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Inhibitory Effects of Eutigosides Isolated from *Eurya emarginata* on the Inflammatory Mediators in RAW264.7 Cells

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The anti-inflammatory activity of *Eurya emarginata* (Thumb) Makino, of which leaves have been traditionally used to treat ulcers or diuretic in Jeju Island, has been investigated in the present study. Through the phytochemical study from the methanol extract of *E. emaginiata*, eutigosides B and C were isolated as the active components. Sseveral inflammatory markers including TNF-α, IL-1β, IL-6, NO, iNOS, and COX-2 were examined. Eutigosides B and C potentially inhibited production of pro-inflammatory cytokines (IL-6 and TNF-α) in a dose-dependent manner. Additionally, the intracellular contents of iNOS protein were markedly decreased after treatment with eutigosides B and C. The inhibition of iNOS activity was correlated with the decrease in nitrite levels. These results suggest that eutigoside B and C from *E. emarginata* may have anti-inflammatory activity through the inhibition of pro-inflammatory cytokines (TNF-α and IL-6), iNOS and COX-2.

Key words: Eutigoside B, Eutigoside C, Pro-inflammatory cytokines, iNOS, COX-2

INTRODUCTION

Cytokines are soluble mediators of inter- and intracellular communications. They contribute to a chemical signaling language that regulates development, tissue repair, hemopoiesis, inflammation, and the specific and non-specific immune responses (Nicod, 1993; Rouveix, 1997; Boraschi *et al.*, 1998; Dinarello, 2000; Oppenheim, 2001; Holloway *et al.*, 2002).

Inflammation is the process by which the human body attempts to counteract potential injurious agents such as invading bacteria, viruses, and other pathogens (Henderson *et al.*, 1996; Ulevitch and Tobias, 1995; Hersh *et al.*, 1998). Although it is essential process in the living organisms, inflammation can also produce a harmful effect to the host through the multiple levels of biochemical, pharmacological, and molecular controls involving a diverse array of cell types and some soluble mediators including cytokines (Nicod, 1993; Rouveix, 1997; Boraschi *et al.*, 1998; Dinarello, 2000; Turcanu *et al.*, 2001). When the proinflammatory cytokines like IL-1β, IL-6, and TNF-α are administered to humans, they produce fever, inflammation, tissue destruction, and in some cases, shock and death (Dinarello, 2000).

Nitric oxide (NO) has been identified as an important molecule involved in regulating biological activities in the vascular, neural, and immune systems (Moncada *et al.*, 1992). NO produced by activated macrophages has been shown to mediate host defense functions such as antimicrobial and antitumor activities, but its excess production causes tissue damage associated with acute and chronic inflammation (MacMicking *et al.*, 1997).

Cyclooxygenase (COX) is the key enzyme for the conversion of arachidonic acid to prostaglandins (PGs) (Vane *et al.*, 1998). There are two isoforms of COX; one is the COX-1, constitutive enzyme, which is responsible for the production of PGs with general housekeeping functions such as maintenance of renal perfusion and a protective effect on the gastric mucosa against ulceration; and the other is the COX-2, inducible enzyme, which is responsible for the production of PGs (Dubois *et al.*, 1998; Needleman *et al.*, 1998; Vane *et al.*, 1994). COX-2 is expressed during

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inflammation by cytokines or bacterial products such as lipopolysaccharide (LPS), and it produces PGs that contribute to the pain and swelling of inflammation (Hla *et al.*, 1992; O'Sullivan *et al.*, 1992).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are able to reduce the pain and swelling associated with inflammation by inhibiting the COX enzyme. Drugs which inhibit selectively COX-2 might block inflammation, pain, and fever with low side effects including gastric erosions and ulcers associated with inhibition of COX-1 (Hawkey, 1999). Celecoxib, specific COX-2 inhibitor, has similar efficacy as conventional NSAIDs in improving the signs and symptoms of osteoarthritis and rheumatoid arthritis.

Eurya emarginata (Thumb) Makino (Theaceae) is a dioecious, insect-pollinated tree, which combines both sexual reproduction and colonial spread. The distribution is restricted in coastal areas from Southern China, Southern Korea, to Central and Southern Japan (Chung and Epperson, 2000). Though leaves of *E. emarginata* have been traditionally used to treat ulcers or diuretic in Jeju Island, studies on the biological activity and constituents of *E. emarginata* are rare. Therefore, this study is performed to identify the active anti-inflammatory components from its leaves as well as to examine their effects on the production of inflammatory-biomarkers (TNF-α, IL-1β, IL-6, iNOS, and COX-2) in murine macrophage cell line RAW264.7.

