A THESIS

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Anti-inflammatory effect of physical damage-induced zebrafish model and identification of zebrafish mechanism about H_2O_2 induced oxidative stress

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Anti-inflammatory effect of physical damage-induced zebrafish model and identification of zebrafish mechanism about H_2O_2induced oxidative stress

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CONTENTS

국문초록……iv

List of Figures……vii

Part I Protocol development for Anti-inflammatory effect evaluation on physical damage-induced zebrafish model

Abstract……2

Introduction……3

MATERIALS AND METHODS……4

Origin and maintenance of parental zebrafish……4

Waterborne exposure of embryos to chemicals and cutting……4

Estimation of tail cutting induced intracellular ROS and NO generation and image analysis……5

Estimation of tail cutting induced intracellular ROS and NO generation and image analysis……5

Measurement of pro-inflammatory cytokines (TNF-α, IL-1ß) production……6

Determination of PGE_2production……6

Western blotting……7

RESULT AND DISCUSSION……7

Part II Anti-inflammation effect of fucoidan from *Eclonia cava* on physical damage and LPS-induced zebrafish

ABSTRACT……18

INTRODUCTION……19

Material and methods……20

Materials……20

Crude fucoidan separation from Ecklonia cava ……21

Cell culture……21

Determination of NO production……22

Western blot analysis……22

Origin and maintenance of parental zebrafish……23

Estimation of intracellular NO generation and image analysis……23

Estimation of intracellular ROS generation and image analysis……24

Western blotting of zebrafish……25

Result and discussion……25

Part III Protective effects of phlorofucofuroeckol-A against H_2O_2-induced oxidative stress in zebrafish embryo

Abstract……38

Introduction……39

Material and mathods……40

Origin and maintenance of parental zebrafish……42

Waterborne exposure of embryos to compounds and H_2O_2……42

Measurement of heart-beat rate……42

Estimation of intracellular ROS generation and image analysis……43

Lipid peroxidation inhibitory activity and image analysis……43

Western blotting……44

Result and discussion……44

REFERENCE……..51

국문초록

현재 in vivo 실험에서 가장 널리 쓰이는 동물은 마우스, 쥐와 토끼이다. 그러나 최근에 동물학대로 인한 윤리적인 문제가 대두되면서 이들의 이용과 관련하여 법적인 제약이 생겨났다. 이로 인해 2000년대에 in vivo 실험에서 제브라피쉬가 등장하여 점차 많이 사용되고 있다. 제브라피쉬는 마우스와 비슷하게 수명은 2년 정도이고 생후 3개월이면 번식이 가능하다. 계절에 관계없이 암컷은 일주일 간격으로 200-300개의 알을 낳고 체외수정을 하며 발생배가 투명하기 때문에 일반 해부현미경 하에서 쉽게 발생의 모든 과정을 관찰할 수 있다.이들은 발생이 매우 빨라 초기 세포분열이 대장균 보다도 빠른 15분 간격으로 진행되며, 발생 6 시간째에 gastrulation 이 시작되어4시간 만에 마치고, 12 시간이 지나면 눈의 형태형성이 이미 시작된다. 수정 후 24 시간이 지나면 심장의 박동과 혈액순환을 관찰할 수 있다. 제브라피쉬는 허파를 제외하고는 간과 췌장 그리고 지라와 흉선 등 면역계를 포함한 대부분의 기관을 가지며, 특히 돌연변이 연구에서 밝혀지는 여러 결과들이 인간의 유전질환과 매우 유사한 것으로 밝혀졌고, 인간과의 유전적 상동성도 현재 약 90%까지 일치하는 것으로 밝혀졌다.

제브라피쉬 모델의 장점

▶ 제브라피쉬의 수정란을 대량으로 쉽게 확보할 수 있음

▶ 발생이 매우 빨라 대부분의 조직 및 장기가 하루 만에 형성

▶ 투명한 난-현미경으로 발생단계, 혈류 흐름 및 형태적 관찰이 용이

▶ 척추동물로서 유전체 구성이 인간과 비슷함.

마우스와 인간과 아주 유사한 게놈 구조를 가지면서도 선충이나 초파리에서나 가능한 유전학적, 세포생물학적인 실험실이나 대규모 연구가 가능하다. 제브라피쉬의 발생배는 직경 0.7mm 정도로 작기 때문에 96-well microplate 에 3-5 embryo 정도씩 분주가 가능하며 소량의 화합물에 대해서도 형태 형성, 혈관형성 등 생체를 이용한 생리활성물질 검출이 가능하다.

1, 일반적으로 제브라피쉬에서 염증실험은 형질전환 제브라피쉬를 사용하여 측정하는 것이 주요 실험방법이다. 그러나 wild type zebrafish를 사용하여 항염증을 측정하는 방법은 아직까지 나와있지 않다. 이 연구에서는 wild type 제브라피쉬에서 물리적인 데미지를 입혀 염증을 유발하는 방법을 사용하였다. 물리적인 데미지를 입혀서 제브라피쉬를 실험한 결과 이미 항염활성이 있는 덱사메타손과 레스베라트롤이 이미 알려진 것과 거의 같은 항염 활성을 보였다. 그리고 웨스턴블롯과 사이토카인 키트에서 COX-2와 iNOS가 감소되었고 PGE2, IL-1beta, TNF-alpha 가 감소되었다. 이러한 결과 첫번째로 wild type 제브라피쉬를 사용하여 항염증 활성을 확인 할 수 있는 것이고, 두번째로 기존의 자극인자를 사용하지 않고 물리적 데미지를 입혀서 염증을 측정할 수 있었던 것이다.

2, 푸코이단은 생리활성이 뛰어나기에 여러 가지로 사용된다. 그러나 제주도에서 많이 채취되는 감태유래의 후코이단의 경우는 현재 후코이단의 원료로 거의 사용되지 않는다. 따라서 이 감태유래 후코이단의 항염증활성을 제브라피쉬를 사용하여 기존에 판매되어지고 있는 후코이단과 비교하여 기존 후코이단에 대용으로 사용할 수 있는지 알기위해 항염증 활성을 측정하였다. 그 결과 RAW264.7 세포와 제브라피쉬에서 동일한 결과가 나왔고, 기존의 후코이단과 비교하여 비슷하거나 더 좋은 항염증 활성을 나타내었다. 따라서 이 감태 유래 후코이단의 활성을 기존의 후코이단과 비교하여 기존 후코이단 대용으로 사용할 수 있을 것이라 생각된다.

3. 제브라피쉬를 사용하여 독성의 측정은 wild type 제브라피쉬로 가장 쉽고 빠르게 확인 할 수 있는 방법이다. 이 연구에서는 제브라피쉬를 사용하여 PFF-A의 항산화 활성을 측정하였다. 그 결과 PFF-A는 과산화수소의 제브라피쉬 발생배에 대한 독성에 대한 뛰어난 보호효과를 보였고, 형광 시약인 DCF-DA와 DPPP를 사용하여 분석한 결과도 항산화 활성이 뛰어남을 알 수 있었다. 이 결과 PFF-A는 항산화 활성을 갖고 있음을 확인할 수 있었다.

기존의 제브라피쉬를 사용한 염증활성에 대한 연구는 모두 형질전환 제브라피쉬를 사용하여 측정하였다. 하지만 이는 형질전환 제브라피쉬를 만들거나 따로 구입을 하지 못하는 실험실의 경우 제브라피쉬 사육 시스템을 갖추고도 제대로 필요한 실험을 못하는 경우가 발생하는 것이 된다. 따라서 wild type의 제브라피쉬를 사용하여 염증을 스크리닝 할 수 있게 된다면 보다 더 제브라피쉬를 사용한 염증 연구가 활발해 질 것으로 기대할 수 있다. 실험의 결과 wild type의 제브라피쉬에서도 충분히 염증을 스크리닝 할 수 있었고, 기존에 염증 유발물질로 많이 쓰이는 LPS 뿐만 아니라 물리적 데미지를 제브라피쉬에 가하여 염증을 유발하여도 충분히 염증 활성을 측정할 수 있음을 확인였다.

따라서 이 제브라피쉬를 사용하여 항염증 활성과 항산화 활성을 측정하였고, 이 실험방법들을 다른 실험에도 충분히 적용시킬 수 있었다. 앞으로도 이 방법들을 사용하여 항염증과 항산화 스크리닝을 할 것이다.

LIST OF FIGURE

Fig. 1-1. Inhibition effect of compounds on tail cutting induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of tail cutting induced NO generation intensity, as follows (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone and (iv) pretreated with resveratrol. (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone (0.2 μg/ml); (iv) pretreated with Dexamethasone (0.4 μg/ml); (v) pretreated with resveratrol (5 μg/ml) and (vi) pretreated with resveratrol (10 μg/ml).

Fig. 1-2. Inhibition effect of compounds on tail cutting induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of tail cutting induced ROS generation intensity, as follows (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone and (iv) pretreated with resveratrol. (B) After DCF-DA staining a fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone (0.2 μg/ml); (iv) pretreated with Dexamethasone (0.4

μg/ml); (v) pretreated with resveratrol (5 μg/ml) and (vi) pretreated with resveratrol (10 μg/ml).

Fig. 1-3. Expression of iNOS and COX-2 and ß -actin in zebrafish embryo. The embryos were treated to each of compounds and co-treated cutting tail. (I) control; (II) zebrafish embryo tail cutting only; (III) pretreated with Dexamethasone (0.2 μg/ml); (IV) pretreated with Dexamethasone (0.4 μg/ml); (V) pretreated with resveratrol (5 ug/ml) and (VI) pretreated with resveratrol (10 μg/ml)

Fig. 1-4. Inhibitory effects of dexamethasone and resveratrol on PGE_2 production in zebrafish. Zebrafishes were stimulated by tail cutting 3 hours later, and introduction of (i) dexamethasone (0.5μg/ml), (ii) resveratrol (10 μg/ml). Supernatants were collected, and the PGE_2 production in the supernatants was determined by ELISA.

Fig. 1-5. Inhibitory effects of dexamethasone and resveratrol on pro-inflammatory cytokines (TNF-α, IL-1ß) production in zebrafish. Zebrafishes were stimulated by tail cutting 3 hours later, and introduction of (i) dexamethasone (0.5 μg/ml), (ii) resveratrol (10 μg/ml). (A) Supernatants were collected, and the TNF-α production in the supernatants was determined by ELISA. (B) Supernatants were collected, and the IL-1ß production in the supernatants was determined by ELISA.

Fig. 2-1. Effects of Dexamethasone, SF and ECF on NO production in LPS-induced RAW264.7 cells. NO production was measured. Cells were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. Cytotoxicity was determined using the LDH method. Values are expressed as means ± S.D. of triplicate

experiments. *Pb 0.05 and **Pb 0.01 indicate significant differences from the LPS-stimulated group.

Fig. 2-2. Expression of iNOS and COX-2 and ß -actin in RAW264.7 cells. Cells were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml)

Fig. 2-3. LPS exposed each concentration (A) survival rate, (B) Heart beating, and (C) pericardial edema of zebrafish embryos. Zebrafish embryos were pretreated for 1 h with ECF (200 ug/ml) and then each LPS (2, 5, 10 μg/ml) was added the effect on toxicity were scored at 48 hpf.

Fig. 2-4. Inhibition effect of compounds on LPS induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and LPS (5 ug/ml) added. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of LPS induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 ug/ml); (IV) pretreated with SF (100 ug/ml); (V) pretreated with SF (200 ug/ml); (VI) pretreated with ECF (100 ug/ml) and (VI) pretreated with ECF (200 ug/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 ug/ml) and (v) pretreated with ECF (100 ug/ml).

Fig. 2-5. . Inhibition effect of compounds on LPS induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and LPS (5 ug/ml) added. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of LPS induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 ug/ml); (IV) pretreated with SF (100 ug/ml); (V) pretreated with SF (200 ug/ml); (VI) pretreated with ECF (100 ug/ml) and (VI) pretreated with ECF (200 ug/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 ug/ml) and (v) pretreated with ECF (100 ug/ml).

Fig. 2-6. Inhibition effect of compounds on tail cutting induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of tail cutting induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 ug/ml) and (V) pretreated with ECF (100 ug/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 ug/ml) and (v) pretreated with ECF (100 ug/ml).

Fig. 2-7. Inhibition effect of compounds on tail cutting induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. ROS generation

levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of tail cutting induced ROS generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 ug/ml) and (V) pretreated with ECF (100 ug/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 ug/ml) and (v) pretreated with ECF (100 ug/ml).

