

Comparative Study of Extraction Solvents on the Anti-inflammatory Effects of *Scutellaria baicalensis*

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Objectives: This study was performed to evaluate the influence of different extracting solvents (water, methanol, ethanol, or n-hexane) on the anti-inflammatory efficacy of *Scutellaria baicalensis* (Lamiaceae), which has been used widely as a traditional herbal medicine for its anti-inflammatory properties.

Methods: The ability of each extract to inhibit the production of pro-inflammatory mediators such as NO, TNF- α , and PGE₂ by lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells was measured.

Results: The results showed that extraction solvents (except n-hexane) for *S. baicalensis* showed significant inhibitory effects on NO, TNF- α and PGE₂ production. Especially, methanol was the solvent with the greatest activity against NO and PGE₂ production. However, there was no difference between the extracts for inhibitory activity of TNF- α .

Conclusion: The present study suggests that methanol is a superior extraction solvent than water, ethanol, or n-hexane for maintaining the anti-inflammatory effects of *S. baicalensis*.

Key Words : *Scutellaria baicalensis*, anti-inflammatory, extracting solvent, NO, TNF- α , PGE₂

Introduction

Inflammation is defined clinically as a pathophysiological process characterized by redness, edema, fever, pain and loss of function¹. Doctors have prescribed steroidal anti-inflammatory drugs (SAID) and non-steroidal anti-inflammatory drugs (NSAID) to treat acute inflammatory disorders, but these conventional drugs cannot cure chronic inflammatory disorders such as rheumatoid arthritis (RA) and atopic dermatitis (AD). Gaps in knowledge about the causes and mechanisms of these inflammatory disorders have delayed the development of new

drugs and increased the demand for safe, plant-derived anti-inflammatory agents². Medicinal plants or their crude extracts are gaining popularity as complements or alternatives to traditional treatment regimens for inflammatory disorders³.

Scutellaria baicalensis Georgi (Labiatae) is well known as *Hwang-keum* in Korea and is one of the most widely used traditional herbal medicines and food additives that are listed officially in the Korean Pharmacopoeia. The roots of *S. baicalensis* have been used for the treatment of various diseases such as inflammation, cancer, bacterial and viral infections of the respiratory and gastrointestinal tracts, toxicosis,

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and hyperlipidemia^{4,7}).

S. baicalensis contains a variety of phenylethanoids, amino acids, sterols, essential oils, and flavonoids. Flavonoids, as natural anti-inflammatory agents⁸), are thought to neutralize and/or modulate oxidative stresses such as superoxide, nitric oxide and peroxynitrite radicals, and protect cells during inflammation⁹). Flavonoids of *S. baicalensis* include baicalin, baicalein, wogonin, wogonin 7-O-glucuronide, oroxylin A, oroxylin A 7-O-glucuronide, chrysin, apigenin, and scutellarein. Baicalin, baicalein, wogonin, and oroxylin A are the most active¹⁰⁻¹²), and baicalin is the most abundant. Baicalin has anti-allergic¹³), anti-inflammatory¹⁴), anti-HIV^{15,16}), anti-tumor¹⁷⁻¹⁹), antioxidant and free radical scavenging²⁰), and anti-SARS coronavirus²¹) effects. Baicalein has anti-HIV^{15,16}), anti-tumor¹⁸), and antioxidant and free radical scavenging effects²⁰). Wogonin has anti-respiratory syncytial virus²²), anti-hepatitis B virus²³), anti-tumor¹⁸), and antioxidant and free radical scavenging effects²⁴). Oroxylin A has anti-respiratory syncytial virus effects²²). Chrysin has anti-inflammatory²⁵), anti-tumor²⁶), and radioprotective effects²⁷).

