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Thesis for the Degree of Master of Engineering

Anti-bacterial activity and anti-inflammatory activity of the phlorotannins from *Eisenia bicyclis* against *Propionibacterium acnes*

by

Eun Hye Lee

Department of Food Science & Technology

The Graduate School

Pukyong National University

August 2014

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여드름 균에 대한 대황 추출물의 항균 효과 및 여드름 균으로 염증 유도된 **HaCaT cell** 에 대한 **phlorotannin compounds** 의 항염증 효과

Advisor: Prof. Young-Mog Kim

by

Eun Hye Lee

A thesis submitted in partial fulfillment of the requirements for the degree of

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in the Department of Food Science and Technology,

the Graduate School,

Pukyong National University

August 2014

Anti-bacterial activity and anti-inflammatory activity of the phlorotannins from *Eisenia bicyclis* against *Propionibacterium acnes*

A dissertation

by

Eun Hye Lee

Approved as to style and content by:

(Chairman) Yang-Bong Lee

(Member) Byung-Soo Chun

(Member) Young-Mog Kim

August, 2014

Contents

PUKYONG NATIONAL UNIVERSITY
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List of Figures

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List of Tables

List of Abbreviation

BSA	**Bovine serum albumin**
DMEM	**Dulbecco's Modified Eagle medium**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
FBS	**Fetal bovine serum**
IL-1β	**Interleukin-1 beta**
IL-6	**Interleukin-6**
LPS	**Lipopolysaccharide**
MAPK	**Mitogen-activated protein kinase**
mRNA	**Messenger ribonucleic acid**
MTT	**3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide**
NF-κB	**Nuclear factor kappa B**
NO	**Nitric oxide**
PCR	**Polymerase chain reaction**
RNA	**Ribonucleic acid**
iNOS	**Inducible nitric oxide synthase**
ROS	**Reactive oxygen species**

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MMP-2	**Matrix metalloproteinase-2**
MMP-9	**Matrix metalloproteinase-9**
TNF-α	**Tumor necrosis factor alpa**
Akt	**Protein Kinase B**

여드름 균에 대한 대황 추출물의 항균 효과 및 여드름 균으로 염증 유도된 HaCaT cell 에 대한 phlorotannin compounds 의 항염증 효과

이은혜

부경대학교 대학원 식품공학과

요약

여드름은 털 피지선샘 단위에서 생기는 만성 염증 질환으로 피지선이 모여있는 얼굴, 가슴, 목, 등에서 주로 발병한다. 여드름이 발생한 부위엔 다양한 피부변화가 발생하며 이에 따른 후유증으로 오목한 흉터 또는 확대된 흉터, 색소침착을 남기기도 한다. 대황 (*Eisenia bicyclis*)은 갈조류의 미역과로 다시마 대용으로 식용 되어지며 조간대 하부에서 서식한다. 그 효능으로는 변비치료에 도움이 되고 항균 소염작용이 뛰어나며 지혈작용과 피부병에 좋다고 보고 되어 있다. 현재 대황에 대한 강한 항균 및 항염증 효과는 많이 보고 되어지고 있으나 아직 여드름 균에 대한 대황의 항균 및 항염증 연구는 미흡한 실정이다. 따라서 본 연구에서는 대황을 Methanol 로 추출하여 극성별로 분획한 후 Disc diffusion assay 와 Minimum Inhibitory Concentration 을 통해 항균효과를 알아보았다. 또한 각질형성 세포인 HaCaT cell 에 여드름균을 직접

주입하여 염증을 유발시킨 후 대황 속 Phlorotannin coumpounds 를 이용하여 염증억제 효능을 검토하였다.

그 결과, 염증반응매개 효소인 iNOS 와 COX-2, 염증성 사이토카인인 IL-6, IL-1β, TNF-α 의 유전자 및 단백질 발현이 억제됨을 확인할 수 있었고 Phlorotannin compounds 중 Eckol 이 가장 항염증 효과가 우수하였고 그 다음 Dioxinodehydroeckol 이 항염증 효과가 뛰어남을 확인 할 수 있었다. 또한 이러한 compounds 는 염증 신호전달 경로인 NF-κB 의 활성화를 억제함으로서 염증 반응을 제어하는 것을 확인 할 수 있었다.

Introduction

Acne vulgaris is one of the most common skin diseases, affecting nearly 80% of young adults aged to 11 to 30 years. Not only it can cause disfiguration and permanent scarring, but also have an adverse effect on psychological development, resulting in profound emotional scarring, which may lead to social phobias, withdrawal from society, and clinical depression (J.Leyden et al., 1995). *Propionibacterium acne* has been described as an obligate anaerobic organism. It is implicated in the development of inflammatory acne by its capability to activate complements and by its ability to metabolize sebaceous triglycerides into fatty acids, which chemotactically attract neutrophils (Chomnawang et al., 2005).

P. acne stimulates keratinocytes to produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (ILs), and the formation of other inflammatory mediators, including nitric oxide (NO). NO is endogenously produced from L-arginine and molecular oxygen by the action of NO synthases (NOSs). In mammals, there are three isoforms of NOS; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed in neuronal and endothelial, respectively. In contrast, iNOS is inducible and its expression is increased in cells that are exposed to LPS or cytokines (Vane et al., 1994). Accordingly, high expression and activity of iNOS are observed in chronic diseases, such as inflammation and cancer (Maeda et al., 1998., Liu et al., 1998).

Prostaglandins (PGs) are synthesized by the action of cyclooxygenase (COX) in arachidonic acid metabolism (Vane et al., 1994). COX has two isoforms; COX-1 and COX-2. COX-1 is constitutively expressed in most cells and the COX-1-produced prostaglandins which has involved in normal physiological functions. COX-2 is inducible in many types of cells including macrophages after the exposure of LPS, growth factors, and tumor promoters (Prescott et al., 2000, Hinz et al., 2002). In view of the importance of iNOS and COX-2 as plausible targets for the treatment of inflammatory disorders, I was interested in the effect of *E. bicyclis* on the suppression of iNOS and COX-2 expressions in *P.acne*-induced HaCaT cells.

MMPs are a group of zinc-dependent endopeptidases that selectively degrade the components of various extracellular matrix as well as non-matrix proteins (Chakraborti et al., 2003). Thus, MMPs are implicated in the remodeling of the extracellular matrix in both physiological and pathological conditions such as wound healing, inflammation, and tumor metastasis (Kleiner and Stetler- Stevenson . 1999; Cook et al., 2000; Kim et al., 2005).

Eisenia bicyclis is a perennial brown alga which belongs to the Laminareaceae family and it is distributed in the middle Pacific coast around Korea and Japan. It is consumed as a raw material for sodium alginate and phlorotannin-rich raw materials (Okada ea al., 2004). Although individual phlorotannins contained in the edible brown algae have been reported to possess strong anti-bacterial and anti-inflammatory activity, but the responsible components of *Eisenia bicyclis* have yet to be fully studied (Jung et al., 2013). Therefore, the objective of this study was to examined the antimicrobial activity

of *Eisenia bicyclis* against *P. acne* and anti-inflammatory activity of *Eisenia bicyclis* and its possible mechanism was to investigate in *P.acnes*-induced HaCaT cells.

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Materials and Methods

1. Raw materials and extraction

The fresh *E. bicyclis* was purchased from Ulleung Trading Co. A voucher specimen was deposited in the author's laboratory in late August 2012. Fresh *E. bicyclis* was washed three times with water to remove salt. Dried *E. bicyclis* was ground and then finely powdered with a food mixer (HMF -1000A; Hanil Electronics, Seoul, Korea). The dried powder was stored at -20℃ until required. The dried *E. bicyclis* powder (1.0 Kg) was extracted with methanol (MeOH; 10L x 3) at 70℃ for 3 hrs (3 times) and the solvent was evaporated *in vacuo*. The combined crude MeOH extract (164.3g) was suspended in 10% MeOH (1.0 L) and then partitioned in turn with *n*-hexane (Hexane; 1.0 L x 3), dichloromethane (DCM; 1.0 L x 3), ethyl acetate (EtOAc; 1.0 L x 3), and *n*-butanol (BuOH; 1.0 L x 3) portions. Each extract was evaporated using rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 45℃.

2. Preparation of *P. acne*

Propionibacterium acne (KCTC 3314) which was used for evaluation of anti-related bacterial effect, was obtained from the Korea Collection for Type Cultures (KCTC; Daejeon, Korea).

P. acnes was anaerobically cultivated in brain heart infusion broth (BHI ; Difco Inc., Detroit, MI) supplemented with 1.0% glucose, and incubated at 37℃ for 72 hrs in a CO_2 incubator (NAPCO 5400; General Laboratory Supply, Pasadena, TX), in a 10% CO_2- humidified atmosphere. At determined time intervals, cultures of *P. acnes* were diluted and plated on Blood Agar Base (Difco Inc., Detroit MI). The plates were incubated at 37℃ for 72 hrs in a 10% CO_2 incubator and the number of colony-forming units (CFU) was determined (data not shown).

3. Disk diffusion assay

The disc diffusion assay described by the National Committee for Clinical Laboratory Standards (NCCLS, 2004) was carried out with some modifications. *Propionibacterium acnes* was incubated in brain heart infusion medium (BHI) with 1% glucose for 72 hrs under anaerobic conditions and adjusted to yield approximately 1.0×10^8 CFU/ mL. 1 mL of bacterial culture was spread on Blood Agar Base plate and a paper disc (6 mm in diameter) containing 1-5 mg of each extract was then placed on the agar surface. After incubating for 24 hrs at 37℃, the diameter of inhibition zone was measured. The experiment was done three times and the mean values were presented.

4. Measurement of minimum inhibitory concentration (MIC)

The MIC can be defined as the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation (Grierson and Afolayan, 1999). The MIC values were determined by microdilution assay. The cultures were prepared at 72 hrs broth cultures of *Propionibacterium ances* (Chomnawang et al., 2005). The MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37℃ for 20-24 hrs and the experiment was performed in triplicates (Grierson andAfolayan, 1999).

5. Isolation and purification of phlorotannins from *E. bicyclis*

UV spectra were obtained with a Hitachi U-2000 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were measured on a Varian VNS600 instrument operating at 600 and 150 MHz, respectively. The chemical shifts are given in δ (ppm) values relative to that of the solvent DMSO-d_6 (δ_H 2.49; δ_c 39.7) on a tetramethylsilane (TMS) scale. The standard pulse sequences programmed into the instruments were used for each 2D measurement. The J_{CH} value was set at 8Hz in the Heteronuclear Multiple Bond Correlation (HMBC) spectra. Fast Atom Bombardment (FAB-MS) using 3-nitrobenzyl alcohol as the matrix agent was performed on a Micro Mass Auto Spec OA-TOF spectrometer. High-performance liquid chromatography (HPLC) analysis was carried out on a YMC-Pack ODS A-302 column (4.6 mm i.d. x 150 mm; YMC Co., Ltd., Kyoto, Japan) and

developed at 40℃ with 1% formic acid (HCOOH : MeCN = 8 : 2; Detection : 280 nm). Column chromatography was performed using LiChroprep RP-18 (Merck, Darmstadt, Germany) and Sephadex LH-20 (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates (0.25 mm layer thickness, Merck, Darmstadt, Germany) and the spots were detected by UV irradiation (254, 365 nm) and by spraying with 10% H_2SO_4 reagent.

