A DOCTORAL DISSERTATION

Anti-Cancer and Anti-Inflammatory Effects of Eutigosides from *Eurya emarginata*

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우묵사스레피의 eutigosides에 의한 항암 및 항염증 효과

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이 논문을 이학 박사학위 논문으로 제출함

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Anti-Cancer and Anti-Inflammatory Effects of Eutigosides from *Eurya emarginata*

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(Supervised by Professor **Se Jae Kim)**

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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The incidence of cancer has been rapidly increased and it is one of the main causes of death. Recent advances of chemotherapy have contributed to improve life quality and prolong the life span of cancer patients.

The major chemotherapy agents contain alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, and steroid hormones (Kaufmann and Earnshaw, 2000). But cancer chemotherapy has a limitation because most of anticancer agents don't have the discrimination between normal cells and cancer cells (Sporn, 1994; Greenwald *et al*., 1990). The chemotherapy leads to death of normal cells as well as cancer cells with lots of side effects (Wattenberg, 1996; Greenwald *et al*., 1990; Boone, 1990). To overcome the limitation of chemotherapy, it has been tried to isolate new anticancer compounds from natural products that were used in the folk for a long time. Extracts of the natural plants are important sources for development of new drug. However, these extracts suffer from a lack of reproducible bioactivity and chemical composition caused by the highly inducible, variable, and transitory nature of plant secondary metabolism (Poulev *et al*., 2003). Recently a large number of new anticancer agents, which target one or more of the extracellular, transmembrane, or intracellular (but extranuclear) processes involved in the malignant transformation of cells or carcinogenesis have been developed from natural resources (Pezzuto, 1997; Wattenberg, 1996; Sporn, 1994). The crude extracts of plants grown in Jeju were screened for cytotoxic effect. As a result, it was found that the extract of the leaves from *E. emarginata* markedly inhibited the growth of human leukemia HL-60 cells (Park

and Kang, 2000).

Eurya emarginata (Thunb.) 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). The leaves of *Eurya* are used in the traditional medicine with the treatment of ulcers or diuretic in the coastal areas of Jeju Island. Nevertheless, there are few reports on the biological activity and constituents of *E. emarginata*. Therefore, the present study investigated the anticancer effects and action mechanism of the extract from the leaves of *E. emarginata* to identify constituents of its leaves with anti-cancer activity. And, the components which displayed anti-cancer activity were isolated from the leaves of *E. emarginata.* In addition, in order to identify whether the isolated components have chemopreventive effect, we examined the anti-oxidative and anti-inflammatory activities.

Part Ⅰ

Anti-cancer effects of eutigosides in human leukemia HL-60 cells

ABSTRACT

The present study investigated the anticancer effects and action mechanism of eutigosides from the leaves of *E. emarginata*. The crude extract of *E. emarginata* markedly inhibited the growth of leukemia cells such as HL-60. When HL-60 cells were treated with the ethyl acetate fraction, several apoptotic events like as DNA fragmentation, morphologic changes and increase of the population of sub-G1 hypodiploid cells were observed. The growth inhibitory effect of *E. emarginata* seems to be a result from the induction of apoptosis. Moreover, the ethyl acetate fraction markedly reduced c-Myc expression in a time-dependent manner. The eutigoside C showing the cytotoxic effect was isolated from the leaves of *E. emarginata*. Furthermore, when the HL-60 cells were treated with eutigoside C, the Bcl-2 expression decreased in a time-dependent manner, whereas the expression of Bax was increased in a time-dependent manner compared to the control, and the release of cytochrome c from mitochondria into the cytosol was also observed. In addition, the expression of the active forms of caspase 9 and 3 were increased and the cleavage of Poly(ADP-ribose) polymerase, a vital substrate of effector caspase, was observed. The results indicate that the eutigoside C from *E. emarginata* induce apoptosis of HL-60 cells via the down-regulation of Bcl-2 expression and activation of caspases.

This study also investigated the mechanism by which the eutigoside C of *E. emarginata* induces cell growth arrest in human leukemia HL-60 cells. The data showed that the eutigoside C lead to the decrease of cyclin D1/E and the induction of Cdk inhibitor $p21^{WAF1/CIP1}$. In addition, the eutigoside C inhibited Cdk activity, and this inhibition was associated with the induction of Cdk

inhibitor $p21^{WAF1/CIP1}$ and its binding to the Cdk, similar to the induction of $p21^{WAF1/CIP1}$ by certain anticancer drugs. These findings suggest that the down regulation of cyclin D1/E and the induction of $p21^{WAF1/CIP1}$ play a key role in the negative regulation of cell cycle progression by the eutigoside C from *E. emarginata* in the HL-60, human leukemia cells.

Key word: *Eurya emarginata,* Eutigoside C, HL-60, Apoptosis, Bcl-2, Bax, Cytochrome c, Caspase 3, PARP, Cdk, $p21^{WAF1/CIP1}$, pRB

INTRODUCTION

Cancer is a disease, which can be characterized by the reduction or loss of effective controlling to maintain cellular organization in tissues. Cancer cells display temporally unrestricted growth preference over their normal counterparts (Clark, 1991). The basic features of cancer cells can be summarized as (a) uncontrolled cell proliferation, (b) lack of cellular differentiation features, (c) the ability to metastasize (Buick and Pollak, 1984).

Chemotherapy, or the use of chemical agents to destroy cancer cells, is a mainstay in the treatment of malignancies. The possible role in treating illness was discovered when the bone marrow suppressive effect of nitrogen mustard was noted in the early 1900's. Since that time, the search for drugs with anticancer activity has continued, and the goal of treatment with chemotherapy has evolved from relief of symptoms to cure. A major advantage of chemotherapy is its ability to treat widespread or metastatic cancer, whereas surgery and radiation therapies are limited to treating cancers that are confined to specific areas (Wattenberg, 1996; Mesner *et al*., 1997; Kaufmann and Earnshaw, 2000).

Previous studies have demonstrated that a wide range of anticancer agents, including chemotherapeutic agents, hormones, and various biologicals, induce apoptosis in malignant cells *in vitro* (Arends and Wyllie, 1991; Mesner *et al*., 1997) It is important to emphasize that this treatment-induced apoptosis is not merely a phenomenon in tissue cultures. Serial examination of peripheral blood mononuclear cells from acute leukemia patients undergoing induction therapy has demonstrated that various agents, including cytarabine, mitoxantrone, etoposide,

paclitaxel, and topotecan, cause a marked increase in the number of apoptotic blasts (Li *et al*., 1994). Characteristic apoptotic changes have also been described in solid tumors after treatment of mice with various cytotoxic drugs, including cytarabine, 5-fluorouracil (5FU), fludarabine, doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycin, and camptothecin (Mesner *et al*., 1997; Li *et al*., 1994). Although the occurrence of apoptosis after treatment of solid tumors is not as well documented in the clinical setting, this might reflect the difficulty in securing sequential biopsies at appropriate times after therapy. Unfortunately, the appropriate timing for such biopsies, even if they were feasible, is not clear, for different agents induce apoptosis with different kinetics even in leukemia cells (Li *et al*., 1994; Kaufmann, 1989). On the other hand, the possibility that other cell death processes also play a major role in the regression of solid tumors after drug treatment (Houghton, 1999) cannot be completely ruled out at the present time. If the assertion that anticancer drugs kill target cells by inducing apoptosis is correct, some potentially important implications immediately follow. Because apoptosis is a regulated process (Earnshaw *et al*., 1999), biochemical alterations that make cells more or less susceptible to apoptosis might affect their sensitivity to a wide range of unrelated antineoplastic agents. In other words, changes that decrease the ability to activate the apoptotic machinery might play a role in resistance to a wide variety of drugs (Kaufmann and Earnshaw, 2000).

Apoptosis, a programmed cell death (Wyllie *et al*., 1980; White, 1996), also plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells, initiated cells or cells progressed to malignancy (Schulte-Hermann *et al*., 2000). Induction of

apoptosis thus is a highly desirable mode as a chemotherapeutic as well as a chemopreventive strategy for cancer control. Indeed, many chemopreventive agents act through the induction of apoptosis as a mechanism to suppress carcinogenesis (Kelloff *et al*., 1996; Taraphdar *et al*., 2001). Apoptosis is known as an important biologic mechanism that contributes to the maintenance of the integrity of multicellular organisms. It is induced by a wide variety of cellular stresses such as DNA damage, UV radiation, ionizing radiation and oxidative stress (Nagata, 1997), and is morphologically distinct from necrosis in many of its characteristic changes as follows; DNA fragmentation, chromatin condensation, cytoplasmic membrane blebbing, and cell shrinkage. Antitumor agents also induce apoptosis in some cancer cells both *in vitro* and *in vivo*, indicating that apoptosis plays a very important role in cancer chemotherapy (Kaufmann, 1989; Meyn *et al*., 1995).

In the present study, the crude extracts of plants grown in Jeju Island were screened for cytotoxic effect and the methanol extracts were prepared and used for the evaluation of growth inhibitory activity. As results, it was found that the extract of the leaves from *E. emarginata* markedly inhibited the growth of human leukemia HL-60 cells (Park and Kang, 2000).

Eurya emarginata (Thunb.) 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). The present study investigated the constituents of the leaves of *E. emarginata,* grown in Jeju Island, Korea. The leaves of *Eurya* are used in the traditional medicine of the coastal areas of Jeju Island for the treatment of

ulcers or diuretic. Nevertheless, there are few reports on the biological activity and constituents of *E. emarginata*. With these facts as backgrounds, the present study investigated the anticancer effects and action mechanism of the extract from the leaves of *E. emarginata* to identify constituents of its leaves with anti-cancer activity. And, the components which displayed anti-cancer activity were isolated from the leaves of *E. emarginata.*

MATERIALS AND METHODS

1. Purification of biologically active components from *E. emarginata*

1-1. Preparation of extracts from leaves of *E. emarginata*

The leaves from *Eurya emarginata* (Thunb.) Makino were collected in May, 2000 at Cheju National University, Jeju Island, Korea. The leaves were cleaned, dried at room temperature for 2 weeks and ground into a fine powder. The dried leaves were extracted with the 80% methanol (MeOH) after standing a week at room temperature and then concentrated under a vacuum. The resulting MeOH extract was suspended in water (1L), and successively partitioned with hexane (1L × 3), ethylacetate (EtOAc; 1L × 3), and n-butanol (BuOH; 1L × 3), to give hexane, EtOAc, *n*-BuOH and H_2O layer, respectively. Each partition layer was evaporated, and freeze-dried (Scheme 1).

1-2. Characterization procedures

^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JNM-LA 400 (FT NMR system, Nuclear Magnetic Resonance, Japan) spectrometer. TLC (Thin layer chromatography) was carried out on Merck precoated silica gel 60 F_{254} plates and normal-phase column chromatography was carried out on Kieselgel 60 (230-400 mesh ASTM, Merck, Germany) silica gels. Reverse-phase column chromatography was carried out with Silica gel 100 C18-Reversed phase (3×15, Silica gel 100 C18-Reversed phase, Merck, Germany). C18 HPLC (μBondapak C18, 7.8×300㎜ column, Merck; Waters

2487, Waters) was used for HPLC analysis.

2. Analysis of cytotoxic- or apoptotic events

2-1. Cell cultures

The anticancer assay was performed using sixteen different human cancer and two different normal cell lines, which were obtained from the KCLB (Korean Cell Line Bank). The cells were maintained at 37℃ in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium or DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/㎖ and 100 ㎍/㎖, respectively; GIBCO Inc, NY, USA). The exponentially growing cells were used throughout the experiments (Table 1 and Table 2).

2-2. Cytotoxicity tests

The effect of the solvents extracts on the growth of various cancer cells was determined by measuring the metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Carmichael *et al*., 1987). The MTT assays were performed as follows: The cancer cells ($1.0 \sim 3.0 \times 10^5$cells/㎖) were treated for 4 days with 100 ㎍/㎖ of extracts in 96-microwell plates. After incubation, 0.1 ㎎ (50 ㎕ of a 2 ㎎/㎖ solution) of MTT (Sigma, MO, USA) was added to each well and cells were then incubated at 37℃ for 4 h. The plates were centrifuged at 1500 rpm for 20 min at room temperature and the media was then carefully aspirated. 150 ㎕ of dimethylsulfoxide was then added to each

Table 1. Human cancer cell lines used in this study

Name	Status	Origin
HL-60	leukemia, acute promyelocytic	blood
KG-1	leukemia, acute myelogenous	blood
U-937	histiocytic lymphoma	monocyte
K562	leukemia, chronicacute myelogenous	blood
Jurkat	acute T cell leukemia	T lymphocyte
MCF-7	adenocarcinoma; pleural effusion	breast, mammary gland
HCT-15	adenocarcinoma	colon
A431	carcinoma	skin
A498	carcinoma	kidney
A549	carcinoma	lung
Hep-G2	hepatoblastoma	liver
MIA PaCa-2	carcinoma	pancreas
SNU-16	adenocarcinoma	stomach; ascites
SNU-C5	adenocarcinoma	colon; colorectal
SK-N-SH	neuroblastoma	brain
SK-OV-3	adenocarcinoma, malignant ascites	ovary

Table 2. Normal human cells used in this study

Name	Status	Origin
CCD-25Lu	normal, fibroblast	lung
HEL-299	normal, fibroblast	lung

well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech, UK, USA).

The results are represented as the percentage inhibition that produced reduction of absorbance of each group compared to the untreated controls from triplicated experiments.

2-3. Cell surface antigen analysis

The HL-60 cells (3.0×10^5cells/㎖) were incubated with the extracts from *E. emarginata* (100 ㎍/㎖) for 6 days, the cells were harvested and examined for induction of differentiation by analysis of cell surface antigen using fluorescence-activated cell sorter (FACS). For analysis of cell surface antigens, cells were stained by direct immunofluorescent staining using fluoerescein-isothiocyanate (FITC) conjugated mouse antihuman CD 66b or CD 14, and R-phycoerythrin (PE) conjugated mouse antihuman CD 33. Control studies were performed with a non-binding control mouse Ig G1 isotype antibodies. Flow cytometric ananlysis was performed with EPICS-XL FACScan flow cytometer (Coulter, Miami, FL, USA).

2-4. DNA fragmentation analysis

The HL-60 cells (3.0×10^5cells/㎖) were treated with 100 ㎍/㎖ of extracts for 24 h. For the DNA fragmentation assay, the cells were collected by centrifugation and DNA was extracted with Wizard Genomic DNA purification kit (Promega, WI, USA). The DNA fragmentation pattern was analyzed by electrophoresis on a 1.5% agarose gel containing 0.1 ㎍/㎖ ethidium bromide for 2 h at 50 V.

2-5. Microscopic observation

In order to examine the morphological changes, HL-60 cells (3.0×10^5cells/㎖) were treated with 100 ㎍/㎖ of extracts for 24 h and then harvested. The cells were fixed in 4% paraformaldehyde and incubated in 1 ㎖ of a staining solution containing 2.5 ㎍/㎖ of 4.6-diamidino-2-phenyl-indole (DAPI; Sigma, MO, USA) in the dark place for 30 min. After washing with PBS, 10 ㎕ of stained cells were mounted on a slide glass. The nuclei were observed under a fluorescence microscope (BX-50, Olympus, Japan).

2-6. Cell cycle phase distribution analysis

For the flow cytometric analysis to determine cell cycle phase distribution, 3.0×10^5 cells per sample were incubated with components from *E. emarginata* at the indicated times. After cells were washed twice with phosphate-buffered saline (PBS) and fixed in 70% ethanol for 30 min at 4℃. The cells were then rinsed with PBS and incubated in 50 ㎍/㎖ of a propidium iodide (PI; Sigma, MO, USA) solution and 50 ㎍/㎖ of RNase A in the dark for 30 min at 37℃. Flow cytometry analysis was performed using an EPICS-XL FACScan flow cytometer (Coulter, Miami, FL, USA).

2-7. RNA extraction

The total RNA was extracted from cells with the Tri-Reagent (MRC, OH, USA) method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. The RNA was quantified by reading the absorbance at 260 nm according to the methods

described by Sambrook *et al*. (1989).

2-8. RT-PCR analysis

The reverse transcription of 1 ㎍ RNA was carried out using M-MuLV reverse transcriptase (Promega, WI, USA), oligo (dT) 18 primer, dNTP (0.5 ㎛M) 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 10 min. The polymerase chain reaction (PCR) was performed in a reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 50 ㎛M 3' and 5' each primer 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 amplication was followed by 35 cycles of 94℃ for 45 sec (denaturing), 55-65℃ for 45 sec (annealing) and 72℃ 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 3.

