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Antiinflammatory Activity of the Medicinal Plant *Geum Japonicum**

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G. japonicum is a perennial herb and the flowering plant has been used as a diuretic and an astringent in Japan and China. However, little information is available about the anti-inflammatory action of _G. japonicum_. Therefore, the objective of this study was to investigate the antiinflammatory action of fractions from _G. japonicum_ methanol extract. Inhibition of NO production was observed when cells were cotreated with fractions of _G. japonicum_ and lipopolysaccharide. We observed that ethyl acetate fraction of _G. japonicum_ inhibited NO production by LPS-activated RAW 264.7 cells, and that the suppression induced by ethyl acetate fraction of _G. japonicum_ was associated with antioxidant activity and direct NO clearance. In addition, only ethyl acetate fraction of _G. japonicum_ inhibited stimulated PGE_2, TNF-α, IL-1β production, whereas water and methyl chloride fractions showed no such effects. The ethyl acetate fraction of _G. japonicum_ methanol extract showed a remarkable scavenging activity on the 1,1-diphenyl-2 picrylhydrazyl radical. Based on the results, ethyl acetate fraction of _G. japonicum_ may be useful source as natural antioxidants and antiinflammation. Therefore, the results obtained from this study provide an alternative protective mechanism of ethyl acetate fraction of _G. japonicum_ and provide information on the potential use of ethyl acetate fraction of _G. japonicum_ in chemoprevention or pathogenic conditions related to overproduction of NO and PGE_2. However, the mechanism of the inflammatory effect must be evaluated through various parameters for induction of NO production.

Key words: *G. japonicum*, Antioxidative properties, Antiinflammatory effects, NO

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INTRODUCTION

G. japonicum is a perennial herb and the flowering plant has been used as a diuretic and an astringent in Japan and China.[1-3] Xu *et al.*[2] reported that *G. japonicum* inhibited the human immunodeficiency virus (HIV-1) protease and the potent inhibitors were isolated as ursolic acid and maslinic acid. Shiraki et al. and Hozumi et al. reported that *G. japonicum* had antiviral effects and antiviral compounds had already been isolated. Recently many Chinese medicinal plants have been screened for their antioxidative effects.[4] Powerful antioxidative activities were detected in the extracts of *Agastache rugosa* and *Geum japonicum*.[5] The antioxidant of *A. rugosa* was identified as rosmarinic acid, while in *G. japonicum*, the antioxidant yet remains unknown. In the present work, attempts were made to verify the folk medicinal claim that the crude ethanolic extract of *G. japonicum* has antiinflammatory action and to relate the activity to particular fractions using NO assay.

Numerous studies have previously shown that various compounds have considerable antioxidant ability and antinflammatory action.[5-11] EGCG, a potent antitumor agent with anti-inflammatory and antioxidant properties, inhibited NO generation, as measured by the amount of nitrite released into the culture medium. Inhibition of NO production was observed when cells were cotreated with EGCG and LPS.[6] In the present study, we reported the inhibitory effect of glabridin on nitric oxide (NO) production and induced nitric oxide synthase (iNOS) gene expression in murine macrophages. Glabridin attenuated lipopolysaccharide (LPS)-induced NO production in isolated mouse peritoneal macrophages and RAW 264.7 cells, a mouse macrophage-like cell line.

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Moreover, induced nitric oxide synthase (iNOS) mRNA expression was also blocked by glabridin treatment in LPS-stimulated RAW 264.7 cells.[7]

NO is a short-lived bioactive molecule that participates in the physiology and pathophysiology of many systems.[12] NO is synthesized *in vivo* from L-arginine by NOS with NADPH and oxygen as cosubstrates.[13,14] Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS isoforms. Low levels of concentration of NO are sufficient, in most cases, to affect these functions. However, during infection and inflammation, *in vivo* formation of NO is increased, suggesting that NO concentrations are well above those found in normal physiological function in infected tissues. High concentrations of NO have been shown to cause deamination of deoxynucleotides and bases within intact DNA *in vitro* and are mutagenic *in vivo*.[15] Exposure of human cells to NO under aerobic conditions results in DNA strand breakage and nitrosactive deamination of DNA bases.[16] Therefore, NO can cause DNA damage as well as mutation in human cells.