6. Cell culture and cell viability assay

HaCaT cells were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37ºC in a humidified atmosphere of 5% CO_2. Cytotoxicity of various molecular weight of *E. bicyclis* was evaluated by MTT assay, a method based on the reduction of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Weislow et al., 1989) (Fig. 1). The medium containing HaCaT cells were cultured into a 96-well plate at a density of 1 x 10^5 cells per mL. The plate was incubated overnight and treated with 100 μl of DMEM medium containing different concentrations of *E. bicyclis*. After 24 hrs of incubation, MTT solution (1 mg/ mL) was added to each well and the plate was incubated for another 4 hrs at 37ºC. The blue formazan salt was dissolved in DMSO. Optical density was measured at 540 nm with a GENios microplate reader (Tecan, Austria GmbH, Austria). The optical density of formazan formed by untreated cells was taken as 100% of viability.

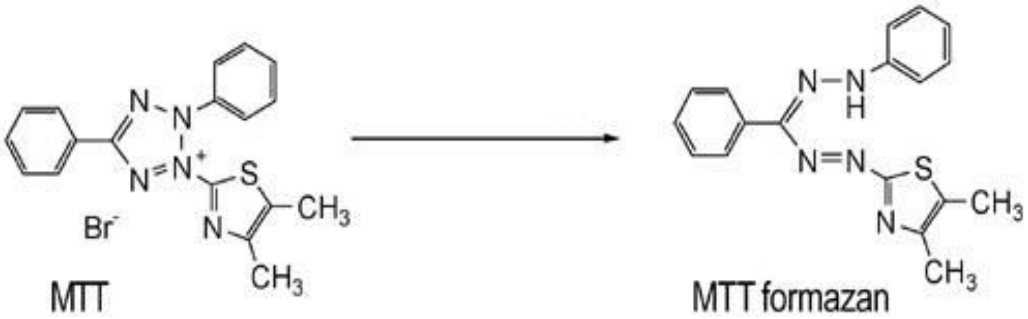

Fig. 1. Molecular structure of MTT and its corresponding reaction product

7. Measurement of NO

HaCaT cells (1 x 10^6) were plated and incubated with absence or presence of *P. acne* (10^6 CFU/ mL) for 72 hrs. After then, 0~300 μg/ mL *E. bicyclis* was added and incubated for 24 hrs. After treatment of *P. acne* and *E. bicyclis*, HaCaT cells culture medium was saved for measured as of nitrite. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction (Kim et al., 1995). One hundred mL of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Infinite F200 pro, TECAN) at 540 nm.

8. RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using a Trizol reagent (Invitrogen Co., CA, USA) following the manufacture's recommendations. Total RNA was digested with RNase-free DNase (Roche, IN, USA) for 15 min at 37ºC and repurified by the RNeasy kit according to the manufacture's protocol (Quiagen, CA, USA). cDNA was synthesized from 2 μg total RNA. By incubation at 37ºC for 1 hr with MLV reverse transcriptase (Promega) with random hexanucleotide according to the manufacture's instruction. Primers to specifically amplify the genes interested were shown in Table 1. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with cycles of denaturation at 95ºC 30 sec, annealing at 60ºC 45 sec, and extension at 72ºC for 1 min, respectively. The amplified PCR products were run in 1.5% agarose gels and visualized by ethidium bromide (EtBr).

9. Western blot analysis

Western blotting was performed according to standard procedures. Briefly, cells were lysed in RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, leupeptin (80 μg/ml), 3 mM NaF and 1 mM DTT at 4°C for 30 min. Cell lysates (50 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene

fluoride membrane (Amersham Pharmacia Biotech., England, UK), blocked with 5% skim milk, and hybridized with primary antibodies (diluted 1:1000). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000® Luminescent Image Analyzer (Fujifilm Life Science, Tokyo, Japan).

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Table 1. Chemicals used for reverse transcription PCR

PCR Chemical	Amount used	Stock
MMLV reverse ranscriptase	0.5 µl	200 U/ µl
dNTP mixture	0.5 µl	10 mM
DTT	1 µl	100 mM
5X reaction buffer	5 µl	
RNase inhibitor	0.5 µl	80 U/ µl

Table 2. RT-PCR conditions

Temperature	Time
37ºC	1h
70ºC	5 min
4ºC	∞

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Table 3. Chemicals used for PCR reaction

PCR Chemical	Amount used (µL)	Stock
Taq polymerase	0.125	5 U/ µl
dNTP	0.5	2.5 mM
Reverse Primer	0.5	25 pmole/ µl
Forward Primer	0.5	25 pmole/ µl
5X reaction buffer	5	
DW	17.375	

Table 4. PCR conditions

Temperature	Time	Cycle
95ºC	2 min	1
95ºC	30 sec	
60ºC	45 sec	30
72ºC	1 min	
72ºC	5 min	1
4ºC	∞	

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Table 5. Gene-specific primers used for the RT-PCR

Gene	Direction	Sequence
iNOS	Forward	5'- CAC CTT GGA GTT CAC CCA GT -3'
	Reverse	5'- ACC ACT CGT ACT TGG GAT GC -3'
COX-2	Forward	5'- TGA AAC CCA CTC CAA ACA CA -3'
	Reverse	5'- GAG AAG GCT TCC CAG CTT TT -3'
TNF-α	Forward	5'-AGG CCT TGT GTT GTG TTT CCA-3'
	Reverse	5'-TGG GGG ACA GCT TCC TTC TT-3'
IL-1β	Forward	5'- CTG TCC TGC GTG TTG AAA GA -3'
	Reverse	5'- TTC TGC TTG AGA GGT GCT GA -3’
IL-6	Forward	5'- AGG AGA CTT GCC TGG TGA AA -3'
	Reverse	5'- CAG GGG TGG TTA TTG CAT CT -3'
MMP-2	Forward	5'-ATG GCA AGT ACG GCT TCT GT-3'
	Reverse	5'-ATA CTT CTT GTC GCG GTC GT-3'
MMP-9	Forward	5'-CTC GAA CTT TGA CAG CGA CA-3'
	Reverse	5'-GCC ATT CAC GTC GTC CTT AT-3'
β-actin	Forward	5'- CCA CAG CTG AGA GGG AAA TC-3'
	Reverse	5'-AAG GAA GGC TGG AAA AGA GC-3'

Results and Discussion

1. Anti- *P. acnes* activity of *E. bicyclis* extract

The methanolic extract of *E. bicyclis* exhibited an antibacterial activity against *P. acnes*, suggesting that the extract contains an antibacterial substance against *P. acnes*. The methanolic extract was further fractioned using organic solvents (Fig 2). A lyophilized powder (1.0 kg) of *E. bicyclis* was percolated in methanol (3 times x 1.0L), followed by partitioning with several organic solvents to yield Hexane-soluble extract (42.3 g), DCM-soluble extract (2.5 g), EtOAc-soluble extract (23.8 g), BuOH-soluble extract (26.5 g) and H_2O-soluble extract (69.1 g) in extract. The anti-*P.acne* activities of hexane, DCM, EtOAc, BuOH and water soluble fractions were appraised by measuring inhibition zones. Among them, the EtOAc soluble fraction showed the strongest anti-*P.acne* activity and followed by Hexane soluble fraction. No anti-*P.acne* activity was observed in BuOH and water soluble fraction (Table 6). EtOAc soluble fraction of *Eisenia bicyclis* exhibited the strongest anti-*P.acne* acitivity.

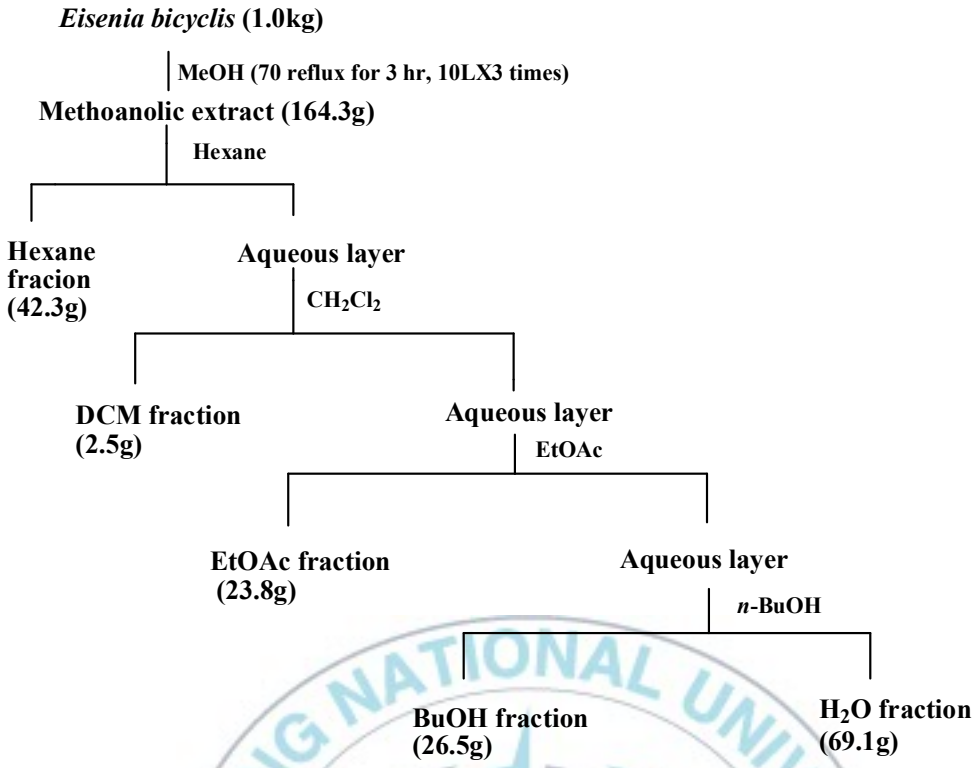

Fig. 2. Scheme of extraction and liquid-liquid partition.

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Table 6. Disk diffusion assay of methanol extract and its soluble fractions from *Eisenia bicyclis* against *Propionibacterium acnes*.

