A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Hepatoprotective Effects of Dieckol Isolated from
Ecklonia cava against Ethanol-induced Liver Damage;
In vitro and *in vivo*

Min Cheol Kang

Department of Marine Life Science

GRADUATESCHOOL
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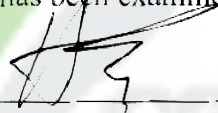JEJU NATIONAL UNIVERSITY

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Hepatoprotective effects of dieckol isolated from *Ecklonia cava* against ethanol-induced liver damage; *in vitro* and *in vivo*

Min Cheol Kang
(Supervised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement
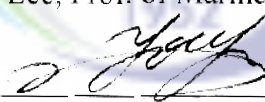For the degree of Master of Science

2011. 02.

This thesis has been examined and approved.

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Thesis director, Moon-Soo Heo, Prof. of Marine Life science

_____
Seungheon Lee, Prof. of Marine Life science

_____
You-Jin Jeon, Prof. of Marine Life science

<u>2011. 02.</u>
Date

Department of Marine Life Science
GRADUATE SCHOOL
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COTENTS

Part Ⅰ. Protective effects of Dieckol isolated from *Ecklonia cava* against ethanol-caused damages in human chang liver cells

Part Ⅱ. Hepatoprotective effects of Ethanol extract from *Ecklonia cava* against ethanol- caused liver damage in Bab/c mice

국문초록

우리나라는 세계에서 5위 안에 속할 정도로 높은 술 소비량을 보이고 있으며, 습관성 음주자의 수는 약 100~200 만 명 정도로 추산되고 있다. 술을 과잉 섭취하여 체내에 혈중 알코올농도가 높아졌을 경우, 활성 산소 종의 유리라디칼을 생성하게 되어, 지질과 산화물을 형성하고 이로 인해, 두뇌와 소화기관 및 심혈관 등의 신체에 악영향을 미친다는 많은 국내외에서 여러 연구 결과가 보고되고 있다. 특히, 간은 알코올 대사과정에서 중요한 역할을 하는 기관으로 알코올성 지방간과 알코올성 간염 및 알코올성 간질환과 간에 치명적인 영향을 받는다고 보고되어 왔다. 따라서, 많은 연구자들은 알코올성 질병의 치료를 위해 다양한 육상식물의 간 보호효과를 연구해왔고, 지금도 간 보호효과를 보이는 새로운 천연 소재를 연구하고 개발하기 위해 노력하고 있다.

해조류는 풍부한 미네랄과 비타민, 다당류 및 polyphenol이 풍부하게 함유되어 있다. 특히 해조류 중, 갈조류에 속하는 감태 (*Ecklonia cava*)는 다당류와 polyphenol 성분이 주로 함유되어 있고, 그 중, polyphenol 성분인 phlorotannin이 항산화, 항암, 항고혈압, 항당뇨, 항균 및 항바이러스 등과 같은 다양한 생리활성효과를 가진다고 보고되어 왔다. 그러나 해양유래의 유용성분을 이용한 간 보호효과에 대한 연구는 미비한 단계이다. 따라서, 이 연구에서는 제주도 연안에 서식하고 있는 20여종의 갈조류로부터 에탄올로 유도된 간 독성에 대해 가장 높은 Chang cell 보호효과를 보인 감태 (*Ecklonia cava*)를 가지는 in

vitro와 in vivo상에서 간 보호효과를 밝히고 그 기전을 밝히고자 했다.

먼저, Part 1에서는 20여종의 갈조류로부터 제조된 70% 에탄올 추출물이 인간 유래 간세포 Chang cell에 대한 에탄올 보호효과를 가지는지를 확인하였다. 그 결과, 감태의 phlorotanin 성분인 dieckol과 4% 에탄올이 처리된 Chang cell에서 Extracellular Signal Regulated kinase (ERK)의 활성화를 유도하면서, apoptosis를 조절하여 세포의 DNA 손상을 감소시켜 세포를 보호한다는 것을 확인하였다. 또한, Part 2에서는 에탄올을 투여한 마우스에서 감태 에탄올 추출물이 간 보호효과를 가지는 지를 확인하였다. 그 결과, 에탄올을 투여한 마우스의 생존율과 체중이 에탄올을 처리하지 않은 마우스에 비해 상당히 감소하였는데, 감태 에탄올 추출물을 경구 투여한 마우스는 생존율과 체중이 상당히 증가하였다. 또한 간 손상 척도로 사용되는 Glutarnic oxaloacetic transaminase (GOT)와 Glutarnic pyruvic transminase (GPT) 및 콜레스테롤 함량은 에탄올 투여 마우스에 비해, 감태 에탄올 추출물 투여 마우스군에서 감소하는 경향을 관찰할 수 있었다. 그리고, 조직학적 변화를 확인한 결과에서도 에탄올 투여 시, 지방이 증가하였으며 세포의 손상이 다소 관찰되었으나 감태 에탄올 추출물 투여군에서는 감소시켰다. 이상의 결과를 종합해 보면, 감태 에탄올 추출물에서 분리한 phlorotanin 계열의 성분인 dieckol이 Chang cell의 보호효과를 보였으며, 또한 에탄올로 간손상을 유발한 Balb/c 마우스에서도 간 보호효과를 나타내는 것으로 볼 때, 감태 에탄올 추출물의 간보호효과를 확인할 수 있었다.

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24 h with 4% ethanol in DMEM medium. Experiments were performed in triplicate and thedata are expressed as mean ± SE. (*, P<0.05; **,P<0.01)

Fig. 1-10. Protective effect of isolated compounds from *E. cava* on ethanol induced chang cell damage of fibroblasts. The fibroblasts were treated with various concentrations of isolated compounds from *E. cava* and after 1h later, ethanol at 4% was applied to the chang cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining A: untreated, B: 4% ethanol C: Dieckol 25 μg/ml, D: Eckol 25 μg/ml E: Phloroglucinol 25 μg/ml.

Fig. 1-11. The hepatoprotective effect of isolated compounds from *E.cava* on ethanol - induced cytotoxicity in chang cells. Cytotoxicity was determined using the LDH method. Cytotoxicity was assessed after incubating for 24 h with 4% ethanol in DMEM medium. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **,P<0.01)

Fig. 1-12. Protective effect of isolated compounds from *E. cava* on ethanol induced chang cell damage of fibroblasts. The fibroblasts were treated with various concentrations of isolated compounds from *E. cava* and after 1h, ethanol at 4% was applied to the chang cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining A: untreated, B: 4%

ethanol C: Dieckol 25 μg/ml, D: : Dieckol 50 μg/ml.

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INTRODUCTION

Alcohol, the substance most frequently abused by humans normally causes harmful effects on several organs; in particular, it promotes gastrointestinal disorders such as gastric mucosa, gastritis, gastric and liver disorders such as alcoholic hepatitiss, steatosis, alcoholic hepatitis and cirrhosis (ALEYNIK SI and LIEBER CS,2003;Tsukamoto H and Lu SC 2001). In addition, chronic excessive ethanol intake has known as a main cause of alcoholic liver disease which is one of the most serious liver disorders throughout the world (Mandayam et al, 2004, Pari and Karthikesan, 2007). With these points, when it considered that the liver is an important organ for the biotransformation and detoxification of endogenous and exogenous harmful substances, researches for the therapy of liver diseases and development of its potential products are required. Interestingly, many researchers have investigated potential products using natural resources such as plants and seaweeds without harmful to treat the liver diseases for several years and recent studies have been also focused on their hepatic-protective effects (Choi et al. 2010, Khanal). Indeed, the recent studies have indicated that saponins, epigallocatechin-3-gallated (-), bicyclol led to hepatic-protective effects against damages caused by treatment of ethanol in liver cells or mice. In addition, many researchers have demonstrated that the hepatoprotective effects of natural

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products were affected by their antioxidant effects such as reduction of ROS and/or increment of antioxidant enzymes and cytoprotective capacities such as inhibition of DNA damages and/or apoptosis (Husain et al.2001; Molina et al., 2003). In particular, previous studies have also reported that polyphenol from seaweed and terrestrial plants have antioxidant and cytoprotective capacities in cells and mice and their capacities affected to induce hepatoprotective effects (Zhao J et al., 2008). Therefore, natural products showing the biological capacities might be good candidates for therapy of liver diseases caused by ethanol treatment.

Among the candidates, seaweeds and their active compounds such as polyphenols and polysaccharides can be potential candidates for natural hepatoprotectors. *Ecklonia cava*, an edible brown seaweed have been indicated various biological activities such as antioxidant, cytoprotective and radioprotective effects (Nagayama et al., 2002; Mayer and Hamann, 2004; Athukorala and Jeon, 2005; Kotake-Nara et al., 2005; Heo et al., 2008). Especially, its polyphenolic compounds consisted of phloroglucinol, eckol, dieckol and phlorofucofuroeckol have recently showed the excellent antioxidant and protective effects against oxidative stresses caused by exposure of radiation, ultraviolet, and hydrogen peroxide (Kang et al., 2005; Ahn et al., 2007). However, there is no report about their hepatoprotective effects until now.

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Therefore, in this study, Part 1 demonstrates that dieckol, a polyphenolic conpoound of *E. cava* induced the hepatoprotective effects by inhibiting damages as regulating apoptosis-related molecules via the activation of ERK signal in human liver chang cells. Then, Part 2 indicates that dieckol rescued mice by enhancement of antioxidant system as inducing the increment of antioxidant and hepatic enzymes against liver damages caused by ethanol.

Part Ⅰ.

Protective effects of Dieckol isolated from *Ecklonia cava* against ethanol-caused damages in human chang liver cells

Protective effects of Dieckol isolated from *Ecklonia cava* against ethanol-caused damages in human chang liver cells

1. ABSTRACT

The protective effects of dieckol isolated from marine brown alga, *Ecklonia cava* against ethanol-induced cell damage and apoptosis were investigated *in vitro*. *E. cava* was selected as our targeting material among twenty brown marine algae due to the less Cytotoxicity and higher cell viability. The cell viability of 80% ethanol extract from *E. cava* was also determined. The cell viability was 61.9 and 67.73% at the concentration of 50 and 100 μg/ml, respectively. Furthermore, *E. cava* ethanol extract showed the protective effect against cell apoptosis induced by ethanol, which was investigated via nuclear staining with Hoechst 33342 and Flow cytometry. Thus, we tried to isolate the active compounds from ethanol. Three compounds including phloroglucinol, eckol and dieckol were successively isolated and identified. The structures of the isolated compounds were determined by analysis of EI-MS and NMR spectra data. Due to the highest antioxidant and lowest cytotoxicity activities,

dieckol was further studied on cell viability, lactate dehydrogenase (LDH) inhibition and protective effect against cell apoptosis. At the concentration of 25 and 50 µg/ml, dieckol showed a concentration-dependent protective effect on cell viability and inhibition of LDH production. From the results of apoptotic body investigated via nuclear staining with Hoechst 33342 and Flow cytometry. Dieckol was also found to protect ethanol-induced cell apoptosis. Finally, the mechanisms of protective effect against ethanol-induced apoptosis were also studied using western blot analysis. From the results, pretreatment with dieckol can protect the cell apoptosis through activation of mitogen activated protein kinase (MAPK) signaling and down-regulation of Bax, activation of Bcl-xL, down-regulation of caspase-3 and PARP. In conclusion, dieckol isolated from *E. cava* could be used to protect the ethanol-induced liver disease.

2. MATERIALS AND METHODS

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), medium, fetal bovine serum (FBS), penicillin–streptomycin and trypsine-EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, Dihydroethidium (DE), propidium iodide (PI), dimethyl sulfoxide (DMSO) and Hoechst 33342 were purchased from Sigma (St. Louis, MO). DNA ladder size markers were bought from Invitrogen (Carlsbad, CA). mitogen activated protein kinase (MAPK) signaling and d own-regulation of Bax, activation of Bcl-xL, down-regulation of caspase-3 and PARP antibodies used in this study were purchased from Cell Signaling Technology (Bedford, Massachusetts). The other chemicals and reagents used were of analytical grade.

2.2. Isolation of phlorotannins from *Ecklonia cava*

Twenty species of brown algae were collected along the coast of Jeju island, Korea, between October 2008 and March 2009 (Table 1-1). To remove salt, sand, and epiphytes

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attached to the surface, the seaweeds were washed three times with tap water and maintained

in a medical refrigerator at -20˚C. The frozen samples were pulverized with a grinder prior

to extraction. Evaporation and dissolved in DMSO, and then used for experiments, adjusting

the final concentration of DMSO in culture medium to <0.1%.

The dried *E. cava* powder (500 g) was extracted with 80% aqueous ethanol, cell and filtered.

The filtrate was evaporated at 40˚C to obtain ethanol extract, which was suspended in water,

then extracted with EtOAc. The EtOAc extract (45.65g) was mixed with celite. The mixed

celite was dried and packed into a glass column, and eluted in the order of hexane,

methylene chloride, diethyl ether, and methanol. The diethyl ether fraction (26.69 g) was

subjected to Sephadex LH-20 column chromatography using stepwise gradient

chloroform/methanol (2/1→0/1) solvents system, and then finally purified by reversed-phase

HPLC to give compound Phloroglucinol, Eckol and Dieckol (Fig. 1-1).

Phloroglucinol : white powder; IR (KBr) v_{max} 3481, 1617 cm-1; ^{1}H-NMR (400MHz,

CD$_3$OD): δ 5.78 (3H, s, H-2, 4, 6); ^{13}C-NMR (100 MHz, CD3OD): δ 160.9 (C-1,3, 5), 96.3

(C-2, 4, 6); EIMS m/z 126 [M]$^+$ (C$_6$H$_6$O$_3$).

