Carbohydrate Polymers 92 (2013) 84–89

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Anti-inflammatory effect of fucoidan extracted from *Ecklonia cava* in zebrafish model

Seung-Hong Lee[a,1], Chang-Ik Ko[b,1], Youngheun Jee[c], Yoonhwa Jeong[d], Misook Kim[d], Jin-Soo Kim[e], You-Jin Jeon[b,f,*]

[a] School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea
[b] Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea
[c] Department of Veterinary Medicine and Applied Radiological Science Institute, Jeju National University, Jeju 690-756, Republic of Korea
[d] Department of Food Science and Nutrition, Dankook University, Gyeonggi 448-701, Republic of Korea
[e] Department of Seafood Science and Technology/Institute of Marine Industry, Gyeongsang National University, Tongyeong 650-160, Republic of Korea
[f] Marine and Environmental Research Institute, Jeju National University, Jeju 695-814, Republic of Korea

ARTICLE INFO

Article history:
Received 23 August 2012
Received in revised form
24 September 2012
Accepted 24 September 2012
Available online 2 October 2012

Keywords:
Fucoidan
Zebrafish
Tail-cutting
LPS-treatment
Anti-inflammatory

ABSTRACT

Fucoidan extracted from *Ecklonia cava* had strong anti-inflammatory activities. However, the direct effects of fucoidan of *E. cava* on anti-inflammatory activities *in vivo* model remained to be determined. Therefore, the present study was designed to assess *in vivo* anti-inflammatory effect of fucoidan extracted from *E. cava* (ECF) using tail-cutting-induced and lipopolysaccharide (LPS)-stimulated zebrafish model. Treating zebrafish model with tail-cutting and LPS-treatment significantly increased the ROS and NO level. However, ECF inhibited this tail-cutting-induced and LPS-stimulated ROS and NO generation. These results show that ECF alleviated inflammation by inhibiting the ROS and NO generation induced by tail-cutting and LPS-treatment. In addition, ECF has a protective effect against the toxicity induced by LPS exposure in zebrafish embryos. This outcome could explain the potential anti-inflammatory activity of ECF, which might have a beneficial effect during the treatment of inflammatory diseases.

1. Introduction

Fucoidans are commonly found in brown seaweeds and some marine invertebrates such as sea cucumbers and sea urchins (Chevolot et al., 1999; Vieira & Mourao, 1988). They mainly consist of fucose and sulfate with small amounts of galactose, xylose, mannose, and uronic acids (Bilan et al., 2002; Chizhov, Dell, Morris, Haslam, & McDowell, 1999; Partankar, Oehninger, Barnett, Williams, & Clark, 1993). Fucoidans isolated from brown algae have been extensively studied because of their diverse biological activities such as anticoagulant, antitumor, immunomodulatory, and anti-inflammatory activities (Li, Lu, Wei, & Zhao, 2008). Because of these activities, fucoidans have been investigated in the recent years to be developed as drugs and functional foods.

We extracted fucoidan in our previous study from an enzymatic hydrolysate of the brown alga, *Ecklonia cava* (*E. cava*), and was mainly composed of fucose, with small amounts of galactose, xylose, and mannose. We also demonstrated that the fucoidan extracted from *E. cava* had anti-inflammatory activities (Lee et al., 2012). However, the direct effects of fucoidan extracted from *E. cava* on anti-inflammatory activities *in vivo* model remained to be determined.

The vertebrate zebrafish (*Danio rerio*) is a small tropical freshwater fish, which has emerged as a useful vertebrate model organism because of its small size, large clutches, transparency, low cost maintenance, morphological and physiological similarity to mammals (Eisen, 1996; Fishman, 1999). Traditionally, zebrafish has been used in the fields of molecular genetics and developmental biology (Driever, Solnica-Krezel, & Schier, 1996; Kimmel, 1989). However, recently the value of the zebrafish as a model organism for drug discovery and toxicological studies has been recognized (den Hertog, 2005; Pichler et al., 2003).

Zebrafish have well-developed innate and acquired immune systems that very similar to the mammalian immune system (Trede, Zapata, & Zon, 2001). In addition, the optical transparency of zebrafish embryos allows noninvasive and dynamic imaging the inflammation *in vivo*. Because of these characteristics, zebrafish are a useful and popular animal model for a variety of inflammation studies. In *in vivo* anti-inflammation test model, zebrafish is

* Corresponding author at: Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea. Tel.: +82 64 754 3475; fax: +82 64 756 3493.
E-mail addresses: youjinj@jejunu.ac.kr, youjinj@gmail.com (Y.-J. Jeon).
[1] These authors contributed equally to this work.