Macrophage inducible nitric oxide synthase is able to generate massive amounts of nitric oxide (NO) which contributes to the host immune defense against viruses and bacteria. Monocyte-macrophages stimulated with the bacterial wall component lipopolysaccharide (LPS) and cytokines such as interferon-gamma (IFN-gamma) express the inducible form of nitric oxide synthase (iNOS).[10,11] Furthermore, tumor necrosis factor-alpha (TNF-alpha) is one of the central regulatory cytokines in macrophage antimicrobial activity and synergizes with IFN-gamma in the induction of NO synthesis. Because of its pivotal role in both antimicrobial and tumoricidal activities of macrophages, a significant effort has focused on developing therapeutic agents that regulate NO production. In the present study, fermented papaya preparation (FPP) is shown to exert both immunomodulatory and antioxidant activity in the macrophage cell line RAW 264.7. However, little information is available about the antinflammatory activity of *G. japonicum*. Therefore, the objective of this study was to investigate the anti-inflammatory activity of fractions from *G. japonicum* methanol extract on the nitrite level of murine macrophage-like cell line RAW 264.7.

MATERIALS AND METHODS

1. Preparation of *G. japonicum* Fractions

G. japonicum THUNBERG plants (Bammu in Korea) were grown at Hamyang Medicinal Plant Experimental Station (Rural Development Office, Gyeongsangnam-do) and harvested in October. The stems and leaves were dried under air condition and used for extracts. Aerial parts of *G. japonicum* THUNBERG were extracted with methanol, and the extracts were dried in rotary evaporator, dissolved in H_2O, and fractionated using hexane, methylene chloride, ethyl acetate, and butanol. The extracts and fractions were dried in a rotary evaporator and dissolved in a proper solvent for the anti-inflammatory activity test. Cell culture materials and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. and Promega, Madison, Wisconsin (U.S.A.).

2. Cell Culture

RAW 264.7 cells (ATCC TIB 67), a mouse macrophage-like cell line was cultured in DMEM (dulbeccos modified eagle's medium) supplemented with 10% heat-inactivated fetal bovine serum, 25 mM HEPES, 2 mM Glutamine, 100 IU penicillin ml-1 and 100 μg streptomycin ml-1 in a CO_2 incubator (5% CO_2, and 95% humidity) at 37 ℃. Cells were studied between passages 7~30. Cells were seeded in 24-well dishes at a density of 2×10^5 cells per well and incubated either with or without the indicated amount of *G. japonicum* THUNBERG for 24 h.

3. Cell Viability

Cell viability was determined using the MTT method and through morphologic observations. RAW 264.7 cells (1.0×10^4 cells/well) in 96-well plates were exposed to various concentrations of fractions with LPS 100 ng/mL and IFN-gamma 10 U/mL at 37 ℃ for 20 h. 3-(4,5-Dimethyl thiazol-2yl)-2.5-diphenyl tereasodium (MTT) 10 ul solution (5 mg/mL) was added, and further incubation followed for 4 hr at 37 ℃. After aspirating the supernatant from the wells, 100 ul of dimethylsulfoxide was added to dissolve formazen crystals. The absorbance of each well was read at 550 nm using an MTP-120 microplate reader (Corona Electric Co., Ltd., Japan)

4. Nitrite Analysis

Nitrite was determined spectrophotometrically by using the Griess reagent (0.5% sulfanilic acid, 0,002% N-1-naphtyl-ethylenediamine dihydrochloride, 14% glacial acetic acid) in supernatants. Absorbance was measured at 550 nm with baseline correction at 650 nm and nitrite concentration was determined using sodium nitrite as a standard.[17] Medium alone was used to calculate the assay background level, and this was subtracted from all data.

5. ELISA Assay for TNF-α, IL-1β, and PGE_2 Analysis

The production and release of TNF-α, IL-1β, and PGE_2

in the culture medium was determined using a TNF-α, IL-1β, and PGE_2 ELISA commercial kit (Amersham Bioscience, Buckinghamshire, UK). All assays were run in triplicate.