Eckol: amorphous powder; IR (KBr) $v$max 3250, 1605 cm^{-1}; UV (MeOH): λ_{max} nm (log ε)

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230 (4.4), 290 (3.4); 1H and 13C NMR; HREIMS m/z 372.0460 [M]+ (calcd for $C_{18}H_{12}H_9$,

372.0481).

Dieckol : amorphous powder; IR (KBr) νmax 3250, 1605 cm-1; UV (EtOH): λmax nm 225;

1H and 13C NMR; FABMS m/z 742 [M]+ ($C_{36}H_{22}H_{18}$).

Table 1-1. List of brown algae used in this study

No.	Scientific name (Korean name)
1	*Dictyota coriacea* (참가죽 그물 바탕말)
2	*Sargassum thunbergii* (지충이)
3	*Myelophycus Caespitosus* (바위수염)
4	*Ishige okamurae* (패)
5	*Ishige foliacea* (넓패)
6	*Padina arborescens* (부챗말)
7	*Hizikia fusiforme* (톳)
8	*Undaria Wrightii* (미역)
9	*Sargassum confusum*(알송이모자반)
10	*Sargassum serratifolium* (톱니모자반)
11	*Sargassum horneri* (괭생이모자반)
12	*Sargassum hemiphyllum* (짝잎모자반)
13	*Sargassum piluiferum* (구슬모자반)
14	*Sargassum patens* (쌍발이모자반)
15	*Sargassum tortile* (꽈배기모자반)
16	*Undariopsis peterseniana* (넓미역)
17	*Myagropsis yendoi* (외톨개모자반)
18	*Sargassum macrocarpum C* (큰열매모자반)
19	*Sargassum ringgoldianum* (큰잎모자반)
20	*Eckloinia cava* (감태)

Fig.1-1. Isolation scheme of phlorotannins from *E. cava*

2.3. Cell culture

Chang liver cell line was grown in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in 5 % CO_2 incubator.

2.4. Assessment of cell viability

Cell viability was then estimated via an MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is also a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/ml. After 16 h, the cells were treated with 80% of ethanol extracts prepared from 20 marine algae and *E. cava* and its phlorotannins containing phloroglucinol, eckol, and dieckol. Then, 10 μl of ethanol (4%) was added to the cell culture medium, and more incubated for 24 h at 37°C. MTT stock solution (50 μl; 2 mg/ml) was then applied to each of the wells, to a total reaction volume of 200 μl. After 4 h of incubation, the plates were centrifuged for 5 min at 800 × *g*, and the

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supernatants were aspirated. The formazan absorbances were measured via ELISA reader at

a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the

quantity of MTT converted to the insoluble formazan salt. The optical density of the

formazan generated in the control cells was considered to present 100% viability. The data

are expressed as mean percentage of the viable cells versus the respective control.

2.6. Nuclear staining with Hoechst 33342

To identify effects of *E. cava* on changes of nuclear morphology of human liver chang cells

after ethanol treatment, Hoechst 33342 staining assay was performed. Normally, cells with

homogenyeously stained nuclei were considered viable, whereas the presence of chromatin

condensation and/or fragmentation was indicative of apoptosis (Lizard et al., 1995). The

Chang liver cells were placed in 24-well plates at a concentration of 1.0×10^5 cells/ml. After

16 h, the cells were treated with 80% ethanol extracts and phlorotannins of *E. cava*

containing phloroglucinol, eckol, and dieckol, and further incubated for 1 h prior to exposure

to 4% ethanol. After 12 h, 1.5 µl of Hoechst 33342 (stock 10 mg/ml), a DNA-specific

fluorescent dye, was added to each well, followed by 10min of incubation at 37˚C. The

stained cells were then observed under a fluorescence microscope equipped with a

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CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear of condensation.

2.7. Cell cycle analysis

To check whether *E. cava* reduces the proportion of apoptotic sub-G1 hypodiploid cells increased by ethanol treatment, propidium iodide (PI) staining assay was used according to a method suggested by Nicoletti et al. 1991. The Chang liver cells were placed in a 6-well plate at a concentration of 4.0×10^5 cells/ml. The cells were treated with 80% ethanol extract and phlorotannins of *E. cava* containing phloroglucinol, eckol, and dieckol and further incubated for 1 h prior to exposure to ethanol (4%). After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS and incubated in the dark in 1 ml of PBS containing PI of 100 μg and RNase of 100 μg A for 30 min at 37°C. Flow cytometric analysis was performed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, U.S.A). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit.

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2.5. LDH cytotoxicity Assay

Then, LDH cytotoxicity Assay was performed to identify cytotoxicity of dieckol on Chang liver cells. For the assay, Chang liver cells (1.5×10^5 cells/ml) pre-incubated in 96-well plates for 16 h were incubated with 80% ethanol extract and phlorotannins of *E. cava* containing phloroglucinol, eckol, and dieckol for 1 h. After treatment of 4% ethanol, the cells were more incubated for 24 h. The medium was carefully removed from each well, and the LDH activity in the medium was determined using an LDH cytotoxicity detection kit (Madison, WI, USA). Briefly, 100 μl of reaction mixture were added to each well, and the reaction was incubated for 30 min at room temperature in the dark. The absorbance of each well was measured at 490 nm using a UV spectrophotometer.

2.8. Western blot analysis

To investigate whether cytoprotective effects of dieckol are related with the expressions of apoptotic molecules such as Bax, Bcl-xL, cleaved caspase-3, PARP and p-ERK Western blot analysis was performed. First, the cells (2×10^5 cells/ml) were treated with dieckol for 1 h before a challenge with 4% ethanol. After incubation of 6 h, the cells were harvested. The

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cell lysates were prepared by using lysis buffer (50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l

NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). The protein concentrations of the

cell lysates were determined by using BCA^{TM} protein assay kit (Thermo SCIENTIFIC,

Rockford, USA). The protein (30 μg/well) preparations were loaded into each lane of

sodium dodecyl sulfate-polyacrylamide gels and electrophoresed under denaturing

conditions. Subsequently, the proteins were electro-transferred onto nitrocellulose transfer

membranes (Schleicher and Schuell, Keene, NH, USA). After blocking with 5% bovine

serum albumin (BSA) for 1 hrs, the blots were incubated with Anti-Bax (1:1000 dilution,

Cell signalling), Bcl-xL (1:1000 dilution, Cell signalling), cleaved caspase-3 (1:1000

dilution, Cell signaling), PARP (1:1000 dilution, Cell signalling), or β-actin (1:5000 dilution,

Sigma) protein antibody for 60 min followed by incubation with horseradish peroxidase-

conjugated anti-mouse or anti-rabbit IgG (1:2000, Santa Cruz Biotechnology, Inc) for as

secondary antibody 45 min. Visualization was achieved by using ECL reagents (Amersham

Life Science, Buckinghamshire, UK).

2.9. Statistical analysis

Data was analyzed using the statistical package for the social science (SPSS) package for

Windows (Version 8). Values were expressed as means ± standard error (SE). A *p*-value of

less than 0.05 was considered significant.

3. Results

3.1. Protective effects of brown algae against ethanol-induced injury to Chang cells.

In the present study, we have screened the cytotoxicity and protective effects against ethanol-induced cell damage of 20 brown algae in Chang cell line by MTT assay. When the Chang cell was treated with the brown algae extracts, the slight cytotoxic effects were observed. Among the algae, *Ecklonia cava* didn't show cytotoxicity to the Chang cells. The result was present in Fig. 1-2. Further, the concentration of ethanol was also examined for ethanol-induced cell damage experiment. At the tested concentration of 4% ethanol, 50% of the cells were damaged. However, when the cells treated with 5% ethanol, around 60% cells were damaged. Therefore, the concentration of ethanol for further study was optimized to 4% ethanol in PBS solution (Fig. 1-3). The hepato-protective effects of 20 brown algae were examined by MTT assay in ethanol-induced on Chang liver cells. The results are shown in Fig.1-4. Here, the cells damage caused by ethanol-induced cellular toxicity, however, was significantly reduced when the Chang liver cells were pretreated with *E.cava* (63% of cell viability). Thus, further study on hepato-protective effect of extract and active compound from *E. cava* will be carried out.

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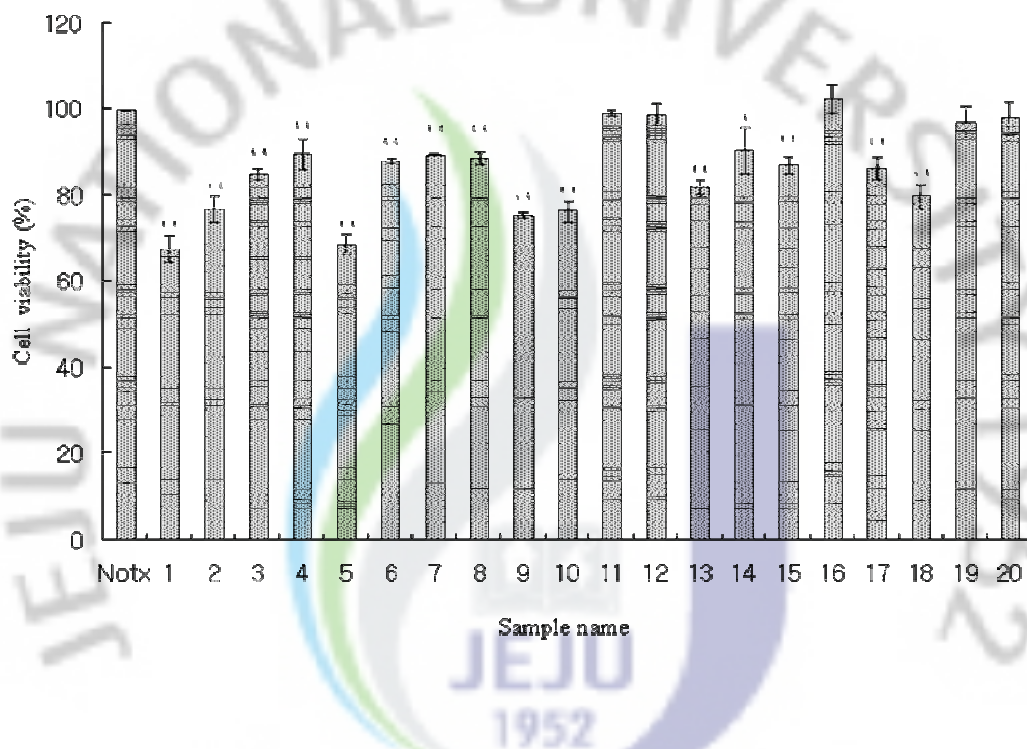
Fig. 1-2. Protective Effect of brown algae in Chang cells by MTT assay. Chang cells were

seeded at a density of 1×10^5 cells/ml in 0.2 ml medium in 96-well plate. Ethanol (4%)

treated for 24 hr. After solubilization of formazan thus formed, absorbance was recorded at

540 nm using microplate reader. Experiments were performed in triplicate and the data are

expressed as mean ± SE. (*, P<0.05; **,P<0.01)
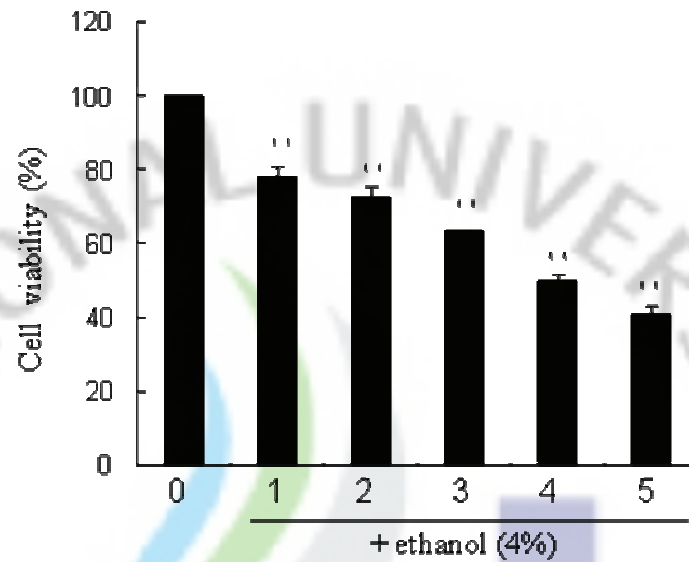
Fig. 1-3. Effect of Ethanol on cell viability in Chang liver cells. (1 x 10^5 cells/ml) were incubated Ethanol for 24hr. The cell viability was measured in three different plates in triplicate using MTT assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **, P<0.01)
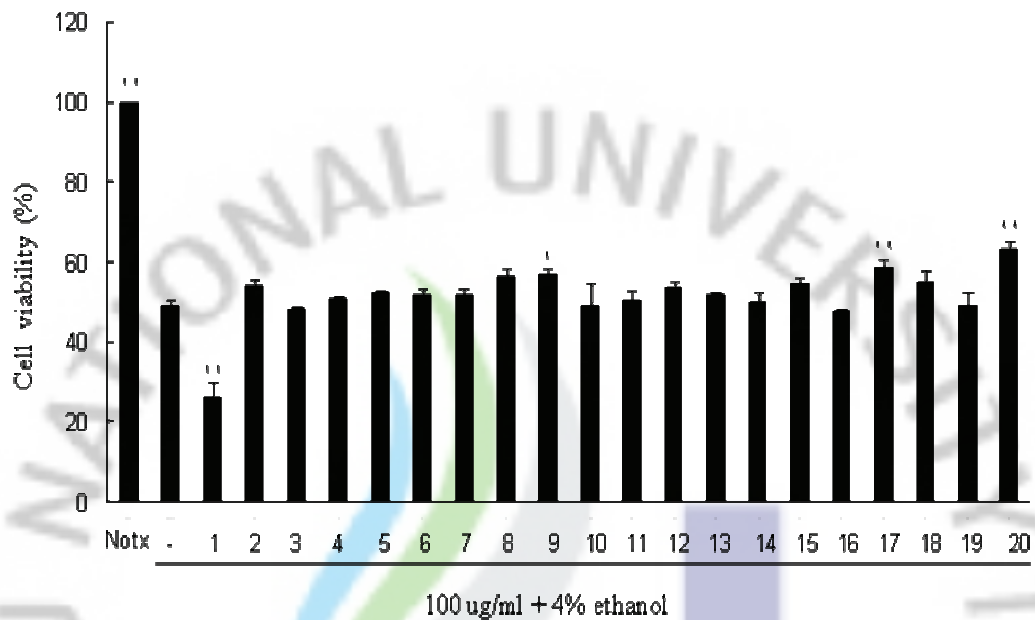
Fig. 1-4. The hepatoprotective effect of brown algae on ethanol-induced cytotoxicity in chang cells. Chang cells were seeded at a density of 1×10^5 cells/ml in 0.2 ml medium in 96-well plate. Brown algae concentrations (100 μg/ml) were treated for 1 hr. After solubilization of formazan thus formed, absorbance was recorded at 540 nm using microplate reader. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **,P<0.01)

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3.2. Protective effect of *Ecklonia cava* against ethanol induced injury to Chang liver cells.

