

Luteolin and Chicoric Acid, Two Major Constituents of Dandelion Leaf, Inhibit Nitric Oxide and Lipid Peroxide Formation in Lipopolysaccharide-Stimulated RAW 264.7 Cells

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Abstract

Luteolin and chicoric acid are the most abundant phytochemicals in dandelion (*Taraxacum officinale*) leaf. In this study, four kinds of extraction methods [hot water, ambient temperature (AT) water, ethanol, and methanol] were applied to analyze the contents of both phytochemicals and verify their anti-inflammatory and antioxidative activities. The methanol extract showed the most potent nitric oxide (NO) inhibitory effect. The luteolin and chicoric acid concentrations were 3.42 and 12.86 µg/g dandelion leaf in the methanol extract. The NO-suppressive effect of luteolin and chicoric acid was identified in a dose-dependent manner with IC₅₀ values of 21.2 µM and 283.6 µM, respectively, in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells without cytotoxicity. Malondialdehyde (MDA) concentration, as an index for free radical injury on cell membrane, was also dose-dependently inhibited by the two compounds. The suppressive effect was further examined using mRNA and protein expression levels, which were attributable to the inhibition of inducible nitric oxide synthase (iNOS). These results suggest that two phytochemicals in dandelion leaf, luteolin and chicoric acid, may play an important role in the amelioration of LPS-induced oxidative stress and inflammation.

Key words: *Taraxacum officinale*, luteolin, chicoric acid, nitric oxide, lipid peroxide

INTRODUCTION

Lipopolysaccharide (LPS) triggers the secretion of a variety of inflammatory products, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, as well as excessive amounts of nitric oxide (NO), which produce the pathophysiologic changes during septic shock (1-3). NO reacts with superoxide to yield peroxynitrite, which contributes to the etiology of cardiovascular disease and cancer by promoting oxidative stress and inflammatory processes (4). Lipid peroxidation generates cell and tissue damage as a result of degradation of the lipid membrane and the formation of new reactive oxygen species, which are implicated in the pathogenesis of inflammatory cascades, and toxic injury by xenobiotics (5). Thus, inhibited NO production by down-regulated inducible nitric oxide synthase (iNOS) expression and lipid peroxide are very important for controlling inflammatory and oxidative responses.

Dandelion (*Taraxacum officinale*) has been widely used as a traditional medicine against various disorders, such as liver diseases, gallbladder disorders, digestive complaints, and arthritic and rheumatic diseases (6-8). Because of this, many researchers worked to identify the

phenolic compounds in dandelion, which are now known to include: luteolin, chrysoeriol, chicoric acid, and chlorogenic acid (9). Among them, luteolin and chicoric acid are the most abundant phenolic compounds. Luteolin is a type of flavonoid, which is a group of phenolic compounds that play a role as an antioxidant, an anti-inflammatory agent, and a free radical scavenger (10). Chicoric acid, a caffeic acid derivative, decreases LPS-stimulated nuclear factor (NF)-κB level as well as TNF-α production in macrophages and strengthens the non-specific immune system in animal studies (11). To further characterize luteolin and chicoric acid in dandelion leaf, this study quantitated the amount of luteolin and chicoric acid derived from four different extraction methods, and also evaluated anti-inflammatory and antioxidative activities of the extracts in LPS-activated RAW 264.7 cells.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), and glutamine were obtained from Gibco-BRL (Gaithersburg, MD, USA). Luteolin, LPS,

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DMSO, SDS, NP-40, and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and chicoric acid was obtained from Chromadex (Santa Ana, CA, USA).

Preparation of dandelion extract

Dried powdered dandelion leaf was obtained from Min-Dle-Leh-Food (Uiryeong, Korea). Dandelion extracts were created using four different techniques: hot water, ambient temperature (AT) water (20~25°C), ethanol, and methanol. The hot water extract was achieved after adding 10× water and cooking in a double boiler with a reflux condenser for 4 hr. Extraction with AT water, ethanol, and methanol was conducted by adding 10× of the corresponding liquids and mixing well for 4 hr. After extraction, water extracts were lyophilized (Biotron, Bucheon, Korea), and the ethanol and methanol extracts were concentrated in a rotary evaporator (Buchi, St. Gallen, Switzerland). The recovery rate of the extracts was 34.2, 34.8, 36.6, and 36.1%, for hot water, AT water, ethanol, and methanol, respectively. All extracts were dissolved in 10% 100 mL methyl alcohol and mixed with the same volume of ethyl acetate. The two layers were shaken thoroughly. Separated ethyl alcohol was gathered three times and extracted in a rotary evaporator. Ethyl acetate extracts were dissolved in methyl alcohol and filtered (0.2 µm nylon; Whatman, Tokyo, Japan).

