

감초의 추출용매별 항염증 효능 평가 연구

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Evaluation of Solvent Extraction on the Anti-Inflammatory Efficacy of *Glycyrrhiza uralensis*

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ABSTRACT : *Glycyrrhiza uralensis* (Leguminosae) is a well-known herbal medicine that has long been valued as a demulcent to relieve inflammatory disorders. To compare the influence of different solvents on the anti-inflammatory efficacy of *G. uralensis*, we measured the inhibition of pro-inflammatory mediators such as NO, TNF- α , and PGE₂ in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells by extracts produced using different solvents (water, methanol, ethanol, or *n*-hexane). The results showed that methanol was the most effective solvent for the inhibition of both NO and PGE₂ production in RAW 264.7 cells. However, there was no difference among the extracts for inhibition of TNF- α . Further study must be performed for the analysis of correlation between the anti-inflammatory activity of extracts produced using different solvents and the content of major bioactive compounds in *G. uralensis*, such as glycyrrhizin and liquiritin. The present study suggests that methanol may be a more appropriate solvent of *G. uralensis* than other solvents (water, ethanol, and *n*-hexane) to yield the greatest anti-inflammatory activity for food additives and medicine.

Key Words : *Glycyrrhiza uralensis*, Anti-Inflammatory, Extracting Solvent, NO, TNF- α , PGE₂, Macrophage, Glycyrrhizin, Liquiritin

INTRODUCTION

Inflammation protects the host against tissue injury and microbial invasion. However, an excessive inflammatory response and failure of immune protection can result in many immune-related diseases (Pulendran *et al.*, 2001; Steinman, 2004). Doctors have prescribed steroidal anti-inflammatory drugs (SAID) and non-steroidal anti-inflammatory drugs (NSAID) to treat acute inflammatory disorders, but these conventional drugs have not been successful against chronic inflammatory disorders such as rheumatoid arthritis (RA) and atopic dermatitis (AD). The lack of knowledge about the critical etiology and exacerbating mechanisms of inflammatory disorders has delayed the development of new anti-inflammatory drugs

(Kim *et al.*, 2004). Therefore, it is noteworthy that the use of traditional medicinal plants or their crude extracts is increasingly becoming an attractive approach as a complement or alternative medicine for treatment of various inflammatory disorders (Lee *et al.*, 2001).

Glycyrrhiza uralensis Fisch (Leguminosae) is well known as 'Kam-cho' in Korea. It is one of the most widely used as a traditional herbal medicine and food additive, and is officially listed in the Korean Pharmacopoeia. The root of *G. uralensis* has been used for the treatment of various diseases as well as a tonic medicine for thousands of years. Meanwhile, the herb has been valued as a demulcent to relieve respiratory and gastrointestinal ailments such as allergies, bronchitis, tuberculosis, gastritis, and hepatitis (Cheng *et al.*, 2008b). The principle pharmacologically

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ingredients in the root of the plant are glycyrrhizin (triterpene) and liquiritin (flavonoid) (Sabbioni *et al.*, 2006; Sun *et al.*, 2008). Glycyrrhizin and liquiritin were reported to have anti-inflammatory, anti-viral, and anti-oxidant activities (Sun *et al.*, 2008).

Although the water extract of *G uralensis* has been used widely in medicine, drug and food industries because of its physical and functional properties (Cheng *et al.*, 2008a), extraction solvents for maximizing anti-inflammatory activity have not been well studied. Presently, most medicinal herbs are extracted with H₂O. Therefore, the anti-inflammatory potentials of extracts by different solvents should be evaluated and compared to determine which preparation has the most efficacy. In the present study, the influences of different solvents on anti-inflammatory efficacy were studied using four pure solvents (water, methanol, ethanol, and *n*-hexane) and two aqueous solvents (70% ethanol and 85% ethanol). Since the production of pro-inflammatory mediators such as nitric oxide (NO), tumor necrosis factor- α (TNF- α), and prostaglandin E₂ (PGE₂) are critical steps in inflammation (Fujiwara and Kobayashi, 2005; Paul *et al.*, 1999), the anti-inflammatory activity of these various extracts was measured via the inhibition of NO, TNF- α , and PGE₂ production in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells. This is the first report describing the use of different solvents for extracting the active components of *G uralensis* and measuring their anti-inflammatory potential.