Strains	Concn.	Zone of inhibition (mm)[a]					
		MeOH[b]	Hexane	DCM	EtOAc	BuOH	H_2O
Propionibacterium acne (KCTC 3314)	1 mg/disk	7.0	7.0	7.0	10.0	-	-
	5 mg/disk	13.0	17.0	10.0	18.0	12.0	-

[a] Methanol extract and its fraction from *Eisenia bicyclis* was loaded onto a disk (6 mm in diameter)

[b] MeOH, methanolic extract; Hexane, *n*-hexane-soluble extract DCM, dichloromethane-soluble extract.; EtOAc, ethyl acetate-soluble extract; BuOH, *n*-butanol-soluble extract; H_2O, water soluble extract.

[c] Data are the averages of duplicate experiments.

[d] –, no detection of antibacterial activity.

2. Determination of MIC of *E. bicyclis* extract

The current study was focused on an antibacterial activity of *E. bicyclis* extracts against *P. acnes*. In order to quantitatively evaluate its antibacterial activity, MIC values of the extract against *P. acnes* (Table 7) was investigated. The highest anti- *P. acnes* activity was observed on the ethylacetate (EtOAc) soluble fraction. These results were also consistent with the results obtained by the disk diffusion assay. The MICs of EtOAc soluble extract was 64 μg per mL. Hexane soluble fraction was 128 μg per mL for MICs. DCM and MeOH were 256 μg per mL respectively. However, no antibacterial activity was observed in BuOH and H_2O-soluble extracts (Table 7). These results thoroughly suggested that an anti- *P. acnes* substance originated from the *E. bicyclis* methanolic extract will be abundant in the EtOAc soluble fraction.

It has been previously reported that the EtOAc extract of this plant also showed the highest antibacterial activity against Gram positive bacterium, *Methicillin Resistance Staphlyococcus Aureus* Eom (2012). Thus, it was investigated that the *E. bicyclis* extract will exhibit an antibacterial activity against several kinds of pathogenic or spoilage bacteria.

Table 7. Minimum inhibitory concentration of *Eisenia bicyclis* extracts against *Propionibacterium acne*.

Strains	MIC (μg/mL)					
	MeOH	Hexane	DCM	EtOAc	BuOH	H_2O
Propionibacterium acne **(KCTC 3314)**	**256**	**128**	**256**	**64**	**1024**	**>1024**

MeOH, methanolic extract; Hexane, *n*-hexane-soluble extract DCM, dichloromethane-soluble extract.; EtOAc, ethyl acetate-soluble extract; BuOH, *n*-butanol-soluble extract; H_2O, water soluble extract.

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3. Effect of *P. acne* on NO production in HaCaT cells

NO is synthesized from the amino acid of arginine by nitric oxide synthase (NOS). Under pathological conditions, NO production is increased by the inducible NOS (iNOS) and, subsequently, brings about cytotoxicity and tissue damage (Kim et al., 1998). *Propionibacterium acne* can induce the formation of iNOS and NO in HaCaT cells (Fig 3). HaCaT cell is incubated with *Propionbacterium acne* for 72 hrs. The concentration of *Propionibacterium acne* is 10^3, 10^4, 10^5 and 10^6 CFU/mL. As an indicator of NO production, nitrite ($NO2^-$) accumulation in the cultured media was determined by the Griess method.

4. Effect of *P. acne* on HaCaT cells viability

The effect of *P. acnes* on the viability of HaCaT cells was determined by MTT assay. HaCaT cells were treated with or without *P. acne* at concentrations of 10^3, 10^4, 10^5, 10^6 CFU per mL. As shown in (Fig 4). *P. acnes* did not show any significant cytotoxicity. These results revealed that *P. acnes* are safe materials for *in-vitro* culture experiments up to a concentration of 10^6 CFU per mL.

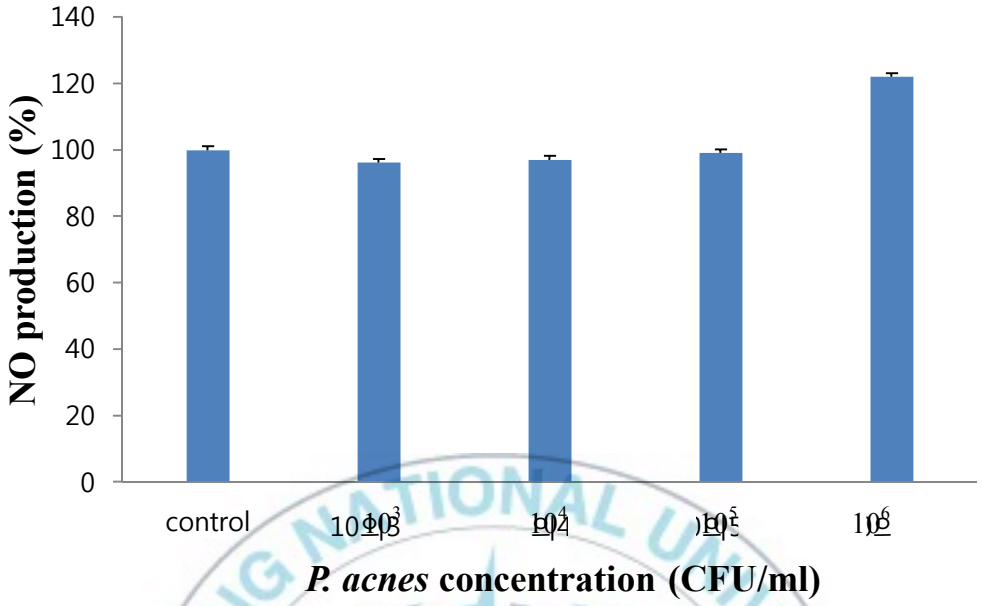

Fig. 3. Effect of *Propionibacterium acne* on nitric oxide (NO) production in HaCaT cells.

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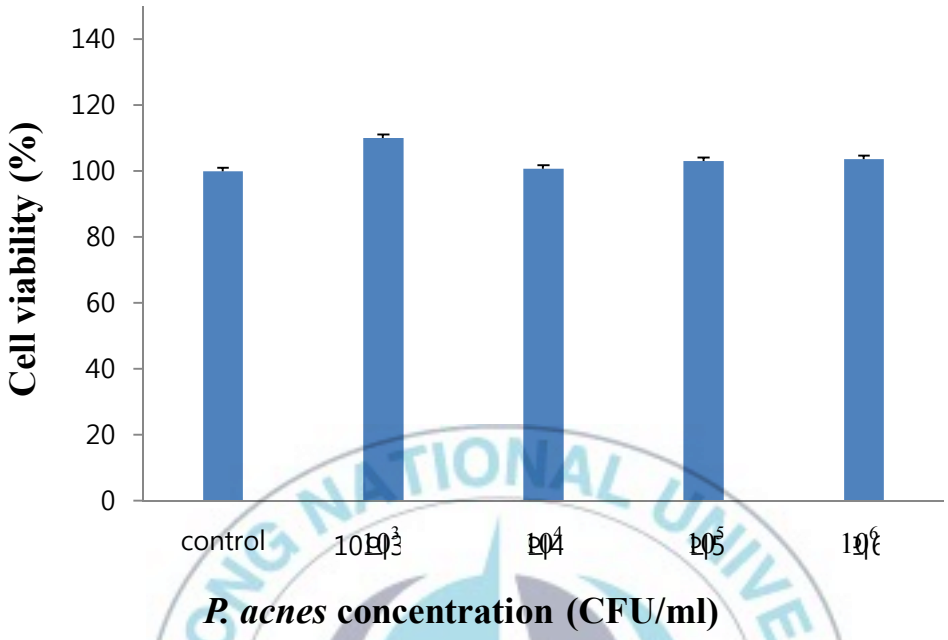

Fig. 4. Effect of *Propionibacterium acnes* on cell cytotoxicity in HaCaT cells.

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5. Effect of *E .bicyclis* on NO production in HaCaT cells

NO is synthesized from the amino acid arginine by nitric oxide synthase (NOS). Under pathological conditions, NO production is increased by the inducible NOS (iNOS) and, subsequently, brings about cytotoxicity and tissue damage (Kim et al., 1998). Extracts which were able to reduce NO production by iNOS may be attractive as anti-inflammatory agents, and for this reason, the effects of polyphenoles on iNOS activity have been intensively studied to develop anti-inflammatory drugs (Santangelo et al., 2007). *P. acnes* can induce the formation of iNOS and NO. Therefore, it was investigated whether extracts from *E. bicyclis* could inhibit NO production in *P. acnes*-induced HaCaT cells. As an indicator of NO production, nitrite ($NO2^-$) accumulation in the cultured media was determined by the Griess method. Extracts from *E. bicyclis* inhibited *P. acnes*-induced NO production in HaCaT cells compared to control group (Fig. 5). Among them, the EtOAc-soluble extract showed the strongest effect reducing the NO production. To my knowledge, this is the first report regarding the inhibitory effects of extracts from *E. bicyclis* on NO production in HaCaT cells. Hence, these findings suggest that the inhibition of NO production by extracts might be due to the suppression of *P. acnes*-induced iNOS transcription.

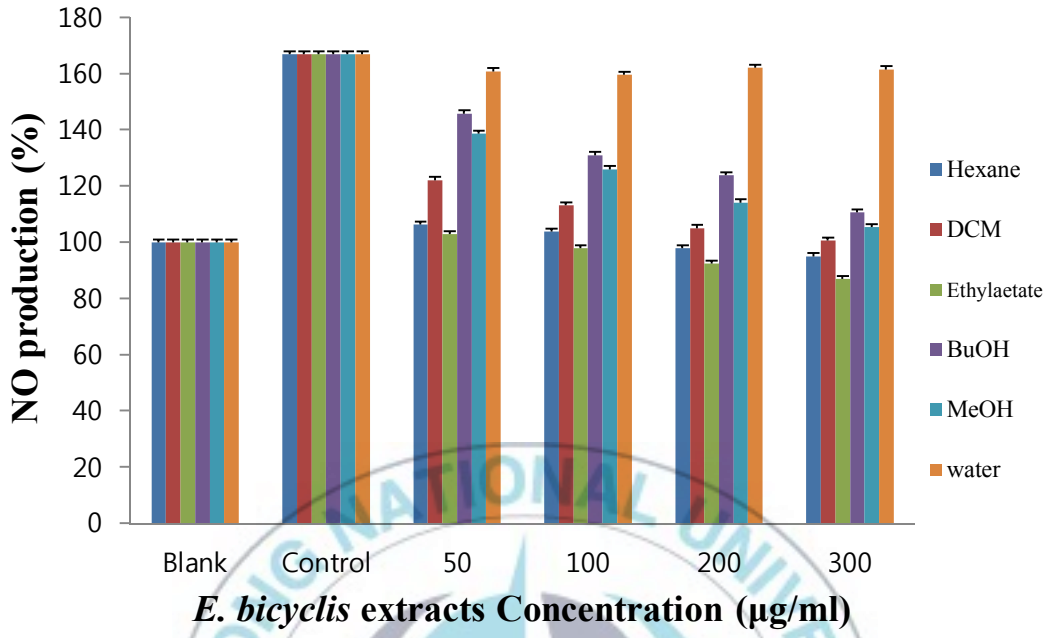

Fig. 5. Effect of *Eisenia bicyclis* extracts on nitric oxide (NO) production in *P. ances*-induced HaCaT cells.

