Quantitative analysis and anti-inflammatory effects of *Gleditsia sinensis* thorns in RAW 264.7 macrophages and HaCaT keratinocytes

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Abstract. *Gleditsia sinensis* thorns have traditionally been used to treat edema and carbuncles and drain abscesses. In the present study, a simultaneous analysis of four flavonoids [(+)-catechin, (-)-epicatechin, eriodictyol and quercetin] and two phenolic compounds (caffeic acid and ethyl gallate), obtained from a 70% ethanol extract of *G. sinensis,* was performed using high-performance liquid chromatography-photodiode array techniques. In addition, the inhibitory activities of the solvent fractions from a *G. sinensis* extract and its major constituents on the lipopolysaccharide-stimulated production of inflammatory mediators by macrophage RAW 264.7 cells and the tumor necrosis factor (TNF)-α and interferon (IFN)-γ (TI)-stimulated production of chemokines by HaCaT keratinocyte cells were investigated. The established analytical method showed high linearity, with a correlation coefficient of ≥0.9998. The limits of detection and quantification of the six compounds were 0.037-0.425 and 0.124-1.418 μg/ml, respectively. The ethyl acetate fraction inhibited nitric oxide and prostaglandin E_2 production in RAW 264.7 cells and the production of thymus- and activation-regulated chemokine (TARC) in HaCaT cells more than did the other fractions. Furthermore, the six compounds reduced the production of TARC, macrophage-derived chemokine and regulated on activation normal T-cell expressed and secreted in TI-stimulated HaCaT cells; in particular, ethyl gallate and quercetin exhibited a significant dose-dependent inhibition. Further elucidation of the signaling pathways involved in the T-helper cell 2 chemokine inhibition by *G. sinensis* is necessary to facilitate the design of therapeutic agents for the inflammatory response.

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Introduction

The inflammatory response is a complex reaction of the immune system that is regulated by numerous inflammatory mediators, such as nitric oxide (NO), cytokines/chemokines and prostaglandins (1). These mediators are known to contribute to the regulation of a wide array of physiological and pathological processes following an immune response and inflammation (1,2). The role of macrophages in mediating numerous different immunopathological phenomena during inflammation, including the overproduction of inflammatory cytokines and mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, NO and prostaglandin E_2 (PGE_2) is crucial. NO is synthesized by inducible NO synthase (iNOS), and PGE_2 is synthesized by cyclooxygenase-2 (COX-2) (3,4).

Inflammatory chemokines play a fundamental role in attracting a diverse set of effector leukocytes to sites of inflammation (5). Various chemokines have been shown to affect the immunopathology of inflammatory skin diseases. The thymus- and activation-regulated chemokine (TARC/CCL17) has been shown to be upregulated by keratinocytes in atopic dermatitis lesional skin (6); therefore, the modulation of TARC production in keratinocytes may contribute to the pathological processes of inflammatory skin diseases, such as atopic dermatitis. The serum level of the macrophage-derived chemokine (MDC/CCL22) has been reported to be significantly elevated in patients with atopic dermatitis, and increased levels of these chemokines were associated with increased disease severity (7). Furthermore, enhanced production of regulated upon activation normal T-cell expressed and secreted (RANTES/CCL5) has been found in inflammatory diseases of the skin, including atopic dermatitis and psoriasis (8).

The thorns of *Gleditsia sinensis* (Leguminosae) are widely used in traditional Chinese and Korean medicine for the treatment of several diseases, including tumor-related diseases, obesity and thrombosis (9). Previous studies have investigated the biological activity of the chemical constituents of

G. sinensis thorns, such as stigmasterol (10), ellagic acid glycosides (11), flavonoids (12) and lupane acid (13); however, the anti-inflammatory effects of *G. sinensis* thorn extracts remain unclear. The aim of the present study, therefore, was to investigate the anti-inflammatory activities of the solvent fractions from a *G. sinensis* extract and its major constituents on the lipopolysaccharide (LPS)-stimulated production of inflammatory mediators by RAW 264.7 macrophages and the TNF-α and IFN-γ (TI)-stimulated production of chemokines by HaCaT keratinocytes.

Materials and methods

Plant material. Thorns of *G. sinensis* were purchased in October 2008 from Omniherb (Yeongcheon, Republic of Korea). The origin of these materials was confirmed taxonomically by Professors Je-Hyun Lee (Dongkuk University, Gyeongju, Republic of Korea) and Young-Bae Seo (Daejeon University, Daejeon, Republic of Korea). A voucher specimen (2008-ST22) has been deposited at the K-herb Research Center, Korea Institute of Oriental Medicine (Daejeon, Republic of Korea).

Extraction and solvent fractionation. The dried thorns of *G. sinensis* (10 kg) were extracted three times with 70% ethanol by sonication for 1 h. The extracted solution was filtered through filter paper and evaporated to dryness (450 g). The ethanol extract was suspended in water (1 liter) and then successively partitioned with *n*-hexane, ethyl acetate and *n*-butanol (1.5 liters each, three times) to give extracts of 33.8, 103.2 and 119.1 g, respectively.

Chemicals and reagents. (-)-Epicatechin, ethyl gallate and quercetin (all with purity ≥99.0%) were purchased from ChromaDex, Inc., (Santa Ana, CA, USA). Caffeic acid, eriodictyol and (+)-catechin (all with purity ≥99.0%) were obtained from Acros Organics (Fair Lawn, NJ, USA), Extrasynthèse S.A. (Genay, France) and Fluka AG (Buchs, Switzerland), respectively. Methanol, high-performance liquid chromatography (HPLC)-grade reagents, water and acetonitrile were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Glacial acetic acid was of analytical reagent grade and was procured from Junsei Chemical Co. (Tokyo, Japan).