MATERIALS AND METHODS

Plant material

The leaves of *E. emarginata* (Thumb) Makino were collected during May 2000 at the campus of Cheju National University, Jeju Island, Korea. The collected material was dried under dark place at room temperature and stored in freezer at -20°C.

Characterization and experimental procedures

^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JNM-LA (400 MHz) spectrometer. TLC was carried out on Merck precoated silica gel 60 F_{254} plates, and silica gel for normal-phase column chromatography was Kieselgel 60 (230-400 mesh ASTM, Merck, Germany). Reverse-phase column chromatography was carried out using silica gel 100 C_{18}-reversed phase (3 × 15, silica gel 100 C_{18}-reversed phase, Merck). C_{18} reverse phase HPLC column (Bondapak C_{18}, 7.8 × 300 nm column, Merck; Waters 2487, Waters) was used for HPLC analysis.

Isolation of eutigosides B and C

The dried leaves (150 g) were extracted using 80% aqueous methanol (1 L × 3) after maintaining a week at room temperature. The liquid layer was obtained by filtration, and the filtrate was concentrated using evaporator under reduced pressure to get the crude extract (48.8 g). Water (1 L) was added to the extract, and then the aqueous layer was successively partitioned into hexane, ethyl acetate (EtOAc) and *n*-butanol. A part (1.5 g) of the ethyl acetate fraction (7.7 g) was purified through reversed phase SiO_2 column using gradient elution of aqueous methanol (20% to 100%) to give five fractions (A – E). The polar fraction A (600 mg) was subjected to sephadex LH-20 column chromatography using CH_2Cl_2/acetone/methanol (3/2/0 to 1/4/0 to 2/7/1) to give 32 fractions. The fraction 26 (13 mg) was further purified by HPLC to give eutigoside C (5.3 mg). The fraction 30 (15 mg) was also purified by HPLC to give eutigoside B (3.8 mg). The structure of eutigosides B and C was identified by comparison of the spectroscopic data to the literature report (Khan, 1992).

Eutigoside B

Pale yellow powder: $[\alpha]_D^{20}$ –18 (MeOH, *c*=0.0028); ^{1}H-NMR (400 MHz, methanol-d_4) δ 7.63 (1H, d, 16.0, H7"), 7.47 (2H, d, 8.6, H2", H6"), 6.97 (2H, m, H2, H6), 6.81 (2H, br d, 8.6, H3", H5"), 6.35 (1H, d, 16.0, H8"), 6.07 (2H, d, 10.0, H3, H5), 4.48 (1H, dd, 12.0, 2.2, H6'), 4.29 (1H, dd, 12.0, 6.0, H6'), 4.24 (1H, d, 7.8, H1'), 3.92 (1H, dt, 11.9, 6.1, H8), 3.64 (1H, dt, 11.9, 6.1, H8), 3.49 (1H, dd, 9.0, 6.0, H5'), 3.31-3.39 (2H, m, H3', H4'), 3.16 (1H, dd, 9.0, 7.8, H2'), 2.04 (2H, t, 6.1, H7); ^{13}C-NMR (100 MHz, methanol-d_4) δ 187.8 (C4), 169.1 (C9"), 161.4 (C4"), 154.4 (C2), 154.3 (C6), 146.8 (C7"), 131.2 (C2", C6"), 128.0 (C3), 127.9 (C5), 116.9 (C3", C5"), 115.0 (C8"), 104.4 (C1'), 77.9 (C3'), 75.5 (C5'), 75.0 (C2'), 71.8 (C4'), 69.2 (C1), 65.9 (C8), 74.6 (C6'), 41.0 (C7).

