A Doctoral Dissertation

Anti-inflammatory effects and action mechanisms of the isolated compounds from *Gracilaria verrucosa*

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꼬시래기로부터 분리한 화합물의 염증억제 작용 및 기전

지도교수 유 은 숙

이 혜 자

이 논문을 의학 박사학위 논문으로 제출함

2007 년 8 월

이혜자의 의학 박사학위 논문을 인준함

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제주대학교 대학원

2007 년 8 월

Anti-inflammatory effects and action mechanisms of the isolated compounds from *Gracilaria verrucosa*

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A thesis submitted in partial fulfillment of the requirement for the degree of doctor of philosophy in medicine

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June 11, 2007

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Abstract

Gracilaria verrucosa Papenfuss, common marine red algae in Korea, has been widely used for agar production together with Gelidium. Because, the life history, reproduction, and phenology of *G. verrucosa* has been studied. However, only a few studies have reported the biological effects of this algae. In this study, we investigated the modulatory effects of *G. verrucosa* on the production of pro-inflammatory markers. We isolated fourteen compounds from *G. verrucosa*; four compounds (GV-c-1~4) were prostaglandins, one (GV-c-5) was ceramide, and the others (GV-c-6~14) were fatty acids. 2-Formamido-1,3-dihydroxyoctadecane (GV-c-5) and 11-oxohexadec-9-enoic acid (GV-c-6) have not yet been reported from this alga. We investigated the anti-inflammatory effects of these compounds from *G. verrucosa* on the production of inflammatory markers in LPS-stimulated RAW 264.7 cells. GV-c-6, GV-c-9 and GV-c-10 dose-dependently inhibited the production and mRNA expression of the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). At 70 μM, these three compounds also inhibited the production of nitric oxide (NO) and inducible NO synthase (iNOS) by up to 70-90%. To investigate their mechanism of action, we examined the effects of GV-c-9 and GV-c-10 on LPS-induced activation of NF-κB and MAPKs such as ERK1/2, p38 and JNK. Luciferase assays revealed that GV-c-9 and GV-c-10 suppressed the transcriptional activity of NF-κB. These two compounds also inhibited phosphorylation of ERK1/2. These results suggest that the anti-inflammatory activity of *G. verrucosa* may be due to modulation of pro-inflammatory cytokines (TNF-α and IL-6) and NO production via suppression of NF-κB activation and

ERK 1/2 phosphorylation.

Key word: *Gracilaria verrucosa*, inflammation, TNF-α, IL-6, NO, NF-κB, ERK

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1. Introduction

Inflammation plays a role as the first line of defense against infection of pathogenic microbes. Macrophages play a central role in the inflammatory response and serve as an essential interface between innate and adaptive immunity (Maruotti *et al*., 2007; An *et al*., 2002). Bacterial endotoxin (lipopolysaccharide, LPS) is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation (Cohen, 2002; Fujihara *et al*., 2003; Guha and Mackman, 2001). LPS activates monocytes and macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1. Although cytokine production is important for the efficient control of growth and dissemination of invading pathogens, overproduction of cytokines have been implicated in many human diseases such as rheumatoid arthritis, septic shock and other chronic inflammatory diseases (Dobrovolskaia and Vogel, 2002; Ishii *et al*., 2003; Lee *et al*., 2004). TNF-α and IL-1 are associated with osteoclast differentiation and activation, resulting in bone loss. In patients with rheumatoid arthritis, treatment with TNF and IL-1 blockers significantly reduced erosions and joint space narrowing (Strand and Kavanaugh, 2004). Also, IL-1 and TNF are produced by microglia during CNS inflammation. Pathogens or pathogen components such as LPS can immediately induce IL-1 and TNF-α in both cultured microglia and LPS-injected rats (Appel *et al*., 1995; Buttini and Boddeke, 1995).

Nitric oxide (NO) is also an important mediator and effector molecule with various biological functions (Cross and Wilson, 2003; Stepien *et al*., 2005; Bove and Vliet, 2006). NO produced by activated macrophages has been shown to mediate host defense functions such as antimicrobial and antitumor activities, but excess production causes tissue damage associated with acute and chronic inflammation (Macmicking *et al*., 1997). LPS-induced NO

production is mediated by a series of signalling pathways, such as nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases (Islam *et al*., 2004; Lee and Schorey, 2005).

NF-κB is a major transcription factor involved in the release of proteins that mediate cardiovascular growth, stress response, and inflammation by controlled gene expression. The degradation or phosphorylation of IκB-α are necessary to release NF-κB from the cytoplasmic NF-κB/ IκB-α complex and allow its subsequent translocation to the nucleus of the cell (Kim *et al*., 2006; Murakami *et al*., 2003; Inoue *et al*., 2007). Many compounds isolated from natural plant exhibit anti-inflammatory activity associated with their potent NF-κB inhibition. For example, a green tea polypenol (epigallocatechin-3-gallate) and rosemary phytopolyphenol (Carnosol) were found to directly block the activity of I-κB kinase (Yang *et al*., 2001; Lo *et al*., 2002). Various upstream activators are involved in LPS signalling may mediate activation of the MAPK pathway, including extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in monocytes and macrophages. ERK 1/2 belongs to a group of serine/threonine-specific mitogen-activated protein kinases (MAPKs). ERK 1/2 is activated by various extracellular stimuli, including LPS, and connects the extracellular signal to intracellular transcription factors and other regulatory proteins, thus participating in regulation of gene expression. LPS-induced inflammatory gene expression inhibited by PD98059, a specific inhibitor of MEK that is an upstream activator of ERK 1/2 (Lahti *et al*., 2000; Su and Karin, 1996). The p38 kinase is an important mediator of stress-induced gene expression (Raingeaud *et al*., 1995). In particular, the p38 kinase plays a key role in the LPS-induced signal transduction pathway (Rafi *et al*., 2007; Lee and Young, 1996). Also, stimulation of RAW 264.7 cells rapidly activates the JNK pathway (Hambleton *et al*., 1996). This pathway contains the c-Jun N-terminal kinase (JNK), which is also known as stress-activated protein kinase (SAPK1). Two isoforms of JNK (54 and 46 kDa) phosphorylate the

N-terminus of c-Jun (Swantek *et al*., 1997). Treatment of either SB203580 (inhibitor of p38 MAPK) or SP600125 (inhibitor of JNK) suppressed NO, TNF-α, and IL-1β production (Kwak *et al*., 2005).

There are various species of marine algae in Jeju Island, and useful secondary metabolites are obtained from marine algae. So, study of the marine algae is very valuable. Therefore, we investigated anti-inflammatory effects of fifty species of marine algae in Jeju Island. Among them, *Gracilaria verrucosa* showed potentially inhibitory effects on the production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells.

Gracilaria verrucosa Papenfuss is a common marine red algae that grows on pebbles or rocks in the shallow littoral, and has been widely used for agar production together with Gelidium (Armisen, 1995). This algae is rich in arachidonic acid (AA) and eicosapentaenoic acid, polyunsaturated fatty acids (PUFA) with 20 carbon atoms that are the precursors of prostaglandins (PGs). Several glycolipid compounds have been isolated from *G. verrucosa* (Son *et al*., 1994) and are reportedly cytotoxic against mouse leukemia and carcinoma cells (Roh *et al*., 1995). Recently, *G. verrucosa* solvent extracts have been shown to have antioxidant activity (Heo *et al*., 2006). However, the cellular and molecular mechanism of this activity has not been sufficiently explained.

In this study, therefore, we isolated several compounds from *G. verrucosa* and investigated their inhibitory effects on the production of inflammatory markers (TNF-α, IL-6, and NO) in LPS-stimulated murine macrophage RAW 264.7 cells. Also, we investigated the action mechanism of active compounds on the activation of NF-κB and phosphorylation of MAPKs (ERK1/2, JNK and p38). And, to study on the functional group from active compounds, we examined inhibitory effects of prostaglandins (PGE and PGA) on the production of inflammatory markers.

2. Material and Methods

2-1. Reagents

Lipopolysaccharide (LPS, E. coli 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) and DMEM were obtained from GIBCO (Grand Island, NY, USA). The enzyme-linked immunosorbent assay (ELISA) kit for TNF-α and IL-6 were obtained from R&D Systems (Minneapolis, MN, USA) and the lactate dehydrogenase (LDH) Cytotoxicity Detection kit from promega (Medison, WI, USA). Dual-Luciferase Reported Assay Kit from Promega (Madison, WI, USA). Antibodies against to IκB-α, p38, ERK1/2, phospho–ERK 1/2, JNK and phospho–JNK obtained from Cell Signaling Technology (Beverly, MA, USA) and antibody to phospho-p38 from BD Biosciences (San Diego, USA). All other chemicals were of Sigma grade.

2-2. Isolation of compounds from *Gracilaria verrucosa*

The *G. verrucosa* were collected in May at Jeju Island, Korea. The collected material was dried under dark place at room temperature and stored in freezer at –20℃. *G. verrucosa* (50 g) were pulverized using milling machine and extracted with methanol (MeOH) for 24 h three times at room temperature. The extract was filtered, and the filtrate was concentrated in vacuum and lyophilized. The lyophilized MeOH extract (F1, 15.0 g) was suspended in distilled water and extracted with methylene chloride (CH_2Cl_2). A part of CH_2Cl_2 fraction (F3, 7.50 g) extracted with aqueous MeOH (F4, 3.8 g) and *n*-hexane (F5, 3.6 g).

Aqueous MeOH fraction was purified through reversed-phase flash column chromatography (YMC Gel ODS-A, 60 Å, 220 mesh) using gradient elution of aqueous methanol (50% to 100%) to give twenty fractions (GV1 – 20). The fraction 5 (GV5, 136 ㎎) was purified through prep-HPLC (C18-5E Shodex packed) using 60% acetonitrile (ACN) to

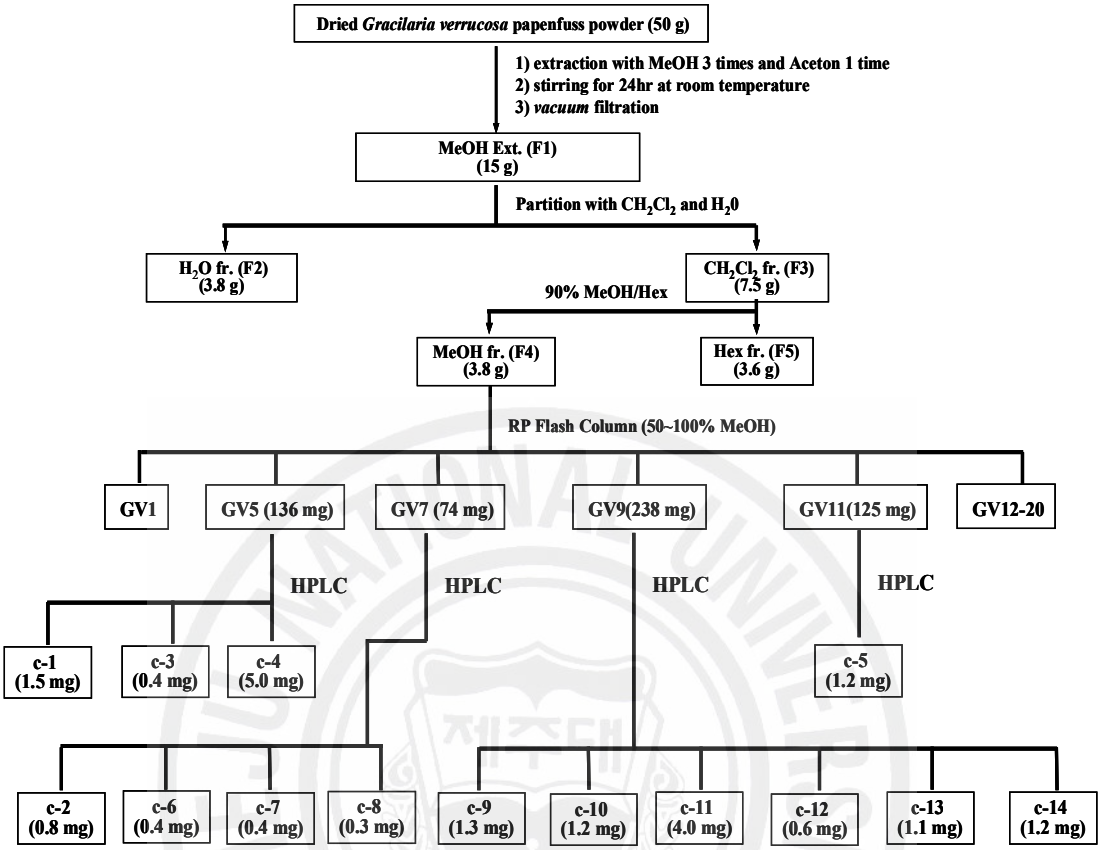
give compound **1** (GV-c-1, 1.5 ㎎), **3** (GV-c-3, 0.4 ㎎), **4** (GV-c-4, 5 ㎎). The fraction 7 (GV7, 74 ㎎) was purified through prep-HPLC (C18-5E Shodex packed) using 81% MeOH to give compound **2** (GV-c-2, 0.8 ㎎), **6** (GV-c-6, 0.4 ㎎), **7** (GV-c-7, 0.4 ㎎), **8** (GV-c-8, 0.3 ㎎). The fraction 9 (GV9, 74 ㎎) was purified through prep-HPLC (C18-5E Shodex packed) using 81% MeOH to give compound **9** (GV-c-9, 1.3 ㎎), **10** (GV-c-10, 1.2 ㎎), **11** (GV-c-11, 4.0 ㎎), **12** (GV-c-12, 0.6 ㎎), **13** (GV-c-13, 1.1 ㎎), **14** (GV-c-14, 1.2 ㎎). And, the fraction 11 (GV11, 125 ㎎) was purified through prep-HPLC (YMC ODS-H80) using 88% ACN to give compounds **5** (GV-c-5, 1.2 ㎎) (Scheme 1).

