Full Paper

Anti-inflammatory Activity of Methylene Chloride Fraction From *Glehnia littoralis* Extract via Suppression of NF-κB and Mitogen-Activated Protein Kinase Activity

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Abstract. *Glehnia littoralis* (Umbelliferae) has been used traditionally in Korean, Japanese, and Chinese medicine for the treatment of immune-related diseases; however, its anti-inflammatory activity and underlying mechanism remain to be defined. We investigated the anti-inflammatory effect and inhibitory mechanism on inflammation by the methylene chloride fraction from *Glehnia littoralis* extract (MCF-GLE), which was more effective than *Glehnia littoralis* extract (GLE). MCF-GLE inhibited 12-O-Tetradecanoyl-phorbol-13-acetate (TPA)–induced inflammation in an inflammatory edema mouse model. Also, MCF-GLE strongly inhibited the releases of nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) and significantly suppressed the mRNA and protein expression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW 264.7 macrophage cells in a dose-dependent manner. Furthermore, MCF-GLE suppressed NF-κB activation and IκB-α degradation. MCF-GLE also attenuated the activation of ERK and JNK in a dose-dependent manner. These results indicate that MCF-GLE has an inhibitory effect on the in vivo and in vitro inflammatory reaction and is a possible therapeutic agent. Our results suggest that the anti-inflammatory properties of MCF-GLE may result from the inhibition of pro-inflammatory mediators, such as NO, PGE2, TNF-α, and IL-1β via suppression of NF-κB– and mitogen-activated protein kinases-dependent pathways.

Keywords: inflammation, *Glehnia littoralis*, NF-κB, mitogen-activated protein kinase (MAPK)

Introduction

*Glehnia littoralis* (Umbelliferae) is a perennial herb that is distributed along the coastline of northern Pacific countries. The dried roots and rhizomes of this plant are listed in the Korean, Japanese, and Chinese Pharmacopoeia and have been used in traditional oriental medicine as diaphoretic, antipyretic, and analgesic agents. Previous studies reported that *Glehnia littoralis* has anti-oxidant (1, 2), anti-tumor (3, 4), anti-amnesic (5), blood circulation-promoting (6), immunomodulatory (7), and anti-microbial (8) activities. Quercetin, isoquercetin, rutin, chlorogenic acid, and caffeic acid have been isolated as the major anti-oxidative constituents in the underground parts of *Glehnia littoralis* (9). In addition, imperatorin and isoimperatorin, which are the main coumarins in both the root and the fruit of *Glehnia littoralis*, show anti-tumor activity (10). Ferulate and polyine compounds have been reported as the anti-amnesic and anti-microbial compounds in *Glehnia littoralis*, respectively (5, 11). However, although several previous studies, as well as its traditional uses, implicate the possible anti-inflammatory properties of this herb, the pharmacological activity and mechanism of *Glehnia littoralis* on inflammatory conditions remain uninvestigated.

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Our study focuses on the anti-inflammatory activity and mechanism of *Glehnia littoralis*, with respect to understanding its traditional medicinal applications, its medicinal uses in the modern society, and potential uses in drug development.

Inflammation is defined clinically as a pathophysiological process. Macrophages are activated in inflammatory conditions, and they initiate many intracellular cascades of cytokines and chemokines (12). 12-O-Tetradecanoylphorbol-13-acetate (TPA), a well-characterized protein kinase C activator and tumor promoter, has been used to induce the inflammatory animal models. Also, lipopolysaccharide (LPS), a well-known endotoxin (13), activates the inflammatory signaling pathway, resulting in increased production of inflammatory mediators, such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), from macrophages (14 – 16). The LPS-induced inflammatory pathways involve the mitogen-activated protein kinases (MAPK), including ERK, JNK, and p38. MAPK plays an important role in inflammation (17) and their activations bring about over-expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes (18 – 20). In addition, the activation of the transcription factor NF-κB pathway regulates the over-expression of proinflammatory mediators in response to several different signals and finally leads to the production of NO, PGE2, TNF-α, and IL-1β (21 – 23).

In the present study, we investigated the anti-inflammatory effect and molecular mechanism underlying the pharmacological properties of the methylene chloride fraction from *Glehnia littoralis* extract (MCF-GLE), which was more effective than *Glehnia littoralis* extract (GLE).