Chromatographic conditions. This analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), which consisted of an on-line degasser, a solvent-delivery unit, an autosampler, a photoiode array (PDA) detector and a column oven. The data processor employed the LC solution software (version 1.24; Shimadzu Co.). A Gemini® C18 analytical column (250x4.6 mm; particle size 5 μm; Phenomenex, Torrance, CA, USA) was used. Solvent A (1.0%, v/v, aqueous acetic acid) and solvent B (acetonitrile with 1.0%, v/v, acetic acid) comprised the mobile phases. The gradient flow was as follows: 0-50 min, 5-70% solution B; 50-55 min, 70-100% solution B; 55-60 min, 100% solution B; 60-65 min, 100-105% solution B, 65-80 min, 5% solution B. The column temperature was maintained at 40˚C. The analysis was carried out at a flow rate of 1.0 ml/min with PDA detection from 280 nm. The volume of the injection was 10 μl.

Preparations of sample and standard solutions. Standard stock solutions of four flavonoids [(+)-catechin, (-)-epicatechin, eriodictyol and quercetin)] and two phenolic compounds (caffeic acid and ethyl gallate) were dissolved in methanol at a concentration of 1.0 μg/ml and maintained at <4˚C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. The extracts (40 mg) of each lyophilized fraction, such as 70% ethanol, *n*-hexane, ethyl acetate, *n*-butanol and water fractions, were each dissolved in 70% ethanol (20 ml, respectively). The solutions were filtered through a 0.2-μm syringe filter (Woong Ki Science Co., Ltd, Seoul, Republic of Korea).

Calibration curves and limits of quantification (LOQ) and detection (LOD). All calibration curves were obtained by the assessment of peak areas from standard solutions in the seven different concentration ranges, from 0.78 to 50.00 μg/ml. The LOD and LOQ data were determined based on signal-to-noise ratios of 3 and 10, respectively.

Cell culture. The HaCaT human keratinocyte and RAW 264.7 murine macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (HaCaT) or 5.5% (RAW 264.7) heat-inactivated fetal bovine serum (Gibco-BRL), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37˚C in a 5% CO_2 incubator.

Cytotoxicity assay. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. RAW 264.7 ($3x10^3$ cells/well) and HaCaT ($1x10^3$ cells/well) cells were incubated in 96-well plates with various concentrations of the test materials for 24 h. Following the addition of CCK-8 reagent to each well the cells were further incubated for 4 h. A Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure absorbance at 450 nm, and the cell viability percentage was calculated using the following formula: Cell viability (%) = [(mean absorbance in test wells - mean absorbance in blank wells)/(mean absorbance in control wells - mean absorbance in blank wells)] x 100.

Measurement of PGE_2 and NO production. RAW 264.7 cells were plated in 48-well plates at a density of $2.5x10^5$ cells/well and incubated overnight. The cells were then treated with LPS (1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of various concentrations of the test materials. Following incubation for 24 h, ELISA kits were used to analyze the supernatants for the levels of PGE_2 (Cayman Chemical Co., Ann Arbor, MI, USA) and NO (Griess Reagent System; Promega Biotech Co., Ltd., Madison, WI, USA), according to the manufacturers' instructions. N(G)-Monomethyl-L-arginine (L-NMMA; Sigma-Aldrich) and indomethacin (Sigma-Aldrich) were used as positive controls to inhibit the production of NO and PGE_2, respectively.

Measurement of chemokine production. HaCaT cells ($1x10^6$ cells/well) were cultured in six-well plates in medium

Table I. Linearities, correlation coefficients, LODs and LOQs of the six constituents (n=3).

Compound	Linear range (μg/ml)	Regression equation[a]	Correlation coefficient (r^2)	LOD[b] (μg/ml)	LOQ[c] (μg/ml)
(+)-Catechin	0.78-50.00	y=2,779.71*x*+274.75	0.9999	0.425	1.418
Caffeic acid	0.78-50.00	y=34,405.46*x*-9,908.57	0.9998	0.039	0.132
(-)-Epicatechin	0.78-50.00	y=7,859.71*x*+1,909.87	0.9999	0.130	0.433
Ethyl gallate	0.78-50.00	y=24,543.65*x*+2,271.17	0.9999	0.051	0.171
Eriodictyol	0.78-50.00	y=34,836*x*+6,410.25	0.9999	0.037	0.124
Quercetin	0.78-50.00	y=13,439.20*x*-676.53	0.9998	0.118	0.395

[a]*y*, peak area (mAU) of constituents; *x*, concentration (μg/ml) of constituents. [b]LOD = 3 x signal-to-noise ratio. [c]LOQ = 10 x signal-to-noise ratio. LOD, limit of detection; LOQ, limit of quantification.

containing 10% fetal bovine serum. After reaching confluence, the cells were washed and treated with the test materials in 1 ml serum-free medium containing TI (10 ng/ml TNF-α and 10 ng/ml IFN-γ; R&D Systems Inc., Minneapolis, MN, USA) for 24 h. The supernatants of the cells were harvested, and the production of TARC, MDC and RANTES was quantified using ELISA (R&D Systems, Inc.), which was performed according to the manufacturer's instructions.