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.09.066

Table 1
Chemical composition of fucoidan extracted from *E. cava*.[a]

Component	Fucoidan
Total carbohydrate (%)	51.8 ± 1.3
Sulfate content (%)	20.1 ± 0.7
Uronic acid (%)	11.3 ± 0.5
Protein (%)	8.7 ± 0.3
Proportion of monosaccharide (%)	
Fucose	61.1 ± 1.6
Rhamnose	3.9 ± 0.4
Galactose	27.2 ± 1.2
Glucose	0.8 ± 0.1
Xylose	7.0 ± 0.3

[a] Information from Lee et al. (2012).
Experiments were performed in triplicate and the data are expressed as mean ± SE.

widely accepted as the best method for effective anti-inflammation assay (Liao et al., 2011; Novoa, Bowman, Zon, & Figueras, 2009; Park & Cho, 2011). Therefore, the present study was designed to assess *in vivo* anti-inflammatory effect of fucoidan extracted from *E. cava* using tail-cutting-induced and lipopolysaccharide-stimulated zebrafish model.

2. Materials and methods

2.1. Materials

The brown alga *E. cava* (Phylum Phaeophyta, Class Phaeophyceae, Order Laminariales, Family Alariaceae) was collected from the coast of Jeju Island, South Korea. A voucher specimen has been deposited in the author's laboratory and taxonomic identification of *E. cava* was performed by Prof. Ki-Wan Lee at Jeju National University, Republic of Korea. Salt, sand and epiphytes were removed with tap water. The samples were then rinsed carefully with fresh water and freeze-dried. The dried alga sample was ground and sifted through a 50-mesh standard testing sieve. All chemicals and reagents used were of analytical grade and obtained from commercial sources.

2.2. Preparation of fucoidan from E. cava

Fucoidan was extracted using the previously reported methods (Lee et al., 2012). A 10 g sample of the ground, dried *E. cava* powder was homogenized with 1 L of distilled water (dH_2O) and mixed with 100 μL of Celluclast (Novo Nordisk, Bagsvaerd, Denmark). This reaction 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 enzymatic hydrolysate was adjusted to pH 7.0 and precipitated with 3 volumes of ethanol. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the precipitate was re-dissolved in dH_2O and sequentially treated with 4 M $CaCl_2$. The resulting precipitate was removed by centrifugation and the supernatant was treated with cetylpyridinium chloride. The pyridinium salts were solubilized with 3 M $CaCl_2$ and reprecipitated with ethanol. The precipitate was re-dissolved in dH_2O, dialyzed (M_WCO, 10–12 kDa) against water at 4 °C for 72 h, and then lyophilized; the lyophilized sample was used as fucoidan sample. The chemical composition of the fucoidan was shown in Table 1 (Lee et al., 2012).

2.3. Origin and maintenance of parental zebrafish

Ten adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea) and were kept in a 3 L acrylic tank at 28.5 °C with a 14:10 h light:dark cycle. The zebrafish were fed three times a day, 6 days/week, with tetramin flake food supplemented with live brine shrimps (*Artemia salina*; SEWHAPET food Co., Seoul, Korea). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. The collection of embryos was completed within 30 min.

2.4. Preparation of inflammation-induced zebrafish model by tail-cutting and application of test fucoidan

The zebrafish larvae were anesthetized in tricaine methanesulfonate solution (Sigma, St. Louis, MO, USA) before tail-cutting. After anesthesia, to trigger tail-cutting-induced inflammation, tail of zebrafish larvae was half-cutted, around 50% of tail area was removed. To make wounds as consistently sized as possible, tail-cutting was performed using blade under a dissecting microscope. As soon as tail-cutting was completed, the zebrafish larvae were transferred in the fresh embryo medium.

The zebrafish larvae ($n = 15$) were randomly divided into following four groups: (1) tail-uncutted control group (negative control); (2) tail-cutted only group; (3) tail-cutted with fucoidan extracted from *E. cava* (ECF)-treated group; and (4) tail-cutted with commercial fucoidan (CF, Sigma, St. Louis, MO, USA)-treated group. ECF and CF-treated group was exposed to test samples at 28.5 °C for 3 h.

2.5. Preparation of inflammation-induced zebrafish model by LPS treatment and application of test fucoidan

Synchronized zebrafish embryos were collected and arrayed by pipette, 10–15 embryos/well, in 12-well plates containing 2 mL embryo medium for 7–9 h post-fertilization (hpf), and then incubated without or with the test samples for 1 h. To induce inflammation, 5 μg/mL LPS (final concentration) was added to the embryo medium for 15–17 hpf at 28.5 °C. Thereafter, the zebrafish embryos were transferred in the fresh embryo medium.

