Research Article

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Polyphenol-rich fraction from *Ecklonia cava* (a brown alga) processing by-product reduces LPS-induced inflammation *in vitro* and *in vivo* in a zebrafish model

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Ecklonia cava is a common edible brown algae that is plentiful in Jeju Island of Republic of Korea. Polyphenols from *E. cava* have strong anti-inflammatory activity. However, a large number of the by-products from *E. cava* processing are discarded. In the present study, to utilize these by-products, we assessed the anti-inflammatory activity of the polyphenol-rich fraction (PRF) from *E. cava* processing by-product (EPB) in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells. Four compounds, namely eckol, eckstolonol, dieckol, and phlorofucofuroeckol-A, were isolated and identified from PRF. We found that PRF suppressed the production of nitric oxide (NO), inducible nitric oxide synthase, and cyclooxygenase-2 in the LPS-induced cells. Furthermore, the protective effect of PRF was investigated *in vivo* in LPS-stimulated inflammation zebrafish model. PRF had a protective effect against LPS-stimulated toxicity in zebrafish embryos. In addition, PRF inhibited LPS-stimulated reactive oxygen species and NO generation. According to the results, PRF isolated from EPB could be used as a beneficial anti-inflammatory agent, instead of discard.

Key Words: anti-inflammation; by-product; *Ecklonia cava*; polyphenol; seaweeds

INTRODUCTION

Seaweeds are rich in vitamins, minerals, proteins, polyphenols, and polysaccharides. Therefore, several species of seaweeds are used in cosmetics, food, and pharmaceuticals. Many studies have shown that seaweeds have diverse bioactivities such as antioxidant, anticoagulant, anti-inflammation, antibacterial, and anticancer activities (Heo et al. 2003, Shin et al. 2006, Kim et al. 2007, Erbert et al. 2012, Ko et al. 2012, Yoon et al. 2013). In particular, *Ecklonia cava* is a common edible brown algae that is plentiful in Jeju Island of Republic of Korea. *E. cava* has been identified to contain various natural substances such as carotenoids, fucoidans, and phlorotannins, which have beneficial biological activities including antioxidant, anti-inflammation, anticoagulant, antiviral, and antidiabetic effects (Kang et al. 2010, 2012*b*, Lee et al. 2011, 2013*a*, Kwon et al. 2013). Dried forms of *E. cava* are consumed, but steam heated *E. cava* products sometimes are manufactured. When steaming the alga, a large amount of by-product is produced and discarded without utilization. Some studies have suggested that fermenting

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E. cava processing by-product (EPB) has both antioxidant and anti-inflammation effects and rich in polyphenols (Wijesinghe et al. 2012, 2013). However, the anti-inflammatory effects of EPB have not yet been assessed.

Inflammation is a normal host defense response by the organism to remove the tissue injury caused by pathogens, damaged cells, or irritants, and to initiate the healing process (Dewanjee et al. 2013). The inflammatory process is mediated by inflammatory cells, such as macrophages, neutrophils, eosinophils, and mononuclear phagocytes. Macrophages play an important role in a variety of disease processes, and are involved in the pathogenesis of autoimmune diseases, infection, and inflammatory disorders (Choi et al. 2012). And macrophages secrete inflammatory mediators such as reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin mediators, which are generated by activated inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-6) in response to an activating stimulus, such as, the bacterial endotoxin, lipopolysaccharide (LPS) (Kim et al. 2013, Ma et al. 2013).

The vertebrate zebrafish (*Danio rerio*) is a small tropical freshwater fish that has emerged as a useful vertebrate model organism owing to its small size, large clutches, transparency, low cost, and physiological similarity to mammals (Leite et al. 2012, Yang et al. 2012). Because of these advantages of zebrafish as an *in vivo* model, zebrafish is widely used in molecular genetics and developmental biology studies (Pogoda and Hammerschmidt 2007, Meunier 2012). Furthermore, zebrafish have recently been used in toxicology and drug discovery studies investigating oxidative stress and inflammation, because the embryos and juveniles are transparent. This property allows the visualization of specific cells, tissues, and organs under the microscope (Park and Cho 2011, Zhang et al. 2013, Kang et al. 2014*b*, Ko et al. 2014). In this study, therefore, the polyphenol-rich fraction (PRF) was obtained from EPB and was examined for anti-inflammatory activities *in vitro* RAW264.7 cells and *in vivo* zebrafish model.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Gibco/BRL (Burlington, ON, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2,7-dichlorofluorescein diacetate (DCF-DA), diaminofluorophore 4-amino-5-methylamino-2′7′-difluorofluorescein diacetate (DAF-FM DA), acridine orange were purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade solvents were purchased from Burdick & Jackson (Muskegon, MI, USA). The other chemicals and reagents used were of analytical grade.

