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A THESIS  
FOR THE DEGREE OF MASTER OF SCIENCE

Antidiabetic activity of phlorotannins isolated from  
*Ecklonia cava* in zebrafish model

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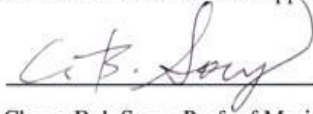
Antidiabetic activity of phlorotannins isolated from  
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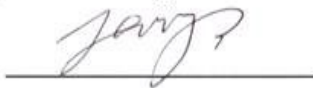
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## 국문초록

제브라피쉬는 인간과 유전적 상동성과 허파를 제외하고는 간과 췌장, 지라와 흉선 등 면역계를 포함한 대부분의 기관을 가지고 있는 척추동물로 최근 실험동물 모델로써 각광받고 있다. 이들은 체외수정을 하며, 수정란을 대량으로 쉽게 확보할 수 있다. 그리고 난이 투명하여 배아의 발생과정, 혈류 흐름 및 형태적 관찰이 용이하며, 크기가 작아 공간의 제약을 받지 않는다. 또한 발생이 매우 빨라 대부분의 조직 및 장기가 하루 만에 형성된다. 이러한 장점을 이용하여 유전자 대신에 수천 수만의 천연물, 화합물을 대상으로 곧바로 생체기능조절물질을 발굴하려는 시도가 전세계적으로 진행되고 있으며 특히 암, 비만 등 퇴행성 뇌질환 및 심혈관계질환 질병 등에 가장 적합한 모델로 연구되고 있다. 현대사회에 들어서 극심한 스트레스와 운동부족으로 인해 과도한 영양섭취가 과다한 에너지의 저장으로 인하여 대사성질환의 발현을 필연적으로 증가시키고 있다. 이에 따라 복합적으로 여러 요소가 존재하지만 가장 중요한 요소로 받아들여지고 있는 것은 인슐린 저항성이다. 따라서 대사성질환의 발병과 깊은 상관관계를 보여주는 당뇨병의 증가추세가 두드러짐에 따라 당뇨병은 현재 전세계적으로 급격하게 증가하고 있다. 당뇨병의 발병이 증가함에 따라 당뇨 치료제의 수요 또한 증가하고 있다. 하지만 이들은 저혈당, 심장손상, 망막증, 발가락등과 같은 부작용을 일으

키고 있어 부작용이 없고 우수한 효과를 나타내는 당뇨병의 예방 치료물질로써 천연물 유래 소재의 발굴에 관한 연구가 많이 진행되고 있다. 천연물 유래 소재 중 해조류는 풍부한 미네랄과 비타민, 다당류, peptides 및 polyphenol이 풍부하게 함유되어있다. 특히 갈조류에 속하는 감태 (*Ecklonia cava*)는 다당류와 polyphenol이 많이 함유되어있으며, 이들은 항산화, 항암, 항염, 항고혈압, 항당뇨등과 같은 다양한 생리활성효과를 가진다고 보고되어 있다. 따라서, 이번 연구에서는 제주도 연안에 서식하는 감태에서 phlorotannin의 4가지의 물질(6,6 bieckol, Phloroeckol, Dieckol, Phlorofucofuroeckol)을 분리하였다. 4가지의 물질이 high glucose로 산화적스트레스가 유도된 제브라피쉬 모델에서 high glucose에 대해 보호효과가 얼마나 있는지 확인하였다. 이에 대한 관찰은 생존율, 심박수, 활성산소 (Reactive oxygen species), 일산화질소 (Nitric oxide), 지질과산화 (Lipid peroxidation), 세포사멸 (cell death)을 통해서 확인하였다. 활성에 관한 확인은 항산화물질로 알려진 Resveratrol과 비교하여 확인하였으며, 감태의 Phlorotannin 물질 중 Dieckol이 high glucose에 대한 보호효과를 보였다. 이에 대한 결과는 Dieckol이 high glucose로 산화적스트레스를 유도시킨 혈관내피세포에서의 보호효과를 지닌다는 이전논문과 동일한 결과를 보임에 따라 high glucose로 산화적스트레스를 유도시킨 제브라피쉬 모델이 *in vivo* 실험으로 가능함을 확인하였다.

또한, 제브라피쉬 항당뇨모델을 만들기 위해서 당뇨 유발성물질인 Alloxan을 처

리하였다. 이에 대한 과정은 Alloxan을 처리한후 1% glucose에 담궈둔 후에 1시간 동안 순치시켰다. 그 결과 제브라피쉬의 혈당이 증가함을 확인하였고, 활성 확인을 위하여 Dieckol과 metformin을 투입하였다. 시간대별 혈당체크를 통해 항당뇨 효능을 확인하였으며, 가장 좋은 혈당감소 효능이 보인 시간대를 선택하여 인슐린 측정과 간에서 글루코즈 대사과정에 작용하는 효소들 (GK, G6pase, PEPCK)을 확인하였다. 또한 작용기전을 확인하기 위해서 근육에서 AMPK와 Akt signaling pathway을 확인하였다. DK에 의한 포도당 흡수 촉진 효과가 어떠한 pathway로 이루어지는지 Western blot을 통해 살펴본 결과 AMPK는 활성화시키지 않지만, Akt는 활성화 시킴을 확인하였다.

이 모든 결과를 종합해 볼 때, 제브라피쉬에서 high glucose로 유도한 산화적 스트레스 모델과 항당뇨 모델에서 dieckol이 우수한 효과를 가지고 있음을 확인하였다. 이에 대한 확인은 이전 dieckol의 항당뇨 *in vitro* and *in vivo* 실험과 동일한 결과로 확인 할 수 있었다. 그러므로 항당뇨 모델로써 제브라피쉬가 사용될 수 있으며, dieckol은 항당뇨성 물질로 잠재적인 기능성 식품 및 천연의약 소재로서 충분한 가능성이 있으리라 사료되어진다.



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**Table 1.** The list of phlorotannins isolated from *E. cava*

## Introduction

Zebrafish (*Danio reiro*) has become a popular model to study vertebrate development due to their comparatively small size, that makes them easier to handle in large numbers in the laboratory environment (Kishi et al., 2003). One female can spawn about transparent 100 eggs per day which are fertilized by sperm release by the male into the water and optical clarity of the embryo (Scholz et al., 2008; Olsen et al., 2010). Also zebrafish have a very short development processes that organogenesis occurs rapidly, and major organs are appear in larvae by 5 to 6 days post- fertilization (dpf) and they attain maturity at the age of about 3 months (Rubinstein 2003). The zebrafish model is a fresh water teleost that has become a popular and informative developmental research because of its fecundity and its genetic and physiological similarities to mammals (Eames et al., 2010; Elo et al., 2007). These advantages are leading to use of the zebrafish model in drug discovery, toxicological studies and human disease (Hertog, 2005; Pichler, 2005). A lot of human disease resulting from oxidative stress can be caused by free radicals and natural antioxidants, can act as free radical scavengers (Chang et al., 2007). Free radicals can cause a damage on DNA, protein and tissue by reacting with other chemicals in the body (Fang et al., 2002; Kang et al., 2012a). Oxidative stress is a well-recognized mechanism in many pathological conditions

like insulin resistance, endothelial dysfunction and abnormal serum lipids (Holvoet et al., 2008). So, Antioxidants can prevented the pathological damage caused by hyperglycemia-induced oxidative stress associated with diabetes (Yokozawa et al., 2007). And zebrafish regulated of glucose metabolism, through the production of insulin, similar to mammalian models, and many of the genes involved in regulating blood glucose levels. Therefore recently, human diseases studies using zebrafish models have been established diversity a wide range of human pathologies.

Contemporary society, diabetes mellitus is the most serious disease that is developing with an increasing obesity and aging in the general population over the world (Lee et al., 2012). Diabetes mellitus is appeared by dysfunction of the action of glucose, due to in part of the resistance to action of insulin in peripheral tissues. When insulin is present, glucose is transported into cells, metabolic processes are activated and the amount of glucose in the blood decreases. But ultimately results in blood vessel damage leading to complications such as cardiovascular disease and stroke, retinopathy, nephropathy, neuropathy, and impaired wound healing. In type 1 diabetes mellitus autoimmune destruction of beta islet cells occurs and is no longer produced. And then it previously known as juvenile diabetes, this form generally develops in children and young adults. In type 2 diabetes mellitus cells develop insensitivity to the presence of insulin. And it considered a disease of middle and old age,

type 2 is also becoming more common in youths as the incidence of childhood obesity grows (Olsen., 2011; Riaz et al., 2009). Type 1, type 2 and gestational diabetes all lead to increased blood glucose levels due to either the loss of production or function of insulin, a hormone that promotes the transport of glucose from blood cells (Gleeson et al., 2007). Without enough insulin, the glucose stays in the blood. Increased blood glucose, or hyperglycaemia, leads to many complications, including cardiovascular and microvascular disease, periodontal disease, retinopathy, nephropathy, neuropathy, and impaired wound healing (Gleeson et al., 2007; Olsen, 2011). The diabetes is determined by the difference between the amount of glucose entering and leaving the circulation. Glucose released from the liver is derived from both glycogenolysis and gluconeogenesis. (Basu et al., 2001). Abnormal hepatic glucose metabolism is a major symptom of diabetes and it contributes to postprandial hyperglycemia. And then Skeletal muscle, a key insulin sensitive tissue, has a paramount role in energy balance and is the principal site for postprandial glucose utilization and disposal.

Recently available drugs for diabetes have a lot of limitation, such as side effects and high rates of secondary failure (Lee et al., 2012). Therefore, currently, the therapeutic use of natural products for diabetes. Specially, those derived from herbs (Chang et al., 2006). Seaweeds are known to compose a variety of bioactive substances (polysaccharides,



pigments, minerals, peptides and polyphenols) with valuable pharmaceutical and biomedical potentials. In particular brown seaweeds contain a diversity of biological benefits such as antioxidant, anticoagulant, antihypertension, antibacterial, and antitumor activities (Heo et al., 2005.; Nagayama, 2002; Mayer, 2004; Athukrala, 2005; Kotake-Nara, 2005). Seaweeds are classified into three divisions such as brown seaweed (phaeophyta), red seaweed (rhodophyta) and green seaweed (chlorophyta) according to their composition of pigment. In particular, brown seaweeds are plentifully present around Jeju Island, Korea, where these valuable brown algae have various biological compounds, such as xanthophyll, pigments, fucoidans, phycocolloids, phlorotannins, and fucoxanthin (Halliwell and Gutteridge, 1999). *Ecklonia cava* (*E. cava*) has a variety of compounds including sulfated polysaccharides and phlorotannins with different biological activities (Heo et al., 2009) Several studies have demonstrated the variety of biological benefits associated with phlorotannins, including antioxidant, anticoagulant, antibacterial, anti-inflammatory, anti-cancer and anti-diabetes activities (Kang et al., 2012b; Mayer and Hamann, 2004; Heo et al., 2008; Lee et al., 2012)

In previous studies, a type of phlorotannin from the brown algae *E. cava* of jeju Island, Korea evaluated anti-diabetic effects *in vitro* (Lee et al 2010a) and *in vivo* mouse model (Lee et al., 2010b, 2012). However, anti-diabetic effects have not been studied *in vivo* zebrafish model. Therefore, in this study, we confirmed to measurement of phlorotannins isolated from

*E. cava* against high glucose-induced oxidative stress embryo zebrafish model and we demonstrated its anti-diabetic activities adult zebrafish model.