2-3. Cell culture

The mouse macrophage RAW 264.7 was purchased from ATCC (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-activated fetal bovine serum, streptomycin (100 ㎍/㎖) and penicillin (100 U/㎖) at 37℃ atmosphere and 5% CO_2.

2-4. Cytotoxicity assay

Lactate dehydrogenase leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate (Fernandez *et al*., 2006). The release of LDH from RAW 264.7 cell was used to detect cytotoxicity and was measured at the end of each proliferation experiment. Briefly, culture medium was centrifuged at 12,000 rpm for 3 min at room temperature to ensure



Scheme 1. Isolation procedure of compounds from *Gracilaria verrucosa* papenfuss

accumulation of cells. The cell-free culture medium (50 ㎕) was collected and then incubated with 50 ㎕ of the reaction mixture cytotoxicity detection kit for 30 min at room temperature in the dark. 1N HCl (50 ㎕) was added into each well to stop the enzymatic reaction. The optical density of the solution was then measured by using an ELISA plate reader with a 490 nm. Percent cytotoxicity was determined relative to the control group.

2-5. Measurement of pro-inflammatory cytokines (TNF-α and IL-6) production

The inhibitory effects of *G. verrucosa* on TNF-α and IL-6 production were determined by the method previously described (Cho *et al*., 2000). The isolated compounds solubilized with EtOH diluted with DMEM. The final concentration of chemical solvents should not exceed 0.1% in the culture medium. In these conditions, none of the solubilized solvents altered TNF-α and IL-6 production in RAW 264.7 cells. Before stimulation with LPS (1 ㎍/㎖) and test materials, RAW 264.7 cells (8.0×10^5 cells/㎖) were incubated for 18 h in 24-well plates with the same conditions. Lipopolysaccharide (LPS) and the test materials were then added to the cultured cells for 6 h incubation. The medium was used for TNF-α and IL-6 assay using mouse ELISA kit (R & D Systems Inc, MN, USA). The inhibitory effects of testing fractions on TNF-α and IL-6 production were determined as previously described.

2-6. Measurement of NO production

After pre-incubation of RAW 264.7 cells (1.5×10^5 cells/㎖) for 18 h, the various concentrations of compounds with LPS (1 ㎍/㎖) were incubated for 24 h. Nitrite in culture supernatants were measured by adding 100 ㎕ of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 ㎕ samples of medium. All measurements were performed in triplicate. The concentration of NO_2^- was calculated by comparison with a standard curve prepared using $NaNO_2$.

2-7. Western blot analysis

Murine macrophage cell line RAW 264.7 were pre-incubated for 18 h, and then stimulated with LPS (1 ㎍/㎖) in the presence of testing materials for the indicated time. After incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 ㎍/㎖ aprotinin, 25 ㎍/㎖ leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at 15,000 rpm at 4℃ for 15 min and then until use, the supernatants were stored at −70℃. Protein concentration was measured using the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 ㎍ of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA), the membrane was then incubated with specific primary antibody at 4℃ for overnight. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersharm-Pharmacia Biotech, NY, USA).

2-8. RNA preparation and RT-PCR

Total RNA was extracted from cells by the Tri-Reagent (MRC, Cincinnati, OH, USA) method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. The reverse transcription of 1 ㎍ RNA was carried out using M-MuLV reverse transcriptase (Promega, WI, USA), oligo $(dT)_{15}$ primer, dNTP (0.5 μM) and 1 U RNase inhibitor. After incubation at 70℃ for 5 min, 37℃ for 5 min, 37℃ for 60 min, and

M-MuLV reverse transcriptase was inactivated by heating at 70℃ for 15 min. The polymerase chain reaction (PCR) was performed in a reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3' and 5' primer 50 μM each and 200 mM dNTP in 200 mM Tris-HCl buffer, pH 8.4, containing 500 mM KCl and 1-4 mM $MgCl_2$]. The PCR was performed with a DNA gene cycler (BIO-RAD, HC, USA), and the amplification was followed by 30 cycles of 94℃ for 45 sec (denaturing), 50-58℃ for 45 sec (annealing) and 72℃ for 1 min (extension). The PCR products were electrophoresed on a 1.2% agarose gel.

2-9. Transient transfection and luciferase assay

Cells were cotransfected with 50 ng of NF-kB promoted luciferase reporter gene plasmid (pNF-kB-Luc, Panomics, Redwood City, CA, USA) and 10 ng of Renilla luciferase reporter plasmid (pRL-null, Promega corp. Madison, WI, USA), which served as the internal standard, using TransFast™ transfection reagent (Promega. Madison, WI, USA). After 24 h, cells were incubated with LPS (1 ㎍/㎖) in the presence of compounds. After 20 h incubation, luciferase activity in the cell lysate was determined using Dual-Luciferase Reported Assay Kit (Promega corp. Madison, WI, USA). The luciferase activity was normalized to the transfection efficiency as monitored by the Renilla luciferase expression vector. The level of luciferase activity was determined as a ratio in comparison to cells with no stimulation.

2-10. Statistical analyses

The student's t-test and one-way ANOVA were used to determine the statistical significance of differences between values for a variety of experimental and control groups. Data are expressed as means ± standard deviation (SD) of at least three independent experiments performed in triplicate. P-values of 0.05 or less were considered statistically

significant.

3. Results

3-1. Compounds isolated from red alga *Gracilaria verrucosa*.

The *G. verrucosa* were collected in May at Jeju Island, Korea. We isolated fourteen compounds from *G. verrucosa* (Fig. 1). Compounds **1 -14** were obtained from crude extract (MeOH ext.) in 0.027-0.033% yield. Compound **1**, **2**, **3** and **4** were obtained as colorless oil, and the ^{1}H and ^{13}C NMR data showed Table 1 and 2. The structure of compound **1** and **3** showed 11-deoxyprostaglandin E_2 and E_1, respectively. Compound **5** was obtained as a white amorphous solid, and the structure showed ceramide series. Compound **6** was obtained as colorless oil, and the structure showed unsaturated-fatty acid. Compound **7, 8, 9, 10, 11, 12, 13**, and **14** were obtained as colorless oil, and the structure of these compounds showed fatty acid (NMR data showed Table 3 and 4). Among the isolated compounds, compound **5** and **6** have not yet reported from *G.verrucosa*. Chemical structure of compound **6, 9,** and **10** are the acyclic enone analogs containing a α,β-unsaturated carbonyl ring, which is characteristic of cyclopentenone prostaglandins.

11-deoxyprostaglandin E2 (1): Yield 0.01%; colorless oil; $[\alpha]^{24}_{D}$ –33.0° (c 0.09, MeOH); ^{1}H and ^{13}C NMR data, see Table 1 and 2; HRFABMS *m/z* 359.2187 $[M + Na]^{+}$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198).

Methyl-(5*Z*,13*E*)-(15*S*)-15-hydroxy-9-oxoprosta-5,13-dienoate (2): Yield 0.0027%; colorless oil; $[\alpha]^{25}_{D}$ –35.0° (*c* 0.05, MeOH); ^{1}H and ^{13}C NMR data, see Table 1 and 2; HRFABMS *m/z* 373.2347 $[M + Na]^{+}$ (calcd for $C_{21}H_{34}O_4Na$, 373.2355).

(*E*)-(15*S*)-15-Hydroxy-9-oxoprost-13-enoic acid (11-deoxyprostaglandin E_1) (3): Yield

0.053%; colorless oil; $[\alpha]^{27}_{D}$ –23.0° (*c* 0.04, MeOH); ^{1}H NMR data, see Table 1; HRFABMS *m/z* 361.2358 $[M + Na]^{+}$ (calcd for $C_{20}H_{34}O_4Na$, 361.2356).

(*Z*)-9,15-Dioxoprost-5-enoic acid (4): Yield 0.0333%; colorless oil; $[\alpha]^{24}_{D}$ –53.0° (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 1 and 2; HRFABMS *m/z* 359.2183 $[M + Na]^{+}$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198).

(2*R*,3*S*)-2-Formamido-1,3-dihydroxyoctadecane (5): Yield 0.0080%; white amorphous solid; $[\alpha]^{27}_{D}$ –2.7° (*c* 0.1, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 0.90 (3H, *t*, *J* = 6.4 Hz, H-18), 1.29 (26H, *m*, H-5 - H-17), 1.54(2H, H-4), 3.62 (1H, *m*, H-3), 3.70 (2H, *d*, *J* = 5.2 Hz, H-1), 3.90 (1H, *q*, *J* = 6.4 Hz, H-2), 8.11 (1H, *s*, CHO); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.8, 30.5, 30.8, 33.1, 34.8, 55.6, 62.0, 72.2, 163.8; HRFABMS *m/z* 352.2834 $[M + Na]^{+}$ (calcd for $C_{19}H_{39}NO_3Na$, 352.2828).

(*E*)-11-Oxohexadec-9-enoic acid (6): Yield 0.0027%; colorless oil; ^{1}H NMR (500 MHz, CD_3OD) δ 0.94 (3H, *t*, *J* = 7.0 Hz, H-16), 1.31-1.36 (10H, m), 1.51 (2H, quint, *J* = 7.0 Hz, H-7), 1.60 (4H, *m*, H-3 and H-13), 2.26 (4H, *m*, H-2 and H-8), 2.60 (2H, *t*, *J* = 7.5 Hz, H-12), 6.13 (1H, *dt*, *J* = 14.0, 1.5 Hz, H-10), 6.94 (1H, *dt*, *J* = 14.0, 7.0 Hz, H-9); FABMS *m/z* 313 $[M – H + 2Na]^{+}$; HRFABMS *m/z* 291.1943 $[M + Na]^{+}$ (calcd for $C_{16}H_{28}NO_3$, 291.1936); CID of *m/z* (rel int) 313 $[M – H + 2Na]^{+}$ (100) *m/z* 283 (5), 269 (29), 173 (52), 159 (31), 145 (12), 131 (7), 117 (33), 104 (33), 90 (29).

10-Oxohexadecanoic acid (7): Yield 0.0027%; colorless oil; ^{1}H NMR data, see Table 3; negative-ion FABMS *m/z* 269 $[M - H]^{-}$; positive-ion FABMS *m/z* 293 $[M + Na]^{+}$, 315 $[M – H + 2Na]^{+}$; CID of *m/z* (rel int) 315 $[M – H + 2Na]^{+}$ (100) *m/z* 299 (9), 285 (14), 271 (23),

257 (75), 187 (77), 173 (30), 159 (34), 145 (18), 131 (14), 117 (61), 104 (69), 90 (69).

(*E*)-(*S*)-9-Hydroxyhexadec-8-enoic acid (8): Yield 0.002%; colorless oil; $[\alpha]^{24}_{D}$ +2.8° (*c* 0.04, MeOH); ^{1}H NMR data, see Table 3; negative-ion FABMS *m/z* 269 $[M - H]^-$; positive-ion FABMS *m/z* 293 $[M + Na]^+$, 315 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 315 $[M – H + 2Na]^+$ (100) *m/z* 271 (12), 257 (10), 243 (14), 229 (20), 159 (24), 145 (12), 131 (10), 117 (78), 104 (90), 90 (71).

(*E*)-10-Oxooctadec-8-enoic acid (9): Yield 0.0086%; colorless oil; ^{1}H NMR data, see Table 3; FABMS *m/z* 319 $[M + Na]^+$, 341 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 341 $[M – H + 2Na]^+$ (100) *m/z* 297 (14), 283 (17), 269 (14), 255 (86), 159 (57), 145 (19), 131 (12), 117 (88), 104 (89), 90 (81).

(*E*)-11-Oxooctadec-9-enoic acid (10): Yield 0.0080%; ^{1}H NMR data, see Table 3; colorless oil; FABMS *m/z* 319 $[M + Na]^+$, 341 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 341 $[M – H + 2Na]^+$ (100) *m/z* 297 (15), 283 (20), 269 (78), 173 (53), 159 (19), 145 (20), 131 (15), 117 (75), 104 (90), 90 (58).