Eutigoside C

Pale yellow powder: $[\alpha]_D^{20}$ –15 (MeOH, *c*=0.0038); ^{1}H-NMR (400 MHz, methanol-d_4) δ 7.71 (1H, d, 16.0, H8"), 7.62 (2H, m, H2" and H6"), 7.40 (3H, m, H3", H4", H5"), 6.97 (2H, m, H2, H6), 6.56 (1H, d, 16.0, H8"), 6.06 (2H, br d, 10.4, H3, H5), 4.50 (1H, dd, 11.8, 2.0, H6'), 4.32 (1H, dd, 11.8, 2.0, H6'), 4.25 (1H, d, 7.8, H1'), 3.92 (1H, dt, 10.2, 6.5, H8), 3.65 (1H, dt, 10.2, 6.5, H8), 3.51 (1H, dd, 9.0, 6.0, H5'), 3.34 (2H, m, H3', H4'), 3.16 (1H, dd, 9.0, 7.8, H2'), 2.04 (2H, t, 6.5); ^{13}C-NMR (100 MHz, methanol-d_4) δ 187.8 (C4), 168.5 (C9"), 154.4 (C2), 154.3 (C6), 146.5 (C7"), 135.7 (C1"), 131.6 (C4"), 130.1 (C2", C6"), 129.3 (C3", C5"), 128.0 (C3), 127.9 (C5), 118.7 (C8"), 104.4 (C1'), 77.9 (C3'), 75.4 (C2'), 75.0 (C5'), 71.7 (C4'), 69.2 (C1), 65.9 (C8), 64.8 (C6"), 41.0 (C7).

Cell culture

Murine macrophage cell line RAW264.7 was obtained from the KCLB (Korean Cell Line Bank). The cells were

maintained at 37°C in a humidified atmosphere of 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; GIBCO Inc, NY, U.S.A.). Throughout the experiments exponential phase cells were used.

Pro-inflammatory cytokine production *in vitro*

The inhibitory effect of *E. emarginata* on TNF-α and IL-6 production was determined by the method previously described (Cho *et al.*, 1998c). The isolated compounds solubilized with PBS and EtOH were 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 RAW264.7 cells. Before stimulation with LPS (1 μg/mL) and test materials, RAW264.7 cells (1.0×10^6 cells/mL) were incubated for 18 h in 24-well plates with the same conditions. Lipopolysaccharide (LPS) and the 100 μg/mL of 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, U.S.A.). The inhibitory effect of testing fractions on TNF-α and IL-6 production was determined as previously described.

RNA isolation and RT-PCR analysis

Total RNA was extracted from cells by the Tri-Reagent (MRC, Cincinnati, OH, U.S.A.) method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook *et al.* (1989). The reverse transcription of 1 mg RNA was carried out using M-MuLV reverse transcriptase (Promega, WI, U.S.A.), oligo (dT) 18 primer, dNTP (0.5 μM) and 1 U RNase inhibitor. After incubation at 70°C for 5 min, 37°C for 5 min, 37°C for 60 min, and M-MuLV reverse transcriptase was inactivated by heating at 70°C for 10 min. The polymerase chain reaction (PCR) was performed in a reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, U.S.A.), 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, U.S.A.), and the amplification was followed by 35 cycles of 94°C for 45 sec (denaturing), 60-65°C for 45 sec (annealing) and 72°C for 1 min (primer extension). The PCR products were electrophoresed on a 1.5% agarose gel. The nucleotide sequence of each primers and the size of product were shown in Table I.

Western blot analysis

Murine macrophage cell line RAW264.7 were pre-incubated for 18 h, and then stimulated by LPS (1 μg/mL) and IFN-γ (50 U/mL) in the presence of testing materials for 24 h. 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 μg/mL aprotinin, 25 μg/mL leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at 12,000×*g* at 4°C for 15 min and then until use, the supernatants were stored at –70°C. Protein concentration was measured using the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 μg of protein) were separated on a 8-12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, U.S.A.) 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 5% nonfat dried milk, the membrane was then incubated with specific primary mouse monoclonal anti-mouse iNOS Ab (1:1000, Santa-Cruz, CA, U.S.A.), or rabbit polyclonal anti-rabbit COX-2 Ab (1:1000, Santa-Cruz, CA, U.S.A.) at 4°C for overnight. The membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Santa-Cruz, CA, U.S.A.) to mouse or rabbit. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersharm-Pharmacia Biotech., NY, U.S.A.).

Table I. The sequences of primers used in RT-PCR analysis and the sizes of RT-PCR products

Gene		Primer sequences	Fragment size(bp)
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'	364
	R	5'-CCTGTAGCCCACGTCGTAGC-3'	
IL-1β	F	5'-CAGGATGAGGACATGAGCACC-3'	447
	R	5'-CTCTGCAGACTCAAACTCCAC-3'	
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'	308
	R	5'-TGCTGGTGACAACCACGGCC-3'	
β-Actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

Nitrite assay

The production of nitric oxide (NO) was determined by measuring the amount of nitrite from cell culture supernatant (Ryu *et al.*, 2000) using the Griess reagent (Sigma, MO, U.S.A.). Briefly, the RAW 264.7 cells were stimulated with LPS (1 μg/mL), and 100 μL of the supernatant was mixed with 100 μL of the Griess reagent (0.1% naphthlyene diamine dihydrochloride, 1% sulphanilamide, 2.5% H_3PO_4). This mix was incubated for 10 min at room temperature

(light protected). Absorbance at 540 nm was measured using an ELISA reader (Amersham Pharmacia Biotech, UK, U.S.A.) and the results were compared against a calibration curve using sodium nitrite as the standard.