# **Antidiabetic activity of phlorotannins isolated from *Ecklonia cava* in zebrafish model**

## **1. ABSTRACT**

The zebrafish model system is one of the most widely used animal models, and it now becomes an attractive model for molecular genetics, development biology, drug discovery and human disease. Recently, alternative therapeutic has been a growing interest of natural products for diabetes, especially those derived from marine algae. Because plant sources are usually considered to be less toxic with fewer side effects than synthetic ones. Therefore, in this study, we evaluated anti-diabetic activities using embryos and adults zebrafish model of phlorotannins (6,6 bieckol (6,6BK), phloroeckol (PK), dieckol (DK), phlorofucofuroeckol (PFFK)) isolated from *Ecklonia cava* (*E. cava*). We confirmed that the effect of phlorotannins against high glucose-induced oxidative stress in the embryos. Among the phlorotannins, DK significantly reduced in zebrafish model reactive oxygen species (ROS), nitric oxide (NO), lipid peroxidation generation and cell death, induced high glucose oxidative stress. Moreover, diabetic zebrafish model was established following steps, adults

zebrafish were exposed of 2 mg/mL alloxan for 1 h and moved in 1% glucose in water during 1 h, and then exposed in water for 1 h. Zebrafish were divided into Normal (wild type), control, dieckol (DK) rich extract of *E. cava*, and metformin groups. The blood glucose, plasma insulin levels and hepatic glucose regulating enzyme activities including glucokinase, glucose-6-phosphate and phosphoenolpyruvate carboxykinase were identified in the zebrafish model. These findings indicate that DK isolated from *E. cava* confirmed to antioxidant activities against high glucose-induced oxidative stress and anti-diabetic effects in diabetic zebrafish model. Therefore, in this study show the anti-diabetes activity with the possible evaluation on zebrafish model.

## **2. Materials and methods**

### **2.1. Materials**

The marine brown alga *E. cava* was collected along the coast of Jeju Island, Korea, between March 2011 and June 2011. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, and then carefully rinsed with fresh water, and maintained in a medical refrigerator at  $-20\text{ }^{\circ}\text{C}$ . Therefore, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

### **2.2. Isolation of phlorotannins from *E. cava***

It exhibited the most efficient separation of 4 fractions from *E. cava* extracts under the solvent condition, n-hexane / ethyl acetate / methanol / water (2:8:3:7). The pure compounds of each fractions were certainly confirmed as 6,6-bieckol, phloroeckol, dieckol and phlorofucofuroeckol-A, respectively comparing with previously reported  $^1\text{H}$  and  $^{13}\text{C}$ -NMR data through their  $^1\text{H}$  and  $^{13}\text{C}$ -NMR data

### **2.3. Origin and maintenance of parental zebrafish**

Adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea)

and 15 fishes were kept in 3.5 L acrylic tank with the following conditions;  $28.5 \pm 1$  °C, and were fed twice times a day (Tetra GmgH D-49304 Melle Made in Germany) with a 14/10 h light/dark cycle. The day before, Breeding 1 female and 2 males interbreed. In the morning (On set of light), embryos were obtained from natural spawning collection of embryos were completed within 30 min in petridishes (containing media).

#### **2.4. Experimental design of high glucose zebrafish**

The embryos (n=15) were transferred to individual wells of 12-well plates containing 950  $\mu$ L embryo media. At 24 hpf, a 150 mM glucose solution of 50  $\mu$ L was treated to the embryo exposed for 24 h. Then, embryos were rinsed using fresh embryo media.

#### **2.5. Waterborne exposure of embryos to phlorotannins and glucose solution**

The embryos (n=15) were transferred to individual wells of 12-well plates containing 900  $\mu$ L embryo media from approximately 7 to 9 hpf, 50  $\mu$ L of samples were added to the wells. At 24 hpf, a 150 mM glucose solutions of 50  $\mu$ L was treated to the embryo exposed for up to 2 dpf. Then, embryos were rinsed using fresh embryo media.

#### **2.6. Measurement of heartbeat rate**

The heartbeat rate of both atrium and ventricle were measured at 2 dpf. Counting and recording of atrial and ventricular contractions were performed for 1 min under a microscope.

### **2.7. Measurement of oxidative stress-induced intracellular ROS generation and image analysis**

Generation of ROS production of zebrafish was analysed using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized to the highly fluorescent compound dichlorofluorescein (DCF) in the presence of cellular peroxides (Rosenkranz et al., 1992). At 4 dpf, the zebrafish larvae were transferred to one well of 24-well plate, treated with DCFH-DA solution (20  $\mu\text{g}/\text{mL}$ ) and incubated for 1 h in the dark at  $28.5\pm 1$  °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

### **2.8. Measurement of oxidative stress-induced NO generation and image analysis**

The generation of NO production of zebrafish was analyzed using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Transformation of DAF-FM DA by NO in the presence of dioxygen generates highly fluorescebt triazole derivatives (Itoh, 2000). At 4 dpf, the zebrafish larvae were transferred to one well of 24-well plate, treated with DAF- FM DA solution (5  $\mu$ M) and incubated for 2 h in the dark at  $28.5\pm 1$  °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

### **2.9. Measurement of oxidative stress-induced lipid peroxidation generation and image analysis**

Lipid peroxidation was measured to assess the membrane damage in zebrafish model. Diphenyl-1-pyrenylphosphine (DPPP) is fluorescent probe for detection of cell membrane lipid peroxidation. DPPP is non-fluorescent, but it becomes fluorescent when oxidized. At 4 dpf, the zebrafish larvae were transferred to one well of 24-well plate, treated with DPPP solution (25  $\mu$ g/mL) and incubated for 1 h in the dark at  $28.5\pm 1$  °C. After the incubation, the



zebrafish larvae were rinsed using fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

#### **2.10. Measurement of oxidative stress-induced cell death and image analysis**

Cell death was detected in live embryos using PI staining. PI is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population. At 4 dpf, the zebrafish larvae were transferred to one well of 24-well plate, treated with PI solution (80  $\mu\text{g}/\text{mL}$ ) and incubated for 30 min under the dark at  $28.5\pm 1$   $^{\circ}\text{C}$ . After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

#### **2.11. Experimental design of diabetic zebrafish**

The adult zebrafish were exposed of 2 mg/mL alloxan for 1 h and moved to 1% glucose

during 1 h. After an adjustment period of approximately 1 h, the zebrafish were anesthetized by placing them in ice-cold water over several minutes. For injection, an insulin syringe was used to deliver saline, DK or metformin to dose of  $0.6 \pm 0.05$  g ( $30 \mu\text{L}$  dependent on weight). Normal and Control zebrafish were injected with a like volume of saline. And the zebrafish were injected with DK or metformin ( $1 \text{ mg/kg}$  body weight)

### **2.12. Collection of blood glucose levels**

Blood samples were taken from the heart at 0, 60, 90, 120, 180 min. Zebrafish were anesthetized by placing them in ice-cold water over several minutes. After, the zebrafish was removed from the solution by Kimwipe. Blood sample were taken from the heart at 0, 60, 90, 120, 180 min. Approximately  $1 \mu\text{L}$  of blood rapidly transferred to a glucometer strip (Roche Diagnostics GmbH, Germany). Areas under the curve (AUC) were calculated using the trapezoidal rule.

### **2.13. Plasma insulin**

Blood sample from the heart were collected into tubes. After centrifugation at  $1000 \text{ g}$  for 15 min at  $4 \text{ }^\circ\text{C}$ , the plasma was carefully removed from the sample and then samples were dilute of 1 : 4. The levels of plasma insulin were determined using radioimmunoassay with

enzyme-linked immunosorbent assay ELISA kit (Milipore, EZRMI-13K).

#### **2.14. Hepatic tissue processing**

The hepatic tissue homogenized in PRO-PREPTM Protein Extraction solution (iNtBiotechnology, Korea) for 30 min on ice. Then the samples centrifuged at 12,000 rpm at 4 °C for 15 min, and transfer supernatants to a fresh tube. The protein concentrations were determined by using BCA<sup>TM</sup> protein assay kit.

#### **2.15. Hepatic glucose regulating enzyme activities**

The glucokinase (GK) activity was determined using glucokinase kit (abcam,ab125967), where the formatin of glucose-6-phosphate was coupled to its oxidation by glucose-6-phosphate (g6pase) activity was determined using glucose-6-phosphatase assay kit (Bio Vision,CA95035,USA). The phosphoenolpyruvate carboxykinase (PEPCK) activity was monitored in the direction of oxaloacetate synthesis using human PCK ELISA kit (CUSABIO, CSB-E09828h).

#### **2.16. Western blot analysis**

The muscle tissue homogenized in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl,

1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO<sub>3</sub>, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 µg/mL aprotinin, 25 µg/mL leupeptin) and using homogenizer. Tissue lysate were washed by centrifugation, and protein concentrations were determined by using BCA<sup>TM</sup> protein assay kit. Aliquots of the lysates (50 µg of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)). After blocking the nonspecific site with 1% bovine serum albumin (BSA) for 4 h. The primary antibodies were used at a 1:500 dilution. Membranes incubated with the primary antibodies at 4 °C for overnight. Then the membranes were washed with TTBT and then incubated with the secondary antibodies used at 1:3000 dilution. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit and exposed to X-ray films.

