ORIGINAL ARTICLE

Phytochemical Analysis on Quantification and the Inhibitory Effects on Inflammatory Responses from the Fruit of *Xanthii fructus*

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ABSTRACT

Objective: *Xanthii fructus* (Compositae) is a traditional herbal medicine used for treating headache, toothache, pruritus, empyema, and rhinitis. In this study of the quality control of *X. fructus*, we performed simultaneous analysis of nine marker compounds: Protocatechuic acid (1), chlorogenic acid (2), caffeic acid (3), 4,5-dicaffeoylquinic acid (4), ferulic acid (5), 3,5-dicaffeoylquinic acid (6), 1,3-dicaffeoylquinic acid (7), 1,4-dicaffeoylquinic acid (8), and 4,5-dicaffeoylquinic acid (9). **Materials and Methods:** Nine components were separated using reversed-phase SunFire™ C_{18} analytical column and analyzed using high-performance liquid chromatography. We examined the biological effects of the nine marker compounds by determining their anti-inflammatory activities in the murine macrophage cell line RAW 264.7. **Results:** Among the nine marker compounds, eight significantly inhibited lipopolysaccharide (LPS)-stimulated tumor necrosis factor-alpha (TNF-α) production. 1, 3, 5 had significant inhibitory effects on LPS-induced prostaglandin E_2 (PGE_2) production in RAW 264.7 cells. None of the tested marker compounds had a significant effect on interleukin-6 production in LPS-treated RAW 264.7 cells. Our data demonstrated that each marker compound from *X. fructus* exerts anti-inflammatory activity by targeting different inflammation-related pathways such as the TNF-α or PGE_2 pathway. **Conclusion:** Further experiments using *in vitro* and *in vivo* models are needed to identify the mechanisms responsible for the anti-inflammatory properties of each marker compound.

Key words: Anti-inflammatory effect, herbal medicine, high-performance liquid chromatography, RAW 264.7, *Xanthii fructus*

SUMMARY
- Simultaneous analysis of nine phenylpropanoids in the Xanthii fructus was established using HPLC-PDA system.
- 1,4-dicaffeoylquinic acid significantly inhibited LPS-stimulated TNF-a production.
- Protocatechuic acid, caffeic acid and ferulic acid had significant inhibitory effects on LPS-induced PGE2 production in RAW 264.7 cells.
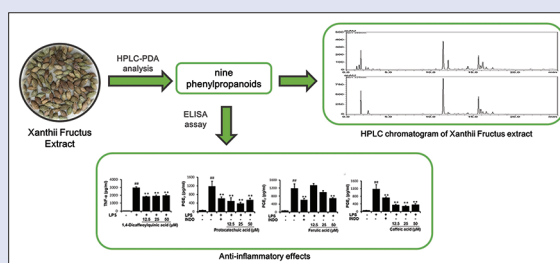
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INTRODUCTION

Xanthii fructus (Compositae) is derived from the fruit of *Xanthium strumarium* L., which is widely distributed in Asian countries such as Korea, Japan, and China.[1] Some investigations showed that *X. fructus* has a variety of pharmacological properties regarding antioxidant, anti-cancer, and analgesic effects.[2,3] Phytochemical studies of *X. fructus* have identified carboxyatractyloside, xanthanol, alkaloids, thiazinediones, and phenylpropanoids.[2-4] Among these compounds, we simultaneously examined nine phenylpropanoid marker compounds for quality control of *X. fructus* extracts using high-performance liquid chromatography (HPLC).

Inflammation is a complex immuno- and biological-reaction against various harmful stimuli.[5] Activated macrophages by inflammatory stimulator lead to produce pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6.[6] Since pro-inflammatory cytokines have been considered as the attractive target molecule(s) for treating inflammatory diseases,[7] it is important to examine the effects of the drug on their production. Prostaglandin E_2 (PGE_2) also plays a crucial role in the regulation of inflammatory response.[8] PGE_2 is synthesized by cyclooxygenase (COX)-2 that is mediated by inflammatory stimuli.

In recent years, nonsteroidal anti-inflammatory drugs are most popularly used for treating inflammatory disorders. However, several adverse effects such as gastrointestinal problems and damage of central nervous system can limit therapeutic effects.[9] To overcome these problems, recent research has been focused on the anti-inflammatory action of phytochemical (s).[10-13] In our study, we investigated anti-inflammatory effects of nine marker compounds from *X. fructus*. Inflammatory reaction in macrophages was induced by stimulating with lipopolysaccharide (LPS) and analyzed production of major inflammation markers such as TNF-α, IL-6, and PGE_2.