6. *In Vitro* NO Clearance Activity

To understand whether the lowered NO concentration in the culture medium was due to direct interaction between NO and derivatives, it was incubated with various concentrations of *G. japonicum* THUNBERG fractions in a cell-free condition. The amounts of NO in the reaction mixture were then determined by the Griess reaction.

7. DPPH Radical Scavenging Method

The DPPH radical scavenging method of Duh and Yen[18] was modified as follows. The soluble compounds isolated from *G. japonicum* THUNBERG fractions were standardized to give stock solutions containing 100 ug soluble solid per 1mL/methanol. The stock solutions were diluted to 5, 10, 20, 40, 60 and 100 ug/mL in MeOH and 100 ul of each concentration was added to the methanol (900ul) of DPPH radical; final concentration of DPPH was 0.1 mM. The mixture was shaken vigorously and kept standing at room temperature for 30 min. The absorbency of the resulting solution was measured using a Beckman spectrophotometer DU 70 at 517 nm.

8. Data Analysis

Each experimental result as shown in the figures is the mean±s.d. for at least three measurements. Statistical analyses were performed using one-way ANOVA and further analyzed by Duncan test for statistical difference. Differences between treatments were considered to be statistically significant at $P<0.05$.

RESULTS

1. Cell Viability Effect of *G. Japonicum*

The MTT assay was used to measure the viability of RAW 264.7 cells exposed to *G. japonicum*. As the result in Fig. 1, the viabilities of cells exposed to *G. japonicum* at ethylacetate and water fraction were 60.7±9.7% and 66.4±4.7%, respectively. However, cell viabilities of methanol, methylchloride and hexane fraction were low.

2. Effects of Fractions Obtained from *G. Jponicum* on NO and PGE_2 Production

LPS treatment in RAW 264.7 cells for 24 h significantly enhanced NO production, and cotreatment with *G. japonicum*

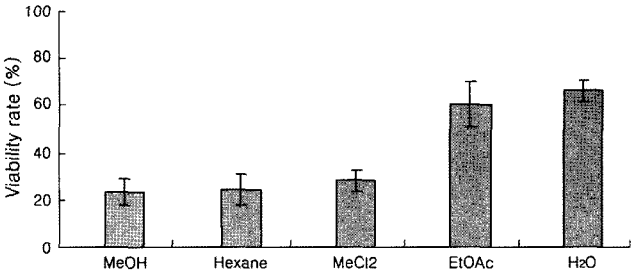

Fig. 1 Cytotoxicity effects of the solvent fractions and methanol extracts derived from Geum japonicum THUNBERG by MTT assay

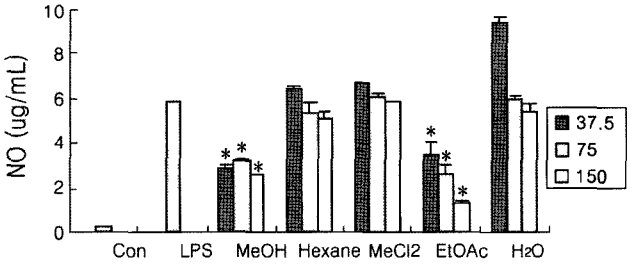

Fig. 2 Effects of fraction of Geum japonicum THUNBERG on LPS-activated nitric oxide released into the medium by RAW 264.7 cells.

Cell were cotreated with LPS (75 ng/ml) and various fractions of Geum *japonicum* THUNBERG (37.5, 70, and 150 ng/ml) for 24 h.
The nitrite content in the medium was then determined as described in MATERIALS AND METHODS. Values represent the mean standard deviation from three separate experiments.
* P<0.05 compared with corresponding LPS alone treated cells.