2-9. Subcellular fractionation

The procedure for the preparation of Mitochondria (MT) and Cytosolic fractions was carried out as described before with minor modfications (Hai *et al*., 2000). Briefly, the cells were washed twice with cold-PBS and lysed in buffer A [20 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 ㎛g/㎖ leupeptin, 10 ㎛g/㎖ aprotinin, 10

mM benzamidine, and 0.2 mM sodium orthovanadate] for 30 min on ice at a concentration of 1.0×10^7cells/㎖. The cell lysates were subjected to homogenization using an ultraschallprozessor (UP-50H; Gmbh, Germany). The cell homogenates were centrifuged twice at 600×g for 5 min at 4°C. The cell pellets were discarded, and the supernatant was again centrifuged at 17,000×g for 30 min at 4°C. The obtained supernatant was further centrifuged at 70,000×g for 1 h at 4°C. Finally, the cytosol fraction was obtained from the supernatant. The pellet from the 17,000×g centrifugation was resuspended in buffer A and recentrifuged at 17,000×g for 30 min at 4°C. The resultant supernatant was discarded, and the pellet containing the mitochondria (MT) fraction was lysed for the preparation of protein extracts by suspending the MT pellets in buffer B [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 ㎍/㎖ aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate] for 30 min on ice. The lysate was centrifuged at 4°C for 30 min at 17,000×g, and the supernatant was labeled as the MT protein extract. Protein concentrations in the cytosol fractions and MT fractions were determined using Bradford method (Bradford, 1976), and aliquots were stored at -70°C.

2-10. Western blot analysis

After treatment, the cells were collected and washed twice with cold-phosphate-buffered saline (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 ㎍/㎖ leupepti n] and kept on ice for 30 min. The cell lysates were centrifuged at 12,000×g at 4℃ for 20 min and the supernatants were then stored at -70 ℃ until used. The protein concentration was measured by the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 ㎍ of protein) were separated on a 8-12% 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 5% nonfat dried milk, the membrane was then incubated with human specific primary anti-body at 4℃ overnight. The membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Santa-Cruz, CA, USA). The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersharm-pharmacia Biotech., NY, USA). The antibodies used in Western blot analysis were shown in Table 4.

Table 3. The sequences of primers used in RT-PCR analysis and the sizes of RT-PCR products

Gene (type)		Primer sequences	Fragment size(bp)
Bcl-2	F	5'-TGCACCTGACGCCCTTCAC-3	292
	R	5'-AGACAGCCAGGAGAAATCAAACAG-3	
Bax	F	5'-ACCAAGAAGCTGAGCGAGTGT-3	364
	R	5'-ACAAAGATGGTCACGGTCTGCC-3	
c-Myc	F	5'-TCTGGATCACCTTCTGCTGG-3'	397
	R	5'-GCTCCTCTGCTTGGACGGAC-3	
Cyclin D1	F	5'-ACCTGGATGCTGGAGGTCTG-3'	402
	R	5'-GAACTTCACATCTGTGGCACA-3'	
Cyclin E	F	5'-GGAAGGCAAACGTGACCGTT-3'	638
	R	5'-GGGACTTAAACGCCACTTAA-3'	
CDK2	F	5'-CATGGAGAACTTCCAAAAG-3'	901
	R	5'-CTATCAGAGTCGAAGATGGGG-3'	
CDK4	F	5'-ATGGCTGCCACTCGATATGA-3'	912
	R	5'-CTCTGGGTTGCCTTCGTCCT-3'	
$p21^{WAF1/CIP1}$	F	5'-ATGTCAGAACCGGCTGGGGA-3'	496
	R	5'-TTAGGGCTTCCTCTTGGAGA-3'	
$p27^{KIP1}$	F	5'-ATGTCAAACGTGCGAGTGTCTAACG-3'	597
	R	5'-TTACGTTTGACGTCTTCTGAGGCCA-3'	
β-Actin	F	5'-ATGGGTCAGAAGGATTCCTAT-3'	588
	R	5'-CAGCTCGTAGCTCTTCTCC-3'	

Table 4. Antibodies used in Western blot analysis

Antibody	Origin	Company
Bcl-2	mouse monoclonal	Santa-Cruz Biotechenology
Bax	rabbit polyclonal	Santa-Cruz Biotechenology
c-Myc	mouse monoclonal	Santa-Cruz Biotechenology
PARP	rabbit polyclonal	Santa-Cruz Biotechenology
Caspase-9	rabbit polyclonal	Cell Signaling
Caspase-3	rabbit polyclonal	Cell Signaling
Caspase-8	mouse monoclonal	Cell Signaling
Cytochrome c	goat polyclonal	Santa-Cruz Biotechenology
pRB	goat polyclonal	Santa-Cruz Biotechenology
$p21^{WAF1/CIP1}$	rabbit polyclonal	Santa-Cruz Biotechenology
β-Actin	mouse monoclonal	Sigma

PARP, poly-(ADP-ribose) polymerase; pRB, retinoblastoma protein; Caspase-3, cystein protease-3 (also designated CPP32); Caspase-9, cystein protease-9 (also designated ICE-LAP6); Caspase-8, cystein protease-8 (also designated pro-MACH α 1, FLICE or Mch5).

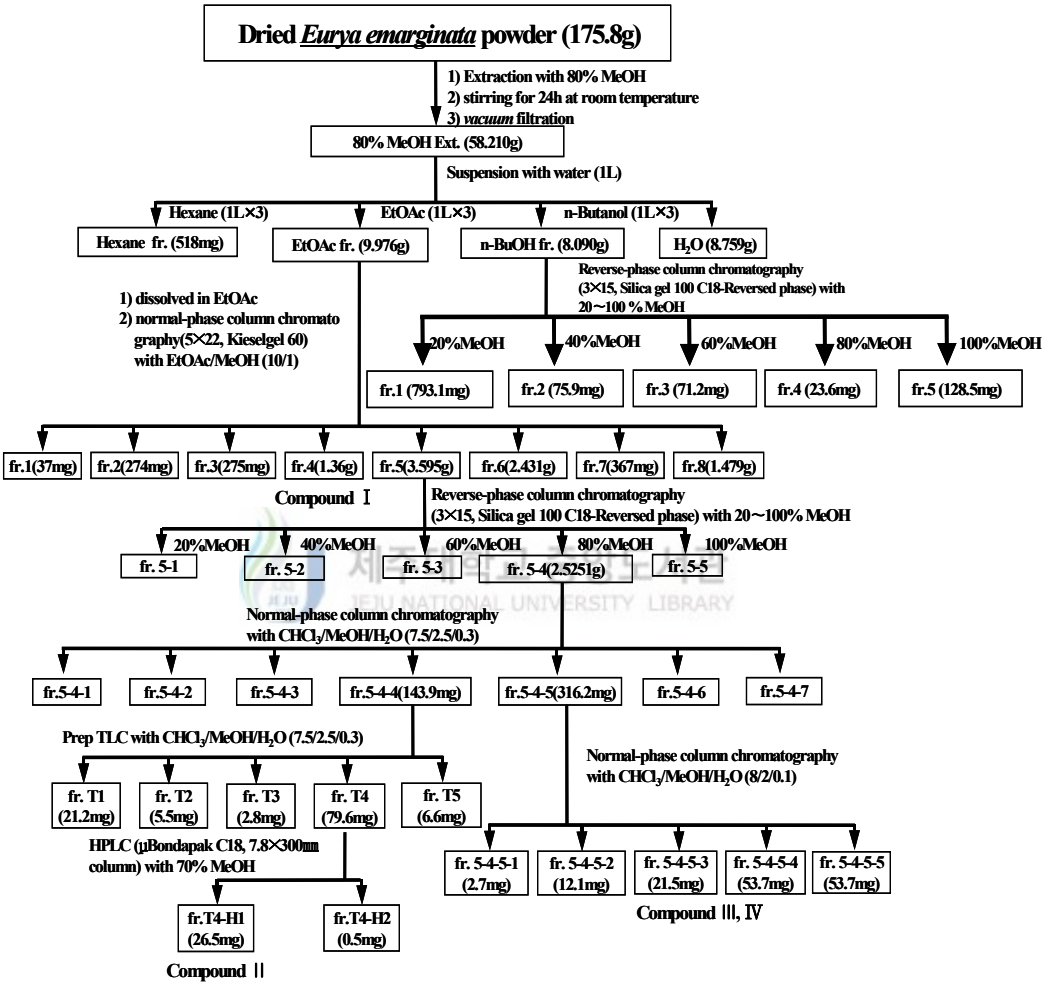
RESULTS

1. Purification of biologically active components from *E. emarginata*

1-1. Extraction and Fractionation of *E. emarginata* leaves

The leaves of *Eurya emarginata* was collected in the campus of Cheju National University, and air-dried in the dark place. The dried leaves (175.8 g) were extracted using the 80% aqueous methanol (1L × 3) after standing a week at room temperature. The liquid layer was by filtrated, and the solvent was evaporated under reduced pressure to give the crude extract (58.21 g). After water (1L) was added to the extract, the aqueous layer was successively partitioned into hexane, ethyl acetate and *n*-butanol (Scheme 1). The EtOAc (9.976 g) fraction was chromatographed over a normal-phase column and eluted with EtOAc/MeOH (10/1) to obatin 8 fractions (fr.1～fr.8). A portion of fraction 4 (1.36 g) was purified by a reverse-phase column chromatography with 20～100% MeOH and normal-phase column chromatography with $CHCl_3$/MeOH/H_2O (7.5/2.5/0.3) to obtain compound Ⅰ (Quercitrin, Figure 1).

A portion of fraction 5 (3.595 g) was purified by a reverse-phase column chromatography with 20～100% MeOH to obtain 5 subfractions (fr.5-1～fr.5-5). A portion of subfraction 5-4 (2.525 g) was purified by normal-phase column chromatography with $CHCl_3$/MeOH/H_2O (7.5/2.5/0.3) to obtain 7 subfractions (fr.5-4-1～fr.5-4-7). A portion of subfraction 5-4-4 (143.9 mg) was purified by Prep TLC with $CHCl_3$/MeOH/H_2O (7.5/2.5/0.3) to obtain 5 Prep TLC subfractions (fr. T1～fr. T5). Prep TLC 5 subfractions fr. T4 (79.6 mg) was purified by HPLC with the 70% MeOH to obtain 2 subfractions (fr. T4-H1 and



Scheme 1. Systematic purification using the solvent partitioning and silica gel chromatography from *Eurya emarginata.*

fr. T4-H2). Further, a subfraction T4-H1 was purified to obtain compound Ⅱ (Cornoside, Figure 2). A subfraction 5-4-5 (316.2 mg) was purified by a normal-phase column chromatography with $CHCl_3$/MeOH/H_2O (8/2/0.1) to obtain 5 subfractions (fr.5-4-5-1～fr.5-4-5-5). A subfraction 5-4-5-3 (21.5 mg) was further purified to compound Ⅲ and Ⅳ (Eutigoside B and C, Figure 3).

1-2. Isolation of the eutigoside B and C

The leaves of *Eurya emarginata* was collected in the campus of Cheju National University, and air-dried in the dark place. The dried leaves (150 g) were extracted using 80% aqueous methanol (1L × 3) after standing a week at room temperature. The liquid layer was filtrated, and the solvent was evaporated under reduced pressure to give the crude extract (48.8 g). After water (1L) was added to the extract, the aqueous layer was successively partitioned into hexane, ethyl acetate 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 (fr.1-5). The polar fraction 1 (603 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 C18 HPLC to give eutigoside C (5.3 mg). The fraction 30 (30 mg) was also purified by C18 HPLC to give the eutigoside B (3.8 mg). The polar fraction 32 (300 mg) was purified by SiO_2 CC to give quecitrin (45 mg) as the major component (Scheme 2).

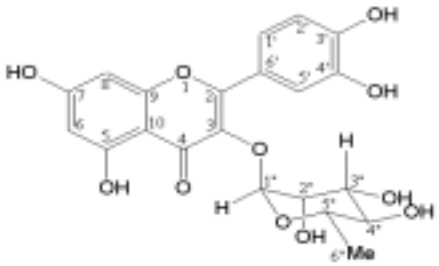


Figure 1. The structure of isolated compound Ⅰ(Quercitrin) from EtOAc subfraction 4.

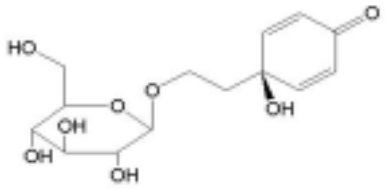
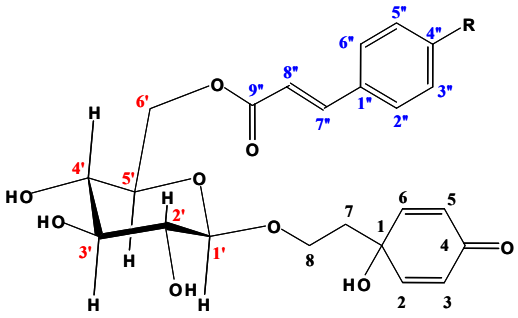


Figure 2. The structure of isolated compound Ⅱ (Cornoside) from EtOAc subfraction 5-4-5-T4-H1.



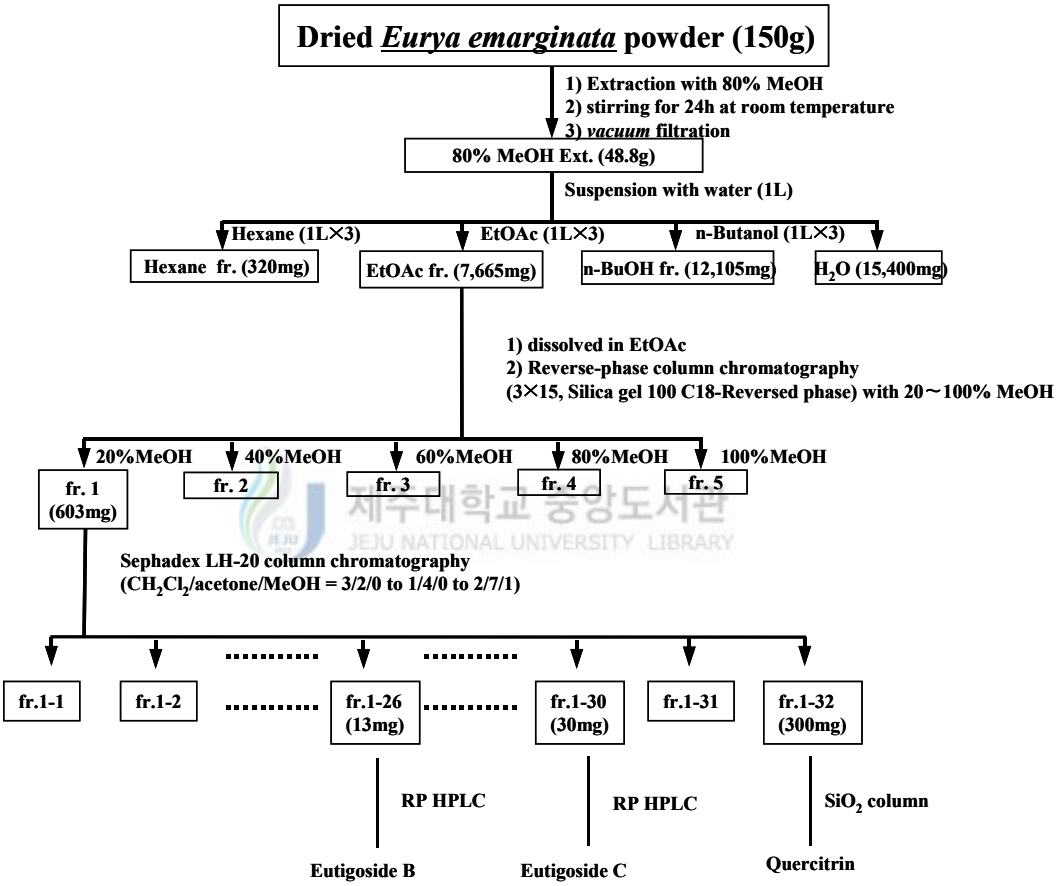
R = H Eutigoside C

R = OH Eutigoside B

Figure 3. The structure of isolated compound Ⅲ and Ⅳ (Eutigosides) from EtOAc subfraction 5-4-5-3.

Eutigoside B (Figure 4) - ^{1}H NMR (400 MHz, methanol-d4) d 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 (methanol-d4) d 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) ; Consistent with $C_{23}H_{26}O_{10}$.

Eutigoside C (Figure 5) - ^{1}H NMR (400 MHz, methanol-d4) d 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 (methanol-d4) d 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) ; Consistent with $C_{23}H_{26}O_{9}$.