**Materials and Methods**

**Chemicals**

TPA, indomethacin, LPS (*Escherichia coli* 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Antibodies for iNOS, COX-2, phospho-ERK1/2, phospho-JNK, phospho-p38, β-actin, and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-IκBα and NF-κB antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Griess reagent for NO and the enzyme immunoassay kits for PGE2, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN, USA). ECL detection reagents were purchased from GE Healthcare (Buckinghamshire, UK). The standard compounds of *Glehnia littoralis* for HPLC analysis were purchased from the following commercial sources: chlorogenic acid, caffeic acid, psoralen, and 8-methoxypsoralen (Sigma); rutin, bergapten, imperatorin, and isoimperatorin (ChromaDex, Aurora, OH, USA); quercetin, ferulic acid, scopoletin, and umbelliferon (Wako Pure Chemical Industries, Ltd., Osaka); nodakenin was obtained from KFDA (Korea Food and Drug Administration).

**Plant material**

*Glehnia littoralis* Fr. SCHMIDT et MIQUEL (Umbelliferae) was collected in Geumhwajeong-ri, Toseong-myeon, Goseong-gun, and Gangwon-do, Korea. The identification of the plant was authenticated based on microscopic 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. We also deposited a voucher specimen (KIO0077036) at the herbarium of the Center of Herbal Resources Research at the Korea Institute of Oriental Medicine (Daejeon, Korea).

**Sample preparation**

The dried roots of *Glehnia littoralis* were extracted three times with 70% ethanol (with 2-h reflux), and the extract was concentrated under reduced pressure and lyophilized. The powder was dissolved in water, and an equal volume of *n*-hexane was added to the solution. After the solution separated into two phases, the fractions of *n*-hexane and water were rescued, and an equal volume of *n*-hexane was added again to the water fraction. Next, the separating step was repeated. The final water fraction was sequencially fractionated by the same method, according to the polarities of solvents using methylene chloride, ethyl acetate, and butanol. The resulting five fractions of *n*-hexane, methylene chloride, ethyl acetate, butanol, and water were lyophilized. The yields of dried extracts from starting plant materials were 4.25% (70% ethanol), 0.98% (*n*-hexane fraction), 0.13% (methylene chloride fraction), 0.28% (ethyl acetate fraction), 0.25% (butanol fraction), and 1.83% (*H2O* fraction), respectively. The lyophilized powders were dissolved in 10% dimethyl sulfoxide (DMSO) and filtered through a 0.2-µm syringe filter to create stock solutions.

**Animals**

The animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Korea Institute of Oriental Medicine.
Specific pathogen–free 5-week-old male C57BL/6J mice were purchased from Dae Han Biolink Co. (Eumseong, Korea) and housed in an air-conditioned specific pathogen–free room under conditions of 20°C – 22°C, 40% – 60% relative humidity, and a 12-h light / 12-h dark cycle. Food and water were available ad libitum.

Assay of TPA-induced inflammatory edema

The ear edema mouse model of acute inflammation was established by the previously described procedure (24). Edema was induced on the right ear of the mouse by topical application of TPA (1 μg/ear in 20 μl acetone). To examine the effect of test sample on ear edema, groups of mice were treated topically with test sample, vehicle (negative control), or indomethacin (0.3 mg/ear, positive control) at 30 min before the application of TPA. The thickness of ear edema was measured with a micrometer (Mitutoyo Series 293; Mitutoyo America Co., Aurora, IL, USA) before and at 6 h after the induction of inflammatory response. To minimize variation due to technique, a single investigator performed all measurements throughout any one experiment.

Cell culture

Mouse macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM containing 10% heat-inactivated FBS, streptomycin (100 mg/ml), and penicillin (100 units/ml) in an incubator with a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability assay

To examine the cytotoxicity of samples, we performed the MTT assay. RAW 264.7 cells (5 × 10⁴ cells/well) in 96-well plates were cultured for 24 h, and the cells were treated with test sample, 1 μg/ml of LPS, or both for 20 h. Next, MTT solution (final 500 μg/ml) was added to each well and cells were further incubated for 1 h at 37°C. Media was discarded, and DMSO was added to each well for the solubilization of formazan (25). The absorbance was measured at 570 nm with a SpectraMax 340 microplate reader (Molecular Devices, Silicon Valley, CA, USA). Percentage viability was calculated by comparison with the control group.