(*E*)-(*R*)-10-Hydroxyoctadec-8-enoic acid (11): Yield 0.0266%; colorless oil; ^{1}H NMR data, see Table 4; $[\alpha]^{24}_{D}$ -3.8° (*c* 0.07, MeOH); negative-ion FABMS *m/z* 297 $[M - H]^-$; positive-ion FABMS *m/z* 321 $[M + Na]^+$, 343 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 343 $[M – H + 2Na]^+$ (100) *m/z* 299 (14), 271 (18), 257 (11), 243 (18), 229 (32), 159 (26), 145 (19), 131 (18), 117 (86), 104 (93), 90 (68).

10-Oxooctadecanoic acid (12): Yield 0.0040%; colorless oil; ^{1}H NMR data, see Table 4;

negative-ion FABMS *m/z* 297 $[M - H]^-$; positive-ion FABMS *m/z* 321 $[M + Na]^+$, 343 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 343 $[M – H + 2Na]^+$ (100) *m/z* 327 (13), 313 (19), 299 (22), 285 (13), 271 (25), 257 (66), 187 (66), 173 (25), 159 (31), 145 (19), 131 (16), 117 (66), 104 (66), 90 (78).

11-Oxooctadecanoic acid (13): Yield 0.0073%; colorless oil; ^{1}H NMR data, see Table 4; negative-ion FABMS *m/z* 297 $[M - H]^-$; positive-ion FABMS *m/z* 321 $[M + Na]^+$, 343 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 343 $[M – H + 2Na]^+$ (100) *m/z* 313 (14), 299 (14), 285 (36), 271 (71), 201 (75), 187 (25), 173 (32), 159 (29), 145 (21), 131 (14), 117 (71), 104 (89), 90 (64).

12-Oxooctadecanoic acid (14): Yield 0.0080%; colorless oil; ^{1}H NMR data, see Table 4; FABMS *m/z* 321 $[M + Na]^+$, 343 $[M – H + 2Na]^+$; CID of *m/z* (rel int) 343 $[M –H + 2Na]^+$ (100) *m/z* 299 (16), 285 (42), 215 (42), 201 (29), 187 (24), 173 (21), 159 (26), 145 (21), 131 (13), 117 (63), 104 (63), 90 (55).

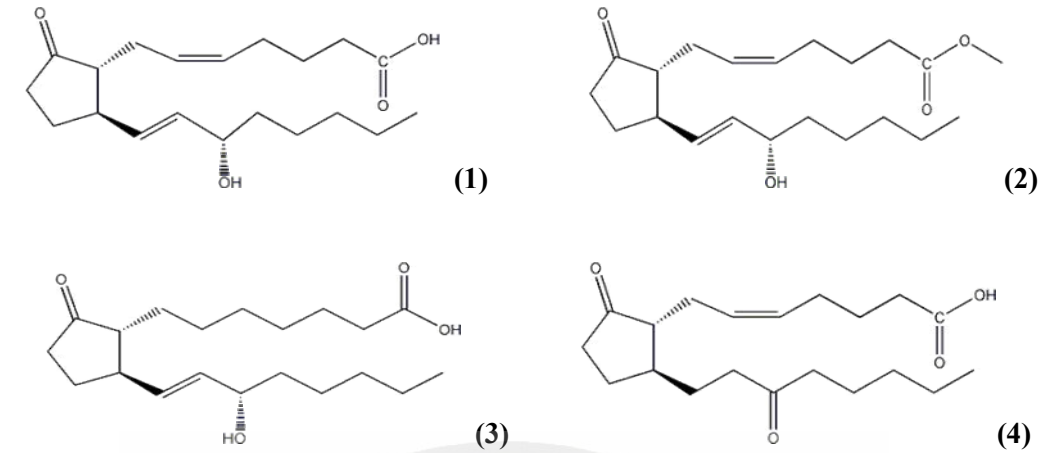


Fig. 1-1. The Structures of Prostaglandins isolated from *G. verrucosa*

Fig. 1-2. The Structure of Ceramide isolated from *G. verrucosa*

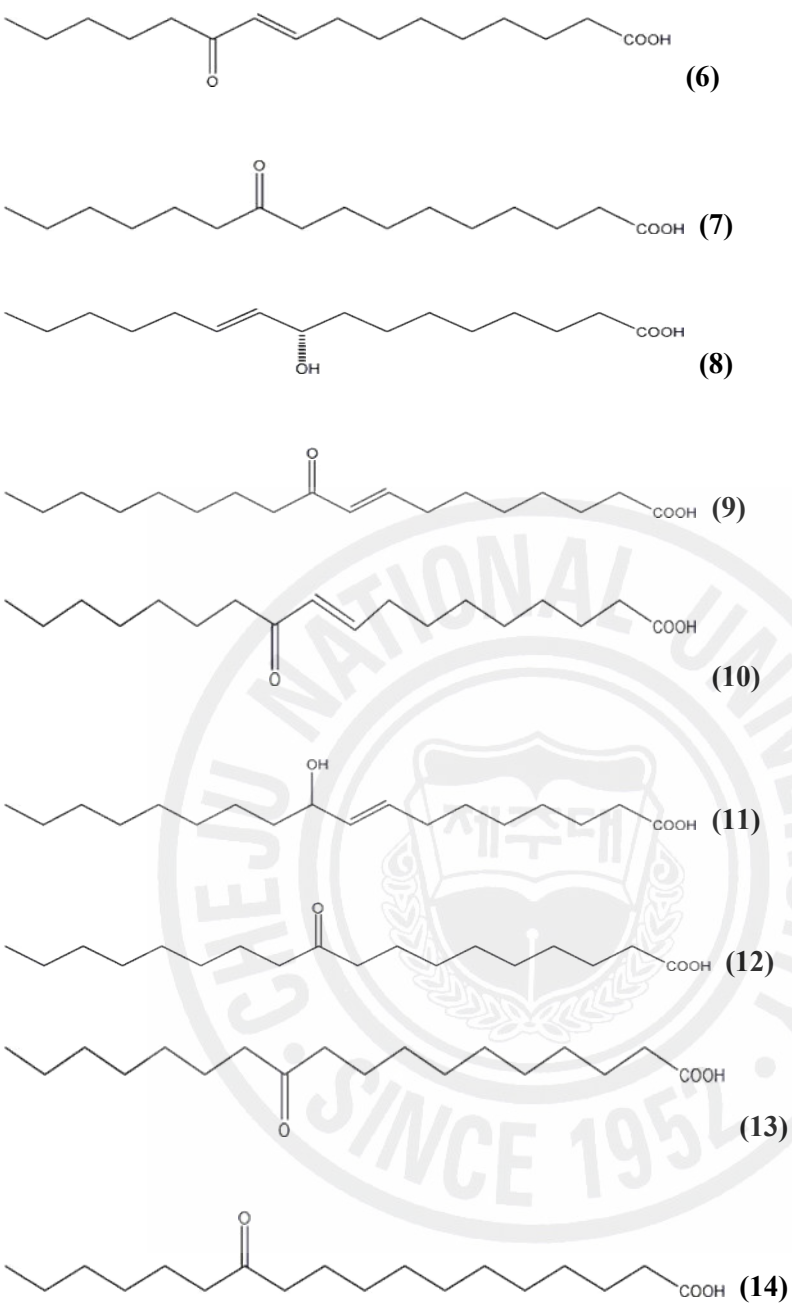


Fig. 1-3. The Structures of Fatty acids isolated from *G. verrucosa*.

Table 1. ^{1}H NMR Data of Compounds 1-4 (CD_3OD)

Position	**1**[a]	**2**[b]	**3**[b]	**4**[a]
2	2.26 (t, 6.8)	2.30 (t, 7.2)	2.19 (t, 7.5)	2.28 (t, 7.6)
3	1.65 (m)	1.65 (m)	1.61 (m)	1.64 (quint, 7.2)
4	2.10 (m)	2.10 (m)	1.28 – 1.43 (m)	2.11 (q, 7.2)
5	5.40 (m)	5.38 (m)	1.28 – 1.43 (m)	5.42 (m)
6	5.33 (m)	5.32 (m)	1.28 – 1.43 (m)	5.33 (m)
7	2.33 (m)	2.32 (m)	1.28 – 1.43 (m)	2.35 (m)
8	2.00 (m)	2.00 (m)	1.88 (m)	1.86 (m)
10	1.65 (m, H-a), 2.33 (m, H-b)	1.65 (m, H-a), 2.32 (m, H-b)	1.61 (m, H-a), 2.31 (m, H-b)	2.50 (ddd, 15.6, 9.2, 6.0, H-a), 2.59 (ddd, 16.0, 9.6, 5.6, H-b)
11	2.10 (m)	2.10 (m)	2.10 (m)	2.02 (m)
12	2.51 (m)	2.51 (m)	2.48 (m)	1.86 (m)
13	5.61 (dd, 15.2, 7.6)	5.61 (dd, 15.5, 7.6)	5.61 (dd, 15.5, 7.5)	1.45 (m)
14	5.53 (dd, 15.2, 6.8)	5.51 (dd, 15.5, 6.5)	5.51 (dd, 15.5, 6.5)	2.28 (t, 7.6)
15	4.00 (q, 6.0)	4.00 (q, 6.0)	4.00 (q, 7.0)	
16	1.54 (m)	1.53 (m)	1.52 (m)	2.46 (t, 7.6)
17-19	1.28 – 1.48 (m)	1.28 – 1.46 (m)	1.28 – 1.43 (m)	1.55 (quint, 7.2)
20	0.90 (t, 7.2)	0.90 (t, 7.0)	0.90 (t, 6.5)	0.90 (t, 7.2)
$COO\underline{CH_3}$		3.67 (s)		

Multipicities and coupling constants are in parentheses.

[a]Measured at 400 MHz. [b]Measured at 500 MHz.

Table 2. ^{13}C NMR Data of Com pounds **1-4** (100 MHz, CD_3OD)

Position	**1**	**2**	**4**
1	178.5	176.0	177.6
2	35.2	34.2	34.5
3	26.3	25.9	26.1
4	27.9	27.7	27.6
5	131.7	131.4	131.8
6	128.0	128.2	128.3
7	25.8	25.8	26.3
8	55.9	55.9	56.0
9	221.7	221.0	222.5
10	38.7	38.7	41.0
11	28.9	28.9	27.7
12	46.1	46.1	42.0
13	135.6	135.6	29.4
14	134.4	134.4	38.7
15	73.6	73.6	213.6
16	38.5	38.5	43.5
17	26.4	26.4	24.6
18	33.0	33.0	32.6
19	23.8	23.7	23.5
20	14.4	14.4	14.3
$COO\underline{CH_3}$		51.2	

Table 3. ^{1}H NMR Data of Compounds 7-10 (500 MHz, CD_3OD)[a]

Position	7	8	9	10
2	2.23 (t, 7.5)	2.29 (t, 7.5)	2.27 (m)	2.25 (m)
3	1.59 (m)	1.62 (m)	1.62 (m)	1.58 (m)
4	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.33 - 1.40 (m)	1.28 - 1.33 (m)
5	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.33 - 1.40 (m)	1.28 - 1.33 (m)
6	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.51 (m)	1.28 - 1.33 (m)
7	1.29 - 1.31 (m)	2.06 (m)	2.27 (m)	1.49 (m)
8	1.53 (m)	5.61 (dt, 14.0, 7.0)	6.94 (dt, 16.0, 6.5)	2.25 (m)
9	2.43 (t, 7.5)	5.42 (ddt, 15.0, 6.5, 1.5)	6.13 (dt, 16.0, 1.5)	6.91 (dt, 14.0, 7.0)
10		3.97 (q, 7.0)		6.10 (dt, 14.0, 1.5)
11	2.43 (t, 7.5)	1.55 (m)	2.58 (t, 7.5)	
12	1.53 (m)	1.32 - 1.42 (m)	1.62 (m)	2.58 (t, 7.5)
13	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.33 - 1.40 (m)	1.58 (m)
14	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.33 - 1.40 (m)	1.28 - 1.33 (m)
15	1.29 - 1.31 (m)	1.32 - 1.42 (m)	1.33 - 1.40 (m)	1.28 - 1.33 (m)
16	0.89 (t, 7.5)	0.91 (t, 7.5)	1.33 - 1.40 (m)	1.28 - 1.33 (m)
17			1.33 - 1.40 (m)	1.28 - 1.33 (m)
18			0.92 (t, 6.5)	0.90 (t, 7.0)

[a]Multipicities and coupling constants are in parentheses.