S. baicalensis has been used as an anti-inflammatory agent in traditional oriental medicine for many years²⁸), however, solvents used for extracting its bioactive components to maximize anti-inflammatory activity have not been well studied. Most herbal medicines customarily are extracted with water. Therefore, the anti-inflammatory potentials of extracts generated using other solvents need to be tested and compared for more effective application. In the present study, we used four pure solvents (water, methanol, ethanol, and n-hexane) and two aqueous solvents (70% ethanol and 85% ethanol) for extracting *S. baicalensis*. Since the production of pro-inflammatory mediators such as nitric oxide (NO), tumor necrosis factor- α (TNF- α), and prostaglandin E₂ (PGE₂) are considered critical steps of the inflammatory process^{29,30}), the anti-inflammatory activity of the different *S. baicalensis* extracts was measured by the inhibition of NO, TNF- α , and PGE₂ production from lipopolys-

accharide (LPS)-stimulated mouse macrophage RAW 264.7 cells. This is the first report describing the effect of extraction of *S. baicalensis* using various solvents on anti-inflammatory activity.

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 NO detection 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 purchased from Sigma Chemical Co. (St. Louis, MO).

2. Plant material

The 1-year-old roots of *Scutellaria baicalensis* Georgi (Labiatae) were obtained from Omniherb Co. (Yeongcheon, Korea) and authenticated based on macroscopic characteristics according to the Classification and Identification Committee of the Korea Institute of Oriental Medicine. The committee was composed of nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. A voucher specimen (KIOM 0077004) was deposited at the herbarium of Center of Herbal Resources Research at the Korea Institute of Oriental Medicine (Daejeon, Korea).

3. Sample preparation for evaluation of anti-inflammatory activity

The dried roots (20 g) of *S. baicalensis* were extracted twice with 2 hrs reflux using 200 mL of H₂O, MeOH, EtOH, 70% EtOH, 85% EtOH, or

n-hexane, and the extracts were then concentrated under reduced pressure, lyophilized, and stored at 4°C. The lyophilized powder was dissolved in 10% dimethyl sulfoxide (DMSO) and filtered through a 0.2-µm syringe filter to create each stock solution.

4. Cell culture

The mouse monocyte/macrophage cell line, RAW 264.7, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

5. Cell viability

To examine the cytotoxicity of extracts from different solvents on cell viability of RAW 264.7 cells, the MTT assay was used to measure the amount of formazan due to mitochondrial dehydrogenase in viable cells³¹. RAW 264.7 cells (5.0×10^5 cells/mL) were cultured in 96-well plates for 24 hrs after treatment with sample extracts. Next, MTT solution (final 500 µg/mL) was added to each well and incubated for 1 hr at 37°C. Media were discarded and DMSO was added to each well to dissolve the generated formazan. Light absorbance at 570 nm was measured using a SpectraMax 340 reader (Molecular Devices, Silicon Valley, CA, USA) and the percent survival from each group was compared with a control group.

6. Measurement of NO generation

RAW264.7 cells in 10% FBS-DMEM without phenol red were plated in 96-well plates (5×10^5 cells/mL), and incubated for 24 hrs. Subsequently, the medium was replaced with new medium containing 1 µg/mL of LPS and test extracts of various concentrations (0, 1, 10, 50, or 100 µg/mL). After an additional 20 hrs incubation, the medium was

removed and the generation of NO was analyzed by an NO colorimetric assay (R&D Systems Inc., Minneapolis, MN, USA) based on the Griess reaction³² according to the manufacturer's instructions. 100 % 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. Percent 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 in 96-well plates were seeded at a concentration of 5×10^5 cells/mL in 10% FBS-DMEM, and incubated for 24 hrs. Next, the medium was replaced with new medium containing 1 µg/mL LPS and test extracts of various concentrations (0, 1, 10, 50, or 100 µg/mL), and incubated for 20 hrs. Finally, the medium was harvested and TNF-α and PGE₂ were measured with an enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. 100 % activity was defined as the difference in accumulation of TNF-α and PGE₂ between the absence (blank) and the presence (control) of LPS for 20 hrs in triplicate. Percent inhibition of TNF-α and PGE₂ were 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

All the measurements were performed in triplicate. The results are presented as mean ± S.D. Statistical significance was analyzed by Student's t-test and ANOVA using SPSS package (version 10.0). A P-value less than 0.05 was considered to be statistically significant.