MATERIALS AND METHODS

Plant materials

The *X. fructus* used in this study was purchased from HMAX (Jecheon, Korea) in October 2008. The botanical origin of this sample was taxonomically confirmed by Professor Je-Hyun Lee, Dongguk University, Gyeongju, Republic of Korea. A voucher specimen (2008-ST-25) has been deposited at the Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine.

Chemicals and reagents

Chlorogenic acid and caffeic acid were purchased from Acros Organics (Pittsburgh, PA, USA). 4,5-Dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, and 1,5-dicaffeoylquinic acid were obtained from Chengdu Biopurify Phytochemicals Ltd., (Chengdu, China). Protocatechuic acid, ferulic acid, and 1,4-dicaffeoylquinic acid were obtained from ChromaDex (Irvine, CA, USA), Wako Chemicals (Osaka, Japan), and ChemFaces (Wuhan, China), respectively. The purity of these reference standards was >98.0% by HPLC analysis. The chemical structures of these components are shown in Figure 1. The HPLC-grade solvents methanol, acetonitrile, and water were obtained from JT Baker (Phillipsburg, NJ, USA). Glacial acetic acid (analytical grade) was purchased from Merck KGaA (Darmstadt, Germany).

Preparations of seventy percentage ethanol extract

Dried *X. fructus* (200 g) was extracted three times with 70% (v/v) ethanol (2 L) by sonication for 60 min. The extracted solution was filtered through filter paper, evaporated at 40°C using a Büchi R-210 rotary evaporator (Flawil, Switzerland) under vacuum to dryness and freeze-dried. The yield of the freeze-dried 70% ethanol extract obtained was 5.86% (11.72 g).

Quantitative analysis of the marker components in *Xanthii fructus*

The sample was analyzed using a Shimadzu Prominence LC-20A series HPLC (Shimadzu Co., Kyoto, Japan) comprising a solvent delivery unit (LC-20AT), online degasser (DGU-20A$_3$), column oven (CTO-20A), auto sample injector (SIL-20AC), and photodiode array detector (PDA) (SPD-M20A). Data were collected and processed using LC solution software (Version 1.24, Shimadzu Co., Kyoto, Japan). The stationary phase for the separation of the nine components used a reversed-phase SunFire™ C$_{18}$ analytical column (Waters, Milford, MA, USA; 150 mm × 4.6 mm and 5 μm particle size). The mobile phase comprised 1.0% aqueous acetic acid (eluent A) and 1.0% acetic acid in acetonitrile (eluent B). The gradient flow of the two-solvent system was as follows: 5–5% B (3 min), 5–40% B (15 min), 40% B (20 min), and 40–5% B (25 min). The flow rate was 1.0 mL/min, the column temperature was maintained at 40°C, and the injection volume was 10 μL. The detection wavelength of quantification was set over the range of 190–400 nm and was recorded at 260 and 325 nm.

Cell culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Carlsbad, CA) supplemented with 5.5% fetal bovine serum (Gibco BRL), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C.

Cytotoxicity assay

To test the cytotoxicity of the nine markers, RAW 264.7 cells were incubated for 24 h in the presence or absence of each marker compound from *X. fructus*. Cell counting kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added, and the cells were incubated for 4 h. After the incubation, the absorbance was read at 450 nm in a Microplate Reader (Benchmark Plus, Bio-Rad, MN, USA). Cell viability was calculated as the percentage of viable cells in the *X. fructus*-treated group versus untreated controls.

Measurement of tumor necrosis factor-alpha, interleukin-6, and prostaglandin E$_2$ production

RAW 264.7 cells were plated at a density of 2.4×10^4 cells on 24 well and incubated for 24 h. Cells were treated with various concentration of each marker compound from *X. fructus* for 4 h prior to LPS (1 μg/mL) stimulation. After an additional incubation for 20 h, the concentrations of TNF-α (BD Biosciences, Mountain View, CA, USA), IL-6 (BD Biosciences), and PGE$_2$ (Cayman Chemical Co., Ann Arbor, MI, USA) in the supernatants were measured by enzyme-linked immunosorbent assays (ELISAs) according to manufacturers' instructions.