Scheme 2. Isolation procedure of eutigoside B and eutigoside C from *Eurya emarginata*

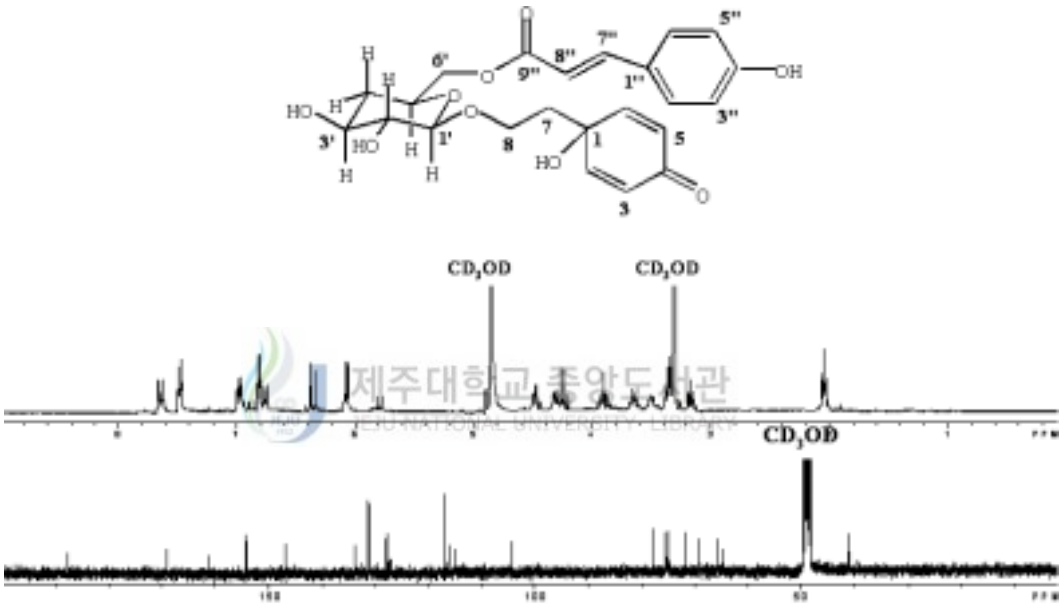


Figure 4. The ^{1}H-NMR and ^{13}C-NMR spectrum of eutigoside B from *Eurya emarginata.*

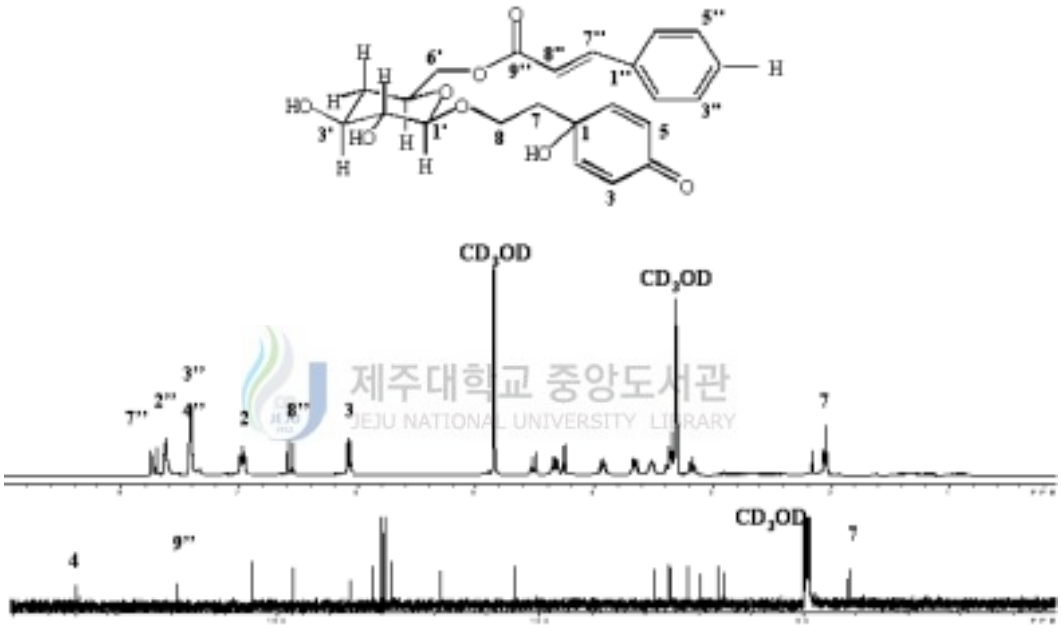


Figure 5. The ^{1}H-NMR and ^{13}C-NMR spectrum of eutigoside C from *Eurya emarginata.*

2. Analysis of cytotoxic- or apoptotic events

2-1. Effects of the extracts of *E. emarginata* on the growth of tumor cells

The effects of extracts from *E. emarginata* on the growth of tumor cells were assessed using a MTT assay. Briefly, tumor cells were cultured for 4 days in a medium containing 100 ㎍/㎖ of the MeOH extract, the hexane fraction, the EtOAc fraction, the BuOH fraction or the H_2O fraction. The MTT result showed (Table 5) that the EtOAc fraction markedly inhibited the growth of leukemia cells including HL-60 (acute promyelocytic leukemia cells), KG-1 (acute myelogenous leukemia cells), U937 (histiocytic lymphoma cells), K562 (chronic myelogenous leukemia cells) and Jurkat (acute T cell leukemia cells) by 70, 63, 72, 45 and 66%, respectively. However, the extract or solvent fractions only marginally inhibited the growth of A549 (lung carcinoma cells), SNU-C5 (colon cancer cells), HCT-15 (colon cancer cells), SNU-16 (stomach cancer cells), MCF-7 (breast cancer cells), SK-OV-3(ovary cancer cells), A498 (kidney cancer cells), MIA PaCa-2 (pancreas cancer cells), Hep-G2 (hepatoblastoma cells), SK-N-SH (neuroblastonema cells), and A431 (epidermoid carcinoma cells). In the following experiments, HL-60 cells were used to investigate the action mechanism of the components of *E. emarginata*, because the EtOAc fraction of *E. emarginata* was found to be the most cytotoxic against the HL-60 cells among the tested cell lines.

Table 5. Inhibitory effects of the several extracts from *Eurya emarginata* on the growth of tumor cells

Cell line	Inhibition (%)				
	80% MeOH	Hexane	EtOAc	n-BuOH	H_2O
HL-60	53.46±1.645*	31.33±3.271*	70.11±3.683*	34.65±2.342*	11.78±2.251*
KG-1	62.98±2.054*	40.48±3.196*	63.07±2.019*	64.78±3.007*	19.74±4.481*
U937	23.45±3.801*	8.11±2.358*	72.25±3.188*	69.89±2.299*	37.40±4.957*
K562	28.52±3.145*	17.96±4.614*	45.18±2.851*	16.96±3.828*	19.66±3.568*
JurKat	65.87±4.102*	17.74±3.986*	66.34±2.814*	58.83±4.573*	45.16±4.259*
MCF-7	2.49±1.135*	33.86±4.471*	12.34±3.483*	10.45±3.193*	17.28±4.636*
HCT-15	25.14±2.045*	40.69±3.639*	- 18.37±6.560	12.20±2.384*	39.10±3.588*
SK-OV-3	8.07±3.789*	16.50±4.993*	- 8.76±4.245*	- 6.81±1.529*	- 2.71±1.601*
A498	3.47±1.234*	- 0.71±2.488*	4.29±2.156*	20.68±3.204*	- 8.35±2.845*
SNU-16	4.07±4.148*	28.70±3.203*	23.62±5.370	23.62±5.244	10.85±4.627*
SNU-C5	19.12±2.546*	8.75±5.235	31.76±3.888*	49.46±3.705*	5.50±6.074
MIA PaCa-2	1.92±1.255*	16.76±2.064*	9.33±2.254*	7.85±1.833*	0.51±4.602*
Hep-G2	3.96±1.947*	2.98±3.198*	6.83±4.403*	4.13±4.415*	5.85±7.748
A431	0.03±1.526*	10.31±8.188	1.47±3.539*	3.98±4.430*	4.33±4.837*
SKN-SH	30.39±4.762*	65.77±1.457*	28.36±3.077*	5.45±2.718*	7.20±6.372
A549	3.94±4.180*	6.97±4.842*	7.35±4.536*	7.65±2.870*	15.46±4.305*

The cancer cells ($1.0 \sim 3.0 \times 10^5$cells/㎖) were treated for 4 days with 100 ㎍/㎖ of the extracts in 96-microwell plates. After incubation, 3-(4,5-dimehtylthiazol)-2,5-diphenyltetrazolium bromide (MTT) was added and incubation was continued for further 4 hours. The formazan salt formed was dissolved in dimethylsulfoxide, and quantified using a microplate reader at 540 nm. The mean absorbance value for each extract was calculated. Results are expressed as the percentage inhibition that produced a reduction in the absorbance in extract-treated cells compared to the untreated controls. All the experiments were performed in triplicate. The data is presented as a mean ±SD. *$P< 0.05$ compared to the control.

2-2. Cell surface antigen analysis

The present study examined the differentiation induction of HL-60 cells by observing change of the expression of cell surface antigen. When HL-60 cells were treated with 100 ㎍/㎖ of the crude extraction, the ability of HL-60 cells to express CD 66b or CD 14 antigens was unchanged when compared with the control (Figure 6). The result indicates that the HL-60 cells are not differentiated to mature granulocytes or monocytes by *E. emarginata*.

2-3. Effects of the EtOAc subfraction of *E. emarginata* on the growth of HL-60 cells

The effects of components from *E. emarginata* on the growth of HL-60 cells were assessed using the MTT assay. Briefly, the HL-60 cells were cultured for 4 days in a medium containing 100 ㎍/㎖ of the components from *E. emarginata*. As shown in Figure 7, the eutigoside C of its leaves markedly inhibited the growth of HL-60 cells.

2-4. Induction of apoptosis by *E. emarginata* in HL-60 cells

The degree of the chromosomal DNA fragmentation was determined to assess the onset of apoptosis of HL-60 cells by the extracts of *E. emarginata.* When the HL-60 cells was treated with 100 ㎍/㎖ of the MeOH extract or EtOAc fraction, and 100 ㎍/㎖ of the eutigoside C for 24 h, internucleosomal cleavage of genomic DNA resulting in DNA ladder was observed (Figure 8). When the HL-60 cells were stained with the DNA-specific fluorochrome, propidium iodide, the EtOAc fraction or eutigoside C was found to increase the proportion of sub-G1 hypodiploid cells (Figure 9 and Figure 10).

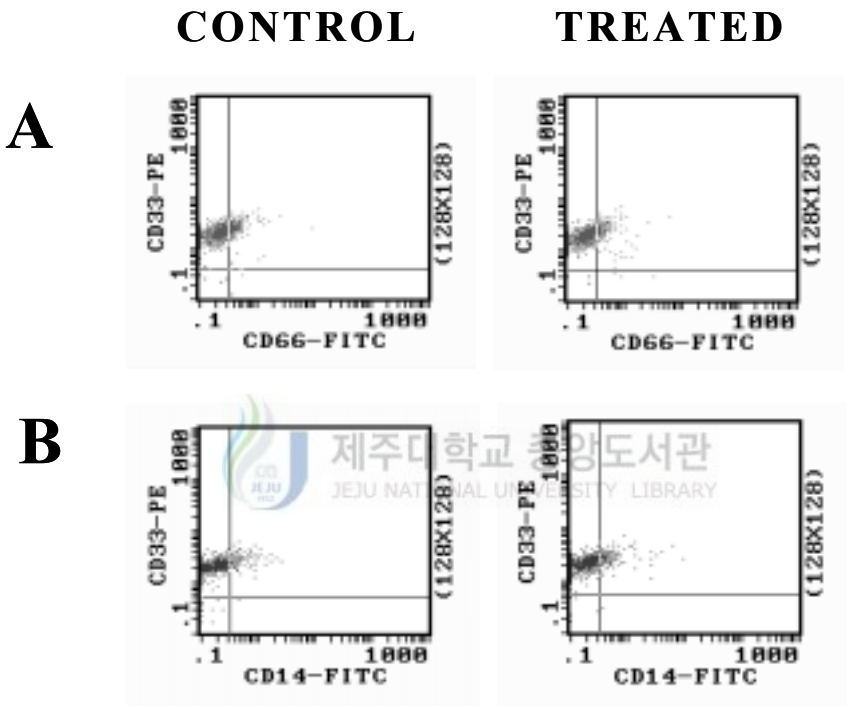


Figure 6. Cell surface antigen analysis in HL-60 cells.

The cells were treated for 6 days with the 80% MeOH extract of *Eurya marginata* (100 ㎍/㎖) and expressions of CD 66b (A) and CD 14 (B) antigens were analyzed by FACS.

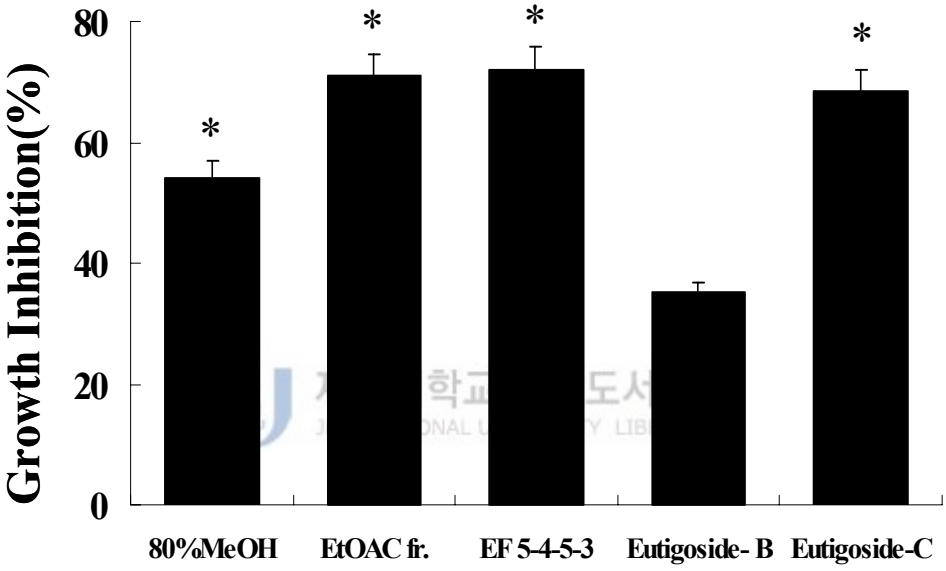


Figure 7. Inhibitory effects of *Eurya emarginata* on the growth of HL-60 cells. The HL-60 cells were treated for 4 days with 100 ㎍/㎖ of components of *E. emarginata*.

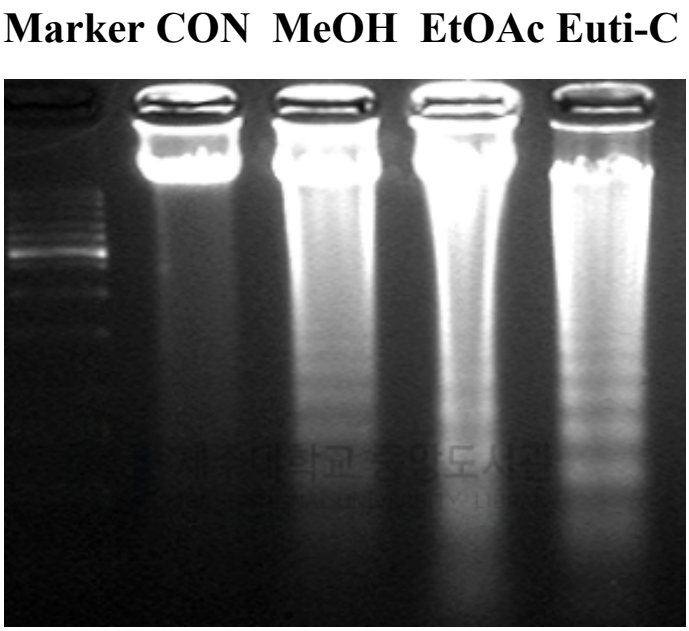


Figure 8. DNA fragmentation by the components of *Eurya emarginata* in HL-60 cells. The DNA was isolated and subjected to 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining. The HL-60 cells (3.0×10^6cells/㎖) were treated with several extracts (100㎍/㎖) for 24 hours. Lane M: DNA marker, Lane 1: Control, Lane 2: 80% MeOH extract, Lane 3: EtOAc fraction, Lane 4: Eutigoside C.

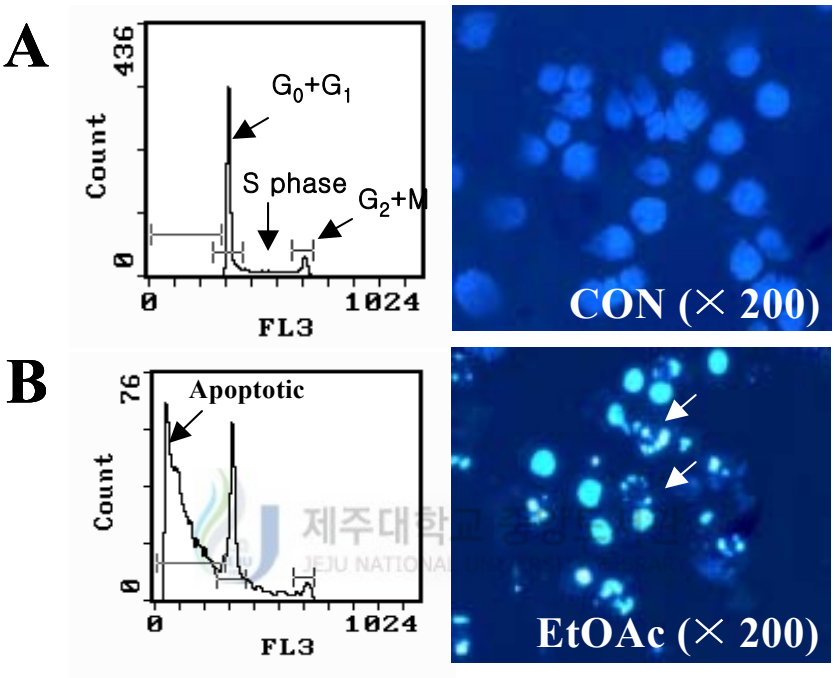


Figure 9. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis (left panel) and the fluorescence micrographs (right panel) of cells with highly condensed nuclei stained with 4.6-diamidino-2-phenyl-indole (DAPI). The HL-60 cells (3.0×10^5cells/㎖) were treated without (A) or with EtOAc fraction (100 ㎍/㎖) of *Eurya emarginata* (B) for 24 hours.