NO, PGE₂, TNF-α, and IL-1β assay

RAW 264.7 cells (5 × 10⁴ cells/well) in 96-well plates were cultured for 24 h. The cells were treated with the test sample and 1 μg/ml of LPS for 20 h, and the medium was collected. NO production was determined using Griess reagent, and the concentrations of TNF-α, IL-1β, and PGE₂, secreted into the medium were measured using a specific enzyme immunoassay, according to the manufacturer’s instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine the expression of iNOS and COX-2 mRNA, RAW 264.7 cells were stimulated with the test sample and LPS for 6 h. After washing twice with PBS, total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. RT-PCR was performed using 1 μg of total RNA, 10 μM of target gene-specific primers, and the OneStep-RT-PCR kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instructions. β-Actin was used as an internal standard. RT-PCR products were separated using a 1% agarose gel electrophoresis and stained with ethidium bromide, which was visualized by UV transillumination. The primers used in this experiment are indicated in Table 1.

Western blot analysis

Equal amounts of protein (10 – 30 μg), as determined with the Bio-Rad Protein assay kit (Bio-rad, Hercules, CA, USA), were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue, pH 7.6), incubated at 100°C for 5 min, and loaded onto 10% SDS-polyacrylamide gels. The proteins separated on the gels were transferred onto a nitrocellulose membrane (Scheicher & Schnell BioScience, Dassel, Germany), and the membranes were incubated with a blocking

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>Mouse COX-2 Sense</td>
<td>5′-GGAGAGACTATCAAGATAGTGATC-3′</td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-ATGGTCAGTAGACTTTTACAGCTA-3′</td>
</tr>
<tr>
<td>Mouse iNOS Sense</td>
<td>5′-ATGTCGGAAGCAACATCAC-3′</td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-TAATGTCCAGGAAGTGAGTG-3′</td>
</tr>
<tr>
<td>Mouse β-actin Sense</td>
<td>5′-TGTGATGTTGGGAATGGGTCAG-3′</td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-TTTGATGTCAAGCAGATTCC-3′</td>
</tr>
</tbody>
</table>

Table 1. Primer sequences used in the present study
buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 3% nonfat dry milk, overnight at 4°C. The membranes were then incubated for 2 h at room temperature with the primary antibodies. After washing three times for 10 min each, the membranes were probed with the secondary antibodies for 1 h. The membranes were washed three times for 10 min and developed with the ECL Western blotting detection system. Immunoreactive proteins (iNOS, COX-2, NF-κB p65, IκB-α, ERK, JNK, p38, and β-actin) were detected by a LAS-3000 Luminescent image analyzer (Fuji Photo Film, Tokyo).

**HPLC analysis**

One gram of sample was dissolved in 100 ml of 70% ethanol or 100% methanol for the pattern analysis using high performance liquid chromatography (Waters 2695; Waters Co., Milford, MA, USA), with a photodiode array detector (Waters 2996). Separation was carried out using a Eclipse XDB-C18 column (2.1 × 150 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA) with the following solvent ratios for the mobile phase, where solvent A is water and solvent B is acetonitrile: A : B = 100 : 0 (0 min) → 70 : 30 (5 min) → 55 : 45 (15 min) → 20 : 80 (30 min) → 10 : 90 (50 min) → 0 : 100 (60 min). The detection wavelength was scanned at 190 – 400 nm with 0.19 ml/min of flow. The peak analysis and assignment were performed using the standard compounds, which were identified in accordance with their UV spectra and retention time in the HPLC chromatogram.

**Statistical analyses**

All data were presented as the mean ± S.D. of three or more independent experiments, and were compared using Student’s t-test, with P-values less than 0.05 considered statistically significant.

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Fig. 1. Effects of GLE and five fractions from GLE on inflammatory mediator production in LPS-induced RAW 264.7 macrophages. Cells were treated with 100 μg/ml of sample and LPS (1 μg/ml) for 20 h. A) The NO concentration in the medium was measured using Griess reagent. B – D) PGE2, TNF-α, and IL-1β productions were determined using an ELISA kit. Each bar represents the mean ± S.D. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, compared to LPS alone.

GLE, Glehnia littoralis; HF-GLE, n-hexane fraction of GLE; MCF-GLE, methylene chloride fraction of GLE; EAF-GLE, ethyl acetate fraction of GLE; BF-GLE, butanol fraction of GLE; WF-GLE, water fraction of GLE.
Results

Effects of GLE and five fractions from GLE on LPS-induced productions of NO, PGE₂, TNF-α, and IL-1β

GLE was sequentially fractionated according to the polarities of solvents using n-hexane, methylene chloride, ethyl acetate, butanol, and water. We measured the effects of GLE and five fractions from GLE on LPS-induced production of NO, PGE₂, TNF-α, and IL-1β in RAW 264.7 macrophages in order to rescue the anti-inflammatory fractions. Cells were treated with 100 μg/ml of sample and 1 μg/ml of LPS for 20 h. The productions of these pro-inflammatory mediators were reflected in their accumulations in the cell culture medium. The anti-inflammatory activities of the resultant fractions and GLE were compared with one another for screening more active fractions. Among them, MCF-GLE was the most effective on the suppression of the LPS-induced release of NO, PGE₂, TNF-α, and IL-1β in RAW 264.7 macrophages (Fig. 1). Therefore, MCF-GLE was used for further studies to demonstrate the anti-inflammatory mechanism by this herb.