Statistical analysis

All values are expressed as the mean ± standard error of the mean of three independent samples of each marker compound from *X. fructus*. One-way analysis of variance was used to identify significant differences between the treatment groups. Dunnett's test was used for multiple group comparisons. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

### Quantitative determination of nine compounds in *Xanthii fructus*

An optimized HPLC method was applied for the simultaneous analysis of nine phenylpropanoids in the *X. fructus* extract. Five components were eluted within 20 min using the two mobile phases. Each compound in the chromatogram was identified by comparing the retention time and UV spectra compared with those of the standards [Figure 2]. The retention times of protocatechuic acid, chlorogenic acid, caffeic acid, 1,4-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, ferulic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid were 6.51, 11.45, 11.80, 12.16, 13.22, 14.67, 15.63, 15.89, and 16.55 min, respectively. Representative HPLC chromatograms of standards and the extracts are shown in Figure 2. The coefficients of determination (r^2) of the calibration curves for the nine

Table 1 : The concentrations of nine marker compounds in the *Xanthii fructus* extract (*n*=3)

| Compound	Concentration (mg/g)		
	Mean	**SD**	**RSD (%)**
Protocatechuic acid	ND	-	-
Chlorogenic acid	33.56	0.59	1.75
Caffeic acid	0.30	0.01	1.96
1,4-Dicaffeoylquinic acid	1.20	0.02	1.43
1,3-Dicaffeoylquinic acid	0.82	0.01	0.55
Ferulic acid	ND	-	-
1,5-Dicaffeoylquinic acid	10.12	0.16	1.60
3,5-Dicaffeoylquinic acid	3.01	0.01	0.30
4,5-Dicaffeoylquinic acid	2.46	0.02	0.86
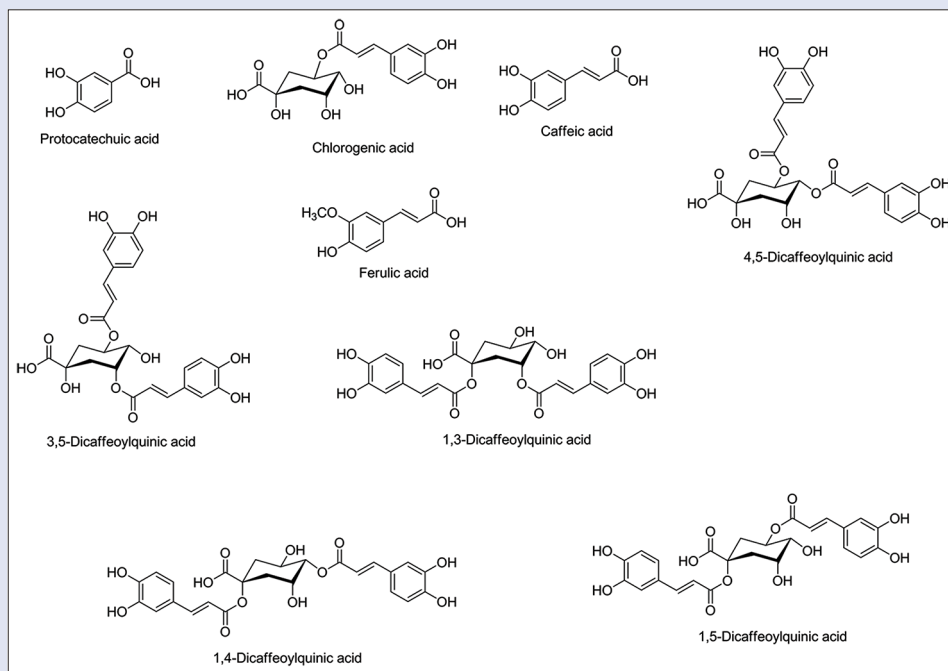
Figure 1: Chemical structures of nine marker compounds in *Xanthii fructus*
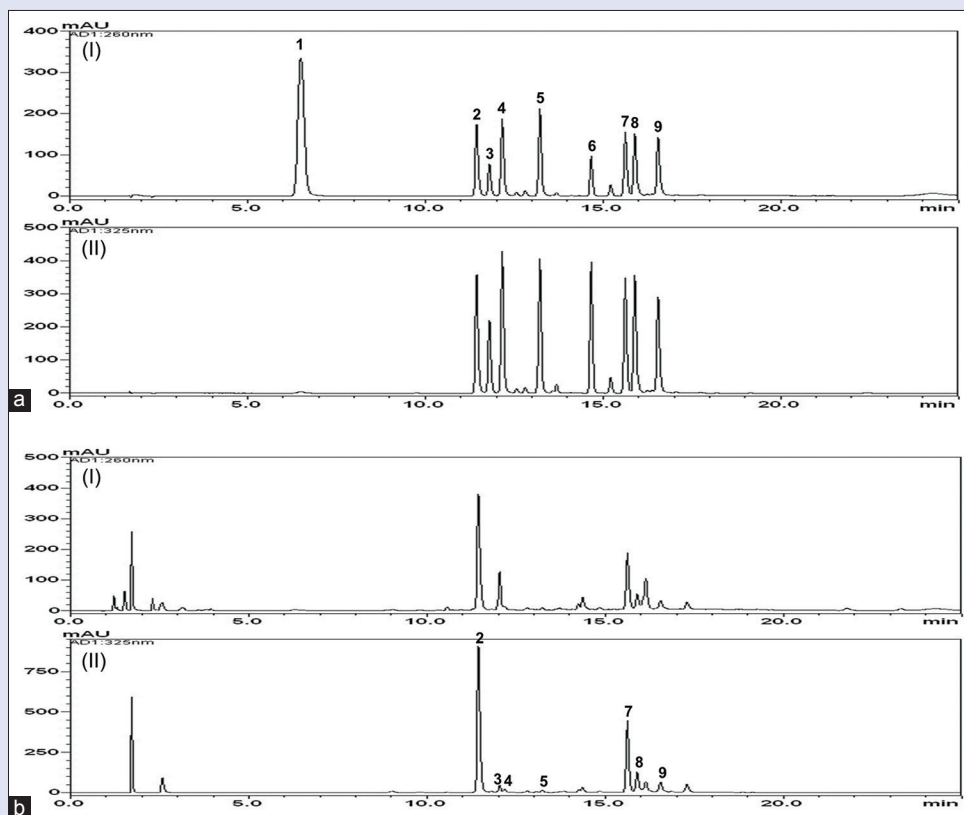