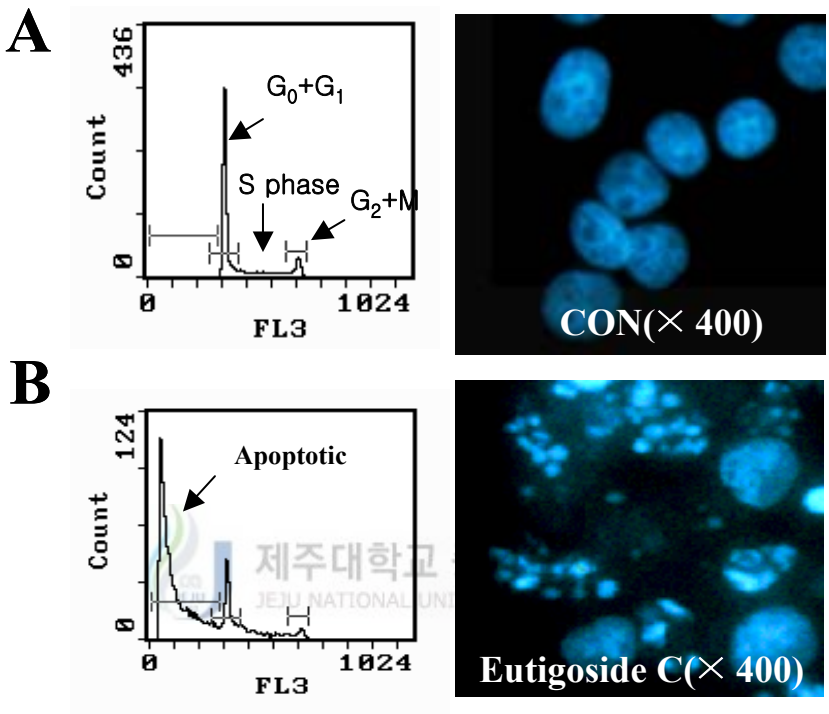


Figure 10. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis (left panel) and the fluorescence micrographs (right panel) of cells with highly condensed nuclei stained with 4.6-diamidino-2-phenyl-indole (DAPI). The cells (3.0×10^5cells/㎖) were treated without (A) or eutigoside C (100 ㎍/㎖) of *Eurya emarginata* (B) for 24 hours.

These results showed that the EtOAc fraction or eutigoside C induces the apoptosis of HL-60 cells, and were supported by the increase in the number morphologic changes (Figure 9 and Figure 10) (cell shrinking, the condensation of chromatin, and the membranous apoptotic bodies), which are characteristic of apoptosis.

2-5. Expressions of c-Myc by the EtOAc fraction in HL-60 cells

This study examined the ability of *E. emarginata* to affect the expression of c-Myc using RT-PCR analysis and Western blot analysis. The treatment of the HL-60 cells with 100 ㎍/㎖ of the EtOAc fraction resulted in the decrease of c-Myc mRNA expression as well as c-Myc protein in a time-dependent manner (Figure 11-A and Figure 11-B).

2-6. Expressions of Bcl-2 and Bax by the eutigoside C in HL-60 cells

In order to understand the induction mechanism of apoptosis by the EtOAc fraction of *E. emarginata*, the present study examined expression levels of Bcl-2 family using RT-PCR analysis and Western blot analysis. The eutigoside C showing the cytotoxic effect was isolated from the leaves of *E. emarginata*. Bcl-2, an anti-apoptotic protein, forms homo- or hetero-dimer with the pro-apoptotic protein (i.e., Bax) of Bcl-2 family within the cell and their ratio eventually determines whether the cell takes into the apoptotic pathway. Bcl-2 is known to suppress the apoptosis by controlling the membrane potential of mitochondria and/or preventing calcium depletion of endoplasmic reticulum (Distelhorst *et al*., 1996; Shimizu *et al*., 1996). As shown in Figure 12-A and Figure 12-B, the eutigoside C induced the decrease of Bcl-2 level in a

time-dependent manner. Interestingly, however, the Bax level was increased in a time-dependent manner in HL-60 cells with eutigoside C.

2-7. Release of mitochondrial cytochrome c into cytosol in HL-60 cells

This study examined the release of cytochrome c from mitochondria into the cytosol in HL-60 cells using Western blot analysis. It has been reported that the release of cytochrome c from mitochondria to cytosol is a key step to the activation of caspase 9 (Li *et al*., 1997; Srinivasula *et al*., 1998).

As shown in Figure 13, the treatment with eutigoside C at 100 ㎍/㎖ induced the release of cytochrome c from mitochondria into the cytosol fraction on HL-60 cells in a time-dependent manner.

2-8. Degradation of PARP, an endogenous substrate of caspase-3 in HL-60 cells

From the results showing apoptotic-events by eutigoside C (100 ㎍/㎖), it was determined whether caspases were stimulated. Time-dependent processing of caspase-3 or 9 was shown Figure 14. In untreated HL-60 cells, caspase-3 was present primarily as its intact 32 kDa proform. Induction of apoptosis by the eutigoside C resulted in the appearance of two fragments of 19 kDa and 17 kDa. Also, caspase-9 of 46 kDa was also cleaved into fragments of 37 kDa and 35 kDa in a time-dependent manner. However, caspase-8 was not detected after 100 ㎍/㎖ with the eutigoside C (not shown).

The DNA repair enzyme PARP is a substrate for caspase-3, which hydrolysis the full-length form of 116 kDa PARP to an apoptotic 85 kDa fragment. To further confirm the apoptotic cell death, the protein level of PARP was

analyzed. As shown in Figure 15, PARP cleavage was detected after 6 h treatment with eutigoside C (100 ㎍/㎖), and increased in a time-dependent manner. As shown in Figure 16, PARP cleavage was markedly increased at concentration higher than 75 ㎍/㎖ of eutigoside C, paralleled with an increase of caspase-3 activity.

These results suggest the involvement of caspase-3 in the onset of apoptosis by the eutigoside C.

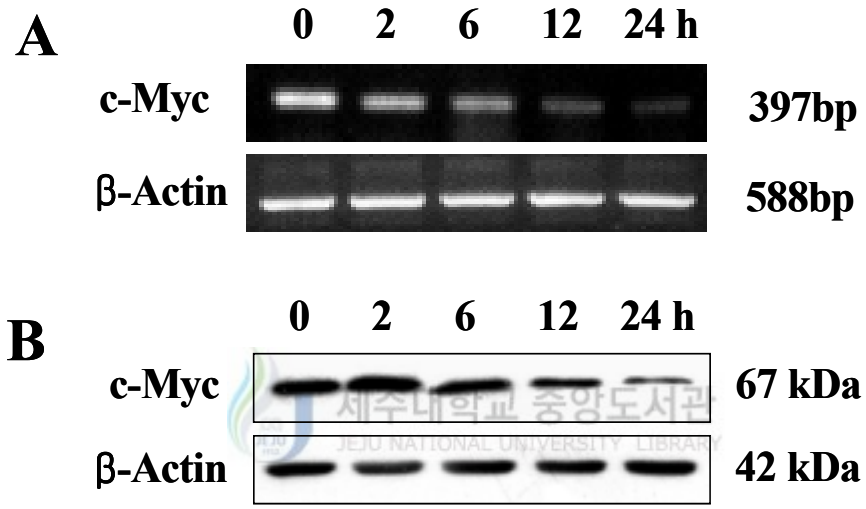


Figure 11. Expression of c-Myc and β-actin in HL-60 cells by a treatment with the EtOAc fraction. The cells (3.0×10^5cells/㎖) were treated with EtOAc fraction (100 ㎍/㎖) of *Eurya emarginata.* (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of c-Myc performed after synthesizing the cDNA as described in the "materials and methods". (B) Western blot analysis of c-Myc using its antibody (67 kDa).

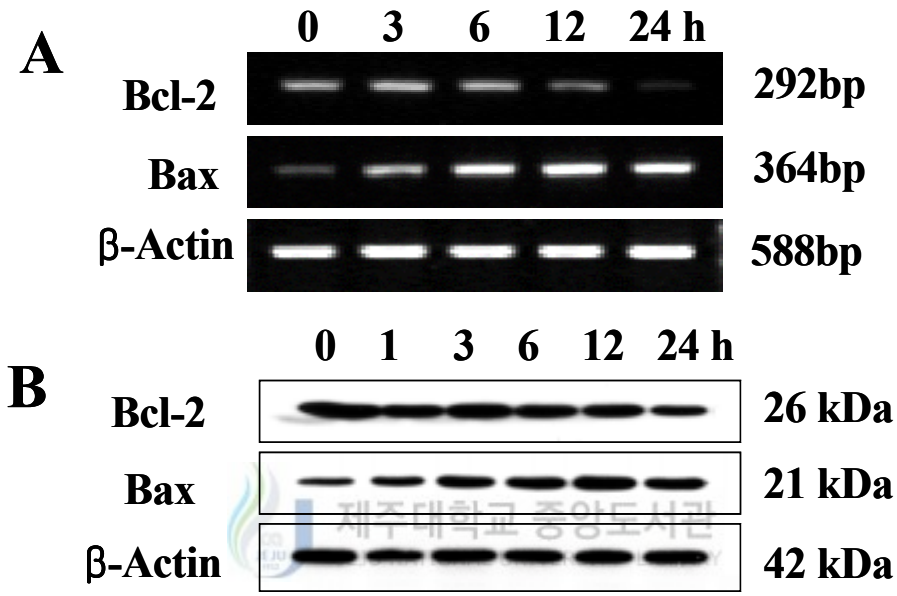


Figure 12. Expression of Bcl-2 and Bax in HL-60 cells by a treatment with the eutigoside C. The cells (3.0×10^5cells/㎖) were treated with eutigoside C (100 ㎍/㎖) of *Eurya emarginata.* (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of Bcl-2 and Bax were performed after synthesizing the cDNA as described in the "materials and methods". (B) Western blot analysis of Bcl-2 or Bax using the antibodies against Bcl-2 recognized a protein at 26 kDa and a 21 kDa protein for Bax.

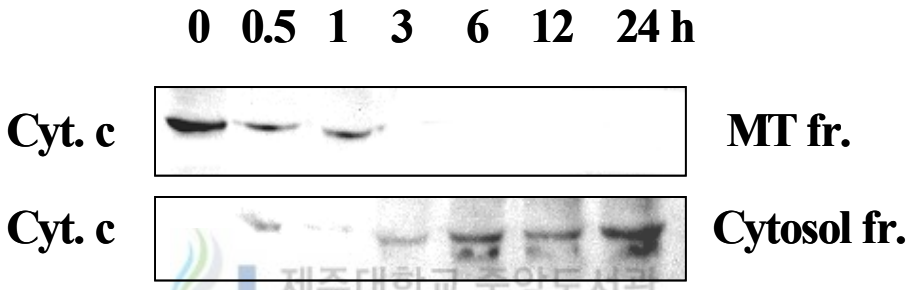


Figure 13. Expression of cytochrome-c in the HL-60 cells by a treatment with eutigoside C. The cells (3.0×10^5cells/㎖) were treated with the eutigoside C (100 ㎍/㎖) of *Eurya emarginata*. Western blot analysis of cytochrome-c using its antibody (11 kDa).

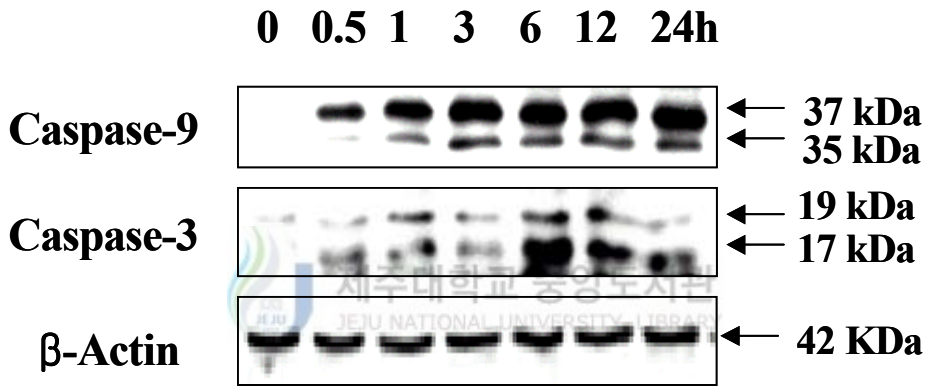


Figure 14. Immunoblot analysis of caspase-9 and caspase-3 in the HL-60 cells by a treatment with eutigoside C. The cells (3.0×10^5cells/㎖) were treated with the eutigoside C (100 ㎍/㎖) of *Eurya emarginata*.

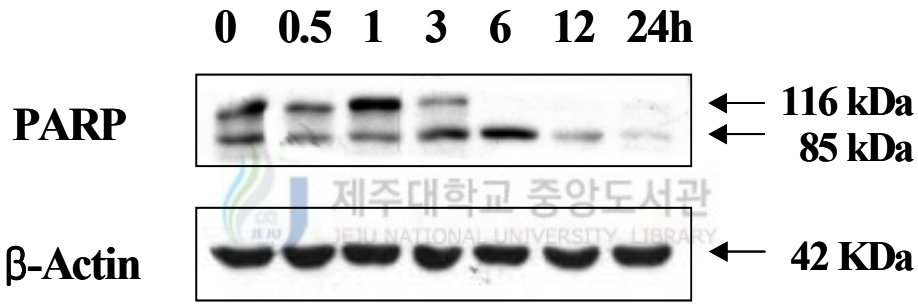


Figure 15. Immunoblot analysis of PARP in the HL-60 cells by a treatment with eutigoside C. The cells (3.0×10^5cells/㎖) were treated with the eutigoside C (100 ㎍/㎖) of *Eurya emarginata*.

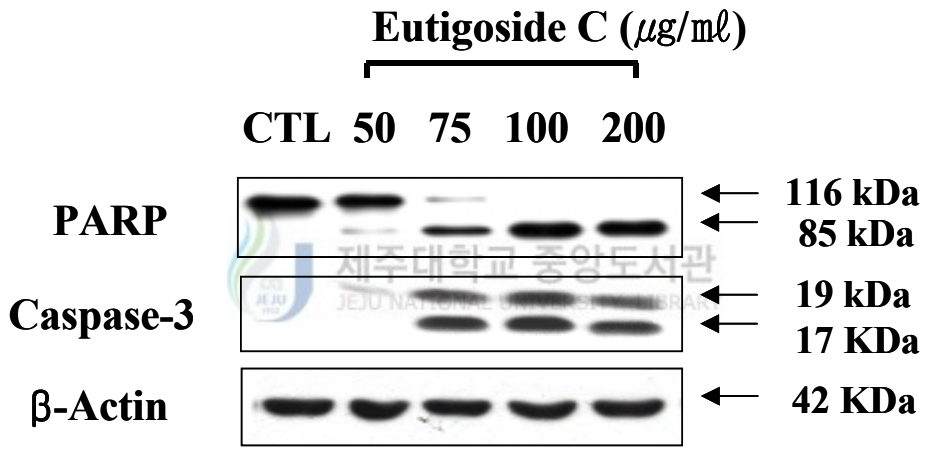


Figure 16. Immunoblot analysis of PARP and caspase-3 in the HL-60 cells by a treatment with different concentrations of eutigoside C.

3. Cell cycle regulation by the eutigoside C in HL-60 cells

3-1. Inhibition of cell growth by eutigoside C in HL-60 cells

The effects of eutigoside C from *E. emarginata* on the growth of HL-60 cells were assessed using the MTT assay. This result that markedly inhibited the growth of HL-60 cells in a dose-dependent manner (Figure 17). The IC_{50} values were found to be 70.66 ㎍/㎖ in acute promyelocytic leukemia HL-60 cells.

3-2. Cell growth arrest induced by the eutigoside C from *E. emarginata*

Since the eutigoside C of *E. emarginata* at 75 ㎍/㎖ induced growth arrest, it was examined whether the expression of mRNA or protein related with growth arrest might be changed with eutigoside C treatment. The G1/S transition is regulated by D-type cyclins that bind to and activate Cdk4 and Cdk6 and cyclin E, which activate Cdk2. It was investigated whether eutigoside C affected the expression of these G1-related genes. The HL-60 cells were treated with the eutigoside C at 75 ㎍/㎖ for the times indicated and the RT-PCR analysis was performed (Figure 18). The expression of Cdk4 was markedly diminished in a time-dependent manner when treated with eutigoside C at 75 ㎍/㎖, while mRNA expression of cyclin D1 was decreased only after 48 h (components of the cyclin-Cdk complex that are active in early G1 phase). In addition, the expression of cyclin E was relatively unaffected by eutigoside C under the same conditions, although there was a very slightly decrease in the level of cyclin E after 48 h. and the expression of Cdk2 was markedly diminished in a

time-dependent manner when treated with eutigoside C at 75 ㎍/㎖ (components of the cyclin-Cdk complex that are active in late G1 phase).