Effect of MCF-GLE on TPA-induced inflammatory edema

We assessed the anti-inflammatory activity of MCF-GLE in a mouse model of irritant contact dermatitis by TPA. Exposure to TPA resulted in marked increases in skin thickness, which is one indicator of a number of processes that occur during skin inflammation. Ear edema was measured in the dorsal skin prior to and at 6 h following treatment of TPA. Topical application of vehicle did not significantly alter skin thickness; however, MCF-GLE significantly inhibited the TPA-induced increase of skin thickness, indicating the therapeutic effect of this extract (Fig. 2).

Effect of MCF-GLE on cell viability

Figure 3 presents the viability of RAW 264.7 macrophages in the presence of MCF-GLE. Cytotoxicity of MCF-GLE was assessed using the MTT assay. Cell viability of the RAW 264.7 macrophages was not significantly altered by 20-h incubation with MCF-GLE at concentrations less than 200 μg/ml. Thus, in subsequent experiments, cells were treated with MCF-GLE in the concentration range of 5 – 200 μg/ml.

Effects of MCF-GLE on LPS-induced productions of NO, PGE₂, TNF-α, and IL-1β

To measure the effects of MCF-GLE on LPS-induced production of NO, PGE₂, TNF-α, and IL-1β in RAW 264.7 macrophages, cells were treated with various concentrations of MCF-GLE and 1 μg/ml of LPS for 20 h. As shown in Fig. 3, the stimulation of LPS alone caused a marked accumulation of NO, PGE₂, TNF-α, and IL-1β in the culture medium; however, MCF-GLE significantly reduced these productions in a dose-dependent manner (Fig. 4). MCF-GLE inhibited the release of NO, PGE₂, TNF-α, and IL-1β with IC₅₀ values of 42.1, 24.4, 36.3, and 4.68 μg/ml, respectively.
Anti-inflammatory Mechanism of MCF-GLE

Effects of MCF-GLE on LPS-induced mRNA and protein expression of iNOS and COX-2

To investigate whether the inhibitory effects of MCF-GLE on NO and PGE2 production resulted from decreased mRNA and protein expression of iNOS and COX-2, we performed the semi-quantitative RT-PCR and Western blot analysis of iNOS and COX-2 in LPS-induced RAW 264.7 macrophages. Figure 5 indicates that the expressions of iNOS and COX-2 mRNA and proteins were not detectable in unstimulated cells; however, their expressions were markedly increased following LPS treatment. Consistent with the findings shown in Fig. 4, A and B, MCF-GLE had a significant, concentration-dependent inhibitory effect on iNOS and COX-2 mRNA and protein expression in LPS-induced RAW 264.7 macrophages (Fig. 5).

Effects of MCF-GLE on LPS-induced activation of NF-κB

We examined the effects of MCF-GLE on the nuclear translocation of NF-κB to understand the mechanisms underlying the inhibition of LPS-induced expression of iNOS and COX-2. NF-κB p65 is the major component of the NF-κB activated by LPS in macrophages. When the p65 levels in nuclear extracts was evaluated by Western blot analysis, MCF-GLE inhibited LPS-induced nuclear translocation of NF-κB p65 in a dose-dependent manner (Fig. 6A). Because phosphorylation of IκB-α results in its degradation and the release of NF-κB, which then translocates to the nucleus, it was confirmed whether MCF-GLE could suppress the phosphorylation of IκB-α, when induced by LPS treatment. As shown to Fig. 5B, IκB-α phosphorylation was significantly inhibited in the presence of MCF-GLE, similar to the results for the nuclear translocation of NF-κB p65. Taken together, these data suggest that treatment with MCF-GLE resulted in the cytosolic accumulation of IκB-α via the suppression of IκB-α phosphorylation and therefore blocked the DNA-binding activity of NF-κB through the inhibition on LPS-induced translocation of NF-κB p65.
To investigate further whether the inhibition of NF-κB activation and inflammatory mediators by MCF-GLE is modulated through the MAPK pathway, we evaluated the effects of MCF-GLE on the LPS-induced phosphorylation of ERK, JNK, and p38. MCF-GLE suppressed LPS-induced phosphorylation of ERK and JNK in a dose-dependent manner, while the p38 activation was only inhibited by 200 μg/ml of MCF-GLE (Fig. 7). These results suggest that MCF-GLE blocks ERK and JNK phosphorylation in MAPK pathways to suppress the inflammatory response in LPS-induced RAW 264.7 macrophages.