Results and Discussion

Water is used most commonly for extraction of medicinal herbs for therapeutic preparations regardless of their chemical content, without regard to effect on preservation of optimal anti-inflammatory efficacy. This study compared how well a variety of solvents can be used to extract the anti-inflammatory potential from *S. baicalensis*. As an Oriental medicine, the root of *S. baicalensis* has been used for treatment of variety of inflammatory diseases because of the pharmacological efficacy based on the cold and bitter properties of heat-clearing and dampness-drying herbs according to the traditional herbological classification. To our knowledge, this is the first investigation which has been performed under standardized experimental conditions for extracting solvents of *S. baicalensis*.

In this study, we compared how different extraction solvents (water, methanol, ethanol and n-hexane) affect the anti-inflammatory activity of *S. baicalensis*. First of all, we evaluated cytotoxicity of extracts according to different solvents. To investigate whether the extracts of different solvents had cytotoxic effects on the viability of RAW 264.7 cells, we performed an MTT assay. Figure 1 shows that the extracts at a concentration of 100 $\mu\text{g/mL}$ induced no significant decrease in cell viability compared with control (100% viable). Cell viability was greater than 90%

for all extracts. We conclude that the different solvents are not cytotoxic below a concentration of 100 $\mu\text{g/mL}$.

The production of NO, TNF- α , and PGE₂ from macrophage cells treated with *S. baicalensis* extracts were measured because the release of these inflammatory mediators play important roles in diseases such as cancer, multiple sclerosis, Parkinson's syndrome and Alzheimer's disease³³.

The level of nitrite, the metabolite of NO and used as an indicator for NO generation, was monitored in cultured LPS-stimulated RAW 264.7 cells to evaluate the effects of the *S. baicalensis* extracts on inflammatory NO formation. The stimulation of LPS caused the increased generation of NO to 40.7 μM from 0 μM in the culture medium (data not shown). The inhibition of LPS-induced NO production by the different extracts was 89.5%, 72.5%, 71.9%, 70.9%, and 70.1% for MeOH, 85% EtOH, 70% EtOH, EtOH, and H₂O respectively (Fig. 2). However, n-hexane did not show inhibitory activity on LPS-induced generation of NO. The MeOH extract of *S. baicalensis* showed the greatest inhibitory effect on generation of NO.

To evaluate the inhibitory activities of the *S. baicalensis* extracts on inflammatory TNF- α production, we measured the level of TNF- α in LPS-stimulated RAW264.7 cells. The stimulation of LPS increased

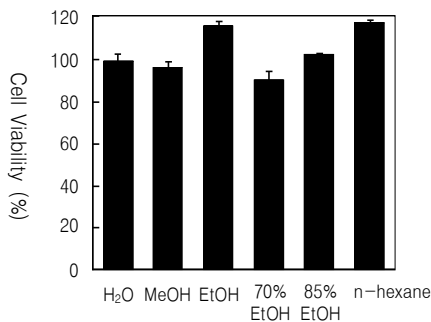


Fig. 1. Cytotoxicity of the different extracts.

An MTT assay was performed after treatment of cells with the extracts (100 $\mu\text{g/mL}$) for 24 hrs. Cell viability is expressed as a mean \pm S.D. (N = 3) and was >90% for individual groups.

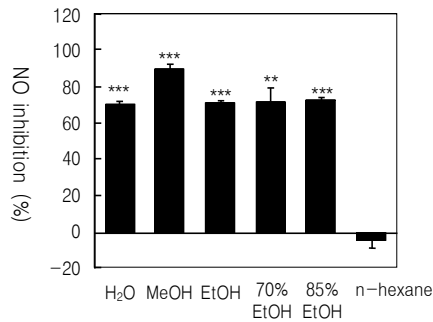


Fig. 2. Effects of the *S. baicalensis* extracts on NO generation in LPS-stimulated RAW 264.7 cells.

For this assay, cells were treated with 50 µg/mL extract and 1 µg/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

the production of TNF-α to 48526.9 pg/mL from 0 pg/mL (data not shown). Figure 3 shows the inhibition of LPS-induced TNF-α synthesis as 28.9%, 25.9%, 20.1%, 18.9%, and 13.1% for 70% EtOH, H₂O, 85% EtOH, MeOH, and EtOH, respectively. All extracts except n-hexane inhibited LPS-induced production of TNF-α.