Table 4. ^{1}H NMR Data of Compounds 11-14 (500 MHz, CD_3OD)[a]

Position	11	12	13	14
2	2.26 (t, 7.5)	2.26 (t, 7.5)	2.26 (t, 7.5)	2.25 (t, 7.0)
3	1.59 (m)	1.61 (m)	1.59 (m)	1.59 (m)
4	1.28 - 1.48 (m)	1.30 - 1.34(m)	1.29 - 1.31(m)	1.28 - 1.31(m)
5	1.28 - 1.48 (m)	1.30 - 1.34(m)	1.29 - 1.31(m)	1.28 - 1.31(m)
6	1.28 - 1.48 (m)	1.30 - 1.34(m)	1.29 - 1.31(m)	1.28 - 1.31(m)
7	2.03 (m)	1.30 - 1.34(m)	1.29 - 1.31(m)	1.28 - 1.31(m)
8	5.61 (dt, 15.0, 7.5)	1.55 (m)	1.29 - 1.31(m)	1.28 - 1.31(m)
9	5.40 (ddt, 15.0, 6.5, 1.5)	2.46 (t, 7.5)	1.54 (m)	1.28 - 1.31(m)
10	3.94 (q, 6.5)		2.43 (t, 7.0)	1.54 (m)
11	1.50 (m)	2.46 (t, 7.5)		2.37(t, 7.0)
12	1.28 - 1.48 (m)	1.55 (m)	2.43 (t, 7.0)	
13	1.28 - 1.48 (m)	1.30 - 1.34 (m)	1.54 (m)	2.37(t, 7.0)
14	1.28 - 1.48 (m)	1.30 - 1.34 (m)	1.29 - 1.31(m)	1.54 (m)
15	1.28 - 1.48 (m)	1.30 - 1.34 (m)	1.29 - 1.31(m)	1.28 - 1.31(m)
16	1.28 - 1.48 (m)	1.30 - 1.34 (m)	1.29 - 1.31(m)	1.28 - 1.31(m)
17	1.28 - 1.48 (m)	1.30 - 1.34 (m)	1.29 - 1.31(m)	1.28 - 1.31(m)
18	0.89 (t, 7.0)	0.91 (t, 7.5)	0.89 (t, 6.5)	0.89 (t, 6.5)

[a]Multipicities and coupling constants are in parentheses.

3-2. *In vitro* cytotoxicity of solvent fractions and isolated compounds from *G. verrucosa*

The isolated compounds from *G. verrucosa* did not show the cytotoxicity at the concentration of 20 ㎍/㎖ according to lactate dehydrogenase release assay. However, GV-c-10 showed 30.5% cytotoxicity in RAW 264.7 cells at 40 ㎍/㎖ (Fig. 10).

3-3. Effect of *G. verrucosa* on LPS-stimulated pro-inflammatory cytokine production

3-3-1. Inhibitory effects of solvent fractions and MeOH sub-fractions from *G. verrucosa* on the LPS-stimulated pro-inflammatory cytokine (TNF-α, IL-6 and IL-1β) expression

Cytokines are soluble mediators of inter- and intra-cellular communications. These proteins are secreted from cells and act on other cells to coordinate and appropriate immune responses (Eigler *et al*., 1997). Therefore, using RT-PCR, we examined whether solvent extracts and sub-fractions of *G. verruocsa* inhibit pro-inflammatory cytokine production in RAW 264.7 macrophages. There was no effect of *G. verrucosa* itself on the pro-inflammatory cytokine mRNA expression in normal RAW 264.7 macrophages (data not shown). However, in RAW 264.7 cells incubated with 100 ㎍/㎖ of solvent extracts of *G. verrucosa* in the presence of LPS, the aqueous MeOH fraction (F4) inhibited mRNA expression of IL-6 and IL-1β by 35.1% and 50.1%, respectively (Fig. 2). In addition, 50 ㎍/㎖ of various aqueous MeOH sub-fractions (GV-3, GV-5, GV-7, GV-9 and GV-11) inhibited the mRNA expression of pro-inflammatory cytokines by up to 80-90% (Fig. 3).

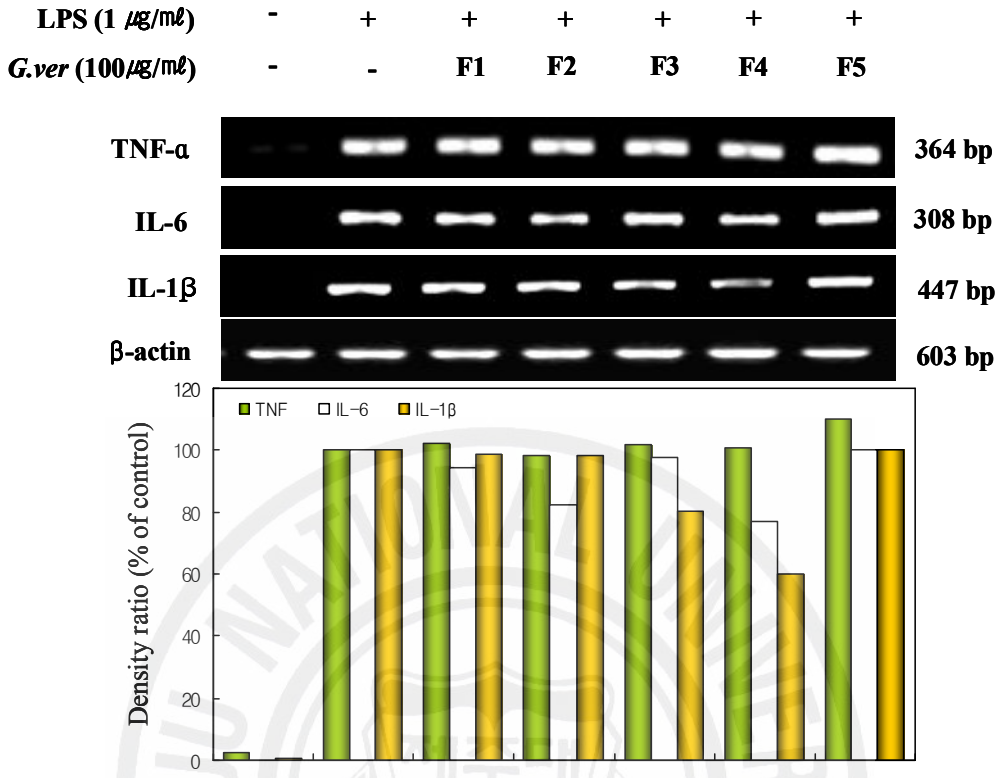


Fig. 2. Effects of solvent extracts purified from *G. verrucosa* on the mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds (100 ㎍/㎖) for 6 h. The RNA extraction was carried out in RNase-free environment and the mRNA expression of TNF-α, IL-6 and IL-1β were determined by RT-PCR.

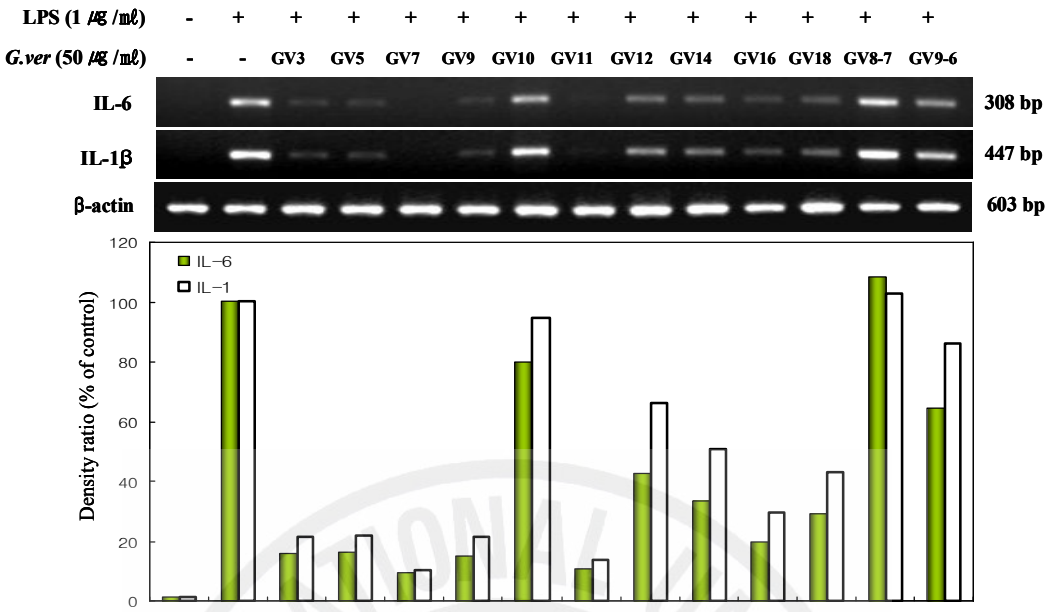


Fig. 3. Effects of MeOH sub-fractions purified from *G. verrucosa* on the mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds (50 ㎍/㎖) for 6 h. The RNA extraction was carried out in RNase-free environment and the mRNA expression of IL-6 and IL-1β were determined by RT-PCR.

3-3-2. Inhibitory effects of compounds isolated from *G. verrucosa* on the LPS-stimulated pro-inflammatory cytokine production

We isolated fourteen compounds from *G. verrucosa* and investigated their inhibitory effect on the production of pro-inflammatory cytokines (TNF-α and IL-6) in LPS-stimulated RAW 264.7 cells. Among them, GV-c-6 [(E)-11-oxohexadec-9-enoic acid], GV-c-9 (10-oxooctadec-8-enoic acid) and GV-c-10 (11-oxooctadec-9-enoic acid) inhibited the production of TNF-α and IL-6 up to 60-90% at 20 ㎍/㎖ (Table 5). Also, these two compounds dose-dependently inhibited the production of pro-inflammatory cytokines (Table 6).

We next investigated the effect of *G. verrucosa* on the mRNA expression of pro-inflammatory cytokines. As shown in Fig. 4 and 5, GV-c-9 and GV-c-10 dose-dependently suppressed the mRNA expression of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells.

Table 5. Effects of compounds isolated from *G. verrucosa* on the production of TNF-α and IL-6 in LPS- stimulated RAW 264.7 cells.

Compounds (20 ㎍/㎖)	Inhibition (%)	
	TNF-α	IL-6
c-1	54.8 ± 4.0	44.3 ±2.2
c-2	57.2 ± 6.7 **	41.6 ± 1.4
c-3	53.8 ± 6.5 **	37.0 ± 0.3
c-4	20.7 ± 12.5	41.4±1.8
c-5	N.D.	N.D.
c-6	47.5 ± 5.7	75.7 ± 1.9**
c-7	17.3 ± 6.3	38.4 ± 2.1
c-8	40.7 ± 3.5	44.8 ± 1.1
c-9	70.1 ± 3.1 **	96.1 ± 2.5 **
c-10	68.0 ± 7.2 **	91.8 ± 1.6 **
c-11	17.8 ± 10.4	N.D.
c-12	33.1 ± 7.8	N.D.
c-13	37.5 ± 6.3	28.1 ±0.7
c-14	18.0 ± 6.9	19.2 ±0.9

N.D. , not detected.

Data represent mean ± SD of independent observations performed in triplicate. *$p<0.05$, **$p<0.01$ compared with the LPS alone.

Table 6. Effects of GV-c-6, GV-c-9 and GV-c-10 isolated from *G. verrucosa* on the production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells.

	sample	Inhibition (%)		
		10 ㎍/㎖	20 ㎍/㎖	40 ㎍/㎖
TNF-α	prednisolone	19.3±0.3	27.3±2.1	51.1±1.0*
	c-6	26.0±1.5	42.9±3.9*	59.3±0.2*
	c-9	34.7±2.2	69.7±1.1**	80.0±1.0**
	c-10	38.4±0.5	53.7±1.0*	92.6±1.5 **
IL-6	prednisolone	33.9±1.3	50.3±0.4*	56.5±4.0 *
	c-6	67.3±0.3*	89.5±0.6**	96.1±0.5**
	c-9	44.8±1.6*	87.8±2.4**	99.4±0.2 **
	c-10	72.4±0.2**	96.1±0.5**	99.0±0.1**

Data represent mean ± SD of independent observations performed in triplicate. *$p<0.05$, **$p<0.01$ compared with the LPS alone.