Figure 2: High-performance liquid chromatography chromatograms of the standard mixture (a) and *Xanthii fructus* extract (b) at ultraviolet detection of 260 nm (I) and 325 nm (II). Protocatechuic acid (1), chlorogenic acid (2), caffeic acid (3), 1,4-dicaffeoylquinic acid (4), 1,3-dicaffeoylquinic acid (5), ferulic acid (6), 1,5-dicaffeoylquinic acid (7), 3,5-dicaffeoylquinic acid (8), and 4,5-dicaffeoylquinic acid (9)

analytes were ≥0.9996, and the calibration curves had good linearity within the tested concentration range.

As determined by the HPLC–PDA method, the amounts of the nine phenylpropanoids, protocatechuic acid, chlorogenic
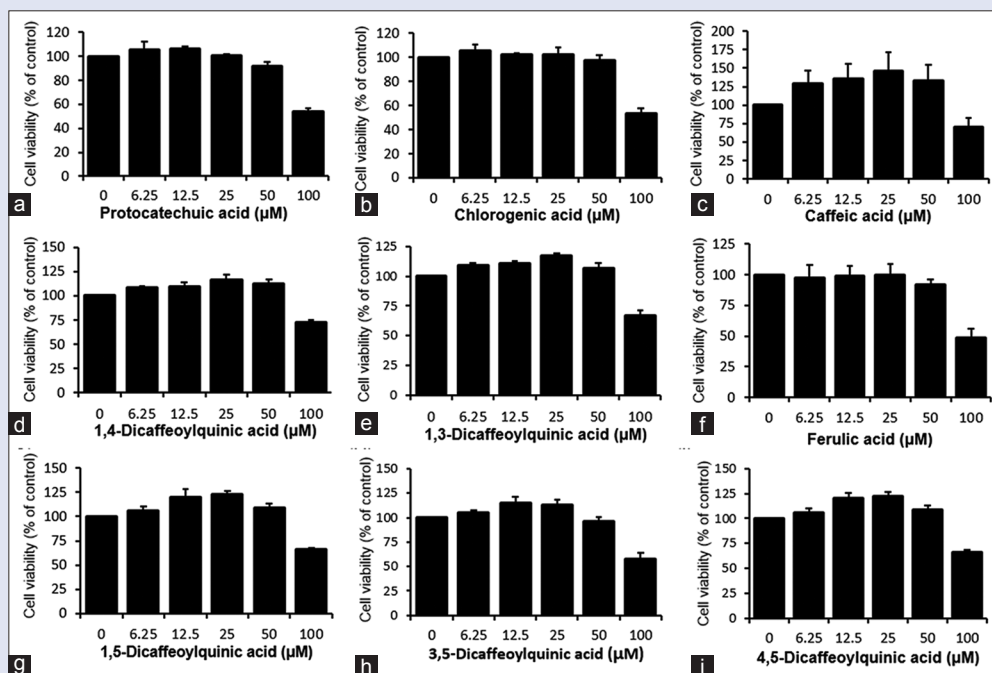
Figure 3: Cytotoxicity of nine marker compounds from *Xanthii fructus* in RAW 264.7 cells. Cells were seeded into 96-well plates and treated with nine marker compounds from *Xanthii fructus* for 24 h. Cell viability was assessed using a cell counting kit-8 assay. (a) Protocatechuic acid, (b) chlorogenic acid, (c) caffeic acid, (d) 1,4-dicaffeoylquinic acid, (e) 1,3-dicaffeoylquinic acid, (f) ferulic acid, (g) 1,5-dicaffeoylquinic acid, (h) 3,5-dicaffeoylquinic acid, and (i) 4,5-dicaffeoylquinic acid