### **2.17. Statistical analysis**

The data are expressed as the mean ± standard error (S.E.) and one-way ANOVA test (using SPSS 12 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by the Student's t-

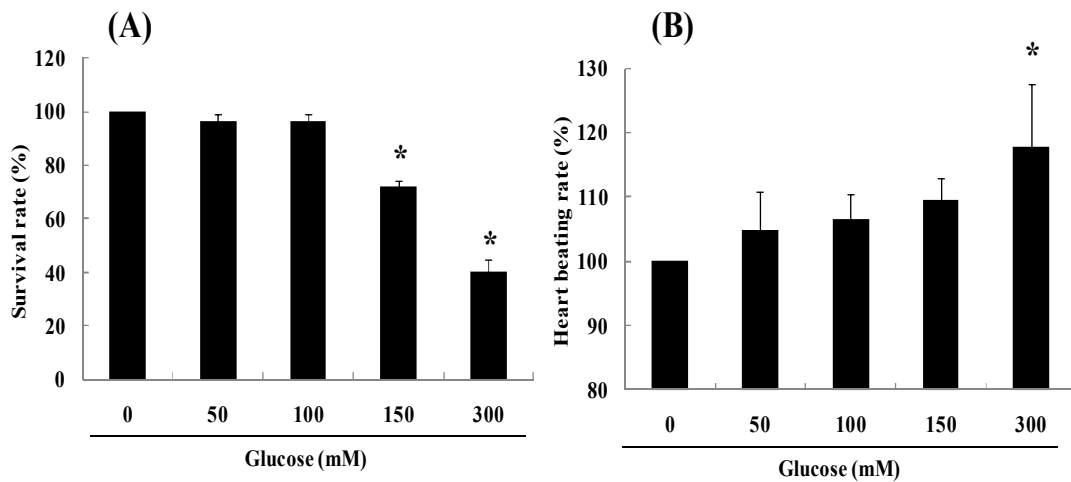
test (\*  $p < 0.05$ , \*  $p < 0.01$  ).

### **3. Results**

#### **3.1 Embryo toxicity of glucose**

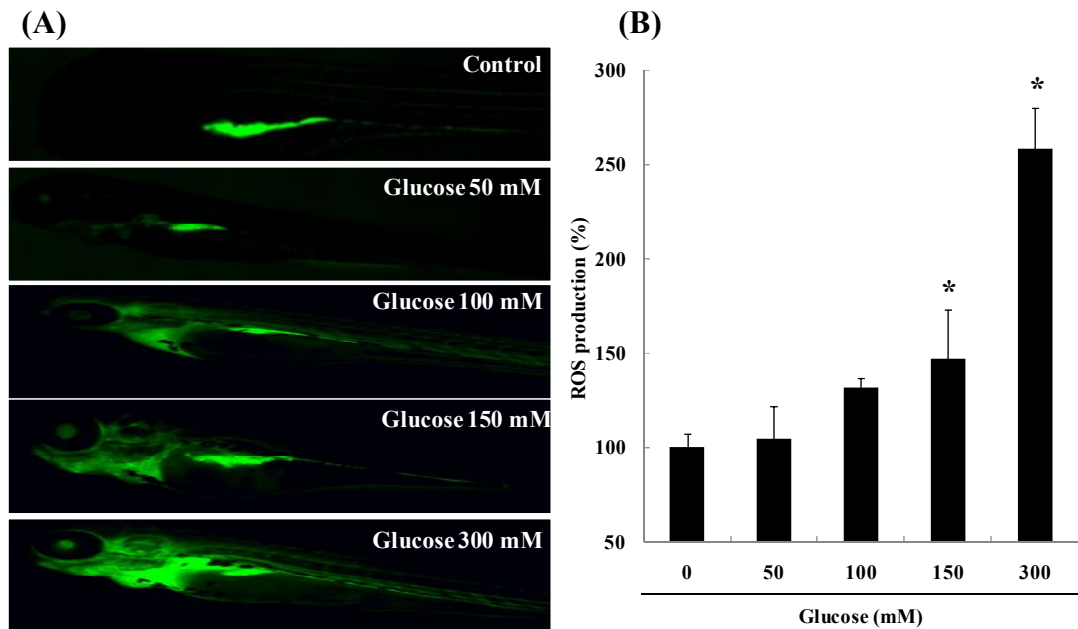
In order to determine the toxicity of the glucose, we observed the survival rate, heart beating rate, ROS production and cell death in zebrafish model. Zebrafish exposed to glucose at 24 hpf until 2 dpf. And then zebrafish were monitored time for survival rate until 5 dpf. At this time of complete yolk consumption and start of external feeding organogenesis of major organs is completed (Scholz et al., 2008). Therefore, we decided that 5 dpf was the endpoints of this point of this experiment for the assessment of toxicity of glucose with survival rate. The survival rate were 100%, 100%, 71% and 40% in the concentration at 50, 100, 150 and 300 mM glucose-treated zebrafish, respectively (Fig.1-A). The zebrafish hatch with 2 ~ 3 dpf and heart develops rapidly; a beating heart forms within 26 hpf and has a complex repertoire of ion channels and functional metabolism (Scholz et al., 2008; Ma et al.,). Therefore heart beating was measured at 2 dpf. The heart-beating rate was observed during the first min while the zebrafish was put on the glass plate. The heart beating rate of the glucose-treated zebrafish increased to 100%, 105%, 106%, 110% and 118% at 0, 50, 100, 150, 300 mM, respectively (Fig.1-B). And then we observed image analysis at 4 dpf. Because, organ development including the liver and pancreas is completed by 4 (96 dpf) of zebrafish

development (Elo et al., 2007). The level of ROS was measured 105%, 132%, 147% and 259% in the 50, 100, 150 and 300 mM glucose concentration group compared to control group (Fig. 2). And, both late apoptotic cells and necrotic cells allow PI to pass through the membrane staining the DNA red, indicating a loss of plasma membrane integrity (Negron et al., 2004). As shown in Fig. 3, the cell death was recorded 100%, 115%, 230% and 283% at 50, 100, 150 and 300 mM, respectively. These result indicated that glucose had toxicity at high concentration (150 mM and 300 mM).



**Fig. 1. Effects of glucose on the survival rate and heart beating rate for measurement of the toxicity. (A) survival rate; (B) heart beating rate.** The embryos were exposed to of Glucose for 24 h (24 hpf – 48 hpf). The results are expressed as percentage of survival rate of controls at various concentration at 5 dpf and the number of heart beating in 1 min was counted at 2 dpf under the microscopy. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .





**Fig. 2. Estimation of reactive oxygen species (ROS) generation in zebrafish. (A)**

**The ROS levels were measured by image analysis and fluorescence microscope. (B) The**

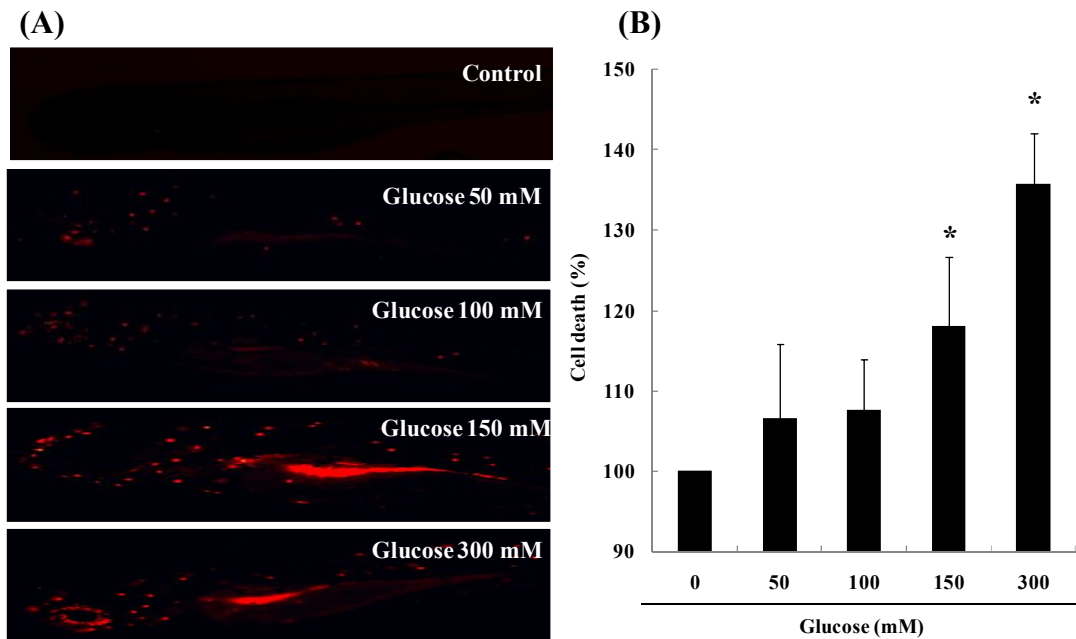
**ROS levels were measured by image J. The embryos were treated to various concentration**

**of glucoses. After incubation, the intracellular ROS detected by fluorescence**

**spectrophotometer after DCFH-DA staining. Experiments were performed in triplicate and**

**the data are expressed as  $\pm$  SE. Statistical evaluation was expressed to compare the**

**experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**



**Fig. 3. Estimation of cell death in zebrafish.** (A) The cell death levels were measured by image analysis and fluorescence microscope. (B) The cell death levels were measured by image J. The embryos were treated to various concentrations of glucoses. After incubation, the cell death was detected by fluorescence spectrophotometer after PI staining. Experiments were performed in triplicate and the data are expressed as  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish.

\* $p < 0.05$ .