HPLC analysis for luteolin and chicoric acid

The analysis was performed using two identical Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA, USA). A reverse phase Agilent Zorbax XDB-C18 column AAA (4.6×150 mm, 3.5 µm) was used for the chromatographic separation. Two Phenomenex C18 security guard columns (4.0×3.0 mm; Phenomenex, Torrance, CA, USA) were used to protect the column at room temperature using a linear gradient elution [solvent A=10% acetonitrile/0.1% phosphoric acid (v/v); solvent B=100% acetonitrile/0.1% phosphoric acid (v/v)]. Solvent B increased from 1 to 100% in 30 min and was kept at 100% for 5 min. Samples were dissolved in the mobile phase, and 5 µL was injected. The UV-Vis spectrophotometer detector was set at 350 nm for luteolin, and 320 nm for chicoric acid. The flow rate was 1.0 mL/min. Luteolin and chicoric acid were used as external standards. Phenolic compounds were calculated from the areas under the respective peaks according to individual standard curves. Chemstation Plus Family for LC software (Agilent Technologies) was used for data acquisition and analysis.

Cell culture and treatment

The RAW 264.7 cell line was obtained from American

Type Culture Collection (TIB-71; Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. Cells in 100-mm dishes (5×10^6 cells/dish) or 24-well plates (4×10^5 cells/well) were pre-incubated with and without indicated concentrations of luteolin or chicoric acid each for 2 hr, and then incubated with LPS (1 µg/mL) for 18 hr at 37°C in a humidified atmosphere containing 5% CO₂. In the remainder of this report, “untreated” refers to a negative control without LPS.

Nitrite assay, cell viability, and lipid peroxidation

The nitrite accumulated in the culture medium as an indicator of NO production was measured according to the Griess reaction (12). Cell viability was assessed through measuring the uptake of the supravital dye neutral red by viable cells according to the procedure of Fautz et al. (13). Lipid peroxidation was measured by thiobarbituric acid reactive substance (TBARS) production as described by Fraga et al. (14).

RT-PCR analysis

Total RNA was isolated using Trizol-reagent (Invitrogen, Carlsbad, CA, USA) according to the method of manufacturer and first strand cDNA was synthesized using the MMLV first strand cDNA synthesis kit (Invitrogen). Primers for iNOS were 5'-GCC TTC AAC ACC AAG GTT GTC TGC A-3' (sense) and 5'-TCA TTG TAC TCT GAG GGC TGA CAC A-3' (antisense), and primers for GAPDH were 5'-CAA TGC CAA GTA TGA TGA CAT-3' (sense) and 5'-CCT GTT ATT ATG GGG GTC TG-3' (antisense). The expected sizes of PCR products were 920 bp for iNOS, and 375 bp for GAPDH. The amplification profile consisted of an initial denaturation at 94°C for 1 min followed by denaturation at 94°C for 3 min, annealing at 59°C for 2 min (iNOS), and 49°C for 2 min (GAPDH), and extension at 72°C for 2 min. Twenty-seven cycles for iNOS and GAPDH proved to be the best amplification profiles to recognize differences among samples. Expression of the housekeeping gene, GAPDH, served as the control.

Western blot analysis

Cell lysate was prepared using Pro-Prep protein extraction solution (Intron Biotechnology, Seongnam, Korea) according to a manufacturer's instruction. Protein samples (50 µg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked for 2 hr at room temperature with 5% nonfat dry milk in TBST solution. The reactions were then incubated at 4°C overnight with a 1:1,000 dilution of antibodies in blocking buffer. After the mem-

branes were washed, they were further incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated secondary antibody for 2 hr at room temperature. The blots were developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) color developing solution, and data were quantified using the Gel Doc EQ System (Bio-Rad). All signals were normalized to protein levels of GAPDH and expressed as a ratio.

Statistical analysis

All data are expressed as the means \pm SD. The statistical analyses were performed using SPSS version 10.0 (SPSS Institute, Chicago, IL, USA). One-way ANOVA with Duncan's multiple range test was used to examine the difference between groups. Probability values <0.05 were considered significant if not otherwise stated.