MATERIALS AND METHODS

1. Chemicals

HPLC grade methanol (MeOH), ethanol (EtOH), water (H₂O), and *n*-hexane were purchased from Burdick and Jackson (Muskegon, MI, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY). Griess reagent for measuring NO and the enzyme immunoassay kits for TNF- α , and PGE₂ were obtained from R&D Systems (Minneapolis, MN). LPS (*Escherichia coli* 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2. Plant material

Roots of *Glycyrrhiza uralensis* Fisch (Leguminosae) were

purchased from Omniherb (Oriental drug store, Yeongcheon, Korea) and were authenticated on microscopic characteristics according to the 'Classification and Identification Committee of the Korea Institute of Oriental Medicine'. This committee was composed of nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. In addition, a voucher specimen (KIOM0079014) was deposited at the herbarium of Center of Herbal Resources Research at Korea Institute of Oriental Medicine (Daejeon, Korea).

3. Sample preparation for anti-inflammatory evaluation

The dried roots (20 g) of *G uralensis* were refluxed twice for 2 hrs with 200 ml of H₂O, MeOH, EtOH, 70% EtOH, 85% EtOH, or *n*-hexane. After extraction, the solvent was evaporated under a vacuum, lyophilized. The yields of dried extracts from starting plant materials were 38.2% (H₂O), 34.2% (MeOH), 30.8% (EtOH), 59.8% (70% EtOH), 31.0% (85% EtOH), and 3.0% (*n*-hexane), respectively. The lyophilized powder was dissolved in 10% dimethyl sulfoxide (DMSO) and then filtered through a 0.2-mm syringe filter to generate the stock solution.

4. Cell culture

Mouse macrophage RAW 264.7 cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 mg/ml) and penicillin (100 units/ml) at 37°C in an incubator with a humidified atmosphere of 5% CO₂ at 37°C.

5. Cell viability

MTT assay, which is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenase in viable cells, was performed to examine the effect of the extracts from different solvents on the viability of RAW 264.7 cells (Carmichael *et al.*, 1987). RAW 264.7 cells (5.0×10^5 cells/ml) were cultured in 96-well plates for 24 hrs after the treatment with samples. MTT solution (final 500 μ g/ml) was added to each well and incubated for 1 hr at 37°C. Media were discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve the generated formazan. The absorbance was measured at 570 nm by a spectrMAX 340 reader (Molecular Devices, Silicon Valley, CA, USA), and the percentage of survival was determined by comparison with the control group.

6. Measurement of NO generation

RAW264.7 cells (5×10^5 cells/ml) were cultured in 10% FBS-DMEM without phenol red in 96-well plates for 24 hrs. Subsequently, the medium was replaced with new medium containing $1 \mu\text{g/ml}$ LPS and test sample. After an additional 20 hrs of incubation, the medium was harvested and NO production was measured using a NO colorimetric assay (R&D Systems Inc., Minneapolis, MN, USA) based on the Griess reaction (Green *et al.*, 1982) according to the manufacturer's instructions. One hundred percentage activity was defined as the difference between NO formation in the absence (blank) and in the presence (control) of LPS for 20 hrs in triplicate. The percentage inhibition was calculated as $[1 - (\text{NO level of sample} - \text{NO level of blank}) / (\text{NO level of control} - \text{NO level of blank})] \times 100$.