Statistical analysis. All experiments of the present study were performed at least in triplicate. Significant differences between the treatment groups were identified using one-way analysis of variance and multigroup comparisons were performed using the Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Calibration curves, LOD and LOQ. The linearity of the peak area (y) versus concentration (x, μg/ml) curve for each component was used to calculate the contents of the six constituents in each fraction of the *G. sinensis* extract. The correlation coefficients (r^2) of the calibration curves of the six constituents were ≥0.9998. The line equations and r^2 of the calibration curves are shown in Table I. The ranges of LOD and LOQ were 0.037-0.425 and 0.124-1.418 μg/ml, respectively (Table I).

HPLC analysis. Good separation chromatograms were obtained using mobile phases consisting of 1.0% (v/v) aqueous acetic acid (solvent A) and acetonitrile with 1.0% (v/v) acetic acid (solvent B). Quantification was achieved using PDA detection at 280 nm, based on retention times and ultraviolet spectra. Fig. 1 shows the representative HPLC chromatogram of the standards and each fraction of the *G. sinensis* extract, with detection of eluents at 280 nm. Reproducibility was assessed by measuring repeatedly the retention times and peak areas of six independently prepared samples of analytes. The reproducibility of the six compounds [(+)-catechin, caffeic acid, (-)-epicatechin, eriodictyol, ethyl gallate and quercetin] was less than the relative standard deviation (RSD) 1.5% for peak responses and less than the RSD 0.2% for retention times (data not shown). The contents of the six constituents are summarized in Table II.

G. sinensis extract inhibits NO and PGE_2 production in RAW 264.7 cells and TARC production in HaCaT cells. The cytotoxic effect of the *G. sinensis* extract on RAW 264.7 and HaCaT cells was measured first. The cells were exposed to various concentrations (2-200 μg/ml) of the *G. sinensis* extract for 24 h. The non-toxic concentrations of the test materials were used in subsequent experiments (data not shown). To determine the effects of the *G. sinensis* extract on NO and PGE_2 production in LPS-stimulated RAW 264.7 cells, the cells were treated with different concentrations of the *G. sinensis* extract (25, 50 and 100 μg/ml) and then stimulated by LPS (1 μg/ml) for 24 h. The *G. sinensis* extract suppressed LPS-stimulated NO production in a dose-dependent manner (Fig. 2A). LPS greatly stimulated NO production (5.846±0.220 nM) in RAW 264.7 cells, whereas the *G. sinensis* extract significantly decreased NO production [1.872±0.513 μM (P<0.01) at a dose of 25 μg/ml, 0.974±0.128 μM (P<0.01) at a dose of 50 μg/ml and 0.205±0.020 μM (P<0.01) at a dose of 100 μg/ml] compared with that observed in LPS-stimulated RAW 264.7 cells. Furthermore, LPS-stimulated RAW 264.7 cells exhibited increased PGE_2 production compared with the controls, whereas the *G. sinensis* extract decreased the production of PGE_2 relative to the production observed in the LPS-stimulated RAW 264.7 cells (Fig. 2B). The effects of the *G. sinensis* extract on TARC production in TI-stimulated HaCaT cells were assessed by treating the cells with different concentrations of the *G. sinensis* extract (50, 100 and 200 μg/ml) and then initiating TI stimulation for 24 h. The *G. sinensis* extract suppressed TI-stimulated TARC production in a dose-dependent manner (Fig. 2C). TI-treated cells showed significantly increased production of TARC (28.27±0.900 ng/ml) relative to that observed in the control cells (P<0.01). This increase was inhibited to 22.44±0.030 ng/ml (P<0.01) and 10.23±0.480 ng/ml (P<0.01) by the administration of the *G. sinensis* extract at 100 and 200 μg/ml, respectively.

Fractions of the G. sinensis extract inhibit NO and PGE_2 production in RAW 264.7 cells and TARC production in HaCaT cells. Fractions of the *G. sinensis* extract were tested to determine whether each fraction inhibited the production of NO and PGE_2 in LPS-treated RAW 264.7 cells and the production of TARC in HaCaT cells treated with TI. NO production is the hallmark of the activation of macrophages. In the present experiment, NO production (4.308±0.225 μM) was