RESULTS AND DISCUSSION

Inhibition of NO and lipid peroxide formation by four kinds of extracts

This study was conducted to quantitate two abundant phenolic compounds, luteolin and chicoric acid, in four kinds of dandelion leaf extracts and to examine anti-inflammatory and antioxidative activities of luteolin and chicoric acid in LPS-stimulated RAW 264.7 cells.

To verify the most effective extraction way, four kinds of solvents (hot water, AT water, ethanol, and methanol) were applied and the four extracts were evaluated for their inhibitory effects on NO and lipid peroxide formation. As shown in Fig. 1, NO production was reduced with an IC_{50} of 354.7, 505.3, 143.3, 77.8 $\mu\text{g/mL}$ without

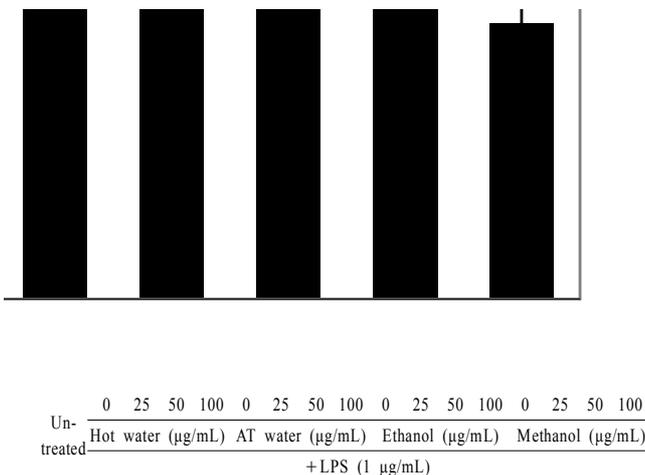


Fig. 1. Effect of four kinds of extracts from dandelion leaf on NO production in LPS-stimulated RAW 264.7 cells. Un-treated signifies the negative control without LPS treatment. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$. AT: ambient temperature.

cytotoxicity (in the order of hot water, AT water, ethanol, and methanol, respectively; cytotoxic data not shown). Among the four extracts, the methanol extract showed the most potent suppressive effect on NO production. Moreover, lipid peroxidation was also dose-dependently reduced by the four extracts, and they showed similar inhibitory fashion (Fig. 2).

Concentration of luteolin and chicoric acid by extraction methods

The concentration of luteolin and chicoric acid were analyzed to evaluate why methanol extract showed the most potent NO inhibition activity. As shown in Table 1, both compounds were found to be relatively higher in the organically-derived extracts (ethanol and methanol) compared to the inorganic (water) solvent extracts. Especially, luteolin and chicoric acid concentrations were quantitated as 34.20 ± 0.08 and 128.59 ± 2.13 $\mu\text{g/g}$ of dried dandelion leaf in the methanol extract. The higher NO inhibitory activity of methanol extract in LPS-stimulated RAW 264.7 cells could be attributed to the higher concentration of each phytochemical.

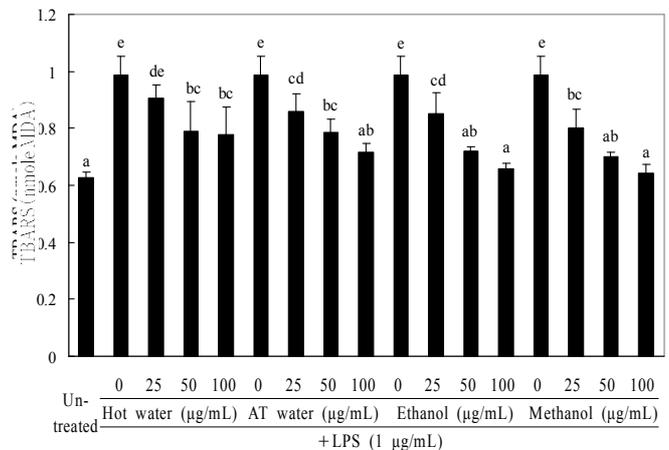


Fig. 2. Effect of four kinds of extracts from dandelion leaf on TBARS generation in LPS-stimulated RAW 264.7 cells. Untreated signifies the negative control without LPS treatment. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$. AT: ambient temperature.