7. Measurement of TNF- α and PGE₂ production

RAW264.7 cells were seeded in 96-well plates at a concentration of 5×10^5 cells/ml with 10% FBS-DMEM, and incubated the cultures for 24 hrs. Subsequently, the medium was replaced with new medium containing $1 \mu\text{g/ml}$ LPS and test samples, and incubated for 20 hrs. Finally, the medium was harvested and TNF- α and PGE₂ production was measured with an enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. One hundred percentage activity was defined as the difference of the accumulation of TNF- α and PGE₂ between the absence (blank) and the presence (control) of LPS for 20 hrs in triplicate. The percentage inhibition of TNF- α and PGE₂ was calculated as $[1 - (\text{TNF-}\alpha \text{ level of sample} - \text{TNF-}\alpha \text{ level of blank}) / (\text{TNF-}\alpha \text{ level of control} - \text{TNF-}\alpha \text{ level of blank})] \times 100$, and $[1 - (\text{PGE}_2 \text{ level of sample} - \text{PGE}_2 \text{ level of blank}) / (\text{PGE}_2 \text{ level of control} - \text{PGE}_2 \text{ level of blank})] \times 100$.

8. Statistical analysis

Assay was performed in triplicate and the value was expressed as mean \pm S.D. Statistical significance was analyzed by Student's *t*-test and ANOVA using Tukey test. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

When medicinal herbs are used for therapeutic

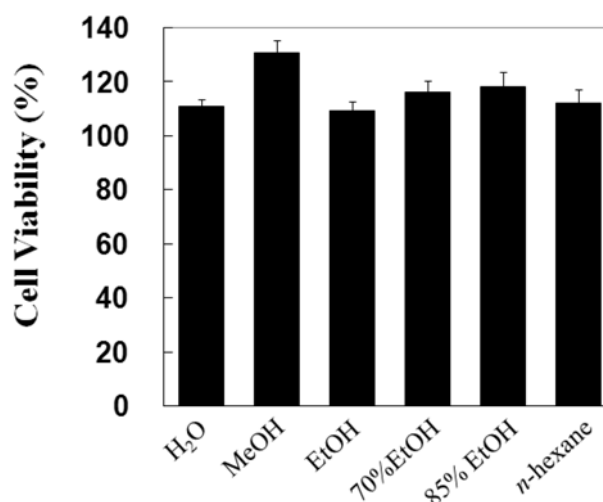


Fig. 1. Cytotoxicity of extracts using different solvents. MTT assay was performed after treatment with extracts ($100 \mu\text{g/ml}$) for 24 hrs. Cell viability is expressed as a mean \pm S.D. and was $> 90\%$ for individual groups.

preparations, they normally extracted by water regardless of their chemical content without regard to effect on preservation of optimal anti-inflammatory efficacy. This study was the first to compare various solvents on extraction efficiency and preservation of anti-inflammatory potential under standardized experimental conditions.

In this study, we investigated how the different extraction solvents (water, methanol, ethanol and *n*-hexane) influence the anti-inflammatory capacity of *G. uralensis*. First of all, we evaluated cytotoxicity of extracts according to different solvents. The MTT assay was performed to investigate any cytotoxic effects of the extracts on the viability of RAW 264.7 cells. Compared to untreated cells (100% viable), there was no significant decrease in cell viability at solvent concentrations up to 100 mg/ml (Fig. 1). The percentage of viable cells was greater than 90% for all extracts. The results indicated that the extracts from different solvents are not cytotoxic at the concentration of $100 \mu\text{g/ml}$.

Since pro-inflammatory mediators, NO, TNF- α , and PGE₂ play important roles in the inflammatory-related diseases such as cancer, multiple sclerosis, Parkinson's syndrome and Alzheimer's disease (Lin *et al.*, 2008), we chose to measure the levels of secreted NO, TNF- α , and PGE₂ from macrophage cells treated with *G. uralensis* extracts.