As shown in fig. 1-5, Ethanol extract of *E. cava*, appeared to protect the Chang liver cells against ethanol-induced cell damage. The results of MTT assays showed that ethanol treatment induced cytotoxicity in the Chang liver cells. When cell viability assays were performed on cells after exposure to 50 and 100 μg/ml of 80% ethanol extract of *E. cava* for 24h, followed by exposure to 4% ethanol for 1h, *E. cava* extract was found to have a concentration-dependent protective effect. The cell viability was 61.9 and 67.73% at the concentration of 50 and 100 μg/ml, respectively (Fig.1-6).

The protective effect of 80% ethanol extract of *E. cava* on Chang liver cells apoptosis induced by ethanol were investigated via nuclear staining with Hoechst 33342. Hoechst 33342 dye specifically stains DNA, and is widely used to detect nuclear shrinkage such as chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, which are all indicative of apoptosis. As shown in Fig.1-6, the normal control, which contained no sample or ethanol, possessed intact nuclei (Fig.1-6A), and the negative control (ethanol treated cells) exhibited significant nuclear fragmentation, indicating apoptosis (Fig. 1-6B). However, the addition of 80% ethanol extract of *E. cava* with ethanol reduced the

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apoptotic bodies (Figs. 1-6C and D), which suggested event of an ethanol challenge, and that

the cells were protected from cells damage –related cellular injuries (Fig.1-6). Therefore, the

photographs (Figs. 1-6C and D) clearly suggest the ability of 80% ethanol extract of *E. cava*

to protect cell damage against ethanol attack. The addition to the morphological evaluation,

the protective effects of *E. cava* was confirmed by flow cytometry. Analysis of the DNA

contents following ethanol treatment of Chang liver cells revealed an increase in the

proportion of cells with sub-G_1 DNA content, to 44.3% (Fig. 1-7B). This result indicates that

apoptosis was induced by ethanol. However, cells that were pretreated with 80% ethanol

extract of *E. cava* showed significantly reduced sub-G_1 DNA content, while the percentages

of the *E. cava* extracts treated cells showed 42.2 and 32.2% at 50 and 100 μg/ml respectively

(Fig. 1-7). These results indicate the *E. cava* pretreatment is protective against ethanol-

induced cell death.

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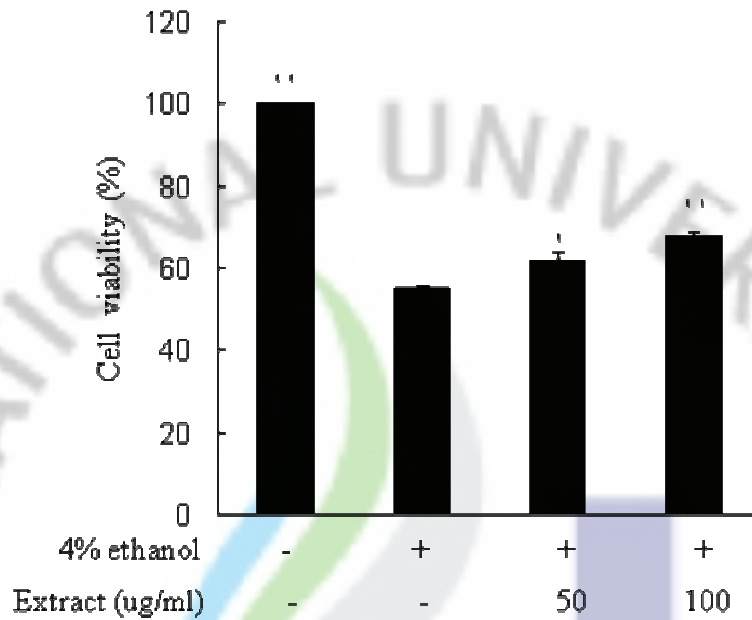
Fig. 1-5. The hepatoprotective effect of *Ecklonia cava* extract on ethanol-induced

cytotoxicity in chang liver cells. Cytotoxicity was assessed after incubating for 24 h with 4%

ethanol in DMEM medium. Experiments were performed in triplicate and the data are

expressed as mean ± SE. (*, P<0.05; **,P<0.01)

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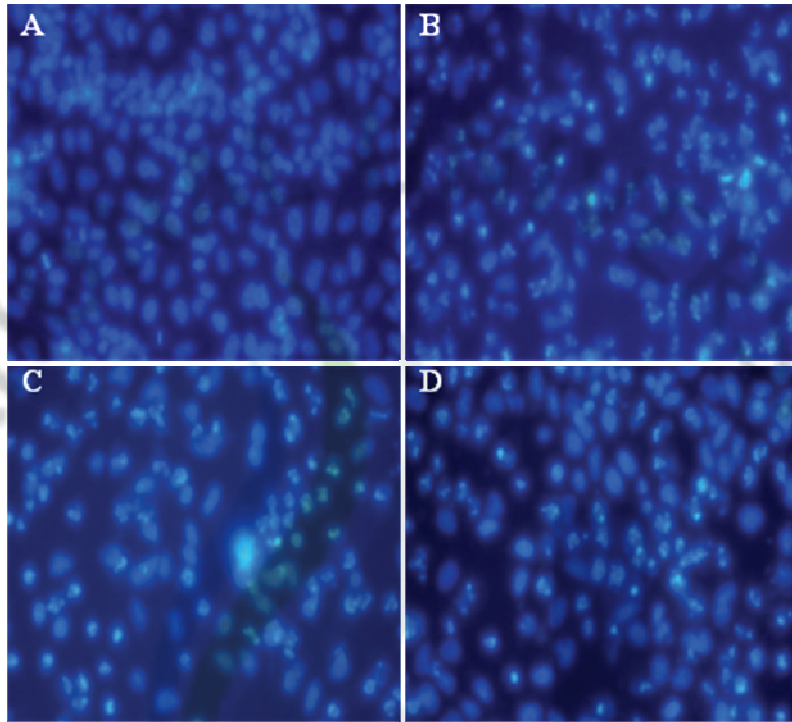
Fig. 1-6. Protective effect of *Ecklonia cava* extract on ethanol induced chang cell damage of fibroblasts. The fibroblasts were treated with various concentrations of *Ecklonia cava* extract and after 1h , 4% ethanol was applied to the chang cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining A : untreated , B: 4% ethanol C: *E. cava* extract 50 μg/ml D: : *E. cava* extract 100 μg/ml.

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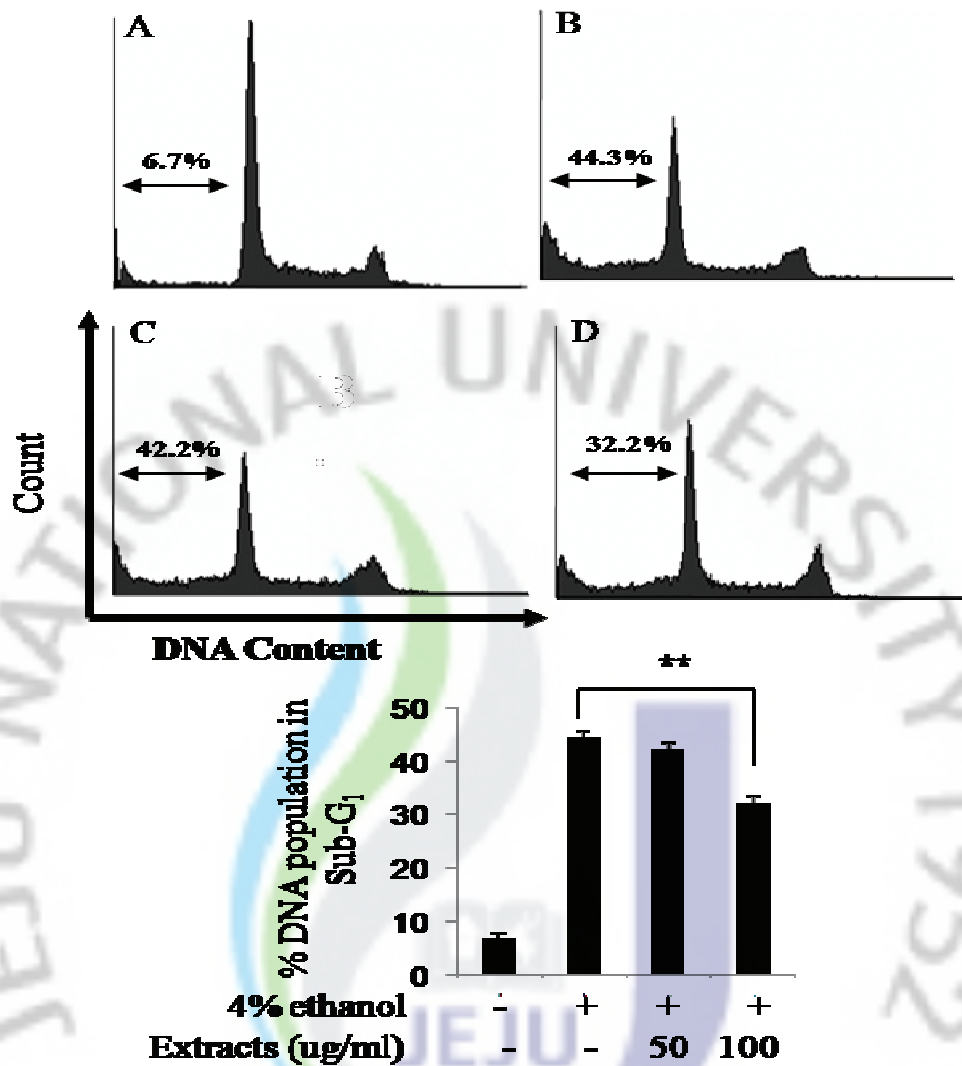
Fig. 1-7. Flow cytometry analysis of propidium iodide stained Chang liver cells treated with

ethanol alone and in presence of ethanol + *Ecklonia cava* extract. (A), control (B), Ethanol

(C), Ethanol + *Ecklonia cava* 50 ug/ml (D), Ethanol + *Ecklonia cava* 100 ug/ml

Experiments were performed in triplicate and the data are expressed as mean ± SE. (*,

P<0.05; **,P<0.01)

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3.3. Isolation and structure determination of phlorotannin compounds from *Ecklonia cava*.

The present study clearly demonstrated that the EtOAc extract was fractionated by celite and Sephadex LH-20 open column chromatography and the active compounds were purified by reversed-phase HPLC.

Phloroglucinol (**1**) was assigned as $C_6H_6O_3$ by combined EIMS and NMR spectrometry. The ^{1}H NMR spectrum exhibits characteristic peak at δ_H 5.78 (H-2, 4, 6) attributable to three phenolic hydroxy protons, and the IR spectrum of 1 showed the presence of hydroxyl group (3481 cm^{-1}).

Eckol (**2**) was isolated as amorphous powder, and its molecular formula deduced as $C_{18}H_{12}O_9$ based on NMR (Table 1-2) and HREI-MS analyses (M$^+$, *m/z*: 372.0460 calcd for $C_{18}H_{12}O_9$ *m/z*: 372.0481), and the IR spectrum of **2** showed the presence of 3250 (OH) and 1605 (aromatic) cm^{-1}. The ^{13}C-NMR spectrum indicated the presence of six non-substituted and twelve O-bearing aromatic carbons, whereas the ^{1}H-NMR spectrum contained signals characteristic of six aromatic protons as well as six phenolic hydroxyl protons.

Dieckol (**3**) was obtained as amorphous powder, and the FABMS revealed a fragment ion as *m/z* 742 [M]$^+$, corresponding to a molecular formula of $C_{36}H_{22}H_{18}$. A combination of 2D

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NMR experiments readily defined the structure of **3** as a dimmer of 2. (Table 1-3)

The only difference between the ^{1}H NMR spectrum of **2** and **3** is that the former lacks the

signals for one phenolic OH and one aromatic proton. The chemical structures of the three

phlorotannins isolated from *E. cava* were indicated in Fig. 1-8.