Table 1. Concentration of luteolin and chicoric acid in four kinds of dandelion leaf extracts

	Concentration ($\mu\text{g/g}$ dried dandelion)	
	Luteolin	Chicoric acid
Hot water	3.53 ± 0.04	18.95 ± 0.07
AT water	9.28 ± 0.19	Not detected
Ethanol	26.52 ± 0.17	101.25 ± 0.95
Methanol	34.20 ± 0.08	128.59 ± 2.13

Each value is the mean \pm standard deviation of triplicate experiments.

Inhibition of NO and lipid peroxide formation by luteolin and chicoric acid

Up to this point, the methanol extraction protocol was determined to be the best of the four methods in terms of having the highest potency of NO production inhibition. Luteolin and chicoric acid, abundant in dandelion leaf, were examined for their anti-inflammatory and antioxidative effects through NO production and lipid peroxidation, as well as iNOS gene expression. NO is a free radical generated from L-arginine. Three major NOS isoforms have been identified so far: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Appropriate amounts of NO production regulate various physiological conditions, but excessive NO formation in macrophages, caused by iNOS upregulation induced by inflammatory stimuli such as interleukin, interferon, and LPS, promotes chronic diseases such as atherosclerosis and cancer (15-17). Plant phenolic compounds have a great capacity to scavenge free radicals and NO production, and several epidemiological studies have indicated a possible association between the dietary

intake of polyphenols and the risk of coronary heart disease and cancer (18-22). Moreover, several reports have suggested that dandelion extracts possess anti-inflammatory, anti-oxidative, and anticarcinogenic properties (23-25). In another study, luteolin and luteolin-7-O-glycoside from dandelion flower extract inhibited NO production in LPS-activated RAW 264.7 cells without cytotoxicity (26). This is in accordance with our results, which showed that luteolin and chicoric acid from dandelion leaf dose-dependently suppressed NO production in LPS-stimulated RAW 264.7 cells without any cytotoxicity (Fig. 3, Cytotoxic data not shown).

Suppressed lipid peroxide production, as indicated by malondialdehyde (MDA) content, was attenuated in LPS-stimulated macrophages by luteolin and chicoric acid treatment (Fig. 4). Lipid peroxidation has been used as a marker of cellular oxidative stress and has been attributed to oxidative stress as the result of xenobiotic compounds and inflammatory processes (27). In this study, dandelion leaf contains higher contents of polyphenol, and thereby shows effective antioxidant property as a

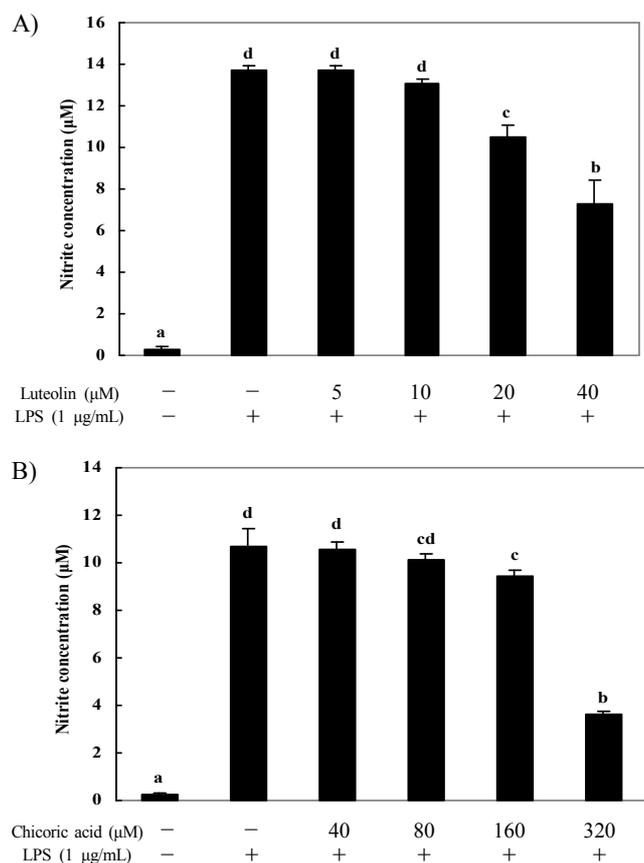


Fig. 3. Effect of luteolin (panel A) and chicoric acid (panel B) on NO production in LPS-stimulated RAW 264.7 cells. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$.