Each extract were treated with the presence of P .ac*nces* (10^6 CFU/ mL).

Blank: - *P. acne*; Control: *P. acnes* (10^6 CFU/ mL).

6. Cytotoxicity of *E. bicyclis* fractions

The cytotoxicity of E. bicyclis extract fractions and isolated compounds were evaluated by MTT assay. Confluent cells in DMEM were incubated in the absence or presence of diluted samples (50, 100, 200, 300 μg per ml) for 24 hrs and absence or presence of diluted compounds (5 μM, 10 μM, 20 μM per ml) for 24 hrs, as well. After MTT reagents were added to the cells. The extracts from *E. bicyclis* did not show any significant cytotoxicity up to a concentration of 300 μg per ml (Fig. 6). But the compounds exerted strong influence to the cells so it did not show significant cytotoxicity up to a concentration of 10 μM per mL (Fig. 7).

7. Isolation of phlorotannins from *E. bicyclis*

Several reports suggest that marine brown algae has various biologically active components specifically, phenols and polyphenols as secondary metabolites and these are responsible their biological activities (Kim ea al., 2006; Okada et al., 2004; Holdt and Kraan, 2011). The previous reports on *E. bicyclis* have revealed that it contains abundant to phlorotannin derivatives with various bioactivities, such as antioxidant (Kwon et al., 2013), anti-inflammation (Jung et al., 2013), anti-diabetes (Broadhurst et al ., 2000; Eon et al., 2012) and antibacterial (Eom et al., 2012). However, there have been few researches regarding the antimicrobial activities and anti-inflammatory activities of isolated phlorotannins from *E. bicyclis* against *P. ance.* Hence, this study attempted to

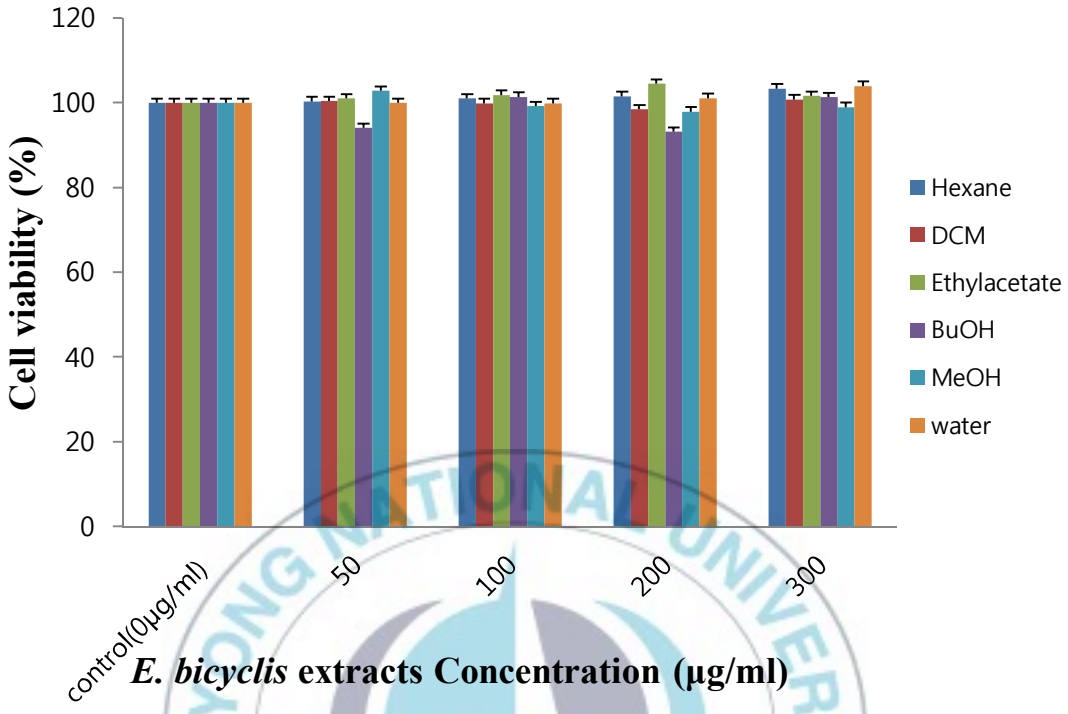

Fig. 6. Effect of *Eisenia bicyclis* extracts on cell cytotoxicity in HaCaT cells.

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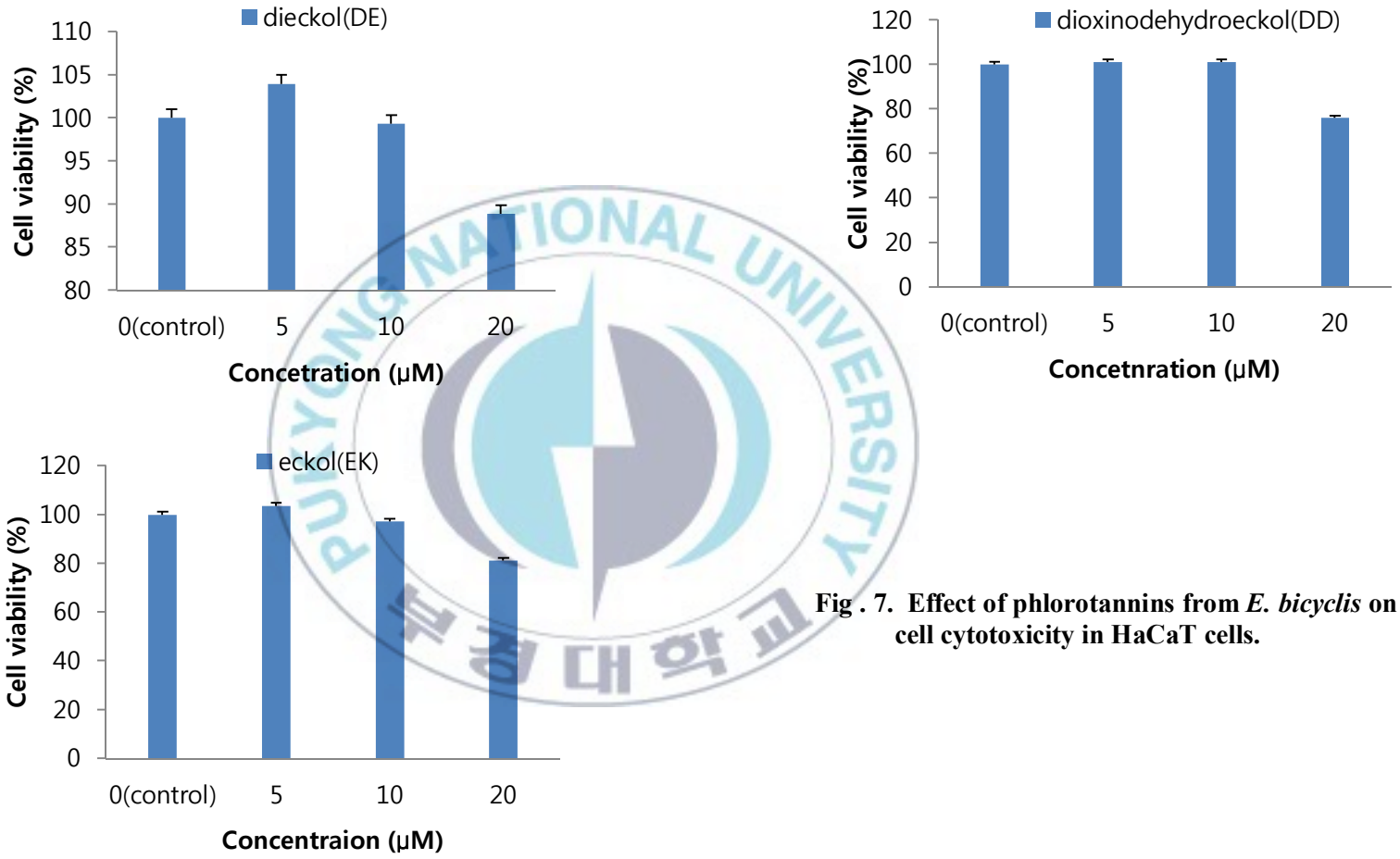

Fig . 7. Effect of phlorotannins from *E. bicyclis* on cell cytotoxicity in HaCaT cells.

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isolate phlorotannins from *E. bicyclis* for evaluating its anti-inflammatory effects against *P. acne*. For the purpose, the EtOAc-soluble extract of *E. bicyclis* was subjected to isolate active compounds.

The EtOAc-soluble extract (23.8 g) was chromatographed on Sephadex LH-20 column using MeOH as a solvent to yield 7 subfracions (Fig 8). A portion (23.8 g) of the EtOAc extract was chromatographed on Sephadex LH-20 column (4.0 cm i.d. x 50 cm) with MeOH and fractioned into seven subfractions (EF01-EF07). Subfractions EF02 and EF03 were subjected to column chromatography over a LiChroprep RP-18 column (1.1 cm i.d. x 37 cm) with aqueous MeOH to yield pure compounds **1** (55.4 mg) and **2** (3.6 mg). Subfractions of EF04 and EF05 were subjected to Sephadex LH-20 column (1.1 cm i.d. x 38 cm) chromatography and LiChroprep RP-18 (1.1 cm i.d. x 37 cm) with aqueous MeOH to yield pure compounds **3** (6.3 mg) and **4** (5.6 mg). Similarly, subfraction of EF07 was chromatographed over Sephadex LH-20 column (1.1 cm i.d. x 38 cm) chromatography and LiChroprep RP-18 (1.1 cm i.d. x 37 cm) with aqueous MeOH to yield pure compounds **5** (3.6 mg) and **6** (32.9 g).