### **3.2 Measurement of phlorotannins isolated from *E. cava* against high glucose-induced oxidative stress *in vivo* zebrafish model**

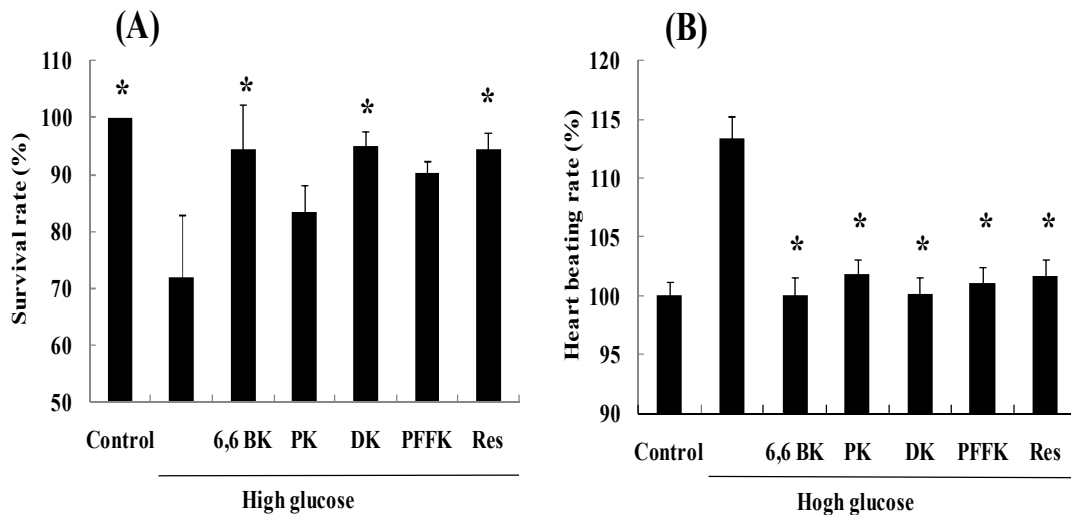
Embryo toxicity of glucose experiments showed that 150 mM and 300 mM glucose induced oxidative stress. Thus we decided to cause 150 mM glucose-induced oxidative damage of the zebrafish. To confirm the reduction of oxidative stress of the phlorotannins (6,6 BK, PK, DK and PFFK), resveratrol (Res), we monitored the survival rate and heart beating rate in zebrafish model. Zebrafish exposed to phlorotannins or resveratrol from 7 ~ 9 hpf. At 24 hpf, 150 mM glucose treated until 2 dpf. The survival rate of zebrafish embryos treated with glucose or co-treated with phlorotannins, resveratrol showed in Fig. 4-A. The survival rate was observed only glucose treatment embryos about 70%. However, the treated of phlorotannins with the glucose-treated zebrafish recorded 95%, 83%, 95% and 90% (6,6 BK, PK, DK and PFFK). And then resveratrol with glucose-treated zebrafish survived about 95%. On the other hand, the heart beating rate of the glucose-treated zebrafish increased to 113% compared with the control (without phlorotannins, resveratrol and glucose) groups (Fig. 4-B). But phlorotannins were record to 100%, 102%, 100% and 101%. And then resveratrol were reduced to 102%. Resultingly, phlorotannins were similar to rate with the control group. In simultaneous survival rate and heart beating rate tests, phlorotannins and resveratrol were protected to induced high glucose oxidative stress. And the scavenging efficacy of

phlorotannins including 6,6 BK, PK, DK, PFFK and resveratrol on ROS production in the glucose-induced zebrafish was measured. Treatment of the embryo with phlorotannins significantly inhibited the ROS production (Fig. 5). Thus, it was shown mostly similar ROS level of the embryos compared with the control at the presence of 20  $\mu$ M phlorotannins. The level of ROS was 156% in the glucose-treated zebrafish compared to control group. In contrast, the zebrafish were exposed to glucose and phlorotannins and resveratrol, a reduction in the levels of ROS (123%, 129%, 122% and 130%) and 123% were significantly reduced. This result reflects a reduction of ROS generation by the phlorotannins and resveratrol treatment. And we observed NO by DAF-FM DA fluorescent dye and its results were shown in Fig. 6. Only glucose-treated zebrafish was observed of 116% compared to control group. However, phlorotannins and resveratrol dramatically reduced NO production (102%, 128%, 101%, 106% and 103%). And the glucose-induced lipid peroxidation verified by DPPH fluorescent dye and its results were shown in Fig. 7. Only glucose-treated zebrafish was observed of 125% compared to control group. But, phlorotannins and resveratrol decreased lipid peroxidation (110%, 117%, 110% and 114%) and 117%. Lastly to evaluate whether phlorotannins protect against glucose treatment, cell death induced by glucose treatment was measured via PI as fluorescence intensity in the zebrafish (Fig. 8). The glucose-induced cell death in zebrafish was measured as 109% compared to control group.

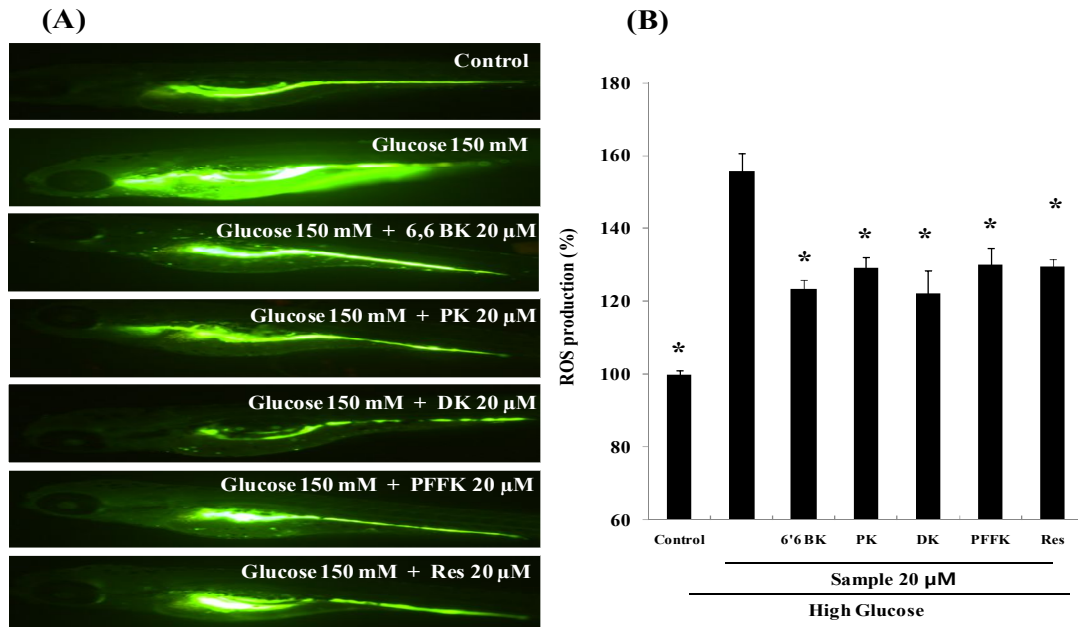
However, the cell death was reduced by the addition of phlorotannins and resveratrol. A phlorotannins and resveratrol showed cell death (110%, 105%, 95% and 101%) and 107%. All these results showed that DK among the phlorotannins had protective effects against high glucose oxidative stress.

**Table 1. The list of phlorotannins isolated from *E. cava***

	<b>Sample name</b>
6,6 BK	6,6 bieckol
PK	Phloroeckol
DK	Dieckol
PFFK	Phlorofucofuroeckol
Res	Resveratrol

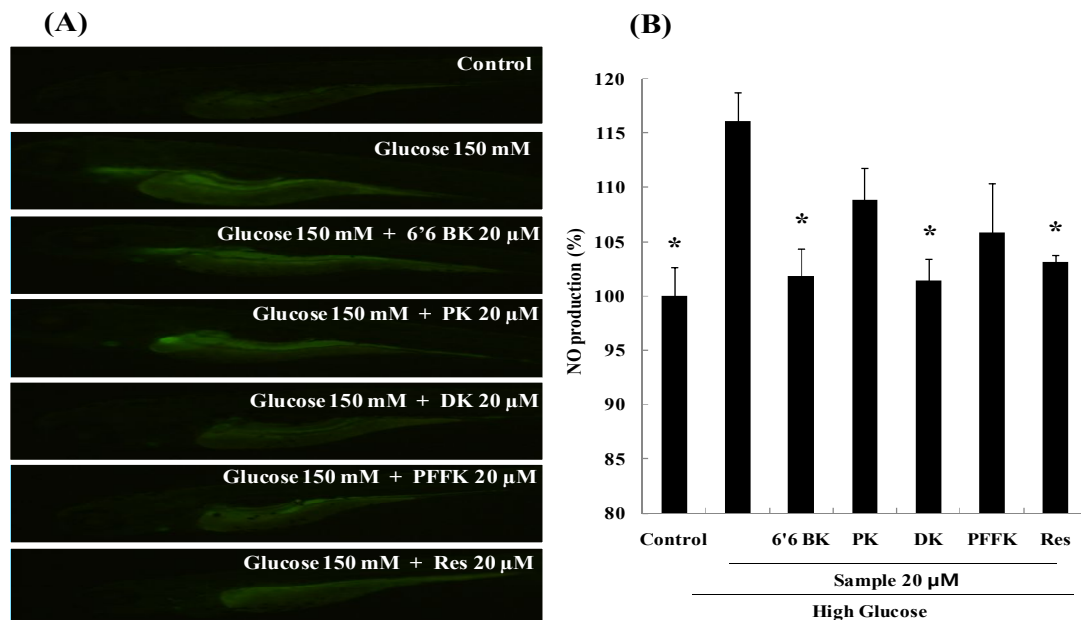


**Fig. 4. Measurement of toxicity of glucose, glucose-co treated phlorotannins by survival rate and heart beating rate. (A) survival rate; (B) heart beating rate.** The embryos were exposed to phlorotannins at 7-9 hpf until 24 hpf. At 1 dpf, a 150 mM glucose was treated to the embryo for up to 2 dpf. The results are expressed as percentage of survival rate of controls at 5 dpf. And the number of heart beating in 1 min was counted at 2 dpf under the microscopy. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p$ <0.01.