Table 1-2. ^{1}H and ^{13}C NMR assignments for eckol

Position	^{13}C	^{1}H (mult. J =Hz)
1	143.8	
2	99.9	
3	147.7	6.13 (1H, s)
4	126.1	
4a	139	
5a	125.3	
6	147.6	5.94 (2H, s)
7	100.3	
8	150	5.94 (2H, s)
9	96.3	
9a	144.7	
10a	125.1	
1'	162.4	
2'	95.9	5.93 (3H, s)
3'	160.7	
4'	98.2	5.93 (3H, s)
5'	160.7	
6'	95.9	5.93 (3H, s)

* 400 MHz for ^{1}H and 100 MHz for ^{13}C

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Table 1-3. ^{1}H and ^{13}C NMR assignments for dieckol

Position	^{13}C	^{1}H (mult. J =Hz)	Position	^{13}C	^{1}H (mult. J =Hz)
1	126.46		1	158.61	
2	148.09		2	148.14	
3	100.3	6.13(1H, s)	3	153.19	6.15 (1H, s)
4	144.1		4	144.2	
4a	125.38		4a	125.45	
5a	144.95		5a	145.1	
6	96.57	5.95 (1H, d, J =2.76)	6	97.02	6.05 (1H, d, J =2.85)
7	156.81		7	155.33	
8	100.56	6.06 (1H, d, J =2.85)	8	100.67	5.98 (1H, d, J =2.76)
9	147.92		9	147.71	
9a	127		9a	125.67	
10a	139.44		10a	139.27	
1'	162.67		1'	126.41	
2'	96.17	5.92 (3H, s)	2'	97.02	6.09 (2H, s)
3'	160.95		3'	100.19	
4'	98.47	5.92 (3H, s)	4'	127.28	
5'	160.95		5'	153.19	
6'	96.17	5.92 (3H, s)	6'	96.65	6.09 (2H, s)

＊ 400 MHz for ^{1}H and 100 MHz for ^{13}C

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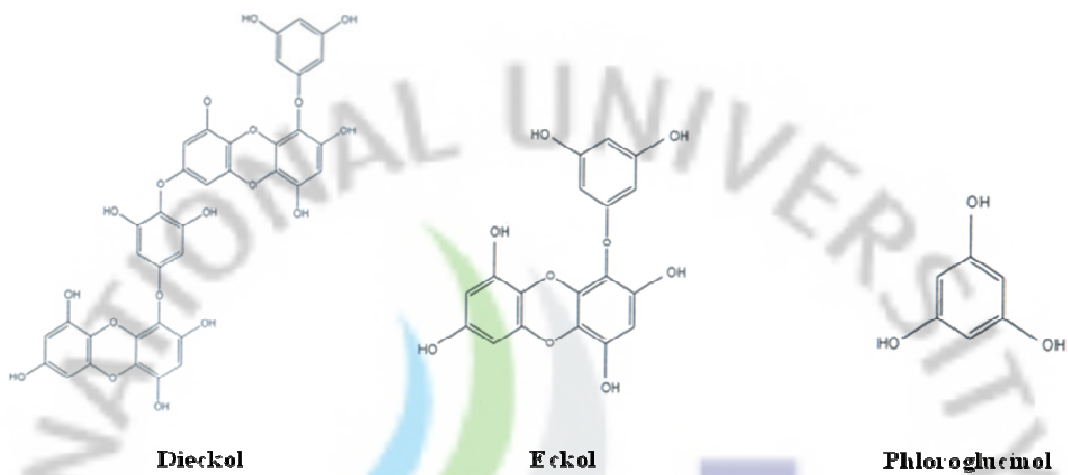
Fig. 1-8. Chemical structures of the three phloratannins isolated from *E. cava*

3.4. DPPH radical scavenging activity of the phlorotannins isolated from *Ecklonia cava*.

DPPH radical is scavenged by antioxidants through the donation of hydrogen to form the stable reduced DPPH molecule. The antioxidant radicals formed are stabilized through the formation of non radical products (Argolo et al., 2004). The scavenging activities of isolated compounds from *E. cava* **1**, **2** and **3** on DPPH free radicals are provided. The IC_{50} of three isolated compound (1, 2 and 3) were 99.14, 9.13 and 3.03 μM, respectively. This result indicated that the compound 2 and 3 could be effective antioxidant. Especially, compare to the other two compound, dieckol showed very strong scavenging activity against DPPH radical. (Table 1-4)

Table 1-4. Scavenging activity of the active compounds isolated from *E. cava* against DPPH

radical

	IC$_{50}$ (μm)
Phloroglucinol	99.14
Eckol	9.13
Dieckol	3.03

3.5. Protective effects of phlorotannins isolated from *E. cava* against ethanol-induced injury to Chang liver cells.

The hepatoprotective effects of dieckol, eckol and phloroglucinol on cell survival in ethanol-treated Chang liver cells were measured via the MTT assay. As shown in Fig. 1-9 treating with 4% ethanol induced a reduction in the cell survival rate, to 51.03%, while dieckol, eckol and phloroglucinol (25, 50 μg/ml) prevented the ethanol-induce damage, restoring cell survival to dieckol (66.72, 73.64%), eckol (55.39, 58.62%) and phloroglucinol (40.93, 45.37%), respectively. The cytoprotective effects of dieckol, eckol and phloroglucinol on apoptosis induced by ethanol were investigated via nuclear staining with Hoechst 33342. Hoechst 33342 dye specifically stains DNA, and is widely used to detect nuclear shrindage such as chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, which are all indicative of apoptosis. As shown in Fig. 1-10, the negative control, which contained no compound or 4% ethanol, possessed intact nuclei (Fig.1-10A), and the positive control (ethanol treated cells) exhibited significant nuclear fragmentation, indicating apoptosis (Fig. 1-10B). However, the addition of phloroglucinol, eckol and dieckol with ethanol reduced the apoptotic bodies (Figs.1-10C, D, E). Which suggested event of an ethanol challenge, and that the cells were protected from cells damage–related cellular

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injuries Therefore, the photographs (Fig. 1-10C) clearly suggest the ability of dieckol to

protect cell damage against ethanol attack.

Fig. 1-9. The hepatoprotective effect of isolated compounds from *E. cava* on ethanol-induced cytotoxicity in chang cells. Cytotoxicity was assessed after incubating for 24 h with 4% ethanol in DMEM medium. Experiments were performed in triplicate and thedata are expressed as mean ± SE. (*, P<0.05; **,P<0.01)

Fig. 1-10. Protective effect of isolated compounds from *E. cava* on ethanol induced chang cell damage of fibroblasts. The fibroblasts were treated with various concentrations of isolated compounds from *E. cava* and after 1h later, ethanol at 4% was applied to the chang cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining A: untreated, B: 4% ethanol C: Dieckol 25 μg/ml, D: Eckol 25 μg/ml E: Phloroglucinol 25 μg/ml

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3.6. Protective effects Dieckol of isolated from *E.cava* against ethanol-induced injury to Chang liver cells.

The hepatoprotective effects of dieckol on cell survival in ethanol-treated Chang liver cells were measured via the MTT assay and LDH assay (Fig. 1-11). When cell viability assays were performed on cells after exposure to 25 and 50 μg/ml of dieckol isolated from *E. cava* for 24h, followed by exposure to 4% ethanol for 1h, dieckol isolated from *E. cava* was found to have a concentration-dependent protective effect. As shown in Fig. 1-11, treating with 4% ethanol induced a reduction in the cell survival rate, to 49.10%, while dieckol prevented the ethanol-induce damage, restoring cell survival to dieckol (58.62, 70.67%) respectively. On the other hand, the cell treated with ethanol only, 42.96% LDH was released. While, at the concentrations of 25 and 50 μg/ml of dieckol, the cytotoxic effects were 27.4% and 16.72%.

The protective effect of dieckol isolated *E. cava* on Chang liver cells apoptosis induced by ethanol was investigated via nuclear staining with Hoechst 33342. Hoechst 33342 dye specifically stains DNA, and is widely used to detect nuclear shrinkage such as chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, which are all indicative of apoptosis. As shown in Fig.1-12, the negative control, which contained no sample or ethanol, possessed intact nuclei (Fig.1-12A), and the positive control (ethanol

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treated cells) exhibited significant nuclear fragmentation, indicating apoptosis (Fig. 1-12B).

However, the addition of dieckol isolated *E. cava* with ethanol reduced the apoptotic bodies

(Figs. 1-12C and D), which suggested event of an ethanol challenge, and that the cells were

protected from cells damage –related cellular injuries. Therefore, the photographs (Figs. 1-

12C and D) clearly suggest the ability of dieckol isolated dieckol to protect cell damage

against ethanol attack.

The addition to the morphological evaluation, the protective effects of dieckol isolated *E.

cava* was confirmed by flow cytometry. Analysis of the DNA contents following ethanol

treatment of Chang liver cells revealed an increase in the proportion of cells with sub-G_1

DNA content, to 52.06% (Fig. 1-13). This result indicates that apoptosis was induced by

ethanol. However, cells that were pretreated with 80% ethanol extract of *E. cava* showed

significantly reduced sub-G_1 DNA content, while the percentages of the dieckol isolated

from *E. cava* treated cells showed 48.71 and 36.92% at 25 and 50 μg/ml, respectively (Fig.

1-13). These results indicate the dieckol isolated from *E. cava* pretreatment is protective
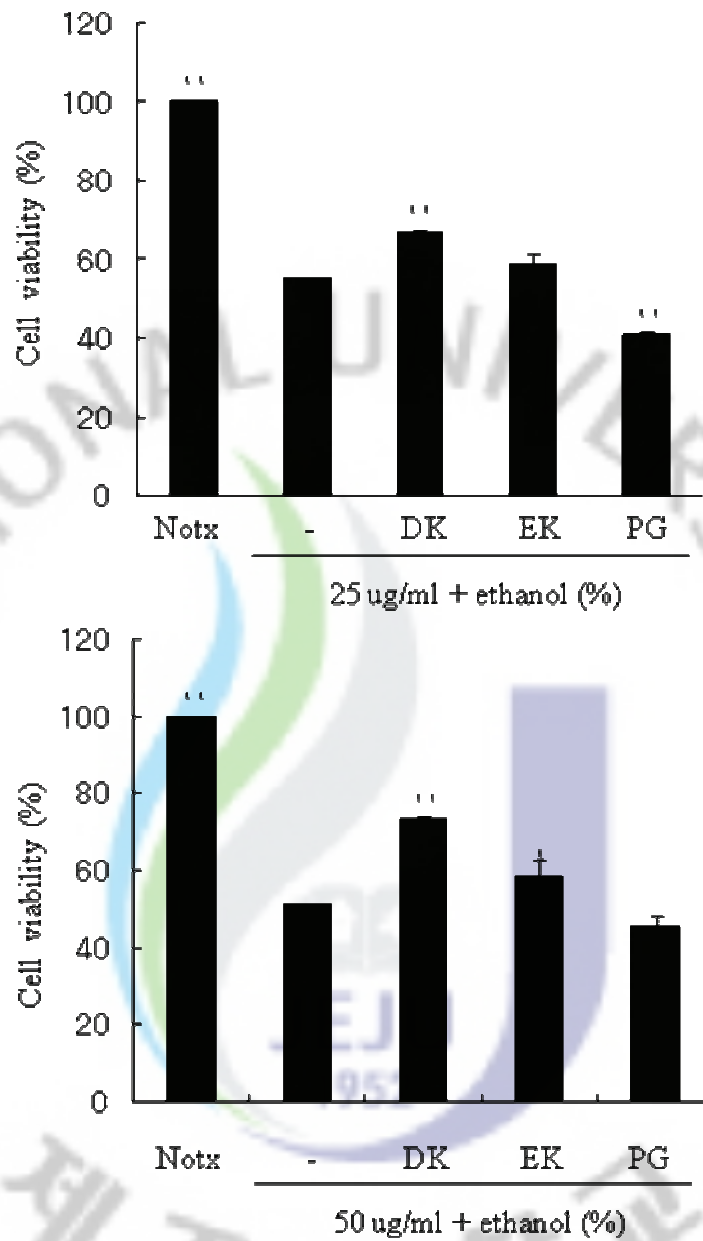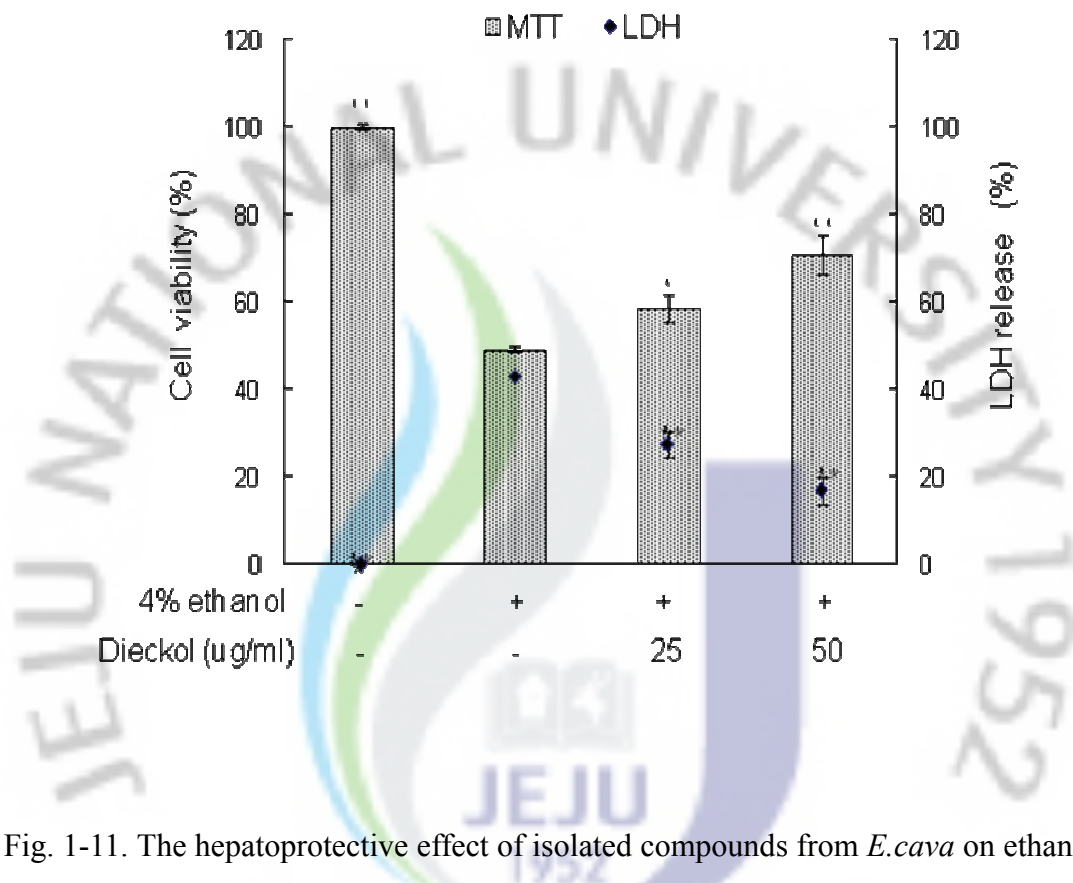
against ethanol-induced cell death.