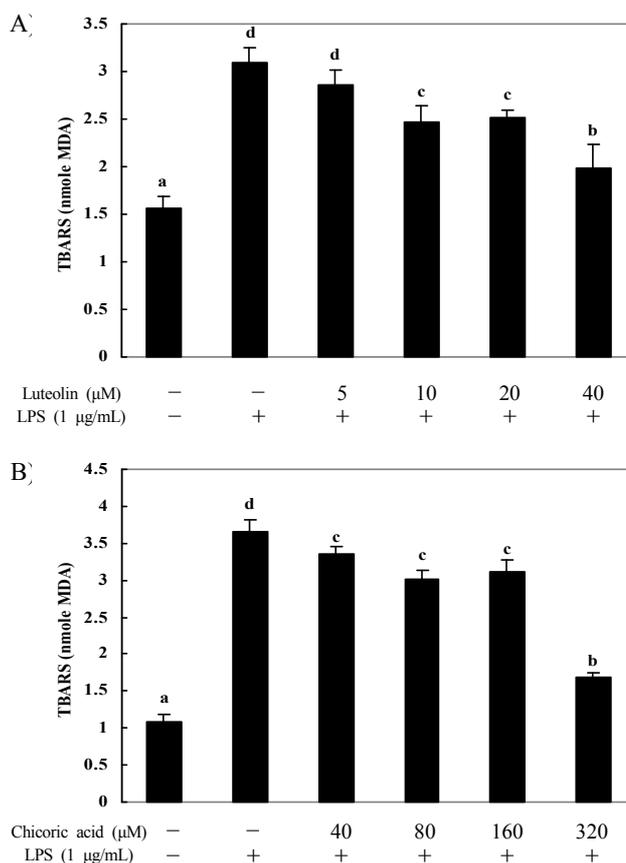


Fig. 4. Effect of luteolin (panel A) and chicoric acid (panel B) on TBARS generation in LPS-stimulated RAW 264.7 cells. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$.

free radical scavenger (28).

Fig. 5 and 6 show altered gene expression levels of iNOS in RAW 264.7 cells. LPS-stimulated murine macrophages were incubated with luteolin and chicoric acid for 20 hr. iNOS mRNA and protein expression showed a dose-dependent reduction when macrophages were incubated with increasing amounts of luteolin and chicoric acid. GAPDH was used as a control, as its expression level remains constant, despite exposure to either luteolin or chicoric acid. This assay showed that luteolin- and chicoric acid-induced iNOS inhibition is not associated with a generalized decrease in mRNA and protein expression. Other researchers reported that luteolin in dandelion flower and chicoric acid from *Echinacea* suppressed iNOS protein expression and NO release in LPS-treated RAW 264.7 cells (6,26). In the present experiment, iNOS gene expression level showed a dose-

pendent decrease in the presence of luteolin and chicoric acid from dandelion leaf. This suggests that both of the phenolic compounds, which are abundantly expressed in dandelion leaf, possess anti-inflammatory and antioxidative properties by regulating iNOS gene expression in LPS-stimulated macrophage cells.

In conclusion, our present study shows that the ethanol and methanol extracts of dandelion leaf, but not the extracts from either hot or AT water, dramatically inhibit NO and lipid peroxide formation in LPS stimulated RAW 264.7. This inhibition is mainly attributed to the luteolin and chicoric acid present in the extracts. These results support the idea that these two phytochemicals contained in dandelion leaf may play critical roles in amelioration of inflammatory and oxidative responses induced by LPS, and therefore could be effective plant flavonoids in the area of nutraceuticals.