Fig. 2-8. Expression of iNOS and COX-2 and ß -actin in zebrafishes. zebrafishes were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml)

Fig. 3-1. (A) phlorofucofuroeckol-A (PFF-A), (B) Dieckol (DK), (C) resveratrol

Fig. 3-2. (A) Survival rate after treated with H_2O_2 or co-treated with compounds. The embryos were exposed to 5mM H_2O_2 and compounds treated. Resveratrol 10uM, Dieckol 50uM(DK) and Phlorofucofuroeckol-A 50uM(PFF-A). (B) Effects of compounds on the heart-beat rate for measurement of the toxicity of the tested samples. The embryos were exposed to 5mM H_2O_2 and compounds teated. The heart-beat was measured at 48hpf, under the microscopy. The number of heartbeat in 3min was counted, and the results are

expressed as the beats/min. Resveratrol 10uM ,Dieckol 50uM(DK) and Phlorofucofuroeckol-A 50uM(PFF-A). (C) Effects of compounds on the Precardial edema size for measurement of the toxicity of the tested samples. The embryos were exposed to 5mM H_2O_2 and compounds teated. The Precardial edema size was measured at 48hpf, under the microscopy. Resveratrol 10uM, Dieckol 50uM(DK) and Phlorofucofuroeckol-A 50uM(PFF-A).

Fig. 3-3. Inhibition effect of compounds on H_2O_2 induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and H_2O_2(5uM) added. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescencemicrographs of H_2O_2 induced ROS generation intensity, as follows (I) control; (II) H_2O_2 only; (III) pretreated with resveratrol (10ug/ml); (IV) pretreated with Dieckol (50uM) and (V) pretreated with PFF-A (50uM). (B) After DCF-DA staining a fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. Resveratrol 10uM ,Dieckol 50uM (DK) and Phlorofucofuroeckol-A 50uM (PFF-A).

Fig. 3-4. Inhibition effect of compounds on H_2O_2 induced lipidperoxidation level in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and H_2O_2 (5uM) added. ROS generation levels were measured after staining with DPPP. (A) Fluorescencemicrographs of H_2O_2 induced lipidperoxidation level intensity, as follows (I) control; (II) H_2O_2 only; (III) pretreated with resveratrol (10ug/ml); (IV) pretreated with Dieckol (50uM) and (V) pretreated with PFF-A (50uM). (B) After DPPP staining a fluorescence spectrophotometer was used for the quantitative analysis of lipidperoxidation level. Resveratrol 10uM ,Dieckol 50uM(DK) and Phlorofucofuroeckol-A 50uM(PFF-A).

Fig. 3-5. Expression of caspase-3, Bcl-xL, PARP and ß -actin in zebrafishes. The zebrafish embryos were pretreated with each of compounds and H_2O_2(5uM) added. Zebrafish embryos hatchis then harvested. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml)

PART. I

Protocol development for Anti-inflammatory effect evaluation on physical damage-induced zebrafish model

Protocol development for anti-inflammatory effect evaluation on physical damage-induced zebrafish model

Abstract

Zebrafish (*Danio rerio)* is an important vertebrate model organism in scientific research. In this study, anti-inflammatory effect of fucoidan isolated from *Ecklonia cava* was assessed via inhibitory effect of nitric oxide (NO) and reactive oxygen species (ROS) production and expression of iNOS and COX-2 in physical damage induced zebrafish model. Anti-inflammation material known dexamethasone, resveratrol was used as compounds. To identify the active compounds, the inhibitory effect of NO and ROS production. Zebrafish is the inhibitory effect of NO, ROS production by fluorescent image and fluorescence spectrophotometer after DCF-DA or DAF-FM DA staining. Western blot assay was observed the inhibitory effects on zebrafish by iNOS, COX-2 results. Results of the experiment with the previous papers data showed the same tendency. In order to determine the potential NO production of anti-inflammation agents, we observed the growth pattern of zebrafish and compounds showed inhibitory effects against NO production on tested embryos. And we can used screening anti-inflammation to wild type zebrafish not used other stimulator and transgenic zebrafish.

Keyword: Physical Damage, Fucoidan, *Ecklonia cava*, Zebrafish, Anti-inflammation,

Introduction

NO is easily spread, short-lived unstable free radical and an important physiological mediator. Although the excess of NO expression has been showed that the cause in the pathogenesis of various inflammatory diseases and immunologically mediated diseases, for example, graft-versus-host disease (Langrehr et al., 1992), diabetes (Cobertt et al., 1991), viral infections (Zheng et al., 1993), and arthritis (Farrel et al., 1992). NO is derived from oxidation of a guanidine nitrogen of L-arginine (palmer et al., 1988, Schmidt et al., 1988) and this reaction is catalyzed by nitric oxide synthase (NOS) (EC1.14.13.39). The third NOS isoform, the iNOS produces lager amounts of NO when the cells are stimulated with bacterial secretion and cytokines (IL-, IL-6, TNF-, IFN-). This enzyme is expressed nearly in macrophages, monocytes (Hibbs et al., 1988), neutrophils (Wright et al., 1989, Wheeler et al., 1997), and hepatocytes (Curran et al. 1989). The second COX isoform, by some new organization and specificity physiological environment COX-2 is the increase in activity at the site of inflammation. This COX-2 worsening inflammation acts on the secretion of prostaglandins.

The zebrafish (*Danio rerio*) is a small tropical freshwater fish which has emerged as a useful vertebrate model because of its small size Large number of offspring (200~300 eggs/ wk), transparent embryo, Low cost maintenance, Rapid generation cycle (2-3 months), Low space requirement, Rapid development or organogenesis and physiological similarity to mammals (Eisen., 1996, Fishman., 1999). The zebrafish is utilized to screening test of drug activation, because the early stage embryo rapidly absorbs small molecular compounds diluted in the bathing media through the skin and gills (Cha et al., 2010).

This experiment on zebrafish is being develop and studied, although there are a few to measure the anti-inflammation experiments have been reported using the wild type zebrafish. Therefore, in this study we use a wild type zebrafish was measured the anti-inflammation experiments.

Materials and Methods

Origin and maintenance of parental zebrafish

Adult zebrafishes were obtained from a commercial dealer (Seoul aquarium, Korea) and 10 fishes were kept in 3 l acrylic tank with the following conditions; 28.5℃, with a 14/10 h light/dark cycle. Zebrafishes were fed three times a day, 6 d/week, with Tetramin flake food supplemented with live brine shrimps (Artemia salina). Embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 min and staged as described by (Kimmel et al., 1995)

Waterborne exposure of embryos to chemicals and cutting

After hatching, chemicals were administrated into embryo media. Three replicate treatment groups were exposed to each dose in 24 well polystyrene multi-well plates (20 to 30 embryos per well). After 1 hour, fishs were anaesthetized in tricaine methanesulfonate 75 - 125 mg/L (Sigma, MS-222) before tail cutting. Surgical knife and microscope, were used to cut the tail of the zebra fish. As soon as tail cutting was completed, the zebrafish embryos

were rinsed in fresh zebrafish embryo medium. Then, medium was changed and chemicals were introduced.

Estimation of tail cutting induced intracellular ROS and NO generation and image analysis

Generation of ROS and NO in zebrafish embryos was analysed modify method previously reported (Cha et al., 2011) using a fluorescent probe dye, 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) and diaminofluorophore 4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate (DAF-FM DA). Two hours after tail cutting and introduction of chemicals, Later 2 hours, the zebrafish embryos were transferred into 96-well plates and treated with DCF-DA (20μg/ml) or DAF-FM DA solution (5 μM). Then the plates were incubated for 1 h in dark at 28.5 ℃. After incubation, anaesthetized in tricaine methanesulfonate 75 - 125 mg/L (Sigma, MS-222) before observation. The zebrafish embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin - Elmer LS-5B, Austria) individually and the image of stained embryos were observed using a fluorescent microscope, which was equipped with a moticam color digital camera (motix, Xiamen, China).

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(DAF-FM DA). Two hours after tail cutting and introduction of chemicals, Later 2 hours, the zebrafish embryos were transferred into 96-well plates and treated with DCF-DA or DAF-FM DA solution. Then the plates were incubated for 1 h in dark at 28.5 ℃. After incubation, anaesthetized in tricaine methanesulfonate 75 - 125 mg/L before observation. The zebrafish embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin - Elmer LS-5B, Austria) individually and the image of stained embryos were observed using a fluorescent microscope, which was equipped with a moticam color digital camera (motix, Xiamen, China).

Measurement of pro-inflammatory cytokines (TNF-α, IL-1ß) production

Following tail cutting and introduction of chemicals, 3 hours later, each zebrafish embryos as harvested, sonicated in lysis buffer. The lysate was clarified by centrifuging at 10,000 rpm for 5 min. The inhibitory effect of chemicals (dexamethasone and resveratrol) on the pro-inflammatory cytokines (TNF-α, IL-1ß) production from tail cut zebrafish was determined as described in the (Cho et al,. 2000)

Determination of PGE_2 production

Following tail cutting and introduction of chemicals, 3 hours later, each zebrafish embryos as harvested, sonicated in lysis buffer. The lysate was clarified by centrifuging at 10,000 rpm for 5 min. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immuno assay kit according to the manufacturer's in structions, The production of PGE_2 was measured relative to that following control treatment.

Western blotting

Following tail cutting and introduction of chemicals, 3 hours later, each zebrafish embryos as harvested, sonicated in 70 μl lysis buffer. The lysate was clarified by centrifuging at 10,000 rpm for 5 min. Then, the supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 mg of protein) were boiled for 5 min and electrophoresed in 7.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Subsequently, the blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, USA). After blocking with 5% Probumin BSA (Millipore, Massachusetts, United States) for 1 h, the blots were incubated with iNOS (1:1500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or COX-2 (1:1000 dilution, Cell Signaling, Massachusetts, United States) for 60 min. The membranes were incubated for 45 min with the anti-mouse or anti-rabbit IgG (1:2,000, Santa Cruz Biotechnology, Inc) horseradish peroxidase conjugates (Pierce, United States). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, UK), and then exposed onto X-ray film.

Results and Discussion

Generally after the in vitro tests for compounds screening in vivo experiments was go through, all in vivo experiments was not entirely consistent with match in vitro experimental results. And the most widely used in mouse experiments case at in vivo experiments because it requires a lot of time and money, it needed in vitro experiments and at in vivo

experiments, or experiments a replaceable between the mouse experiments. Here is reasonable is to use zebrafish. Because zebrafish was used at in vivo experiments similar human genes, a short hatching time, due to the external development of the transparent embryo and etc. These zebrafish used anti-inflammatory experimental method is to make the transgenic zebrafish in pursuance of to observe the movement of leukocyte (Mathias et al., 2006). This method is very good methods. However, if laboratory do not have test apparatus to create these zebrafish, buy experiment machine or to purchase the transgenic zebra fish, this poor conditions in laboratory also need experiment at screening for anti-inflammatory activity replace the mouse experiment in the in vivo or its predictable experiment the results. For this reason, use a wild type zebrafish to measure the anti-inflammation was initiated this study.

Inflammation is the body's defense due to certain stimuli (injury, burns, invasion of pathogen etc.). Inflammation is migration of leukocytes into infected or damaged tissue and involves the activation, and Macrophage acts an important role for inflammatory diseases relating to over production of inflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis fector (TNF)-α, and reactive oxygen species (ROS), and nitric oxide (NO), and prostaglandin E2 (PGE2), generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (20, 21). In this experiment, compounds were for identify the effect on inflammation used positive control by dexamethasone and resveratrol (Tsai et al. 1999). Dexamethasone is synthetic compound of the glucocorticoid class of steroid drugs. Dexamethasone employded as anti-inflammation drug and medicine of autoimmune disease, such as rheumatoid arthritis and bronchospasm. Dexamethasone employded as anti-inflammation drug and medicine of autoimmune disease, such as rheumatoid arthritis and bronchospasm.

Resveratrol is a type of polyphenol, and produced naturally by several plants when under attack by pathogens(bacteria, fungi), UV etc (Dixon., 2001). Its revealed that biological activities of resveratrol such as anticoagulation (Dixon et al., 1995), antioxidant (Fremont et al., 1999), anti-inflammation (Subbaramariah et al., 1999, MacCarrone et al., 1999), antitumor(Fontecative et al., 1998).