Fig. 1-11. The hepatoprotective effect of isolated compounds from *E.cava* on ethanol - induced cytotoxicity in chang cells. Cytotoxicity was determined using the LDH method. Cytotoxicity was assessed after incubating for 24 h with 4% ethanol in DMEM medium. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **,P<0.01)
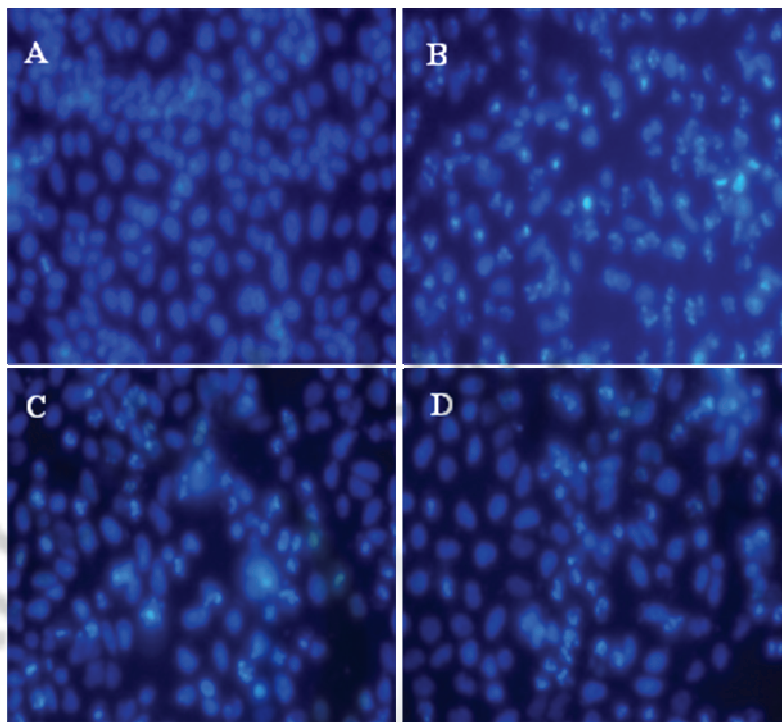
A: Notx
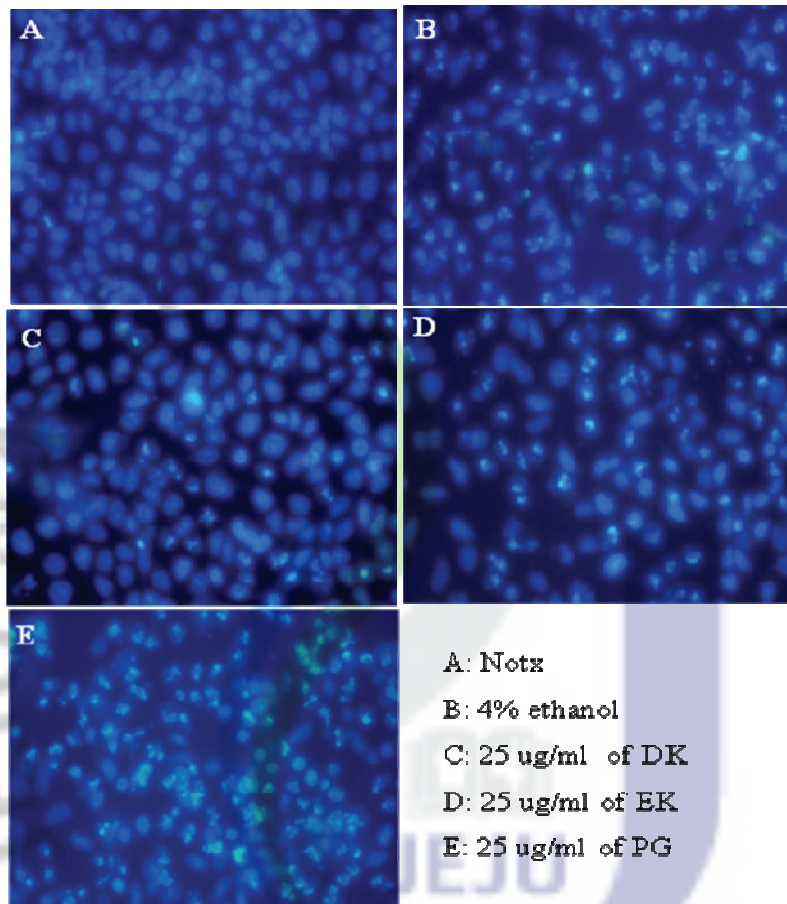B: 4% ethanol
C: 25 ug/ml of DK
D: 50 ug/ml of DK

Fig. 1-12. Protective effect of isolated compounds from *E. cava* on ethanol induced chang cell damage of fibroblasts. The fibroblasts were treated with various concentrations of isolated compounds from *E. cava* and after 1h, ethanol at 4% was applied to the chang cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining A: untreated, B: 4% ethanol C: Dieckol 25 μg/ml, D: : Dieckol 50 μg/ml
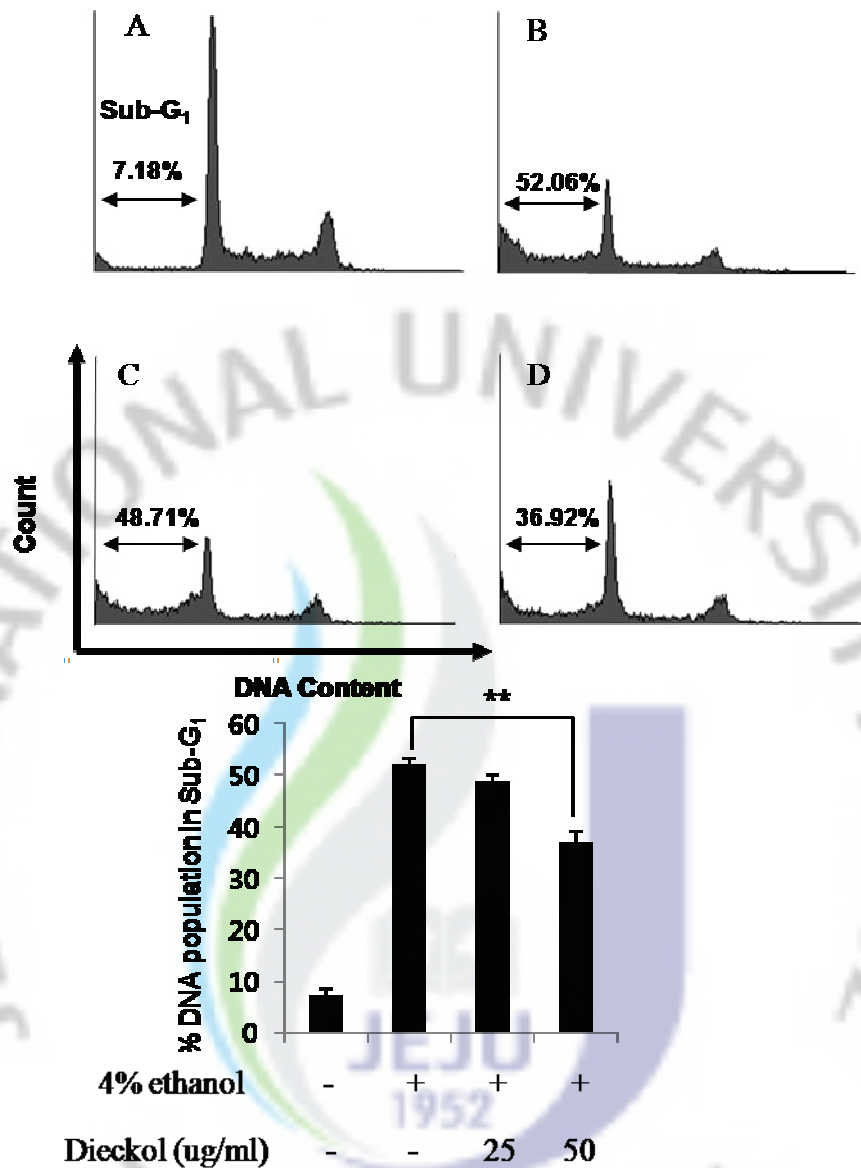
Fig. 1-13. Flow cytometry analysis of propidium iodide stained Chang liver cells treated with ethanol alone and in presence of ethanol + Dieckol isolated compounds from *E. cava* (A), control (B), Ethanol (C), Ethanol + Dieckol 25 ug/ml (D), Ethanol + Dieckol 50 ug/ml. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **,P<0.01)

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3.7. Hepatoprotective of dieckol isolated from *E.cava* on phosphorylation of MAPKs in the ethanol induce Chang liver cells.

Ethanol-induce oxidative stress-responsive signaling pathways involve ERK and MAPK signaling (Nair et al., 2004). Therefore we assessed the expression of MAPK family proteins in Chang liver cells treated with ethanol + dieckol alone. Treatment with ethanol alone induced ERK phosphorylation, but not JNK or p38 activation. Thus, ethanol-induced cytotoxicity involves the activation of ERK, but not of p38 or JNK. However, co-treatment with ethanol and dieckol caused an even greater increase in ERK phosphorylation (Fig. 1-14), further suggesting that ERK phosphorylation is involved in the effect of dieckol on cell survival.

Taken together, these results indicate the although ERK signaling is associated with ethanol-induced cell death, it is not sufficient to mediate the cytotoxicity of ethanol. Thus, we propose that another signaling pathway may be linked to ethanol-induced cytotoxicity, and that this unknown pathway plays a more important role than ERK signaling.

Ethanol (4%)	-	+	+	+
Dieckol	-	-	25	50

P-ERK

Total-ERK

ß-actin
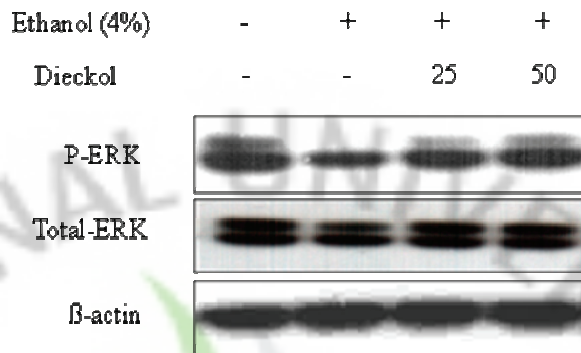
Fig. 1-14. Effects of Dieckol on MAPK signaling pathway in chang cells. Cell were pretreated Dieckol with ethanol (4%) or ethanol only (4%) after incubation time for 6hr Whole – cell extracts were prepared and analyzed by western blotting using anti- phospho-ERK1/2 and anti – β-actin antibodies.

3.8. Dieckol modulated the expression levels of apoptosis-related proteins in ethanol induced chang liver cells.

To assess whether dieckol can inhibit apoptosis by regulating mitochondrial signal pathway including Bax, Bcl-xL , Cleaved Caspase 3 and PARP in ethanol induced chang liver cells, Western blot assay was performed. As shown in Figure 1-15, exposure of ethanol induce considerably up regulated the expression level of Bax known as a pro-apoptotic molecule, whereas markedly down regulated Bcl-xL, PARP, Cleaved Caspase-3 Known as anti-apoptotic molecules in Chang liver cell, comparing with only ethanol cells. However, dieckol treatment dose dependently decreased the expression levels of Bax a pro-apoptotic molecule at all concentrations from 25 to 50 μg/ml compared to only ethanol cells. In addition, the expression levels of Bcl- xL were markedly up-regulated at all concentrations comparing with ethanol treatment cells. Especially, 50 μg/ml of dieckol markedly decreased Bax expression, whereas increased the Bcl-xL expressions in comparison with only ethanol treatment cells. These data indicates that dieckol inhibited apoptosis caused by ethanol treatment as modulating apoptosis-related molecules.