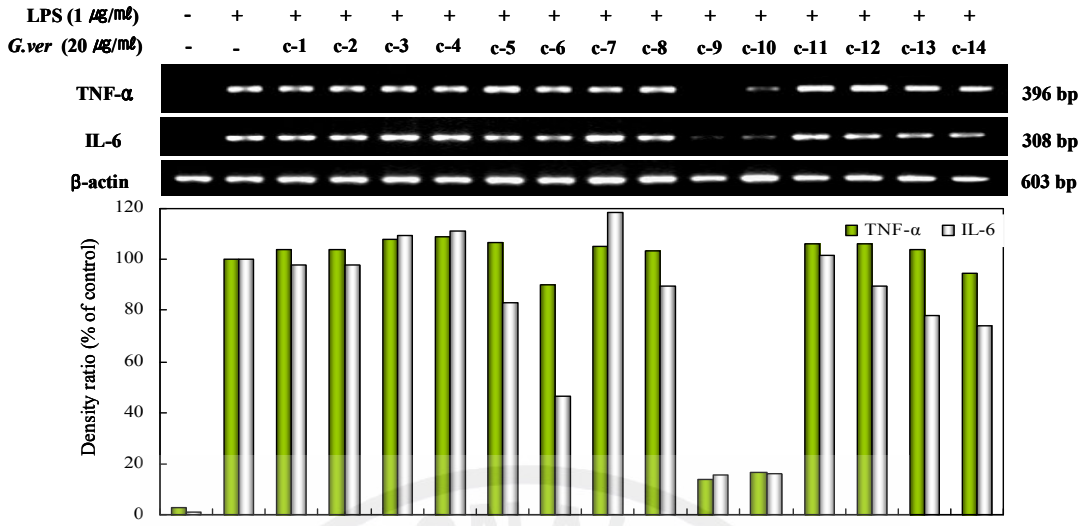


Fig. 4. Effects of compounds isolated from *G. verrucosa* on the mRNA expression of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds (20 ㎍/㎖) for 6 h. The RNA extraction was carried out in RNase-free environment and the mRNA expression of TNF-α and IL-6 were determined by RT-PCR.

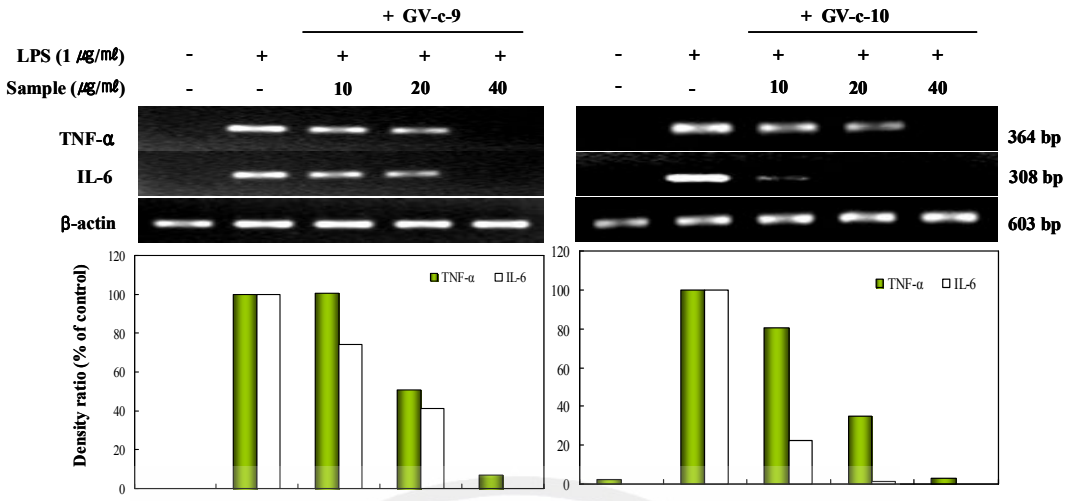


Fig. 5. Effects of GV-c-9 and GV-c-10 isolated from *G. verrucosa* on the mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds at the indicated concentrations for 6 h. The RNA extraction was carried out in RNase-free environment and the mRNA expression of TNF-α and IL-6 were determined by RT-PCR.

3-4. Inhibitory effects of compounds isolated from *G. verrucosa* on the LPS-stimulated NO production

NO produced by activated macrophages is involved in various harmful responses including tissue injury, septic shock, and apoptosis (James, 1995). Stimulation of RAW 264.7 macrophage with LPS for 24 h increased the NO production in the medium compared to cells treated with vehicle alone (Fig. 6). We first examined the suppressive effects of fourteen compounds isolated from *G. verrucosa* on LPS-induced NO production and iNOS mRNA expression at 20 ㎍/㎖. Among them, GV-c-6, GV-c-8, GV-c-9 and GV-c-10 significantly inhibited NO production by 60.2%, 74.8%, 86.5% and 84.5%, respectively (Fig. 6). To examine whether the suppression of NO production by *G. verrucosa* was due to reduced iNOS mRNA expression, RT-PCR analysis from LPS-stimulated cells were conducted. The results showed that these four compounds significantly inhibited iNOS mRNA expression by 70-90% (Fig. 7). Also, GV-c-6, GV-c-9 and GV-c-10 inhibited LPS-stimulated NO production and iNOS mRNA expression in a dose-dependent manner (Fig. 8, 9 and 10). However, GV-c-10 increased LDH release at the concentration of 40 ㎍/㎖. These results were indicated that compounds isolated from *G. verrucosa* controlled NO production via iNOS mRNA expression.

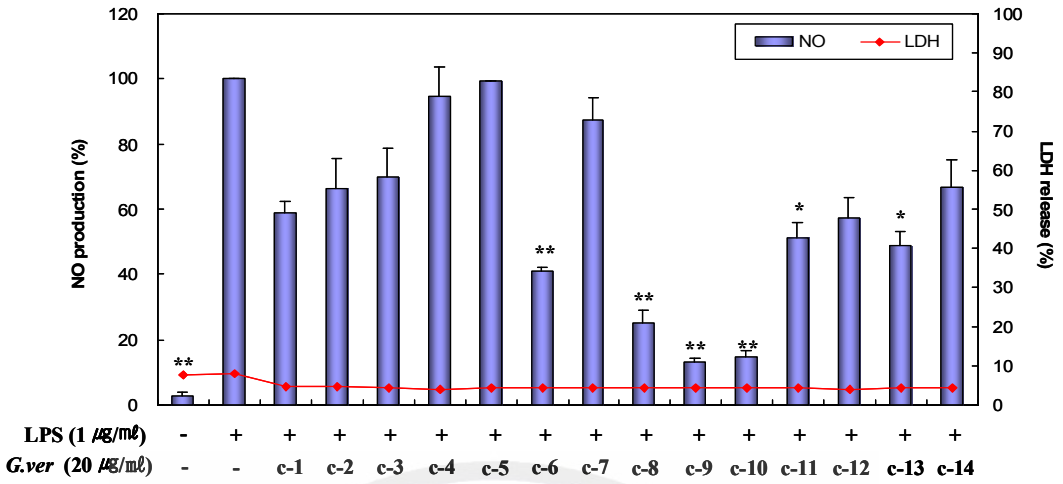


Fig. 6. Effects of compounds isolated from *G. verrucosa* on the production of nitric oxide in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds (20 ㎍/㎖) for 24 h. Cell cytotoxicity was determined using LDH release method. The data represent the mean ± SD of triplicate experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with the LPS alone.

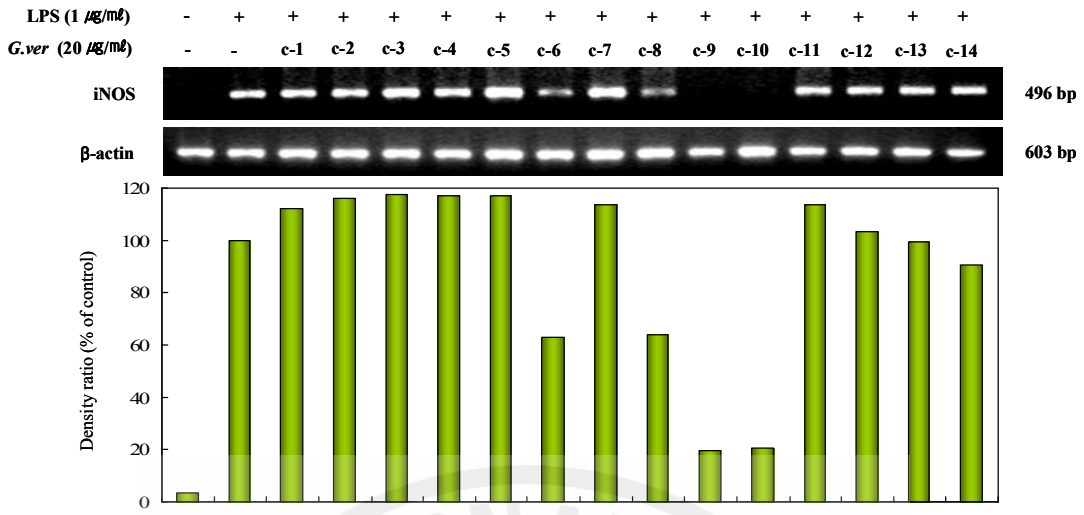


Fig. 7. Effects of compounds isolated from *G. verrucosa* on the mRNA expression of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of compounds (20 ㎍/㎖) for 18 h. The mRNA expression of iNOS was determined by RT-PCR.

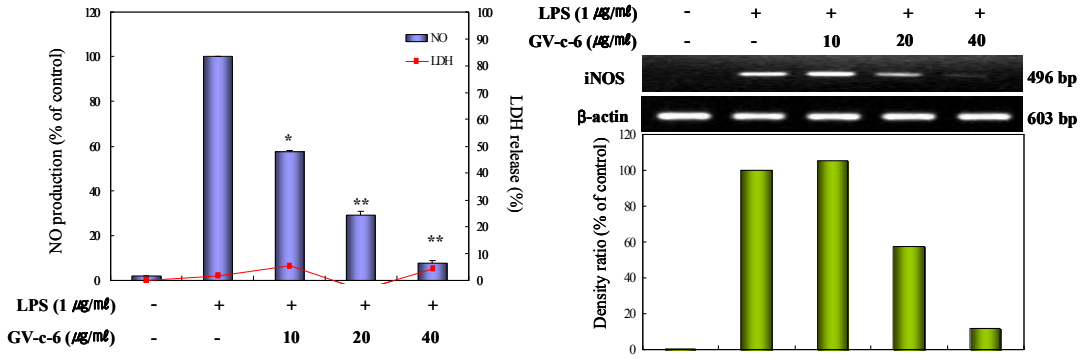


Fig. 8. Effects of GV-c-6 isolated from *G. verrucosa* on the production of nitric oxide and the mRNA expression of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of GV-c-6 at the indicated concentrations for 24 h. Cell cytotoxicity was determined using LDH release method. The data represent the mean ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS alone (Left panel). RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of compounds at the indicated concentrations. The mRNA expression of iNOS was determined by RT-PCR (Right panel).

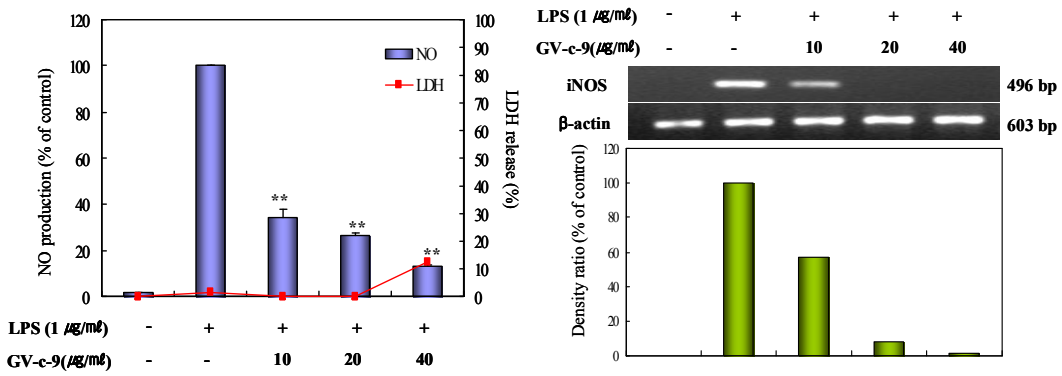


Fig. 9. Effects of GV-c-9 isolated from *G. verrucosa* on the production of nitric oxide and the mRNA expression of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of GV-c-9 at the indicated concentrations for 24 h. Cell cytotoxicity was determined using LDH release method. The data represent the mean ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS alone (Left panel). RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of compounds at the indicated concentrations. The mRNA expression of iNOS was determined by RT-PCR (Right panel).

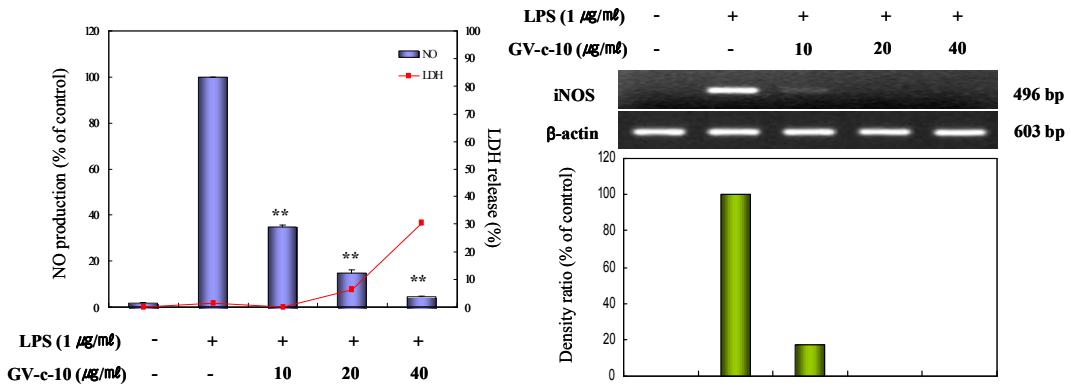


Fig. 10. Effects of GV-c-10 isolated from *G. verrucosa* on the production of nitric oxide and the mRNA expression of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence of GV-c-10 at the indicated concentrations for 24 h. Cell cytotoxicity was determined using LDH release method. The data represent the mean ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS alone (Left panel). RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of compounds at the indicated concentrations. The mRNA expression of iNOS was determined by RT-PCR (Right panel).