In this study, induces inflammation to cut tail zebrafish. After 3 hour cutting tail measured NO on the basis of once hurt to zebrafish, approximately 180 min leukocyte migration in response to a wound (Mathias et al., 2006). For validate of study, analysed using DCF-DA the generation of ROS in zebrafish embryos and fluorescent images, and analysed using DAF-FM DA the generation of NO in zebrafish embryos and fluorescent images (Cha et al., 2011). And using western blot was measured inflammatory cytokine iNOS, COX-2 expression. Generation of NO in zebrafish embryos and fluorescent image was analysed using a DAF-FM DA. A simple and sensitive assay and a cellular bioimaging method for NO were using a diaminofluorescein DAF-FM and its diacetate. DAF-FM is converted via an NO-specific mechanism to an intensely fluorescent triazole derivative. (Itoh et al., 2000).

(Fig. 1-1.(A)) in the fluorescent image, can be found of NO generation degree with a fluorescent difference of the zebrafish and the wild type zebrafish that a tail is cut. Each of compounds treatment of (iii)-(vi) groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results. The result, the level of NO in the tail cut zebrafish embryos was 2642 absorbance compared with the negative control embryos. Intracellular NO accumulations were 2297, 2184,

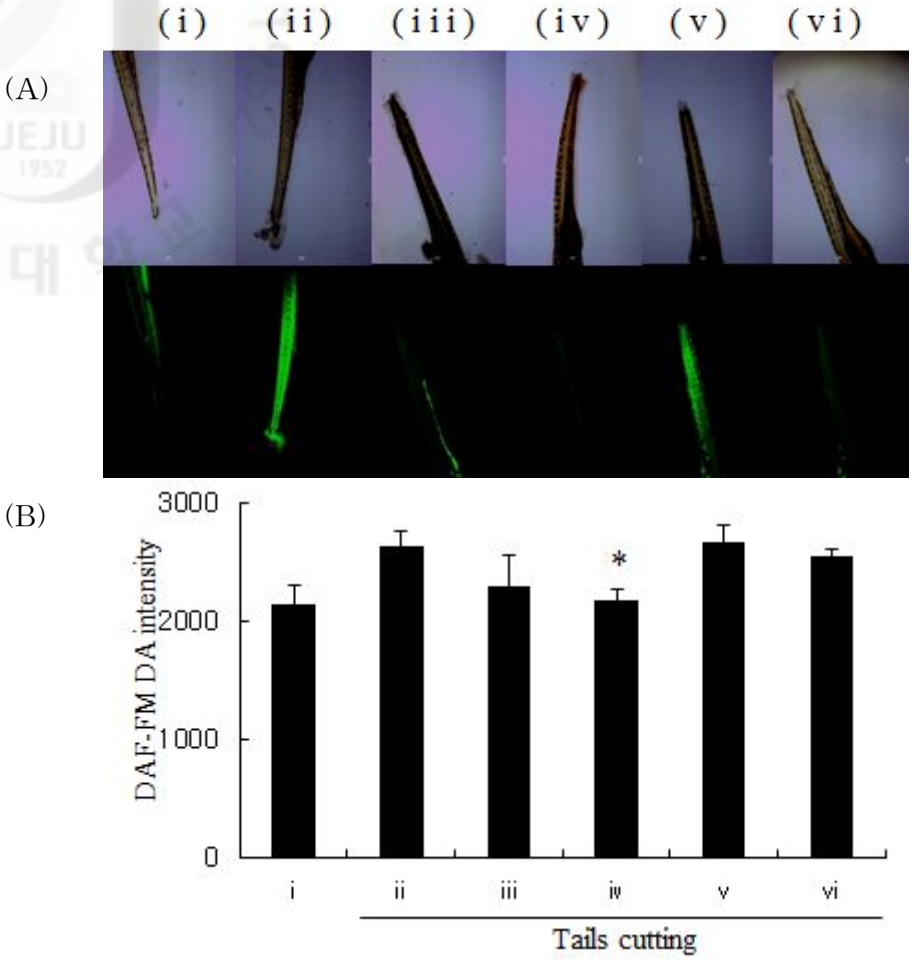


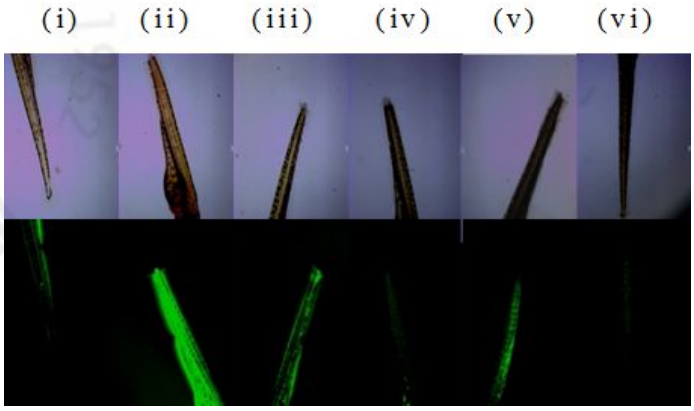
Fig. 1-1. Inhibition effect of compounds on tail cutting induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of tail cutting induced NO generation intensity, as follows (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone and (iv) pretreated with resveratrol. (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone (0.2 μg/ml); (iv) pretreated with Dexamethasone (0.4 μg/ml); (v) pretreated with resveratrol (5 μg/ml) and (vi) pretreated with resveratrol (10 μg/ml).

2679 and 2550 absorbance in the embryos pretreated with dexamethasone 0.2 μg/ml, dexamethasone 0.4 μg/ml, resveratrol 5 μg/ml and resveratrol 10 μg/ml, respectively.(Fig. 1-1.(B)).

(Fig. 1-2.(A)) in the fluorescent image, can be found of ROS generation degree with a fluorescent difference of the zebrafish and the wild type zebrafish that a tail is cut. Each of compounds treatment of (iii)-(vi) groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results. The result, the level of ROS in the tail cut zebrafish embryos was 2358 absorbance compared with the negative control embryos. Intracellular ROS accumulations were 2231, 2190, 2121 and 2004 absorbance in the embryos pretreated with dexamethasone 0.2 μg/ml, dexamethasone 0.4 μg/ml, resveratrol 5 μg/ml and resveratrol 10 μg/ml, respectively (Fig. 1-2.(B)). As shown in (Fig. 1-3), by the Western blotting results, the expression of iNOS and COX-2 confirm tail cutting zebrafish increase express of iNOS and COX-2 then wild type zebrafish. Check Protein bands results, chemical treatment of (iii)-(vi) groups, iNOS and COX-2 than negative expression of all of groups small. (Fig. 1-4.) inhibitory effects of dexamethasone and resveratrol on PGE_2 production in zebrafishes were stimulated by tail cutting(Fig.1-4.). Tail cut zebrafish increase on PGE_2 production then normal zebrafish. dexamethasone and resveratrol treatment groups, on PGE_2productiondecreasethanonlytailcuttinggroup. (Fig. 1-5.) inhibitory effects of dexamethasone and resveratrol on pro-inflammatory cytokines (TNF-α, IL-1ß) production in zebrafishes were stimulated by tail cutting. (Fig. 1-5.) tail cut zebrafish increase on pro-inflammatory cytokines (TNF-α, IL-1ß) production then normal zebrafish. (Fig. 1-5.(A)) dexamethasone and resveratrol treatment groups, on TNF-α production decrease than only tail cutting group, and IL-1ß production also shoes the similar results.

As this experiment, we can instead induce inflammation to tail cut of stimulator. And a teansgenic zebrafish as wild type zebrafish had screened anti-inflammation. Study result, dexamethasone and resveratrol showed similar tendency to the results of previous studies. Therefore, this method can be used screening for anti-inflammatory seems to be. Finally, this way want to be assist to other scientists in anti-inflammation experiment.

(A)



(B)

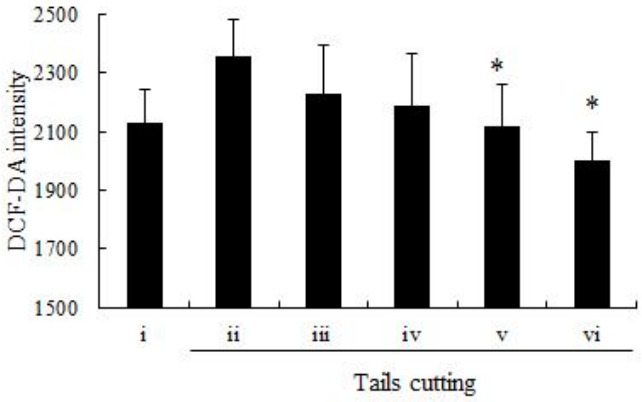


Fig. 1-2. Inhibition effect of compounds on tail cutting induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of tail cutting induced ROS generation intensity, as follows (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone and (iv) pretreated with resveratrol. (B) After DCF-DA staining a fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. (i) control; (ii) zebrafish embryo tail cutting only; (iii) pretreated with Dexamethasone (0.2 μg/ml); (iv) pretreated with Dexamethasone (0.4 μg/ml); (v) pretreated with resveratrol (5 μg/ml) and (vi) pretreated with resveratrol (10 μg/ml).

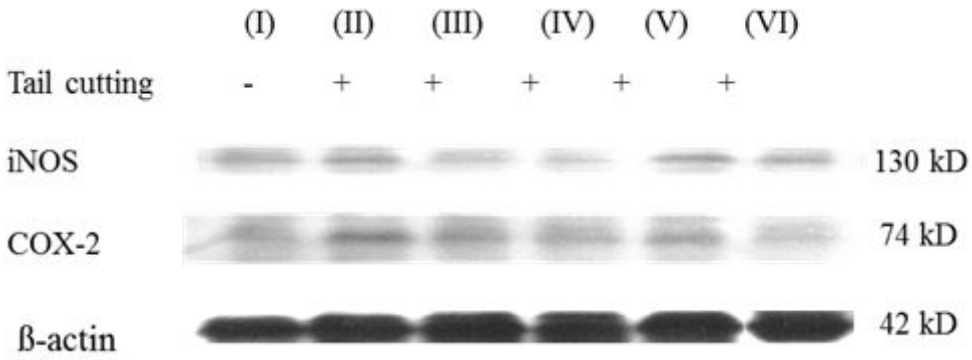


Fig. 1-3. Expression of iNOS and COX-2 and ß -actin in zebrafish embryo. The embryos were treated to each of compounds and co-treated cutting tail. (I) control; (II) zebrafish embryo tail cutting only; (III) pretreated with Dexamethasone (0.2 μg/ml); (IV) pretreated with Dexamethasone (0.4 μg/ml); (V) pretreated with resveratrol (5 μg/ml) and (VI) pretreated with resveratrol (10 μg/ml).

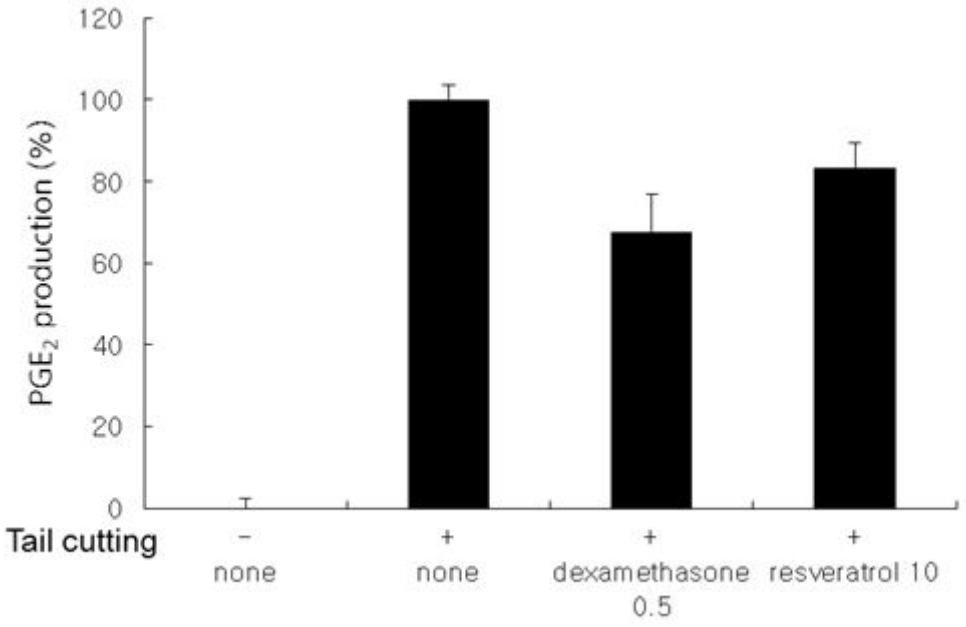


Fig. 1-4. Inhibitory effects of dexamethasone and resveratrol on PGE_2 production in zebrafish. Zebrafishes were stimulated by tail cutting 3 hours later, and introduction of (i) dexamethasone(0.5μg/ml), (ii) resveratrol (10 μg/ml). Supernatants were collected, and the PGE_2 production in the supernatants was determined by ELISA.