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| 4% ethanol | - | + | + | + |
| Dieckol (ug/ml) | - | - | 25 | 50 |

Bax

Bcl-xL

Cleaved Caspase 3
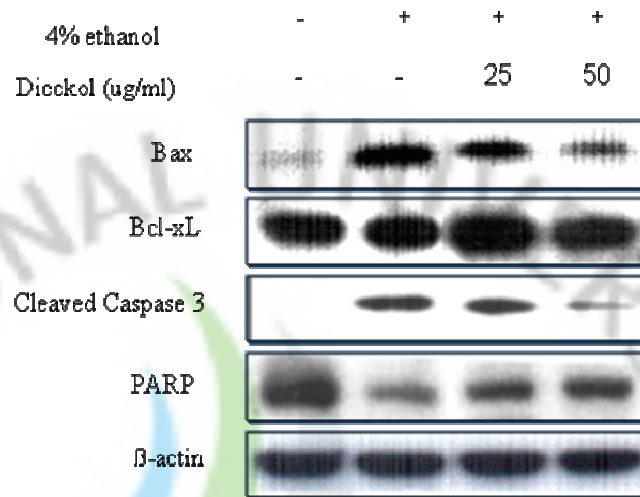
PARP

ß-actin

Fig. 1-15. Analysis of ethanol (4%) induced cell death in chang cell extracts. Whole-cell extracts were prepared and analyzed by Western blotting using anti Bax, Bcl-xL,Caspase - 3, PARP and ß-actin antibodies.

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4. Discussion

The abused of ethanol could induce alcohol-related liver disease, which is the second leading cause of death among all liver diseases. Mechanisms of ethanol toxicity have been most extensively studied in the liver. Oxidative stress has been considered as one of the key mechanism responsible for alcoholic liver damage.

Marine algae contain diverse bioactive compounds with unique structure. That was possibly caused by extreme marine environment. Many studies have reported their biological effects. Nowadays, more researchers paid attention to the protective effects of natural compounds against ethanol induced cytotoxicity. Choi reported the protective effect of a polysarccharide from *Hizikia fusiformis* against ethanol-induced cytotoxicity in IEC-6 cells and ethanol toxicity in rats. *Ecklonia cava* is a brown alga that widely distributed in korea and Japan. Our research group has reported the antioxidant, anti-hypertension and anti-coagulant activities of its extract and compounds.

Therefore, the objective of this study was to isolate and identify secondary metabolites from the ethanol extract of *E. cava*. And investigate the hepatoprotective activity of the compound isolated from *E. cava.*

The cytotoxicity and cell viability of 80% ethanol extract from twenty brown algae were

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screened by MTT assay. Among them, the extract of *E. cava* showed the non cytotoxicity

and highest cell viability in the Chang cell line. Thus, the brown alga, *E. cava* was selected

for further study.

The cell viability of ethanol extract of *E. cava* against ethanol induced toxicity was also

determined via MTT assay. The results showed the cell viability was significantly increased

by addition of test samples. The *E. cava* ethanol extract also showed the protective effect

against ethanol induced cell apoptosis. The similar result was observed by cell cycle analysis.

Therefore, the ethanol extract of *E. cava* could protect Chang cells against ethanol-induced

cell death.

The ethanol extract of *E. cava* showed protective effect against ethanol-induced cell damage.

The extract was separated and purified by silica gel, sephadex LH-20 column and prep-

HPLC chromatography. Three compounds were successively isolated. The structures of three

compounds were determined by analysis of EI-MS and NMR spectra data. The isolated

compounds were identified as phloroglucinol, eckol and dieckol.

To determined antioxidant activity of three isolated compounds, DPPH scavenging assay has

been used. Among the three compounds, dieckol showed highest DPPH scavenging activity.

The cell viability of three isolated compounds was determined by MTT assay. At the

concentrations of 25 μg/ml and 50 μg/ml, dieckol showed highest cell viabilities compared

to those of phloroglucinol and eckol. The protective effects of three pholotannins against

ethanol induced cell damage in the Chang cell line were estimated by nuclear staining with

Hoechst 33342. At the concentration of 50 μg/ml, dieckol could reduce the apoptosis in

Chang cell line. Therefore, the further investigation of dieckol on protective effect against

ethanol-induced cell damage was carried out.

The cell viability of dieckol showed a dose-dependent manner. Pretreatment with different

concentration of dieckol could significantly increased cell survival compared to the ethanol

treated group. Additionally, dieckol could reduce LDH production in a dose-dependent

manner. Pretreatment with different concentration dieckol was found to protect the Chang

cells against nuclear fragmentation induced by ethanol. From the results of cell cycle

analysis, dieckol could reduce accumulation of cells in the sub-G1 peak by 4% and 16%.

MAPK signaling pathway has been considered as an important mechanism of ethanol-

induced cell damage. Due to the excessive production of reactive oxygen species and other

free radicals, MAPKs in the hepatocytes could be affected. The MAPK family includes three

kinases: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38

kinase. Some report showed that ERK activation was proposed as protective role against

apoptosis. Hwang reported that the protective effects of *Hizikia fusiformis* are due to the

activation of ERK. In the present study, we investigated whether ERK was activated by

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dieckol. And our results showed that the protective effect of dieckol was caused by up-regulation of the ERK.

Apoptosis was associated to intrinsic (i. e., the mitochondrial pathway) and extrinsic (i. e., the death receptor). BCL-2 family, including anti-apoptotic (BCL-2 and Bcl-xL) and pro-apoptotic (BAX and BAD), are responsible to the cell apoptosis and cell death. Our study has determined BAX, Bcl-xL, cleaved caspases -3 and PARP regulation induced by ethanol in the dieckol treated Chang cells. Pretreatment with dieckol could down-regulate BAX, activate Bcl-xL, down-regulate caspase-3 and PARP. This result suggested that dieckol protects Chang cell against ethanol-induced apoptosis by regulation of BAX, Bcl-xL caspase-3 and PARP.

In conclusion, our research demonstrated that dieckol isolated from *E. cava* could protect cell damage and apoptosis by activation of MAPK signaling and regulation of BCL-2. Thus, dieckol isolated from *Ecklonia cava* could be useful for preventing ethanol relative liver diseases.

Part Ⅱ.

Hepatoprotective effets of Dieckol isolated from *E.cava* against Ethanol induced liver damage in Bab/c mice

Part Ⅱ.

Hepatoprotective effets of Dieckol isolated from *E.cava* against Ethanol induced liver damage in Bab/c mice

1.ABSTRACT

The *in vivo* hepatoprotective effect of 80% ethanol extract of *E. cava* on ethanol induced hepatic damage in Bab/C mice liver were investigated. After administration of ethanol extract of *E. cava*, the body weight and survival rate were significantly increased. The glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels in the serum were significantly lower than ethanol treated group. Furthermore, *E. cava* ethanol extract exhibited a significant inhibition of the total cholesterol. When the mice fed with 5 and 25 mg/kg, mouse of 80% ethanol extract of *E. cava*, the lower levels of SOD enzyme and reduction of the formation of malondialdehyde (MDA) were observed. Finally the protective effect on improvement of fatty liver induce by ethanol administration was also investigated by

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taking out the liver immediately after dissecting the mouse. However, no significant

changes on hepatic histophathological changes were observed. In conclusion, this

study indicated that *E. cava* ethanol extract could protect liver injury induced by

ethanol *in vivo*. The brown alga, *E. cava* possesses the beneficial effect to the human

against ethanol-induced liver injury.

2. MATERIALS AND METHODS

2.1. Materials

The brown seaweed, *Ecklonia cava* was collected along the Coast of Juju Island in Korea, during the period from March to May 2008. Salt, sand and epiphytes were removed using tap water. Finally, the samples were rinsed with fresh water and stored at -20°C for further experiments.

2.2. 80% Ethanolic extracts from *E. cava*

In order to obtain 80% ethanol extracts from *E. cava* one gram of *E. cava* powder was mixed with 80% ethanol and kept in a shaking incubator for 24hr at room temperature. Then, the mixture was centrifuged at 3500 rpm for 20 min at 4°C and filtered using Whatman filter paper to remove the precipitate, thereafter evaporated under vacuum at 40°C to remove all ethanol, and then dissolved in water. All samples were kept at -20°C for further experiments.

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2.3. Determination of total dieckol content by HPLC system.

The extracts were analyzed by HPLC according to the method of Ahn et al. (2007). The HPLC system (YoungLin Instrument, Korea) comprising the following components: Acme 9000 UV/Vis spectrometer, Gradient pump, Vacuum degasser & Mixer were used for the determination of the component of ethanol extract from *E. cava*. The column used a RP-C18 column (250 x 4.6mm,5 µM, Waters, USA). The flow rate was 1 ml/min, the column temperature was room temperature and sample volume injected was 20 µl. The component was determined by comparison of the retention time with the previously isolated standard.

2.4. Animals

Male Balb/c mice, weighing 20-25 g, were obtained from KUMSUNG HITEC. Animals were acclimated to temperature (22± 2˚C) and humidity (55±5%) controlled rooms with a 12-h light/dark cycle for 1 week prior to use. Laboratory pellet and tap water were allowed *ad libitum*. The binge drinking mice model developed by Carson and Pruett (1996) was utilized for ethanol challenge.

2.5. Animal grouping

Six-week-old, male Balb/c mice were randomly divided into 4 groups, control (saline, 200 μl), ethanol treatment (4 g/kg), *E. cava* ethanol extract (5 mg/kg, mouse) treatment, *E. cava* (25 mg/kg, mouse) ethanol extract treatment, with 9 mice in each group. During the experiment period, the body weight and survival rate were investigated every day. After 14 days, the mice were anesthetized and blood samples were collected to determine biochemical parameters. Livers from each group were collected, weighed and a thin slice preserved in 10% buffered formalin solution for histopathological analysis. The remaining livers were frozen in liquid nitrogen and stored at -70˚C for biochemical assays.

The blood samples of mouse were collected for serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) concentration and total cholesterol content respectively, and livers were excised from the animals to analyze malondialdehyde (MDA) formation and antioxidant enzyme activity.

Control group

Mice were supplied with normal laboratory diet for 14 days.

Ethanol-treatment group

Mice were orally administered with 0.2 ml of ethanol (4 g/kg) for 14 days.

Group receiving of 80% ethanol extract from *E. cava*

Mice were orally administered with alcohol and *E. cava* extract at the concentrations

of (5, 25 mg/kg, mouse) for 14 days.

Experimental groups for investigation of dose-dependent hepatoprotective effect of ethanol extract of *E. cava* against ethanol - induced toxicity.

Groups	No. of Mouse treated	Oral Treatment
Normal control	9	Saline (200 μl)
Ethanol-treatment group	9	Ethanol + Saline (4g/kg)
Test group1	9	Ethanol + *E. cava* 5 mg/kg, mouse
Test group2	9	Ethanol + *E. cava* 25 mg/kg, mouse

Ethanol administration mouse (n=9) of each experimental group, administered

ethanol (4 g/kg) and then saline (control) or *E. cava* ethanol extract (5, 25 mg /kg, mouse).

2.6. Biochemical assays

Early acute hepatic damages were determined by detecting serum GOT and GPT activities .The activity of GOT and GPT in the plasma samples was determined using an enzymatic analysis kit (Asan Pharmaceuticals, Hwasung, Korea), according to the Reitman-Frankel method (Reitman & Frankel, 1957). The livers were weighed, minced into small pieces, and rinsed twice with ice-cold homogenization buffer [250 mM sucrose, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA)] at 4 ℃. The livers were homogenized in five volumes of ice-cold homogenization buffer with a motor-driven Teflon pestle. The homogenates were centrifuged at 1000 g for 10 min. The pellets were discarded, and supernatants were centrifuged at 12,000 g for 30 min. The final supernatant protein contents were determined by using the bicinchoninic acid protein assay reagents.

Lipid peroxidation products were estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) in fluorescence at 530 nm ex/552 nm.

Activities of superoxide dismutase (SOD) and catalase (CAT) were measured by the method (Kakkar et al and Sinha, 1984), respectively. The liver homogenates were

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diluted 1:20 (g ml^{-1}) in 5% metaphosphoric acid. The homogenates were then

centrifuged at 2500×g and 4 $^{\circ}$C, for 10 min. Reduced glutathione content (GR) is

expressed as micro-molar per milligram of protein (μ mg protein^{-1}).

The total cholesterol content in the serum was determined using a commercial kit

(Asan Pharmaceuticals, Hwasung, Korea) according the method of Allain et al.

2.7. Hematoxylin and eosin staining

Liver tissues were fixed in 10% (v/v) phosphate buffer formalin and embedded in

paraffin wax. Sections (3 μm thick) were cut, and each section was stained with

hematoxylin and eosin Stain. All the sections were examined by light microscopy

(Olympus DP70, Olympus Optical Co., Japan).

2.8. Statistical analysis

Data was analyzed using the statistical package for the social science (SPSS) package

for Windows (Version 8). Values were expressed as means $\pm$ standard error (SE). A

p-value of less than 0.05 was considered significant.

3. Results

3.1. The HPLC chromatography of ethanolic extract of *E. cava*.

The chemical constituent of 80% ethanolic extract of *E. cava* was analyzed by reverse-phase HPLC using a gradient condition (0% - 100% MeOH/H$_2$O). The chromatogram was shown in Fig. 2-1. The main compound was identified as dieckol by compared the retention time with previously purified standard. Furthermore, our research group has reported that the major compounds of ethanol extract of *E. cava* were phlorotaninns.
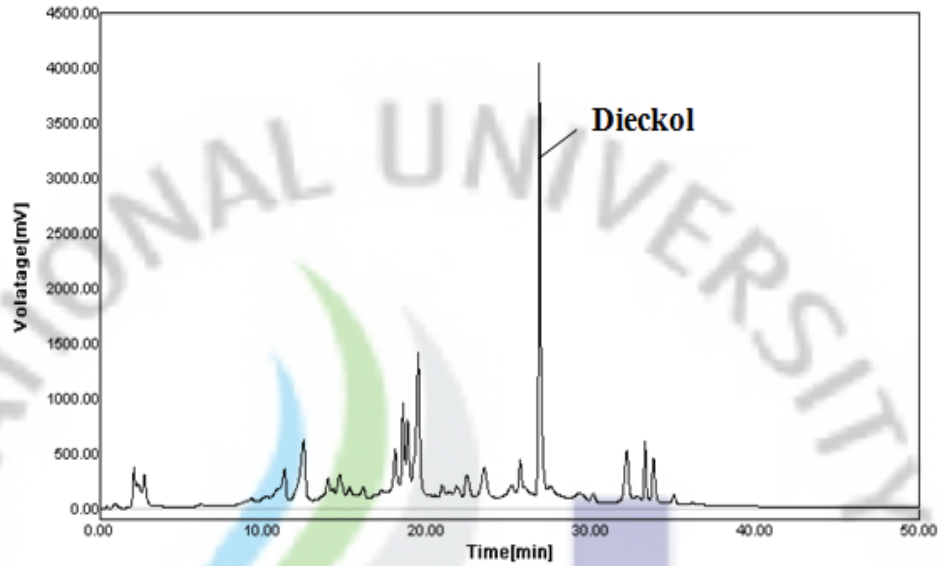
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Fig.2-1. The HPLC chromatography of ethanol extract of *E. cava*. Solvent A was MeOH, Solvent B was distilled water. The system was eluted using linear gradient as 0% A increased to 100% A from 0 to 50 min. The flow rate was 1 mL/min, and detector was set at 220 nm.