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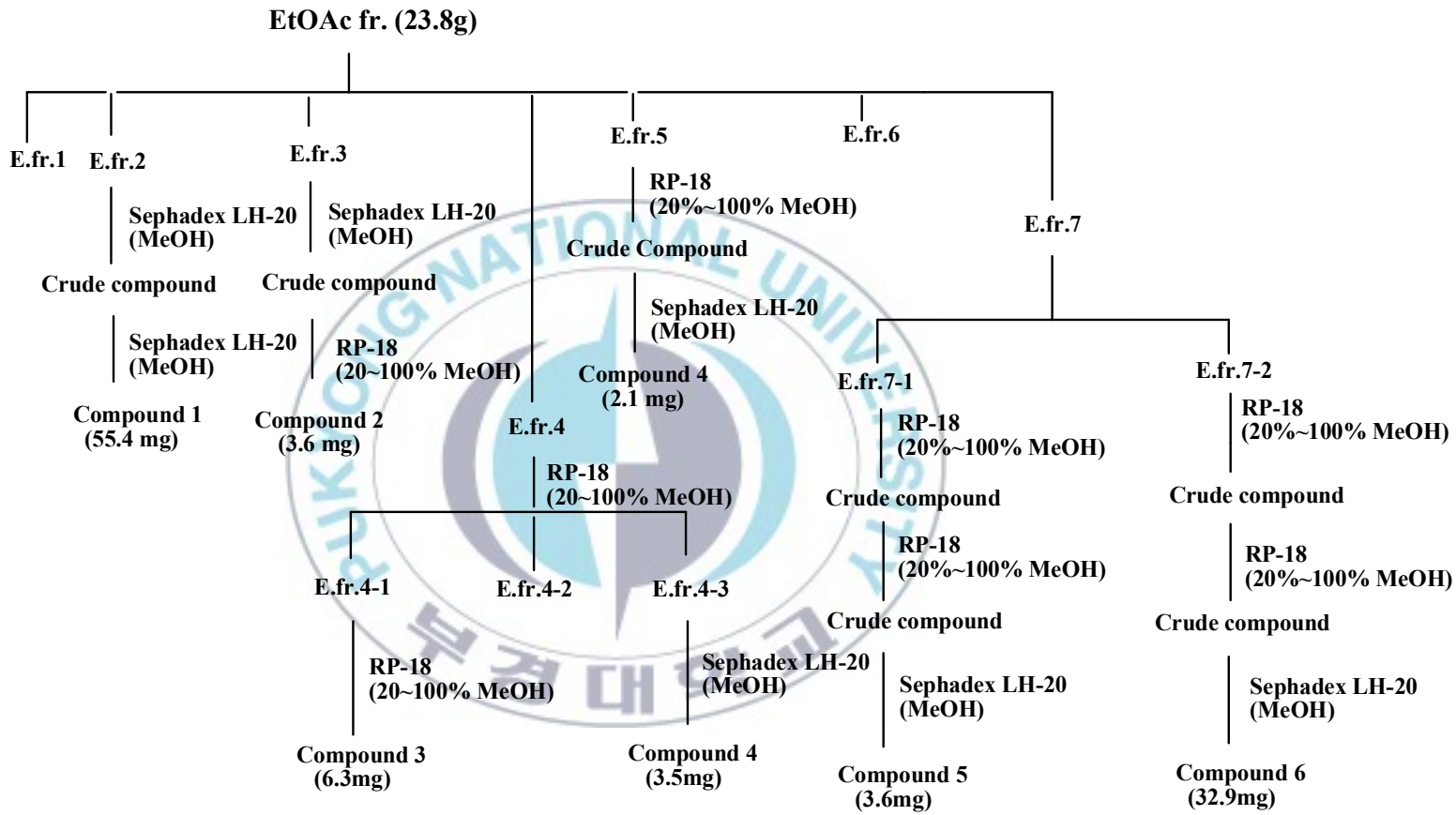

Fig. 8. Isolation of compounds 1-6 from the ethyl acetate-soluble extract of *Eisenia bicyclis*.

8. Identification of compounds isolated from *E. bicylcis*

Successive chromatographic purification of the EtOAc-soluble extract led to the isolation and characterization of six phloroglucinol derivatives: eckol, fucofuroeckol A, 7-phloroecko, dioxinodehydroeckol, PFF, and dieckol. The isolated compounds (Fig. 9) were identified through comparisons of the physicochemical and spectroscopic data (^{1}H and ^{13}C NMR, FAB-MS, HPLC) with those of authentic samples and reference data (Fukuyama et al., 1989; Glombitza et al., 1989; Kang et al., 2003a; Lee et al., 2009; Okada et al., 2004).

Compound 1 (eckol, EK); pale brown powder, FAB-MS *m/z* 373 $[M + H]^{+}$. $C_{18}H_{12}O_9$. ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 9.46 (1H, s, OH-9), 9.41 (1H, s, OH-4), 9.14 (2H, s, OH-2, 7), 9.11 (2H, s, OH-3′, 5′), 6.14 (1H, s, H-3), 5.96 (1H, d, *J* = 2.4 Hz, H-8), 5.80 (1H, d, *J* = 1.8 Hz, H-6), 5.79 (1H, d, J = 3.0 Hz, H-4′), 5.72 (2H, d, *J* = 1.8 Hz, H-2′, 6′). ^{13}C-NMR (DMSO-*d6*, 100MHz) δ: 160.6 (C-1′), 159.0 (C-3′, 5′), 153.2 (C-7), 146.3 (C-9), 146.1 (C-2), 142.8 (C-5a), 142.1 (C-4), 137.4 (C-10a), 123.4 (C-1), 122.9 (C-9a), 122.5 (C-4a), 98.7 (C-8), 98.4 (C-3), 96.4 (C-4′), 93.9 (C-2′), 93.8 (C-6), 93.7 (C-6′); see Table 8 and Fig. 10.

Compound 4 (dioxinodihydroeckol, DD); pale brown powder, FAB-MS *m/z* 371 $[M + H]^{+}$. $C_{18}H_{10}O_9$. ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 9.73 (1H, s, OH-1), 9.59 (1H, s, OH-9), 9.56 (1H, s, OH-6), 9.24 (1H, s, OH-3), 9.23 (1H, s, OH-11), 6.10 (1H, s, H-7), 6.04 (1H, d, *J* = 2.7, Hz, H-2), 6.01 (1H, d, *J* = 2.7 Hz, H-10), 5.84 (1H ,d, *J* = 2.7 Hz, H-4), 5.82 (1H ,d, *J* = 2.7 Hz, H-12). ^{13}C-NMR (DMSO-*d6*, 100MHz) δ: 153.3 (C-3), 153.0

(C-11), 146.3 (C-1), 146.1 (C-9), 142.1 (C-4a), 141.7 (C-12a), 140.1 (C-6), 137.2 (C-7a), 131.6 (C-13b), 125.9 (C-5a), 122.6 (C-8a), 122.4 (C-13a), 122.2 (C-14a), 98.8 (C-2, 10), 97.5 (C-7), 93.9 (C-4, 12); see Table 8 and Fig. 11.

Compound 6 (dieckol, DE); pale brown powder, FAB-MS *m/z* 743 $[M + H]^+$. $C_{36}H_{22}O_{18}$. ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 9.65 (1H, s, OH-9), 9.55 (1H ,s, OH-9"), 9.45 (1H, s, OH-4"), 9.40 (1H ,s, OH-4), 9.31 (2H, s, OH-3□, 5□), 9.23 (1H ,s, OH-2"), 9.18 (1H, s, OH-2), 9.17 (1H, s, OH-7"), 9.10 (2H, s, OH-3′, 5′), 6.16 (1H, s, H-3"), 6.14 (1H, s, H-3), 6.02 (1H ,d, *J* = 3.0 Hz, H-8), 5.99 (1H, d, *J* = 3.0 Hz, H-8"), 5.95 (2H ,s, H-2□, 6□), 5.82 (1H, d, *J* = 3.0 Hz, H-6), 5.81 (1H, d, *J* = 3.0 Hz, H-6"), 5.80 (1H, d, *J* = 1.8 Hz, H-4′), 5.72 (2H ,d, *J* = 1.8 Hz, H-2′, 6′). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 160.2 (C-1′), 158.7 (C-3′), 158.6 (C-5′), 155.8 (C-1□), 154.2 (C-7), 153.0 (C-7"), 151.1 (C-3□ ,5□), 146.0 (C-2, 9"), 145.8 (C-2" , 9), 142.5 (C-5a"), 142.3 (C-5a), 141.9 (C-4"), 141.8 (C-4), 137.2 (C-10a), 137.0 (C-10a"), 124.2 (C-4□), 124.0 (C-9a), 123.2 (C-4a), 123.1 (C-4a"), 122.6 (C-9a"), 122.2 (C-1, 1"), 98.3 (C-3), 98.2 (C-3"), 98.0 (C-8, 8"), 96.1 (C-4′), 94.4 (C-2□ , 6□), 93.8 (C-6"), 93.6 (C-2′, 6′), 93.5 (C-6); see Table 8 and Fig. 12.

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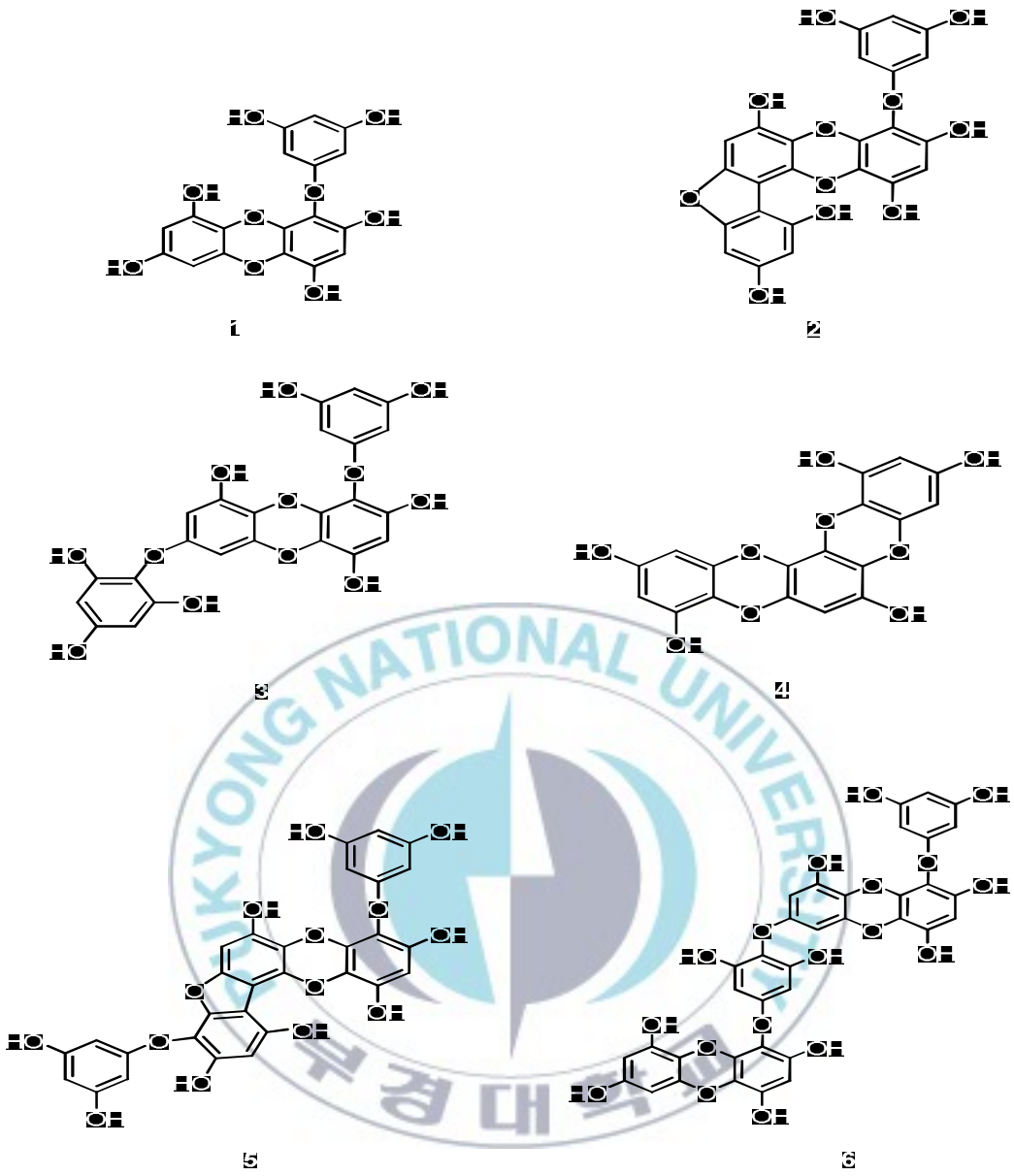