As shown in Figure 18～20, the eutigoside C from *E. emarginata* markedly increased the level of $p21^{CIP1/WAF1}$ and dramatically inhibited Cdk2 activity. Moreover, the $p27^{KIP1}$ mRNA level was markedly increased in a time-dependent manner by eutigoside C treatment. This data suggested a possible binding between Cdk2 and CDK inhibitor protein, $p21^{CIP1/WAF1}$. The association of $21^{CIP1/WAF1}$ and Cdk2 by the eutigoside C treatment suggests that the eutigoside C from *E. emarginata* treatment-induced $p21^{CIP1/WAF1}$ blocks Cdk2 activity by direct binding of $p21^{CIP1/WAF1}$ to Cdk2.

pRB acts as a potent inhibitor of cell growth and is thought to play a crucial role in the decision-making at the G1/S restriction point (Sherr and Roberts, 1995; Weinberg, 1995). The functional activation of pRB depends on its phophorylation status. In cells treated with the eutigoside C, the amount of pRB was markedly diminished in a time-dependent manner after 48 h treatment with eutigoside C at 75 ㎍/㎖ (Figure 20). These results suggest that induction of $p21^{CIP1/WAF1}$ and down regulation of cyclin D1 may cause hypophosphorylation of pRB after the eutigoside C treatment.

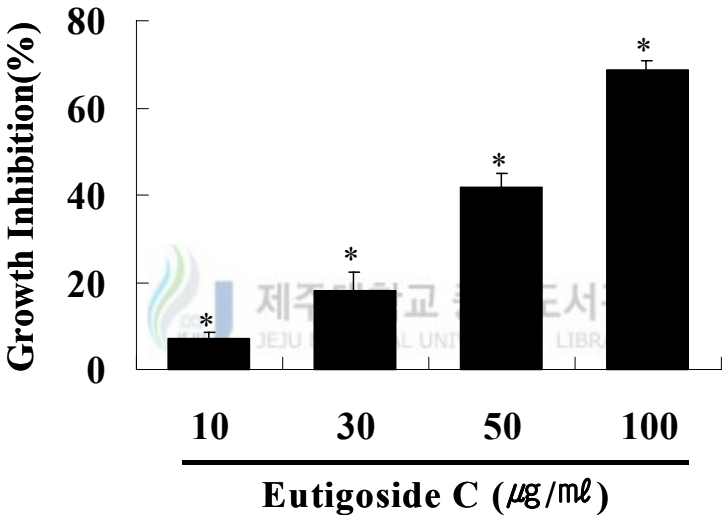


Figure 17. Effect of eutigoside C on the growth of HL-60 cells.

HL-60 cells were treated with the eutigoside C from *E. emarginata* for 4 days.

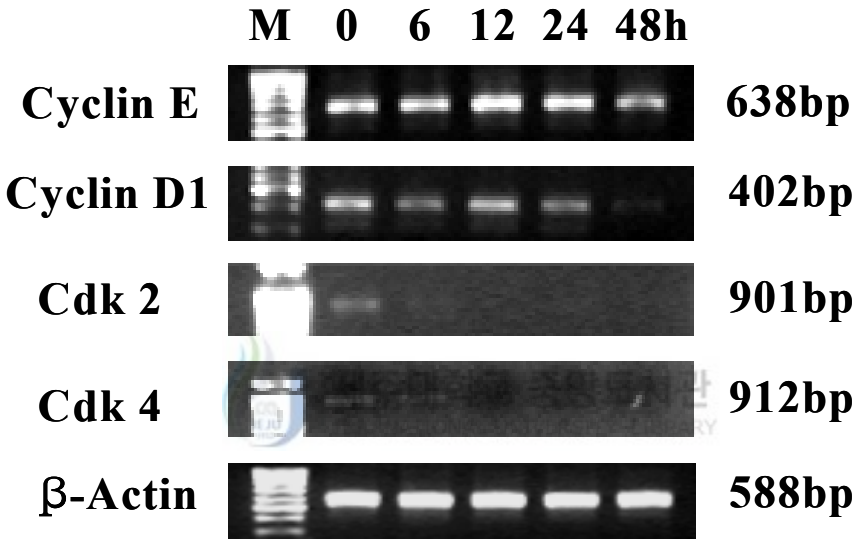


Figure 18. Effects of eutigoside C on the levels of cyclins E, cycline D1, cdk 2 and cdk 4 in HL-60 cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis of cyclins or cdks performed after synthesizing cDNA as described in the "materials and methods". The cells were incubated with the eutigoside C at the indicated times, total RNA was extracted for RT-PCR analysis.

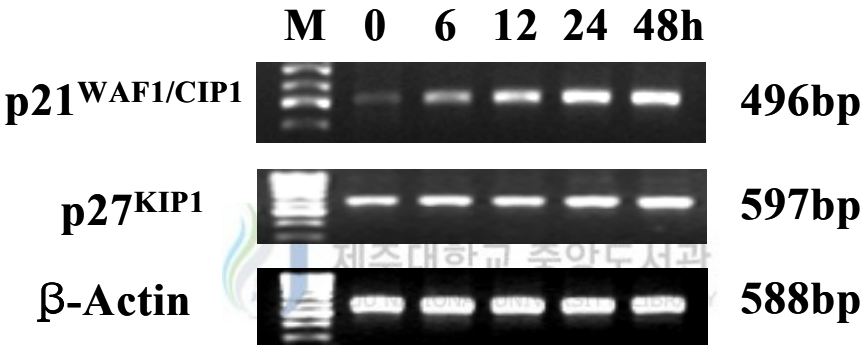


Figure 19. Effects of eutigoside C on the levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in HL-60 cells. RT-PCR analysis was performed as described in the "materials and methods". The cells were incubated with the eutigoside C at the indicated times, total RNA was extracted for RT-PCR analysis.

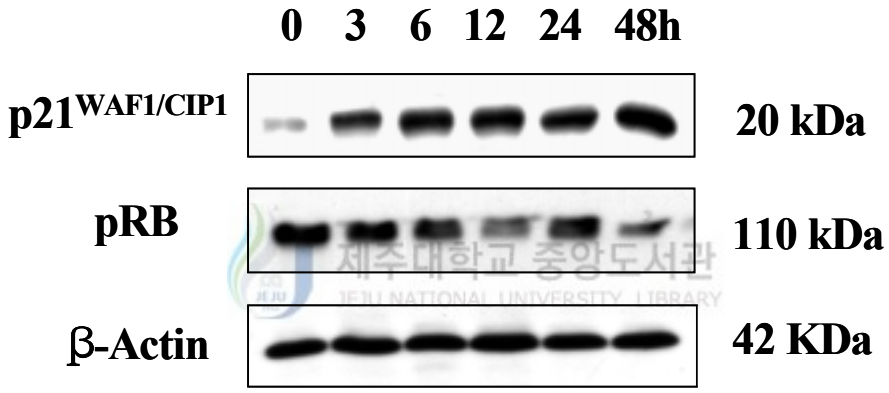


Figure 20. Effects of eutigoside C on pRB and p21$^{WAF1/CIP1}$ proteins in HL-60 cells. pRB and p21$^{WAF1/CIP1}$ was detected by Western blot analysis using anti-pRB and anti-p21$^{WAF1/CIP1}$ antibody in cell lysates from untreated cells (0 h) and in cells 3, 6, 12, 24 and 48 hours after treatment with 75 ㎍/㎖ of the eutigoside C.

DISCUSSION

Eurya emarginata (Thunb.) 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). At first, the present study characterized the constituents of the leaves of *E. emarginata,* grown in Jeju Island, Korea. The leaves of *Eurya* have been used as traditional medicine with the aim of treatment of ulcers or diuretic in the coastal areas of Jeju Island. Four components were isolated (quercitrin, cornoside, eutigoside B and eutigoside C) from the fresh leaves of this plant (Scheme 1～2, Figure 1, Figure 2 and Figure 3). Morita *et al*. (1974) reported the presence of quercitrin in *E. emarginata,* however, three other constituents (cornoside, eutigoside B and eutigoside C) have known to exist in other species not in *E. emarginata* (Jensen *et al*., 1973, Kahn *et al*., 1992). Previous studies suggested that flavonoids including quercitrin might have several biological activities like as anticancer, antioxidative and anti-inflammation. The cornoside has a novel chemical structure as constituents of higher plants, reported from many species of *Cornus* (Jensen *et al*., 1973). Otherwise, the eutigoside B and C are very rare compounds only described from *Eurya tigang* (Theaceae) (Khan *et al*., 1992). Furthermore, the biological function of cornoside, eutigoside B and C has been not defined yet. In the present study, eutigosides from *E. emarginata* was able to induce apoptosis of human leukemia HL-60 cells. The induction of apoptosis is known to be an efficient strategy for the

treatment of cancer cells. Various stimuli including ionizing radiation, toxins, cytokines and anticancer drugs have been used to induce the apoptosis in tumor cells (Michael *et al*., 1997; Lehmann *et al*., 2001; Devireddy *et al*., 2001; Geller *et al*., 2001; Mansat *et al*., 1997). Recently, a variety of fractions from different plants have been discovered to exhibit ability to trigger the apoptotic process (Seo *et al*., 2001; Bussing *et al*., 1999; Yoon *et al*., 1999).

The present investigated the molecular action mechanism by which *E. emarginata* inhibited proliferation of HL-60 leukemia cells. The EtOAc fraction of *E. emarginata* inhibited the growth of leukemia cells including HL-60, KG-1, U937, K562 and Jurkat, suggesting its ability to induce differentiation and/or apoptosis. But, it failed to affect the growth of CCD-25Lu and HEL-299, which are normal cell lines (not shown). It was also investigated whether a fraction of *E. emarginata* can induce the differentiation of HL-60 cells by measuring the expression of cell surface antigens. When the HL-60 cells were treated with 100 ㎍/㎖ of the crude extract, the ability of HL-60 cells to express CD 66b or CD 14 antigens was unchanged with the control (Figure 6). This result indicates that HL-60 cells do not differentiate into mature granulocytes or monocytes by *E. emarginata*. Besides, the HL-60 cells exhibited the apoptotic characteristics such as internucleosomal DNA cleavage, chromatin condensation and a blebbing of the plasma membrane after treatment with components of *E. emarginata.* The HL-60 cells have known to express a large amount of c-Myc, a proto-oncogene (Iguchi-Ariga *et al*., 1987; Kato and Dang, 1992). The alteration of c-Myc was also shown to be involved in

the apoptosis of HL-60 cells (Kimura *et al.*, 1995) and the expression of c-Myc was diminished during adenosine-induced apoptosis in HL-60 cells (Hong *et al.*, 1997). In order to understand the nature of molecular events that occurred in HL-60 cells by the treatment of EtOAc fraction of *E. emarginata,* the degree of c-Myc expression was evaluated (Figure 11). The expressions of c-Myc were dramatically decreased where the HL-60 cells underwent apoptosis by *E. emarginata*. The decrease of c-Myc expression was occurred before the induction of DNA fragmentation, suggesting that the decrease of c-Myc expression might be associated with the apoptosis induction in human leukemia cells. A number of recent studies have focused on how c-Myc affects apoptosis. The c-Myc is generally known as a key regulator of cell proliferation in normal and neoplastic cells, but until recently its apoptotic properties, which appear to be intrinsic, were not fully appreciated (Thompson, 1998; Prendergast, 1999). Bcl-2 is known to suppress apoptosis by controlling the membrane potential of mitochondria and/or preventing calcium depletion of endoplasmic reticulum (Distelhorst et al., 1996; Shimizu and Pommier, 1996). The cytotoxic component of *E. emarginata* decreased the amount of Bcl-2 protein as well as Bcl-2 mRNA in a time-dependent manner. On the contrary, the amount of Bax protein or mRNA was increased with eutigoside C treatment at 100 ㎍/㎖ (Figure 12). The cytotoxic effects of anticancer drugs are known to be linked with the regulation of oncogene or tumor suppressor genes such as p53 and Bcl-2. Recent studies on the Bcl-2 oncogene have demonstrated its role in preventing apoptosis induced by a wide variety of stimuli (Chiou *et al.*, 1994; Dole *et al.*, 1994). The

anti-apoptotic activity of Bcl-2 correlates with its intracellular ratio to Bax. High levels of Bax have been shown to favor apoptosis in cells subjected to growth factor deprivation, whereas high levels of Bcl-2 prolong cell survival (Baffy *et al*., 1993). A number of studies have shown that constitutive expression of Bcl-2 proto-oncogene in a variety of cells results in a heightened resistance to chemotherapeutic agents that function by inducing apoptosis (Hanada *et al*., 1993). Upon apoptotic stimulation, Bax forms oligomers and translocates from the cytosol to the mitochondrial membrane (Jurgensmeier *et al*., 1998). By interacting with pore proteins on the mitochondrial membrane, Bax increases the membrane's permeability and leads to the release of cytochrome c from mitochondria, which activates caspase-9 and initiates the caspase activation pathway to promote apoptosis (Narita *et al*., 1998). In response to various apoptotic stimuli, caspase cascades are activated leading to an irreversible commitment to cell death. One principle issue to be clarified concerns the activation mechanism of caspase cascades. Two regulatory pathways of the caspase cascades have been well studied so far. The first begins with the assembly of a death-inducing signaling complex (DISC) at the Fas receptor (Fas) (Walker *et al*., 1998; Mizutani *et al*., 1998; Uslu *et al*., 1996). Triggering of Fas by its natural ligand (FasL) induces the formation of a DISC consisting of a Fas-associated death domain and caspase-8. Activated caspase-8 in the Fas/FasL-initiated DISC activates caspase-3 and activated caspase-3 then directly initiates certain caspase-activated deoxyribonucleases. The second pathway begins with the release of cytochrome c from the mitochondria, which subsequently causes apoptosis by the activation of

caspase-9 and caspase-3 (Kojima *et al*., 1998; Walker *et al*., 1998; Salvesen *et al*., 1997; Cohen, 1997; Kuwahara *et al*., 2000; Nijhawan *et al*., 1997; Srinivasula, *et al*., 1998). In the presence of dATP and cytochrome c, released from mitochondria by a number of apoptotic stimuli, Apaf-1 binds to caspase-9 and causes its activation and activated caspase-9 then activates caspase-3 (Nijhawan *et al*., 1997; Srinivasula *et al*., 1998). Caspase-9 is an important member of the cysteine aspartic acid protease (caspase) family (Duan *et al*., 1996; Srinivasula *et al*., 1999). The present study examined the release of cytochrome c from mitochondria, the amounts of cleaved caspase-9 (37kDa/35kDa) and cleaved caspase-3 after the eutigoside C at 100 ㎍/㎖ in HL-60 cells. Eutigoside C (100 ㎍/㎖) induced release of cytochrome c from mitochondria into the cytosol fraction on HL-60 cells in a time-dependent manner (Figure 13).

Activity of Caspase-9 was increased in a time-dependent manner with the eutigoside C (100 ㎍/㎖) treatment in HL-60 cells. Caspase-3 was also increased in a time-dependent manner (Figure 14).

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri *et al*., 1994). To further confirm apoptotic cell death, the protein levels of poly (ADP-ribose) polymerase (PARP) were measured. PARP, a caspase-3 substrate, is important for maintaining cell viability and appears to be involved in DNA repair in response to environmental stress. PARP cleavage facilitates cellular disassembly and serves as a marker for the cells undergoing apoptosis (Oliver *et al*., 1998). This protein can be

cleaved by many ICE-like caspases *in vitro* and is one of the main cleavage targets of caspase-3 *in vivo*. In human PARP, the cleavage occurs between Asp-214 and Gly-215, which separates PARP's N-terminal DNA binding domain (24 kDa) from its C-terminal catalytic domain (89 kDa) (Oliver *et al*., 1998; Lazebnik *et al*., 1994). Proteolysis of the 116 kDa full-length PARP to an 85 kDa PARP and 89 kDa cleaved PARP activated fragment was monitored using anti-PARP antibodies. The cleaved PARP fragment was increased with eutigoside C (100 ㎍/㎖) in a time-dependent manner (Figure 15).