3-5. Inhibitory effects of GV-c-9 and GV-c-10 on the LPS-stimulated NF-κB activation

NF-κB can be activated in cells stimulated with LPS or other inflammatory insults, and is involved in the transcriptional activation of responsive genes (Mukaida, 1996). Therefore, reporter gene analysis was carried out using luciferase reporter plasmids containing the minimal NF-κB binding sequences to determine if the suppressive effect of *G. verrucosa* occurred through inhibition of NF-κB reporter activation. In cells stimulated with LPS (1 ㎍/㎖) for 20 hr, NF-κB reporter activity increased 5-fold (Fig. 11), and treatment with GV-c-9 and GV-c-10 significantly inhibited the LPS-induced NF-κB reporter activity. A control drug, pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF-κB activation) also inhibited the NF-κB reporter activity by 80% at 10 ㎍/㎖.

3-6. Inhibitory effects of GV-c-9 and GV-c-10 on the LPS-stimulated IκB-α degradation

NF-κB exists in latent form in the cytoplasm of unstimulated cells and is bound to the inhibitory protein, IκB. Phosphorylation of IκB leads to its degradation and the subsequent translocation of NF-κB to the nucleus where it activates the transcription of target genes (Ghosh and Karin, 2002). We analyzed the degradation of IκB-α in RAW 264.7 cells stimulated with LPS in the presence or absence of compounds isolated from *G. verrucosa.* Stimulation of cells with LPS (1 ㎍/㎖) for 10 min decreased the IκB-α band intensity and recovered to basal level at 30-60 min after LPS-induced IκB-α degradation. We investigated the degradation of IκB-α in the presence of GV-c-9 and GV-c-10, these compounds did not inhibit LPS-induced IκB-α degradation (Fig. 12).

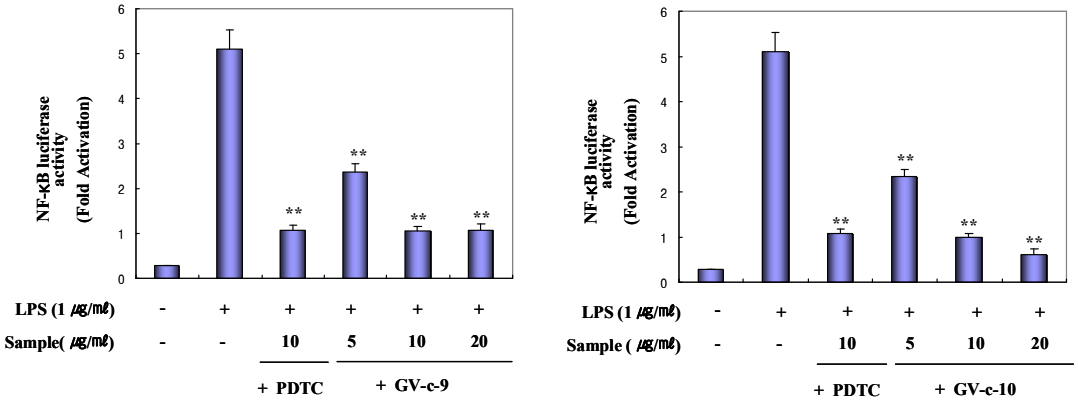


Fig. 11. Effects of GV-c-9 and GV-c-10 on the activation of NF-κB in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (2.0×10^5 cells/㎖) were transiently cotransfected with NF-κB promoted luciferase reporter plasmid (pNF-κB-Luc) and Renilla luciferase reporter plasmid (pRL-null) as internal control for 24 hr, and then treated with LPS (1 ㎍/㎖) in the presence of GV-c-9 and GV-c-10 for 20 hr. The luciferase activity was measured and data were normalized by Renilla luciferase expression vector. The data represent the mean ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS alone.

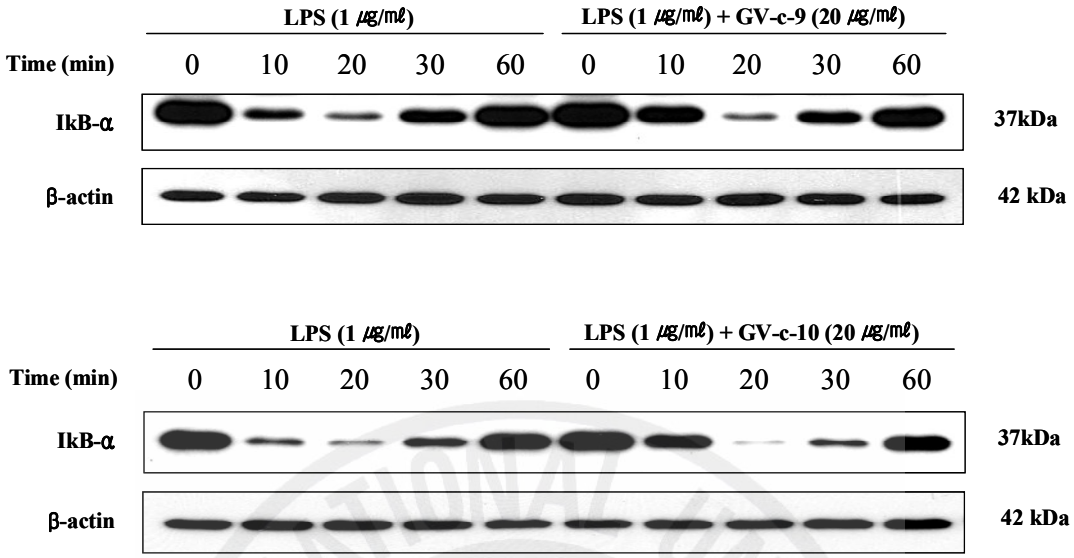


Fig. 12. Effects of GV-c-9 and GV-c-10 on the degradation of IkB-α in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of GV-c-9 and GV-c-10 (20 ㎍/㎖) for indicated times. Whole-cell lysates were prepared and the protein level was determined by western blotting.

3-7. Inhibitory effects of GV-c-9 and GV-c-10 on the LPS-stimulated MAPK activation

Several early signalling pathways have been identified in response to LPS stimulation of macrophages, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase, and p38/stress-activated protein kinase. We assayed the effect of GV-c-9 and GV-c-10 on MAPK activation by examining the phosphorylation of ERK 1/2, JNK, and p38 MAPKs through western blot analysis. Stimulation of cells with LPS increased the phosphorylation of ERK 1/2 at 20 min (Fig. 13), and treatment with GV-c-9 and GV-c-10 decreased the phosphorylation of ERK 1/2 (Fig. 13 and 14). However, GV-c-9 and GV-c-10 did not affect LPS-induced phosphorylation of JNK and p38 (Fig 13 and 14). These results indicated that the anti-inflammatory effect of *G. verrucosa* occurs through inhibiting the activation of ERK 1/2 pathway, but not inhibition of the JNK and p38 pathways.

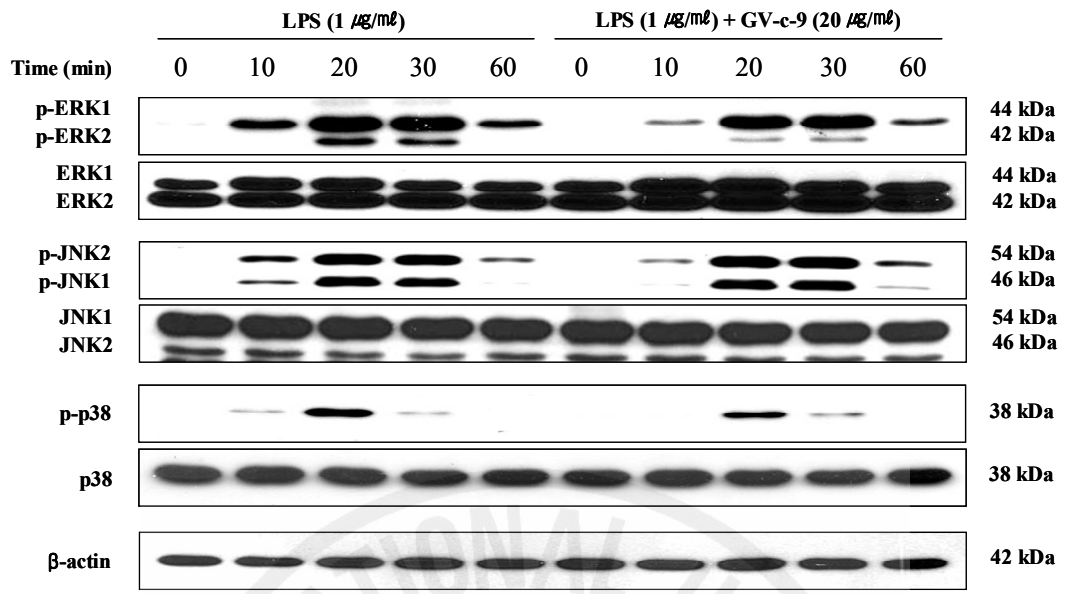


Fig. 13. Effect of GV-c-9 on the activation of ERK1/2, JNK1/2 and p38 MAPK in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of GV-c-9 (20 ㎍/㎖) for indicated times. Whole-cell lysates were prepared and the protein level was determined by western blotting

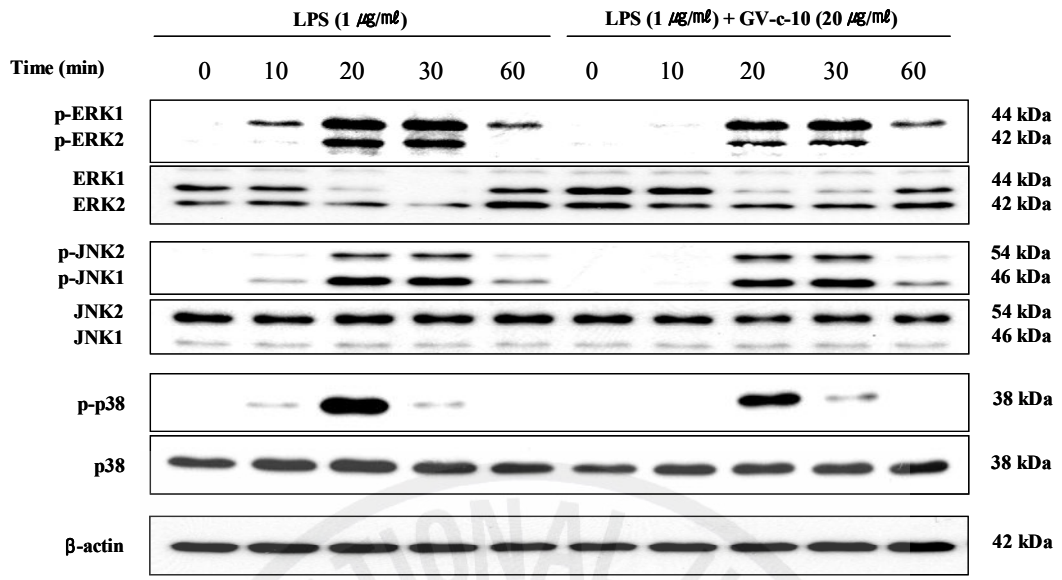


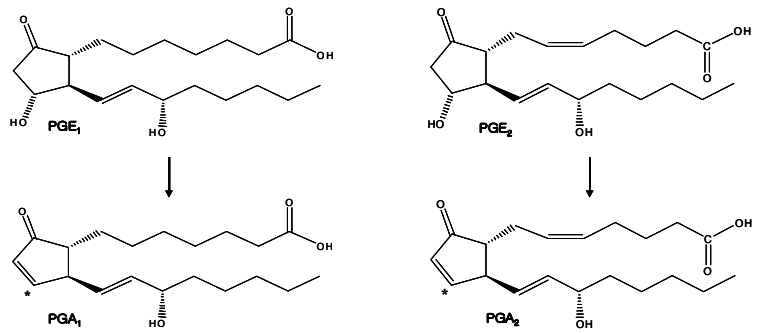
Fig. 14. Effect of GV-c-10 on the activation of ERK1/2, JNK1/2 and p38 MAPK in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of GV-c-10 (20 ㎍/㎖) for indicated times. Whole-cell lysates were prepared and the protein level was determined by western blotting.