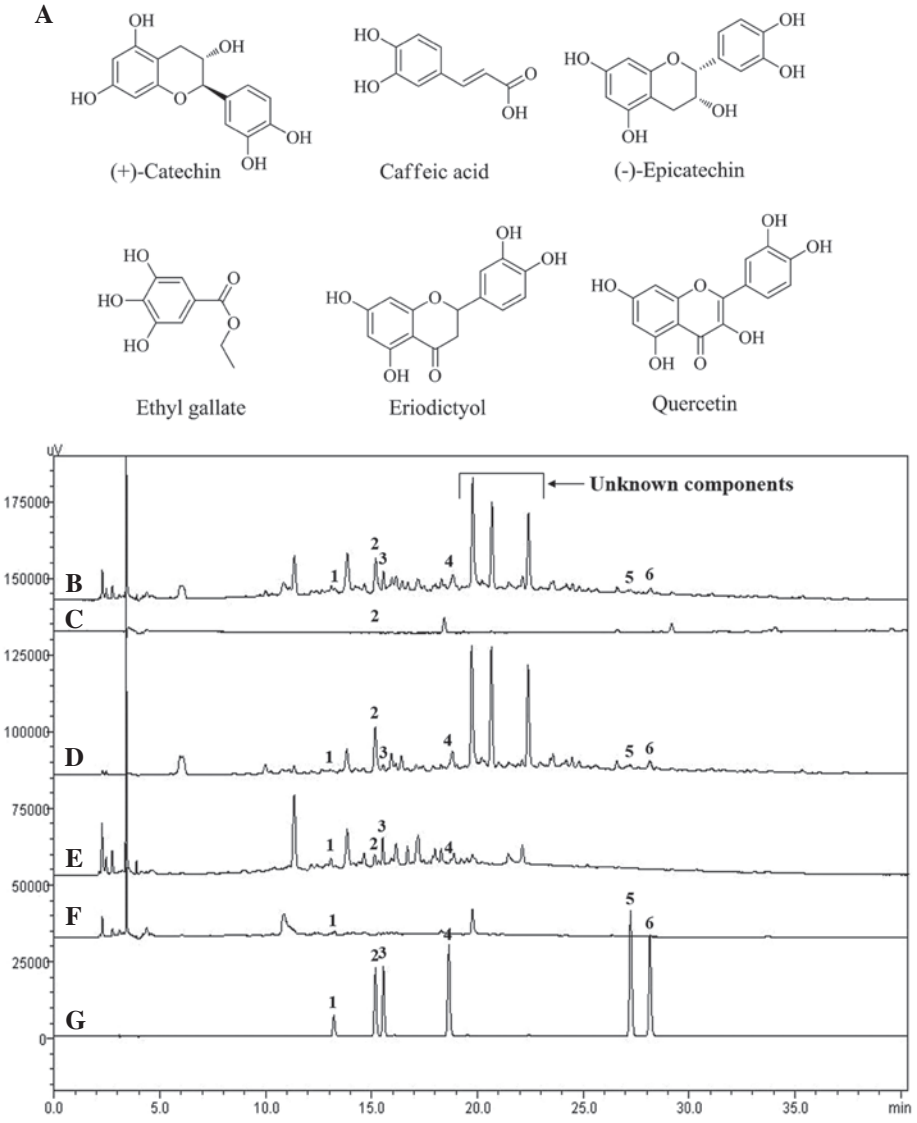

Figure 1. Chemical structures of the six constituents and high-performance liquid chromatography chromatogram of *Gleditsia sinensis*. (A) Chemical structures of the six constituents. (B-G) Chromatogram of (B) 70% ethanol extract, (C) *n*-hexane, (D) ethyl acetate, (E) *n*-butanol, (F) water fractions and (G) standard mixture. Peaks are labeled as follows: 1, (+)-Catechin; 2, caffeic acid; 3, (-)-epicatechin; 4, ethyl gallate; 5, eriodictyol; and 6, quercetin.

observed after 24 h of LPS incubation (1 μg/ml). As shown in Fig. 3A, treatment with the *n*-hexane, ethyl acetate and *n*-butanol fractions suppressed the NO production. In addition, the *n*-hexane and ethyl acetate fractions decreased PGE_2 production compared with that observed in LPS-stimulated RAW 264.7 cells (P<0.01, Fig. 3B). TARC production was increased 2.7-fold in the TI-treated cells (28.30±0.820 ng/ml) compared with that in the control cells (10.16±1.540 ng/ml) (P<0.01), whereas cells treated with the ethyl acetate fraction showed the most significant reductions in TARC production compared with the TI-treated cells (P<0.01, Fig. 3C). These results showed that the biological effects of *G. sinensis* were maximized by the ethyl acetate fraction.

Major constituents of the G. sinensis extract inhibit NO and PGE_2 production in RAW 264.7 cells and chemokine production in HaCaT cells. To assess the effects of the major constituents of the *G. sinensis* extract on LPS-stimulated NO and PGE_2 production in RAW 264.7 macrophages, cells were treated with various concentrations of these major constituents and 1 μg/ml LPS for 24 h. LPS stimulation caused a marked accumulation of proinflammatory mediators (NO and PGE_2) in the culture medium. Of note, all major constituents of the *G. sinensis* extract significantly reduced the LPS-stimulated production of NO (Fig. 4A) and PGE_2 (Fig. 4B) in a dose-dependent manner. Treatment of HaCaT cells with TI for 24 h led to a 1.9-fold increase in TARC levels (6.85±0.51 ng/ml) compared with that observed in the vehicle-treated control group (3.52±0.29 ng/ml) (P<0.01); however, TARC production was significantly inhibited in a dose-dependent manner in the ethyl gallate-, eriodictyol- and quercetin-treated cells (P<0.01) (Fig. 4C). In addition, MDC production was increased in the TI-treated cells (264.80±12.76 ng/ml) compared with that in the vehicle-treated control group (P<0.01), and its levels were significantly reduced following ethyl gallate and quercetin treatment (P<0.01) (Fig. 4D). TI-treated cells exhibited a significantly increased production of RANTES

Table II. Contents of six constituents of *G. sinensis* (n=3).

	Content (μg/g)													
	(+)-Catechin			Caffeic acid			(-)-Epicatechin			Ethyl gallate			Eriodictyol			Quercetin		
Fraction	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
70% EtOH	8.63	0.18	2.07	2.79	0.04	1.44	4.92	0.03	0.55	2.89	0.06	2.04	0.39	0.01	3.55	2.54	0.06	2.21
n-Hexane	N.D.	-	-	0.18	0.00	0.65	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
EtOAc	5.33	0.08	1.45	6.07	0.02	0.33	4.88	0.06	1.20	1.26	0.04	3.06	1.13	0.03	2.24	7.00	0.03	0.37
n-BuOH	5.28	0.05	0.98	1.04	0.01	1.30	5.61	0.06	0.99	0.96	0.02	1.79	N.D.	-	-	N.D.	-	-
Water	3.78	0.06	1.54	0.28	0.00	1.18	0.73	0.03	3.69	N.D.	-	-	N.D.	-	-	N.D.	-	-

SD, standard deviation; RSD, relative standard deviation; EtOH, ethanol; EtOAc, ethyl acetate; *n*-BuOH, *n*-butanol; N.D., not detected.

