Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Protective effect of fucoidan against AAPH-induced oxidative stress in zebrafish model

Eun-A Kim [a], Seung-Hong Lee [b], Chang-ik Ko [c], Seon-Heui Cha [d], Min-Cheol Kang [a], Sung-Myung Kang [e], Seok-Chun Ko [f], Won-Woo Lee [a], Ju-Young Ko [a], Ji-Hyeok Lee [a], Nalae Kang [a], Jae-Young Oh [a], Ginnae Ahn [g], Young Heun Jee [h], You-Jin Jeon [a,*]

[a] Department of Marine Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea
[b] Division of Food Bioscience, Konkuk University, Chungju, Chungbuk 380-701, Republic of Korea
[c] Research Institute of Processing from Jeju Fisher Food, Choung Ryong Fisheries Co., Ltd., Jeju 697-943, Republic of Korea
[d] Depatment of Pharmacology, School of Medicine, Ajou University Suwon, Republic of Korea
[e] Pediatric Oncology Experimental Therapeutics Investigators Consortium (POETIC) Laboratory for Pre-Clinical and Drug Discovery Studies, University of Calgary, Calgary, AB, Canada
[f] Department of Food and Nutrition, Sungshin Women's University, Seoul 136-742, Republic of Korea
[g] Laboratory of Comparative Animal Medicine, Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
[h] Department of Veterinary Medicine, Applied Radiological Science Research Institute, Jeju National University, Jeju 690-756, Republic of Korea

ARTICLE INFO

ABSTRACT

Fucoidan, extracted from Ecklonia cava, has been extensively studied because of its wide biological activities. However, antioxidative activities have not been yet examined. Therefore we evaluated in vitro and in vivo studies on antioxidative activities of E. cava fucoidan (ECF). ECF exhibited more prominent effects in peroxyl radical scavenging activity, compared to the other scavenging activities. Thus, ECF was further evaluated for its protective ability against 2,2′-azobis dihydrochloride induced oxidative stress in Vero cells and ECF strongly reduced the AAPH-induced oxidative damage through scavenging intracellular reactive oxygen species. Furthermore, we evaluated protective effect of ECF against AAPH-induced oxidative stress in zebrafish model. ECF significantly reduced ROS generation, lipid peroxidation and cell death in zebrafish model. These findings indicate that ECF has antioxidant activities in vitro Vero cells and in vivo zebrafish model, even though ECF is not a polyphenol or flavonoid compound and does not contain benzene rings or conjugated structures.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Reactive oxygen species (ROS) or high levels of free radicals cause oxidative stress leading to degradation of DNA, cell membranes, proteins and other cellular constituents (Fang, Yang, & Wu, 2002; Lopaczynski & Zeisei, 2001). As a result, different kinds of serious human diseases are created including atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, some neurological disorders, some types of cancers and aging (Kovatcheva et al., 2001; Ruberto, Baratta, Biondi, & Amico, 2001).

Zebrafish (Danio reiro) have definite advantages. For example, their comparatively small size makes them easier to administer in large numbers in a laboratory environment (Kishi et al., 2003). Furthermore zebrafish have a very short development, as the basic body plan is laid out 24 h post-fertilization (hpf),

embryos hatch approximately 2–3 days post-fertilization (dpf) and they attain maturity at about 3 months. Additionally, one female can spawn about 100 eggs per day, which are fertilized by sperm released into the water by males (Scholz, Fischer, & Gundel, 2008). Zebrafish have been traditionally used in the fields of molecular genetics and development biology, as a model organism for drug discovery and toxicological studies because of their physiological similarity to mammals (Driever et al., 1996; Hertog, 2005; Kimmel, 1989; Pichler et al., 2003). Moreover, zebrafish have been used as a model for human disease and development and have been the target of a large-scale funding program undertaken by the European Union (Zebrafish Model for Human Development and Disease ZF-MODELS) (Bradbury, 2004).

Seaweeds are composed of a variety of bioactive substances (polysaccharides, pigments, minerals, peptides and polyphenols) with valuable pharmaceutical and biomedical potential. In particular brown seaweeds contain various biological benefits such as antioxidant, anticoagulant, antihypertension, antibacterial, and

antitumor activities (Athukorala & Jeon, 2005; Heo, Park, Lee, & Jeon, 2005; Kotake-Nara, Asai, & Nagao, 2005; Mayer & Hamann, 2004; Nagayama, Iwamura, Shibata, Hirayama, & Nakamura, 2002). *Ecklonia cava* (class, Phaeophyceae; family, Lessoniaceae; order, Laminariales, *E. cava*) contains a variety of unique compounds including sulfated polysaccharides and phlorotannins with different biological activities (Heo et al., 2009). In recent years, sulfated polysaccharides and isolated compounds from seaweeds have been demonstrated to be potential ROS scavengers, and to possess anticoagulant, antithrombotic, antiviral, anticancer, anti-inflammation and antioxidant activities (Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Lee et al., 2012; Li et al., 2009; Shi, Nie, Chen, Liu, & Tap, 2007; Wang, Zhang, Zhang, & Li, 2008). Nevertheless, the antioxidative activities of polysaccharides such as fucoidans have not been evaluated properly, because they do not have benzene rings or conjugated structures which are generally the functional groups for antioxidant activity.