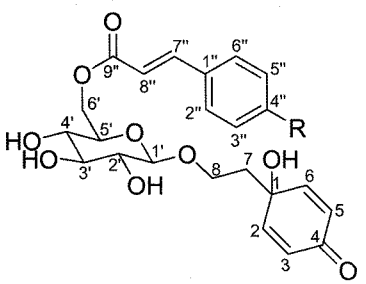
RESULTS AND DISCUSSION

E. emarginata (Thumb) Makino (Theaceae) is distributed in coastal areas of Jeju island. The leaves of *Eurya* have traditionally been used for ulcers or diuretic in the island. Nevertheless, there are only few reports on the pharmacological activity and constituents of *E. emarginata*. Therefore, the present study is performed to investigate the anti-inflammatory effects of the active constituents in the leaves of *E. emarginata* in order to get the information for the potential development of its constituents to anti-inflarmmatory agent. We have isolated eutigoside B and eutigoside C, the cytotoxic compounds from the EtOAc-soluble fraction of *E. emarginata* (Fig. 1).

The use of traditional medicine is widespread, and a number of plants are the major source of natural antioxidants that might contribute to the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their pharmacological activities (Perry *et al.*, 2002; Repetto and Llesuy, 2002).

Lipopolysaccharide (LPS) derived from gram-negative bacteria stimulates macrophage such as RAW264.7 to produce an array of pro-inflammatory mediators, which include the potent vasodilator nitric oxide (NO) and the cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-12 (IL-12) (Wei *et al.*, 1995; Axtelle and Pribble, 2001; Schutt and Schumann, 1993; Mukaida *et al.*, 1995; Lazarov *et al.*, 2000; Scott and Hancock, 2000). Many of these contribute to the pathogenesis of endotoxic shock.

In order to understand the anti-inflammatory effects by *E. emarginata*, the inhibitory effects of the eutigosides B and C isolated from the leaves of *E. emarginata* on the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) activated with lipopolysaccaride (LPS) as well as on the production of nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) activated with LPS/Interferon-γ (IFN-γ) were examined in murine macrophage cell line RAW264.7. Further evidences on the anti-inflammatory function were obtained from RT-PCR analysis and Western blot analysis. The protein content and the mRNA levels were determined using ELISA and RT-PCR, respectively.



R = H Eutigoside C
R = OH Eutigoside B

Fig. 1. The structure of eutigoside B and eutigoside C

The eutigosides B and C potentially inhibited the mRNA expression of TNF-α and IL-6 in a dose-dependent manner (Fig. 2). Also, the eutigoside B and eutigoside C potentially inhibited the production of pro-inflammatory cytokines (IL-6 and TNF-α) in a dose-dependent manner (Fig. 3 and Fig. 4).

In order to determine whether the eutigoside B and the eutigoside C can reduce LPS-induced iNOS and COX-2 production, the protein content of iNOS or COX-2 was measured by Western blot analysis. The eutigoside B and the eutigoside C potentially inhibited the iNOS and COX-2 expression (Fig. 5 and Fig. 6).

The Griess reaction (Ryu *et al.*, 2000), a spectrometric determination for nitrite, was carried out to quantify the nitrite levels in the conditioned medium of RAW 264.7 cells treated with LPS. In murine macrophage RAW 264.7 cells, LPS stimulation alone could induce iNOS transcription and its protein synthesis, and following NO production. Furthermore, LPS stimulation was also well known to induce I kappa-B proteolysis and NF-kappa B nuclear translocation (Xie *et al.*, 1994; Henkel *et al.*, 1993).