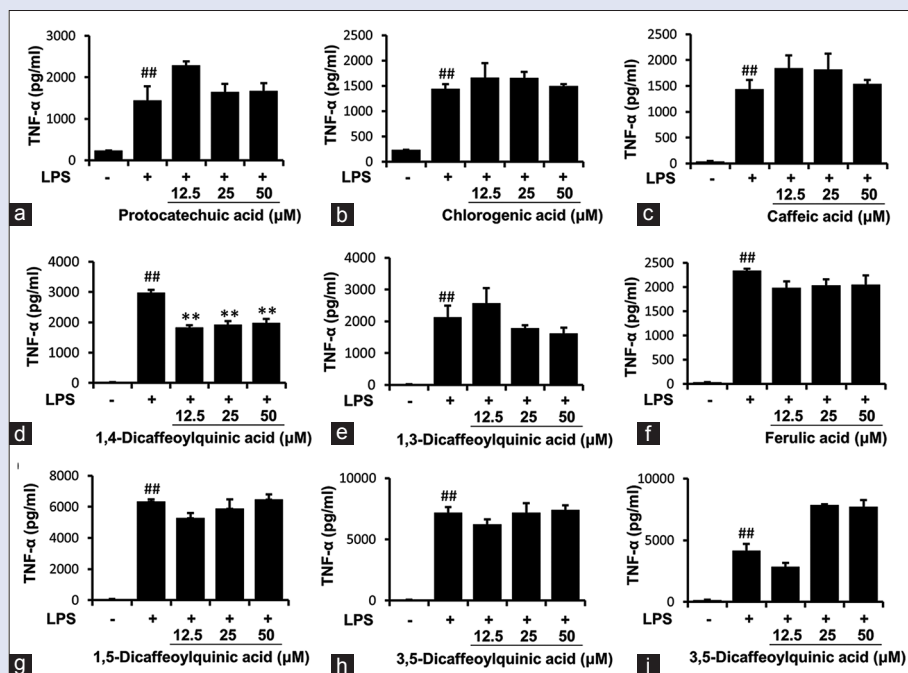
Figure 4: Effect of nine marker compounds from *Xanthii fructus* on lipopolysaccharide-stimulated tumor necrosis factor-alpha production in RAW 264.7 cells. Each bar represents the mean of three independent experiments (#$P < 0.05$ and ##$P < 0.01$ versus untreated control; **$P < 0.01$ versus lipopolysaccharide-treated cells). (a) Protocatechuic acid, (b) chlorogenic acid, (c) caffeic acid, (d) 1,4-dicaffeoylquinic acid, (e) 1,3-dicaffeoylquinic acid, (f) ferulic acid, (g) 1,5-dicaffeoylquinic acid, (h) 3,5-dicaffeoylquinic acid, and (i) 4,5-dicaffeoylquinic acid

acid, caffeic acid, 1,4-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, ferulic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid in *X. fructus* extract ranged from 0 to 33.56 mg/g [Table 1]. Among these components, chlorogenic acid and 1,5-dicaffeoylquinic acid were detected as the main constituents (33.56 mg/g and 10.12 mg/g) of *X. fructus*. Protocatechuic acid and ferulic acid, which have been isolated and analyzed as bioactive compounds from *X. fructus*,[2,14,15] were not detected in this sample.