In this study, therefore, the antioxidative activities of *E. cava* fucoidan (ECF) were evaluated against 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress *in vitro* cell experiments and *in vivo* zebrafish model.

2. Materials and methods

2.1. Materials

Brown seaweed, *E. cava* was collected from the coast of Jeju Island, South Korea, between February and April 2011. The sample was rinsed carefully with fresh water and freezedried. The dried seaweed sample was ground and shifted through a 50 mesh standard testing sieve. All chemicals and reagents used were of analytical grade and obtained from commercial sources (Lee et al., 2012).

2.2. Extraction of fucoidan from E. cava

Fucoidan was extracted and fractionated according to the previously reported methods with slight modifications (Lee et al., 2012). Ten grams sample of the ground, dried *E. cava* powder was homogenized with 1000 mL of distilled water (dH$_2$O) and mixed with 100 μL of Celluclast (Novo Nordisk, Bagsvaerd, Denmark). This reaction was continued for 24 h at 50 °C and then the digest was boiled for 10 min at 100 °C to inactivate the enzyme. The product was clarified by centrifugation (3000 × g for 20 min) to remove the unhydrolyzed residue. Then the optimal pH for the enzymatic hydrolysis was adjusted to 7.0 and the crud ECF was as addition of precipitated with 3 volumes of ethanol after the hydrolytic reaction. Subsequently the centrifugation at 10,000 × g for 20 min at 4 °C, the precipitate was re-dissolved in dH$_2$O and sequentially treated with 4 M CaCl$_2$ for purification. The resulting precipitate was removed by centrifugation and the resultant supernatant was treated with cetylpyridinum chloride. The pyridinium salts were solubilized with 3 M CaCl$_2$ and reprecipitated with ethanol. The precipitate was re-dissolved in dH$_2$O, dialyzed (MwCO, 10–12 kDa) against water at 4 °C for 72 h, and then lyophilized; the lyophilized sample was used as crude fucoidan sample. The fucoidan consisted of mostly carbohydrates (51.8%), sulfates (20.1%), uronic acid (11.3%) and small amounts of protein (8.7%). Monosaccharide composition analysis showed that fucose (61.1%), galactose (27.2%) were the major sugars in the fucoidan, with minor amounts of xylose (7.0%), rhamnose (3.9%) and glucose (0.8%) (Lee et al., 2012).

2.3. Radical scavenging assays by using electron spin resonance (ESR) spectrometer

2.3.1. DPPH radical scavenging assay

This assay is based on the scavenging ability of stable DPPH radicals by the radical scavenging constituents in the extracts. Method described by Nanjo et al. (1996) was used to investigate the DPPH scavenging activity by ESR spectrometer (JES-FA ESR, JEOL, Tokyo, Japan). DPPH was prepared in MeOH at the concentration of 60 μM. A 60 μL of ECF was added to the same volume of freshly prepared DPPH solution. Then, the reactants were thoroughly mixed and transferred to 50 μL glass capillary tube and fitted into the ESR spectrometer. The spin adduct was measured after 2 min. The measurement conditions were as follows; central field 2 min. The measurement conditions were as follows; central field 3475 G, modulation width 0.8 mT, amplitude 500 mT, scan width 10 mT, microwave power 5 mW.

2.3.2. Peroxyl radical scavenging assay

Peroxyl radicals were generated by AAPH and their scavenging effects were investigated by the method described by Hiramoto, Johkoh, Sako, and Kikugawa (1993). The reaction mixture containing 20 μL of distilled water, 20 μL of the extract, 40 mM AAPH and 20 μL of 40 mM POBN was incubated at 37 °C for 30 min. The spin adduct was recorded on JES-FA ESR spectrometer. The measurement conditions were as follows; central field 3475 G, modulation width 0.2 mT, amplitude 500 mT, scan width 10 mT, microwave power 8 mW.

2.3.3. Hydrogen peroxide scavenging assay

The ability of ECF to scavenge hydrogen peroxide scavenging activity was determined according to the method of Müller (1985). A 100 μL of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96-well plate. A 20 μL of hydrogen peroxide was added to the mixture, and then incubated at 37 °C for 5 min. After the incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, and then incubated at 37 °C for 10 min. The absorbance was read with an ELISA reader 405 nm.

2.4. In vitro cell experiment

2.4.1. Cell culture

The monkey kidney fibroblast cell line (Vero, KCKB No. 10081) was maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO$_2$. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated feral bovine serum, streptomycin (100 μg/mL), and penicillin (100 unit/mL).