Preparation of PRF from EPB

EPB was kindly provided by Taerim Co., Ltd. (Jeju, Korea) and then was freeze-dried. The dried EPB powder (5 g) was suspended in 500 mL of 80% ethanol and mechanically stirred for 24 h at room temperature. The solution was filtered and the filtrate was concentrated under reduced pressure to give the oily extract. After the extract was suspended in 200 mL of distilled water, ethyl acetate fraction was obtained with 0.06 g as a yield and the obtained ethyl acetate fraction was used for the further experiments. Total polyphenol content was quantified by Folin-Ciocalteu methods.

HPLC and HPLC-diode array detection / electrospray ionization mass spectrometry (DAD-ESI / MS) analysis PRF from EPB

HPLC and HPLC-DAD-ESI / MS analyses were performed using the protocol described by Lee et al. (2014). For processing HPLC, the mobile phase comprised of acetonitirile-water in gradient mode as follows: acetonitrile with 0.1% formic acid-water with 0.1% formic acid (0-40 min: 10 : 90 → 40 : 60, v/v; 40-50 min: 50 : 50, v/v; 50-60 min: 100 : 0, v/v). The flow rate was 0.2 mL min^{-1} and the UV absorbance was detected at 290 nm. HPLC-DAD-ESI / MS analyses were carried out using a Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump, a DAD detector, and a degasser (Hewlett-Packard, Waldbronn, Germany) coupled to a Finningan electrospray source and was capable of analyzing ions up to m/z 2000. MS was controlled by Xcalibur software (Finnigan MAT, San Jose, CA, USA).

Cell culture

Murine macrophage cell line RAW264.7 was obtained from the Korean Cell Line Bank (KCLB). The cells were maintained at 37°C in humidified atmosphere of 5% CO_2

in DMEM medium supplemented with 10% FBS and penicillin/streptomycin (100 U mL^{-1} and 100 μg mL^{-1}, respectively; Gibco Inc., Grand Island, NY, USA). Exponential phase cells were used throughout the experiments.

Effects of EPB and PRF on LPS-induced NO production and cytotoxicity

To evaluate the effect of EPB and PRF on NO production and cytotoxicity, RAW264.7 cells were stimulated with LPS (1 μg mL^{-1}). NO production was detected using the protocol described by Samaroakoon et al. (2013). Furthermore, cytotoxicity was assessed using the protocol described by Kim et al. (2013).

Western blot analysis of iNOS and COX-2 in RAW264.7 macrophages

Western blot analysis was carried out using the protocol described by Kang et al. (2011). iNOS and COX-2 expression were used specific primary rabbit polyclonal anti-rabbit iNOS Ab (1 : 1,000, Cell Signaling Technology, Danvers, MA, USA), or rabbit polyclonal anti-rabbit COX-2 Ab (1 : 1,000, Cell Signaling Technology) and goat anti-rabbit IgG HRP conjugated secondary antibody (1 : 3,000) in TBST.

Origin and maintenance of zebrafish

Adult zebrafish were obtained from a commercial dealer (Green Fish, Seoul, Korea; Stock obtained from Singapore fish dealer) and 10 fish were kept in a 3-L acrylic tank under the following conditions: 28.5°C, with a 14 : 10 h light : dark cycle. Fish were fed three times a day, 6 days a week, with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 min.

Preparation of inflammation-induced zebrafish model by LPS-treatment and application of PRF

From approximately 7-9 h post-fertilization (7-9 hpf), embryos (groups = 15 embryos) were transferred to individual wells of a 24-well plate and maintained in embryo medium containing 1 mL of PRF (25, 50, and 100 μg mL^{-1}) for 1 h. Then embryos were treated with 10 μg mL^{-1} LPS or co-treated with LPS and PRF for up to 24 hpf.

