0), 150 mM NaCl, 0.02 % sodium azide, 100 µg/mL phenylmethanesulfonyl fluoride (PMSF), 1 µg/mL aprotinin and 1 % Triton X100 and centrifuged at 12,000 rpm for 0.5 h at 4 ℃. Supernatants were then transferred to new tubes and 30 µg of soluble protein was subjected to 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a sample buffer containing 1 M *tris*, 50 % glycerol, 10 % SDS, mercaptoethanol and 1 % bromophenol blue. Samples were heated at 95 ℃ for 5 min prior to gel loading. For inducible nitric oxide synthase and cyclooxygenase-2 detection, separated proteins in gels were transferred to nitrocellulose membranes (Osmonics, Milwaukee, WI) at 0.16 A for 1 h. Membranes were washed 3 times with *Tris*-buffered saline Tween-20 (TBS/T), blocked with 5 % skimmed milk for 1 h at room temperature, incubated overnight at 4 ℃ with either goat anti-human inducible nitric oxide synthase, cyclooxygenase-2 and β-actin polyclonal anti-bodies (1:1000 in 5 % bovine serum albumin, Santa Cruz, Delaware, CA) and then washed 3 times with TBS/T. The secondary mouse anti-goat peroxidase conjugated anti-bodies (1:2000 in blocking solution, Santa Cruz, Delaware, CA) were incubated for 1 h at room tempe-rature. After washing membranes with TBS/T, they were developed with electrochemiluminescence (ECL) solution (Santa Cruz, Delaware, CA) for 3 min and then exposed to X-ray film (Roche, Indianapolis, IN). The OD values for the channel signals, measured by Gelquant software (MiniBIS Pro, Jerusalem, Israel), were normalized to the OD values for the GAPDH signals; the ratios are expressed as arbitrary units for quantitative comparison.

Reverse transcription polymerase chain reaction: Poly-merase chain reaction primers were synthesized using Gene Bank data and a DNA synthesizer (Pharmacia, Björkgatan, Uppsala, Sweden). Their sequences were as follows: inducible

nitric oxide synthase (123bp): 5'-ACC AAC TGA CGG GAG ATG AG -3' (sense), 5'- ATA GCG GAT GAG CTG AGC AT-3' (anti-sense) / cyclooxygenase-2 (210bp): 5'-TGA AAC CCA CTC AAA CAC A-3' (sense), 5'-AAC TGA TGC GTG AAG TGC TG-3' (anti-sense) / GAPDH (593bp): 5'-ATT GTT GCC ATC AAT GAC CC-3' (sense), 5'-AGT AGA GGC AGG GAT GAT GT -3' (anti-sense).

HaCaT human keratinocyte cells were treated as described above for Western blotting. Total RNA was isolated from cultured HaCaT cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and cells were added to 1 mL of TRIzol re-agent in a cultured dish. After 5 min at room temperature, 0.2 mL of chloroform was added per 1 mL of TRIzol reagent, tubes were shaken vigorously by hand for 15 sec and then incubated at 15 ℃ to 30 ℃ for 3 min. Mixtures were centrifuged at 12,000 rpm for 15 min at 4 ℃, after which upper aqueous phases were transferred to fresh tubes and same amounts of 2-propanol were added. Mixtures were then incubated at 4 ℃ for 15 min and centrifuged at 12,000 rpm for 15 min at 4 ℃. The supernatant was removed and then RNA pellet was washed in 500 µL of 70 % ethanol with 12,000 rpm at 4 ℃ for 5 min. The purified RNA samples obtained were dissolved in 30 µL of diethyl pyrocarbonate-distilled water (DEPC-DW) and 3 µg aliquots of total cellular RNA was reverse-transcribed at 42 ℃ for 0.5 h in 20 µL volumes containing 1 µL reverse transcriptase (Takara, Shiga, Japan), 10X buffer 2 µL, 10 mM dNTP 2 µL (dNTP mix), oligo dT primer 1 µL, RNase inhibitor 0.5 µL and 25 mM $MgCl_2$ 4 µL. cDNA samples (2 µL) obtained by RT-PCR were amplified by PCR in a 25 µL-vessel containing 10X buffer 2.5 µL, 25 mM $MgCl_2$ 2.5 µL and 10 pmol 0.75 µL primer. Thermal cycle profiles were as follows 94 ℃ for 5 min: 35 cycles of 94 ℃ for 1 min, 59 ℃ for 1 min, 72 ℃ for 1 min: final extension step of 72 ℃ for 10 min. A densitometer was used for quantitative analysis of the PCR products of inducible nitric oxide synthase, cyclooxygenase-2 and GAPDH on DIG chemiluminescent film (volume of inducible nitric oxide synthase, cyclooxygenase-2/volume of GAPDH).

Statistical analysis: All data are expressed as means ± SD. Analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (S-N-K) test. Results were considered significantly different when p values were less than 0.05. Values bearing different superscripts indicate significant differences.