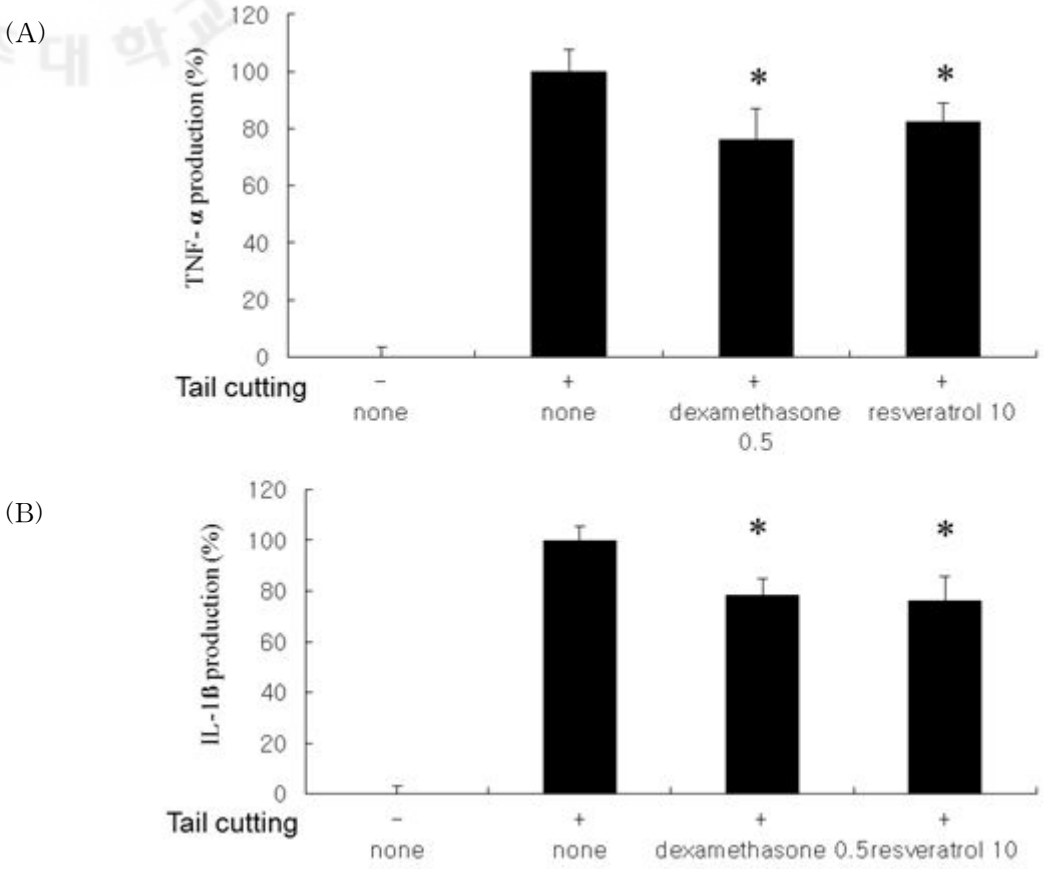


Fig. 1-5. Inhibitory effects of dexamethasone and resveratrol on pro-inflammatory cytokines (TNF-α, IL-1ß) production in zebrafish. Zebrafishes were stimulated by tail cutting 3 hours later, and introduction of (i) dexamethasone (0.5 μg/ml), (ii) resveratrol (10 μg/ml). (A) Supernatants were collected, and the TNF-α production in the supernatants was determined by ELISA. (B) Supernatants were collected, and the IL-1ß production in the supernatants was determined by ELISA.

PART. II

Anti-inflammation effect of fucoidan from *Eclonia cava* in physical damage and LPS-induced zebrafish

Anti-inflammation effect of fucoidan from *Eclonia cava* in physical damage and LPS-induced zebrafish

ABSTRACT

Fucoidan is a sulfated polysaccharide found mainly in various species of brown seaweed. Zebrafish, *Danio rerio*, is an important vertebrate model organism in scientific research. In this study, potential anti-inflammatory effect of fucoidan isolated from brown algae was assessed via inhibitory effect of nitric oxide (NO) production in lipopolysaccharide (LPS) induced RAW 264.7 macrophage cells and zebrafish model. To identify the active compounds, the inhibitory effect of NO production. The isolated fucoidan enhance the cell viability against LPS-induced NO damage in RAW 264.7 cells. Zebrafish is the inhibitory effect of NO production by fluorescence spectrophotometer after DAF-FM DA and DCF-DA staining. Western blot assay was observed the inhibitory effects on RAW 264.7 cells by iNOS, COX-2 results. Results of the zebrafish experiment also showed the same tendency. In order to determine the potential NO product of anti-inflammation agents, we observed the growth pattern of zebrafish and fucoidan showed inhibitory effects against NO production on tested embryos. On the other hand, the presence of LPS embryo also showed NO products.

Key word: zebrafish, inflammation, fucoidan, NO

INTRODUCTION

NO is easily spread, short-lived unstable free radical and an important physiological mediator. Although the excess of NO expression has been showed that the cause in the pathogenesis of various inflammatory diseases and immunologically mediated diseases, for example, graft-versus-host disease (Kim et al., 2006), diabetes (Eisen., 1996), viral infections (Fishman., 1999), and arthritis (DrieverW et al., 1996). NO is derived from oxidation of a guanidine nitrogen of L-arginine (Kimmel et al., 1989, den., 2005) and this reaction is catalyzed by nitric oxide synthase (NOS) (EC1.14.13.39). The third NOS isoform, the iNOS produces lager amounts of NO when the cells are stimulated with bacterial secretion and cytokines (IL-1β, IL-6, TNF-α, IFN-ɣ). This enzyme is expressed nearly in macrophages, monocytes (Pichler et al 2003), neutrophils (Langheinrich et al., 2003, Choi et al., 2007), and hepatocytes (Cha et all., 2010). The second COX isoform, by some new organization and specificity physiological environment COX-2 is the increase in activity at the site of inflammation. This COX-2 worsening inflammation acts on the secretion of prostaglandins.

The zebrafish (*Danio rerio*) is a small tropical freshwater fish which has emerged as a useful vertebrate model because of its small size, large number of offspring (200~300 eggs/ wk), transparent embryo, low cost maintenance, rapid generation cycle (2-3 months), low space requirement, rapid development or organogenesis and physiological similarity to mammals (Eisen 1996; Fishman 1999). The zebrafish is utilized to screening test of drug activation, because the early stage embryo rapidly absorbs small molecular compounds diluted in the bathing media through the skin and gills.

Fucoidans, polysaccharides containing considerable proportion of L-fucose and sulfate ester groups, are constituents of brown seaweed and some marine invertebrates (Chizhov et al., 1999, Bilan et al., 2002). The polysaccharide was named as "fucoidin" when it was first isolated from marine brown algae by Kylin in 1913. Now it is named as "fucoidan" according to IUPAC rules, but some also called it fucan, fucosan or sulfated fucan (Choi et al., 1999). Previous papers about fucoidans isolated from different species have been extensively studied due to their varied biological activities such as Anticoagulant (Grauffel et al., 1981, Mauray et al., 1995, Pereira et al., 1999, Kuznetsova et al., 2003), antiproliferative, antiadhesive (McCaffrey et al., 1992), anti-inflammation (Kubes et al., 1995, Omata et al., 1997, Granert et al., 1999, Foxall et al 1992, Game et al., 1998), antineoplastic, antitumor, antimetastatic (Yamamoto et al., 1984, Coombe et al., 1987, Riou et al., 1996) etc.

However, there are a few studies on inflammatory experiments have been reported using the zebra fish. In this study we have focused the fucoidan from *E. cava* against anti-inflammatory experiments

Material and methods

Material

The E. cava was collected along the coast of Jeju Island, Korea, between October 2009 and March 2010. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface. After then carefully rinsed with fresh water, and maintained in a medical

refrigerator at −20°C. Finally, the frozen samples were lyophilized and homogenized with a grinder prior to extraction. Dexamethasone (D8893, sigma), and fucoidan from *Fucus vesiculosus* (F5631, sigma), and other chemicals used were of 99% or greater purity.

Crude fucoidan separation from Ecklonia cava

The enzymatic digest followed the method previously reported (**Heo et al., 2003**). 5 g of the ground dried E. cava powder was homogenised with 200 mL of distilled water and mixed with 50 μL of Celluclast (Novo Nordisk, Bagsvaerd, Denmark). The reaction with this enzyme was conducted at 50 ○C for 24 h. As soon as the enzymatic reaction was completed, the digest was boiled for 10 min at 100 ○C to inactivate the enzyme. The product was clarified by centrifugation (3000 rpm, for 20 min) to remove any unhydrolysed residue. The enzymatic digest, obtained after filtration of the supernatant, was adjusted to pH 7.0. The enzymatic extract was (200 ml) mixed well with 10 ml of Calcium chloride ($CaCl_2$).Then, the mixture was allowed to stand for 6hr at a 2 supernatant were collected by centrifugation at 10,000 rpm for 20 min at 4. The supernatant was (200 ml) mixed well with 600 ml of 99.5% ethanol. Then, the mixture was allowed to stand for 6hr at a 4 crude fucoidan were collected by centrifugation at 10,000 rpm for 20 min at 4 (**Kuda et al., 2002, Matsubara et al., 2000**).

Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Inc., NY,

USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (FBS; GIBCO). The cells were then incubated in an atmosphere of 5% CO2 at 37 °C and were subcultured every 3 days.

Determination of NO production

After a 24 h pre-incubation of RAW 264.7 cells (1.5×105 cells/ml) with LPS (1 μg/ml), the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. In brief, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid], the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

Western blot analysis

Murine macrophage RAW 264.7 cells were pre-incubated for 18 h, then stimulated with LPS (1 μg/ml) in the presence of FX for the indicated times. After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mMTris - HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin] and maintained on ice for 30 min. The cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCATMprotein assay kit. Aliquots of the lysates (30 - 50 μg of protein) were separated on 12% SDS-polyacrylamide gel and

transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer [192 mM glycine, 25 mM Tris - HCl (pH 8.8), 20% methanol (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA), the membrane was incubated overnight with specific primary antibody at 4 ℃. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

Origin and maintenance of parental zebrafish

Adult zebrafishes were obtained from a commercial dealer (Seoul aquarium, Korea) and 10 fishes were kept in 3 l acrylic tank with the following conditions; 28.5℃, with a 14/10 h light/dark cycle. Zebrafishes were fed three times a day, 6 d/week, with Tetramin flake food supplemented with live brine shrimps (Artemia salina). Embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 min and staged as described by Kimmel et al (1995) (32).

Estimation of intracellular NO generation and image analysis

Generation of NO in zebrafish embryos was analysed modify method previously reported (34) using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate (DAF-FM DA). First method, two hours after tail cutting and introduction of chemicals, Later 2 hours, the zebrafish embryos were transferred into 96-well plates and

treated with DAF-FM DA solution (5 uM). Second method, after hatching pretreated chemical and then LPS was added incubated for 24hr. next , the zebrafish embryos were transferred into 96-well plates and treated with DAF-FM DA solution (5 uM). Then the plates were incubated for 1 h in dark at 28.5 ℃. After incubation, anaesthetized in tricaine methanesulfonate 75 - 125 mg/L (Sigma, MS-222) before observation. The zebrafish embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin - Elmer LS-5B, Austria) individually and the image of stained embryos were observed using a fluorescent microscope, which was equipped with a moticam color digital camera (motix, Xiamen, China).