2.6. Estimation of inflammation-induced intracellular reactive oxygen species (ROS) generation and image analysis

The generation of ROS in inflammatory zebrafish model was analyzed using an oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). The DCF-DA is deacetylated intracellular by nonspecific esterase and is further oxidized to the highly fluorescent compound, dichlorofluorescein (DCF) in the presence of cellular peroxides (Rosenkranz et al., 1992). Following tail-cutting and LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with DCF-DA solution (20 μg/mL), and incubated for 1 h in the dark at 28.5 °C. After incubation, the zebrafish larvae and embryos were rinsed in the fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution before observation. Individual zebrafish larvae and embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.7. Estimation of inflammation-induced intracellular nitric oxide (NO) generation and image analysis

Generation of NO in inflammatory zebrafish model 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 fluorescent triazole derivatives (Itoh et al., 2000). Following tail-cutting and LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with DAF-FM DA solution (5 μM), and incubated for 1 h in the dark at 28.5 °C. After incubation, the zebrafish larvae and embryos were

rinsed in the fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution before observation. Individual zebrafish larvae and embryos fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.8. Measurement of heart-beating rate and yolk sac edema size

The heart-beating rate of both atrium and ventricle was measured at 48 hpf after LPS-exposure (Choi et al., 2007). Counting and recording of atrial and ventricular concentration were performed for 3 min under the microscope, and results are represented as the average heart-beating rate per min.

For size analysis of yolk sac edema at 48 hpf after LPS-exposure, lateral views of anesthetized embryos were imaged using a microscope (Na et al., 2009). The outlines of the yolk sac edema were traced, and the area within each tracing was determined by Moticimages plus V. 2.0 for windows (Motix, Xiamen, China).

2.9. Statistical analysis

The data are presented as means ± standard error. Statistical comparisons were performed using the SPSS package for Windows (Version 14). p-Values of less than 0.05 were considered significant.

3. Results and discussion

3.1. Inhibitory effect of fucoidan extracted from E. cava (ECF) on tail-cutting-induced and LPS-stimulated ROS generation in inflammatory zebrafish model

Inflammation is a complex stereotypical response of the body to cell damage and vascularize tissues. The inflammatory responses are controlled by cytokines, products of the plasma enzyme systems, and lipid mediators (Ross & Auger, 2002). However, chronic and uncontrolled inflammations are detrimental to the tissues, which may cause chronic inflammation-derived diseases, such as cardiovascular diseases and cancers (Frostegard et al., 1999; Karin, Lawrence, & Nizet, 2006). We have previously reported that the fucoidan extracted from *E. cava* had excellent anti-inflammatory activities (Lee et al., 2012). However, the direct effects of fucoidan extracted from *E. cava* on anti-inflammatory activities *in vivo* model remained to be determined. In *in vivo* anti-inflammation test model, zebrafish is widely accepted as the best method for effective anti-inflammation assay (Liao et al., 2011; Novoa et al., 2009; Park & Cho, 2011). Therefore, the present study we first investigated *in vivo* anti-inflammatory effect of fucoidan (ECF) extracted from *E. cava* using zebrafish model.

Inflammation is essential for physiological responses to tissue injury and infection. The wound-induced inflammation involves dynamic regulation of pro-inflammatory mediators (Mathias et al., 2006). To mimic acute inflammatory states in cells or tissues, treatment with lipopolysaccharide (LPS) has been widely used. LPS is a gram-negative bacterial pathogen and a potent activator of innate immune response (Park & Cho, 2011). Recent studies have reported that zebrafish model was used to rapidly and simply assess the anti-inflammatory activity on tail-cutting-induced and LPS-stimulated inflammation *in vivo* (Liao et al., 2011; Park & Cho, 2011).