Fig. 9. Structures of the compounds 1-6 isolated from *Eisenia bicyclis*

(Compound **1**, Eckol; Compound **2**, Fucofuroeckol-A;

Compound **3**, 7-Phloroeckol; Compound **4**, dioxinodehydroeckol;

Compound **5**, Phlorofucofuroeckol-A; Compound **6**, Dieckol)

Table. 8. ^{13}C NMR (600MHz) data for isolated phlorotannins (1-6) in DMSO-d_6

Position	Compund **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6**
1	**123.4**	**122.4**	**122.2**	**146.3**	**122.2**	**122.2**
2	**146.1**	**146.8**	**145.8**	**98.8**	**146.5**	**146.0**
3	**98.4**	**98.2**	**98.3**	**153.3**	**97.8**	**98.3**
4	**142.1**	**141.9**	**141.8**	**93.9**	**141.6**	**141.8**
4a	**122.5**	**122.6**	**123.1**	**142.1**	**122.2**	**123.2**
5a	**142.8**	**133.6**	**142.3**	**125.9**	**133.5**	**142.3**
6	**93.8**	**103.1**	**93.4**	**140.1**	**102.8**	**93.5**
7	**153.2**	**102.4**	**154.5**	**97.5**	**103.0**	**154.2**
7a				**137.2**		
8	**98.7**	**150.2**	**98.1**		**146.1**	**98.0**
8a				**122.6**		
9	**146.3**	**98.0**	**145.9**	**146.1**	**98.7**	**145.8**
9a	**122.9**		**123.9**			**124.0**
10		**157.6**		**98.8**	**150.4**	
10a	**137.4**		**137.0**			**137.2**
11		**90.5**		**153.0**	**119.7**	
11a		**158.2**			**149.1**	
12				**93.9**		
12a		**150.4**		**141.7**	**149.9**	
13		**94.6**			**94.4**	
13a				**122.4**		
13b				**131.6**		
14		**144.3**			**144.3**	
14a		**126.1**		**122.2**	**125.9**	
15a		**136.8**			**136.4**	
1′	**160.6**	**160.2**	**160.2**		**159.8**	**160.2**
2′	**93.9**	**93.7**	**93.6**		**93.3**	**93.6**
3′	**159.0**	**158.8**	**158.7**		**158.4**	**158.7**
4′	**96.4**	**96.3**	**96.2**		**95.9**	**96.1**
5′	**159.0**	**158.8**	**158.7**		**158.4**	**158.6**
6′	**93.7**	**93.7**	**93.6**		**93.3**	**93.6**

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1″	122.5	159.5	122.2
2″	151.3	93.1	145.8
3″	94.8	158.6	98.2
4″	154.7	96.1	141.9
4a″			123.1
5″	94.7	158.6	
5a″			142.5
6″	154.8	93.1	93.8
7″			153.0
8″			98.0
9″			146.0
9a″			122.6
10a″			137.0
1‴			155.8
2‴			94.4
3‴			151.1
4‴			124.2
5‴			151.1
6‴			94.4

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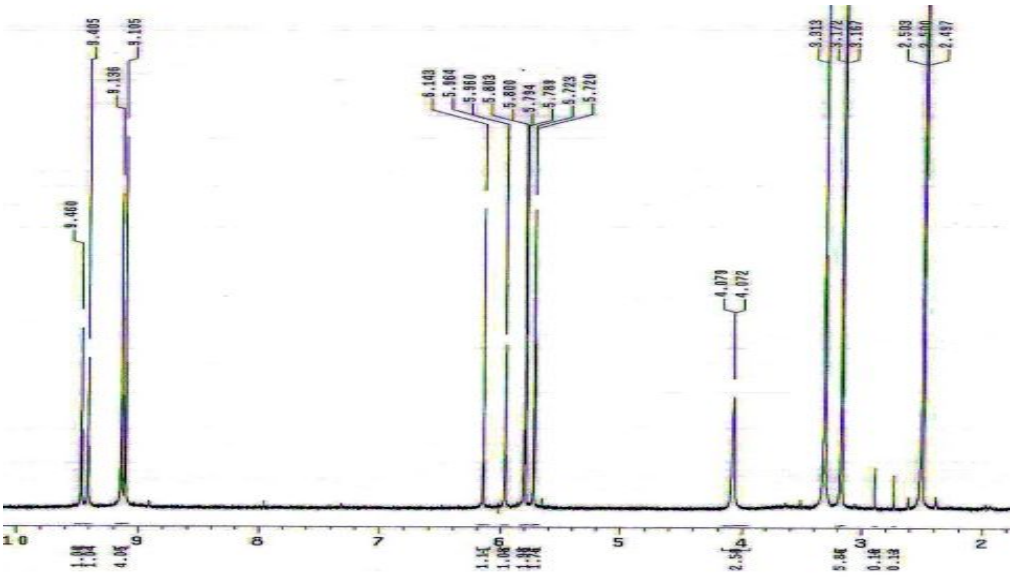
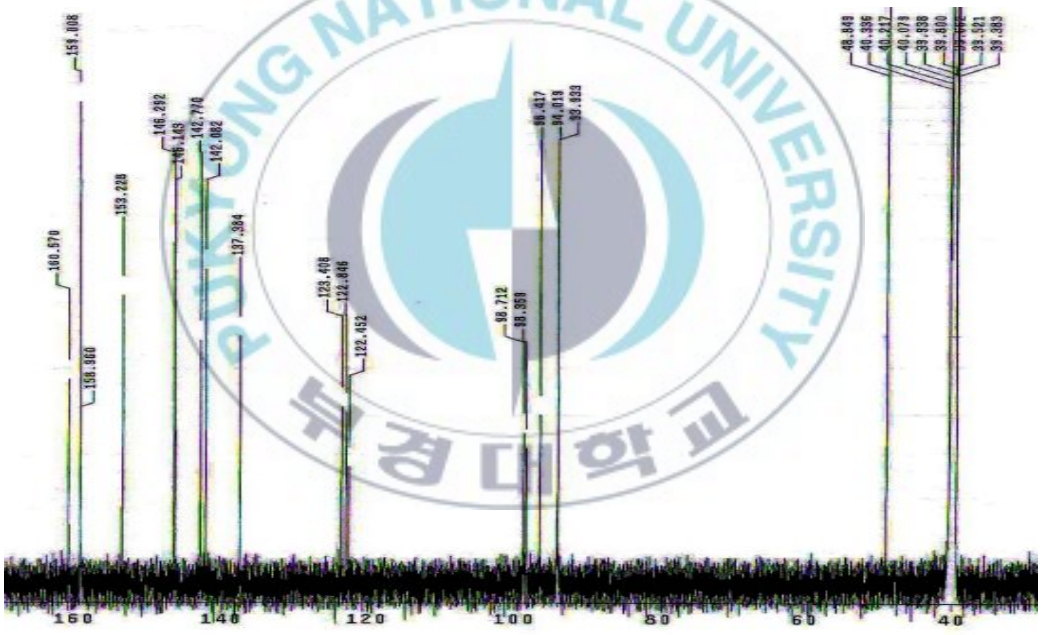
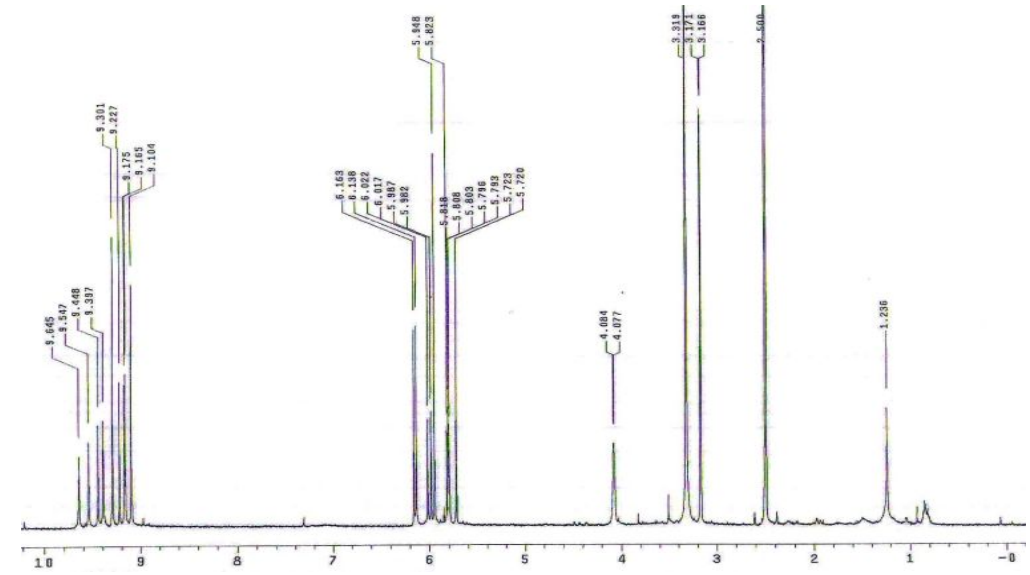


(a)