Transition from G1 to S phase is regulated by D-type cyclins and their cognate kinases Cdk4 and Cdk6, which act by phosphorylating and inactivating pRB prior to the restriction point at which cells commit to DNA synthesis (Pardee and Keyomarsi, 1989), and by cyclin E-Cdk2, which acts later in G1 (Resnitzky, 1997). Genetic changes associated with G1 cyclins and Cdks in human cancers include overexpression of cyclin D1, overexpression or mutation of Cdk4, loss of expression of the cyclin D-directed inhibitor p16, mutation or deletion of p53 or pRB, and over expression of cyclin E (Keyomarsi , 1993; Nobori *et al*., 1994; Wolfel *et al*., 1995). Since previous studies suggested that modulation of phosphorylation of the pRB regulated by Cdk4/cyclin D1 complex, play a key role in regulating G1/S transition (Resnitzky *et al*., 1995; Sherr and Roberts, 1995; Sidle *et al*., 1996; Weinberg, 1995), changes in the expression of cyclin D1, Cdk4, and pRB, and observed Cdk4 activity were determined. A reduction in the phosphorylation of pRB was observed (Figure 20) in the eutigoside C-treated the HL-60 cells, which correlated with the suppressed expression of cyclin D1 and the reduction of Cdk4 kinase activity (Figure 18). This can be a result in an

increase from the relative abundance of hypophosphorylated pRB, by binding to the transcription factor E2F-1, which might restrict the transcription of genes critical to the G1 to S progression. These suggest a mechanism to explain changes in the cell cycle profiles in eutigoside C-treated HL-60 cells. Regulation of the phosphorylation of pRB is associated with the synthesis of Cdk-inhibitory proteins, as well as induction or degradation of cyclin proteins. The present study showed that the eutigoside C caused a significant induction of Cdk-inhibitor $p21^{WAF1/CIP1}$ (Figure 19 and Figure 20). This induction of $p21^{WAF1/CIP1}$ leads to its increased association with G1 target, Cdk2. It has been previously reported that $p21^{WAF1/CIP1}$ can bind to and inhibit the kinase activity of both cyclin D-Cdk4 and cyclin E-Cdk2 complexes (Harper, 2001; Zhang *et al*., 1994). Thus, the induction of $p21^{WAF1/CIP1}$ and its association with Cdk2 upon the eutigoside C treatment may be sufficient to inhibit the activities of these complexes. In support of this, a decrease in the kinase activity of Cdk2 was also observed following the eutigoside C addition (Figure 18). These results, taken together with previous findings which implicate a similar involvement of $p21^{WAF1/CIP1}$ in DNA damage induced cell cycle arrest (Dulic *et al*., 1994; El-Deiry *et al*., 1994), suggest that the induction of $p21^{WAF1/CIP1}$ by the eutigoside C may play a causative role in the eutigoside-mediated inhibition of cell growth. The data presented here demonstrate that in the human leukemia cell line HL-60, the eutigoside C inhibits cell growth by blocking cell cycle progression, through at least two different mechanisms, by inhibition of cyclin D1 expression and by induction of negative growth regulatory protein $p21^{WAF1/CIP1}$.

In conclusion, the inhibitory effects of *Eurya emarginata* on the growth of HL-60 appear to arise from the induction of apoptosis. These results

can be helpful to develop eutigoside C of purpose from *E. emarginata* for a chemotherapeutic as well as a chemopreventive purpose for the treatment of human leukemia.

Part Ⅱ

Anti-inflammatory effect of eutigosides in murine macrophage RAW264.7

ABSTRACT

Eurya emarginata (Thunb.) Makino (Theaceae) is distributed in coastal areas of island. The leaves of *Eurya* have been traditionally used treat ulcers or diuretic in the coastal areas of Jeju Island. Nevertheless, there are few reports on the pharmacological activity and constituents of *E. emarginata*. The present study investigated the anti-inflammatory activity of the isolated constituents (Eutigoside B, C) from EtOAc fraction by examining activities of the several inflammatory markers (TNF-α, IL-1β, IL-6, NO, iNOS and COX-2). Also, the antioxidizing effect of the solvent extracts was also investigated by measuring DPPH radical-scavenging activity. Among the solvent fractions, EtOAc and BuOH fractions showed potent radical scavenging activity (RC_{50} = 10.9 and 12.7 respectively). The eutigoside B and eutigoside C isolated from the EtOAc fraction potentially inhibited the pro-inflammatory cytokines (IL-6 and TNF-α) in a dose-dependent manner. Additionally, the protein expression levels of iNOS and COX-2 were inhibited after a treatment with the EtOAc and BuOH fractions from *Eurya emarginata* (100 ㎍/㎖). Also, the intracellular contents of iNOS protein were a markedly inhibited after a treatment with the eutigoside B and C. The inhibition of iNOS was correlated with the decrease of nitrite level. These results suggest that eutigoside B and C from *E. emarginata* may have anti-inflammatory activity through the inhibition of pro-inflammatory cytokine (TNF-α and IL-6), iNOS and COX-2.

Key word: *Eurya emarginata*, Inflammation. RAW264.7, Eutigoside B, Eutigoside C, LPS, TNF-α, IL-1β, IL-6, NO, 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 potentially 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, inflammation can be harmful to the host and therefore it is subjected to multiple levels of biochemical, pharmacological, and molecular controls involving a diverse and potentially huge array of cell types and soluble mediators including cytokines (Nicod , 1993; Rouveix, 1997; Boraschi *et al*., 1998; Dinarello, 2000; Turcanu *et al*., 2001). IL-1β, IL-6 and TNF-α are proinflammatory cytokines, and when they were administered to humans, they produced 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, including 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 of the production of PGs (Dubois *et al*., 1998; Needleman *et al*., 1998; Vane *et al*., 1994). COX-2 is expressed during inflammation by cytokines and 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 selectively inhibit 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 arthrtis.

Eurya emarginata (Thunb.) 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). Studies on the biological activity and constituents of *Eurya emarginata* are rare. Therefore, this study examined the to find the active constituents from its leaves having anti-oxidative and anti-inflammatory effects and 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

1. DPPH radical-scavenging activity

DPPH radical-scavenging activities of the solvent (hexane, ethyl acetate, n-butanol) fractions obtained from methanol extract were measured. The experiments were carried out according to the method of Blois (1958) with a slight modification. Briefly, a 2 ㎎ of DPPH (1,1-Diphenyl-2-picrylhydrazyl; Sigma, MO, USA) radical solution in 15 ㎖ ethanol was prepared and then, this solution was mixed with 1 ㎎/㎖ of sample solutions in ethanol. Finally, after 10 min, the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. This activity is given as % DPPH radical scavenging that is calculated in the equation:

$$\%\ DPPH\ \ radical\ \ scavenging = \frac{(control\ \ absorbance - sample\ \ absorbance)}{control\ \ absorbance} \times 100$$

The DPPH solution without sample solution was used as control.

2. Cell culture

Murine macrophage cell line RAW264.7 was obtained from the KCLB (Korean Cell Line Bank). The cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/㎖ and 100 ㎍/㎖, respectively; GIBCO Inc, NY, USA). The exponentially growing cells were used throughout the experiments.

3. Pro-inflarmmatory cytokines production *in vitro*

The inhibitory effect of *E. emarginata* on TNF-α and IL-6 production was determined as previously described (Cho *et al*., 1998c). The isolated constituents and fractions solubilized with PBS and EtOH were diluted with DMEM. The final concentration of chemical solvents never exceeded 0.1% in the culture medium. In these conditions, none of the solubilization solvents altered TNF-α and IL-6 production in RAW264.7 cells. Before stimulation with LPS (1 ㎍/㎖) and test materials, RAW264.7 cells (1.0×10^6cells/㎖) were incubated for 18 h in 24-well plates with the same conditions. Lipopolysaccharide (LPS) and the 100 ㎍/㎖ 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, USA). The inhibitory effect of testing fractions on TNF-α and IL-6 production was determined as previously described.

4. RNA extraction

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. RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook *et al*. (1989).

5. RT-PCR analysis

The reverse transcription of 1 μg RNA was carried out using M-MuLV reverse transcriptase (Promega, WI, USA), oligo (dT) 18 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 10 min. The polymerase chain reaction (PCR) was performed in a reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 50 μM 3' and 5' each primer 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 amplication was followed by 35 cycles of 94℃ for 45 sec (denaturing), 60-65℃ for 45 sec (annealing) and 72℃ 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 7.

Table 6. 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'	
IL-10	F	5'-TGCTATGCTGCCTGCTCTTA-3'	385
	R	5'-TTTTCACAGGGGAGAAATCG-3'	
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	496
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'	
COX-2	F	5'-CACTACATCCTGACCCACTT-3'	696
	R	5'-ATGCTCCTGCTTGAGTATGT-3'	
β-Actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

6. Western blot analysis

Murine macrophage cell line RAW264.7 were pre-incubated for 18 h, and then stimulated by LPS (1 ㎍/㎖) and IFN-γ (50 U/㎖) 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 ㎍/㎖ aprotinin, 25 ㎍/㎖ leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at 12,000×g at 4℃ for 15 min and the supernatants were then stored at -70℃ until used. Protein concentration was measured using the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 ㎍ of protein) were separated on a 8-12% 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 5% nonfat dried milk, the membrane was then incubated with specific primary mouse monoclonal anti-mouse iNOS Ab (1:1000, Santa-Cruz, CA, USA), or rabbit polyclonal anti-rabbit COX-2 Ab (1:1000, Santa-Cruz, CA, USA) at 4℃ overnight. The membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Santa-Cruz, CA, USA) to mouse or rabbit. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersharm-pharmacia Biotech., NY, USA).

7. 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, USA). Briefly, The RAW 264.7 cells were stimulated with LPS (1 ㎍/㎖), and 100 ㎕ of the supernatant was mixed with 100 ㎕ 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, USA) and results were compared against a calibration curve using sodium nitrite as the standard.

RESULTS

1. Isolation of eutigosides from *E. emarginata* leaves and measurement of DPPH radical-scavenging activity

The leaves of *Eurya emarginata* was collected in the campus of Cheju National University, and air-dried in the dark place. The dried leaves (175.8 g) were extracted by 80% aqueous methanol (1L × 3) after standing for a week at room temperature. The liquid layer was filtrated, and the solvent was evaporated under reduced pressure to give the crude extract (58.21 g). After water (1L) was added to the extract, the aqueous layer was sequentially partitioned into hexane, ethyl acetate and *n*-butanol. A part (3.6 g) of the ethyl acetate fraction (9.97 g) was purified through reversed phase SiO_2 column by gradient elution of aqueous methanol (20 % to 100 %) to give 5 fractions. The polar fraction 5-4 (2.53 g) was subjected normal-phase column chromatography with $CHCl_3$/MeOH/H_2O (7.5/2.5/0.3) to obtain 7 subfractions (fr.5-4-1～fr. 5-4-7). A subfraction 5-4-5 (316.2 mg) was purified by a normal-phase column chromatography with $CHCl_3$/MeOH/H_2O (8/2/0.1) to obtain 5 subfractions (fr.5-4-5-1～fr.5-4-5-5). A subfraction 5-4-5-3 (21.5 mg) was further purified to compound Ⅲ and Ⅳ (Eutigoside B and C, Scheme 1, Figure 3).

DPPH radical-scavenging activities of each of fractions obtained from *E. emarginata* were measured. The highest DPPH radical-scavenging activity was shown in the EtOAc and the n-BuOH fraction (Table 7). All fractions were subjected to the TLC (Thin layer chromatography) and tested for DPPH radical-scavenging activities. As can be seen from Table 7, the subfractions (fr.5-4-5～5-4-7) exhibited the highest DPPH radical-scavenging

activity.

2. The effect of *E. emarginata* on LPS-induced IL-1β, IL-6, and TNF-α secretion in RAW264.7

Lipopolysaccharide (LPS) derived from gram-negative bacteria stimulates macrophage such as RAW264.7 to produce an array of pro-inflammatory mediators. The present study determined its effect on the expression of pro-inflammatory cytokines by using RT-PCR analysis. Murine macrophage cell line RAW264.7 cells were pre-incubated for 18 h, and then the degree of mRNA expression of pro-inflammatory cytokines was determined after the 6 h stimulation of cells with LPS (1 ㎍/㎖) in the presence of testing materials. The *E. emarginata* extracts in the absence of LPS did not alter the basal IL-1β, IL-6, and TNF-α (not shown). *E. emarginata* extracts (100 ㎍/㎖) did not affect viability of the cells (not shown).

As shown in Figure 21, solvent fractions of *E.emarginata* induced a marked decrease in the level of pro-inflammatory cytokines. The mRNA levels of TNF-α and IL-6 were diminished after treatment of the EtOAc fraction from *E. emarginata*. However, the IL-1β was diminished after treatment with n-BuOH fraction (100 ㎍/㎖) of *E. emarginata* treatment. In addition, the eutigoside B and the eutigoside C from *E. emarginata* markedly inhibited the mRNA levels of TNF-α and IL-6 in a dose-dependent manner whereas the IL-1β mRNA level exhibited no changes (Figure 22). However, the mRNA levels of IL-10, a anti-inflammatory cytokine increased with the eutigoside B and C treatment (100 ㎍/㎖) (Figure 23).

Table 7. Comparison DPPH radical-savenging activities of several extracts of *E. emarginata*

sample	*RC_{50}(㎍/㎖)
	DPPH radical scavenging effect
Vitamine C	3.0
Vitamine E	17.0
Pycnogenol	6.3
Butylated hydroxy anisole (BHA)	9.04
80% MeOH Ext.	32.3
Hexane fr.	89.0
EtOAc fr.	10.9
n-BuOH fr.	12.7
H_2O fr.	89.3
EtOAc fr.5-4-1(EF 5-4-1)	＞100
EtOAc fr.5-4-2(EF 5-4-2)	＞100
EtOAc fr.5-4-3(EF 5-4-3)	＞100
EtOAc fr.5-4-4(EF 5-4-4)	78.9
EtOAc fr.5-4-5(EF 5-4-5)	17.9
EtOAc fr.5-4-6(EF 5-4-6)	10.09
EtOAc fr.5-4-7(EF 5-4-7)	9.09

The experiments were carried out according to the method of Blois (1958) with a slight modification. Briefly, a 2 ㎎ of DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical solution in 15 ㎖ ethanol was prepared and then, this solution was mixed with 1 ㎎/㎖ of sample solutions in ethanol. Finally, after 10 min, the absorbance was measured at 517 nm.

* RC_{50} : Concentration for 50% radical-scavenging

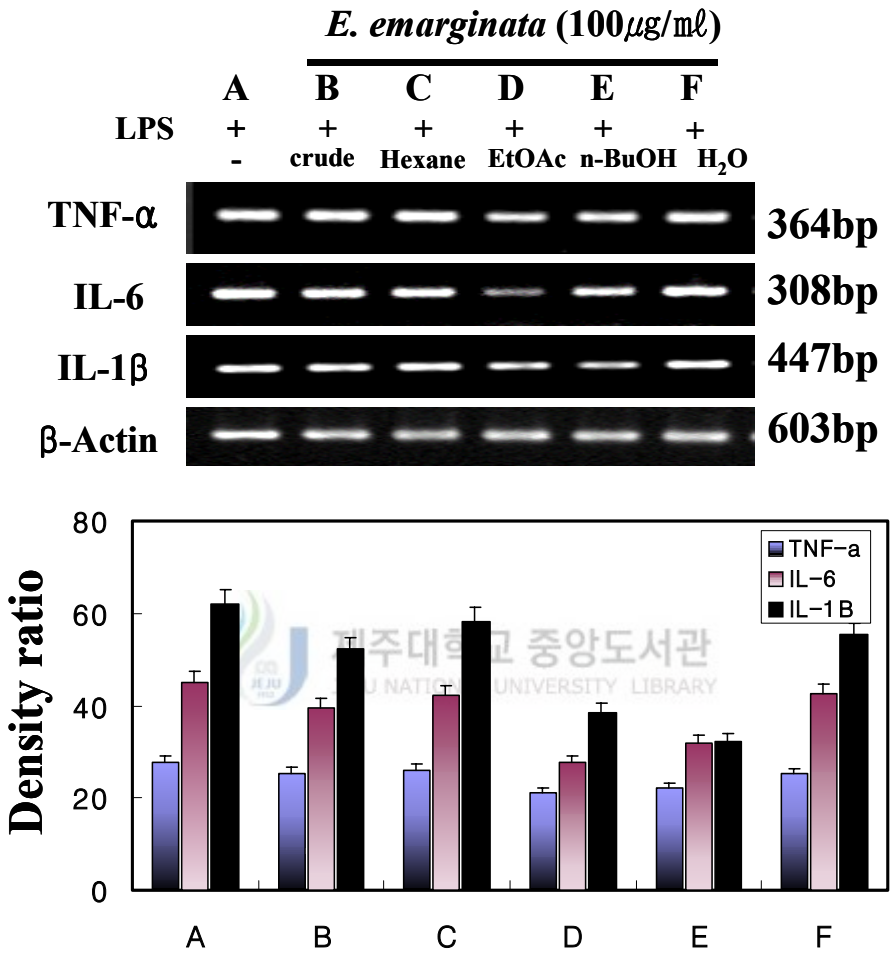


Figure 21. Inhibitory effects of the several extracts of *Eurya emarginata* on the mRNA expression of pro-inflammatory cytokines in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and the levels of mRNA expression were determined after 6 hr stimulation with LPS (1 ㎍/㎖) in the presence of testing materials (100 ㎍/㎖). The product of PCR were electophoresed and stained with ethidium bromide. A: Control (LPS+), B: 80% MeOH extract, C: Hexane fraction, D: EtOAc fraction, E: n-Butanol fraction, F: H_2O.