RESULTS AND DISCUSSION

Activity guided isolation of the hot water extract of *Zea mays* Linn afforded three flavonoid derivatives (**1-3**). Compounds (**1-3**) were identified as chrysoeriol-6-*C*-β-boivino pyranosyl-7-*O*-β-glucopyranoside[18], chrysoeriol[19] and luteolin[20], respectively, by comparing their spectral (MS, NMR) data with literature values (Fig. 1).

Anti-oxidative activities of compounds (**1-3**) were evaluated by determining their DPPH and SOD-like superoxide anion scavenging activity. DPPH radicals readily accept hydrogen due to the unstable nature of the nitrogen, thus anti-oxidative activity can be measured by quantifying the loss of a violet colour when DPPH accepts hydrogen of an anti-oxidant[16]. And, SOD-like superoxide anion scavenging activity was determined by the nitro-blue tetrazolium (NBT) reduction method. In this

method, superoxide reduces the yellow dye (NBT^{2+}) to produce blue formazan. Anti-oxidants are able to inhibit the purple nitro-blue tetrazolium formation[17]. Among these compounds, compound **3** exhibited a potent DPPH free radical scavenging activity with an IC_{50} value of 13.85 ± 0.23 μM, compared with the positive control, L-ascorbic acid ($IC_{50} = 18.31 \pm 1.29$ μM) (Table-2). Compound **3** also exhibited potent SOD-like superoxide anion scavenging activity with an IC_{50} value of 1.64 ± 0.07 μM, compared with the positive control, allopurinol ($IC_{50} = 1.47 \pm 0.14$ μM) (Table-2). These anti-oxidative activities might be due to the *ortho*-dihydroxy-phenolic moiety, which enables a hydrogen atom to be donated to an active free radical[21].

Fig. 1. Chemical structures of compounds **1-3** isolated from *Zea mays* Linn
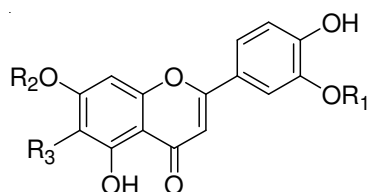
	R₁	R₂	R₃
1	CH₃	Glucose	Boivinose
2	CH₃	H	H
3	H	H	H

TABLE-2
DPPH RADICAL SCAVENGING ACTIVITIES AND SUPEROXIDE ANION SCAVENGING ACTIVITIES BY COMPOUNDS **1-3.** VALUES REPRESENT MEANS ± SD OF THREE DETERMINATIONS. VALUES BEARING DIFFERENT SUPERSCRIPTS IN THE SAME COLUMNS ARE SIGNIFICANTLY DIFFERENT ($p < 0.05$)

Compounds	DPPH radical scavenging activity IC₅₀ (μM)	Superoxide anion scavenging activity IC₅₀ (μM)
1	100 <[a]	100 <[a]
2	100 <[a]	4.99 ± 0.06[b]
3	13.85 ± 0.23[b]	1.64 ± 0.07[b]
L-Ascorbic acid	18.31 ± 1.29[b]	-
Allopurinol	-	1.47 ± 0.14[b]

The NO production is related to induction of cyclooxygenase-2 and inducible nitric oxide synthase expression in various inflammatory diseases. Therefore, in order to elucidate the mechanism responsible for the expressions, inducible nitric oxide synthase, cyclooxygenase-2 proteins and its mRNA were evaluated to prove the anti-inflammatory activities of the compounds (**1-3**) by western blotting and RT-PCR, respectively. L-ascorbic acid, which is known to inhibit expressions of inducible nitric oxide synthase and cyclooxygenase-2, was used as a positive control[22,23].

The results showed that luteolin (**3**) inhibited the expressions of inducible nitric oxide synthase and cyclooxygenase-2 proteins and mRNAs. Among the compounds, luteolin (**3**), which is a flavonol, has a double bond at position C2-C3 of the C ring with a ketone-function at position 4, along with OH groups at positions 3' and 4' of the B ring (Fig. 1), which have been related to the inhibition of LPS-stimulated TNF-α release, inhibited the expressions of inducible nitric oxide synthase and cyclooxygenase-2 proteins and its mRNAs (Figs.

2 and **3**)[22,24]. Furthermore, the results showed that aglycone types (**2-3**) have more potent suppressive effects on inducible nitric oxide synthase and cyclooxygenase-2 than glycoside type (**1**) (Figs. 2 and 3) and these compounds (**2-3**) might play important roles in the anti-inflammatory effects of *Zea mays* Linn.