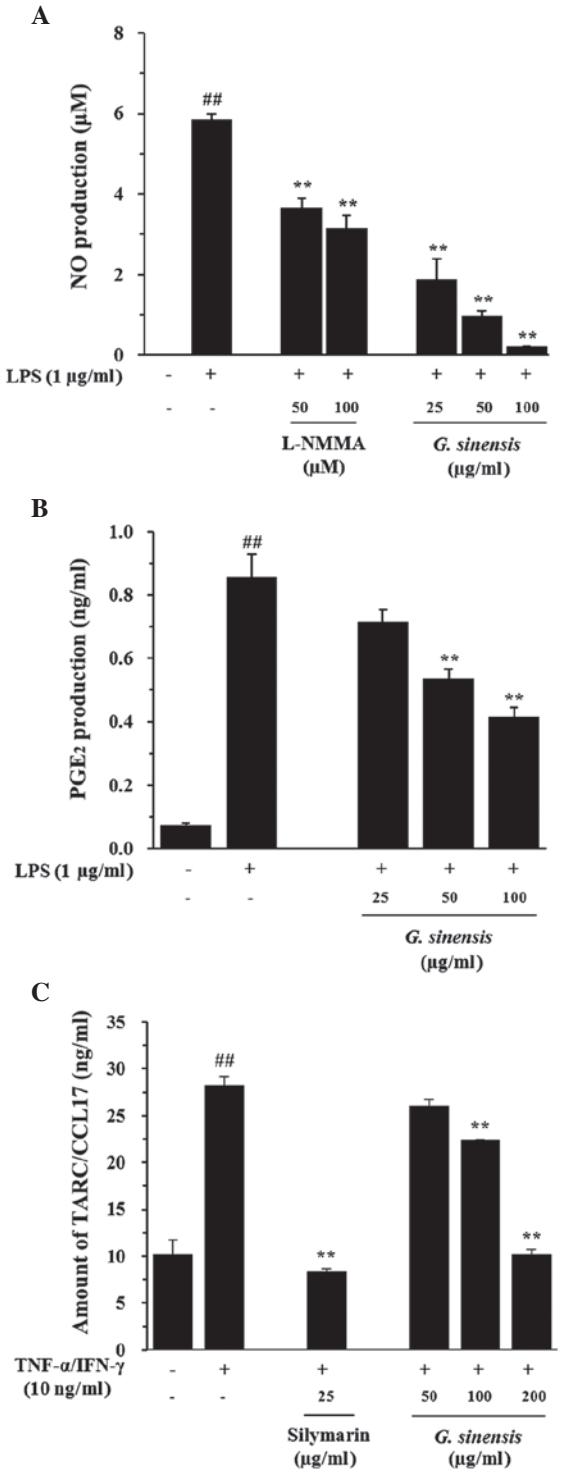

Figure 2. Effects of the extract of *Gleditsia sinensis* on the LPS-stimulated production of NO and PGE_2 in RAW 264.7 cells and the TI-stimulated production of TARC in HaCaT cells. The production of (A) NO and (B) PGE_2 was assayed in the culture medium of cells treated with extracts of *G. sinensis* (25, 50, and 100 μg/ml) and then with LPS (1 μg/ml) for 24 h. L-NMMA (25 μM) was used as a positive control drug. (C) The production of TARC was assayed in the culture medium of cells treated with extracts of *G. sinensis* (50, 100 and 200 μg/ml), followed by TI (TNF-α, 10 ng/ml; IFN-γ, 10 ng/ml) for 24 h. Silymarin (25 μg/ml) was used as a positive control drug. Each bar represents the mean values obtained from three independent experiments. $^{\#\#}$P<0.01 vs. the vehicle control group; **P<0.01 vs. LPS/TI-treated cells. LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2, TARC, thymus- and activation-regulated chemokine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TI, TNF-α and IFN-γ; L-NMMA, N(G)-monomethyl-L-arginine.