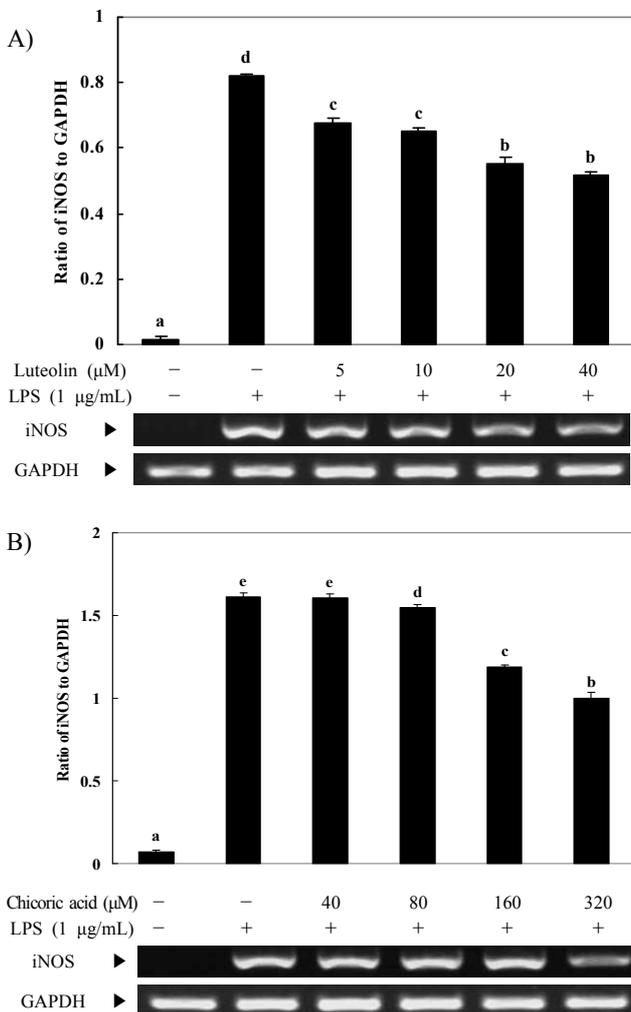


Fig. 5. Effect of luteolin (panel A) and chicoric acid (panel B) on iNOS mRNA expression in LPS-stimulated RAW 264.7 cells. GAPDH was used as an internal control. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$.

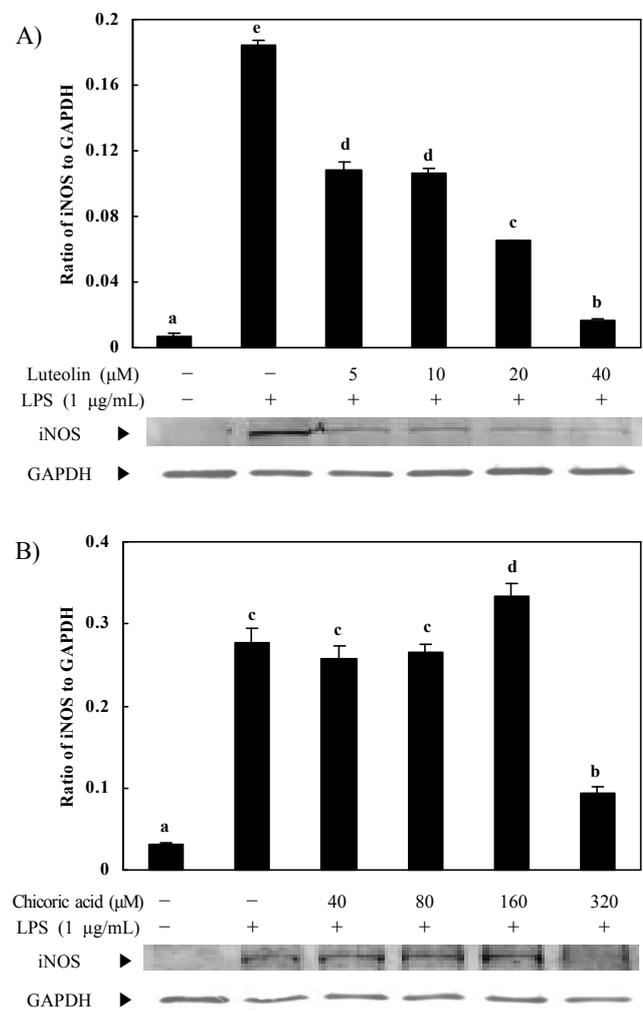


Fig. 6. Effect of luteolin (panel A) and chicoric acid (panel B) on iNOS protein expression in LPS-stimulated RAW 264.7 cells. GAPDH was used as an internal control. Data represent the means \pm SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p < 0.05$.

CONCLUSION

Luteolin and chicoric acid, abundant phytochemicals in dandelion leaf, were measured in four kinds of extracts (hot water, AT water, ethanol, and methanol) and analyzed for their inhibitory effects on NO and lipid peroxide formation in LPS-stimulated RAW 264.7 cells. The ethanol and methanol extracts showed potent NO and lipid peroxide inhibitory effects and contained much higher amount of luteolin and chicoric acid than the water extracts. Significant anti-inflammatory and anti-oxidative activities of both phytochemicals were also identified in LPS-stimulated RAW 264.7 cells. These results suggest that luteolin and chicoric acid in dandelion leaf may play crucial inhibitory roles in LPS-induced oxidative stress and inflammation.

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