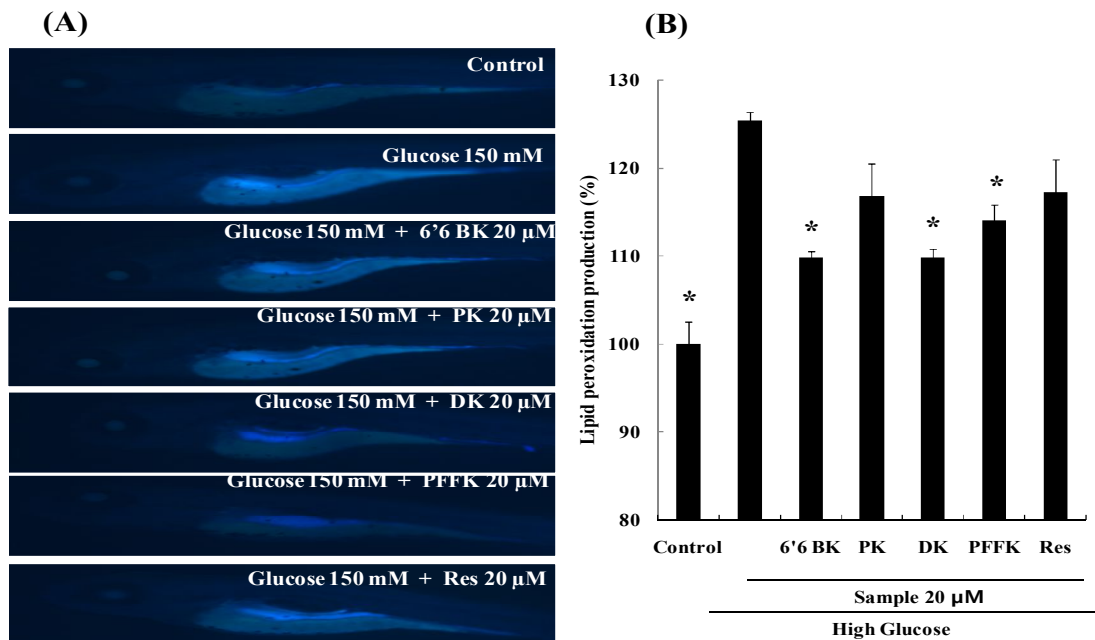


**Fig. 5. Protective effect of phlorotannins on glucose-induced reactive oxygen species (ROS) production in zebrafish. (A) The ROS levels were measured by image analysis and fluorescence microscope. (B) The ROS levels were measured by image J. The embryos were exposed to glucose and glucose-co treated phlorotannins. After incubation, the intracellular ROS was detected by fluorescence spectrophotometer after DCFH-DA staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**

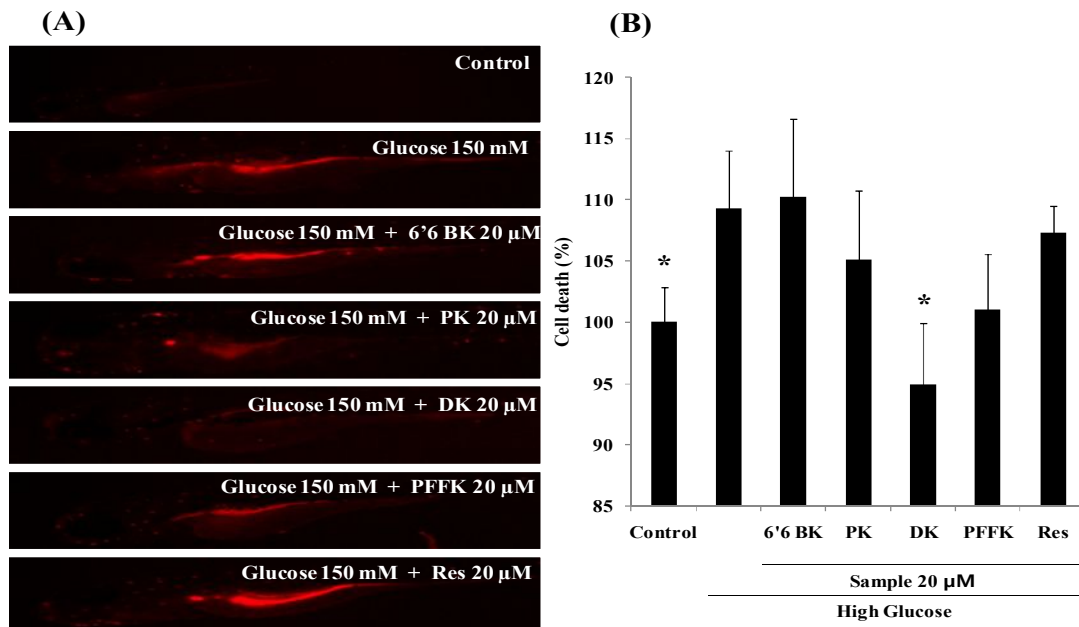




**Fig. 6. Protective effect of phlorotannins on glucose-induced intracellular nitric oxide (NO) production in zebrafish. (A) The NO levels were measured by image analysis and fluorescence microscope. (B) The NO levels were measured by image J. The embryos were exposed to glucose and glucose-co treated phlorotannins. After incubation, the intracellular NO was detected by fluorescence spectrophotometer after DAF-FM DA staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**



**Fig. 7. Protective effect of phlorotannins on glucose-induced lipid peroxidation production in zebrafish. (A) The lipid peroxidation levels were measured by image analysis and fluorescence microscope. (B) The lipid peroxidation levels were measured by image J. The embryos were exposed to glucose and glucose-co treated phlorotannins. After incubation, the lipid peroxidation was detected by fluorescence spectrophotometer after DPPP staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**



**Fig. 8. Protective effect of phlorotannins on glucose-induced cell death in zebrafish.**

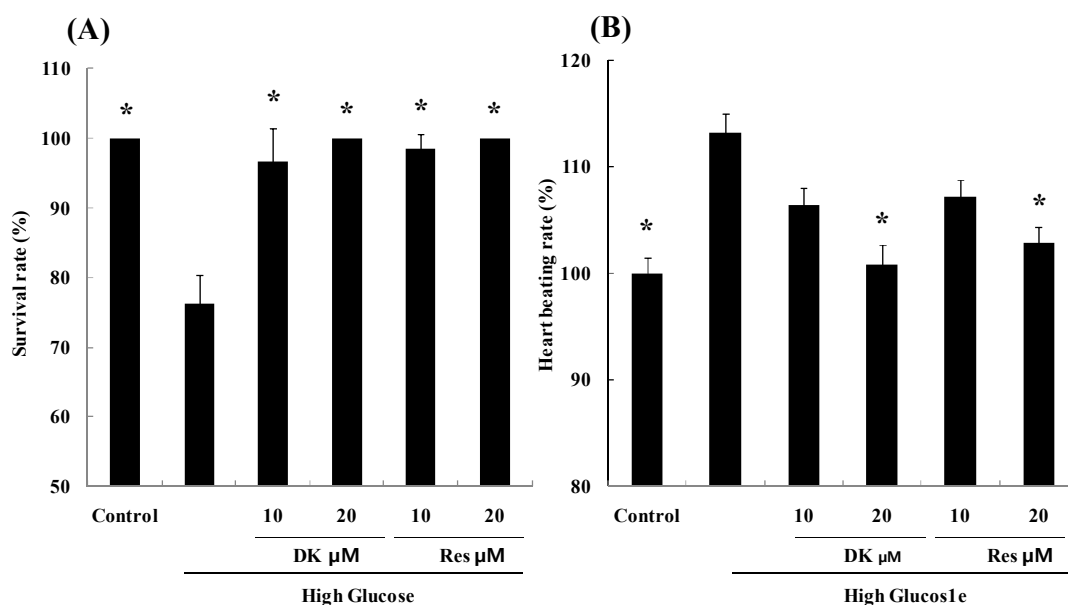
**(A)** The cell death levels were measured by image analysis and fluorescence microscope.

**(B)** The cell death levels were measured by image J. The embryos were exposed to glucose and glucose-co treated phlorotannins. After incubation, the cell death was detected by fluorescence spectrophotometer after PI staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .

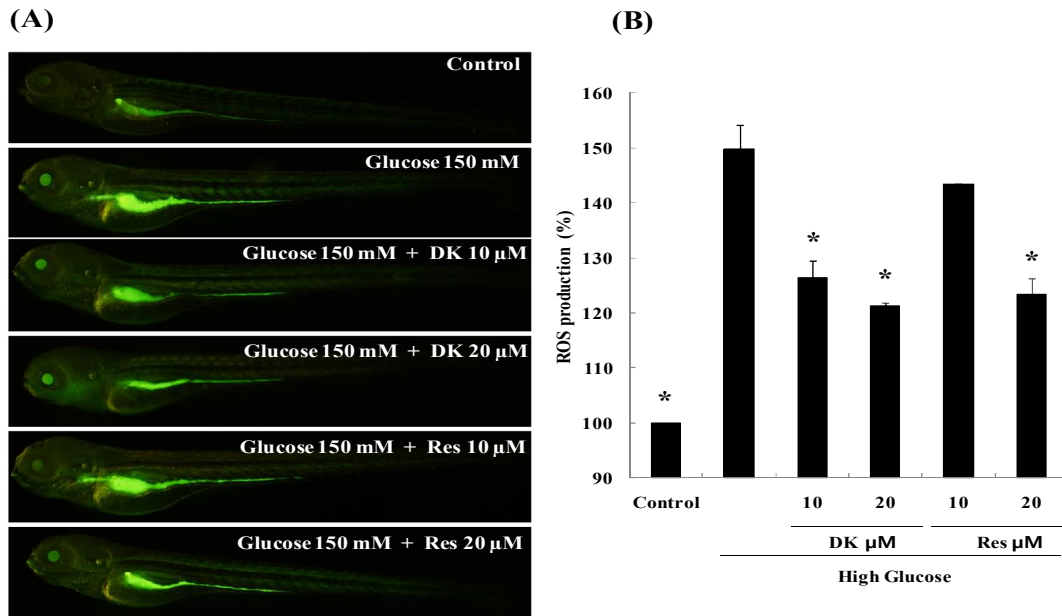
### **3.3 Measurement of DK isolated from *E. cava* against high glucose-induced oxidative stress *in vivo* zebrafish model**

In previous studies showed that DK among the phlorotannins had protective effects against high glucose oxidative stress. Therefore, we identified that DK had glucose-induced oxidative stress compared to resveratrol (Res). In order to determine the toxicity of glucose or glucose co-treated with DK and Res, we observed the survival rate and heart beating rate in zebrafish model. The survival rate was 76 % in the glucose-treated zebrafish comparing to the control (without DK, Res and glucose) group (Fig. 9-A). However, on the addition of DK at the concentrations of 10  $\mu$ M and 20  $\mu$ M, the survival rates recorded 96% and 100%, respectively. And Res at concentration of 10  $\mu$ M and 20  $\mu$ M, the survival rates recorded 98% and 100%, respectively. Therefore, the heart beating rate of the glucose-treated zebrafish increased to 113% compared with the control group (Fig. 9-B). However, the treated concentration of DK and Res at 10  $\mu$ M and 20  $\mu$ M in the glucose-treated zebrafish reduced to 106%, 101% and 107%, 103% in heart beating rate. For this result, it is proved that DK can protect the zebrafish damage induced by glucose. We tested the capacity of DK and Res to detect changes in physiological state using DCFH-DA measurement for the accumulation of ROS, NO measurement for Nitric oxide, DPPP measurement for lipid peroxidation and PI measurement for cell death caused by the glucose treatment. The level of ROS was 150% in