**Discussion**

Although *Glehnia littoralis* has long been used to treat the immune-related diseases in oriental traditional medicine, its anti-inflammatory activity and molecular mechanism have not been demonstrated yet.

Biosynthesis of NO, PGE₂, TNF-α, and IL-1β have been accepted to play important roles in the processes of inflammation and carcinogenesis. Their productions are regulated by activities of NF-κB and MAPK. Many previous studies have examined the production of NO, PGE₂, TNF-α, and IL-1β; mRNA and protein
expression of iNOS and COX-2; activation of NF-κB; and phosphorylation of MAPK for the demonstration of anti-inflammatory activity. Therefore, in this study, we examined the effect of MCF-GLE, which was more effective than GLE, on the inflammatory edema in the TPA-induced edema mouse model and on the production of inflammatory mediators in LPS-induced RAW 264.7 macrophage cells. To understand the molecular mechanisms underlying the suppression of the inflammatory response, the effects of MCF-GLE on LPS-induced activation of NF-κB and MAPK pathways were investigated.

Our results showed that MCF-GLE ameliorated cutaneous inflammation and exerts the anti-inflammatory activity via the inhibitions of the mRNA and protein expressions of iNOS and COX-2, as well as the productions of NO, PGE2, TNF-α, and IL-1β in LPS-induced RAW 264.7 macrophage cells. We also revealed that these anti-inflammatory activities of MCF-GLE are mediated through the inhibition of IκB-α phosphorylation, nuclear translocation of NF-κB p65 subunit, and activation of MAPK (ERK and JNK).

The productions of inflammatory cytokines and chemokines are a crucial part of regulating inflammation and tumorigenesis. Recently, several studies provided evidence strongly suggesting that chronic inflammation leads to cancers (26 – 29). Indeed, the selective inhibitors of COX-2 or iNOS enzyme activity have been reported to have cancer-preventing and anti-inflammatory properties (30 – 34). Their inhibitors may be more selective agents via the regulation of genes that might be changed during the inflammation and carcinogenic process. Therefore, the inhibitory effects of MCF-GLE against the productions of NO, PGE2, TNF-α, and IL-1β suggest that MCF-GLE can be a candidate drug that suppresses tumor progression as well as the inflammatory response.

The key signaling pathway for the inflammatory response has been well established in various inflammatory diseases and cancers (35, 36). This signaling pathway is a good target for anti-cancer and anti-inflammatory drug development. Although the relationship between MAPK and NF-κB in inflammation has not been proved fully, previous studies demonstrated that the inflammatory stimuli activate MAPK (ERK, JNK, and p38), followed by the transcriptional activation of pro-inflammatory cytokines and mediators (37 – 39). Moreover, the MAPK pathway turned out to be involved in the activation of transcription factors such as NF-κB and AP-1 (19, 40 – 42). Considering the signaling mechanism of inflammation, our study indicates that the inhibition of the inflammatory mediators (NO, PGE2, TNF-α, and IL-1β) by MCF-GLE is due to a block of NF-κB activation and the MAPK pathway.

Our HPLC results did not clearly demonstrate the compounds that contributed to the anti-inflammatory activity of MCF-GLE among the major 13 constituents. However, the total content of quercetin, ferulic acid, scopoletin, and umbelliferon was increased in MCF-GLE compared to that in GLE. Additionally, further studies must be performed to identify the active components in Glehnia littoralis that contributed to the anti-inflammatory and cancer chemo-preventive effect.

In conclusion, the present study revealed the anti-inflammatory activities and mechanism of Glehnia littoralis for the first time. We demonstrate herein that MCF-GLE is a novel suppressor of the production of inflammatory mediators, NO and PGE2, and pro-inflammatory cytokines, TNF-α and IL-1β. These effects appear to be mediated by inhibition of NF-κB and MAPK activation. Therefore, MCF-GLE may be useful for the development of new potent anti-inflammatory drugs.

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