2.4.2. Intracellular reactive oxygen species (ROS) measurement in vitro Vero cells

For the detection of intracellular ROS, Vero cells were seeded in 96-well plates at a concentration of 1 × 10^5 cells/mL. After 16 h, the cells were treated with various concentrations of ECF then incubated at 37 °C under a humidified atmosphere. After 1 h, AAPH was added at a final concentration of 10 mM, and the cells were incubated for an additional 30 min at 37 °C. Finally the concentration of 5 μg/mL of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was introduced to the cells, and DCFH-DA fluorescence was detected at an excitation wavelength of 485 nm and an emission wave length of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (LS-5B, Perkin-Elmer, CT, USA).
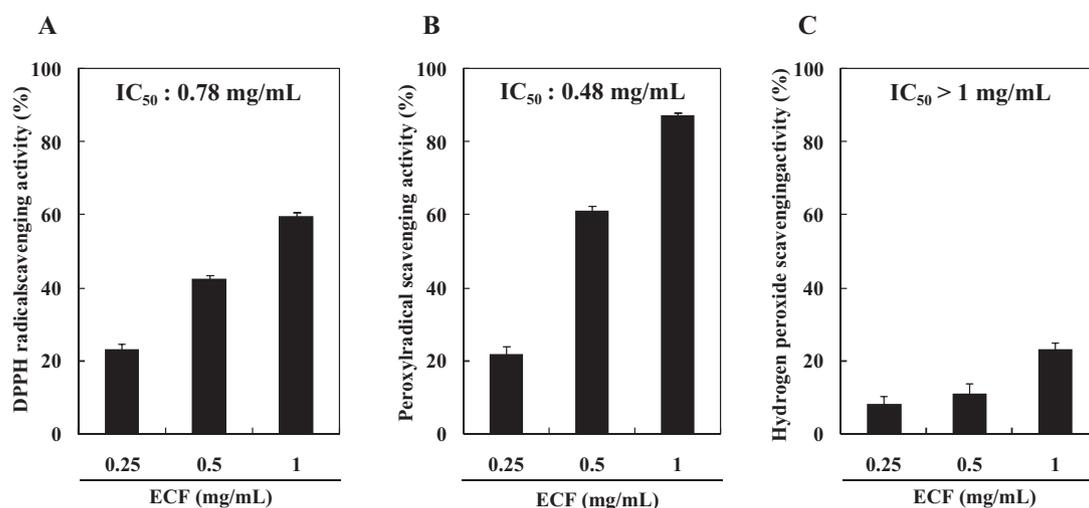
Fig. 1. Measurement of free radical scavenging activities of ECF by ESR. (A) DPPH scavenging activity; (B) peroxyl radical scavenging activity and (C) hydrogen peroxide scavenging activity. Values are means ± SD of triplicate experiments.

2.5. In vivo zebrafish model

2.5.1. 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 petri Dishes (containing media).

2.5.2. Waterborne exposure of embryos to ECF and AAPH

The embryos ($n = 15$) were transferred to individual wells of 12-well plates containing 900 μL embryo media from approximately 7–9 hpf, ECF was added to the wells. After the incubation 1 h, a 15 mM AAPH solution was treated to the embryo exposed with ECF for up to 24 hpf. Then, embryos were rinsed using fresh embryo media.
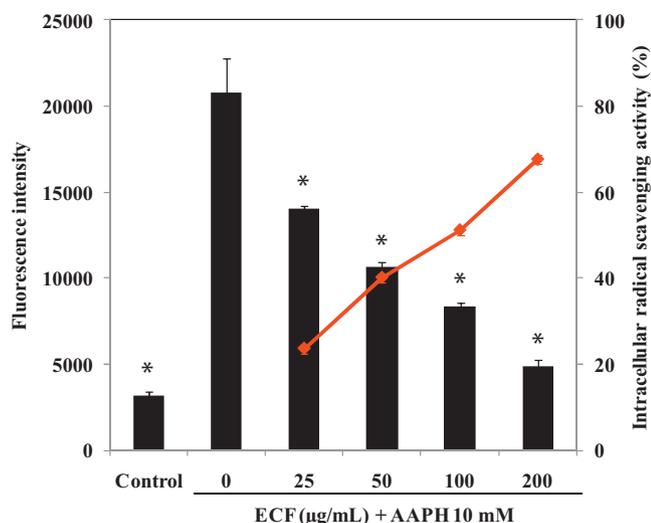

Fig. 2. Effect of ECF on intracellular reactive oxygen species (ROS) scavenging activity. Intracellular ROS were detected by DCFH-DA method. The bar-graph shows fluorescence intensity and the line graph exhibits intracellular radical scavenging activity. Experiments were performed in triplicate and data are mean ± SE. $^{*}p < 0.01$.

2.5.3. Measurement of heart-beat

The heartbeat rate of both atrium and ventricle was recorded at 2 dpf for 1 min under the microscope.