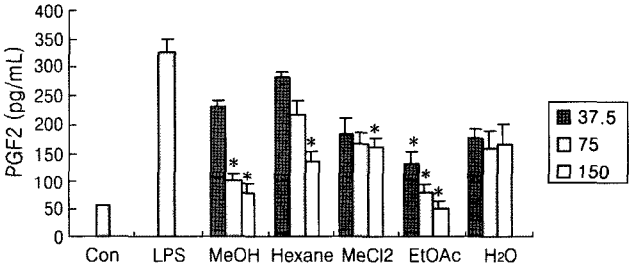

Fig. 3 Effects of fraction of Geum *japonicum* THUNBERG on LPS-activated PGE2 released into the medium by RAW 264. cells.

Cell were cotreated with LPS (75 ng/ml) and various fractions of Geum *japonicum* THUNBERG (37.5, 70, and 150 ng/ml) for 24 h.
Values represent the mean standard deviation from three separate experiments.
* P<0.05 compared with corresponding LPS alone treated cells.

suppressed this enhancement. As shown in Fig. 2, ethyl acetate fraction of *G. japonicum*, at concentrations ranging 37.5 to 150 ng/mL significantly ($P<0.05$) inhibited 31% to 72% of the LPS-induced NO production in a dose-dependent manner. Methanol whole extract (37.5~150 ng/mL) showed similar inhibitory effects, but hexane fraction exhibited the weakest inhibition and 37.5 ng/mL

of water fraction even slightly increased activated NO production. 75 and 150 ng/mL of ethyl acetate fraction of *G. japonicum* inhibiting 53% and 72% of LPS-stimulated cell growth, respectively, suggesting that the NO inhibitory effects of ethyl acetate fraction of *G. japonicum* was not due to cell death. In contrast, these compounds showed different effects on stimulated PGE_2 production. Fig. 3 showed that ethyl acetate and methanol extract of *G. japonicum* inhibited activated PGE_2 production to an extent similar to that of NO, but water fraction of *G. japonicum* and $MeCl_2$ showed no inhibitory effects. Therefore, methanol whole extract, ethyl acetate and water fraction of *G. japonicum* differentially regulated the production of NO and PGE_2 in stimulated RAW 264.7 cells.

3. Effects of Fractions Obtained from *G. Japonicum* on IL-1β and TNF-α Production

Pretreatment with *G. japonicum* dose-dependently reduced the LPS-induced TNF-α, IL-1β formation compared to LPS-stimulated alone cells (Fig 4, 5) As shown in Fig. 4, ethyl acetate fraction of *G. japonicum*, at concentrations ranging 37.5 to 150 ng/mL, significantly

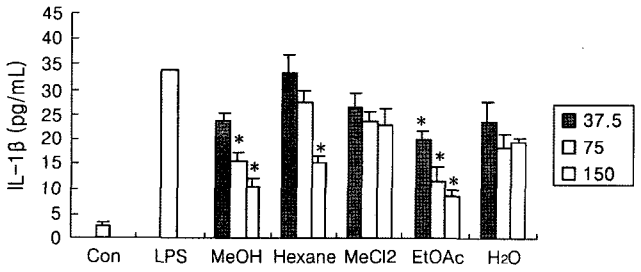

Fig. 4 Effects of fraction of Geum *japonicum* THUNBERG on LPS-activated IL-1ß released into the medium by RAW 264.7 cells.
Cell were cotreated with LPS (75 ng/ml) and various fractions of Geum *japonicum* THUNBERG (37.5, 70, and 150 ng/ml) for 24 h.
Values represent the mean standard deviation from three separate experiments.
* P<0.05 compared with corresponding LPS alone treated cells.

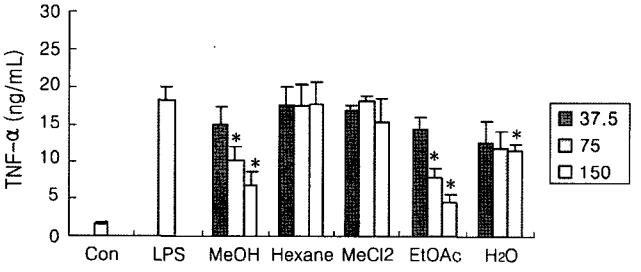

Fig. 5 Effects of fraction of Geum *japonicum* THUNBERG on LPS-activated TNF-α released into the medium by RAW 264.7 cells.
Cell were cotreated with LPS (75 ng/ml) and various fractions of Geum *japonicum* THUNBERG (37.5, 70, and 150 ng/ml) for 24 h.
Values represent the mean standard deviation from three separate experiments.
* P<0.05 compared with corresponding LPS alone treated cells.