Estimation of intracellular ROS generation and image analysis

Generation of ROS in zebrafish embryos was analysed modify method previously reported (34) using a fluorescent probe dye, 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). First method, two hours after tail cutting and introduction of chemicals, Later 2 hours, the zebrafish embryos were transferred into 96-well plates and treated with DAF-FM DA solution (5 uM). Second method, after hatching pretreated chemical and then LPS was added incubated for 24hr. next , the zebrafish embryos were transferred into 96-well plates and treated with DAF-FM DA solution (5 uM). Then the plates were incubated for 1 h in dark at 28.5 ℃. After incubation, anaesthetized in tricaine methanesulfonate 75 - 125 mg/L (Sigma, MS-222) before observation. The zebrafish embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin - Elmer LS-5B, Austria) individually and the image of stained embryos were observed using a fluorescent microscope, which was equipped with a moticam color digital camera (motix, Xiamen, China).

Western blotting of zebrafish

Following tail cutting and introduction of chemicals, 3 hours later or LPS was added incubated for 24hr, each zebrafish embryos as harvested, sonicated in 70 ul lysis buffer. The lysate was clarified by centrifuging at 10,000 rpm for 5 min. Then, the supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 mg of protein) were boiled for 5 min and electrophoresed in 7.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Subsequently, the blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, USA). After blocking with 5% Probumin BSA (Millipore, Massachusetts, United States) for 1 h, the blots were incubated with iNOS (1:1500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or COX-2 (1:1000 dilution, Cell Signaling, Massachusetts, United States) for 60 min. The membranes were incubated for 45 min with the anti-mouse or anti-rabbit IgG (1:2,000, Santa Cruz Biotechnology, Inc) horseradish peroxidase conjugates (Pierce, United States). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, UK), and then exposed onto X-ray film.

Result and discussion

First, sample is for identify the effect on inflammation used dexamethasone and already on the market fucoidan (Sigma, SF) by positivecontrol. In zebrafish induced inflammation was used two ways of LPS(*Lipopolysaccharide*) and physical damage, and in Raw 264.7 cell used

LPS. LPS from many bacterial species will initiate acute inflammatory responses in mammals that are typical of the host reaction to tissue injury or infection. And because hurt of the tissue induce inflammation, it induces inflammation to cut tail of zebrafish. Degree of inflammation analysed using DCF-DA the generation of ROS in zebrafish embryos and fluorescent images, and analysed using DAF-FM DA the generation of NO in zebrafish embryos and fluorescent images. And using western blot was measured inflammatory cytokine iNOS, COX-2 expression. For tail cut method, 3 hour after cutting tail measured NO production, on the basis of on once give the wound zebrafish, approximately 180 min after, leukocyte migration in response to a wound (Mathias et al., 2006). A simple and sensitive assay and a cellular bioimaging method for NO were using a diaminofluorescein DAF-FM and its diacetate. DAF-FM is converted via an NO-specific mechanism to an intensely fluorescent triazole derivative. (Determination et al., 2000) And using western blot was measured inflammatory cytokine iNOS, COX-2 expression.

Effects of Dexamethasone, SF and ECF on NO production in LPS-induced RAW264.7 cells. Cells were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. LPS treatment significantly increased the concentrations of NO. Dexamethasone, SF and ECF inhibited LPS-induced NO production in a concentration-dependent manner: SF 31% and 50%, ECF 42% and 56% at 50 and 100 μg/ml, respectively. And The cytotoxic effects of Dexamethasone, SF and ECF were assessed in the presence or absence of LPS via an LDH assay (Fig.1).

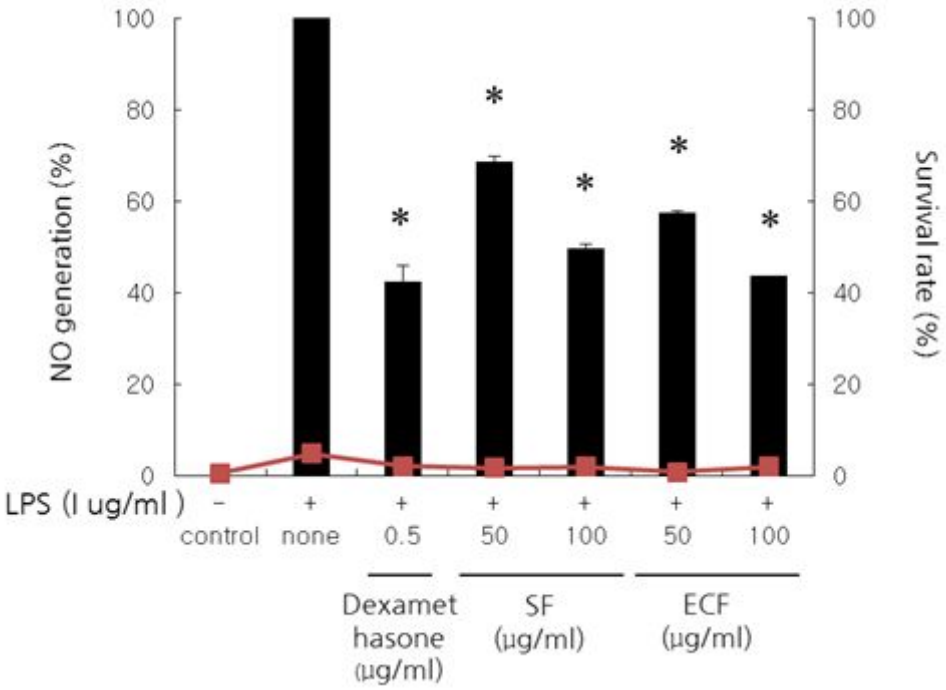


Fig. 2-1. Effects of Dexamethasone, SF and ECF on NO production in LPS-induced RAW264.7 cells. NO production was measured. Cells were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 µg/ml) was added and incubated for 24 h. Cytotoxicity was determined using the LDH method. Values are expressed as means±S.D. of triplicate experiments. *Pb0.05 and **Pb0.01 indicate significant differences from the LPS-stimulated group.

In an effort to characterize the anti-inflammatory activities of SF and ECF, we assessed the effects of SF and ECF on LPS induced iNOS and COX-2 protein upregulation in RAW 264.7 cells, via Western blotting (Fig.2). These results compelled us to evaluate in the effects of fucoidan (SF and ECF) on the expression of the iNOS and COX-2 enzymes, which generate NO as key mediators of inflammation.

The next experiment is toxicity of LPS and protective effects of ECF in zebrafish. (Fig. 3 (A)) survival rate, (Fig. 3 (B)) heart beating, and (Fig. 3 (C)) pericardial edema of zebrafish embryos. Zebrafish embryos were pretreated for 1 h with ECF (200 μg/ml) and then each LPS (2, 5, 10 μg/ml) was added the effect on toxicity were scored after hatching. The results, ECF had known protective effects for toxicity of LPS. (Fig.4.(A)) in the fluorescent image, can be found of NO generation degree with a fluorescent difference of the zebrafish and treated LPS (5 μg/ml) in zebrafishs. Each of compounds treatment of (iii)-(v) groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results. The result, the level of NO treated LPS in zebrafish embryos was 2817 absorbance compared with the negative control embryos. Intracellular NO accumulations were 2216, 2417 and 2307 absorbance in the embryos pretreated with dexamethasone 0.4 μg/ml, SF 100 μg/ml and ECF 100 μg/ml, respectively.(Fig.4.(B)). (Fig.5.(A)) in the fluorescent image, can be found of ROS generation degree with a fluorescent difference of the zebrafish. Each of compounds treatment of (iii)-(v) groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results. The result, the level of ROS in the tail cut zebrafish embryos was 7410 absorbance compared with the negative control embryos.

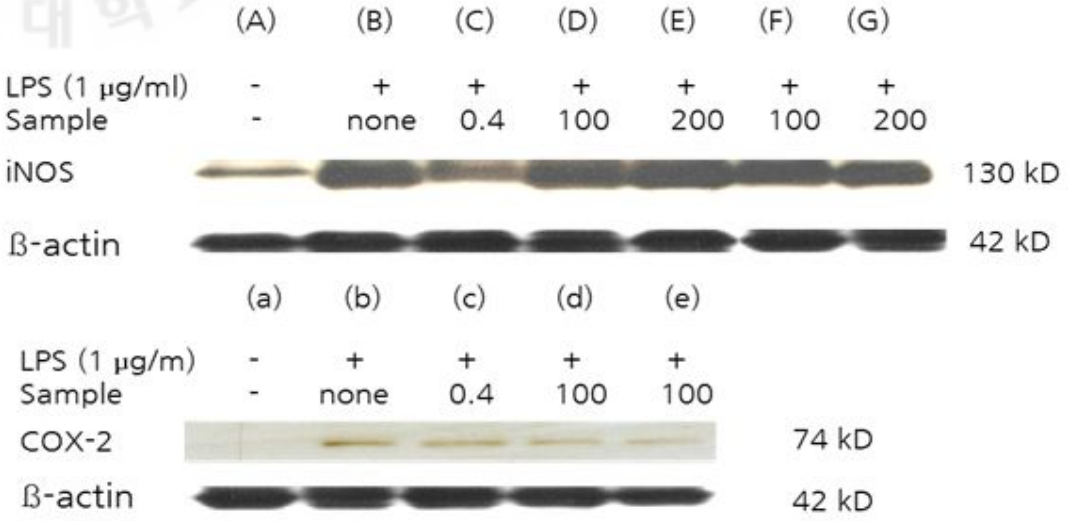


Fig. 2-2. Expression of iNOS and COX-2 and ß -actin in RAW264.7 cells. Cells were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml).

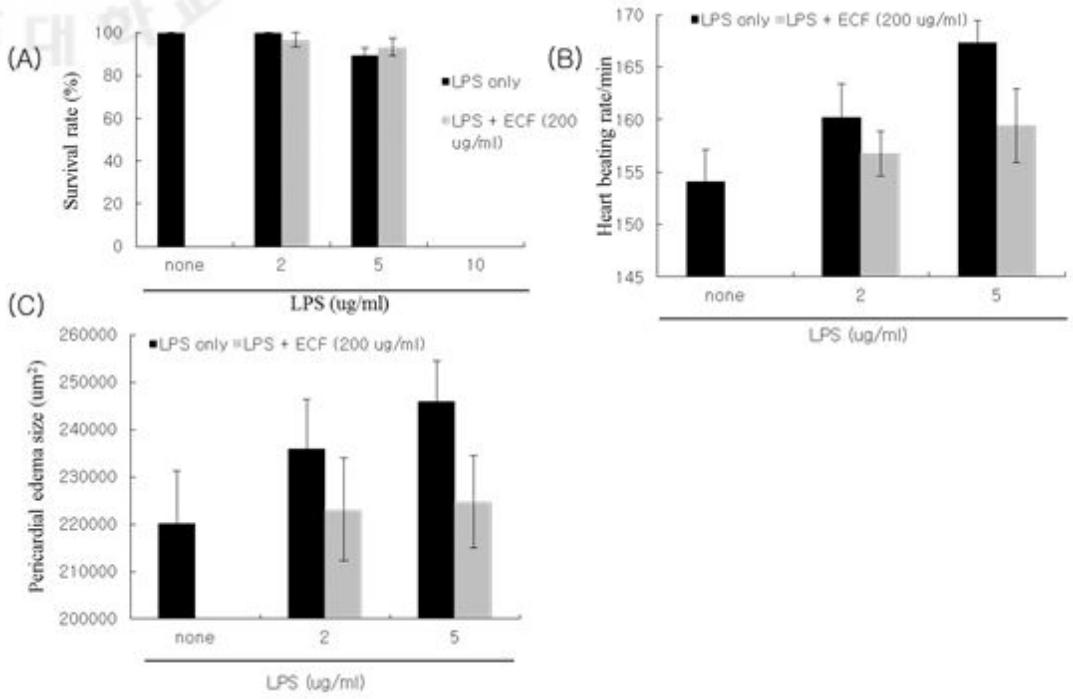
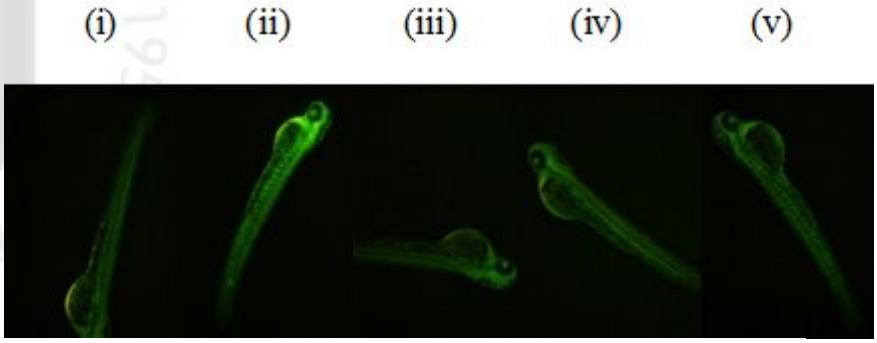


Fig. 2-3. LPS exposed each concentration (A) survival rate, (B) Heart beating, and (C) pericardial edema of zebrafish embryos. Zebrafish embryos were pretreated for 1 h with ECF (200 μg/ml) and then each LPS (2, 5, 10 μg/ml) was added the effect on toxicity were scored at 48 hpf.