2.5.4. Measurement of oxidative stress-induced intracellular ROS generation and image analysis

Generation of ROS production of zebrafish was analyzed using an oxidation-sensitive fluorescent probe dye, DCFH-DA. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plate, treated with DCFH-DA solution (20 μg/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 larva was quantified using the image J program.

2.5.5. Measurement of oxidative stress-induced lipid peroxidation generation and image analysis

Lipid peroxidation was measured to assess the membrane damage in zebrafish model. Diphenyl-l-pyrenylphosphine (DPPP) is fluorescent probe for detection of cell membrane lipid peroxidation. DPPP is non-fluorescent, but it becomes fluorescent when oxidized. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with DPPP solution (25 μg/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.

2.5.6. Measurement of oxidative stress-induced cell death and image analysis

Cell death was detected in live embryos using acridine orange staining. Acridine orange stain cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with acridine orange solution (7 μg/mL) and incubated for 30 min under 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.

2.6. Statistical analysis

The data are expressed as the mean ± standard error (S.E.) and one-way ANOVA test (using SPSS 11.5 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 and discussion

3.1. Radical scavenging activities of ECF

ESR spin trapping provides a sensitive, direct and accurate method to monitor reactive species (Guo et al., 1999). We used the ESR technique to measure DPPH and peroxyl radical scavenging activities of ECF. Hydrogen peroxide and DPPH have been widely utilized to evaluate the antioxidant activity of natural compounds (Kang, Heo, Kim, Lee, & Jeon, 2012). The percentage scavenging activity of ECF against DPPH is shown in Fig. 1A. According to the results, ECF had an IC_{50} value of 0.73 mg/mL for DPPH radical scavenging activity. As shown in Fig. 1B, ECF reduced the ESR signal intensity to scavenge the peroxyl radical in a dose-dependent manner. Peroxyl radical scavenging activity of ECF (IC_{50} value, 0.48 mg/mL) was the highest among other radical scavenging activities. Further, Fig. 1C shows that ECF had concentration-dependent scavenging activity against hydrogen peroxide. However the hydrogen peroxide scavenging activity of ECF was very low. These results indicate that ECF has remarkable scavenging activity on the peroxyl radical. The antioxidant ability of ECF against the peroxyl radical suggests a possible beneficial role preventing various chronic diseases that are linked with oxidative stress.

3.2. Measurement of intracellular ROS in Vero cells in vitro

ECF strongly scavenged peroxyl radical among other radicals. Thus we decided to induce oxidative damage to the cells with AAPH. The level of ROS production in cells was detected via the fluorescent probe DCF to measure whether ECF could prevent AAPH-induced ROS generation and the resulting oxidative stressors (Kang et al., 2012). The intracellular ROS scavenging activity of ECF after administration of AAPH to Vero cells is shown in Fig. 2. The results showed that intracellular radical scavenging of the cells treated with ECF at different concentrations (25–200 μg/mL) decreased with an increase in ECF concentration. In particular the highest concentration (200 μg/mL) of ECF revealed the strongest ROS scavenging activity, and the IC_{50} value was 75 μg/mL. Previous studies have reported that high sulfate and fucose in algal fucoidans are related to radical scavenging effects with greater potential for preventing free radical-mediated disease (Li, Lu, Wei, & Zhan, 2008; Vo & Kim, 2012; Wang et al., 2008; Zhang, Yu, Zhou, Li, & Xu, 2003). In the present study, ECF appeared to be a potential peroxyl radical scavenger on ROS generation. Accordingly ECF could protect the human body against oxidative stress.