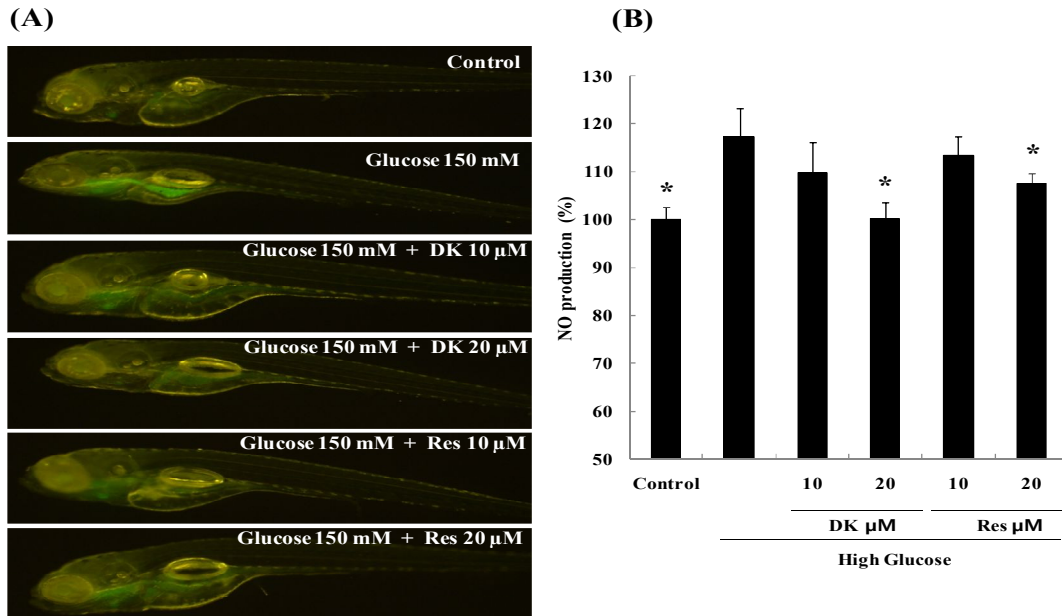
the glucose-treated zebrafish compared the control group. In contrast, the zebrafish exposed to DK and Res at different concentrations (10  $\mu$ M and 20  $\mu$ M) with glucose significantly reduced the levels of ROS production (126%, 121% and 143%, 123%) (Fig. 10). This result implies a reduction of ROS generation by DK and Res treatment. The glucose-induced nitric oxide was shown Fig. 11. The glucose-treated zebrafish indicated 117% of nitric oxide. Whereas, DK and Res zebrafish groups at concentrations of 10  $\mu$ M and 20  $\mu$ M dramatically decreased nitric oxide to 110%, 100%, respectively and 113%, 107%. The glucose-induced lipid peroxidations by DPPP fluorescent dye were shown in Fig. 12. The glucose-treated zebrafish indicated 115% of lipid peroxidation. But, DK or Res zebrafish groups at 10  $\mu$ M and 20  $\mu$ M decreased lipid peroxidation to 112%, 107% and 114%, 108%. Lastly, Fig. 13 showed that the cell death induced by glucose treatment was confirmed via PI as fluorescence intensity. The glucose-induced cell death in zebrafish was measured to 129% compared to the control group. But, the cell death was reduced (122%, 108% and 119%, 109%) by the addition of DK and Res (10  $\mu$ M and 20  $\mu$ M) to glucose-treated zebrafish, respectively.



**Fig. 9. Measurement of toxicity of glucose, glucose-co treated DK and resveratrol by survival rate and heart beating rate. (A) survival rate; (B) heart beating rate.** The embryos were exposed to DK or resveratrol at 7-9 hpf until 24 hpf. At 1 dpf, a 150 mM glucose was treated to the embryo for up to 2 dpf. The results are expressed as percentage of survival rate of controls at 5 dpf. And the number of heart beating in 1 min was counted at 2 dpf under the microscopy. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.01$ .



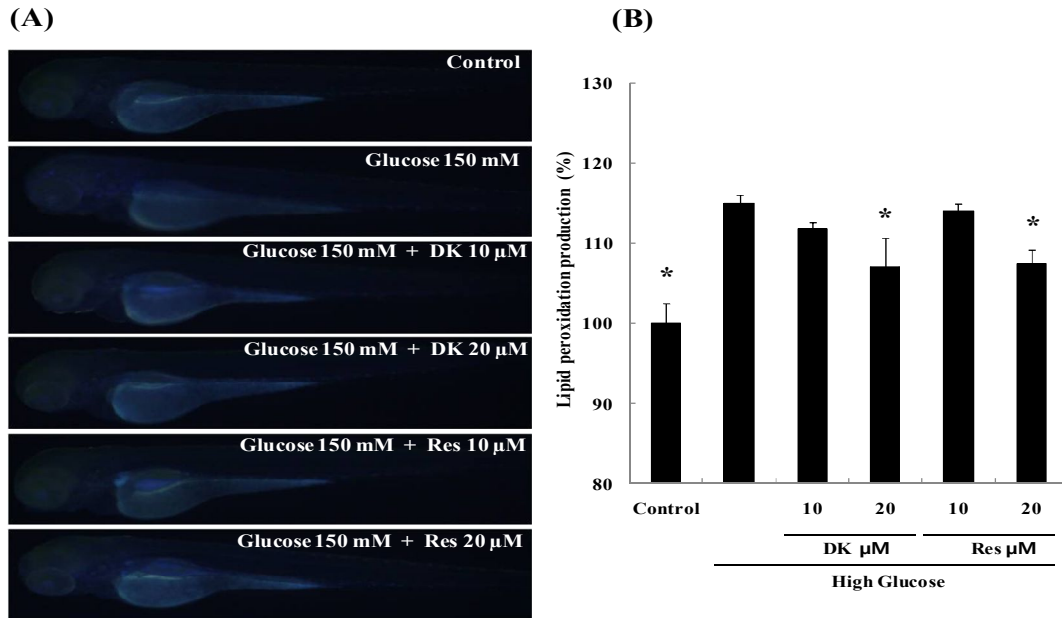
**Fig. 10. Protective effect of DK and resveratrol on glucose-induced reactive oxygen species (ROS) production in zebrafish. (A) The ROS levels were measured by image analysis and fluorescence microscope. (B) The ROS levels were measured by image J.** The embryos were exposed to glucose and glucose-co treated DK or resveratrol. After incubation, the intracellular ROS was detected by fluorescence spectrophotometer after DCFH-DA staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.01$ .



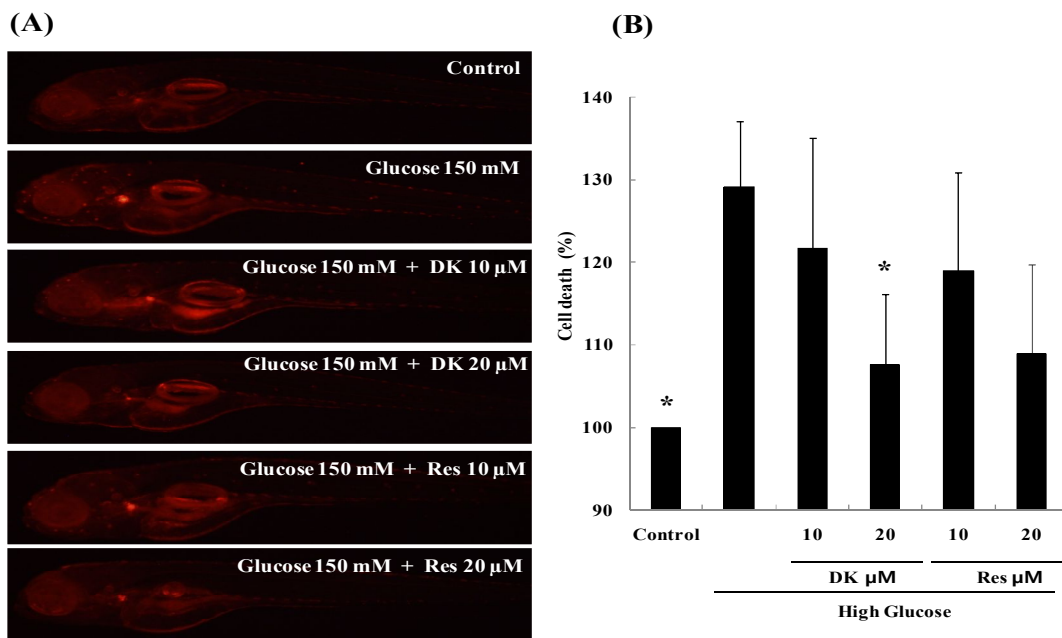
**Fig. 11. Protective effect of DK and resveratrol on glucose-induced intracellular nitric oxide (NO) production in zebrafish. (A) The NO levels were measured by image analysis and fluorescence microscope. (B) The NO levels were measured by image J.**

The embryos were exposed to glucose and glucose-co treated DK or resveratrol. After incubation, the intracellular NO was detected by fluorescence spectrophotometer after DAF-FM DA staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .





**Fig. 12. Protective effect of DK and resveratrol on glucose-induced lipid peroxidation production in zebrafish. (A) The lipid peroxidation levels were measured by image analysis and fluorescence microscope. (B) The lipid peroxidation levels were measured by image J. The embryos were exposed to glucose and glucose-co treated DK or resveratrol. After incubation, the lipid peroxidation was detected by fluorescence spectrophotometer after DPPP staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**



**Fig. 13. Protective effect of DK and resveratrol on glucose-induced cell death in zebrafish. (A) The cell death levels were measured by image analysis and fluorescence microscope. (B) The cell death levels were measured by image J. The embryos were exposed to glucose and glucose-co treated DK or resveratrol. After incubation, the cell death was detected by fluorescence spectrophotometer after PI staining. Experiments were performed in triplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and glucose-treated zebrafish. \* $p < 0.05$ .**

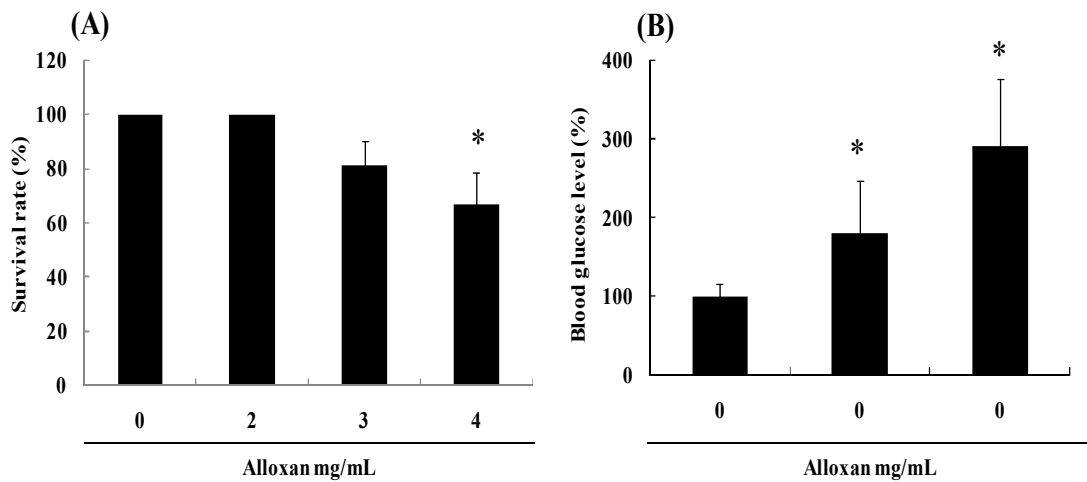
### **3.4 Confirm to anti-diabetic activity of DK isolated from *Ecklonia cava* in adult zebrafish models**