3.2. Changes of the body weight and survival rate of experiment mice

The body weights of the mice were weighed every day during experiment period. The body weight gain of the normal group was 23.52 g and that of ethanol (4g/kg) group was 21.28 g. The mean body weight gains of 80% ethanol extract of *E. cava* administration 5 and 25 mg/kg, mouse groups were 22.13 and 22.85 g, respectively. Compared with ethanol treated group, the body weight gain of the 80% ethanol extract from *E. cava* (5 and 25 mg/kg, mouse) group increased significantly at 14 days of 80% ethanol extract *E. cava* administration (Fig 2-2). The survival rates of experimental mice were also determined. In the negative group (non-treated), the survival rate was 100% during the experiment period. As shown in the Fig. 2-3, when the mice were treated with ethanol (4 g/kg) in the positive group, the survival rate was 66%. In case of ethanolic extract of *E. cava* (5 and 25 mg/mouse) treated groups, the survival rates were 66% and 82%. This result indicated that the ethanolic extract of *E. cava* could increase the survival rate and maybe it has protective effect against ethanol caused toxicity.

Experimental mouse groups

Table 2-1. Experimental groups for investigation of dose-dependent hepatoprotective

effect of ethanol extract of *E. cava* against ethanol-induced toxicity.

Groups	Treatment	No. of Mouse
Notx	Saline	9
Ethanol treated mice	Ethanol + Saline (4g/kg)	9
Group 1	Ethanol + *E. cava* 5 mg/kg Mouse	9
Group 2	Ethanol + *E. cava* 25 mg/kg Mouse	9

Ethanol administration mouse (n=9) of each experimental group, administered

ethanol (4 g/kg) and then saline (control) or *E. cava* (5, 25 mg /kg, mouse)
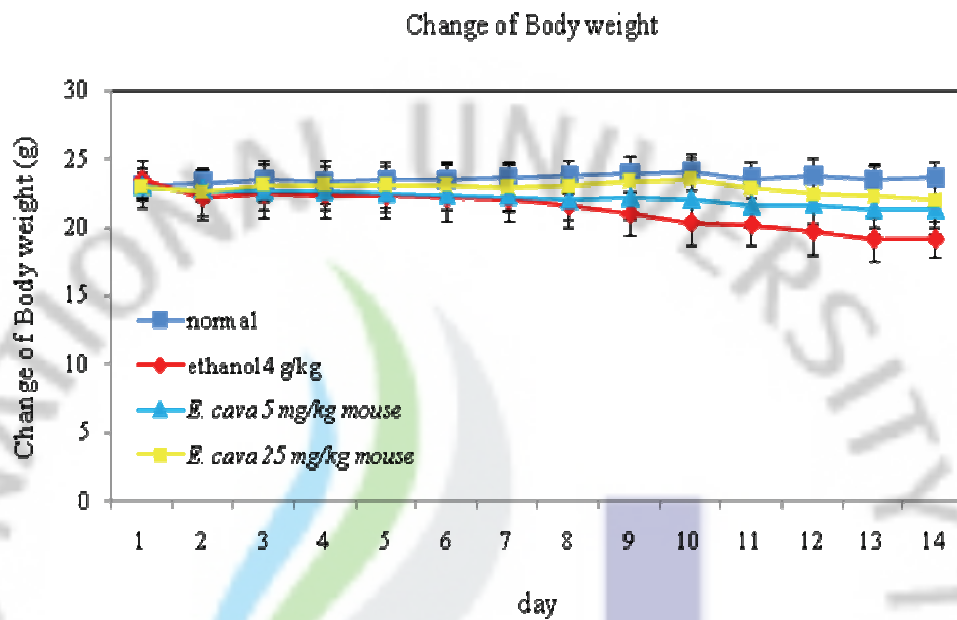
Fig.2-2. Body weight gain of mouse fed experimental diets for 2 weeks.
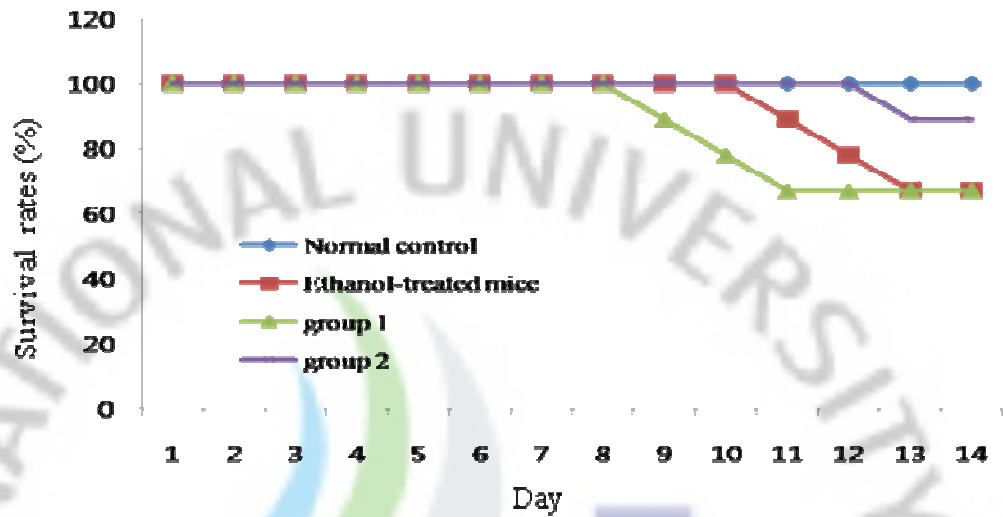
Fig.2-3. Survival rates of experimental mouse

Each value represents the mean ± SD of nine mouse.

Normal control mouse

Negative control: Ethanol (4g/kg) control mouse

Test group1: Mouse treated with *E. cava* (5 mg/kg, mouse) before the ethanol –

administration

Test group2: Mouse treated with *E. cava* (25 mg/kg, mouse) before the ethanol

administration

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3.3. Effect of the 80% ethanol extract of *E.cava* on serum total cholesterol GOT and GPT levels in ethanol-treated mouse.

The results of biochemical indicators of liver function are summarized in Table 2-2. The administration of ethanol caused severe hepatotoxicity in the mice, as evidenced by the significant elevations of serum Glutarnic oxaloacetic transaminase (GOT) and Glutarnic pyruvic transminase (GPT) activities in experimental mice. In the ethanol treated group, the levels of GOT and GPT in the serum were 218.83 and 47.14 Karmen/ml, respectively. However, the mice administrated with different concentration of ethanol extract of *E. cava*, the levels of GOT and GPT were significantly decreased. At the concentration of 25 mg/kg, mouse, almost same level of GOT and GPT were observed in the serum. Thus, the ethanol extract of *E. cava* could protect the liver by decreasing the GOT and GPT level (Table 2-2).

The total cholesterol levels in the serum after 14 days of administration of experimental diets were show in Table 2-2. The serum total cholesterol levels in ethanol group was 69.50 mg/dl. It was significantly higher than those in the normal control group (59.29 mg/dl). While, the mice administrated with 5 mg/kg, mouse of 80% ethanol extract from *E. cava*, the total cholesterol level was slightly decreased

in the serum. However, the level of total cholesterol was significantly decreased by

25 mg/kg, mouse of 80% ethanol extract from *E. cava.* Therefore, the ethanol extract

of *E. cava* could protect the liver by lowering the total cholesterol level.

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Table 2-2. Effect of the 80% ethanol extract of *E.cava* on serum Glutarnic

oxaloacetic transaminase (GOT), Glutarnic pyruvic transminase (GPT) and total

cholesterol levels in ethanol-treated mouse.

Groups	GOT (Karmen/ml)	GPT (Karmen/ml)	Total chlesterol (mg/dL)
Normal control	151.37 ± 18.51**	37.52 ± 5.03*	59.29± 2.14**
Ethanol-treated mice	218.83 ± 25.29	47.14 ± 4.54	69.50 ±5.76
group1	173.56 ± 26.95**	42.25 ± 7.48	64.77 ±3.93
group2	156.54 ± 8.23**	38.34 ± 3.83*	58.77 ± 4.73**

Experiments were performed in triplicate and the data are expressed as mean ± SE. (*,

P<0.05; **,P<0.01)

Normal control mouse

Negative control: Ethanol (4g/kg) control mouse

Test group1: Mouse treated with *E. cava* (5 mg/kg, mouse) before the ethanol –

administration

Test group2: Mouse treated with *E. cava* (25 mg/kg, mouse) before the ethanol

administration

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3.4. Liver antioxidant enzyme activities and MDA levels

The levels of liver antioxidant enzymes SOD, CAT and glutathione (GR) were determined. Table.2-3 showed the hepatic antioxidant enzyme activities in ethanol induced liver damage of mice after 14 days of administration of experimental diets. The level of antioxidant enzymes SOD in the ethanol fed mice was significantly increased than that of non-treated mice. Pretreatment with different concentration of 80% ethanol extract of *E. cava*, the SOD levels were significantly decreased. However, the CAT levels in all experiment groups were not significantly changed. The GR level was significant lower in the ethanol fed mice than that of non-treated group. When the mice fed with 5 and 25 mg/mouse of 80% ethanol extract of *E. cava*, the higher levels of GR were observed. The results of this study suggested that the *E. cava* ethanol extract might inhibit the oxidative stress by reducing reactive oxygen species (ROS).

In order to further evaluate the possible mechanism involved in the protective effect of ethanol extract from *E.cava* on hepatotoxicity, MDA formation in ethanol induced liver damage in mouse was further evaluated (Table. 2-3). Significant increases of MDA level (13.27 mmol/g tissue) as a result of lipid peroxidation were

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found in the ethanol fed mice. However, 80% ethanol extract of *E. cava* pretreatment for 14 days significantly inhibited the lipid peroxidation. The levels of MDA were 10.47 and 10.22 mmol/g tissue at the concentration of 5 and 25 mg/mouse, respectively. The results indicated that the ethanol extract of *E. cava* may protect the liver in the ethanol treated mice by exerting the antioxidant effect.

Table 2-3. Actiyities of catalase, SOD, GR and MDA levels in liver of mouse treated with

ethanol and ethanol extract of *E. cava*

Groups	SOD [1]	CAT [2]	GR [3]	Malondialdehyde (mmol/g tissue)
Normal control	108.83±9.34	19.53 ± 1.40	4.72 ± 1.54	8.28 ± 0.71
Ethanol-treated mice	136.36±0.95	20.43 ± 0.38	4.19 ± 1.57	13.27 ± 0.70
group1	102.27±13.20	19.76 ± 1.00	4.56 ± 1.93	10.47 ± 0.88
group2	93.18±11.82	21.56 ± 0.54	4.61 ± 1.46	10.22 ± 0.70

[1] SOD activity (%) [2] μMol/min/mg protein [3] μ/mg/ protein

Experiments were performed in triplicate and the data are expressed as mean ± SE.