3.3. Measurement of ECF against AAPH-induced oxidative stress in vivo zebrafish model

Zebrafish have become a popular model in pharmacological studies such as chemical toxicity and drug discovery (Ko et al., 2011). We observed the survival rate and heart-beating rate in zebrafish to determine AAPH toxicity in zebrafish co-treated with ECF. The yolk is quickly exhausted after this point, with total absorption occurring by 7 hpf (Jardine & Litvak, 2003). Therefore, we decided that 7 dpf was the endpoint of this experiment to assess survival rate due to ECF toxicity. Survival rate was 83% in the AAPH-treated zebrafish compared to the control (without ECF and AAPH) group (Fig. 3A). However, survival rates were 91% and 97% at 100 and 200 μg/mL ECF, respectively. For this result, it is proved that ECF can protect the zebrafish from damage induced by AAPH. Zebrafish larvae typically hatch within 2–3 dpf at 28.5 °C (Scholz et al., 2008). Therefore the heart beating was measured as soon as the zebrafish hatched at 2 dpf. Heart-beating was observed during the first min as zebrafish lay on a glass plates. The heart-beating rate of the AAPH-treated zebrafish increased to 114%, compared with the control group (Fig. 3B). However, the treated concentration of ECF at 100 and 200 μg/mL in the AAPH-treated zebrafish reduced to 106% and 103% in heart-beating rate, respectively. Thus, survival rate and heart beat rate of ECF-treated zebrafish were similar to the control group. Therefore, ECF could be considered a latent peroxyl radical scavenging agent.
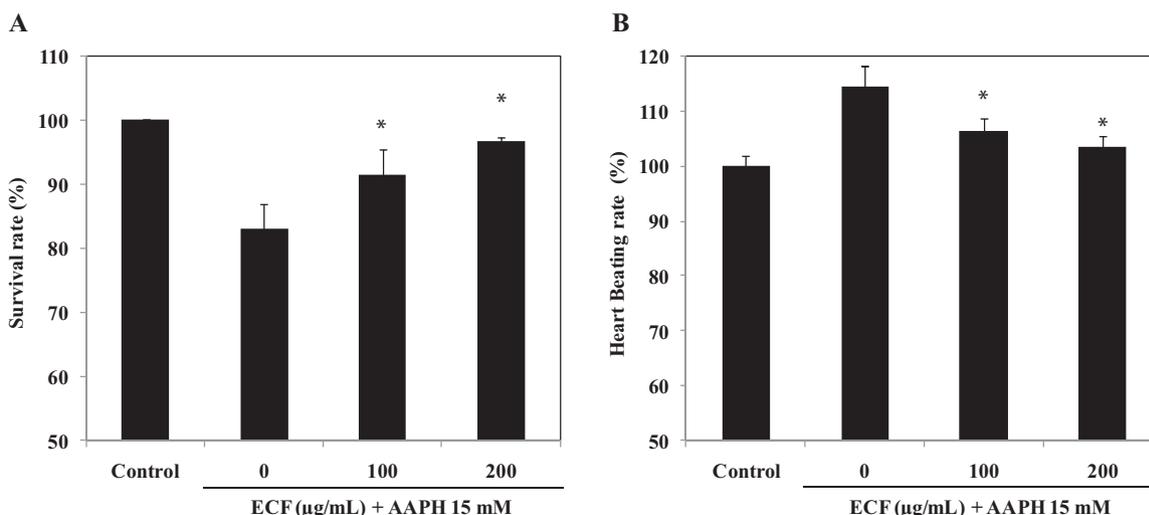

Fig. 3. Measurement of AAPH toxicity and AAPH-co treated with ECF on survival and heart beating rates. (A) Survival rate and (B) heart beating rate. Experiments were performed in triplicate and data are mean ± SE. *$p < 0.01$.
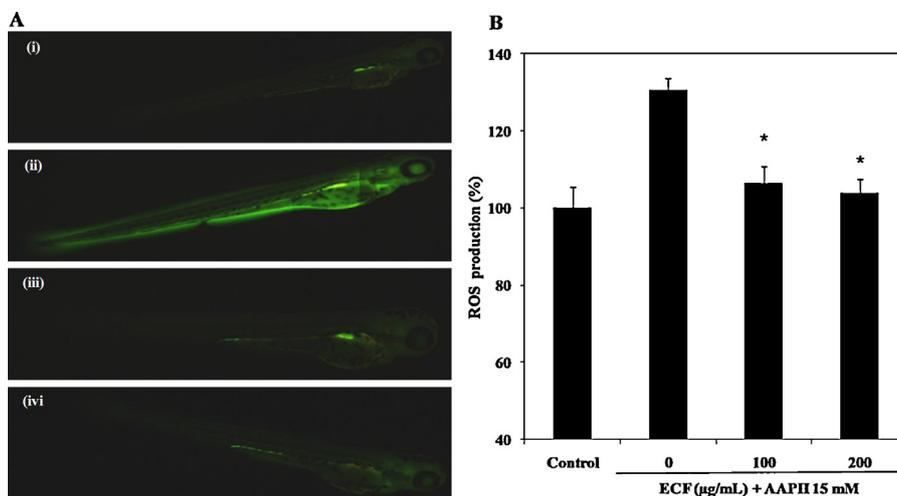
Fig. 4. Protective effect of ECF on AAPH-treated reactive oxygen species (ROS) production in zebrafish. (A) ROS levels were measured by image analysis and fluorescence microscope. (i) Control (without ECF and AAPH); (ii) AAPH only; (iii) AAPH-co treated with 100 μg/ml ECF and (iv) AAPH-co treated with 200 μg/ml ECF. (B) ROS levels were measured by Image J. Experiments were performed in triplicate and data are mean ± SE. *$p < 0.01$.