(A)



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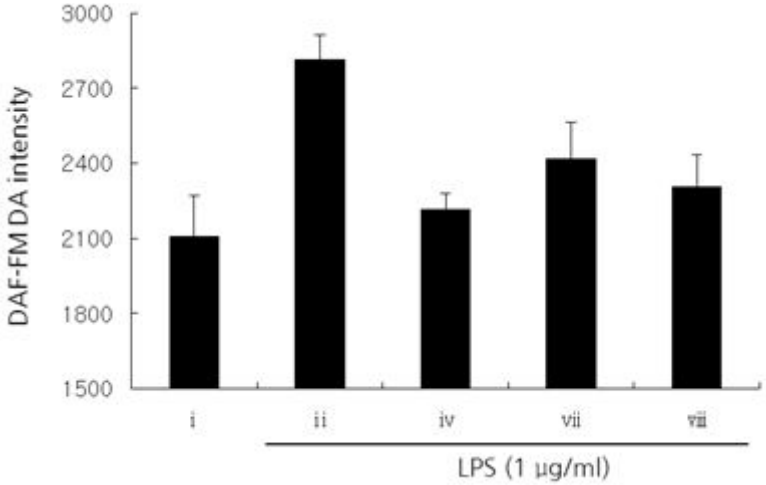


Fig. 2-4. Inhibition effect of compounds on LPS induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and LPS (5 μg/ml) added. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of LPS induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 μg/ml) and (V) pretreated with ECF (100 μg/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 μg/ml) and (v) pretreated with ECF (100 μg/ml).

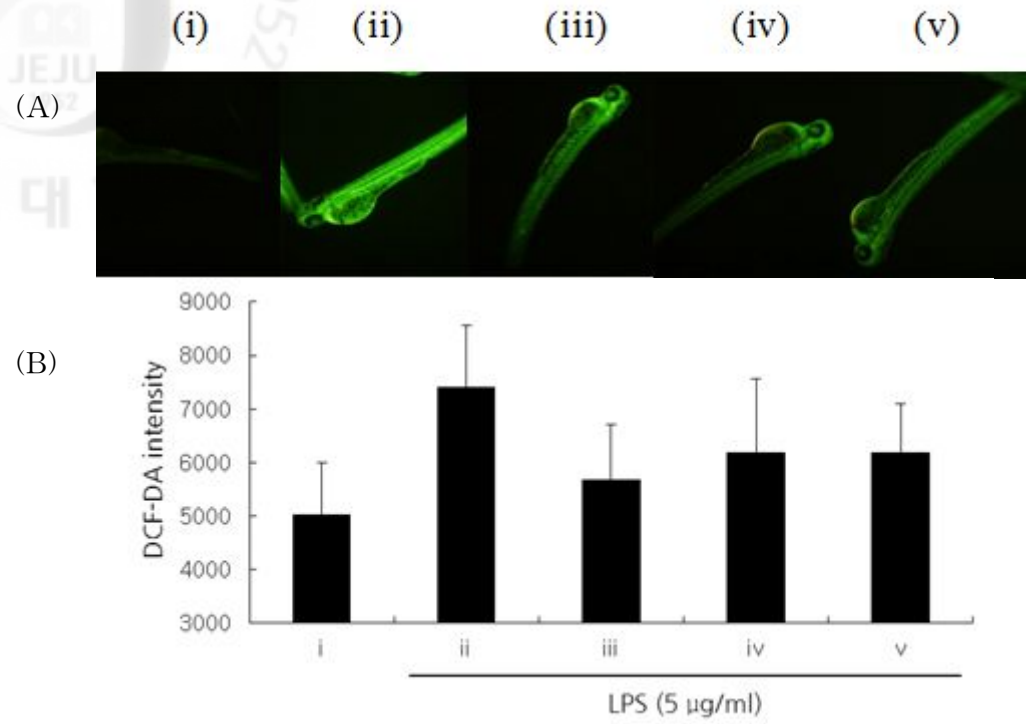


Fig. 2-5. Inhibition effect of compounds on LPS induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and LPS (5 μg/ml) added. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of LPS induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 μg/ml) and (V) pretreated with ECF (100 μg/ml).(B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 μg/ml) and (v) pretreated with ECF (100 μg/ml).

Intracellular ROS accumulations were 5686, 6191 and 6183 absorbance in the embryos pretreated with dexamethasone 0.2 μg/ml, dexamethasone 0.4 μg/ml, resveratrol 5 μg/ml and resveratrol 10 μg/ml, respectively (Fig.5.(B)).

(Fig.6.(A)) in the fluorescent image, can be found of NO generation degree with a fluorescent difference of the zebrafish and the wild type zebrafish that a tail is cut. Each of compounds treatment of (iii)-(v) groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results. The result, treated LPS experiments showed similar pattern(Fig.6.(B)). ROS as well as treated LPS experiments showed similar pattern(Fig. 7.(A, B)).

Leukocytes congregate in the injury site steps, rolling, firm adhesion, diaedesis. The results of experiment, is matched with contents of fucoidans may bind to purified and membrane-exposed P- and L-selectins. The expression of iNOS and COX-2 confirm from the western blot (fig. 2, 8) was stimulated RAW264.7 cell and zebrafish increase express of iNOS and COX-2 then control group. Check Protein bands results, chemical treatment of III-V groups, iNOS and COX-2 than negative expression of all of groups small. Therefore, ECF was Known inhibit expression iNOS and COX-2.

Study result, can be found similer anti-inflammation activity of almost an unused fucoidan from *E. cava* and traditional fucoidan. ECF will be substituted for market fucoidan in results of this study. Due to the results of this study, Again were able to determine the usefulness of E. cava. Therefore, I think the more study need to fucoidan from *E. cava*.

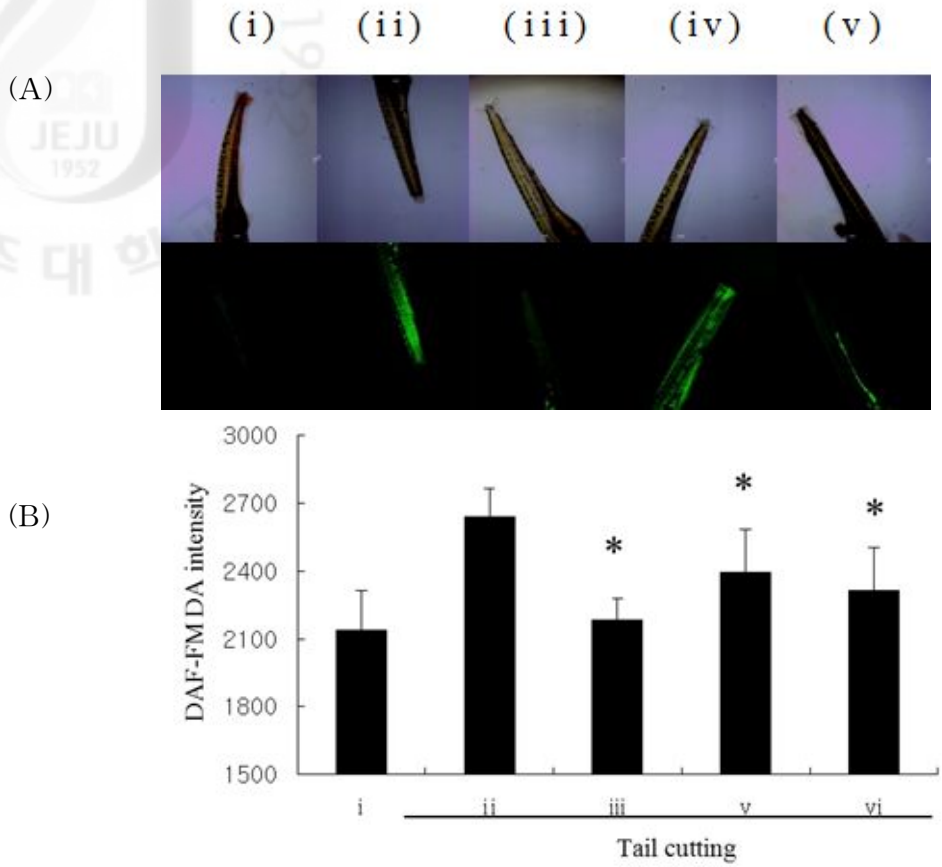


Fig. 2-6. Inhibition effect of compounds on tail cutting induced NO generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. NO generation levels were measured after staining with DAF-FM DA. (A) Fluorescence micrographs of tail cutting induced NO generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 μg/ml) and (V) pretreated with ECF (100 μg/ml). (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 μg/ml) and (v) pretreated with ECF (100 μg/ml).

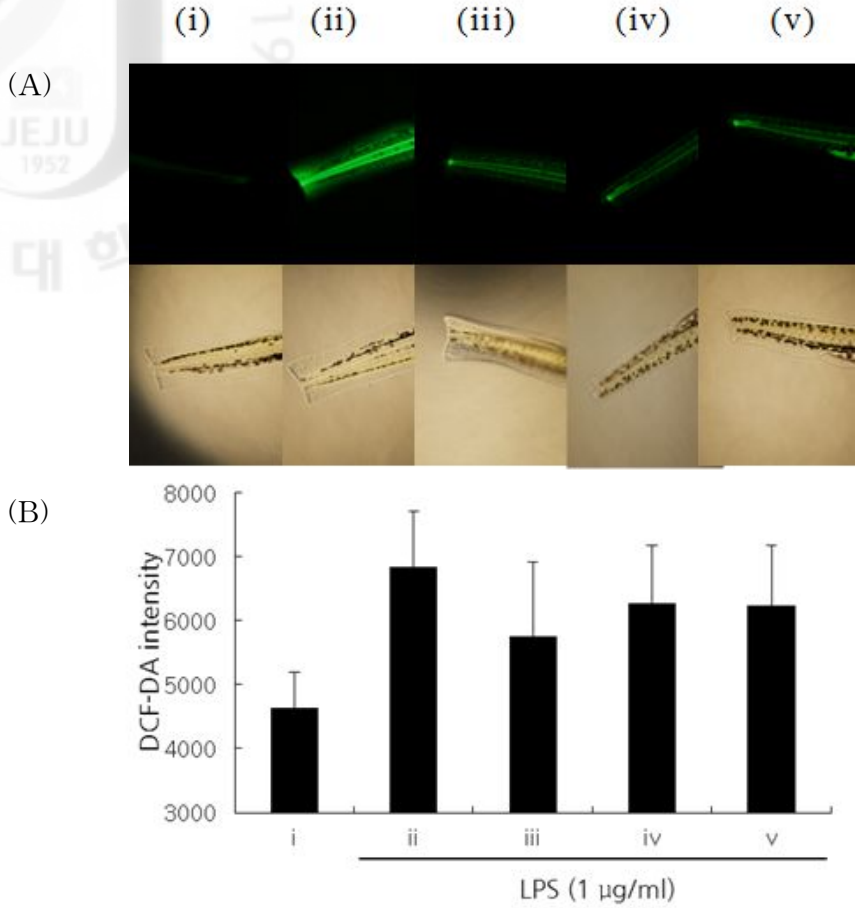


Fig. 2-7. Inhibition effect of compounds on tail cutting induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and cutting tail. ROS generation levels were measured after staining with DCF-DA. (A) Fluorescence micrographs of tail cutting induced ROS generation intensity, as follows (I) control; (II) LPS only; (III) pretreated with Dexamethasone (0.4 μg/ml); (IV) pretreated with SF (100 μg/ml) and (V) pretreated with ECF (100 μg/ml). Fig. 2-7 (B) After DAF-FM DA staining a fluorescence spectrophotometer was used for the quantitative analysis of NO generation. (i) control; (ii) LPS only; (iii) pretreated with Dexamethasone (0.4 μg/ml); (iv) pretreated with SF (100 μg/ml) and (v) pretreated with ECF (100 μg/ml).