Cytotoxic effects of marker compounds from *Xanthii fructus* in RAW 264.7 cells

The cytotoxicity of the nine marker compounds from *X. fructus* was evaluated in RAW 264.7 cells using the CCK-8 assay. Cells were treated with various concentrations (0, 6.25, 12.5, 25, 50, or 100 μM) of each compound for 24 h. As shown in **Figure 3**, none of the tested marker

compounds had a cytotoxic effect at a concentration up to 50 μM, although they caused a marked reduction in the viability of RAW 264.7 cells. Thus, nontoxic concentrations of ≤50 μM were used for subsequent *in vitro* assays.

Inhibitory effects of the marker compounds from *Xanthii fructus* on tumor necrosis factor-alpha and interleukin-6 production by RAW 264.7 cells

To determine the biological activities of the nine marker compounds from *X. fructus*, we used an *in vitro* experimental model of inflammation using the murine macrophage cell line RAW 264.7. We chose these cells because macrophages are major immune cells involved in the initiation, maintenance, and resolution of the inflammatory process.[16,17] Macrophages are activated by various stimuli such as bacterial LPS
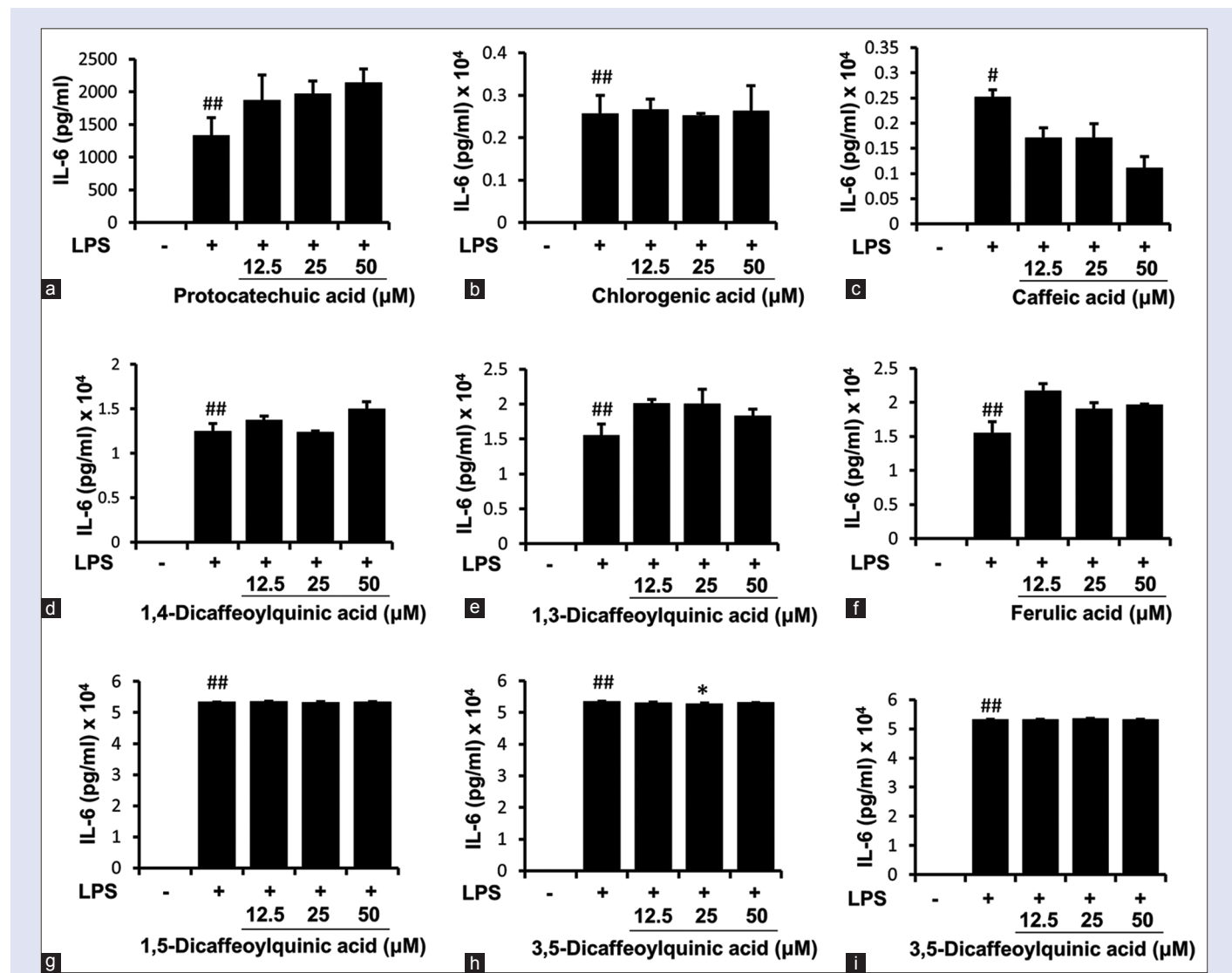