We tested the capacity of ECF to detect changes in a physiological state using DCFH-DA to assess the accumulation of ROS, DPPP to assess lipid peroxidation and acridine orange to assess cell death caused by the AAPH treatment. DCFH-DA is oxidation-dependently converted to non-fluorescent $2',7'$-dichlorodihydrofluorescein ($DCFH_2$) to the fluorescent $2',7'$-dichlorofluorescein (DCF); thus fluorescence intensity increases with ROS production (Walker et al., 2012). ROS level was 130% in AAPH-treated zebrafish compared to the control group. In contrast, zebrafish exposed to AAPH and ECF at different concentrations (100 and 200 μg/mL) showed significantly reduced levels of ROS production (106% and 103%) (Fig. 4). This result suggests a reduction of ROS generation by ECF treatment. Diphenylpyrenylphosphine (DPPP) is a probe that detects hydroperoxides and DPPP oxide is strongly fluorescent (Akasaka, Suzuki, Ohrui, & Meguro, 1987). AAPH-induced lipid peroxidation by DPPP fluorescent dye is shown

in Fig. 5. The AAPH-treated zebrafish revealed 137% of lipid peroxidation, whereas, zebrafish groups treated with 100 and 200 μg/mL ECF showed dramatically decreased lipid peroxidation to 105% and 103%, respectively. This result indicates a reduction in lipid peroxidation production by ECF treatment. Acridine orange is a nucleic acid selective fluorescent cationic dye useful for apoptotic cells. Lastly, Fig. 6 shows that the cell death induced by AAPH treatment was confirmed *via* acridine orange as fluorescence intensity. The AAPH-induced cell death in zebrafish was measured to 118% compared to the control group. However, cell death was reduced (101% and 98%) by adding ECF (100 and 200 μg/mL) to AAPH-treated zebrafish. When zebrafish were treated with ECF prior to AAPH treatment, a dramatic decrease in cell death was observed. These results indicate that zebrafish could be a good animal model for antioxidant material screening. Collectively, our results demonstrate that ECF has excellent antioxidant properties *in vitro* and
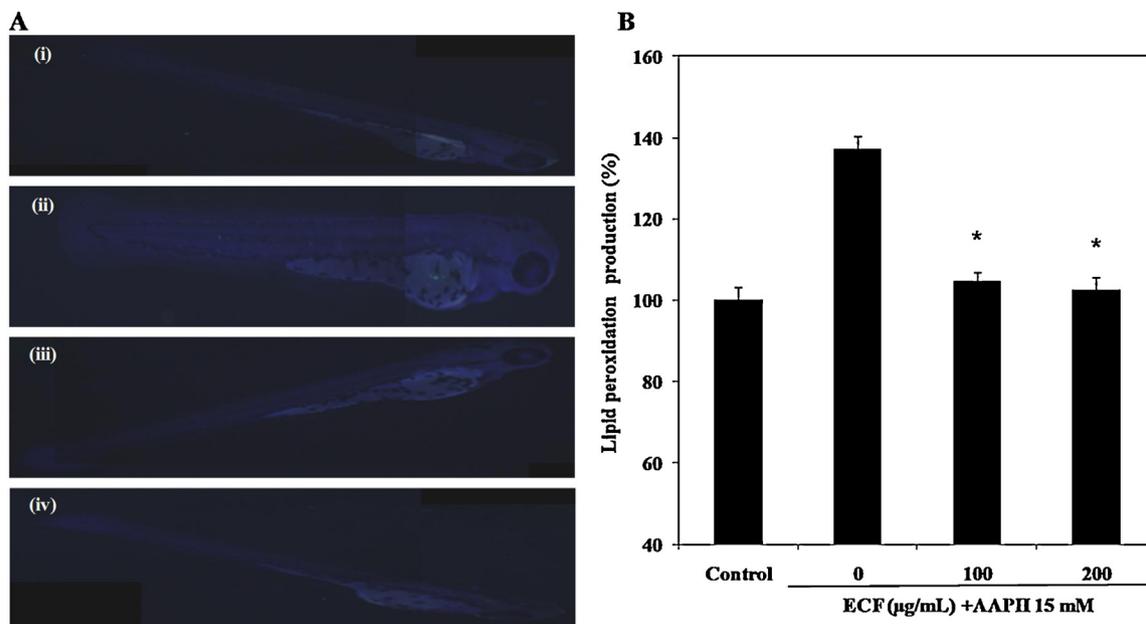