Estimation of LPS-stimulated intracellular ROS and NO production as well as cell death in zebrafish embryos and image analysis

Generation of ROS and NO in zebrafish embryos was analyzed by using the method described by Lee et al. (2013*b*). Production of intracellular ROS in zebrafish embryos were detected using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorofluorescein diacetate (DCF-DA) and 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).Cell death was detected in live embryos using acridine orange staining, a nucleic acid selective metachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attractions. Acridine orange staining was performed by using the method described by Kim et al. (2014) with modification. At 7-9 hpf, the embryos were treated with 25, 50, and 100 μg mL^{-1} PRF and 1 h later, 10 μg mL^{-1} LPS was added to the plate. At 3 day post-fertilization (dpf), the embryos were transferred into 24-well plate and treated with DCF-DA, DAF-FM DA, or acridine orange. Then, the plates were incubated for individual reaction times in the dark at 28.5°C. After incubation, the embryos were rinsed in fresh embryo media and anesthetized before visualization. The images of stained embryos were observed using a fluorescent microscope, which was equipped with Moticam color digital camera (Motix, Xiamen, China) and the fluorescence intensity of individual zebrafish embryos was quantified using the image J program.

Statistical analysis

The data are expressed as the mean ± standard error (SE), and one-way ANOVA test (using SPSS ver. 12 statistical software; SPSS Inc., Chicago, IL, USA) was used to compare the mean values of each treatment. Values with different alphabets are significantly different at $p < 0.05$ as analyzed by Duncan's multiple range test.

RESULTS

Polyphenols of the ethyl acetate fraction from EPB

Total polyphenol content of the ethyl acetate fraction from EPB was 55.81 ± 4.15%. Therefore, this ethyl acetate fraction was named the PRF. The PRF was analyzed using HPLC and HPLC-DAD-ESI / MS analyses to identify algal