Generation of intracellular ROS can be detected using oxidation sensitive dye DCF-DA as the substrate. DCF-DA exhibits no fluorescence without ROS and becomes fluorescent upon interaction with ROS (Handa et al., 2006). Fluorescent probes have been widely employed to monitor oxidative activity cells. During labeling, non fluorescent DCF-DA dye that freely into the cells gets hydrolyzed by intracellular esterase to DCF and trapped inside the cells (Veerman et al., 2004). Therefore, in this study, the generation of ROS in tail-cutting-induced and LPS-stimulated inflammatory zebrafish model was analyzed using an oxidation-sensitive fluorescent probe dye, DCF-DA. Fig. 1A demonstrates that the generation of ROS in zebrafish was significantly increased by the tail-cutting as compared with the negative control (zebrafish without tail-cutting). Tail-cutted zebrafish showed the ROS level to be significantly increased to 145.21%. However, the fucoidan extracted from *E. cava* (ECF) treatment reduced the level of ROS in the zebrafish induced by tail-cutting. In particular, ECF slightly lowered ROS level compared to the commercial fucoidan (CF). Fig. 1B is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no fucoidan or tail-cutting, generated a clear image, whereas the positive control, which was only tail-cutting, generated a fluorescence image, which suggests that ROS took place during tail-cutting in the zebrafish. However, in zebrafish that were treated with ECF prior to tail-cutting, a dramatic reduction in the amount of ROS was observed. Zebrafish incubated for 15–17 hpf with LPS showed ROS generation to be significantly higher relative to the non-LPS treated zebrafish (negative control). Pretreatment with ECF together with LPS significantly inhibited ROS generation, indicating protection against ROS (Fig. 2A). The zebrafish treated with ECF showed ROS generation to be significantly reduced by 121.41%. Fig. 2B is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no fucoidan or LPS treatment, generated a clear image, whereas the positive control, which was only LPS treatment, generated a fluorescence image, which suggests that ROS took place during LPS treatment in the zebrafish.

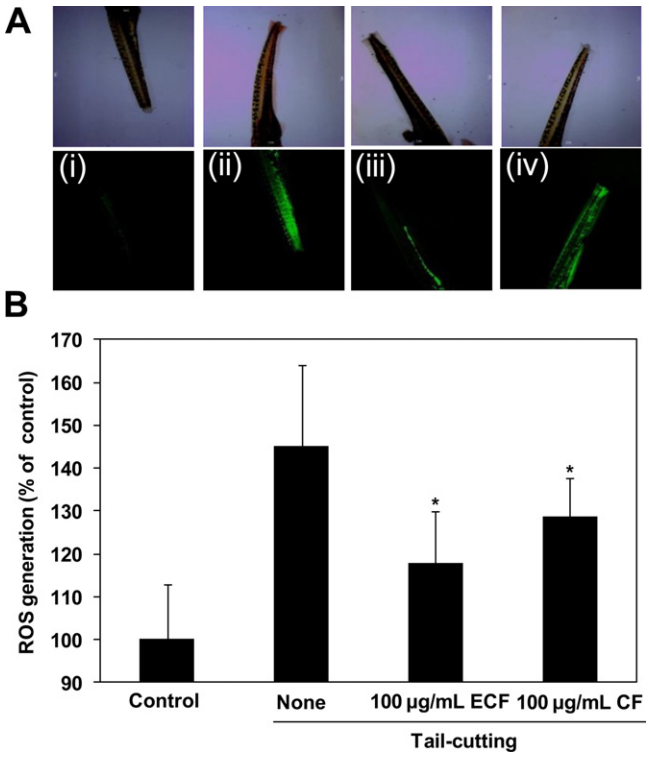

Fig. 1. Inhibitory effect of ECF on tail-cutting-induced ROS generation in zebrafish larvae. The zebrafish larvae were pretreated to tail-cutting and treated with ECF. The ROS generation level was measured after staining with DCF-DA. (A) Fluorescence micrographs of tail-cutting-induced ROS generation, as follows: (i) control; (ii) tail-cutting only; (iii) treated with fucoidan (ECF) extracted from *E. cava* (100 μg/mL); and (iv) treated with commercial fucoidan (CF, 100 μg/mL). (B) A fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. Experiments were performed in triplicate and the data are expressed as mean ± SE. *$p < 0.05$ shows significant difference from the only tail-cutting-treated zebrafish.

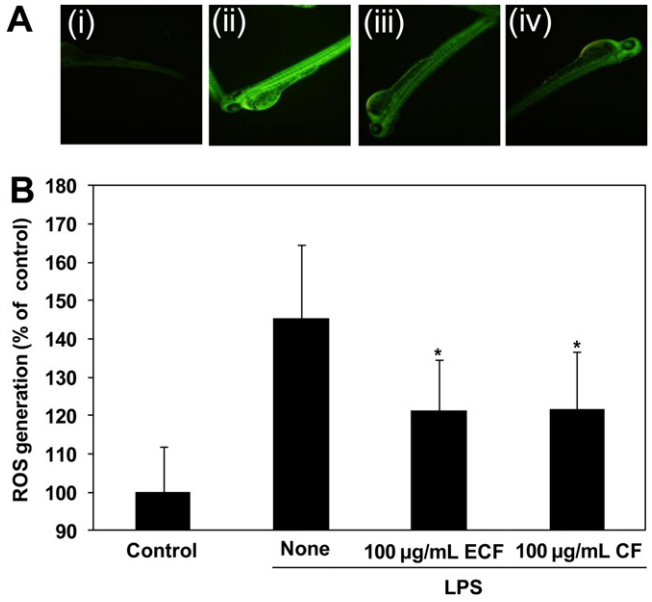

Fig. 2. Inhibitory effect of ECF on LPS-stimulated ROS generation in zebrafish embryos. The zebrafish embryos were pretreated with ECF and exposed to LPS (5 μg/mL). The ROS generation level was measured after staining with DCF-DA. (A) Fluorescence micrographs of LPS-stimulated ROS generation, as follows: (i) control; (ii) LPS-treated only; (iii) pretreated with fucoidan (ECF) extracted from *E. cava* (100 μg/mL); and (iv) pretreated with commercial fucoidan (CF, 100 μg/mL). (B) A fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}p < 0.05$ shows significant difference from the only LPS-treated zebrafish.