We measured the level of PGE₂ in LPS-stimulated RAW264.7 cells to investigate the inhibitory activities of the *S. baicalensis* extracts on inflammatory PGE₂ production. The stimulation of LPS caused the

increased release of PGE₂ to 690.9 pg/mL from 1.3 pg/mL (data not shown). Figure 4 shows the effects of the extracts on PGE₂ production. The inhibition of LPS-induced PGE₂ production was 99.3%, 57.6%, 57.5%, 56.3%, and 50.6% for MeOH, 85% EtOH, 70% EtOH, H₂O, and EtOH, respectively. However, n-hexane did not show inhibitory activity on LPS-induced production of PGE₂. This result indicates that the MeOH extract had the greatest inhibition of production of PGE₂.

Our study indicates that extraction solvents (except

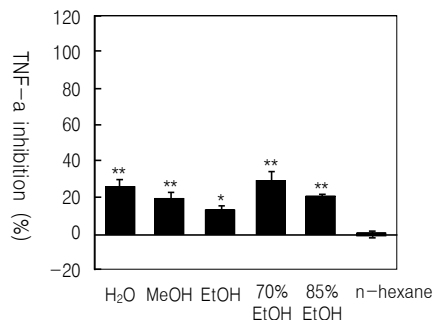


Fig. 3. Effects of the *S. baicalensis* extracts on TNF-α production in LPS-stimulated RAW 264.7 cells.

For this assay, cells were treated with 100 µg/mL extract and 1 µg/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

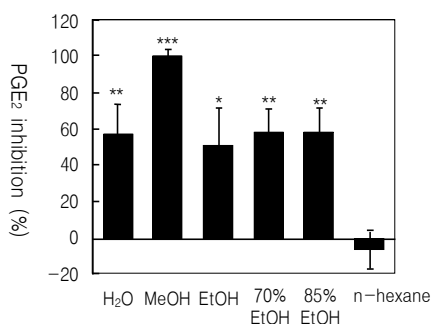


Fig. 4. Effects of the *S. baicalensis* extracts on PGE₂ production in LPS-stimulated RAW 264.7 cells.

For this assay, cells were treated with 1 µg/mL extract and 1 µg/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

n-hexane) for *S. baicalensis* showed significant inhibitory effects on NO, TNF-α and PGE₂ production. Based on NO and PGE₂ analysis, the anti-inflammatory activities in decreasing order were MeOH > 85% EtOH = 70% EtOH = H₂O = EtOH. Thus, MeOH was the solvent that most inhibited the production of NO and PGE₂ among the tested solvents.

We recommend further investigation to identify key elements responsible for suppressing NO and PGE₂. Baicalin, baicalein, wogonin, and chrysin are found in *S. baicalensis* and exert anti-inflammatory action. Baicalein and wogonin can inhibit LPS-induced NO production in macrophages³⁴. Baicalein is the most effective component of *S. baicalensis* for antioxidant and/or free radical scavenging properties³⁵. Moreover, baicalein inhibited the production of leukotriene C₄ (LTC₄) in the same inflammatory model as above, suggesting an inhibitory effect on 5-lipoxygenase (5-LOX)³⁶. Baicalin down-regulates cytokine and PGE₂ expression, nitric oxide formation and neutrophil invasion in a carrageenan-induced paw edema model³⁷. Chrysin has anti-inflammatory effects²⁵. Further study is needed to analyze the correlation between the anti-inflammatory activity and the content of bioactive ingredients of *S. baicalensis*, such as baicalin, baicalein, wogonin, and chrysin, according

to the different solvents. Also, intensive research needs to be performed for the discovery of the new active components in the MeOH extract of *S. baicalensis* responsible for its anti-inflammatory effect. These studies will partially help to understand the cold and bitter properties of the root of *S. baicalensis* according to the traditional herbological classification.

In conclusion, methanol had the greatest activity for inhibiting the production of NO and PGE₂ as compared with other solvents (water, ethanol, and n-hexane), and may be a useful substitute for water in the preparation of *S. baicalensis* used in anti-inflammatory medicines and food. Additionally, a toxicity and safety study of MeOH extract from *S. baicalensis* must be performed for usage of methanol as the proper solvent for *S. baicalensis* for generating extracts with anti-inflammatory activity.

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