The level of Nitrite, the metabolite of NO and used as an indicator for NO generation (Lee and Cho, 2007; Seo *et al.*, 2009), was monitored in cultured LPS-stimulated RAW

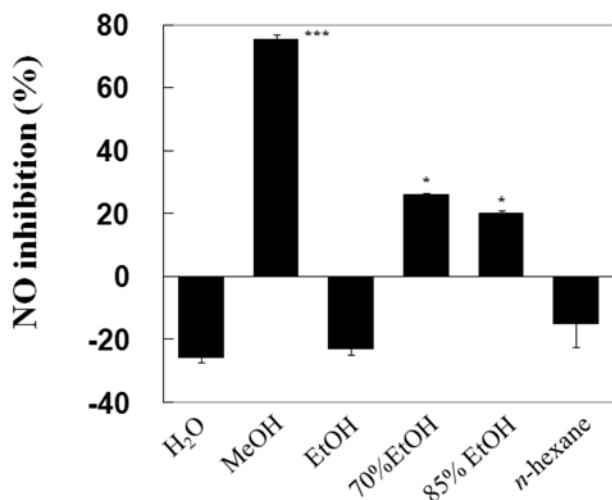


Fig. 2. Effects of the extracts of *G. uralensis* using different solvents on NO generation in LPS-stimulated RAW 264.7 cells. For analysis, cells were treated with 100 $\mu\text{g/ml}$ extract and 1 $\mu\text{g/ml}$ LPS for 20 hrs. * $P < 0.05$; significantly different from the control, ** $P < 0.01$; significantly different from the control, *** $P < 0.001$; significantly different from the control.

264.7 cells to evaluate the effects of the *G uralensis* extracts on inflammatory NO formation. Fig. 2 shows that the inhibition of NO production was 75.4%, 26.0%, and 20.1% for MeOH, 70% EtOH, and 85% EtOH, respectively. The MeOH extract had the greatest inhibitory effect on NO generation. However, water, EtOH, and *n*-hexane were not shown the inhibitory activity on LPS-induced generation of NO. The result indicates that the MeOH extract showed the greatest efficacy for inhibiting NO production.

To evaluate the inhibitory activities of the *G uralensis* extracts on inflammatory TNF- α production, we measured the level of TNF- α in LPS-stimulated RAW264.7 cells. We found that the extracts of *G uralensis* using solvents of 85% EtOH, MeOH, EtOH, H₂O, *n*-hexane, and 70% EtOH inhibited TNF- α production by 38.7%, 29.5%, 24.8%, 23.4%, 12.2%, and 4.6%, respectively (Fig. 3). All extracts except 70% EtOH were found to inhibit LPS-induced generation of TNF- α .

We measured the level of PGE₂ in LPS-stimulated RAW264.7 cells to investigate the inhibitory activities of the *G uralensis* extracts on inflammatory PGE₂ production. The results showed that the inhibition of PGE₂ synthesis were 96.4%, 69.9%, 54.3%, and 27.1% for the extracting solvents of MeOH, 70% EtOH, 85% EtOH, and EtOH, respectively (Fig. 4). However, water and *n*-hexane were not shown the

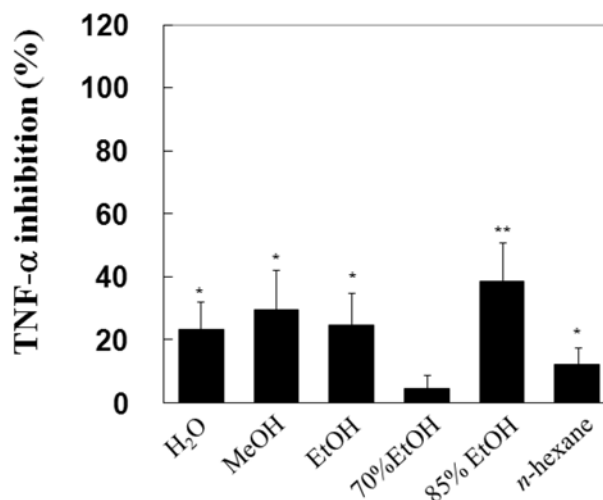


Fig. 3. Effects of the extracts of *G. uralensis* using different solvents on TNF- α production in LPS-stimulated RAW 264.7 cells. For analysis, cells were treated with 100 $\mu\text{g/ml}$ extract and 1 $\mu\text{g/ml}$ LPS for 20 hrs. * $P < 0.05$; significantly different from the control, ** $P < 0.01$; significantly different from the control, *** $P < 0.001$; significantly different from the control.