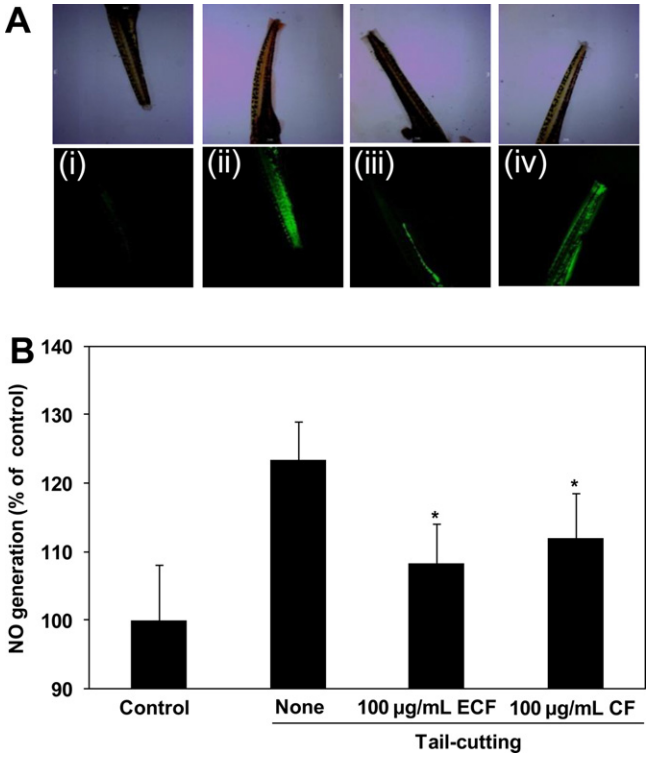

Fig. 3. Inhibitory effect of ECF on tail-cutting induced NO generation in zebrafish larvae. The zebrafish larvae were pretreated to tail-cutting and treated with ECF. The NO generation level was measured after staining with DAF-FM-DA. (A) Fluorescence micrographs of tail-cutting-induced NO generation, as follows: (i) control; (ii) tail-cutting only; (iii) treated with fucoidan (ECF) extracted from *E. cava* (100 μg/mL); and (iv) treated with commercial fucoidan (CF, 100 μg/mL). (B) A fluorescence spectrophotometer was used for the quantitative analysis of NO generation. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}p < 0.05$ shows significant difference from the only tail-cutting-treated zebrafish.

However, in zebrafish that were treated with ECF prior to LPS treatment, a dramatic reduction in the amount of ROS was observed. ECF therefore significantly reduced the elevated ROS level induced by LPS treatment in zebrafish model. Previous studies have indicated that a high ROS level induces oxidative stress which can result in a variety of biochemical and physiological lesions. Such cellular damage frequently impairs the metabolic function and results in cell death and inflammation of tissues (Finkel & Holbrook, 2000). Our results demonstrate that treating zebrafish with tail-cutting and LPS-treatment significantly increased the ROS level. However, ECF inhibited this tail-cutting and LPS-treatment-induced ROS generation. These results show that ECF alleviated inflammation by inhibiting the ROS generation induced by tail-cutting and LPS-treatment.