In previous study, we confirmed that DK decreased high glucose-induced oxidative stress in zebrafish embryos. Therefore, in this study, we checked anti-diabetic activity. Adult zebrafish was measured survival rate and blood glucose level (Fig. 14). Survival rate was recorded 100%, 81% and 67% for the different concentrations treated only alloxan (2, 3, 4, mg/mL) during 1 h. And, on the addition of alloxan at the concentrations of 2 and 3 mg/mL were increased blood glucose level of 180% and 290% compared to the control group. The zebrafish were exposed to various concentration of alloxan for 1 h, after changed to 1% glucose until 1h. After induction, the zebrafish were measured the survival rate and blood glucose level in fig. 15. The survival rate was recorded 100%, 100%, 54% and 40%, at 0, 2, 3 and 4 mg/mL, respectively. And then alloxan after glucose-treated zebrafish was measured 425% and 574% at 2 and 3 mg/mL on blood glucose level, respectively. In these results, alloxan concentration selected 2 mg/mL. The effect of DK on blood glucose level after a meal was investigated in diabetic zebrafish model.

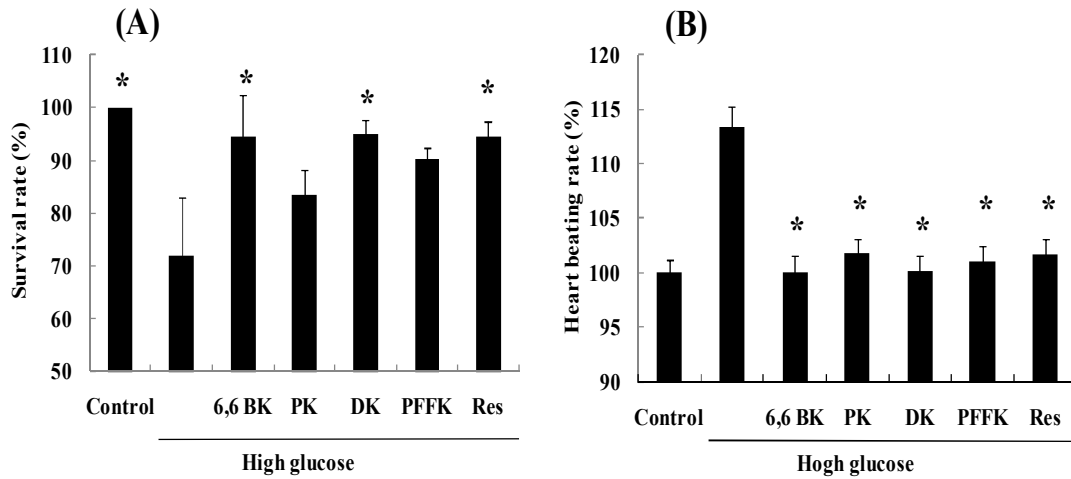
The blood glucose level of control zebrafish increased by 434% compared to the normal zebrafish (Fig. 16). However, blood glucose level was significantly decreased after DK or metformin injection. After the administration, blood glucose recorded of DK as 276, 241,

203 and 91% at 60, 90, 120 and 180 min compared to the normal group. Also, metformin presented as 303, 266, 234 and 196% at 60, 90, 120 and 180 min compared to the normal group. As this result, we selected this time because hourly blood glucose level showed a big difference level at 90 min. The plasma insulin level was presented in Fig 17-B. The plasma insulin levels were high in the DK and Metformin supplemented diabetic zebrafish model as compared to those in the Control zebrafish. Fig. 18 showed hepatic glucose regulating enzyme activities. The hepatic GK activity was significantly higher in the DK supplemented diabetic zebrafish than in the control zebrafish, yet its G6pas and PEPCK activities were significantly lower compared to the control zebrafish. This result showed that the supplementation of DK increased the GK activity. But the activities of G6pase and PEPCK were low activities. The DK group showed almost similar GK, G6Pase, and PEPCK activities to that of Metformin group. Western blotting analysis was the carried out further investigate insulin-mediated signaling pathway in DK action. In accord with the activation of phosphorylated Akt increased significantly by treatment of DK. As shown in Fig. 19, these results appear that DK strongly enhances insulin signaling, and the increase in phosphorylated Akt may plat and important role in this process. We investigated the effects of DK on AMPK activation but DK was not induced in AMPK phosphorylation in diabetic zebrafish model. This result, together with above results, strongly indicates that DK plays a

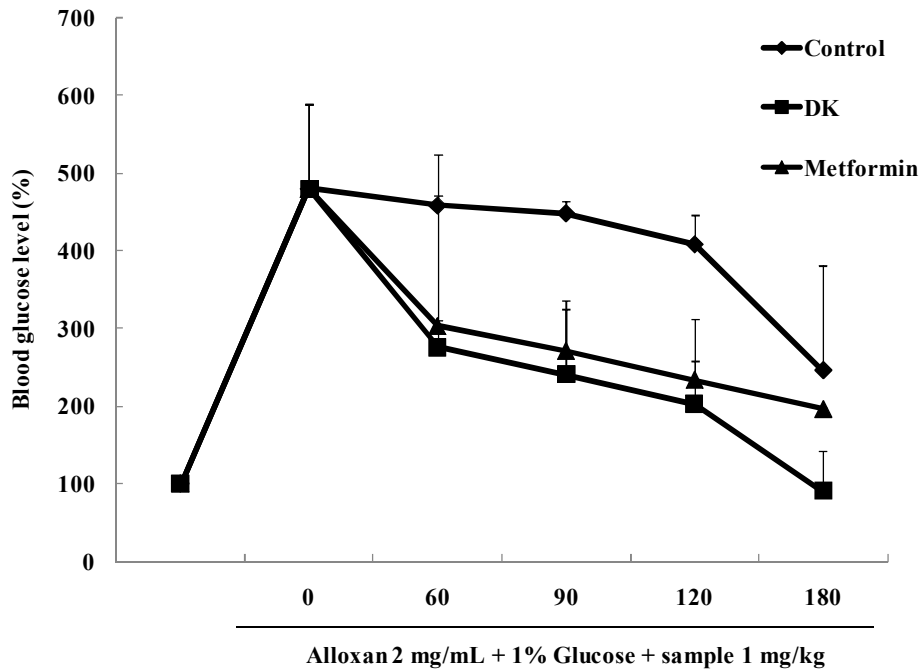
metabolic role in skeletal muscle cells through the Akt pathway.



**Fig. 14. Measurement of survival rate and blood glucose level on alloxan concentration in zebrafish. (A) survival rate; (B) blood glucose level.** The adults zebrafish were exposed to various concentration of alloxan for 1 h. after induction, the zebrafish were measured the survival rate and blood glucose level (n : 20). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and corresponding control groups. \*p<0.05.

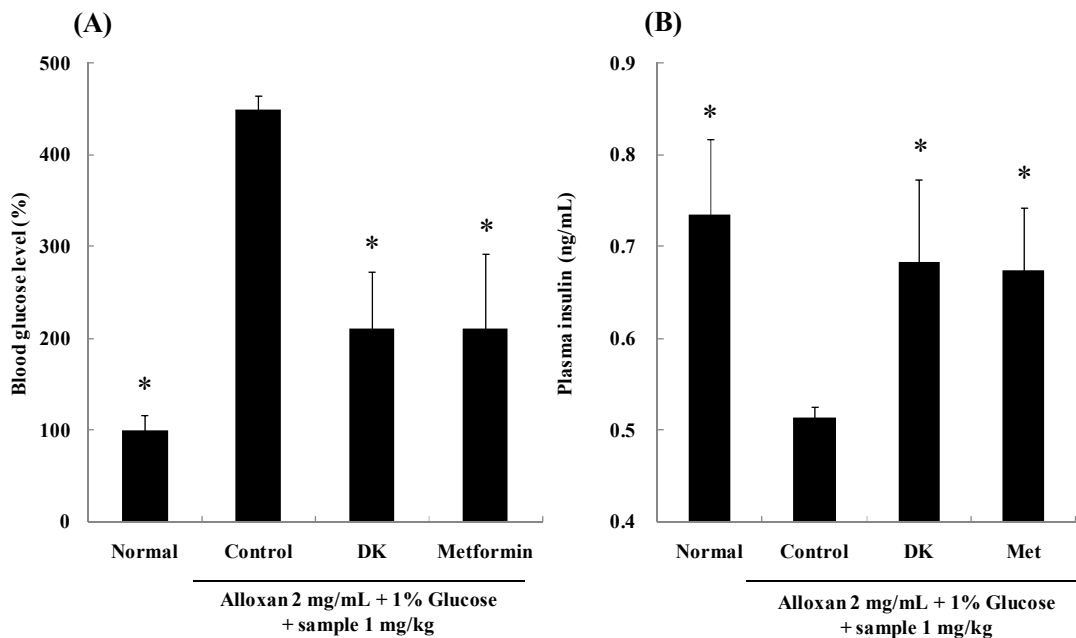


**Fig. 15. Measurement of survival rate and blood glucose level on alloxan concentration with 1% glucose in zebrafish. (A) survival rate; (B) blood glucose level.** The adults zebrafish were exposed to various concentration of alloxan for 1 h. after changed to 1% glucose until 1h. After induction, the zebrafish were measured the survival rate and blood glucose level (n : 20). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and corresponding control groups. \* $p < 0.05$ .