Figure 5: Effect of nine marker compounds from *Xanthii fructus* on lipopolysaccharide-stimulated interleukin-6 production in RAW 264.7 cells. Each bar represents the mean of three independent experiments (*P < 0.05 and ##P < 0.01 versus untreated control; **P < 0.01 versus lipopolysaccharide-treated cells). (a) Protocatechuic acid, (b) chlorogenic acid, (c) caffeic acid, (d) 1,4-dicaffeoylquinic acid, (e) 1,3-dicaffeoylquinic acid, (f) ferulic acid, (g) 1,5-dicaffeoylquinic acid, (h) 3,5-dicaffeoylquinic acid, and (i) 4,5-dicaffeoylquinic acid
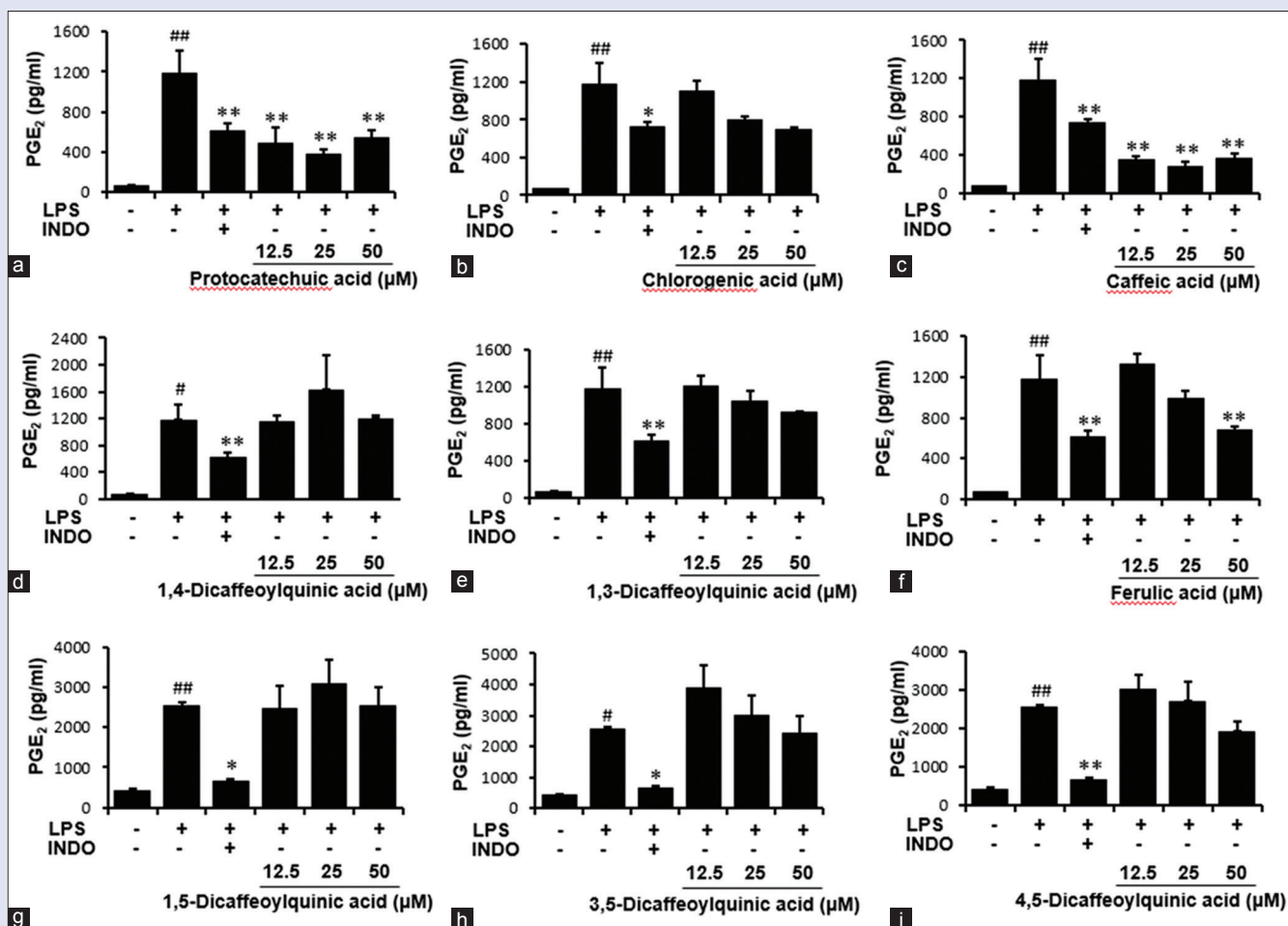
Figure 6: Effect of nine marker compounds from *Xanthii fructus* on lipopolysaccharide-stimulated prostaglandin E_2 production in RAW 264.7 cells. Indomethacin was used as a positive control. Each bar represents the mean of three independent experiments ([#]$P < 0.05$ and [##]$P < 0.01$ versus untreated control; **$P < 0.01$ versus lipopolysaccharide-treated cells). (a) Protocatechuic acid, (b) chlorogenic acid, (c) caffeic acid, (d) 1,4-dicaffeoylquinic acid, (e) 1,3-dicaffeoylquinic acid, (f) ferulic acid, (g) 1,5-dicaffeoylquinic acid, (h) 3,5-dicaffeoylquinic acid, and (i) 4,5-dicaffeoylquinic acid