3-8. Inhibitory effects of cyclopentenone PGs on the LPS-stimulated production of pro-inflammatory mediators (NO, TNF-α and IL-6)

G. verrucosa is rich in polyunsaturated fatty acids (PUFA), which are precursors of prostaglandins (PGs), and known to contain the metabolites, prostaglandin A_2 and E_2 (Imbs *et al*., 2001). Based on their chemical structure and the molecular and cellular mechanisms responsible for their actions, PGs are divided into two groups: cyclopentane (E, F, D) and cyclopentenone (A, B, C) PGs. Within this family, prostaglandins of the A and J series contain a cyclopentenone ring structure, which are characterized by the presence of a chemically reactive α,β-unsaturated carbonyl ring (Hubich and Sholukh, 2006). The cyclopentenone prostaglandins PGA_1 and PGA_2 are formed by dehydration within the cyclopentane ring of PGE_1 and PGE_2, respectively (Fig. 15A). Various compounds in the cyclopentenone prostaglandin family possess potent anti-inflammatory, anti-neoplastic, and anti-viral activity (Straus and Glass, 2001, Straus *et al*., 2000). Therefore, we examined the effect of PGA and PGE on the pro-inflammatory mediators (TNF-α, IL-6 and NO) in LPS-stimulated RAW 264.7 cells. The results showed that PGA_1 and PGA_2 inhibited the LPS-induced gene expression of pro-inflammatory cytokines. However, PGE_1 and PGE_2 did not affect the LPS-induced TNF-α and IL-6 mRNA expressions (Fig. 15B and C).

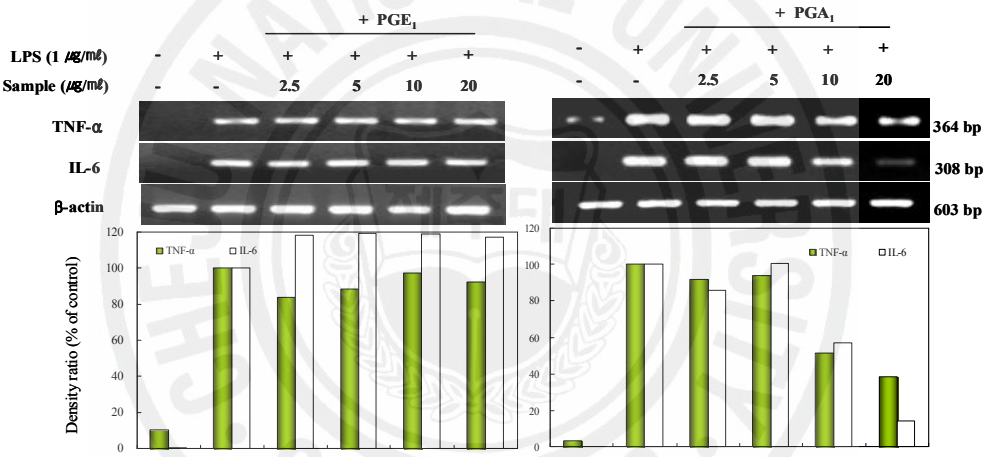
PGA_1 and PGA_2 also dose-dependently suppressed the production of NO and the mRNA expression of iNOS (Fig. 16A and B). PGE_1 did not inhibit the production of NO or iNOS mRNA expression, but PGE_2 inhibited production of NO at 20 ㎍/㎖ (Fig. 16A and C). This result was due to metabolic dehydration of PGE_2 to PGA_2, during 24 h exposure to cells (Ohno *et al*., 1986).

(A)



*** eletrophilic carbon**

(B)



(C)

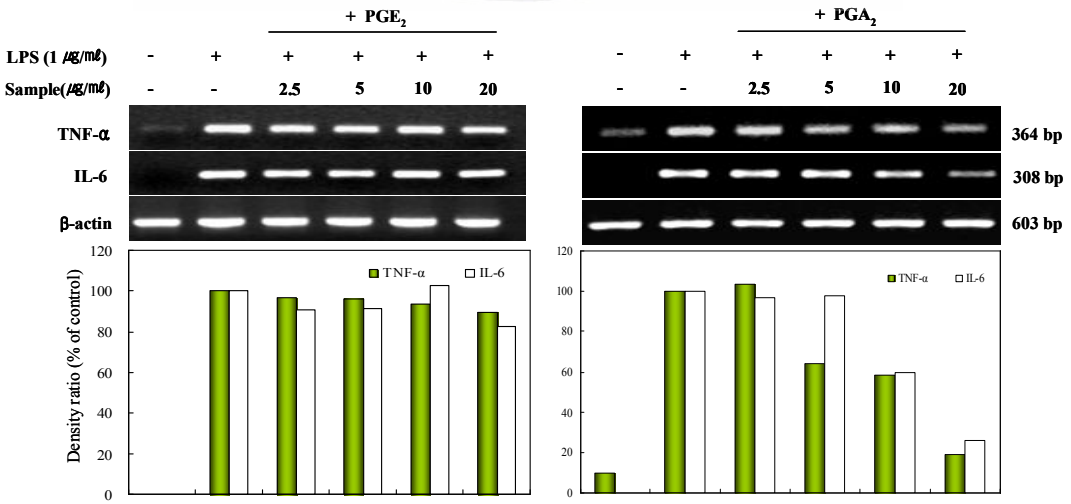
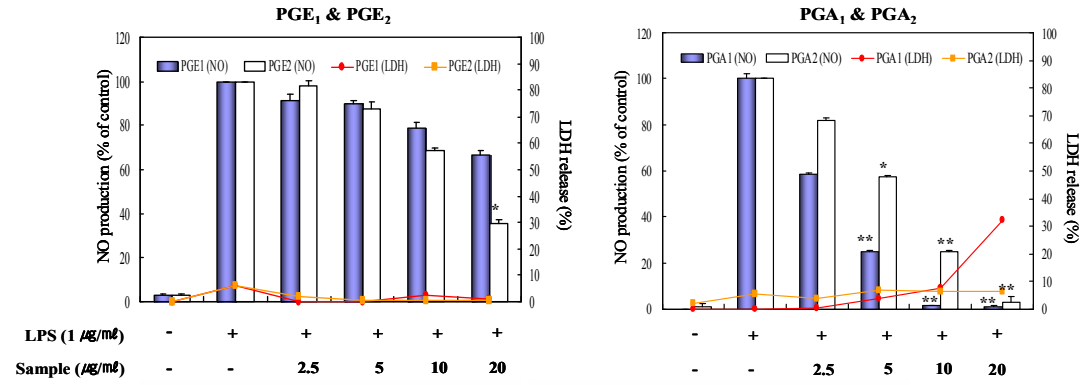
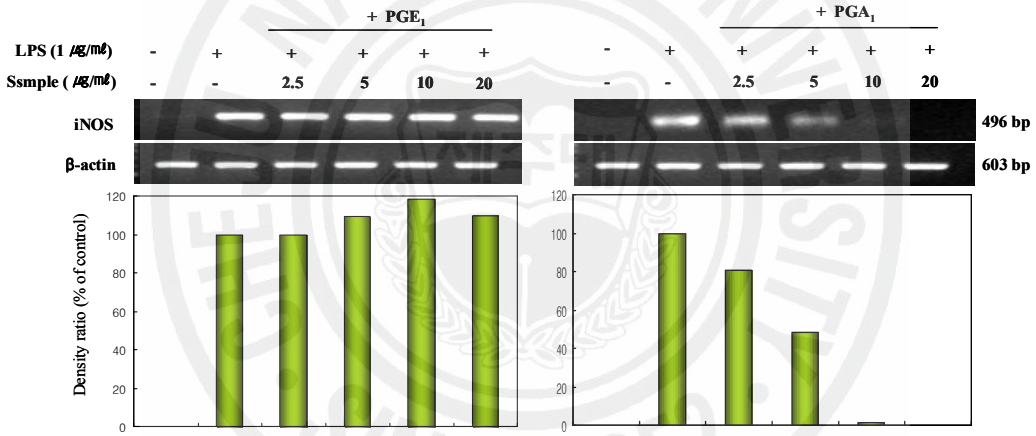


Fig. 15. Effects of PGs on the mRNA expression of pro-inflammatory cytokines in RAW 264.7 cells. (A) Formation of PGA_1 and PGA_2 via dehydration in the cyclopentane ring of PGE_1 and PGE_2 (B) RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of PGE_1 and PGA_1 (2.5, 5, 10 and 20 ㎍/㎖) for 6 h. The mRNA expression of TNF-α and IL-6 were determined by RT-PCR. (C) RAW 264.7 cells (1.5×10^6 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of PGE_2 and PGA_2 (2.5, 5, 10 and 20 ㎍/㎖) for 6 h. The mRNA expression of TNF-α and IL-6 were determined by RT-PCR.

(A)


PGE1 & PGE2
PGE1 (NO)
PGE2 (NO)
PGE1 (LDH)
PGE2 (LDH)
NO production (% of control)
LDH release (%)
PGA1 & PGA2
PGA1 (NO)
PGA2 (NO)
PGA1 (LDH)
PGA2 (LDH)
LPS (1 μg/mℓ) - + + + + +
Sample (μg/mℓ) - - 2.5 5 10 20

(B)


+ PGE1
+ PGA1
LPS (1 μg/mℓ) - + + + + +
Ssmple (μg/mℓ) - - 2.5 5 10 20
iNOS
β-actin
496 bp
603 bp
Density ratio (% of control)

(C)

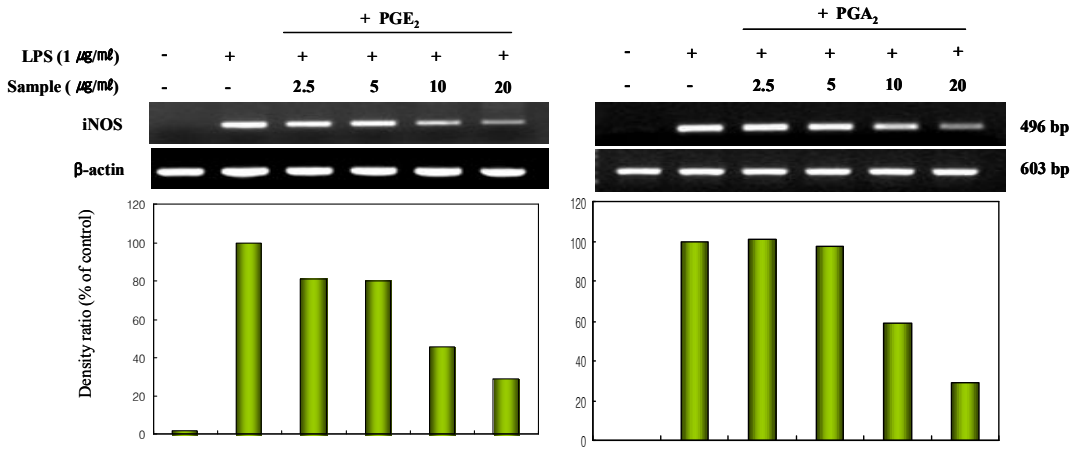

+ PGE2
+ PGA2
LPS (1 μg/mℓ) - + + + + +
Sample (μg/mℓ) - - 2.5 5 10 20
iNOS
β-actin
496 bp
603 bp
Density ratio (% of control)

Fig. 16. Effects of PGs on the production of nitric oxide and mRNA expression of iNOS in RAW 264.7 cells. (A) RAW 264.7 cells (1.5×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of PGA_1, PGA_2, PGE_1, and PGE_2 (2.5, 5, 10 and 20 ㎍/㎖) for 24 h. Cell cytotoxicity was determined using LDH release method. The data represent the mean ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the LPS alone. (B) RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of PGA_1 and PGE_1 (2.5, 5, 10 and 20 ㎍/㎖) for 18 h. The mRNA expression of iNOS was determined by RT-PCR. (C) RAW 264.7 cells (8.0×10^5 cells/㎖) were stimulated with LPS (1 ㎍/㎖) in the presence or absence of PGA_2 and PGE_2 (2.5, 5, 10 and 20 ㎍/㎖) for 18 h. The mRNA expression of iNOS was determined by RT-PCR

4. Discussion

In this study, we investigated the modulatory effects of *G. verrucosa* on the production of pro-inflammatory markers. We isolated fourteen compounds from *G. verrucosa,* among them, Gv-c-9 and GV-c-10 inhibited on the production of TNF-α, IL-6, and NO via suppression of NF-κB activation and ERK phosphorylation. And, to study on the functional group from active compounds, we investigated inhibitory effects of prostaglandins on the production of inflammatory markers. PGA inhibited the production of TNF-α, IL-6, and NO.

There are various species of marine algae in Jeju Island, and useful secondary metabolites are obtained from marine algae. We investigated the anti-inflammatory effects of fifty species of marine algae (data not shown). Among them, *Gracilaria verrucosa* showed potentially inhibitory effects on the production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. *G. verrucosa* is a common marine red alga in Korea, and has been widely used for agar production together with Gelidium. However, only a few studies have reported the biological effects of this algae.