Therefore, this cell system was an excellent model for

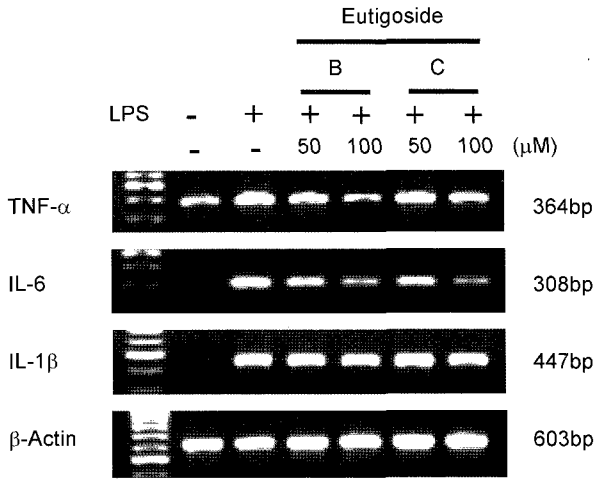


Fig. 2. Inhibitory effects of eutigosides on pro-inflammatory cytokine mRNA expression in activated macrophages. RAW264.7 macrophages (1.0×10^5 cells/mL) were pre-incubated for 18 h, and the levels of mRNA expression were determined after 6 h stimulation with LPS (1 μg/mL) in the presence of testing materials.

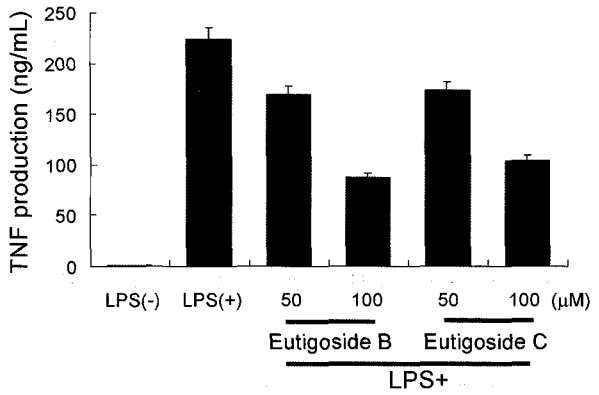


Fig. 3. Inhibitory effects of eutigosides on TNF-α production in RAW 264.7 cells. The content of TNF-α was determined by ELISA from the culture medium of RAW264.7 cells (1.0×10^6 cells/mL) stimulated by LPS (1 μg/mL) in the presence of testing materials.

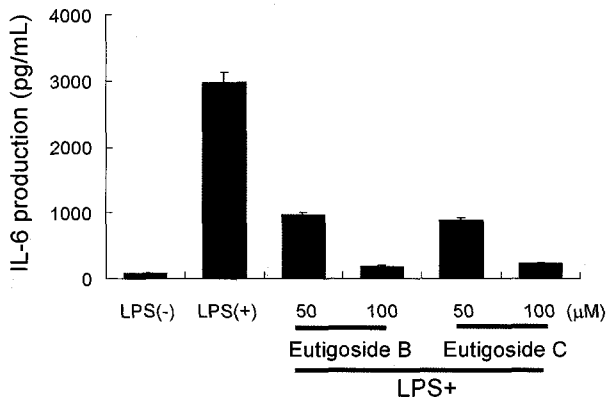


Fig. 4. Inhibitory effects of eutigosides on IL-6 production in RAW 264.7 cells. The content of IL-6 was determined by ELISA from the culture medium of RAW264.7 cells (1.0×10^6 cells/mL) stimulated by LPS (1 μg/mL) in the presence of testing materials.

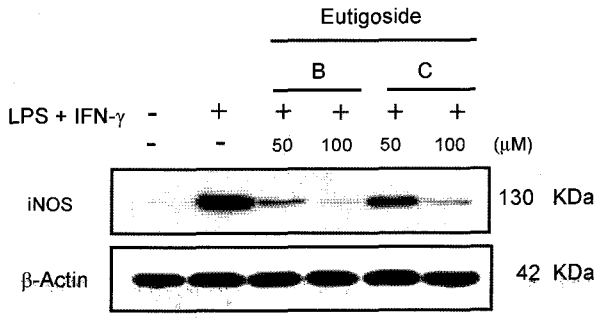


Fig. 5. Inhibitory effects of eutigosides on iNOS protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5 cells/mL) were pre-incubated for 18 h, and then iNOS protein content was determined after the 24 h stimulation with LPS (1 μg/mL) and IFN-γ (50 U/mL) in the presence of testing materials.