Conclusion

Three flavonoids, chrysoeriol-6-*C*-*β*-boivinopyranosyl-7-*O*-*β*-glucopyranoside (**1**), chrysoeriol (**2**) and luteolin (**3**) were isolated from *Zea mays* Linn. These flavonoid (**1-3**) showed inhibitory effects against expressions of inducible nitric oxide synthase and cyclooxygenase-2 proteins and mRNAs., Also, **3** which possess a *ortho*-dihydroxy-phenolic moiety, exhibited
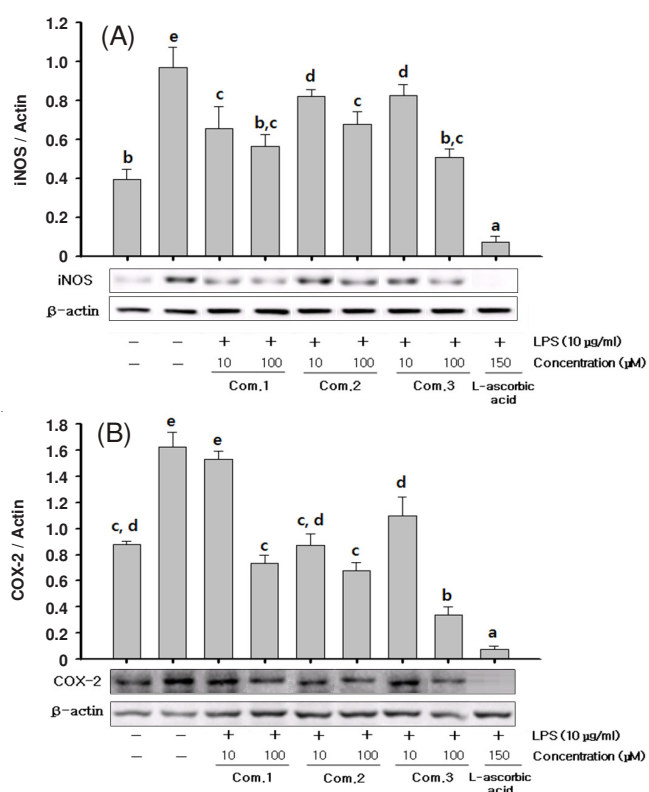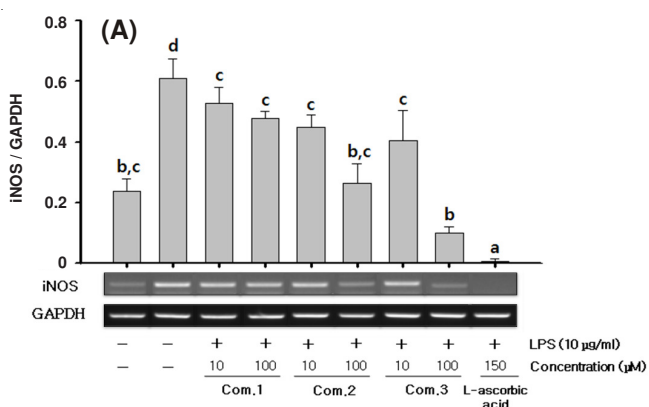

Fig. 2. Effects of compounds **1-3** on the expressions of iNOS and COX-2 proteins in LPS-induced HaCaT cells. HaCaT cells were cultured in the presence of the indicated compounds for 24 h. The expressions of iNOS (A) and COX-2 (B) were quantified by western blotting. Results are expressed as means ± SD for triplicate results. Values bearing different superscripts in the same columns are significantly different ($p < 0.05$)
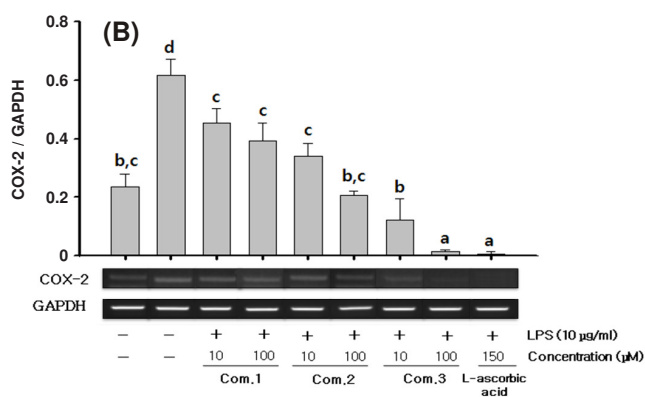
Fig. 3.   Effects of compounds **1-3** on the expressions of iNOS and COX-2 mRNA in LPS-treated HaCaT cells. HaCaT cells were cultured with the indicated samples for 24 h and then the expressions of iNOS (A) and COX-2 (B) mRNA were quantified by RT-PCR. Results are expressed as means ± SD for triplicate results. Values bearing different superscripts in the same columns are significantly different ($p < 0.05$)

potent DPPH radical and SOD-like superoxide anion scavenging activity. Thus, *Zea mays* Linn and the active compound **3** could potentially be developed as anti-oxidative, anti-inflammatory ingredients.

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