3.2. Inhibitory effect of ECF on tail-cutting-induced and LPS-stimulated NO generation in inflammatory zebrafish model

NO is an important inflammatory mediator that is synthesized from arginine by nitric oxide synthase (NOS). Generally, NO plays an important role as a vasodilator, neurotransmitter, and in the immunological system as a defense against tumor cells, parasites, and bacteria (Nakagawa & Yokozawa, 2002). However, under pathological condition, NO production is increased by the inducible NOS (iNOS), subsequently, brings about cytotoxicity, and tissue damage (Kim, Cheon, Kim, Kim, & Kim, 1999). Therefore, NO inhibitors are essential for the prevention of inflammatory diseases. We evaluated in this study the inhibitory effect of ECF on tail-cutting and LPS-treatment-induced NO production in zebrafish by using a fluorescent probe dye, DAF-FM DA. Transformation of DAF-FM DA by NO in the presence of dioxygen generates highly fluorescent triazole derivatives (Itoh et al., 2000). Fig. 3A shows that the level of NO in zebrafish was significantly elevated by the tail-cutting as compared with the negative control (zebrafish without tail-cutting). However, the NO level in the ECF-treated zebrafish was reduced significantly, this effect is similar to the CF. The level of NO in the zebrafish treated with tail-cutting was 123%, but treatment with ECF in conjunction with tail-cutting treatment resulted in a lower NO level of 108%. ECF therefore reduced the elevated NO level induced by tail-cutting in inflammatory zebrafish model. Fig. 3B is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no fucoidan or tail-cutting, generated a clear image, whereas the positive control, which was only tail-cutting, generated a fluorescence image, which suggests that NO took place during tail-cutting in the zebrafish. However, in zebrafish that were treated with ECF prior to tail-cutting, a dramatic reduction in the amount of NO was observed. Fig. 4A shows that the level of NO in zebrafish was significantly elevated by the LPS treatment as compared with the non-LPS treated zebrafish (negative control). However, the NO level in the ECF-treated zebrafish was reduced significantly. The level of NO in the zebrafish treated with LPS was 131.72%, but pretreatment with ECF together with LPS exposure resulted in a lower NO level of 107.87%. It is similar to the CF effect. Fig. 4B is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no fucoidan or LPS treatment, generated a clear image, whereas the positive control, which was only LPS treatment, generated a fluorescence image, which suggests that NO took place during LPS treatment in the zebrafish. However, in zebrafish that were treated with ECF prior to LPS treatment, a dramatic reduction in the amount of NO was observed. ECF therefore significantly reduced the elevated NO level induced by LPS treatment in zebrafish model. Previous studies have indicated that fucoidan of *E. cava*

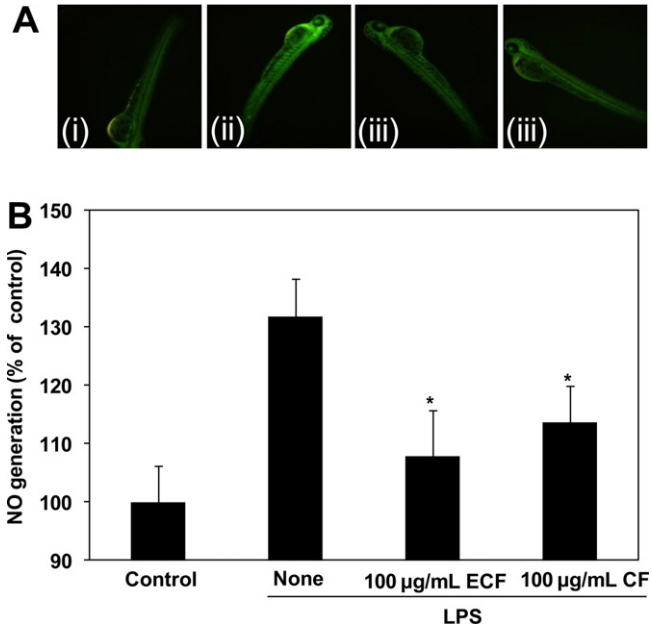

Fig. 4. Inhibitory effect of ECF on LPS-stimulated NO generation in zebrafish embryos. The zebrafish embryos were pretreated with ECF and exposed to LPS (5 μg/mL). The NO generation level was measured after staining with DAF-FM-DA. (A) Fluorescence micrographs of LPS-stimulated NO generation, as follows: (i) control; (ii) LPS-treated only; (iii) pretreated with fucoidan (ECF) extracted from *E. cava* (100 μg/mL); and (iv) pretreated with commercial fucoidan (CF, 100 μg/mL). (B) A fluorescence spectrophotometer was used for the quantitative analysis of NO generation. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}p < 0.05$ shows significant difference from the only LPS-treated zebrafish.