Fig. 5. Protective effect of ECF on AAPH-treated lipid peroxidation production in zebrafish. (A) Lipid peroxidation levels were measured by image analysis and fluorescence microscope. (i) Control (without ECF and AAPH); (ii) AAPH only; (iii) AAPH-co treated 100 μg/ml ECF and (iv) AAPH-co treated 200 μg/ml ECF. (B) Lipid peroxidation levels were measured by Image J. Experiments was performed in triplicate and data are mean ± SE. *$p < 0.01$.
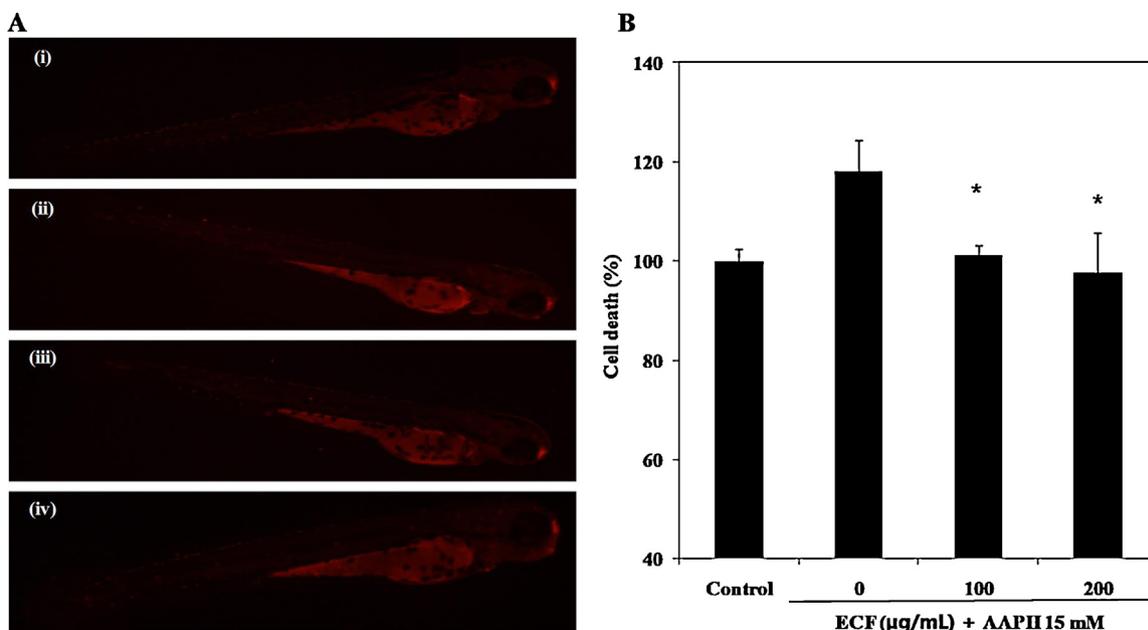
Fig. 6. Protective effect of ECF on AAPH-treated cell death in zebrafish. (A) Cell death levels were measured by image analysis and fluorescence microscope. (i) Control (without ECF and AAPH); (ii) AAPH only; (iii) AAPH-co treated with 100 μg/ml ECF and (iv) AAPH-co treated with 200 μg/ml ECF. (B) Cell death levels were measured by Image J. Experiments was performed in triplicate and data are mean ± SE. $^{*}p < 0.05$.

in vivo. Therefore, ECF can be a potential candidate for future therapeutic applications.

4. Conclusion

ECF exhibited strong peroxyl radical scavenging activity *via* ESR and intracellular ROS scavenging activity in Vero cells *in vitro* induced by AAPH. In addition, antioxidant ability was verified through ROS production, lipid peroxidation production, and cell death in the AAPH-induced oxidative stress zebrafish *in vivo* model. In conclusion, the fucoidan isolated from *E. cava* (ECF) does not include a polyphenol or flavonoid, and does not have benzene rings or conjugated structures. Nevertheless, our studies clearly show that ECF possesses antioxidant verified activities using *in vivo* zebrafish model. Therefore, zebrafish could be an effective *in vivo* model and ECF may be a valuable natural anti-oxidant.

Acknowledgment

References

Akasaka, K., Suzuki, T., Ohrui, H., & Meguro, H. (1987). Study on aromatic phosphines for novel fluorometry of hydroperoxides (II) – The determination of lipid hydroperoxides with diphenyl-1-pyrenylphosphine. *Analytical Letters, 20*, 797–807.

Athukorala, Y., & Jeon, Y. J. (2005). Screening for angiotensin 1-converting enzyme inhibitory activity of *Ecklonia cava*. *Journal of Food Science and Nutrition, 10*, 134–139.

Bradbury, J. (2004). Small fish, big science. *PLoS Biology, 2*(5), 0568–0572.

Driever, W., Solnica-Krezel, L, Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development, 123*, 37–46.

Fang, Y. Z., Yang, S., & Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition, 18*, 872–879.

Guo, Q., Zhao, B., Shen, S., Hou, J., Hu, J., & Xin, W. (1999). ESR study on the structure–antioxidant activity relationship of tea catechins and their epomers. *Biochimica et Biophysica Acta, 1427*, 13–23.

Hayashi, K., Nakano, T., Hashimoto, M., Kanekiyo, K., & Hayashi, T. (2008). Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *International Immunopharmacology, 8*, 109–116.

Heo, S. J., Ko, S. C., Cha, S. H., Kang, D. H., Park, H. S., Choi, Y. U., et al. (2009). Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicology in Vitro, 23*, 1123–1130.

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005). Antioxidant activities of enzymatics from brown seaweeds. *Bioresource Technology, 96*, 1613–1623.

Hertog, J. D. (2005). Chemical genetics: Drug screens in zebrafish. *Bioscience Reports, 25*, 5–6.

Hiramoto, K., Johkoh, H., Sako, K. I., & Kikugawa, K. (1993). DNA breaking activity of the carbon-centered radicalgenerated from 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). *Free Radical Research Communication, 19*, 323–332.