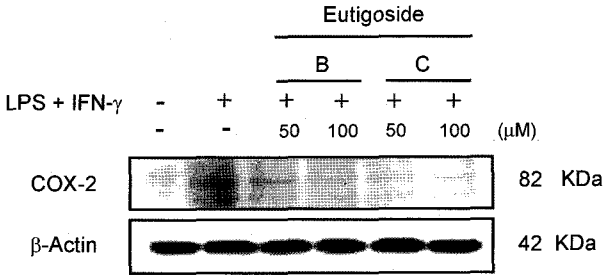


Fig. 6. Inhibitory effects of eutigosides on COX-2 protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5 cells/mL) were pre-incubated for 18 h, and then COX-2 content were determined after 24 h stimulation with LPS (1 μg/mL) and IFN-g (50 U/mL) in the presence of testing materials.

drug screening and the following evaluation of potential inhibitor on the pathways leading to the iNOS induction and NO production. The present results showed that the eutigoside B and C from *E. emarginata* markedly inhibited NO production in a dose-dependent manner. LPS induced a significant increase in nitrite production, and this effect was inversely suppressed by the eutigoside B and eutigoside C in a dose-dependent manner, with an IC_{50} value of 24.41 μM and 24.66 μM, respectively (Fig. 7 and Fig. 8).

It has been shown that induction of iNOS results in the synthesis of μM amounts of NO. Since the eutigoside B or C had a strong inhibition on LPS-induced NO production, the effect of the eutigosides on iNOS protein induction after LPS stimulation was examined. It was observed that the eutigoside B or C blocked the induction of iNOS protein in a dose-dependent manner. Moreover, the inhibition of iNOS activity also could decrease the subsequent NO production after LPS stimulation.

Nitric oxide (NO) plays an important role in the regulation of many physiological processes. It is also an intra- and extracellular mediator of cell function (Marin and Rodriguez-Martinez, 1997; Grisham *et al.*, 1998; Rosselli *et al.*, 1998). NO is synthesized from L-arginine by NO synthase (NOS) with NADPH and oxygen as co-substrates (Marletta *et al.*, 1988). Inducible NOS (iNOS) is the key enzyme that produces large amounts of NO by macrophages stimulated by bacterial endotoxin such as LPS and pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (MacMicking *et al.*, 1997; Nathan and Xie, 1994).

The cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which are produced mainly by activated monocytes or macrophages, stimulate bone resorption as well as enhance PGE_2 production in several type of cells including calvarial osteoblasts (Dinarello, 1988). The IL-6, a potent mitogenic polypeptide, stimulate cell proliferation in a various type of cells (Stein and Sutherland, 1998). It was also reported that TNF-α synergistically potentiate PGE_2 production

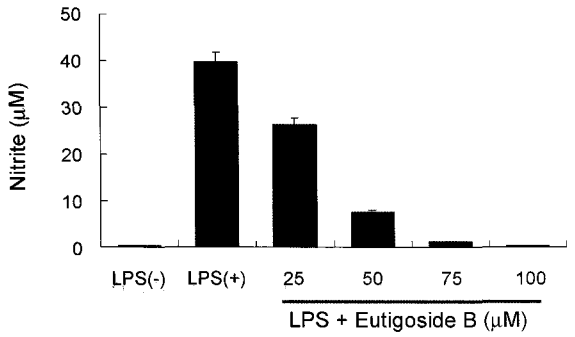


Fig. 7. Inhibitory effects of eutigoside B on nitric oxide production in RAW264.7 macrophages. The cells (1.5×10^5 cells/mL) were pre-incubated for 24 h, and then nitrite production was determined after 24 h stimulation with LPS (1 μg/mL) in the presence of eutigoside B.

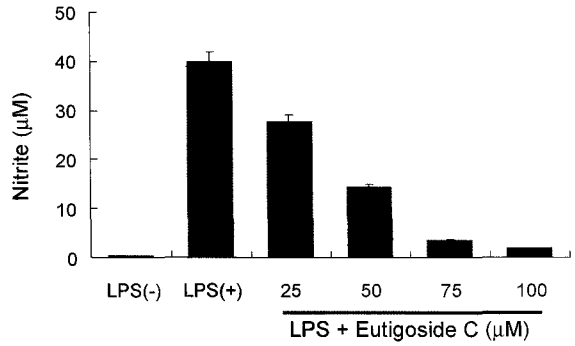


Fig. 8. Inhibitory effects of eutigoside C on nitric oxide production in RAW264.7 macrophages. The cells (1.5×10^5 cells/mL) were pre-incubated for 24 h, and then nitrite production was determined after 24 h stimulation with LPS (1 μg/mL) in the presence of eutigoside C.