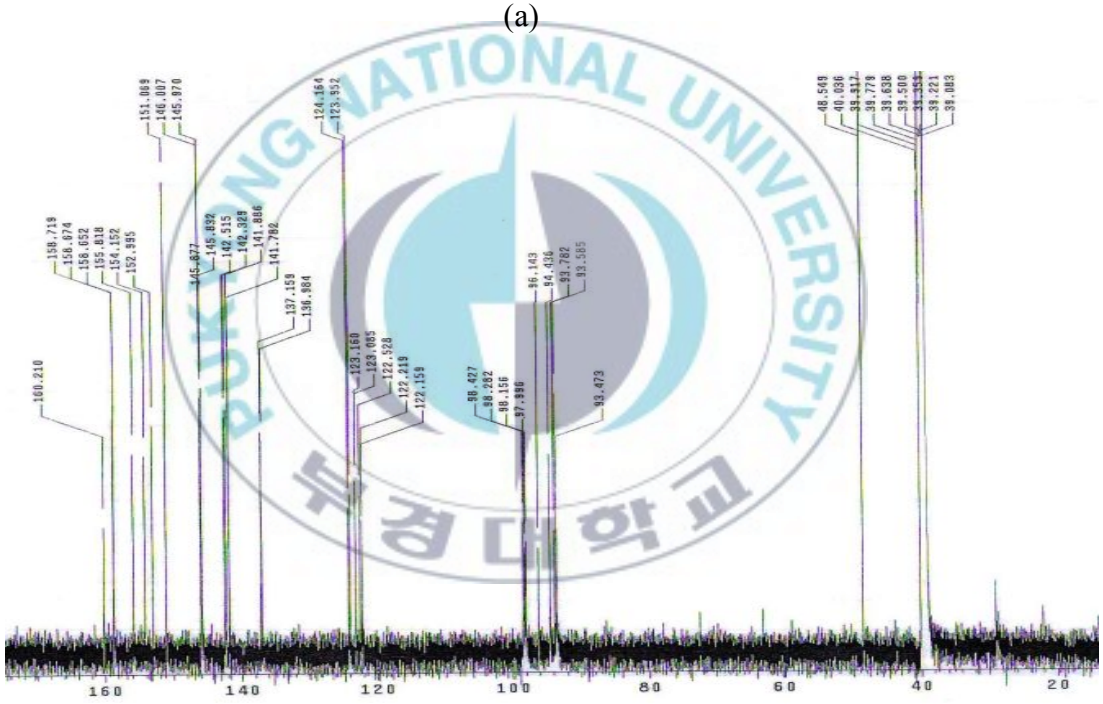


(b)

Fig. 10. ^{1}H-NMR spectrum (a) and ^{13}C-NMR spectrum (b) of compound 1 in DMSO-d_6.

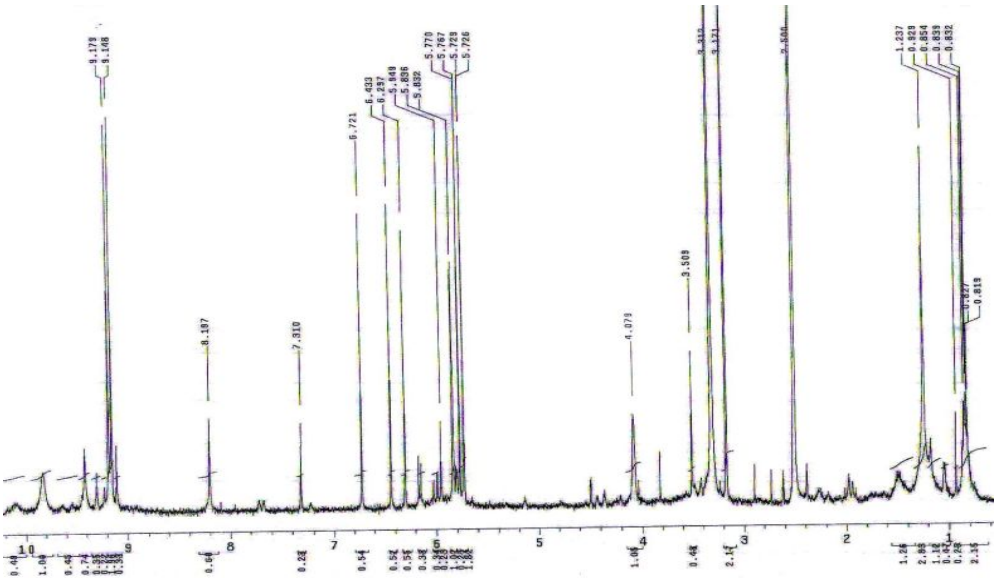


(a)

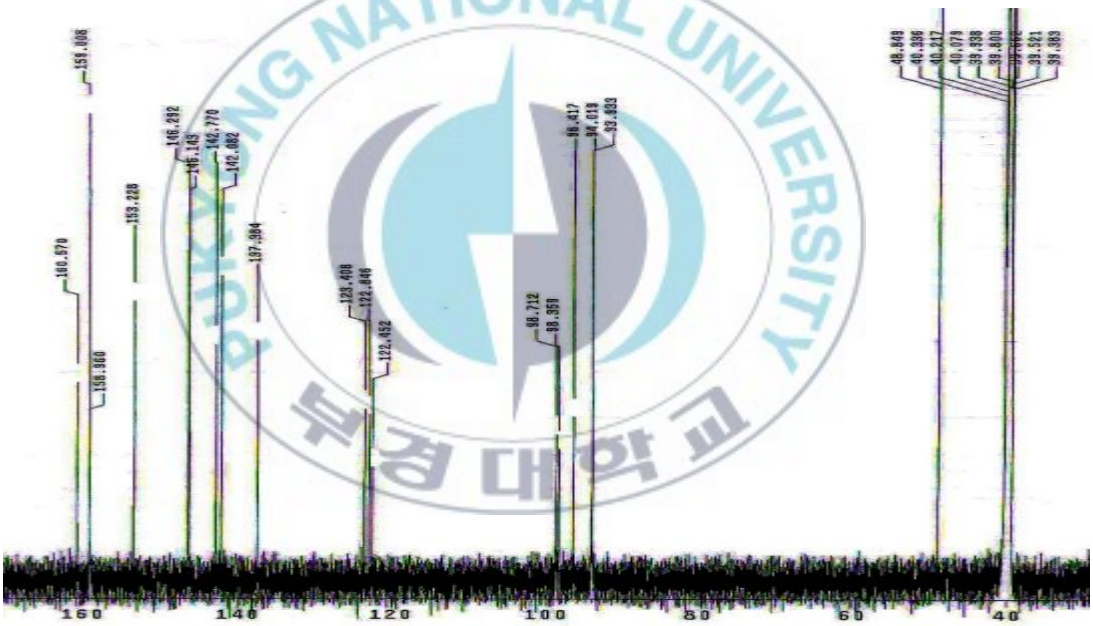


(b)

Fig. 11. ^{1}H-NMR spectrum (a) and ^{13}C-NMR spectrum (b) of compound 4 in DMSO-d_6.



(a)



(b)

Fig. 12. ^{1}H-NMR spectrum (a) and ^{13}C-NMR spectrum (b) of compound 6 in DMSO-d_6.

9. Effect of phlorotannins on the regulation of inflammatory response genes and proteins

Inflammatory processes are mediated by multiple molecular mechanisms. iNOS and COX-2 play a pivotal role in immunity against infectious agents by producing an excess amount of NO and PGE2, respectively; these enzymes have attracted attention for their detrimental roles in inflammation related disease (Yun et al., 1996, Kim et al., 2009) TNF-a, IL-1β and IL-6 are primary inflammatory cytokines which play an essential role during the inflammatory process (Trikha et al., 2003). The pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β are small secreted proteins, which mediate and regulate immunity and inflammation (Huang et al., 2006). TNF-α, one of the pro-inflammatory cytokines produced by *P. acnes* in acne inflammation, plays a critical role in modulating matrix metalloproteinase (MMP) activity in the dermis (Kourbeti and Boumpas, 2005; Numerof and Asadullah, 2006). TNF-α stimulates activation of pro-matrix metalloproteinase (pro MMP)-2 through the activated NF-κB pathway in human dermal fibroblasts (hDF) (Han et al., 2001).

MMPs are group of zinc-dependent endopeptidases that selectively degrade the components of various extracellular matrix as well as non-matrix proteins (Chakrabori et al., 2003). Thus, MMPs are implicated in the remodeling of the extracellular matrix in both physiological and pathological conditions such as wound healing, inflammation, and tumor metastasis (Kleiner and Stetler-Stevenson, 1999; Cook teal., 2000; Kim et al., 2005b). It was evaluated whether phlorotannins from *E. bicyclis* affects the inflammatory

response cytokine mRNA and protein expression in *P. acnes*-induced HaCaT cells, using RT-PCR and Western blot analysis, respectively. The mRNA transcription of MMP-2, MMP-9, iNOS, COX-2, IL-6, IL-1β and TNF-α were reduced by phlorotannins treatment, which was consistent with the results obtained from NO production Dioxinodehydroeckol (DD) has no anti-inflammatory effect against *Propionibacterium acne*. Dieckol (DE) prevented anti-inflammatory cytokines such as IL-6, IL-1β and iNOS by suppressing their mRNA transcription expression in *P. acne*-induced HaCaT cells. Eckol (EK) has the strongest anti-inflammatory effect against *Propionibacterium acne*. MMP-2, MMP-9, TNF-α, COX-2 and iNOS were reduced by eckol treatment (Fig. 13). The protein level of NF-κB, MAPKs and AKt were reduced by phlorotannins treatment as well (Fig. 14).

As a result, *E. bicyclis* was shown to have anti-inflammatory activities by reducing mRNA and protein expression levels of inflammatory cytokines.