released during inflammatory reactions, and secreted proinflammatory cytokines such as TNF-α[18] and IL-6.[19] Thus, we examined whether the marker compounds from *X. fructus* could affect the production of proinflammatory cytokines in RAW 264.7 cells. ELISAs for mouse TNF-α and IL-6 were used to measure the concentrations in the culture supernatant from LPS-treated RAW 264.7 cells incubated with or without each of the marker compounds. Among the nine marker compounds tested, only 1,4-dicaffeoylquinic acid [Figure 4d] had a significant inhibitory effect on TNF-α production by the cells [Figure 4]. None of the marker compounds had an effect on IL-6 production [Figure 5].

Effects of marker compounds from *Xanthii fructus* on prostaglandin E_2 level in RAW 264.7 cells

We also examined effects of nine marker compounds from *X. fructus* on the production of PGE_2 because PGE_2 is the major product of COX-2, a pro-inflammatory mediator.[20] As shown in Figure 6, LPS stimulation significantly increased level of PGE_2 whereas indomethacin, a positive control, significantly reduced LPS-mediated PGE_2 production in RAW 264.7 cells. Among nine marker compounds from *X. fructus*, protocatechuic acid [Figure 6a] and caffeic acid [Figure 6c] had significant inhibitory effects on PGE_2 production ranged from 12.5 to

50 μM. Ferulic acid [Figure 6f] revealed a significant inhibition of PGE_2 production at 50 μM, but not lower concentration.

CONCLUSION

We successfully developed a method to simultaneously identify the nine marker compounds and this method will help to improve the quality control of *X. fructus* extract. In addition, nine marker compounds from *X. fructus* had inhibitory effects on inflammatory responses by targeting different proinflammatory factors, even though some of these compounds have similar chemical structures. However, we found no relationships between the amount detected by HPLC analysis and the *in vitro* anti-inflammatory activities of each of the compounds. Further experiments are needed to identify the major bioactive compound (s) and the molecular mechanisms responsible for the anti-inflammatory effects of each compound.

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Conflicts of interest

There are no conflicts of interest.

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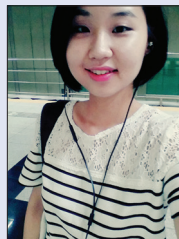
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