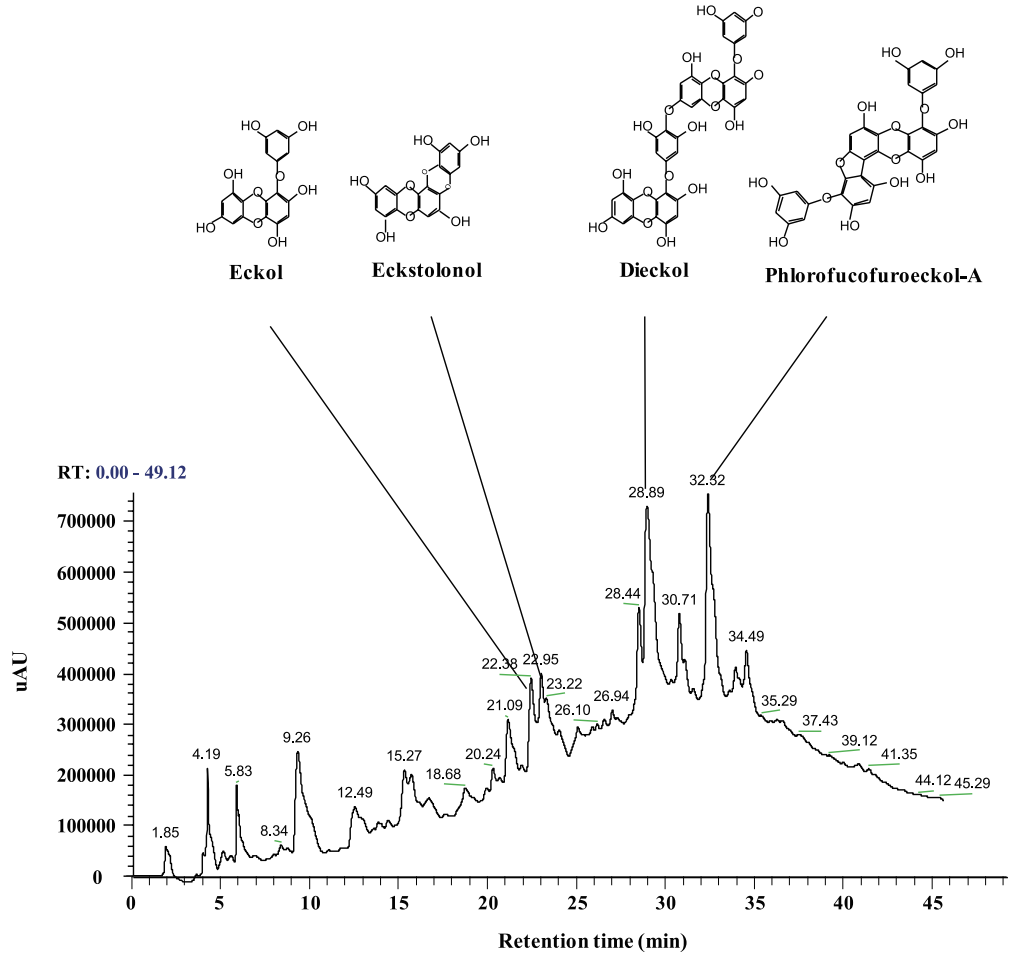

Fig. 1. High-performance liquid chromatography (HPLC) chromatogram and HPLC-diode array detection / electrospray ionization mass spectrometryspectra of ethyl acetate fraction from *Ecklonia cava* processing by-product.

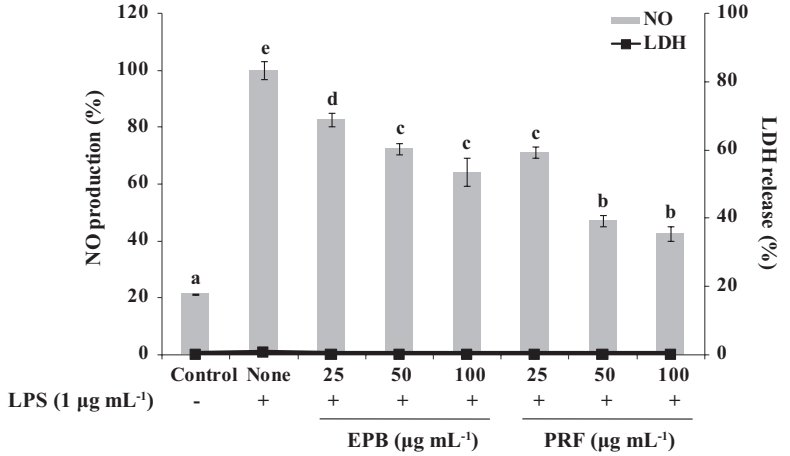

Fig. 2. Nitric oxide (NO) production and cytotoxicity by *Ecklonia cava* processing by-product (EPB) and polyphenol-rich fraction (PRF) on lipopolysaccharide (LPS)-induced RAW264.7 cells. The production of nitric oxide was assayed in the culture medium of cells incubated with LPS (1 μg mL^{-1}) for after 24 h in the presence of EPB and PRF (25, 50, and 100 μg mL^{-1}). Cytotoxicity was determined by lactate dehydrogenase (LDH) assay. ■, % NO production; □, cytotoxicity. Experiments were performed in triplicate and the data are expressed as the mean ± standard error. $^{a-e}$Values with different alphabets are significantly different $p < 0.05$ as analyzed by Duncan's multiple range test.