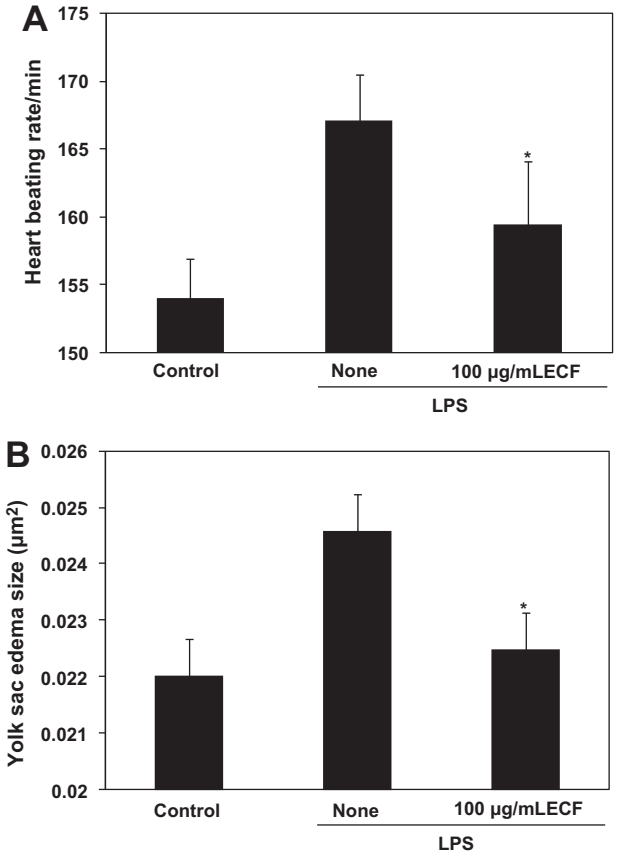

Fig. 5. Effects of ECF on LPS-stimulated the heart-beating rate (A) and yolk sac edema size (B) in zebrafish embryos. The zebrafish embryos were pretreated with ECF and exposed to LPS (5 μg/mL). The data was quantitated by image analysis. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^{*}p < 0.05$ shows significant difference from the only LPS-treated zebrafish.

suppressed NO and the expression of iNOS in murine macrophage cells (Lee et al., 2012). ECF in this study also significantly reduced the elevated NO level induced by tail-cutting and LPS-treatment in zebrafish. Although the expression level of NO synthase was not examined, ECF may therefore inhibit NO synthase in zebrafish based on previously published data. These findings indicate that ECF might confer important protection against the inflammation induced by physical and chemical damage.

3.3. *Effects of ECF on LPS-stimulated the heart-beating rate and yolk sac edema size in zebrafish*

In the zebrafish assay it revealed potential toxicity including swelling of the yolk, small head, tail bending, and a increase of heart-beating rate (Choi et al., 2007). Zebrafish embryos respond to toxicoid sensitively, allowing us to measure heart-beating rate and yolk sac edema size. As shown in Fig. 5A, the heart-beat rate in zebrafish was remarkable increased by the LPS-treatment compared with non-LPS treated zebrafish (negative control). However, the heart-beat rate in the ECF-treated zebrafish was decreased significantly. Zebrafish incubated for 48 hpf without LPS-treatment observed no yolk sac edema. The size of yolk sac edema in zebrafish was elevated by the LPS-treatment compared with negative control group. However, ECF clearly protected zebrafish against LPS-treatment (Fig. 5B). Previously, Na et al. (2009) reported that antioxidant vitamin E protects 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induced toxicity such as pericardial sac edema, yolk sac edema, and growth retardation in zebrafish embryos. In the present study, we found that ECF reduced heart-beating rate and yolk sac edema size produced by LPS exposure in zebrafish embryos. The findings suggest that ECF might confer important protection against the toxicity induced by LPS.

Overall, the above results suggest that ECF could act as strong inhibitors of ROS and NO in tail-cutting-induced and LPS-stimulated inflammatory zebrafish model. In addition, ECF has a protective effect against the toxicity induced by LPS exposure in zebrafish embryos. This outcome could explain the potential anti-inflammatory activity of ECF, which might have a beneficial effect during the treatment of inflammatory diseases.

4. Conclusion

Fucoidan extracted from *E. cava* had strong anti-inflammatory activities. However, the direct effects of fucoidan extracted from *E. cava* on anti-inflammatory activities *in vivo* model remained to be determined. Therefore, the present study we first investigated the anti-inflammatory effect of fucoidan, extracted from *E. cava*, on tail-cutting-induced and LPS-stimulated inflammation, in *in vivo* zebrafish model. Fucoidan from *E. cava* demonstrated strong anti-inflammatory properties against tail-cutting and LPS treatment-induced inflammation. The fucoidan also has a protective effect against the toxicity induced by LPS exposure in zebrafish embryos. The fucoidan from *E. cava* exhibited profound anti-inflammatory effect both *in vitro* as well as *in vivo*, suggesting that the fucoidan might be a strong anti-inflammatory agent.

Acknowledgment

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (Ministry of Education, Science and Technology).

References

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag. *Carbohydrate Research*, *337*, 719–730.

Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A. M., et al. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research*, *319*, 154–165.

Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., & McDowell, A. (1999). A study of fucoidan from the brown seaweed *Chorda filum*. *Carbohydrate Research*, *320*, 108–119.