(*, $P<0.05$; **,$P<0.01$)

Normal control mouse

Negative control: Ethanol (4g/kg) control mouse

Test group1: Mouse treated with *E. cava* (5 mg/kg, mouse) before the ethanol –

administration

Test group2: Mouse treated with *E. cava* (25 mg/kg, mouse) before the ethanol

Administration

3.6. Hepatoprotective effect of 80% ethanol extract from *E. cava* on ethanol induced fatty liver mouse.

The external look of the test mouse liver was observed by taking out them immediately after dissecting the mouse (Fig. 2-4). The hepatoprotective effects of 80% ethanol extract of *E. cava* on ethanol induced fatty liver were observed by comparing the change of liver color. In the normal group, the liver has a bright red color. Dark-red liver and white dots were observed in the liver of ethanol fed mice. This change means that intake of ethanol could cause the fatty liver. In case of ethanol extract of E. cava treated group, the lees deposit of fat were observed. Particularly, at the concentration of 25 mg/mouse, almost no different morphology was observed compared with normal group. **(Fig 2-4. Normal control**). Thus, the ethanol extract of *E. cava* could improve the fatty liver caused by intake of ethanol.
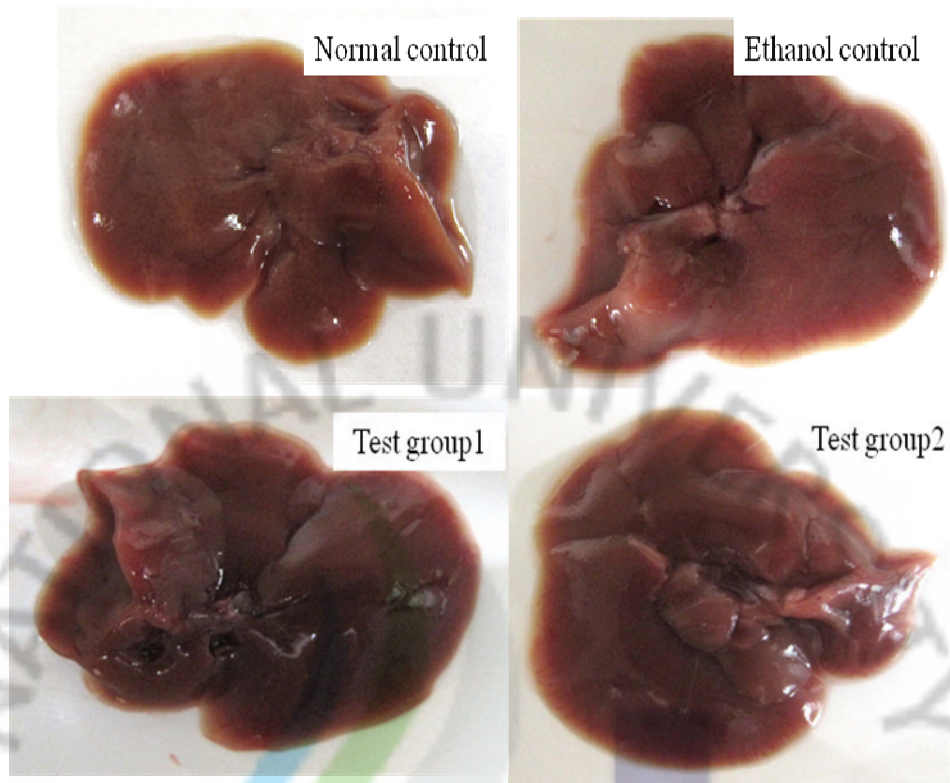
Fig.2-4. Effects of ethanol extract from the *E. cava* on ethanol induced fatty liver

mouse

Experiments were performed in triplicate and the data are expressed as mean ± SE.

(*, P<0.05; **,P<0.01)

Normal control mouse

Negative control: Ethanol (4g/kg) control mouse

Test group1: Mouse treated with *E. cava* (5 mg/kg, mouse) before the ethanol –

administration

Test group2: Mouse treated with *E. cava* (25 mg/kg, mouse) before the ethanol

administration

3.7. Histopathological changes of mouse livers

Liver sections from all experiment groups were examined by H&E staining. The morphological changes in the extracted liver tissues were shown in the Fig. 4. The normal Liver histology was shown in the Fig. 2-5A. However, the presence of faint microvesicular steatosis was found in the ethanol-treated group (Fig.2-5B). Acute ethanol exposure caused degenerative morphological changes in the liver. When the mice were given the test sample at the concentrations of 5 and 25 mg/mouse, these alcohol-induced hepatic pathological changes were slightly inhibited **(Fig.2-5C-D)**.
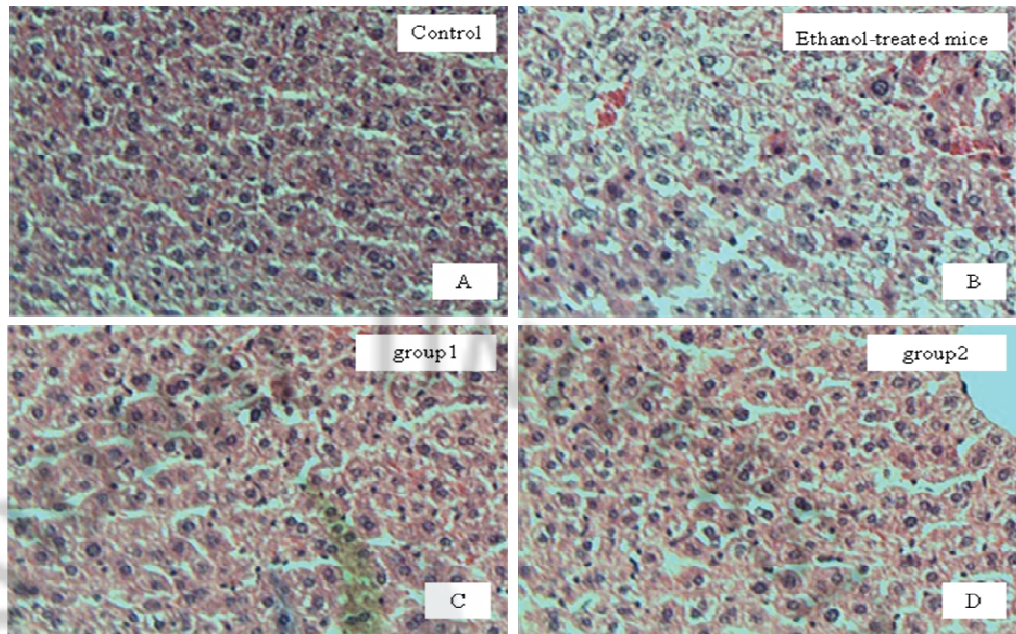
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Fig. 2-5. Hepatic histopathologic changes of central vein and portal area of liver

in ethanol induced fatty liver mouse. The liver sections were stained with

hematoxylin and eosin (H&E stain, x 125).

Experiments were performed in triplicate and the data are expressed as mean ± SE.

(*, $P<0.05$; **,$P<0.01$)

Normal control mouse

Negative control: Ethanol (4g/kg) control mouse

Test group1: Mouse treated with *E. cava* (5 mg/kg, mouse) before the ethanol –

administration

Test group2: Mouse treated with *E. cava* (25 mg/kg, mouse) before the ethanol

administration

Discussion

Ethanol induced liver damage is the substance most often abused by humans, has injurious to health, in particular, it further liver disease. Ethanol-induced liver damage progresses through alcoholic fatty liver, alcoholic hepatitis, fibrosis and cirrhosis. (Yanghee You et al. 2010).

Recently, the extract and compound from marine algae have been attracted more attention due to their diverse biological activities. *E. cava* is an edible brown alga which has been used for long time in Japan and Korea. Our research group already reported the antioxidant, anti-inflammatory and anti-hypertension activities of extracts and compounds from *E. cava*. Many reports have shown that the extract and compounds from marine algae have the protective effect against ethanol induced liver damage.

The aim of the present study is to investigate the protective effects of *E.cava* extract on the liver damage induced by ethanol *in vivo*. Therefore, this study is to evaluation of level of GOT, GPT, total cholesterol, antioxidant enzymes (SOD, CAT and GR) and lipid peroxidation, as well as the hepatoprotective effect on fatty liver and histopathological changes of mouse livers.

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The body weight and survival rate of mice were significantly decreased in the ethanol treated group. This may be caused by ethanol induced oxidative stress. In this study, the ethanol extract of *E. cava* could significantly increase the body weight and survival rate than that of ethanol treated group.

Glutamate oxaloacetate transferase (GOT) and Glutamate pyruvate transferase (GPT) are useful in screening for liver disease. When the hepatocellular injury or death occurs, the levels of GOT and GPT will dispersed in the plasma. Therefore, GOT and GPT activities could be increased. In our study, the levels of GOT and GPT in serum were significantly decreased in the ethanol extract of *E. cava* treated group. This result demonstrated that ethanol extract of *E. cava* could protect liver.

The fatty liver is a reversible condition, which was led by fat accumulation in the hepatocytes. Our result showed, the total cholesterol was significantly increased in the ethanol treated mice compared to the normal mice (non-treated). While, the total cholesterol in the *E. cava* ethanol extract treated groups, the total cholesterol was significantly decreased. Particularly, there was no significant difference the level of total cholesterol in the 25 mg/mouse *E. cava* ethanol extract treated mice was compared with normal group. This result indicated that ethanol extract of *E. cava* could prevent fatty liver induced by ethanol administration.

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Many reports have shown that the alcoholic liver disease was caused by the oxidative stress. The oxidative stress is the result of over production of pro-oxidants. The oxidative stress usually determined by enzyme antioxidant systems, such as SOD, catalase and GR. Our results showed, there were no significant differences in the SOD and CAT activities among the groups. However, the GR activity was significantly decreased in the ethanol treated group compared with normal group. When the mice treated with ethanol extract of *E. cava*, the GR activity was significantly increased. Therefore, the ethanol extract of *E. cava* might protect the liver by the reduction of oxidative stress.

Lipid peroxidation is considered as a mechanism for ethanol-induced liver injury. Alcohol administration can cause the production of reactive oxygen species (ROS). ROS, such as superioxide, hydroxyl radical, and hydrogen peroxide cause lipid peroxidation of cellular membrane, protein and DNA. That finally results in liver injury. In this study, the MDA formation due to the lipid peroxidation in the livers of mice induced by ethanol was increased in the ethanol treated group. The MDA levels in the *E. cava* ethanol extract treated mice were significantly inhibited. This result suggested that the *E. cava* extract can inhibit the lipid peroxidation which induced by ethanol administration. That might be caused by the antioxidant effect of *E, cava*

extract.

Although those of the biochemical assays are important for investigating the protective effect of *E. cava* on ethanol induced liver injury, the external investigation of the test mice livers and hepatic histopathologic changes were carried out. In the ethanol treated mice, the typical fatty liver was observed. *E. cava* ethanolic extract groups, the fatty livers were improved. On the other hand, the hepatic histology was not significantly improved by administration of *E. cava* ethanolic extract. This might be due to the short experimental period. Therefore, the continuous studies on hepatic histological changes could be investigated.

In conclusion, the liver injury induced by intake of ethanol is associated with oxidative stress. Our results indicated that the ethanolic extract of *E. cava* could reduced the ethanol induced liver injury *in vivo* through reducing the total cholesterol, inhibition of ROS generation and reduction of MDA formation. This hepatoprotective effect should due to the presence of bioactive compounds in the *E. cava* ethanolic extract. In addition, this study indicated that intake of *E. cava* could be beneficial to the human health.

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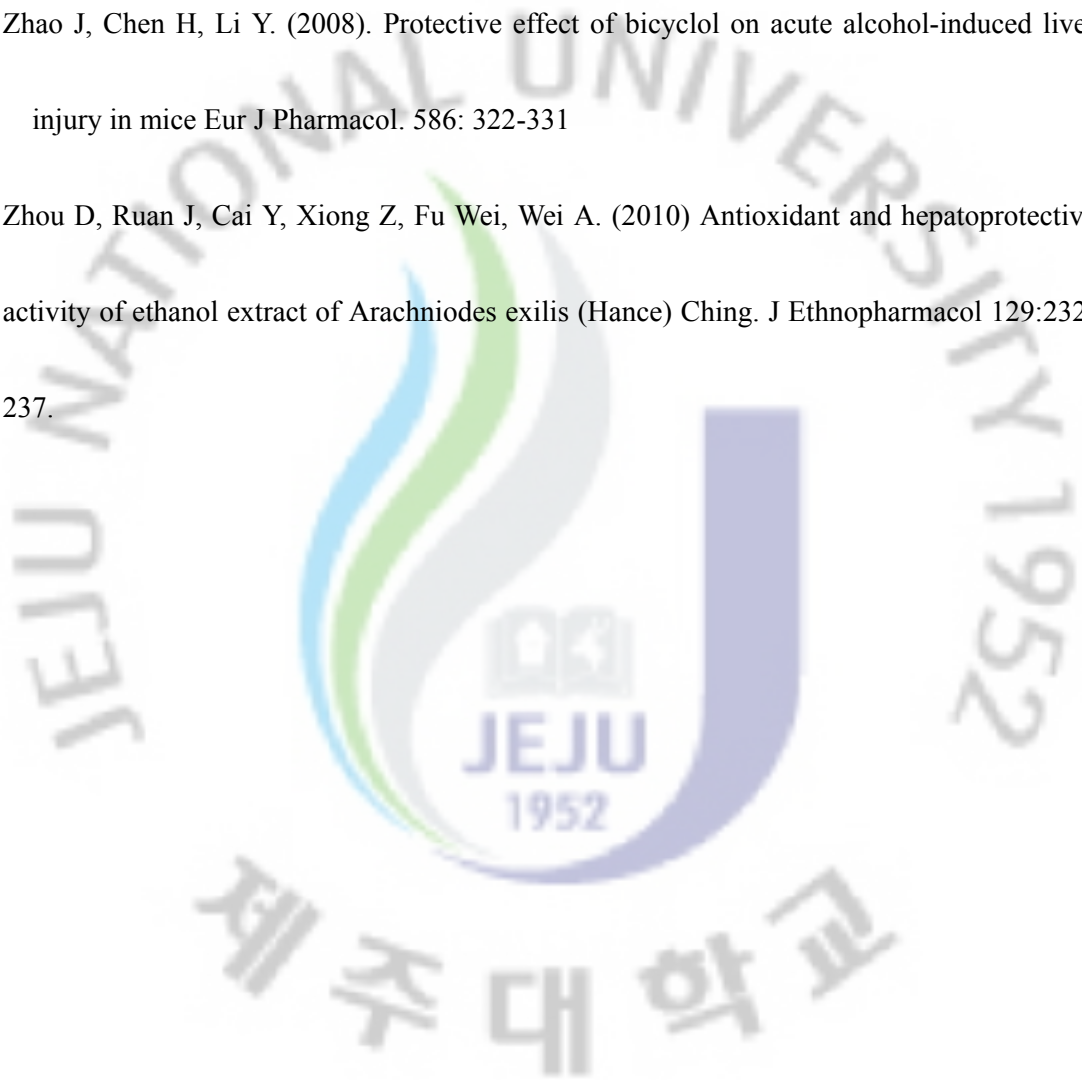
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