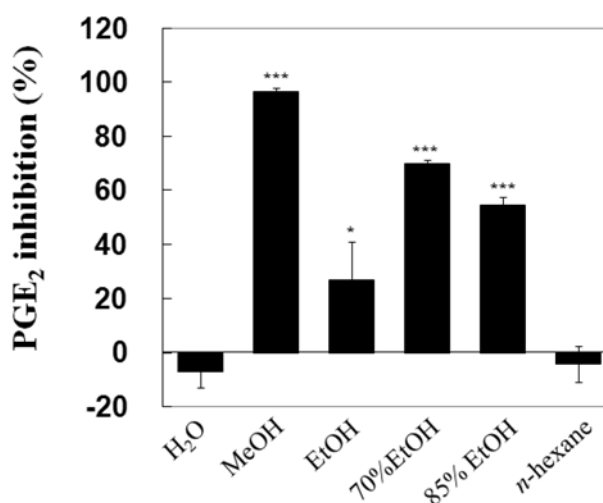


Fig. 4. Effects of the extracts of *G. uralensis* using different solvents on PGE₂ production in LPS-stimulated RAW 264.7 cells. For analysis, cells were treated with 100 $\mu\text{g/ml}$ extract and 1 $\mu\text{g/ml}$ LPS for 20 hrs. * $P < 0.05$; significantly different from the control, ** $P < 0.01$; significantly different from the control, *** $P < 0.001$; significantly different from the control.

inhibitory activity on LPS-induced production of PGE₂. The solvents yielding significant inhibition of PGE₂ were MeOH > 70% EtOH > 85% EtOH > EtOH. Among the extraction solvents, MeOH was the best extracting solvent for inhibition of PGE₂.

Our study indicates that MeOH was proven to be the best solvent among the different solvents (water, methanol, ethanol, and *n*-hexane) for retaining anti-inflammatory activity against both NO and PGE₂ production. To determine the key elements responsible for suppressing NO and PGE₂ production and to clarify the mechanisms involved, further investigations need to be performed. Previous studies showed that glycyrrhizin and liquiritin have anti-viral, anti-inflammatory, and anti-oxidant activities (Cheng *et al.*, 2008a; Sun *et al.*, 2008; Sun and Pan, 2006). Further study must analyze the correlation between the anti-inflammatory activity and the content of bioactive ingredients in *G. uralensis*, such as glycyrrhizin and liquiritin, according to the different solvents. The polar solvents, especially MeOH, may be favorable for the extraction of these active compounds in *G. uralensis*. Also, the intensive researches need to be performed for the discovery of the new active components in MeOH extract from *G. uralensis* responsible for anti-inflammatory effect.

It is routine practice for scientists to investigate medicinal herbs with a view to finding the single compound responsible for the biological activity, which may lead to inconclusive findings. Whole or partially purified plant extracts likely have advantages over a single isolated ingredient. Considering pharmacological properties of herbal medicine, extracts or mixtures of extracts from these plants might be more suitable for the treatment or prevention of diseases than a single chemical entity. Synergistic interactions are of vital importance in phytomedicines to explain that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient. Evidence to support the occurrence of synergy within phytomedicines is now accumulating (Williamson, 2001). The anti-inflammatory effect of *G. uralensis* can be considered as the synergistic effect of its mainly active components of glycyrrhizin and liquiritin and other components. The synergistic effect may be due to multiple different mechanisms of action of the active compounds, and it may be possible that their effect on different targets might account for the synergism. The additional research on the total herbal extract needs to be performed for showing a better effect than an equivalent dose of an isolated compound.

In conclusion, MeOH was proven to be the best solvent for retaining anti-inflammatory activity against both NO and PGE₂ production. Thus, methanol as an extraction solvent

may be superior for *G. uralensis* as compared to conventional use of water for the development of anti-inflammatory food additives and medicines.

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