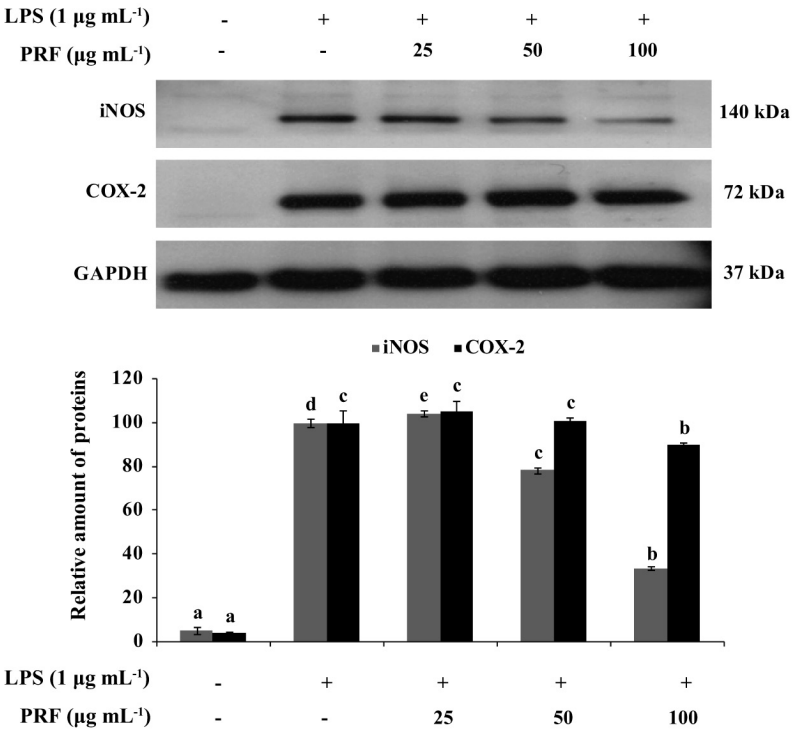

Fig. 3. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression by polyphenol-rich fraction (PRF) in RAW264.7 cells. Cells (1×10^5 cells mL^{-1}) were pre-incubated for 16 h, and the cells were stimulated with lipopolysaccharide (LPS; 1 μg mL^{-1}) in the presence of PRF (25, 50, and 100 μg mL^{-1}) for 24 h. iNOS and COX-2 protein level were determined using a western blot analysis. Equal protein loading was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Experiments were performed in triplicate and the data are expressed as the mean ± standard error. [a-e]Values with different alphabets are significantly different $p < 0.05$ as analyzed by Duncan's multiple range test.

polyphenols (Fig. 1). The four polyphenol compounds, namely eckol, eckstolonol, dieckol, and phlorofucofuroeckol-A were confirmed by comparing their HPLC-DAD-ESI / MS analyses. Thus, this ethyl acetate fraction was used in further experiments.

Inhibition of NO production by EPB and PRF on LPS-induced RAW264.7 cells

To examine the potential anti-inflammatory activities of EPB or PRF in LPS-induced RAW264.7 macrophages, we investigated the inhibitory effect of EPB or PRF on NO production. Cells were treated with or without EPB or PRF (25, 50, and 100 μg mL^{-1}) for 1 h, and then treated with LPS (1 μg mL^{-1}) for 16 h. As shown in Fig. 2, the level of NO production is significantly increased in the LPS-treated cells compared with the untreated cells. However, the NO production was significantly reduced in the cells pretreated with EPB or PRF. In particular, PRF suppressed NO production more than EPB. In addition, as confirmed by the lactate dehydrogenase (LDH) activity using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA), EPB or PRF both showed non-cytotoxic effect on RAW264.7 cells at the tested concentrations (Fig. 2). Thus, PRF may be a used as a potential agent for suppressing NO production without affecting cell viability.

Inhibition of iNOS and COX-2 protein expression by PRF on LPS-induced RAW264.7 cells

iNOS and COX-2 are major inflammatory mediators. To determine the mechanism by which PRF reduces LPS-induced NO production, we investigated the ability of PRF (25, 50, and 100 μg mL^{-1}) to influence the LPS-induced production of iNOS and COX-2. Fig. 3 shows the effect of PRF on iNOS and COX-2 protein expression in RAW264.7 cells by western blot analysis. The iNOS and COX-2 protein expressions were significantly increased in LPS-treated macrophages (1 μg mL^{-1}). However, PRF significantly suppressed the protein expression of iNOS in a concentration-dependent manner and slightly inhibited COX-2 expression at 100 μg mL^{-1}.