stimulated by IL-1 (Lerner and Modeer, 1991). The mechanism by which cytokines such as TNF-α, IL-6, and IL-1 act in concert to stimulate prostaglandin production is, however, not well known. The TNF-α, IL-6, and IL-1, are involved in bone resorption as well as the production of PGE_2 in various type of cells (Smith and Marnett, 1991). Prostaglandins play an important role in the inflammatory processes including periodontitis, osteoporosis and rheumatoid arthritis. The rate-limiting enzymes such as a phospholipase A_2 (PLA_2) and a cyclooxygenase (COX) regulate the production of prostaglandins. The PLA_2 enzyme catalyzes the liberation of arachidonic acid (AA) from membrane phospholipids, whereas the enzyme COX mediates conversion of AA to prostaglandins (Smith and Marnett, 1991). The enzyme COX exists at least in two isoforms, the constitutive COX-1 and the inducible COX-2 (Diaz *et al.*, 1992).

The present study showed that the major constituents of *E. emarginata*, eutigosides B and C, have the anti-inflammatory effect in LPS-activated macrophages by suppressing inflammatory-biomarkers such as TNF-α, paralleled with inhibition of IL-6, NO, iNOS, and COX-2 inductions.

In conclusion, eutigoside B and eutigoside C were isolated from the leaves of *E. emarginata* as the anti-inflammatory components. These components were identified to have anti-inflammatory capacities, and therefore may be useful for the treatment of inflammation.

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REFERENCES

Axtelle, T. and Pribble, J., IC14, a CD14 specific monoclonal antibody is a potential treatment for patients with severe sepsis. *J. Endotoxin Res.*, 7, 3599-3605 (2001).

Boraschi D., Cifone, M. G., Falk, W., Flad, H. -D., Tagliabue, A., and Martin, M. U., Cytokines in inflammation. *Eur. Cytokine Netw.*, 9, 205-212 (1998).

Cho, J. Y., Park, J., Yoo, E. S., Baik, K. U., Lee J., and Park, M. H., Inhibitory effect of sesquiterpene lactones from *Saussurea lappa* on tumor necrosis factor-α production in murine macrophage-like cell. *Planta Med.*, 64, 594–597 (1998).

Chung, M. G. and Epperson, B. K., Clonal and spatial genetic structure in Eurya emarginata (Theaceae). *Heredity.*, 84, 170-177 (2000).

Diaz, A., Reginato, A. M., and Jimenez, S. A., Protein, nucleotide alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by transforming growth factor beta 1,interleukin 1 beta, and tumor necrosis factor-alpha. *J. Biol. Chem.*, 267, 10816-10822 (1992).

Dinarello, C. A., Pro-inflammatory cytokines. *Chest.*, 118, 503-508 (2000).

Grisham, M. B., Granger, D. N., and Lefer, D. J., Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. *Free Radical Biol. Med.*, 25, 404-433 (1998).

Hawkey, C. J., COX-2 inhibitors. *Lancet*, 353, 307-314 (1999).

Henderson, B., Poole, S., and Wilson, M., Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev.*, 60, 316-341 (1996).

Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baeuerle, P. A., Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*, 365, 182-185 (1993).

Hersh, D., Weiss, J., and Zychlinsky, A., How bacteria initiate inflammation: aspects of the emerging story. *Curr. Opin. Microbiol.*, 1, 43-48 (1998).

Hla, T. and Neilson, K., Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U.S.A.,* 89, 7384-7388 (1992).

Holloway, A. F., Rao, S., and Shannon, M. F., Regulation of cytokine gene transcription in the immune system. *Mol. Immunol.,* 38, 567-580 (2002).

Khan, I. A., Erdelmeier, C. A. J., and Sticher, O., New phenolic glucosides from the leaves of *Eurya tigang. J. Nat. Prod.*, 55, 1270-1274 (1992).

Lazarov, S., Balutsov, M., and Ianev, E., The role of bacterial endotoxins, receptors and cytokines in the pathogenesis of septic(endotoxin) shock. *Vutr. Boles.,* 32, 33-40 (2000).

Lerner, U. H. and Modeer, T., Bradykinin B1 and B2 receptor agonists synergistically potentiate interleukin-1 induced prostaglandin biosynthesis in human gingival fibroblasts. *Inflammation.*, 15, 427-436 (1991).