Lipopolysaccharide (LPS) stimulates macrophages such as RAW 264.7 to produce an array of pro-inflammatory mediators, including the potent vasodilator NO and the cytokines, TNF-α, IL-1, and IL-6 (Lapa *et al*., 2000). Increased production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and NO plays a critical role in the process of macrophage activation and is associated with acute and chronic inflammation (Mehra *et al*., 2005; Kofler *et al*., 2005). Our results showed that GV-c-10 from *G. verrucosa* inhibited TNF-α and IL-6, with IC_{50} values of 51.2 μM and 33.1 μM, respectively. Compared with control drug, prednisolone (a steroid compound) inhibited the production of TNF-α and IL-6, with IC_{50} values of 110.9 μM and 40.7 μM, respectively. Also, GV-c-9 and GV-c-10 inhibited the production of NO, with IC_{50} values of less than 30 μM. These results indicate that the anti-

inflammatory effects of *G. verrucosa* may be due to the modulation of macrophage-mediated inflammatory events such as pro-inflammatory cytokines and NO.

The expression of iNOS, COX-2 and the inflammatory cytokines, TNF-α and IL-6, in murine macrophages has been shown to be dependent on NF-κB activity (Shakov et al., 1990; Libermann and Baltimore, 1990; Collart *et al*., 1990). Studies of unstimulated cells have revealed that NF-κB is bound to I-κB in the cytoplasm, which prevents it from entering the nucleus (Sanchez-Perez *et al*., 2002). In most mammalian cells, IκB-α is rapidly degraded (<10 min) following phosphorylation but is quickly resynthesized (50-60 min) in an NF-κB-dependent manner (DiDonato *et al*., 1996; Sun *et al*., 1993). The release of NF-κB from IκB results in the passage of NF-κB into the nucleus, where it binds to specific sequences in the promoter regions of target genes. We investigated effects of GV-c-9 and GV-c-10 on the NF-κB activation in LPS stimulated RAW 264.7 cells. Luciferase assays revealed that GV-c-9 and GV-c-10 inhibited NF-κB activation. But, immunoblot analysis showed that the compounds isolated from *G. verrucosa* did not affect IκB-α degradation. These results suggest that the inhibitory effect of *G. verrucosa* on the pro-inflammatory mediators may involve transcriptional regulation through suppression of NF-κB activation without interfering nuclear translocation of NF-κB in LPS-stimulated RAW264.7 cells (Shin *et al*., 2004).

Numerous studies have suggested that LPS regulates iNOS and TNF-α expression through the mitogen-activated kinase (MAPK) signaling pathway (Suh *et al*., 2006; Raingeaud *et al*., 1995). Also, the activation of NF-κB is regulated by several kinases such as mitogen-activated protein kinase (Carter *et al*., 1998). However, signaling from MAPK to transcription factors that mediate iNOS and pro-inflammatory cytokine expression is not fully understood. LPS stimulation of monocytes/macrophages activates several intracellular signaling pathways, including three MAPK kinase pathways: ERK (p42/44), c-Jun N-

terminal kinase (JNK) and p38 (Guha *et al*., 2001). In the present study, compounds isolated from *G. verrucosa* inhibited phosphorylation of ERK1/2 but not JNK1/2 (Fig. 13 and 14). Several independent groups have reported that LPS-induced NF-κB activation is related to activation of protein kinase C, ERK1/2, but not JNK1/2 in mouse macrophages (Chen and Lin, 2001; Castrillo *et al*., 2001). These results suggest that, in addition to NF-κB inhibition, *G. verrucosa* may inhibit pro-inflammatory genes expression via inactivation of ERK 1/2 pathways in LPS-activated RAW 264.7 cells.

GV-c-6, GV-c-9 and GV-c-10 share a common chemical moiety, the acyclic enone analogs, which have the α,β-unsaturated carbonyl group. Previously, potential anti-inflammatory activities have been reported for compounds containing the cyclic enone group such as cyclopentenone PG (cyPG) and cyclopentenone derivatives in activated-macrophage (Hortelano *et al*., 2000; Musiek *et al*., 2005). However, there is no report showing anti-inflammatory activity of acyclic enone compounds such as GV-c-6, GV-c-9 and GV-c-10. The present study found for the first time that acyclic enone compounds isolated from *G. verrucosa* have remarkably high suppressive activities toward pro-inflammatory gene expression compared to cyclic enone compounds such as PGA. There is ample evidence that the α,β-unsaturated carbonyl group in cyPG inhibits NF-κB activation via direct blockade of IκB kinase (Rossi *et al*., 2000), which phosphorylates IκB protein for its degradation. Some reports have indicated that cyPG directly targets ERK 1/2 (Eligini *et al.,* 2002) and p38 (Murakami *et al*., 2005). Our results showed that GV-c-9 and GV-c-10 blocked phosphorylation of ERK and p38 in LPS-stimulated RAW264.7 macrophage cells (Fig. 13 and 14). Another known target of α,β-unsaturated carbonyl group is peroxisome proliferator-activated receptor γ (PPARγ) (Na and Surh, 2003; Drew *et al*., 2005). PPARγ is a member of the superfamily of nuclear receptors for steroid, thyroid, and retinoid hormones that require ligand binding for regulation of cell cycle control, carcinogenesis, and inflammation

(Zingarelli and Cook, 2005). Thus, we are currently investigating the effect of *G. verrucosa* on PPARγ ligand activity.

PG can be converted non-enzymatically to other compounds, including cyclopentenone PGs (cyPG) such as PGA_1, PGA_2, PGJ_2, and 15d-PGJ_2 (Straus and Glass, 2001). The biological activities of cyPGs have been shown different from those of primary PGs. The cyPGs are actively incorporated into cells independent of PG receptors and are transferred to the nucleus where they exhibit various biological effects including modulation of stress reaction, inhibition of the cell cycle, and suppression of viral replication (Li *et al*., 2001; Hsiang and Straus, 2002; Santoro, 1997). In this study, PGA_1 and PGA_2, two of the metabolites of *G. verrucosa*, suppressed the production of NO and pro-inflammatory cytokines (Fig. 15). Similarly, compounds isolated from *G. verrucosa* had an inhibitory effect on the production of pro-inflammatory mediators. These results suggest that the anti-inflammatory effects of *G. verrucosa* in LPS-stimulated macrophage activation may be due to compounds with an α,β-unsaturated carbonyl structure similar to PGA.

In summary, we have demonstrated that the anti-inflammatory mechanism of *G. verrucosa* may modulate macrophage-mediated inflammatory functions such as the over-production of pro-inflammatory cytokines and NO via suppression of NF-κB activation and ERK1/2 phosphorylation. Moreover, the active compounds isolated from *G. verrucosa* have the α,β-unsaturated carbonyl group in their chemical structures. These results provide new insight into the mechanisms of the anti-inflammatory and pharmacological activities of *G. verrucosa*.

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6. Abstract in Korean

꼬시래기(*Gracilaria verrucosa* Papenfuss)는 한국 연안에 서식하는 홍조류로서, 우뭇가사리와 더불어 전세계적으로 한천의 주요 원료로서 널리 이용되고 있다. 이러한 중요성 때문에 꼬시래기류에 관한 연구는 분류, 생태, 생활사 등 다양한 분야에 걸쳐서 수행되어 왔으나, 이들의 생리활성에 대한 연구는 거의 없는 실정이다. 따라서, 본 논문에서는 꼬시래기로부터 14개의 단일 화합물을 분리해 내고, 분리된 화합물이 염증성 매개인자의 생성에 미치는 영향 및 작용 메카니즘에 대해 조사하였다.

1. 꼬시래기로부터 단일 화합물을 분리하고 그들을 구조 분석한 결과, 4개의 프로스타글란딘, 하나의 세라마이드, 그리고 9개의 지방산 구조를 가지는 화합물들을 확인 할 수 있었으며, 이 중 2-formamido-1,3-dihydroxy-octadecane (GV-c-5) 와 11-oxohexadec-9-enoic acid (GV-c-6)는 이전에 보고된 바 없는 물질이다.
2. 꼬시래기로부터 분리된 화합물들이 LPS로 자극된 RAW 264.7 세포에서 염증성매개 인자의 생성에 미치는 영향을 조사 한 결과, GV-c-6, GV-c-9 그리고 GV-c-10이 염증성 매개인자인 TNF-α 와 IL-6 및 NO 생성을 농도의존적으로 억제함을 알 수 있었다.
3. 높은 활성을 나타내었던 GV-c-9 와 GV-c-10의 작용 메카니즘을 알아보기 위하여, LPS로 자극된 NF-κB 활성화 및 MAPK 활성화에 미치는 영향에 대해 조사하였다. 그 결과, 이 두 화합물이 NF-κB의 전사활성 억제, ERK 1/2 인산화를 억제함을 확인 할 수 있었다.
4. 활성을 나타내었던 화합물의 작용기를 찾기 위하여, 프로스타글란딘류 (PGE 및 PGA)를 LPS로 자극된 RAW 264.7 세포에 처리하여 염증성 매개인자 억제활성을 조사 한 결과, 불포화 카르보닐기를 작용기로

가지는 화합물임을 알 수 있었다.

이러한 결과는, 꼬시래기로부터 분리된 화합물이 불포화 카르보닐기를 작용기로 가지며, NF-κB 와 ERK 활성화 억제를 통하여 염증성 매개인자인 TNF-α, IL-6 그리고 NO 생성을 저해함으로써 염증 억제 효능을 가진다는 것을 시사한다.

주요어: 꼬시래기, 염증, TNF-α, IL-6, NO, NF-κB, ERK

감사의 글

아득하기만 하고 끝나지 않을 것 같던 박사논문을 마무리하며, 아쉬움과 기쁨이 교차합니다. 하지만, 이게 끝이 아니라 시작이라는 마음을 가지며, 이 논문이 완성되는데 많은 도움을 주신 분들께 감사의 마음을 전하고자 이 글을 씁니다.

우선 의대에 들어와 석사과정부터 지금까지 부족한 저를 잘 보듬어주시고 따뜻한 관심 가져주신 유은숙 교수님께 감사 드립니다. 항상 건강하세요. 그리고 논문의 부족한 부분을 세심하게 지적해주신 강희경 교수님, 박덕배 교수님, 조문제 교수님, 현진원 교수님께도 감사의 마음을 전합니다. 대학원 과정 동안 늘 관심과 조언으로 학문의 길을 이끌어주신 이영기 교수님, 강현욱 교수님, 정영배 교수님, 고영상 교수님, 이근화 교수님, 김수영 교수님, 은수용 교수님과 이창현 학장님을 비롯하여 다른 의대 교수님들께도 감사의 마음을 전합니다.

약리학 교실에서 함께 생활하며 다독여 줬던 상철오빠, 바쁘다면서도 짜증 한번 내지 않고 실험과 논문 챙겨주고 있는 원종오빠, 늘 웃는 얼굴로 대해주는 재희, 약방의 힘든 일 마다 않고 열심히 해주는 경진, 정일이와 미소 가득하신 엘비라 선생님, 그리고 약방의 귀염둥이들 은진, 선순, 혜진이와 자주 볼 수는 없지만 만날 때마다 반겨주는 변윤영 선생님, 민경이에게 감사의 마음을 전합니다. 그리고 같이 졸업하며 많은 조언 해주셨던 남권호 선생님... 감사합니다.

의대라는 새로운 곳으로 이끌어주고 늘 도움이 필요할 땐 아낌없이 주시는 생화학 교실 영미언니와 희경 언니, 그리고 지은 언니, 경아, 진영이, 미경 언니, 장예, 힘들고 지칠 때 많이 위로해주던 미생물학 교실 지현 선생님과 정은이에게도 감사의 마음을 전합니다. 그리고 조직학교실 보연이와 지강씨, 임상실험실 수길씨, 병리학교실 이정희 선생님, 보경이, 옆방 연희와 과사에서 많은 도움을 줬던 명선이와 근희 언니에게도 고마운 마음을 전합니다.

늘 옆에 있으면서 힘이 되어줬던 영희, 지영, 복희, 국희, 경화와 태헌오빠, 영복오빠, 논문 쓰느라 힘들거라며 웃음 주던 정아와 지영, 뜬금없이 찾아가 드리는 부탁에 흔쾌히 응해 주시던 지권 선생님과 영준오빠, 지훈 선생님께 감사의 마음을 전합니다. 결혼 생활하느라 바쁜 와중에도 언니 챙긴다며 전화해주던 은아와 혜정에게도 고맙다는 말을 전합니다. 그리고 이 논문이 나올 수 있도록 많은 도움 주신 부산대학교의 정지형 교수님께도 감사의 마음을 전합니다.

끝으로 부족한 딸 믿어주신 아빠, 엄마, 나의 상담자 역할을 해주던 울언니와 혜영, 혜정, 형부와 귀여운 우리 조카들에게 사랑하는 마음을 전합니다. 아프지 말고 늘 건강해요..사랑합니다..