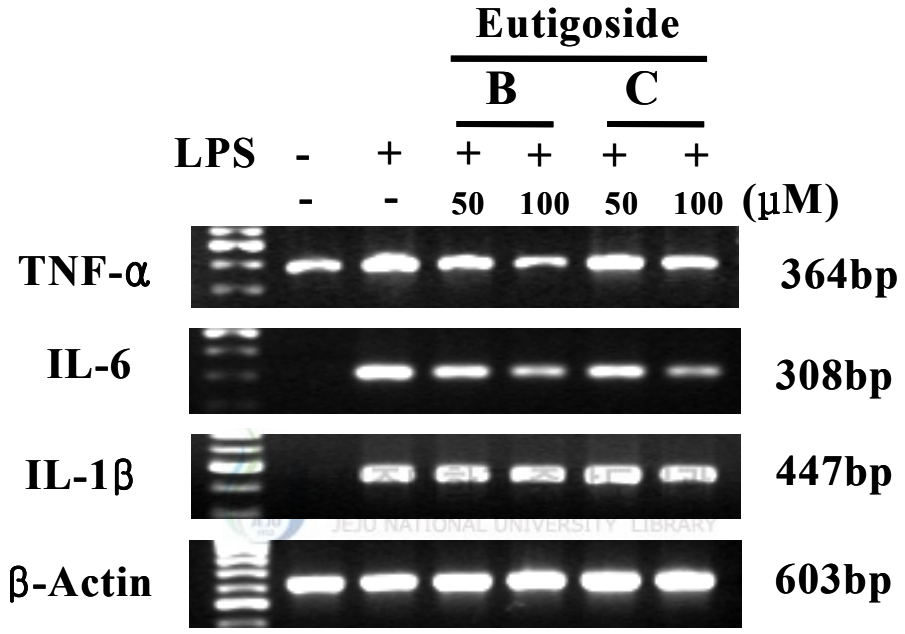


Figure 22. Inhibitory effects of eutigosides on pro-inflammatory cytokine mRNA expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and the levels of mRNA expression were determined after 6 hr stimulation with LPS (1 ㎍/㎖) in the presence of testing materials (100 ㎍/㎖).

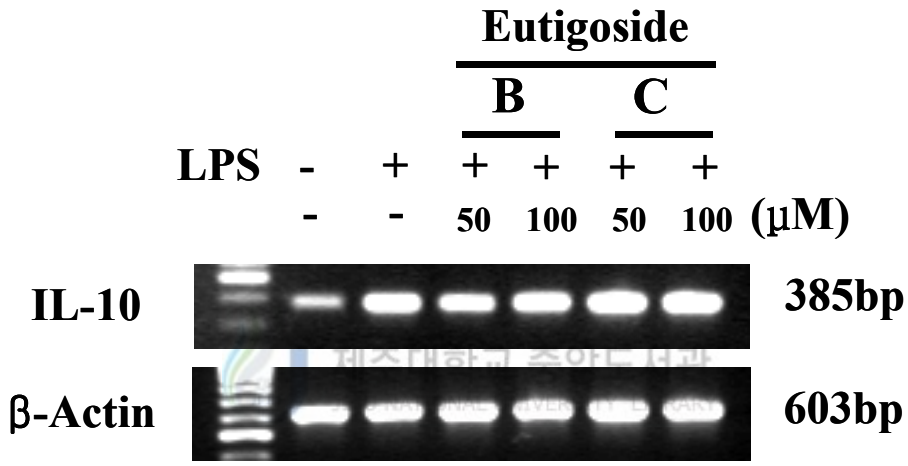


Figure 23. Inhibitory effects of eutigosides on anti-inflammatory cytokine mRNA expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and the levels of mRNA expression were determined after 6 hr stimulation with LPS (1 ㎍/㎖) in the presence of testing materials (100 ㎍/㎖).

3. TNF-α and IL-6 production *in vitro*

The inhibitory effects of the constituents from *E. emarginata* on TNF-α and IL-6 production were determined as previously described (Cho *et al*., 1998c and Schilling *et al*., 1997). As shown in Figure 24-25, the TNF-α and the IL-6 production was markedly suppressed with a subfraction (100 μM) of *E. emarginata,* which were obtained with the eutigoside B and the eutigoside C compared with positive control (LPS+), respectively.

The eutigoside B and eutigoside C isolated from the EtOAc fraction potentially inhibited the production of pro-inflammatory cytokines (IL-6 and TNF-α) in a dose-dependent manner.

4. Inhibition of LPS-mediated iNOS induction in RAW264.7

Lipopolysaccharide (LPS) is known to produce an array of iNOS in murine macrophage cell line RAW264.7. The present study determined the effect of *E. emarginata* on the protein content of iNOS by Western blot analysis.

RAW264.7 cells were pre-incubated for 18 h, and then protein content of iNOS was determined after the 24 h stimulation with LPS (1 ㎍/㎖) in the presence of *E. emarginata* constituents. As shown in Figure 26, The iNOS expression was markedly attenuated in cells co-treated with the EtOAc fraction and the n-BuOH fraction at the 100 ㎍/㎖ in RAW 264.7.

In addition, the eutigoside B and eutigoside C from *E. emarginata* markedly inhibited the production of iNOS in a dose-dependent manner (Figure 27).

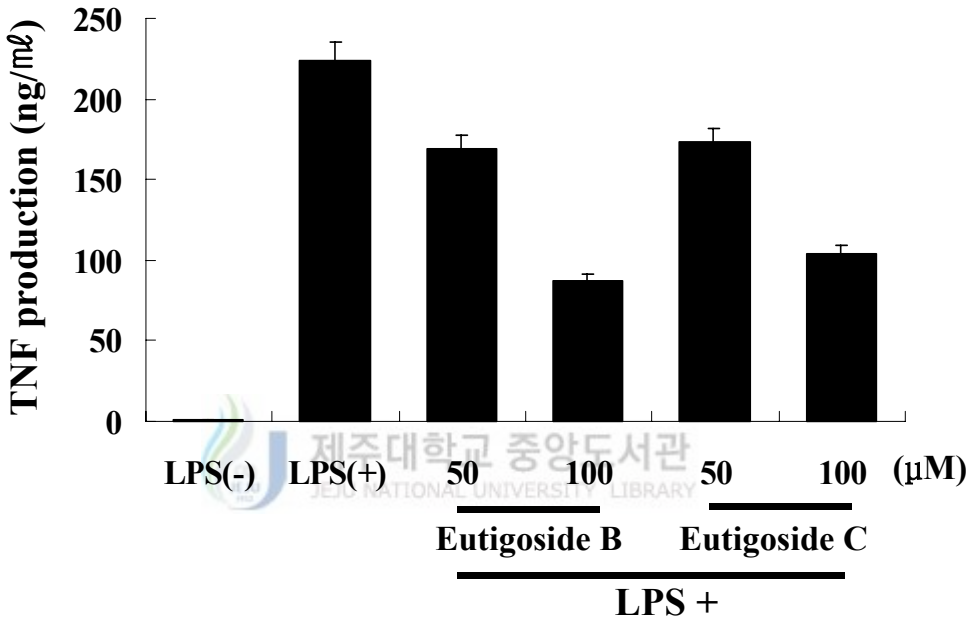


Figure 24. Inhibitory effects of eutigosides on TNF-α production in RAW 264.7 cells. The content of TNF-α was determined by ELISA from the culture medium of RAW264.7 cells (1.0×10^6cells/㎖) stimulated by LPS (1 ㎍/㎖) in the presence of testing materials.

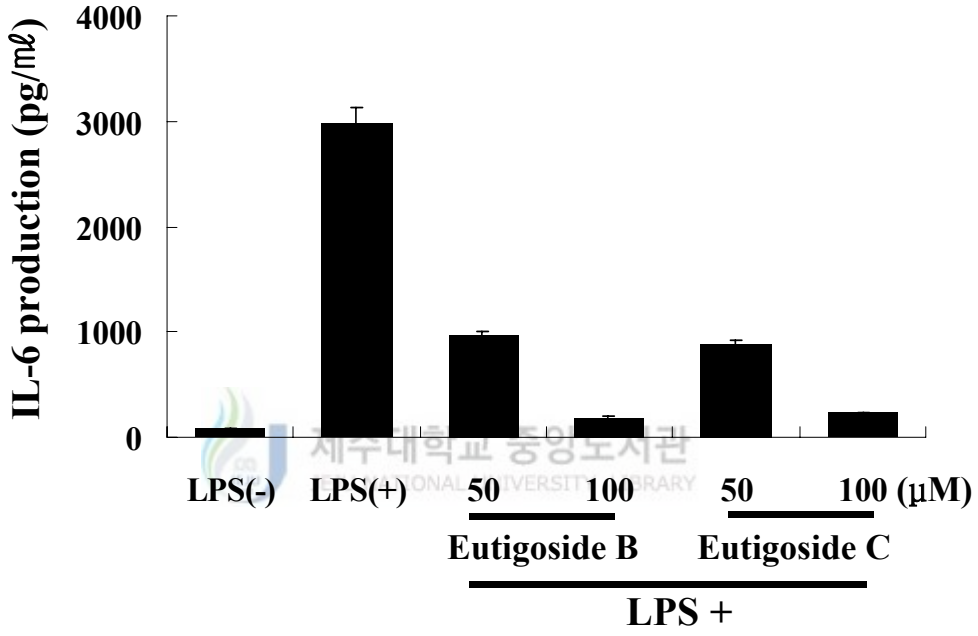


Figure 25. Inhibitory effects of eutigosides on IL-6 production in RAW 264.7 cells. The content of IL-6 was determined by ELISA from the culture medium of RAW264.7 cells (1.0×10^6cells/㎖) stimulated by LPS (1 ㎍/㎖) in the presence of testing materials.

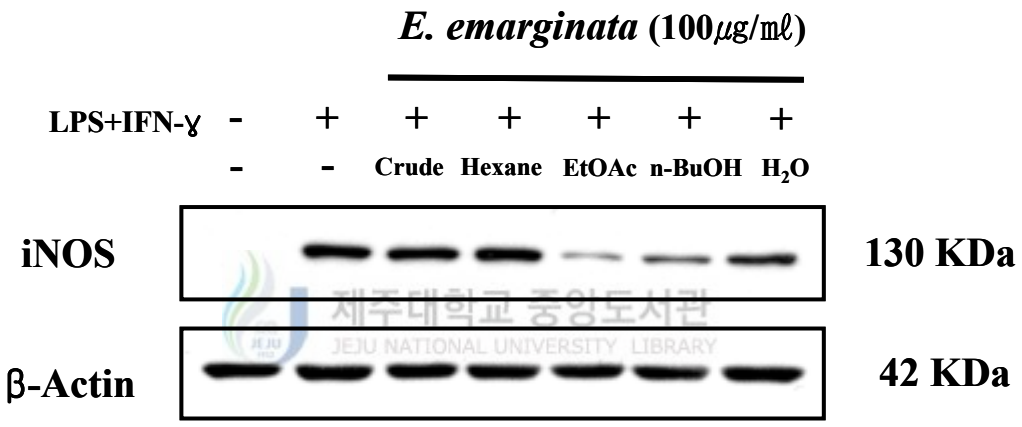


Figure 26. Inhibitory effects of the solvent fractions of *E. emarginata* on iNOS protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and then iNOS production protein expression was determined after 24 hr stimulation with LPS (1 ㎍/㎖) and IFN-γ (50 U/㎖) in the presence of testing materials (100 ㎍/㎖).

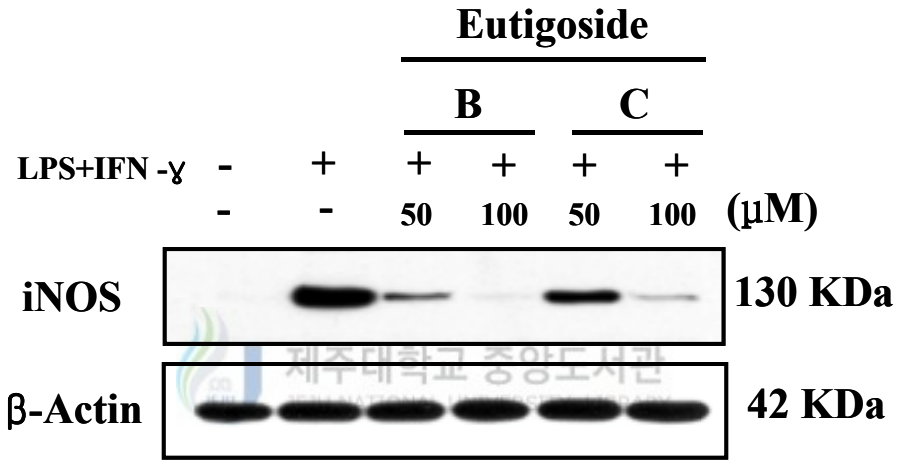


Figure 27. Inhibitory effects of eutigosides on iNOS protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and then iNOS protein content was determined after the 24 hr stimulation with LPS (1 ㎍/㎖) and IFN-γ (50 U/㎖) in the presence of testing materials.

5. Inhibition of LPS-mediated COX-2 induction in RAW264.7

Lipopolysaccharide (LPS) derived from murine macrophage cell line RAW264.7 is known to produce an array of COX-2. The present study determined the effect of *E. emarginata* on COX-2 production by Western blot analysis. COX-2 is expressed during the inflammatory process by cytokines and bacterial, such as LPS, and produces PGs that contribute to the pain swelling of inflammation (Hla *et al*., 1992). As shown in Figure 28, The COX-2 expression was markedly inhibited in cells co-treated with the EtOAc fraction and n-BuOH fraction (100 ㎍/㎖, respectively), in RAW264.7.

In addition, the eutigoside B and the eutigoside C from of *E. emarginata* inhibited the production of COX-2 in a dose-dependent manner (Figure 29).

6. Inhibition of LPS-mediated NO production in RAW264.7

RAW264.7 cells were stimulated with LPS (1 ㎍/㎖) for 24 h to evoke NO synthesis. NO accumulates its stable metabolite, nitrite, in the supernatant. Co-incubation of cells with the *E. emarginata* extracts significantly reduced NO production. *E. emarginata* extracts in the absence of LPS did not alter the basal NO production (data not shown). As shown in Figure 30, the EtOAc fraction inhibited the production of NO in RAW264.7 cells.

In addition, the subfraction of eutigoside B and eutigoside 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 and 24.66 μM, respectively (Figure 31 and Figure 32).

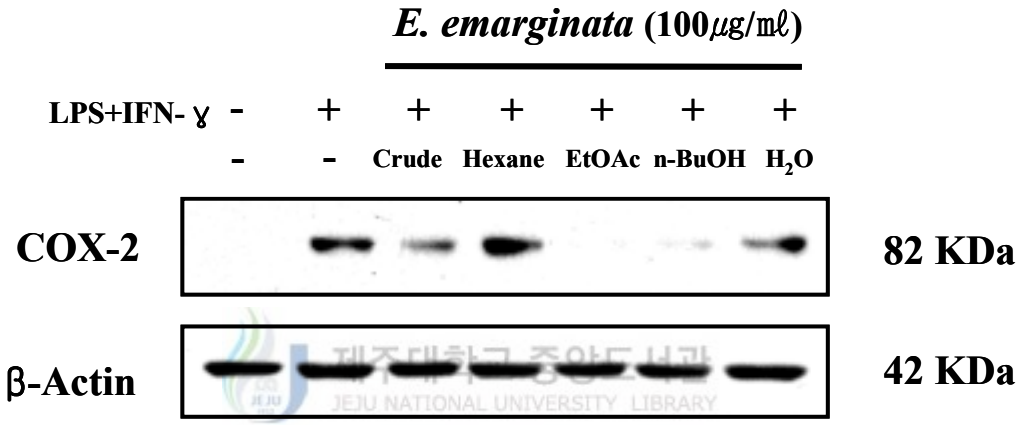


Figure 28. Inhibitory effects of the solvent fractions of *E. emarginata* on COX-2 protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and then COX-2 protein content were determined after 24 hr stimulation with LPS (1 ㎍/㎖) and IFN-γ (50 U/㎖) in the presence of testing materials (100 ㎍/㎖).

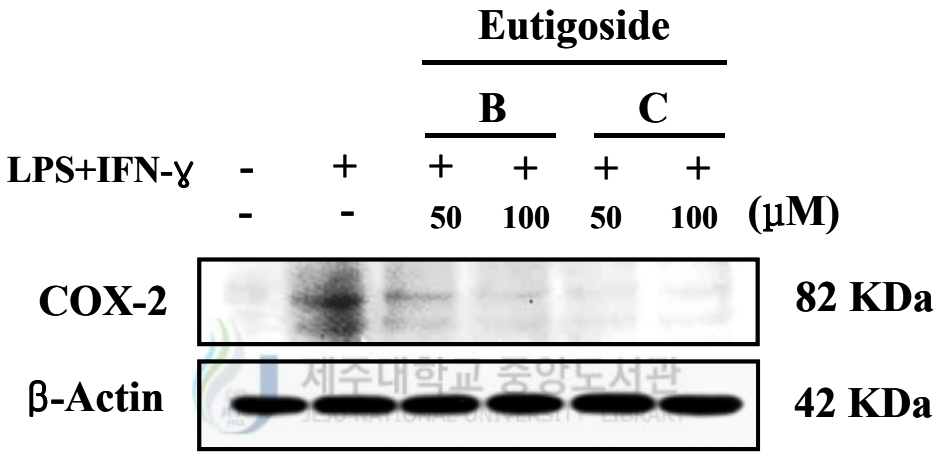


Figure 29. Inhibitory effects of eutigosides on COX-2 protein expression in activated macrophages. RAW264.7 macrophages (1.0×10^5cells/㎖) were pre-incubated for 18 hr, and then COX-2 content were determined after 24 hr stimulation with LPS (1 ㎍/㎖) and IFN-γ (50 U/㎖) in the presence of testing materials (100 ㎍/㎖).