inhibited 31% to 72% of the LPS-induced IL-1β production in a dose-dependent manner. Methanol whole extract (37.5~150~ng/mL) and hexane fraction showed similar inhibitory effects, but water and $MeCl_2$ fraction exhibited the weakest inhibition and 37.5 ng/mL of water fraction even slightly increased activated IL-1β production. 75 and 150 ng/mL of ethyl acetate fraction of *G. japonicum* significantly inhibiting 53% and 72% of LPS-stimulated cell growth, respectively, suggesting that the IL-1β inhibitory effects of ethyl acetate fraction of *G. japonicum* was not due to cell death.

Fig. 5 showed that ethyl acetate and methanol whole extract of *G. japonicum* inhibited activated TNF-α production to an extent similar to that of NO, but water, $MeCl_2$ and hexane fraction of *G. japonicum* showed no inhibitory effects. Therefore, methanol whole extract ethyl acetate and water fraction of *G. japonicum* differentially regulated the production of TNF-α in stimulated RAW 264.7 cells.

4. Free Radical Scavenging Test

DPPH is generally used to determine the antioxidative activity. Under the existence of antioxidant, the free radical was reduced to 1,1-diphenyl-2-picryhydrazine, and the purple color disappeared gradually. At a given time, the change in color was measured at A490. The SC_{50} (Concentration of the sample required to scavenge 50% of 100uM DPPH radical) was calculated on the basis of A490 (Table 1), and the SC_{50} values of α-tocopherol, BHT, BHA, and ethyl acetate fraction of *G. japonicum* were 49, 89, 6, and 50, respectively. The ethyl acetate fraction of *G. japonicum* was similar to or more powerful than the commercial synthetic antioxidant α-tocopherol and BHT, respectively. Considering that ethyl acetate fraction of *G. japonicum* still contain impurities, the antioxidative activity of pure ethyl acetate fraction of *G. japonicum* might be much stronger than this.

Table 1. DPPH radical scavenging effects of five different solvent fractions from Geum *japonicum* THUNBERG

Extracts and fractions	Free radical scavenging activities (SC50, μg/mL)[1)]
Methanolic extract	102
Hexane	250,010
Methylene Chloride	396
EtOAc	50
Water	125
α-Tocopherol	49
BHA(control)	55
BHT(control)	78

[1)] Concentration of the sample required to scavenge 50% of 100 uM DPPH radical

5. Effect of *G. Japonicum* on NO Clearance

Because of *G. japonicum* suppression of NO production was associated with antioxidative activity, direct NO clearance was examined. As shown in Fig. 6, all three compounds decreased the levels of NO generated from the NO donor, indicating that *G. japonicum* interacted with NO and thus decreased its availability. Among fractions, ethylacetate fraction of *G. japonicum* was the least effective at NO clearance.

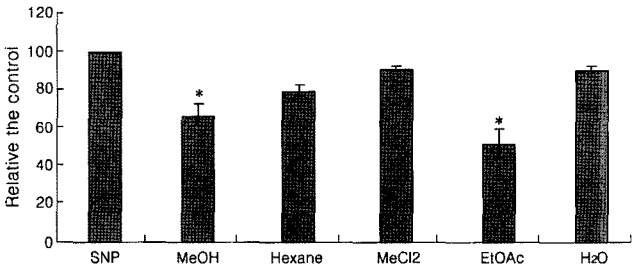

Fig. 6 Effects of fraction of Geum *japonicum* THUNBERG on nitric oxide clearance.

The fractions of Geum *japonicum* THUNBERG were incubated in a 10 mM sodium nitroprusside solution for 60 min, and the nitrite content in the reaction mixture was then determined using the Griess reagent.
Values represent the mean standard deviation from three separate experiments.
* $P<0.05$ compared with corresponding LPS alone treated cells.