Fig. 2-8. Expression of iNOS and COX-2 and ß -actin in zebrafishes. zebrafishes were pretreated for 1 h with different each chemicals (Dexamethasone, SF and ECF) and then LPS (1 μg/ml) was added and incubated for 24 h. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml)

PART. III

Anti-inflammation effect of fucoidan from *Eclonia cava* in physical damage and LPS-induced zebrafish

Protective effects of phlorofucofuroeckol-A against H_2O_2-induced oxidative stress in zebrafish embryo

Abstract

It overload with hydrogen peroxide (H_2O_2) induces oxidative stress and may initiate a cascade of intracellular toxic events leading to oxidation, lipid peroxidation. Phlorofucofuroeckol-A (PFF-A) is a phloroglucinol derivative isolated from the edible brown algae Ecklonia stolonifera and has been shown to possess various biological activities. Here, we have investigated the protective efficacy of PFF-A, dieckol (DK), resveratrol against H_2O_2-induced oxidative stress damage during zebrafish (*Danio rerio*) development. Zebrafish embryo exposed to $H_2O_2$and compared with other groups that were co-exposed with compounds until 2 day post fertilization (dpf). All compounds were found to scavenge intracellular reactive oxygen species (ROS) and prevented lipid peroxidation.

In contrast, compounds coexposed groups did not show any mopological changes. These results clearly indicate that PFF-A from *E. cava* possesses prominent antioxidant activity against H_2O_2-mediated toxicity and which might be a potential therapeutic agent for treating or preventing several diseases implicated with oxidative stress. This study provides a new useful strategy for the protection of H_2O_2-induced oxidative stress in alternative animal model which is zebrafish.

Key word: zebrafish, antioxidant, Phlorofucofuroeckol-A (PFF-A)

Introduction

It is known that many human diseases can be caused by free radicals and natural antioxidants can act as free radical scavengers (Chang et al. 2007; Meisel 1997). The generation of reactive oxygen species (ROS), which include free radicals such as superoxide anion (O_2·−), hydroxyl radicals (·OH) and non free-radical species such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), is associated with life under aerobic conditions and those reactive intermediates are produced under physiological and pathophysiological conditions. There is a balance between the generation of ROS and inactivation of ROS by the antioxidant systems in living organisms. Under pathological conditions, ROS are overproduced and result in lipid peroxidation and oxidative stress. ROS are formed when endogenous antioxidant defenses are inadequate. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules. This is to minimize the generation of ROS desirable to stay healthy but not easy. Because electron transport system more actively of mitochondria induced generation of ROS, exercise or eating is also an increase in ROS. Therefore, antioxidants is needed suppress ROS, and this type of antioxidant carotenoid such as β-carotene, Lycopene and flavonoid such as catechin, resveratrol and Isoflavone, vitamin etc.

Physiologically H_2O_2, a freely diffusible form of reactive oxygen species (ROS), is produced by many intracellular reactions, and an intermediate product of the degradation of ROS and a highly reactive molecule. Extracellular hydrogen peroxide is able to cross membranes, thus directly altering their intracellular concentrations (Li et al., 2000). Increased neurocellular load with ROS induces a number of intracellular events such as

oxidative stress. Calcium is a potent pro-oxidant metal which stimulates production of ROS through inhibition of redox-sensitive enzymes such as catalase (Beyersmann and Hechtenberg, 1997).

E. cava is a brown algae, grows many in Jeju Island South Korea and bettermost inedible because of astringency taste due to tannin. Several components from *Ecklonia* species have been isolated and investigated on the radical scavenging activity, anti-plasmin inhibiting activity, antimutagenic activity, anti-bactericidal activity, HIV-1 reverse transcriptase and protease inhibiting activity, and tyrosinase inhibitory activity.

The zebrafish (*Danio rerio*) is a small tropical freshwater fish which has emerged as a useful vertebrate model because of its small size Large number of offspring (200~300 eggs/ wk), transparent embryo, Low cost maintenance, Rapid generation cycle (2-3 months), Low space requirement, Rapid development or organogenesis and physiological similarity to mammals (Eisen., 1996, Fishman., 1999). The zebrafish is utilized to screening test of drug activation, because the early stage embryo rapidly absorbs small molecular compounds diluted in the bathing media through the skin and gills (Cha et al., 2010). In contrast, relatively late stage zebrafish [from 7 d post-fertilization (dpf) to the adult stage] absorb the compounds orally rather than percutaneously (Langheinrich, 2003).

In the present study, phlorofucofuroeckol A (PFF-A. Fig. 1.(A)) isolated from *E. cava* was characterized in H_2O_2-induced ROS in zebrafish in via inhibition of caspase-3, Bcl-xL, PARP and ROS production.

Material and mathods

(A) (B) (C)

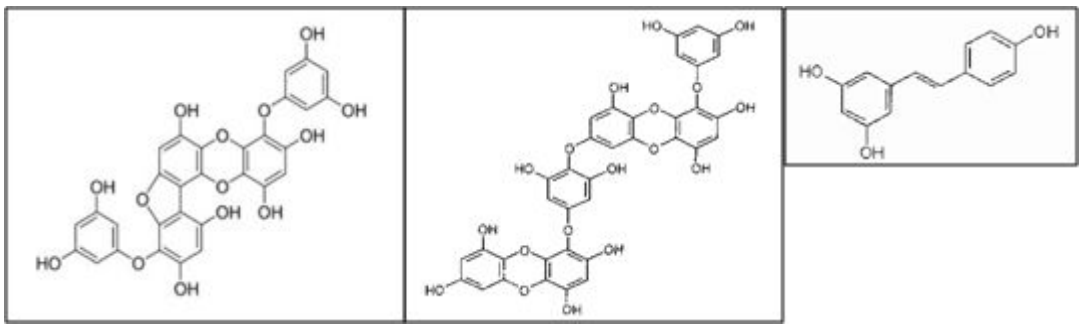


Fig. 3-1. (A) phlorofucofuroeckol-A (PFF-A), (B) Dieckol (DK), (C) resveratrol

Origin and maintenance of parental zebrafish

Adult zebrafishes were obtained from a commercial dealer (Seoul aquarium, Korea) and 10 fishes were kept in 3 l acrylic tank with the following conditions; 28.5℃, with a 14/10 h light/dark cycle. Zebrafishes were fed three times a day, 6 d/week, with Tetramin flake food supplemented with live brine shrimps (Artemia salina).

Embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 min and staged as described by (Kimmel et al., 1995).

Waterborne exposure of embryos to compounds and H_2O_2

From approximately 3 to 4 hour post-fertliztion (3-4 hpf), embryos (n=25) were transferred to individual wells of a 24-well plate and maintained in embryo media containning 1 ml of vehicle (0.1% DMSO) or compound for 1 h. Then treated with 5 mM H_2O_2 or co-treated H_2O_2 and compound for up to 48 hour postfertilization (48 hpf).

Measurement of heart-beat rate

The heart-beating rate of both atrium and ventricle was measured at 35 hpf to determine the sample toxicity (Choi et al., 2007). Counting and recording of atrial and ventricular contraction were performed for 3 min under the microscope, and results were presented as the average heart-beating rate per min.

Estimation of intracellular ROS generation and image analysis

Geneartion of reactive oxygen species (ROS) production of zebrafish embryos was analyzed using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorofluorescein diacetate (DCF-DA). DCF-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized to the highly fluorescent compound dichlorouorscein (DCF) in the presence of cellular peroxides (Rosenkranz et al, 1992). At 3-4 hpf, the embryos were treated with compound and 1 h later, 5 mM H_2O_2 was added to the plate. After treating embryos with 5 mM H_2O_2 for 6 h, the embryo media was changed and the embryos developed up to 2 dpf (day postfertiliztion). The embryos were transferred in to 96 well plate and treated with DCFDA solution (20 μg/ml), and the plates were incubated for 1 h in the dark at 28.5℃. After incubation, the embryos were rinsed in fresh embryo media and anesthesized before visualization. Individual embryo fluorescence intensity was quantified using spectrofluorometer (Perkin - Elmer LS-5B, Austria) and the image of stained embryos were observed using a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan).

Lipid peroxidation inhibitory activity and image analysis

Lipid peroxidation was measured to assess membrane damage according to (Wang et al., 2008). Morphological evaluation of the embryos was performed with Diphenyl-1-pyrenylphosphine (DPPP, Dojindo, Japan) is fluorescent probe for detection of cell membrane lipid peroxidation. DPPP is non-fluorescent, but it becomes fluorescent when oxidized. At 3-4 hpf, the embryos were treated with 50 μM phlorotannins and 1 h later, 5 mM H_2O_2 was added to the plate. After treating 41 embryos with 5 mM H_2O_2 for 6 h, the embryo media was changed and the embryos developed up to 2 dpf. The embryos

were transferred in to 96 well plate and treated with DPPP solution (25 μg/ml), and the plates were incubated for 1 h in the dark at 28.5℃. After incubation, the embryos were rinsed in embryo media and anesthesized before visualization. Individual embryo fluorescence intensity was quantified using spectrofluorometer (Beckman DTX 800, USA) and image of embryos were observed using a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan).

Western blotting

The zebrafish embryos were harvested, washed twice with PBS, lysed on ice for 30 min in 100 ml lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000 X g for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 mg of protein) were boiled for 5 min and electrophoresed in 10% sodium dodecyl sulfatepolyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, USA), which were incubated with the primary antibodies. The membranes were further incubated with the secondary immunoglobulin-Ghorseradish peroxidase conjugates (Pierce, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, UK), and then exposed onto X-ray film.

Result and discussion

Cells or live organism are protected from ROS-induced damage by a variety of endogenous ROS-scavenging enzymes, chemical compounds, and natural products. ROS is associated with life under aerobic conditions and

those reactive intermediates are produced under physiological and pathophysiological conditions. However, in modern society, because therefore the destruction of the ozone layer caused by the ultraviolet or cigarette smoke, environmental pollution the expedite product of ROS, need to be developed of antioxidants. Recently, many researchers have made considerable efforts to search natural antioxidants. Many studies have revealed that seaweeds have potential to be used as a candidate for natural antioxidant. The efficacy of phlorotannins have been studied extensively in which ROS are produced either chemically and the elimination of ROS by *E. cava* are monitored directly or by measuring lipids peroxidation levels.

Ecklonia cava is a brown alga (Laminariaceae) that is abundant in the subtidal regions of Jeju Island in Korea. Recently, it has been reported that Ecklonia species exhibits radical scavenging activity and, anti-plasmin inhibiting activity and, antimutagenic activity and, bactericidal activity, HIV-1 reverse transcriptase and protease inhibiting activity and tyrosinase inhibitory activity. Among the phlorotannins isolated from *E. cava*, PFF-A and Dieckol (DK, Fig. 1.(B)) showed various biological activities such as antioxidant activity, antiplasmin activity, inhibition of acetylcholinesterase, and the hepatoprotective activity against tacrine-induced HepG2 cells. Resveratrol (Fig. 1. (C)) is a type of polyphenol, and produced naturally by several plants when under attack by pathogens(bacteria, fungi), UV etc. Revealed that biological activities of resveratrol such as anticoagulation, antioxidant, anti-inflammation, antitumor.

In this study, we investigated the antioxidant effects of PFF-A from of *E. cava*, after the administration of H_2O_2 in zebrafish embryo. 20,70-Dichlorodihydrofluorescein diacetate was used as a probe for ROS measurement. DCF-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterase to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. It is H_2O_2 is

the principal ROS responsible for the oxidation of DCFH to DCF. PFF-A is has the ROS scavenging effect, it was further evaluated with regard to its protective effects against H_2O_2-induced oxidative stress in zebrafish embryo (Fig. 2). As a result, scavenging activity of PFF-A decreased on intracellular the oxidative damage of H_2O_2. (Fig.3.(A))in the fluorescent image, can be found of ROS generation dgree with a fluorescent difference of the zebrafish and the H_2O_2 treated zebrafish. Conpounds treatment of 3-5 groups, fluorescence than negative expression of all of groups show less, and this quantify graph also shoes the same results (Fig. 4.(B)). And ROS-induced lipid peroxidation was also reduced (Fig. 4.(A)). Also shown fluorescence spectrophotometer same result (Fig. 4.(B)). This results demonstrated that H_2O_2 induces toxicites in the zebrafish embryos and phlorotannisn can protect zebrafish embryos against H_2O_2 by inhibit intracellular ROS formation, lipid peroxidation. As shown (Fig. 5) by the Western blotting results, PFF-A treatment in H_2O_2-treated zebrafish decreased the expression of cell death protein including Bcl-xL and PARP. These data suggest that phlorotannins protects zebrafish from H_2O_2 induced toxicity. These results suggest that PFF-A have ability to protect zebrafish embryo from oxidative stress related cellular injuries.