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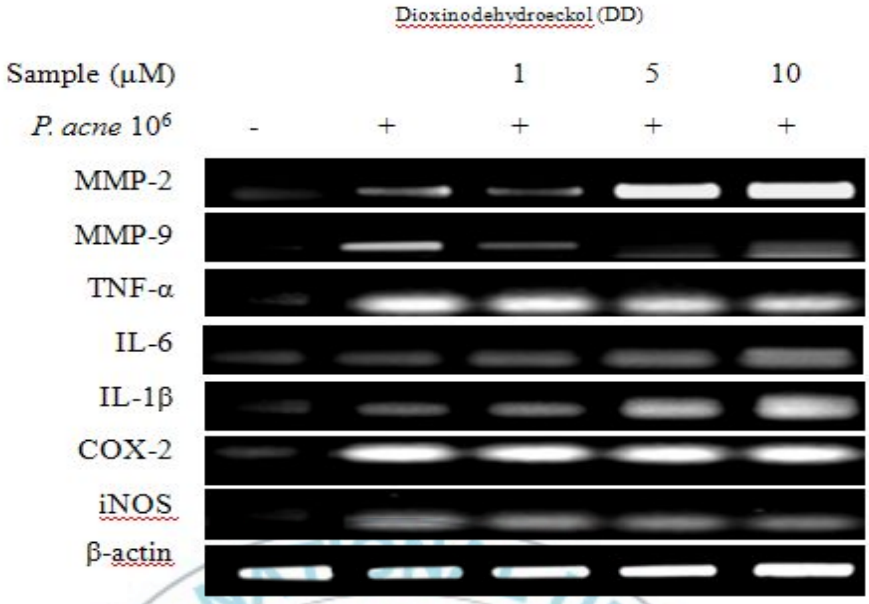
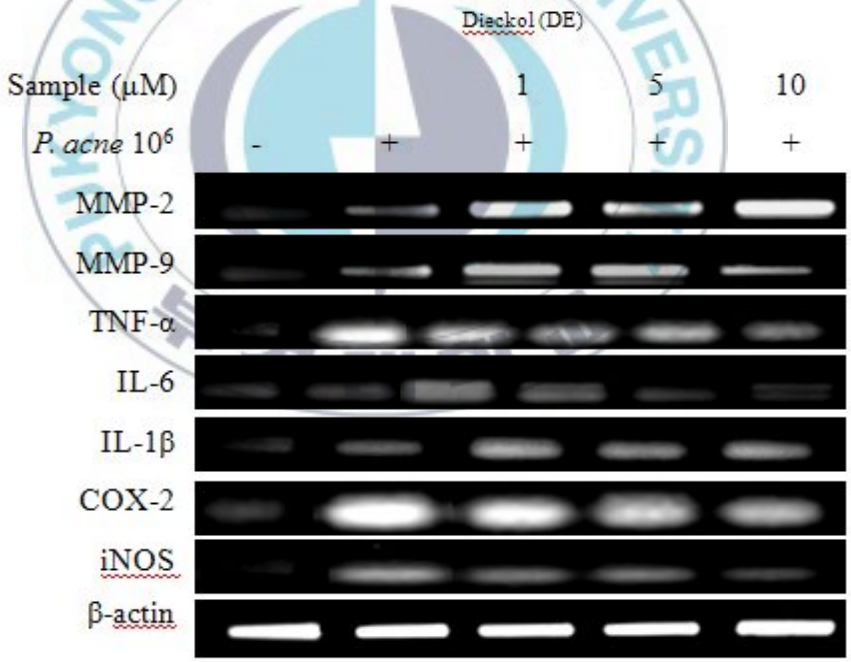
(A)


Dioxinodehydroeckol (DD)
Sample (μM)
1
5
10
P. acne 10^6
-
+
+
+
+
MMP-2
MMP-9
TNF-α
IL-6
IL-1β
COX-2
iNOS
β-actin

(B)


Dieckol (DE)
Sample (μM)
1
5
10
P. acne 10^6
-
+
+
+
+
MMP-2
MMP-9
TNF-α
IL-6
IL-1β
COX-2
iNOS
β-actin

(C)

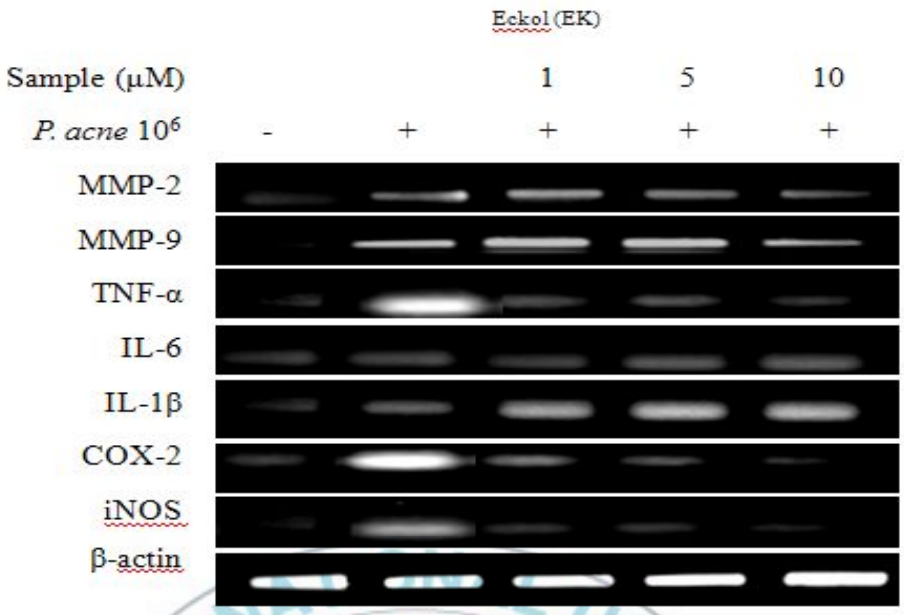


Fig. 13. Effect of phlorotannins on the regulation of MMP-2, MMP-9, TNF-α, IL-6, IL-1β, COX-2 and iNOS expression in *P.acnes* -induced in HaCaT cells.

HaCaT cells were cultured with *Propionibacterium acne* (10^6 CFU/mL) and add various concentrations of phlorotannins. Gene expression levels were determined by RT-PCR. (A): dioxinodehyroeckol (DD); (B): dieckol (DE); (C): Eckol (EK).

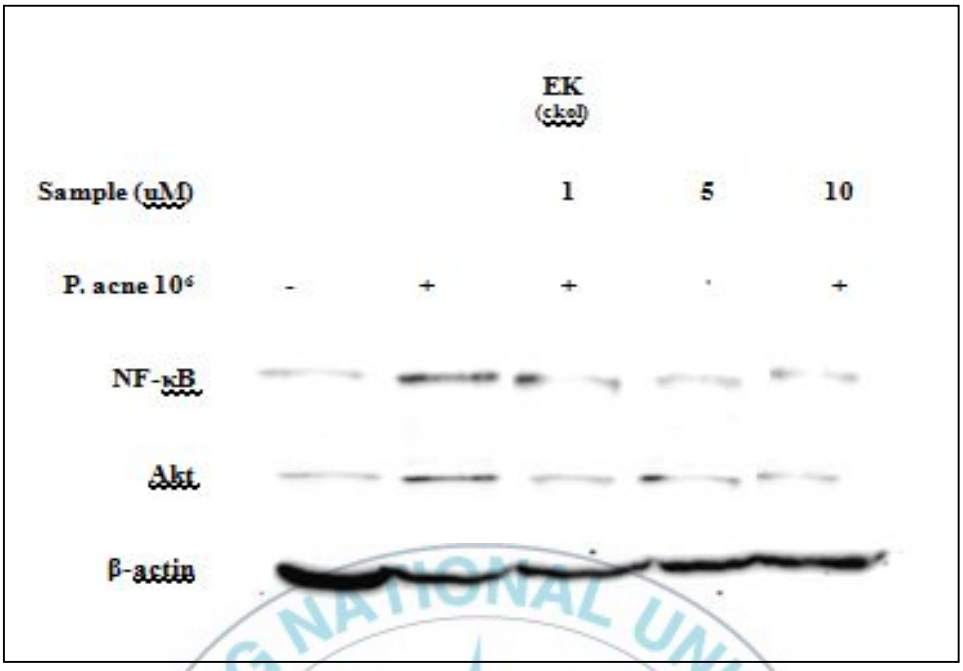


Fig. 14. Effect of Eckol from *E. bicyclis* on the transcriptional activation of nuclear factor kappa B (NF-κB) and AKt in *P. acnes*-induced HaCaT cells.

HaCaT cells were incubated with *Propionibacterium acne* (10^6 CFU /mL) for 72 hrs and then add various concentrations of Eckol. Protein expression levels were determined by Western blot analysis.

10. Effect of phlorotannins on NF-κB signaling pathway

Nuclear factor kappa B (NF-κB) is located predominantly in the cytoplasm in unstimulated cells and is complexed with its inhibitory protein I-κB (IκB). The cytosolic I-κB /NF-κB complex dissociates when IκB is phosphorylated by activated Akt, allowing NF-κB to translocate to the nucleus and promote transcriptional upregulation of various inflammatory mediators including chemokines (M..D. et al.,2006). In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called inhibitor κB (IκB). Various stimuli, such as LPS, cytokines, activators of protein kinase C, oxidants and viruses, activate several signal transduction pathways that all lead to phosphorylation and degradation of IκB and subsequent activation of NF-κB (Hawiger, 2001). Following activation, the NF-κB heterodimer is rapidly translocated to nucleus, where it activates the transcription of target genes, including genes encoding for pro-inflammatory cytokines, adhesion molecules, chemokines and inducible enzymes such as iNOS and COX-2 (Hayden et al., 2006; Ghosh et al., 2008).

The role of the NF-κB signaling pathway in the anti-inflammatory responses in HaCaT cells treated with phlorotannins were confirmed using Western blot analysis (Fig. 14). Inflammatory gene expression can be regulated by NF-κB, which is important for mediating cytokine production in *P. acnes*-induced HaCaT cells. These results indicated that signal transduction of NF-κB might be suppressed by phlorotannins.

Conclusion

This study demonstrated that Eckol phlorotannin effectively inhibited excessive production in inflammatory mediators such as NO, TNF-α, IL-1β and IL-6. These results suggested that Eckol inhibited NO production, biosynthesis of cytokines and expression of inflammatory-related genes in *P. acnes*-activated HaCaT cells. Moreover, these anti-inflammatory profiles of Eckol were mediated through the inhibition of NF-κB transcriptional inductions. Accordingly, these results underscore the nutraceutical value of Eckol as a potential anti-inflammatory agent via attenuation of inflammatory responses or processes.

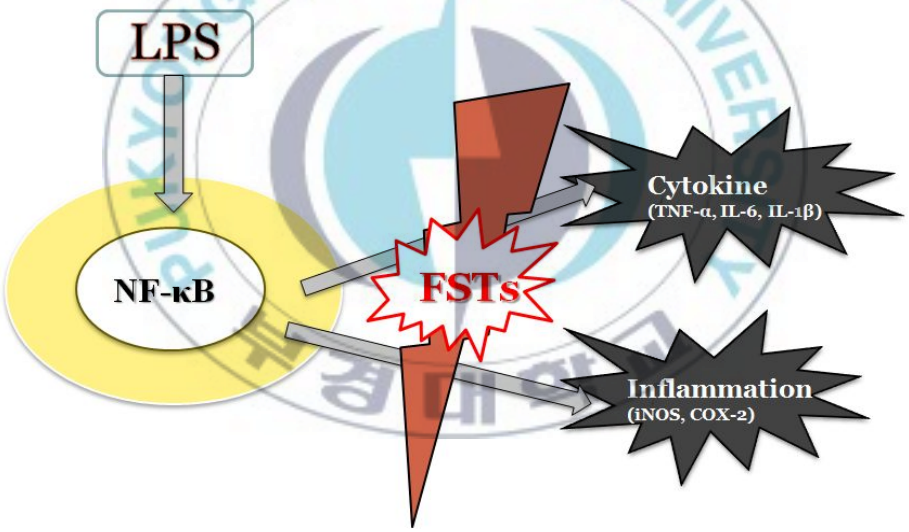


Fig. 15. Effect of phlorotannins against *P.acnes* –induced inflammation in HaCaT cells.

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