MacMicking, J., Xie, Q. W., and Nathan, C., Nitric oxide and macrophage function. *Annu. Rev. Immunol.,* 15, 323-350 (1997).

Marletta, M. A., Yoon, P. S., Iyengar, R., Life, C. D., and Wishnok, J. S., Oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry,* 27, 8706-8711 (1988).

Marin, J. and Rodriguez-Martinez, M. A., Role of vascular nitric oxide in physiological and pathological conditions. *Pharmacol. Therap.,* 75, 111-134 (1997).

Moncada, S., Palmer, R. M., and Higgs, E. A., Nitric oxide: physiology, pathophysiology. *Pharmacol. Rev.,* 43, 109-142 (1992).

Mukaida, N., Ishikawa, Y., Ikeda, N., Fujioka, N., Watanabe, S., Kuno, K., and Matsushima, K., Novel insight into molecular mechanism of endotoxin shock; biochemical analysis of LPS receptor signaling in a cell-free system targeting NF-kapperB and regulation of cytokine production/action through beta2 integrin *in vivo. J. Leukoc. Biol.,* 59, 145-151 (1996)

Nathan, C. and Xie, Q. W., Nitric oxide synthases: roles, tolls and controls. *Cell,* 78, 915-918 (1994).

Needleman, P. and Isakson, P. C., Selective inhibition of cyclooxygenase 2. *Sci. Med.,* 1, 35-36 (1998).

Nicod, L. P., Cytokines: overview. *Thorax,* 48, 660-667 (1993).

Oppenheim, J. J., Cytokines: past, present, and future. *Int. J. Hematol.,* 74, 38 (2001).

O'Sullivan, M. G., Chilton, F. H., Huggins, E. M. Jr., and McCall C. E., Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J. Biological. Chem.,* 267, 14547-14550 (1992).

Perry, E. K., Pickering, A. T., Wang, W. W., Houghton, P. J., and Perru, N. S., Medicinal plants and Alzheimer's disease: from ethnobotany to phytotherapy. *J. Pharm. Pharmacol.*, 51, 527-534 (1999).

Repetto, M. G. and Llesuy, S. F., Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Braz. J. Med. Biol. Res.,* 35, 523–534 (2002).

Rosselli, M., Keller, P. J., and Dubey, R. K., Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum. Reprod. Update.,* 4, 3-24 (1998).

Ryu, S. Y., Oak, M. H., Yoon, S. K., Cho, D. I., Yoo, G. S., Kim, T. S., and Kim, K. M., Anti-allergic and anti-inflammatory triterpenes from the herb of Prunella vulgaris. *Planta Medica,* 66, 358-360 (2000).

Rouveix, B., Clinical pharmacology of cytokines. *Eur. Cytokine Netw.,* 8, 291-293 (1997).

Sambrook, J. E., Fritsch, F., and Maniatis, T., In Molecular Cloning Laboratory Manual (2nd ed.), Cold Spring Harbor Laboratory Press, *United States of America.* 7-8 (1989).

Schutt, C. and Schumann, R., The endotoxin recepter CD14. *Immun. Infekt.,* 21, 36-40 (1993).

Scott, M. G. and Hancock, R. E., Cationic antimicrobial peptides and their imultifunctional role in the immune system. *Crit. Rev. Immunol.,* 20, 407-431 (2000).

Smith, W. L. and Marnett, L. J., Prostaglandin endoperoxide synthase: structure and catalysis. *Biochem. Biophys. Acta,* 1083, 1-17 (1991).

Stein, B. and Sutherland, M. S. K., IL-6 as a drug discovery target. *Drug Discov. Today,* 3, 202-213 (1998).

Turcanu, V. and Williams, N. A., Cell identification and isolation on the basis of cytokine secretion: a novel tool for investigating immune responses. *Nat. Med.,* 7, 373-376 (2001).

Ulevitch, R. J. and Tobias, P. S., Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.,* 13, 437-457 (1995).

Vane, J. R., Bakhle, Y. S., and Botting, R. M.. Cyclooxygenases 1 and 2. *Annual Rev. Pharmacol. Toxicol.*, 38, 97-120 (1998).

Wei, X. C. and Smith, I. G., Altered immune response in mice lacking inducible nitric oxide synthetase. *Nature,* 375, 408-411 (1995).

Xie, Q. W., Kashiwabara, Y., and Nathan, C., Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.,* 69, 4705-4708 (1994).