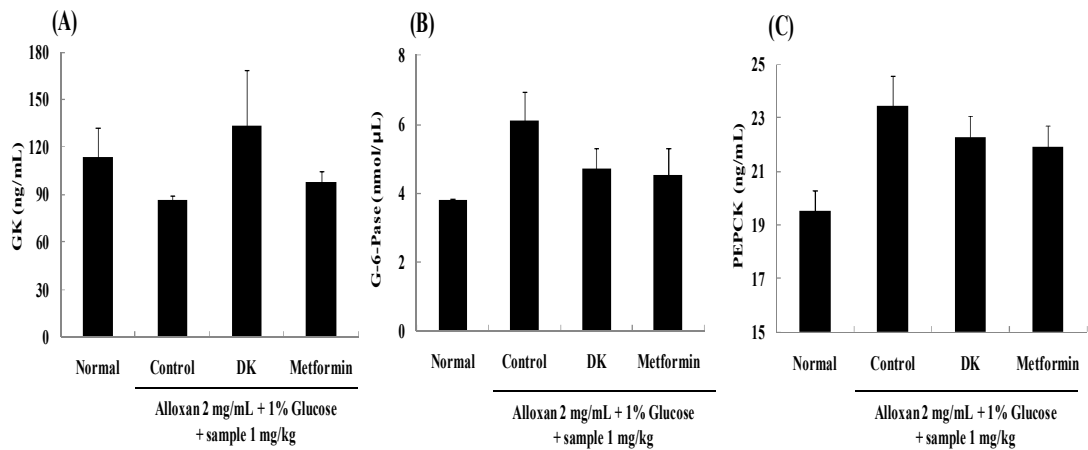
**Fig. 16. Measurement of blood glucose levels in zebrafish.** The adults zebrafish were exposed of 2 mg/mL alloxan for 1 h and moved in 1% glucose in water during 1 h, and then exposed in water until 1 h. After the zebrafish injected with DK or metformin (1 mg/kg body weight) for 0 min, 60 min, 90 min, 120 min, 180 min. After induction, the zebrafish were measured the blood glucose level (n : 20). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE.



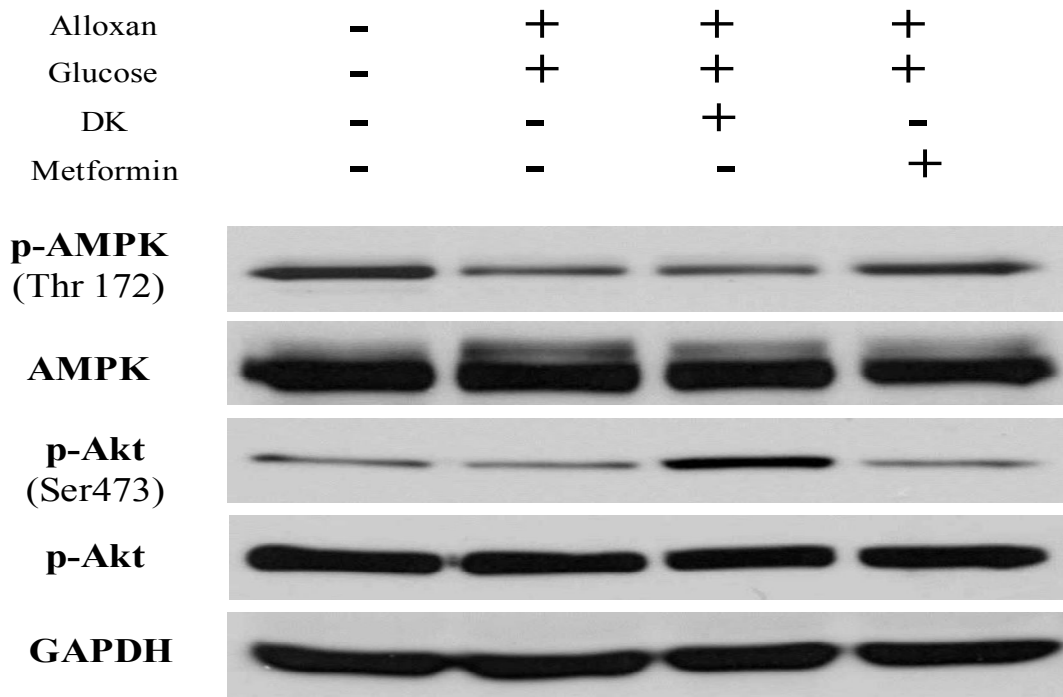


**Fig. 17. Measurement of blood glucose level and plasma insulin in diabetic zebrafish.**

**(A) Blood glucose level; (B) Plasma insulin.** The adults zebrafish were exposed of 2 mg/mL alloxan for 1 h and moved in 1% glucose in water during 1 h, and then exposed in water untill 1 h. The zebrafish injected with DK or metformin (1 mg/kg body weight) after 90 min. The zebrafish were measured the blood glucose level and plasma insulin (n : 20). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE. Statistical evaluation was expressed to compare the experimental groups and corresponding control groups. \* $p < 0.05$ .



**Fig. 18. Hepatic glucose regulating enzyme activities (A) hepatic glucokinase; (B) glucose-6-phosphatase; and (C) phosphoenolpyruvate carboxykinase.** The zebrafish were collected. The livers were collected and immediately stored at  $-70\text{ }^{\circ}\text{C}$  ( $n : 20$ ). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE.



**Fig. 19. Effect of DK on AMPK and Akt pathways.** The zebrafish were collected The muscles were collected and immediately stored at  $-70\text{ }^{\circ}\text{C}$  (n : 20) The muscle lysates were analyzed via Western blotting using anti-phospho AMPK (Thr172) and anti-phospho Akt (Ser 473). Experiments were performed in duplicate and the data are expressed as mean  $\pm$  SE.

## 4. Discussion

The zebrafish is rapidly becoming one of the most widely used animal models for pharmacological, toxicological and human disease testing of new compounds. Modern society, diabetes is big problem among human disease. It is the most serious and chronic disease that is developing with an increasing obesity and aging on the general population over the world. So, we confirmed to diabetes mellitus. Diabetes mellitus is a complex disorder that is characterized by hyperglycemia. Hyperglycemia-induced oxidative stress associated with diabetes and its related complications was caused antioxidants become known to it can prevent pathological damage (Yokozawa et al., 2007; Wang et al., 2011). Therefore, recently, there has been a growing interest in alternative therapies and in the therapeutic use of natural products for diabetes, especially those derived from marine algae (Chang et al., 2006; Jung et al., 2007). Marine algae are known to provide an abundance of bioactive compounds with great pharmaceutical foods and biomedical potential. The physiological benefits of phlorotannins are generally thought to be due to their antioxidant, even though phlorotannins display other biological activities (Heo et al., 2005, Kang et al., 2012a). Although some reports suggest that phlorotannins from algae exhibit antioxidant effects against free radicals, there are no studies on the protective effects against high

glucose in zebrafish model.

Therefore, in this study, zebrafish embryos are appropriated model to test the effect of phlorotannins isolated from *E. cava* in high glucose induced oxidative stress and confirmed to antidiabetic activity of DK isolated from *E. cava* in adult zebrafish model.

First, we tested the capacity of phlorotannins to detect changes in physiological state using DCFH-DA to accumulation of ROS, DAF-FM DA to check of NO and DPPP to measurement of lipid peroxidation to following exposure to glucose (Walker et al., 2012).

High glucose-induced excessive ROS production has been considered to play an important role in the development of diabetes (Wang et al., 2011). DCFH-DA acts by oxidation-dependent conversion of the non-fluorescent DCFH<sub>2</sub> to the fluorescent DCF; fluorescence thus increases connective to ROS production (Keston and Brandt 1965, Walker et al., 2012), A 4-amino-5-methylamino-2',7' – dichlorofluoresceindiacetate (DAF-FM-DA) probe thus increase connective to NO and DPPP is an interesting probe that results in hydroperoxides to give a strong fluorescent DPPP oxide (Akacaka et al., 1987). In addition, many types of stimuli could induce ROS, and the elimination of ROS using antioxidants protects the cells; therefore, ROS are believed to be key mediators of cell death (Martindale and Holbrook., 2002). Both late apoptotic cells and necrotic cells allow PI to pass through the membrane staining the DNA red, indicating a loss of plasma membrane integrity (Negron

et al., 2004). Accordingly, we investigated that measurement of phlorotannins against high glucose-induced oxidative stress via ROS, NO, lipid peroxidation and cell death using zebrafish. Consequently, we demonstrated that DK among the phlorotannins is a potential therapeutic agent that will reduce the damage caused by high glucose-induced oxidative stress associated with diabetes.

Next experiment, we confirmed to anti-diabetes activity in adult zebrafish. Alloxan or streptozotocin is administered to destroy the beta cells (Gleeson et al., 2007). So, we selected that alloxan concentration 2 mg/mL through survival rate and blood glucose level. The effect of DK on blood glucose level after a meal was investigated in diabetic zebrafish model. The present study observed that the supplementation of DK can decreased blood glucose levels and increased plasma insulin levels. And hepatic GK plays a major role in controlling blood glucose homeostasis and its activity is low in diabetes (Postic et al., 1999). G6pase is a key enzyme controlling hepatic gluconeogenesis and glucose output in liver and is normally suppressed by the action of insulin (Nordlie et al., 1993). Both of these enzymes are supposed to be the target of important regulatory mechanisms of hepatic glucose production (Mithieux, 1997). In this present study, hepatic GK activity was higher in the DK group than the control group. Furthermore, hepatic G6pase activity was significantly lowered, compared to the control group. Thereby, reducing hepatic glucose production. Among glucose

regulation genes, an enhanced expression of hepatic PEPCK gene has been identified in most forms of diabetes, and contributes to an increased hepatic glucose output (Davies et al, 2001). In the present study, DK supplements lowered hepatic PEPCK activity compared to the control group. Metformin group also increased GK and lowered G6pase and PEPCK activities. Thus, the anti-diabetic effects of the DK supplements seemed to be mediated through stimulating GK activity and inhibiting G6pase and PEPCK activity in zebrafish model. Skeletal muscle has been identified as the major tissue in glucose metabolism, accounting for nearly 75% of whole-body insulin-stimulated glucose uptake (DeFronzo, 1981). Insulin-stimulated glucose uptake in skeletal muscle is critical for reducing blood glucose levels. In skeletal muscle, glucose transport can be activated via Akt or AMPK by at least two major mechanisms. These results indicate that Akt and AMPK are a principal factor in DK-stimulated glucose uptake.

In conclusion, we confirmed anti-diabetic activity of DK isolated from *E.cava* in zebrafish model through high glucose-induced oxidative stress in embryo zebrafish model and adult diabetic zebrafish model. Thus, zebrafish verified to using anti-diabetic and high glucose-induced oxidative stress model. And then DK might be developed into medicinal preparations, nutraceuticals, or functional foods for diabetes and a may also be applied in other therapeutic fields.

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