We occlude that zebrafish embryos are valuable laboratory alternative *in vivo* model. The antioxidant mechanisms underying the protective efficacies afforded by PFF-A in this experiment remain to elucidate.

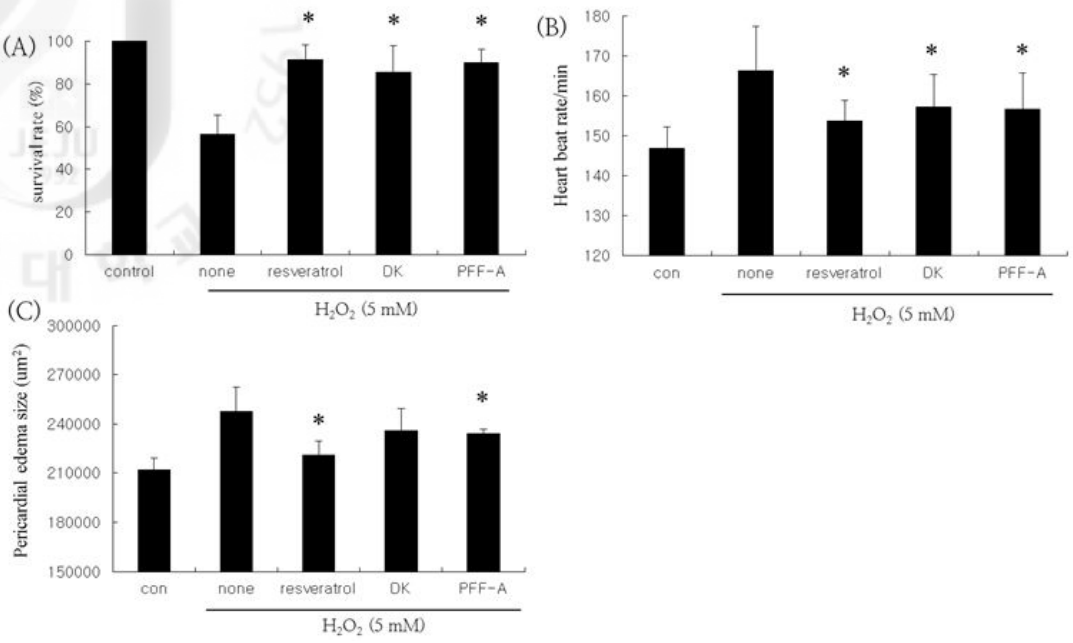
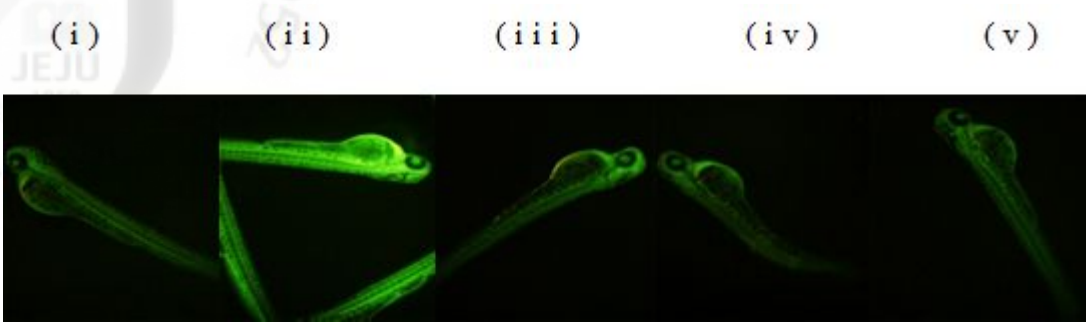


Fig. 3-2. (A) Survival rate after treated with H_2O_2 or co-treated with compounds. The embryos were exposed to 5 mM H_2O_2 and compounds treated. Resveratrol 10 uM, Dieckol 50 uM (DK) and Phlorofucofuroeckol-A 50 uM (PFF-A). (B) Effects of compounds on the heart-beat rate for measurement of the toxicity of the tested samples. The embryos were exposed to 5 mM H_2O_2 and compounds teated. The heart-beat was measured at 48 hpf, under the microscopy. The number of heartbeat in 3 min was counted, and the results are expressed as the beats/min. Resveratrol 10 uM, Dieckol 50 uM (DK) and Phlorofucofuroeckol-A 50 uM (PFF-A). (C) Effects of compounds on the Precardial edema size for measurement of the toxicity of the tested samples. The embryos were exposed to 5 mM H_2O_2 and compounds teated. The Precardial edema size was measured at 48 hpf, under the microscopy. Resveratrol 10 uM, Dieckol 50 uM (DK) and Phlorofucofuroeckol-A 50 uM (PFF-A).

(A)



(B)

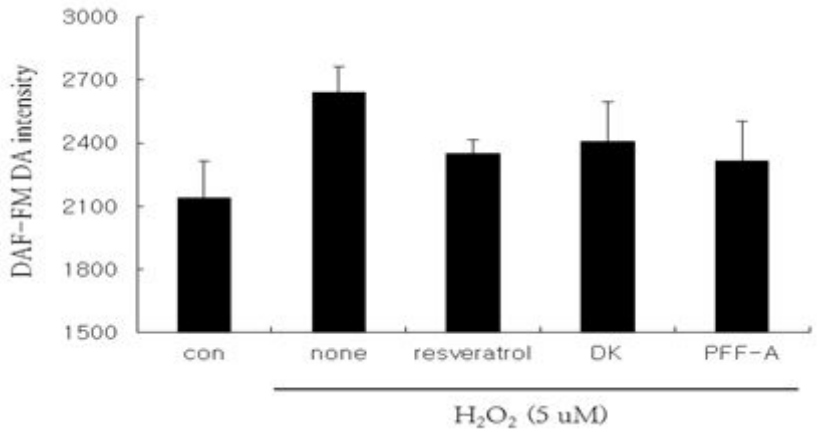
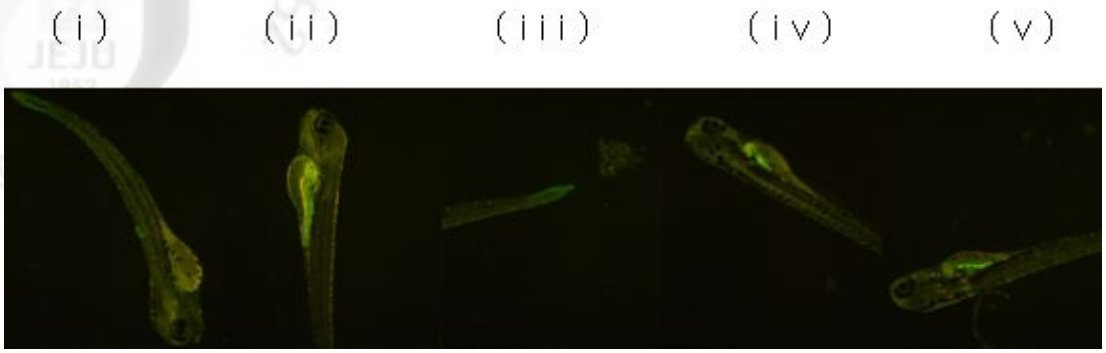


Fig. 3-3. Inhibition effect of compounds on H_2O_2 induced ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and H_2O_2(5uM) added. ROS generation levels were measured afters taining with DCF-DA. (A) Fluorescence micrographs of H_2O_2 induced ROS generation intensity, as follows (I) control; (II) H_2O_2 only; (III) pretreated with resveratrol (10 μg/ml); (IV) pretreated with Dieckol (50 uM) and (V) pretreated with PFF-A (50 uM). (B) After DCF-DA staining a fluorescence spectrophotometer was used for the quantitative analysis of ROSgeneration. Resveratrol 10 uM, Dieckol 50 uM (DK) and Phlorofucofuroeckol-A 50 uM (PFF-A).

(A)



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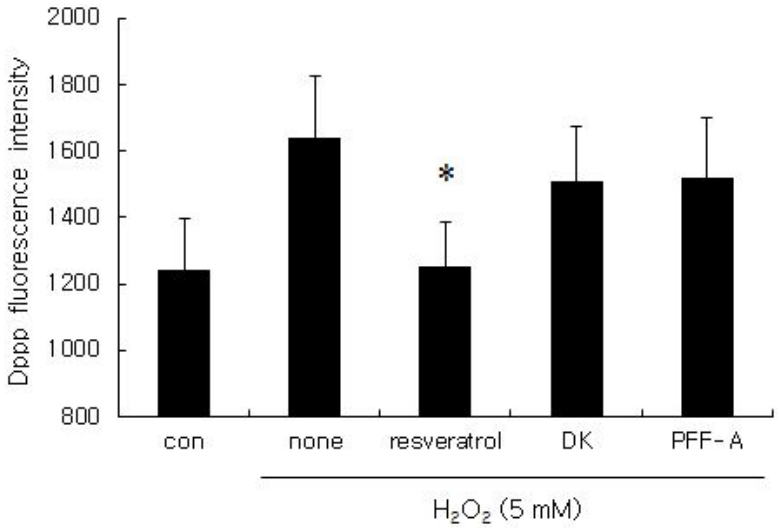

Fig. 3-4. Inhibition effect of compounds on H_2O_2 induced lipid peroxidation level in zebrafish embryos. The zebrafish embryos were pretreated with each of compounds and H_2O_2(5 uM) added. ROS generation levels were measured after staining with DPPP. (A) Fluorescence micrographs of H_2O_2 induced lipid peroxidation level intensity, as follows (I) control; (II) H_2O_2 only; (III) pretreated with resveratrol (10 μg/ml); (IV) pretreated with Dieckol (50 uM) and (V) pretreated with PFF-A (50 uM). (B) After DPPP staining a fluorescence spectrophotometer was used for the quantitative analysis of lipid peroxidation level. Resveratrol 10 uM, Dieckol 50 uM (DK) and Phlorofucofuroeckol-A 50 uM (PFF-A).

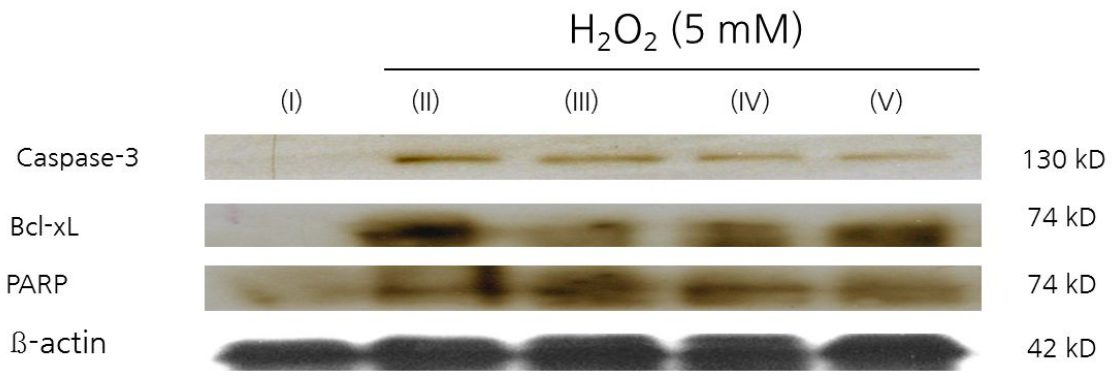

Fig. 3-5. Expression of caspase-3, Bcl-xL, PARP and ß -actin in zebrafishes. The zebrafish embryos were pretreated with each of compounds and H_2O_2(5uM) added. Zebrafish embryos hatch is then harvested. (A) control; (B) LPS only; (C) pretreated with Dexamethasone (0.4 μg/ml); (D) pretreated with SF (100 μg/ml); (E) pretreated with SF (200 μg/ml); (F) pretreated with ECF (100 μg/ml); (F) pretreated with ECF (200 μg/ml).

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