Jardine, D., & Litvak, M. K. (2003). Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. *Journal of Fish Biology, 3*, 388–397.

Kang, S. M., Heo, S. J., Kim, K. N., Lee, S. H., & Jeon, Y. J. (2012). Isolation and identification of new compound, 2,7″-phloroglucinol-6,6′bieckol from brown algae, *Ecklonia cava* and its antioxidant effect. *Journal of Functional Foods, 4*, 158–166.

Kimmel, C. B. (1989). Genetics and early development of zebrafish. *Trends in Genetics, 5*(8), 283–288.

Kishi, S., Uchiyama, J., Baughman, A. M., Goto, T., Lin, M. C., & Tsai, S. B. (2003). The zebrafish as a vertebrate model of functional aging and very gradual senescence. *Experimental Gerontology, 38*, 777–786.

Ko, S. C., Cha, S. H., Heo, S. J., Lee, S. H., Kang, S. M., & Jeon, Y. J. (2011). Protective effect of *Ecklonia cava* on UVB-induced oxidative stress: In vitro and in vivo zebrafish model. *Journal of Applied Phycology, 23*, 697–708.

Kotake-Nara, E., Asai, A., & Nagao, A. (2005). Neoxanthin and fucoxanthin induce apoptosis in PC-3 human prostate cancer cells. *Cancer Letters, 220*, 75–84.

Kovatcheva, E. G., Koleva, I. I., Ilieva, M., Pavlov, A., Mincheva, M., & Konushlieva, M. (2001). Antioxidant activity of extracts from *Lavandula vera* MMcell cultures. *Food Chemistry, 72*, 295–300.

Lee, S. H., Ko, C. I., Ahn, G., You, S. G., Kim, J. S., Heu, M. H., et al. (2012). Molecular characteristics and anti-inflammatory activity of the fucoidan extracted from *Ecklonia cava*. *Carbohydrate Polymer, 89*, 599–606.

Li, B., Lu, F., Wei, X., & Zhan, R. (2008). Fucoidan: Structure and bioactivity. *Molecules, 13*, 1671–1695.

Li, Y., Qian, Z. J., Ryu, B. M., Lee, S. H., Kim, M. M., & Kim, S. K. (2009). Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. *Bioorganic & Medicinal Chemistry, 17*, 1963–1973.

Lopaczynski, W., & Zeisei, S. H. (2001). Antioxidant, programmed cell death, and cancer. *Nutrition Research, 21*, 295–307.

Mayer, A. M. S., & Hamann, M. T. (2004). Marine pharmacology in 2000: Marine compound with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. *Marine Biotechnology, 1*, 37–52.

Müller, H. E. (1985). Detection of hydrogen peroxide produced by microorganisms on an ABTS peroxidase medium. *Zentralbl Bakteriol Mikrobiol Hyg A, 259*(2), 151–154.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I., & Nakamura, T. (2002). Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome. Journal of Antimicrobial Chemotherapy, 50*, 889–893.

Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., & Hara, Y. (1996). Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. *Free Radical Biology and Medicine, 21*, 895–902.

Pichler, F. B., Laurenson, S., Williams, L. C., Dodd, A., Copp, B. R., & Love, D. R. (2003). Chemical discovery and global gene expression analysis in zebrafish. *Nature Biotechnology, 21*, 879–883.

Ruberto, G., Baratta, M. T., Biondi, D. M., & Amico, V. (2001). Antioxidant activity of extracts of the marine algal genus *Cystoseira* in a micellar model system. *Journal of Applied Phycology, 13*, 403–407.

Scholz, S., Fischer, S., & Gundel, U. (2008). The zebrafish embryo model in environmental risk assessment-applications beyond acute toxicity testing. *Environmental Science and Pollution Research, 15*, 394–404.

Shi, B. J., Nie, X. H., Chen, L. Z., Liu, Y. L., & Tap, W. Y. (2007). Anticancer activities of a chemically sulfated polysaccharide obtained from *Grifola frondosa* and its combination with 5-fluorouracil against human gastric carcinoma cells. *Carbohydrate Polymers, 68*, 687–692.

Vo, T. S., & Kim, S. K. (2012). Fucoidans as a natural bioactive ingredient for functional foods. *Journal of Functional Food, 5*, 16–27.

Walker, S. L., Ariga, J., Mathias, J. R., Coothankandaswamy, V., Xie, X., Distel, M., et al. (2012). Automated reporter quantification in vivo: High-throughput screening method for reporter-based assays in zebrafish. *PLoS ONE, 7*, e29916.

Wang, J., Zhang, Q., Zhang, Z., & Li, Z. (2008). Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica. International Journal of Biological Macromolecules, 42*, 127–132.

Zhang, Q. B., Yu, P. Z., Zhou, G. F., Li, Z. E., & Xu, Z. H. (2003). Studies on antioxidnat activities of fucoidan from *Laminaria japonica. Chinese Traditional and Herbal Drugs, 34*, 824–826.