DISCUSSION

In the present study, we demonstrated that ethyl acetate fraction of *G. japonicum* inhibited NO production by LPS-activated RAW 264.7 cells, and that the suppression induced by ethyl acetate fraction of *G. japonicum* was associated with antioxidant activity and direct NO clearance. In addition, only ethyl acetate fraction of *G. japonicum* inhibited stimulated PGE_2, TNF-α, IL-1β production, whereas water and methyl chloride fractions showed no such effects.

Recently, many Chinese medicinal plants have been screened in our laboratory for their antioxidative effects.[5] Powerful antioxidative activities were detected in the extracts of Agastache rugosa and *G. japonicum*. In A. rugosa, the antioxidant was identified as rosmarinic acid, while in *G. japonicum*, the antioxidant yet remains unknown. *G. japonicum* is one of the perennial and flowering plants used as a diuretic and an astrigent in Japan and China.[1-3] Its potent functions have also been reported, such as immunodeficiency virus (HIV-1) protease inhibitors and antiviral effects. However, little information is available on its antiinflammatory effects.

Prolonged overproduction of NO and PGE_2 in response to bacterial endotoxin LPS or cytokines plays important roles in inflammation, which is a risk factor for certain cancers.[19,20] Large amounts of NO and increased formation of reactive oxygen species, including superoxide, by macrophages can lead to the generation of peroxynitrite, which is a powerful oxidant and many are readily decomposed to the highly reactive hydroxyl radical and nitrogen dioxide. These highly reactive and toxic compounds may react with macromolecules, such as proteins, DNA, and RNA in cells and cause cellular or tissue damage. Further, the increased formation of nitrosamines from the induction of iNOS contributes to the carcinogenic effect of NO.[20] In addition iNOS and COX-2 are overexpressed in malignant tumors,[19,21] and COX-2 inhibitors have been reported to posses antitumor activity.[22] Hence, ethyl acetate fraction of *G. japonicum* that suppress the overproduction of NO and PGE_2 may play protective roles in the development of inflammation and carcinogenesis. Also, methanol whole extract, ethyl acetate and water fraction of *G. japonicum* differentially regulated the production of TNF-α and IL-1β in stimulated RAW 264.7 cells.

The SC_{50} is the concentration ug/mL of the sample required to scavenge 50% of free radicals. It is a criterion of the power of antioxidant, lower the value more powerful the antioxidative activity. Based on SC_{50}, the antioxidative activity of ethyl acetate fraction of *G. japonicum* is 7 and 12.5 times more powerful than those of BHT and α-tocopherol, respectively. Considering that the sample contained some impurities, we can speculate that ethyl acetate fraction of *G. japonicum* must have very powerful antioxidants. Rancimat analysis of ethyl acetate fraction of *G. japonicum* also showed good antioxidative activity particularly on palm oil.[5] This study showed that ethyl acetate fraction of *G. japonicum* should be a very useful antioxidant source for unsaturated fatty acids.According to these results, it can be concluded that ethyl acetate fraction of *G. japonicum* has strong antioxidative properties and antiinflammatory effects. Ethyl acetate fraction of *G. japonicum* may differently regulate NO, PGE_2, TNF-α, and IL-1β. Various pathways have been suggested for the anticarcinogenic activity of ethyl acetate fraction of *G. japonicum*, including modulation of xenobiotic-metabolizing enzyme activities,[23-25] inhibition of DNA adduct formation,[26] induction of apotosis,[27] modulation of immune functions,[28] and antioxidation.[29] Therefore, the results obtained from this study provide an alternative protective mechanism of ethyl acetate fraction of *G. japonicum* and provide information on the potential use of ethyl acetate fraction of *G. japonicum* in chemoprevention or pathogenic conditions related to overproduction of NO

and PGE_2. Ethyl acetate fraction of *G. japonicum* may be used in foods and medicines. However, the mechanism of the inflammatory effect must be evaluated through various parameters for induction of NO production. In the future, we will attempt to isolate the substance and determine its structure. Also, its applicability for functional food processing and human health will be considered and tested.

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