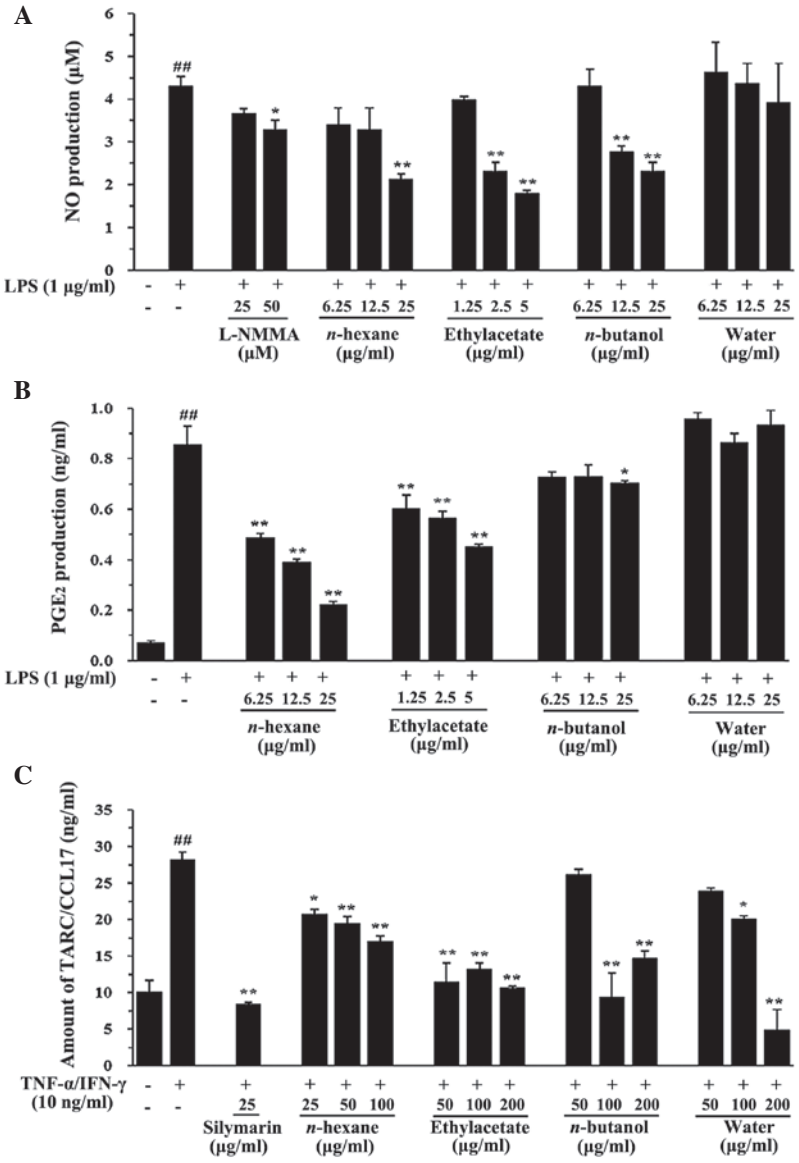

Figure 3. Effects of the fractions of *Gleditsia sinensis* extract on the LPS-stimulated production of NO and PGE_2 in RAW 264.7 cells and the TI-stimulated production of TARC in HaCaT cells. The production of (A) NO and (B) PGE_2 was assayed in the culture medium of cells treated with solvent fractions and then stimulated with LPS (1 μg/ml) for 24 h. L-NMMA (25 μM) was used as a positive control drug. (C) The production of TARC was assayed in the culture medium of cells treated with solvent fractions and then stimulated with TI (TNF-α, 10 ng/ml; IFN-γ, 10 ng/ml) for 24 h. Silymarin (25 μg/ml) was used as a positive control drug. Each bar represents the mean values obtained from three independent experiments. $^{\#\#}$P<0.01 vs. the vehicle control group; *P<0.05 and **P<0.01 vs. the LPS/TI-treated cells. LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2, TARC, thymus- and activation-regulated chemokine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TI, TNF-α and IFN-γ; L-NMMA, N(G)-monomethyl-L-arginine.

(4,652.93±66.95 ng/ml), relative to that observed in the control cells (P<0.01). This increase was inhibited dose-dependently by ethyl gallate (2,530.11±8.67 ng/ml at 50 μg/ml, P<0.01; 1,068.30±95.01 ng/ml at 100 μg/ml, P<0.01), eriodictyol (3,987.98±127.92 ng/ml at 50 μg/ml, P<0.05; 2,433.12±184.17 ng/ml at 100 μg/ml, P<0.01) and quercetin (2,728.46±119.93 ng/ml at 50 μg/ml, P<0.01; 414.34±27.59 ng/ml at 100 μg/ml, P<0.01) (Fig. 4E). Among these compounds, ethyl gallate and quercetin, which were most abundant both in the *G. sinensis* extract and in each solvent fraction (particularly in the ethyl acetate fraction) significantly inhibited the RANTES, MDC and TARC expression in HaCaT cells in a dose-dependent manner, whereas the same expression was not significantly reduced by other constituents in TI-treated cells.

Discussion

The seeds and fruits of *G. sinensis* are widely used in Chinese, Japanese and Korean herbal medicine for the treatment of numerous diseases, such as apoplexy, headache, asthma and scabies (14). Despite the fact that the thorns of *G. sinensis* have been used for the prevention, as well as the treatment, of carbuncles, scabies and other inflammatory diseases (15), the anti-inflammatory effect has not yet been fully elucidated. In the present study, the concentrations of the chemical constituents of *G. sinensis* and their anti-inflammatory effects in *G. sinensis* extracts, based on different solvent fractions and single compounds, were investigated.

Two phenolic compounds (caffeic acid and ethyl gallate) and four flavonoids [(+)-catechin, (-)-epicatechin, eriodictyol