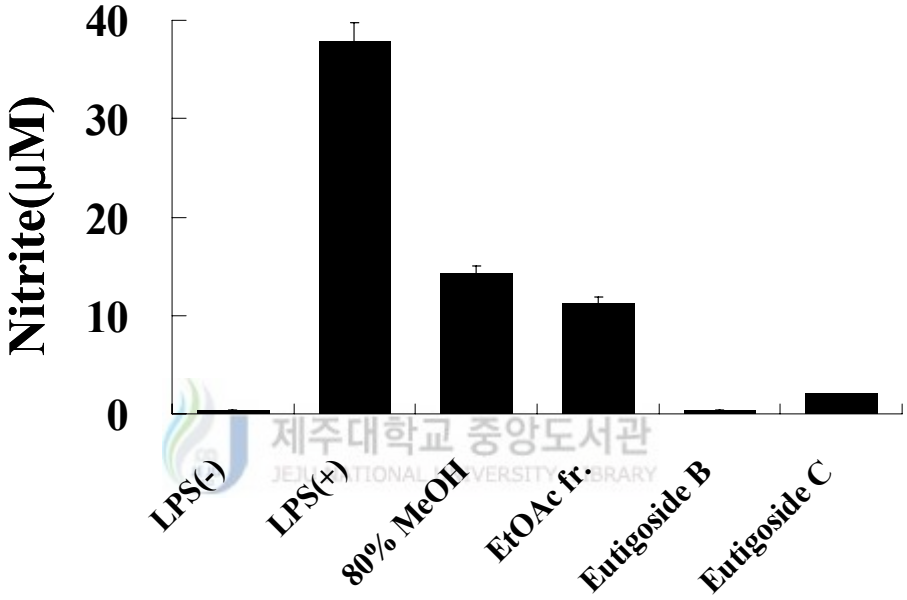


Figure 30. Inhibitory effects of components of *E. emarginata* on nitrite accumulation in RAW264.7 macrophages. The cells (1.5×10^5cells/㎖) were pre-incubated for 24 hr, and then nitrite production was determined after 24 hr stimulation with LPS (1 ㎍/㎖) in the presence of testing materials (100 ㎍/㎖).

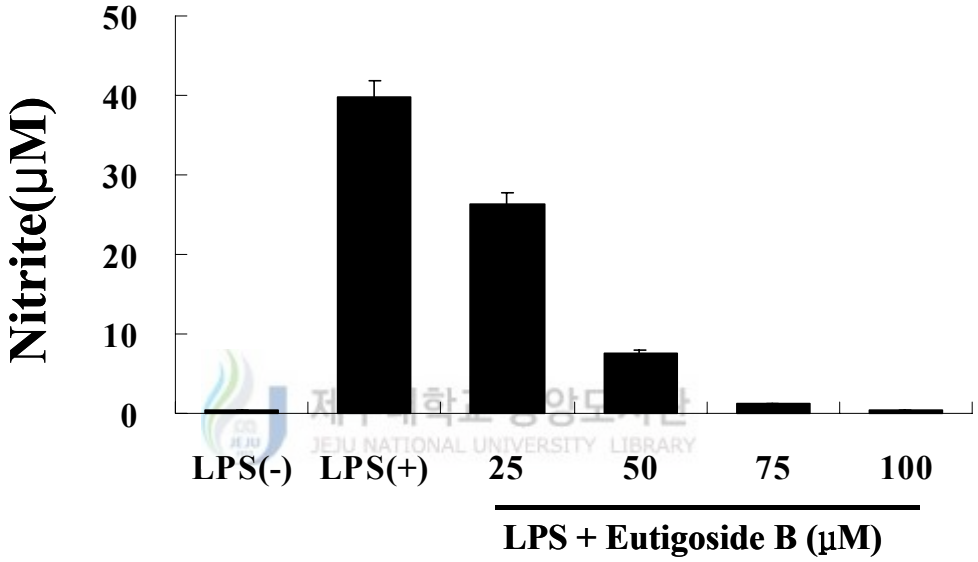


Figure 31. Inhibitory effects of eutigoside B on nitrite accumulation in RAW264.7 macrophages. The cells (1.5×10^5cells/㎖) were pre-incubated for 24 hr, and then nitrite production was determined after 24 hr stimulation with LPS (1 ㎍/㎖) in the presence of eutigoside B.

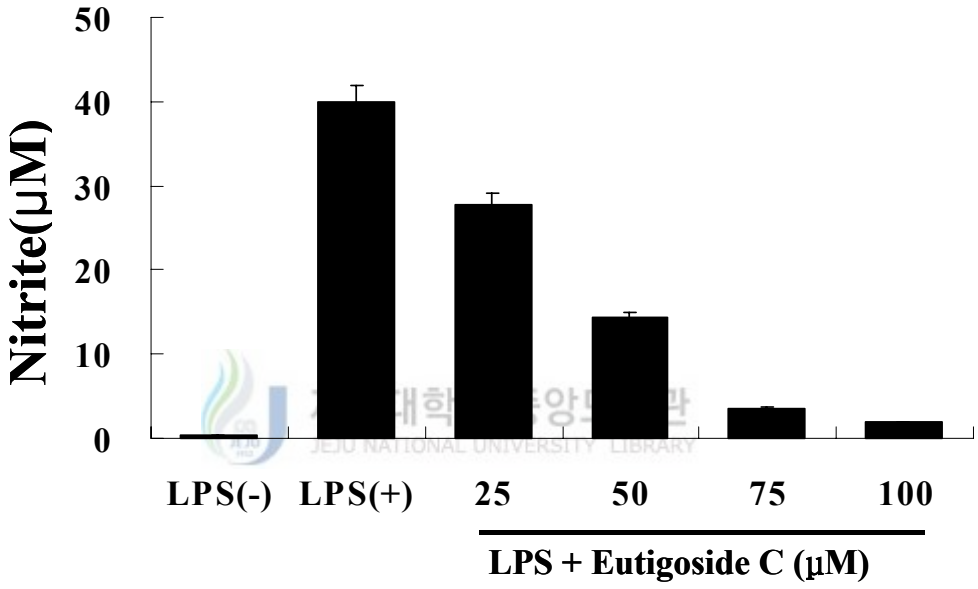


Figure 32. Inhibitory effects of eutigoside C on nitrite accumulation in RAW264.7 macrophages. The cells (1.5×10^5cells/㎖) were pre-incubated for 24 hr, and then nitrite production was determined after 24 hr stimulation with LPS (1 ㎍/㎖) in the presence of eutigoside C.

DISCUSSION

Eurya emarginata (Thunb.) Makino (Theaceae) is distributed in coastal areas of island. The leaves of *Eurya* have been traditionally used treat ulcers or diuretic in the coastal areas of Jeju Island. Nevertheless, there are few reports on the pharmacological activity and constituents of *E. emarginata*. The present study investigated the antioxidant and anti-inflammatory effects of the eutigoside B and eutigoside C from the leaves of *E. emarginata* in order to provide informations to develop the constituents of its leaves an anti-inflarmmatory agent.

The use of traditional medicine is widespread and a number of plants are a large 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 antiradical scavenging mechanism as part of their activity (Perry *et al*., 2002; Repetto and Llesuy, 2002). In the search for sources of natural antioxidants, for some medicinal plants recently have been extensively studied for their antioxidant activity and radical scavenging activity (De las Heras *et al*., 1998; Desmarchelier *et al.*, 2000; Schinella *et al*., 2002; VanderJagt *et al*., 2002).

DPPH radical-scavenging and antioxidant activities of isolated fractions from *E. emarginata* were evaluated. The results showed (Table 7) that the DPPH radical-scavenging activity was higher in the EtOAc and n-BuOH fractions, and the highest activities were observed in the EtOAc subfraction (fr.5-4-5 ~ 5-4-7).

Lipopolysaccharide (LPS) derived from gam-negative bacteria stimulates macrophage such as RAW264.7 to produce an array of pro-inflammatory

mediators. Many of these contribute to the pathogenesis of endotoxic shock, for example the potent vasodilator nitric oxide (NO) and the cytokines (tumour 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; MukIS *et a.l*, 1995; Lazarov *et al.*, 2000; Scott and Hancock, 2000).

In order to understand the anti-inflammatory effects by *E. emarginata*, the inhibitory effect of the solvent extract of *E. emarginata* on the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) activated with lipopolysaccaride (LPS), nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) production 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. The EtOAc fraction of *E. emarginata* inhibited the mRNA expression of TNF-α and IL-6 in RAW 264.7 cells. Additionally, eutigoside B and C from EtOAc fraction of *E. emarginata* potentially inhibited the mRNA expression of TNF-α and IL-6 in a dose-dependent manner (Figure 22). 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 (Figure 24 and Figure 25).

In order to determine whether the EtOAc fraction from *E. emargina* can reduce LPS-induced iNOS and COX-2 production, the protein content of iNOS or COX-2 was measured Western blot analysis. The EtOAc fraction of *E. emarginata* inhibited iNOS and COX-2 production in a dose-dependent manner.

In addition, the eutigoside B and the eutigoside C from *E. emarginata* potentially inhibited the iNOS and COX-2 expression.

The Griess reaction (Ryu *et al.*, 2000), a spectrophotometric 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 the NO production. Furthermore, LPS stimulation is 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 is an excellent model for drug screening and the following evaluation of potential inhibitor on the pathways leading to the induction of iNOS and NO production. The present results showed that the eutigoside B and C dose-dependently inhibited NO production in LPS-stimulated macrophages. 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 profound inhibition on LPS-induced NO production, the effect of eutigoside B or C on iNOS protein induction after LPS stimulation was examined. 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 following 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*., 1998). Inducible NOS (iNOS) is the key enzyme that produces large amounts of NO by macrophages

stimulated by bacterial endotoxin of LPS and proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (MacMicking *et al.*, 1997; Nathan and Xie, 1994).

The cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which are produced mainly by activated monocytes or macrophages, stimulated bone resorption as well as enhanced PGE_2 production in several type of cells including calvarial osteoblasts (Dinarello, 1988). The IL-6, a potent mitogenic polypeptide, stimulated cell proliferation in a various type of cells (Stein and Sutherland, 1998). It was also reported that TNF-α synergistically potentiated PGE_2 production stimulated by IL-1 (Lerner and Modeer, 1991). The mechanism by which cytokines such as TNF-α, IL-6 and IL-1act 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 anti-inflammatory effects of the main components of *E. emarginata* in LPS-activated macrophages, and the eutigoside B or eutigoside C could suppress inflammatory-biomarkers such as TNF-α,

paralled with inhibitions of TNF-α, IL-6, NO, iNOS and COX-2 inductions.

Taken together, the present study identified the anti-oxidative activity and anti-inflammatory capacities of constituents of *E. emarginata.* The eutigoside B or eutigoside C, the components of *E. emarginata* leaves may be useful for the treatment of oxidative damage and inflammation.

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ABSTRACT IN KOREAN

우묵사스레피의 eutigosides에 의한 항암 및 항염증 효과

박 수 영

우묵사스레피 (*Eurya emarginata*)는 차나무과의 상록 관목수로 제주도 같은 따뜻한 도서지방의 바닷가 산지에서 주로 자생하며, 특히 건조, 해풍, 먼지, 대기오염 등 각종 공해에 강하다. 지역적으로 중국, 대만, 일본, 인도 등에 분포하며, 열매가 쥐똥 같고 해변에 자생한다하여 섬쥐똥나무라 불리워지기도 하며 사스레피나무와 생김새가 비슷하나 잎끝이 뒤로 말리어 우묵하게 들어가서 우묵사스레피라 한다.

민간에서는 그 잎이 이뇨와 담 제거 및 종기제거 등의 약재로 쓰이고 있으나, 그 잎의 유효성분이나 생리활성에 대한 보고는 거의 없는 실정이다. 이에 본 연구자는 우묵사스레피 잎에서의 유효활성 성분을 탐색하였고, 또한 유효활성 성분들이 항암 및 항염증 효과에 있어 어떤 작용을 하는지를 알아보았다.

최근 apoptosis가 세포생물학의 주요 주제로 떠오르면서 chemotherapeutic 및 chemopreventive 작용기전 연구로 apoptosis에 대한 많은 연구가 이루어지고 있다. 이에 우묵사스레피 잎에서 분리된 활성성분 중 Eutigoside C를 HL-60 혈액종양암 세포를 이용하여 세포증식억제률, DNA fragmentation, morphologic changes, sub-G1 hypodiploid cells를 관찰한 결과 Eutigoside C에 의해 apoptosis가 유도됨을 확인할 수 있었다. 그리고 Westren blot과 RT-PCR을 통한 apoptosis 관련 유전자 및 세포주기 관련 유전자 변화 등을 통해 유의한 결과를 얻을 수 있었다. 또한, 우묵사스레피 잎에서 분리한 Eutigoside B와 C로부터 염증반응의 주체가 되는 대식세포 모델계에서 murine macrophage cell line RAW264.7 으로부터의 LPS 자극에 의한 TNF-α, IL-6, IL-1β와 같은 pro-inflammatory cytokine과 iNOS 및 COX-2의 발현 억제 효과를 관찰한 결과, TNF-α, IL-6 형성억제 및 iNOS, COX-2의 발현억제, 그리고 높은 NO 형성억제 효과를 얻을 수 있었다. 이러한 결과는 우묵사스레피의 유효성분으로부터 암을 예방하거나 치료할 수 있는 항암제 또는 항염증제 성분의 분리 및 그 작용기전 연구에 중요한 기초 자료가 될 것이라 사료된다.

감사의 글

본 논문이 완성되기까지 항상 사랑으로 옆에서 지켜 봐 주신 많은 분들께 이 지면을 빌어 감사의 말씀을 드립니다.

학위연구를 수행함에 있어 부족하기만 했던 저를 제자로 받아주시고 학문에 임하는 자세를 일깨워 주신 김세재 선생님께 우선 감사의 말씀을 드립니다. 그리고 본 논문에 대한 연구의 수행과 논문의 완성이 있기까지 끊임없는 사랑과 애정을 보여주신 강희경 선생님, 유은숙 선생님께 진심으로 감사드리며, 학문적 지도뿐만 아니라 삶의 밑바탕을 다져주신 허인옥 선생님께도 머리 숙여 감사를 드립니다.

늘 격려와 사랑으로 바쁘신 중에도 기꺼이 심사를 맡아 미흡한 논문을 정성을 다해 다듬어주신 고석찬 선생님, 의학과 강희경 선생님, 유은숙 선생님, 박덕배 선생님께 깊은 감사의 말씀을 드립니다. 아울러 학부와 대학원 과정동안 늘 관심과 조언으로 학문의 길을 이끌어주신 오문유 선생님, 이용필 선생님, 오덕철 선생님, 김원택 선생님, 김문홍 선생님, 이화자 선생님께도 감사의 말씀을 드립니다.

대학원 과정동안 연구를 수행함에 있어 관심과 조언을 통해 늘 옆에서 도움을 주신 김기옥 박사님, 고미희 박사님, 이동헌 박사님, 박지권 박사님, 정용환 박사님 그리고 한라수목원 김철수 선생님, 한태완 선생님과 제주농업시험장 김성철 박사님께도 감사드립니다. 특히, 오랜 시간동안 많은 실험을 함께 하면서 동고동락한 의학과 약리학교실 김상철, 이혜자, 현재희, 윤원종 대학원생, 생명과학과 분자생물학 실험실 정형복, 진영준, 최진영, 현은아 대학원생과 강신해 선생님 그리고 이정배, 송관필, 현화자, 김상범, 김병석 대학원생을 비롯한 생명과학과 학우들에게도 감사의 말씀을 드립니다. 아울러 화학과 이남호 선생님을 비롯한 천연물화학 실험실 백종석 선생님, 윤진석, 양홍철, 오태헌, 문지영 대학원생들에게도 깊은 감사의 말씀을 드립니다.

5년여 조교 기간 동안 언제 어디서나 힘이 되어주셨던 의과대학 홍강의 학장님, 강희경 선생님, 유은숙 선생님, 이영기 선생님, 박덕배 선생님, 이봉희 선생님, 김명주 선생님, 강현욱 선생님, 조문제 선생님, 현진원 선생님, 고영상 선생님, 배종면 선생님, 홍성철 선생님, 이상이 선생님, 정영배 선생님, 최민주 선생님, 염재범 선생님, 조수현 선생님, 강지훈 선생님을 비롯한 모든 선생님들께 감사드리며, 김영미, 임희경, 강윤석, 송지훈 그리고 윤지현, 변경희, 김은희, 오근희, 이정희 선생에게도 감사의 뜻을 전합니다.

지난 일들을 되돌아보면 그토록 힘들고 어려웠던 순간 속에 저 혼자만 있었던 것은 아닌 것 같습니다. 많은 분들의 도움과 그들의 염려하는 마음이 항상 함께하였기에 지금의 위치까지 올 수 있었다고 생각됩니다. 그리고 앞으로도 이 모든 것들을 언제나 감사하고 기억하며 살아가겠습니다.

끝으로 지금까지 저를 낳아 길러 주시고 끊임없는 사랑을 보내주신 부모님과 어려울 때 힘이 되어준 형님과 누님 그리고 가족들에게 감사하다는 말씀과 함께 이 논문을 작으나마 보답으로 드리고 싶습니다.