Choi, T. Y., Kim, J. H., Ko, D. H., Kim, C. H., Hwang, J. S., Ahn, S., et al. (2007). Zebrafish as a new model for phenotype-based screening of melanogenic regulatory compounds. *Pigment Cell Research*, *20*, 120–127.

den Hertog, J. (2005). Chemical genetics: Drug screens in zebrafish. *Bioscience Reports*, *25*, 289–297.

Driever, W., Solnica-Krezel, L., & Schier, A. F. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development*, *123*, 37–46.

Eisen, J. S. (1996). Zebrafish make a big splash. *Cell*, *87*, 969–977.

Finkel, T. N., & Holbrook, J. (2000). Oxidants, oxidative stress and the biology of aging. *Nature*, *408*, 239–247.

Fishman, M. C. (1999). Zebrafish genetics: The enigma of arrival. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 10554–10556.

Frostegard, J., Ulfgren, A. K., Nyberg, P., Hedin, U., Swedenborg, J., Andersson, U., et al. (1999). Cytokine expression in advanced human atherosclerotic plaques: Dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis*, *145*, 33–43.

Handa, O., Kokura, S., Adachi, S., Takagi, T., Naito, Y., Tanigawa, T., et al. (2006). Methylparaben potentiates UV-induced damage of skin keratinocytes. *Toxicology*, *227*, 62–72.

Itoh, Y., Ma, F. H., Hoshi, H., Oka, M., Noda, K., Ukai, Y., et al. (2000). Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. *Analytical Biochemistry*, *287*, 203–209.

Karin, M., Lawrence, T., & Nizet, V. (2006). Innate immunity gone awry: Linking microbial infections to chronic inflammation and cancer. *Cell*, *124*, 823–835.

Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y., & Kim, H. P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure–activity relationships. *Biochemical Pharmacology*, *58*, 759–765.

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

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

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

Liao, Y. F., Chiou, M. C., Tsai, J. N., Wen, C. C., Wang, Y. H., Cheng, C. C., et al. (2011). Resveratrol treatment attenuates the wound-induced inflammation in zebrafish larvae through the suppression of myeloperoxidase expression. *Journal of Food and Drug Analysis*, *19*, 167–173.

Mathias, J. R., Perrin, B. J., Liu, T. X., Kanki, J., Thomas, L. A., & Huttenlocher, A. (2006). Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *Journal of Leukocyte Biology*, *80*, 1281–1288.

Na, Y. R., Seok, S. H., Back, M. W., Lee, H. Y., Kim, D. J., Park, S. H., et al. (2009). Protective effects of vitamin e against 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induced toxicity in zebrafish embryos. *Ecotoxicology and Environmental Safety*, *72*, 714–719.

Nakagawa, T., & Yokozawa, T. (2002). Direct scavenging of nitric oxide and superoxide by green tea. *Food and Chemical Toxicology*, *40*, 1745–1750.

Novoa, B., Bowman, T. V., Zon, L., & Figueras, A. (2009). LPS response and tolerance in the zebrafish (*Danio rerio*). *Fish & Shellfish Immunology*, *26*, 326–331.

Park, K. H., & Cho, K. H. (2011). A zebrafish model for the rapid evaluation of pro-oxidative and inflammatory death by lipopolysaccharide, oxidized low-density lipoproteins and glycated high-density lipoproteins. *Fish & Shellfish Immunology*, *31*, 904–910.

Partankar, M. S., Oehninger, S., Barnett, T., Williams, R. L., & Clark, G. F. (1993). A revised structure for fucoidan may explain some of its biological activities. *The Journal of Biological Chemistry*, *268*, 21770–21776.

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.

Rosenkranz, A. R., Schmaldienst, S., Stuhlmeier, K. M., Chen, W., Knapp, W., & Zlabinger, G. J. (1992). A micro plate assay for the detection of oxidative products using 2′,7′-dichlorofluorescein-diacetate. *Journal of Immunological Methods*, *156*, 39–45.

Ross, J. A., & Auger, M. J. (2002). The biology of the macrophage. In B. Burke, & C. E. Lewis (Eds.), *The macrophage* (pp. 1–72). UK: Oxford.

Trede, N. S., Zapata, A., & Zon, L. I. (2001). Fishing for lymphoid genes. *Trends in Immunology*, *22*, 302–307.

Veerman, E. C., Nazmi, K., Hof, W. V., Bolscher, J. G., Hertog, A. L. D., & Amerongen, A. V. N. (2004). Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5. *Biochemical Journal*, *381*, 447–452.

Vieira, R. P., & Mourao, P. A. (1988). Occurrence of a unique fucose-branched chondroitin sulfate in the body wall of a sea cucumber. *The Journal of Biological Chemistry*, *263*, 18176–18183.