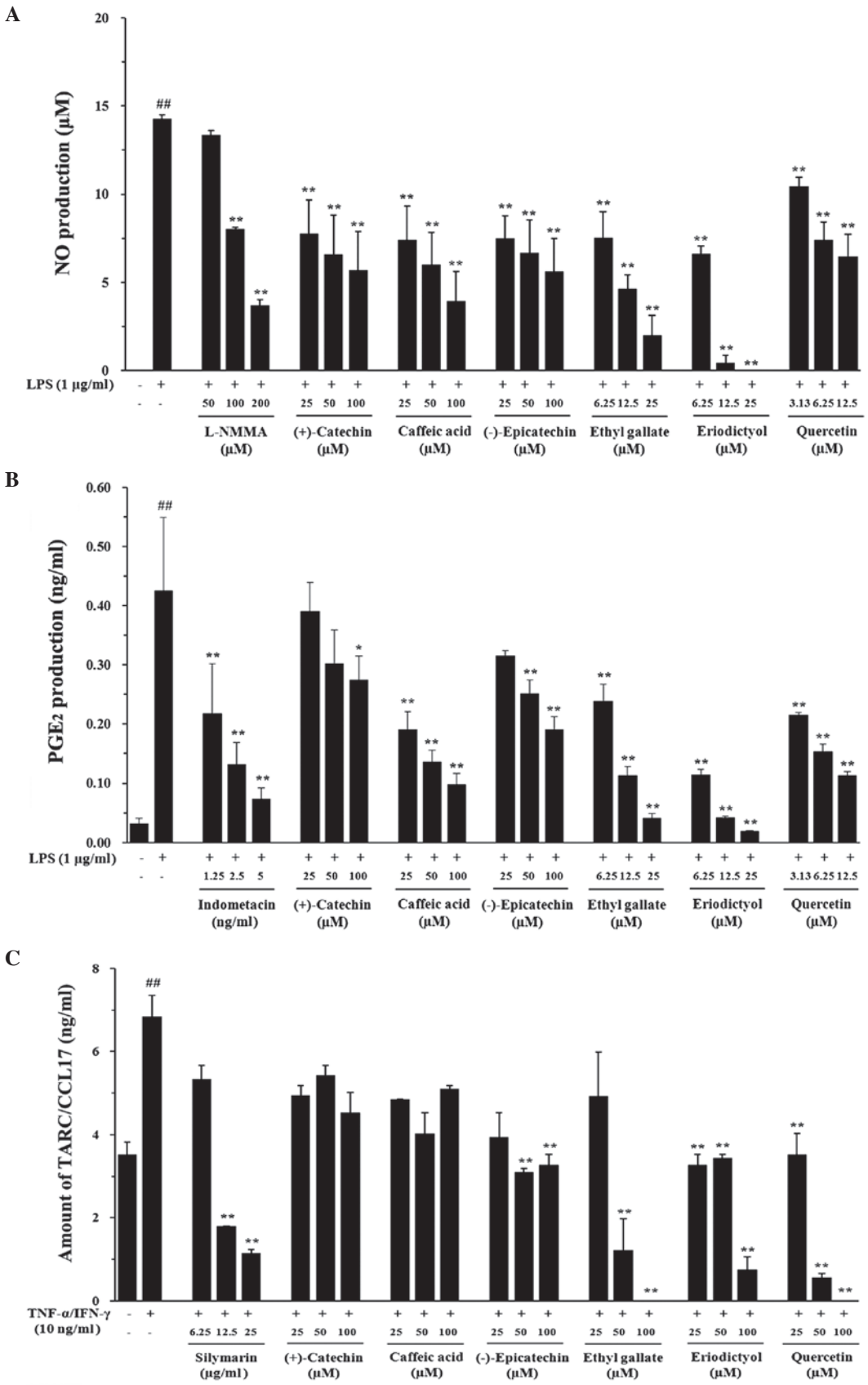

Figure 4. Effects of the major constituents of the *Gleditsia sinensis* extract on the LPS-stimulated production of NO and PGE_2 in RAW 264.7 cells and the TI-stimulated production of chemokines in HaCaT cells. The production of (A) NO and (B) PGE_2 was assayed in the culture medium of cells treated with the major constituents of the *G. sinensis* extract and then stimulated with LPS (1 μg/ml) for 24 h. L-NMMA (25 μM) was used as a positive control drug. (C) The production of TARC was assayed in the culture medium of cells treated with the major constituents of the *G. sinensis* extract and then stimulated with TI (TNF-α, 10 ng/ml; IFN-γ, 10 ng/ml) for 24 h. Silymarin (25 μg/ml) was used as positive control drug. Each bar represents the mean of three independent experiments. $^{\#\#}$P<0.01 vs. the vehicle control group; *P<0.05 and **P<0.01 vs. the LPS/TI-treated cells. LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2, TARC, thymus- and activation-regulated chemokine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TI, TNF-α and IFN-γ; L-NMMA, N(G)-monomethyl-L-arginine.

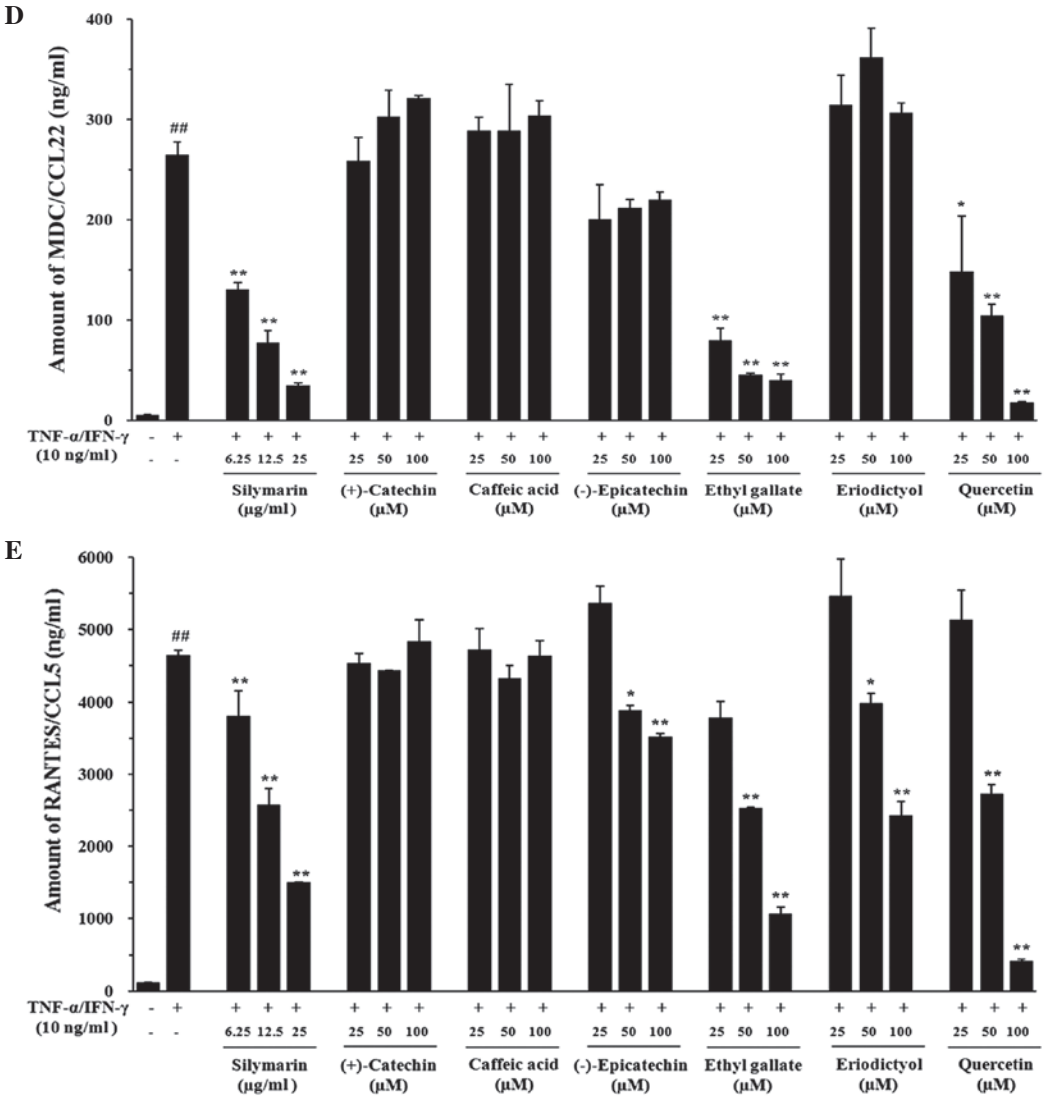

Figure 4. Continued. Effects of the major constituents of the *Gleditsia sinensis* extract on LPS-stimulated production of NO and PGE_2 in RAW 264.7 cells and the TI-stimulated production of chemokines in HaCaT cells. The production of (D) MDC and (E) RANTES was assayed in the culture medium of cells treated with the major constituents of the *G. sinensis* extract and then stimulated with TI (TNF-α, 10 ng/ml; IFN-γ, 10 ng/ml) for 24 h. Silymarin (25 μg/ml) was used as positive control drug. Each bar represents the mean of three independent experiments. $^{\#\#}P<0.01$ vs. the vehicle control group; $^{*}P<0.05$ and $^{**}P<0.01$ vs. the LPS and TI-treated cells, respectively. LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2, TARC, thymus- and activation-regulated chemokine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MDC, macrophage-derived chemokine; RANTES, regulated on activation normal T-cell expressed and secreted; TI, TNF-α and IFN-γ.

and quercetin)] were analyzed in 70% ethanol extracts and solvent fractions (*n*-hexane, ethyl acetate, *n*-butanol and water) of *G. sinensis*; however, the constituents responsible for peaks at the retention times of 19.8, 20.7 and 22.5 min in the 70% ethanol extract and ethyl acetate fraction were not able to be identified. It is imperative that these constituents are identified and quantitatively determined in order for a correlation between the quantities of the major constituents of *G. sinensis* and the biological effects of this herbal medicine to be obtained.

The production of NO and PGE_2, two major mediators of inflammation, plays an important role in the immune response to numerous inflammatory stimuli. The free radical NO is produced by iNOS, and its overproduction has been associated with the pathology of an array of inflammatory disorders, such as septic shock (16). PGE_2, which is generated from arachidonic acid by COX-2, is produced in response to inflammatory stimuli, and abundant COX-2 expression promotes the proinflammatory signaling cascade (17). Thus, potential inhibitors of iNOS and COX-2 have been considered as possible anti-inflammatory agents. It was shown that the extraction solvent and the major constituents in *G. sinensis* reduced the levels of NO and PGE_2 in LPS-stimulated RAW 264.7 cells in a dose-dependent manner.

Exposure of keratinocytes to TI results in an abnormal expression of chemokines and cytokines, which is believed to increase the infiltration of monocytes/T cells into the site of inflammation (18,19). Chemokines are a superfamily of small cytokines that regulate the trafficking of various types of leukocytes (20). TARC/CCL17 is a member of the CC chemokine subfamily that is produced by various cell types, including keratinocytes, and is constitutively expressed in the thymus (21). MDC/CCL22 is closely associated with TARC and is constitutively produced by dendritic cells, B

cells, keratinocytes, epithelial cells and macrophages (22). RANTES/CCL5 belongs to the CC chemokine family, the members of which recruit and activate different subtypes of leukocytes, such as T cells, eosinophils, basophils, monocytes or mast cells (23). The effect of the extract and solvent fractions of *G. sinensis* on TARC production in TI-stimulated HaCaT cells was examined and it was shown that the major constituents of *G. sinensis* reduced the production of TARC, MDC and RANTES in TI-stimulated HaCaT cells; in particular, ethyl gallate and quercetin yielded a significant dose-dependent inhibition.

In conclusion, the *G. sinensis* extract was successively partitioned to identify the fraction that contained the major constituent compounds. It was found that all the reference compounds were present in the ethyl acetate fraction and that larger amounts were present in this fraction than in any other fraction. Furthermore, the ethyl acetate fraction decreased the levels of NO and PGE_2 in RAW 264.7 cells and the expression of TARC, MDC and RANTES in HaCaT cells to a greater extent than the other fractions. Further elucidation of the signaling pathways involved in T-helper cell 2 chemokine inhibition by *G. sinensis* is required for the facilitation of the design of therapeutic agents for the inflammatory reaction.

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