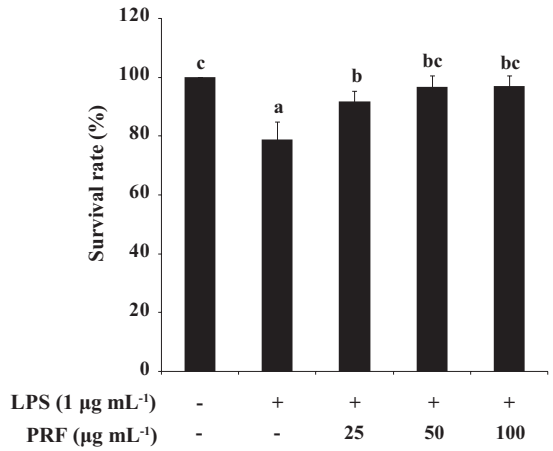

Fig. 4. Toxicity of polyphenol-rich fraction (PRF) or lipopolysaccharide (LPS) in zebrafish embryos. Survival rates were assessed after treatment with LPS or co-treatment with PRF. The embryos were stimulated with 10 μg mL^{-1} LPS and co-treated with PRF (25, 50, and 100 μg mL^{-1}). Experiments were performed in triplicate and the data are expressed as the mean ± standard error. $^{a-c}$Values with different alphabets are significantly different $p < 0.05$ as analyzed by Duncan's multiple range test.

Toxicity of PRF and LPS in zebrafish embryos

To assess the toxicity of LPS (10 μg mL^{-1}) with or without PRF, we determined the survival rates of zebrafish embryos. The survival rates of the positive control group (treated with LPS) were 78.75%, and then the survival rates of the PRF-treated embryos were significantly increased to 91.77, 96.67, and 96.88% at concentrations of 25, 50, and 100 μg mL^{-1}, respectively (Fig. 4).

Protective effect of PRF against LPS-induced ROS and NO production as well as cell death in zebrafish embryos

Production of intracellular ROS can be detected using the oxidation sensitive fluorescent dye, DCF-DA, emits fluorescece upon interaction with ROS (Handa et al. 2006). In the present study, therefore, the production of ROS in the LPS-induced inflammatory zebrafish model was analyzed using DCF-DA. Fig. 5A illustrates the ROS levels in zebrafish with or without LPS and / or PRF. These data show that, the ROS level in LPS-stimulated zebrafish increased to 152.22% compared with the negative control group. However, the ROS productions in zebrafish treated with different concentrations of PRF (25, 50, and 100 μg mL^{-1}) were reduced, and a significant reduction was observed at 100 μg mL^{-1}. We assessed the inhibitory activity of PRF on LPS-stimulated NO production in zebrafish 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, Bölck et al. 2014). The effect of PRF on LPS-stimulated NO production is shown in Fig. 5B. The level of NO production in the positive group (only LPS treated) reached to 129.39% of that observed in controls. However, zebrafish treated with 100 μg mL^{-1} PRF had significantly reduced NO levels.

Acridine orange is a nucleic acid selective methachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attraction. Acridine orange stains cells with disturbed plasma membrane permeability, and can be used to identify necrotic or very late apoptotic cells. Fig. 5C shows that there is a high level of cell death in LPS-treated zebrafish. However, PRF significantly reduced the cell death in a concentration-dependent manner, although this effect was not observed the lowest concentration (25 μg mL^{-1}).

DISCUSSION

In this study, we demonstrated the anti-inflammatory activity of PRF from EPB in LPS-induced RAW264.7 cells. Furthermore, we show that PRF reduced the pro-inflammatory mediators such as NO, iNOS, and COX-2.

Activated RAW264.7 macrophages produce NO, which plays a crucial role in physiological functions. However, overproduction of NO by iNOS can lead to cytotoxicity, inflammation, and the development of autoimmune disorders (Liu and Hotchkiss 1995, Shin et al. 2008). COX-2 is an inflammatory mediator involved in NO production (Kumar et al. 2012, Kim et al. 2013). In the present study, therefore, we investigated the inhibitory activity of PRF on NO production in RAW264.7 cells via the suppression of iNOS and COX-2 expression. We found that PRF inhibits the NO production and significantly suppresses iNOS expression, compared to COX-2 expression in LPS-induced RAW264.7 cells. These results suggest that PRF regulates the NO production via iNOS rather than COX-2.

Many studies have shown that polyphenols from *E. cava* have antioxidant and anti-inflammation effects in mice and zebrafish model (Kang et al. 2012*a*, 2013, 2014*a*, Wijesinghe et al. 2014). However, EPB has not yet been studied for its commercial utilization. In this study, therefore, we first investigated the *in vivo* anti-inflammatory activity of PRF from EPB using the zebrafish model. Recently, zebrafish model have been used to rapidly and

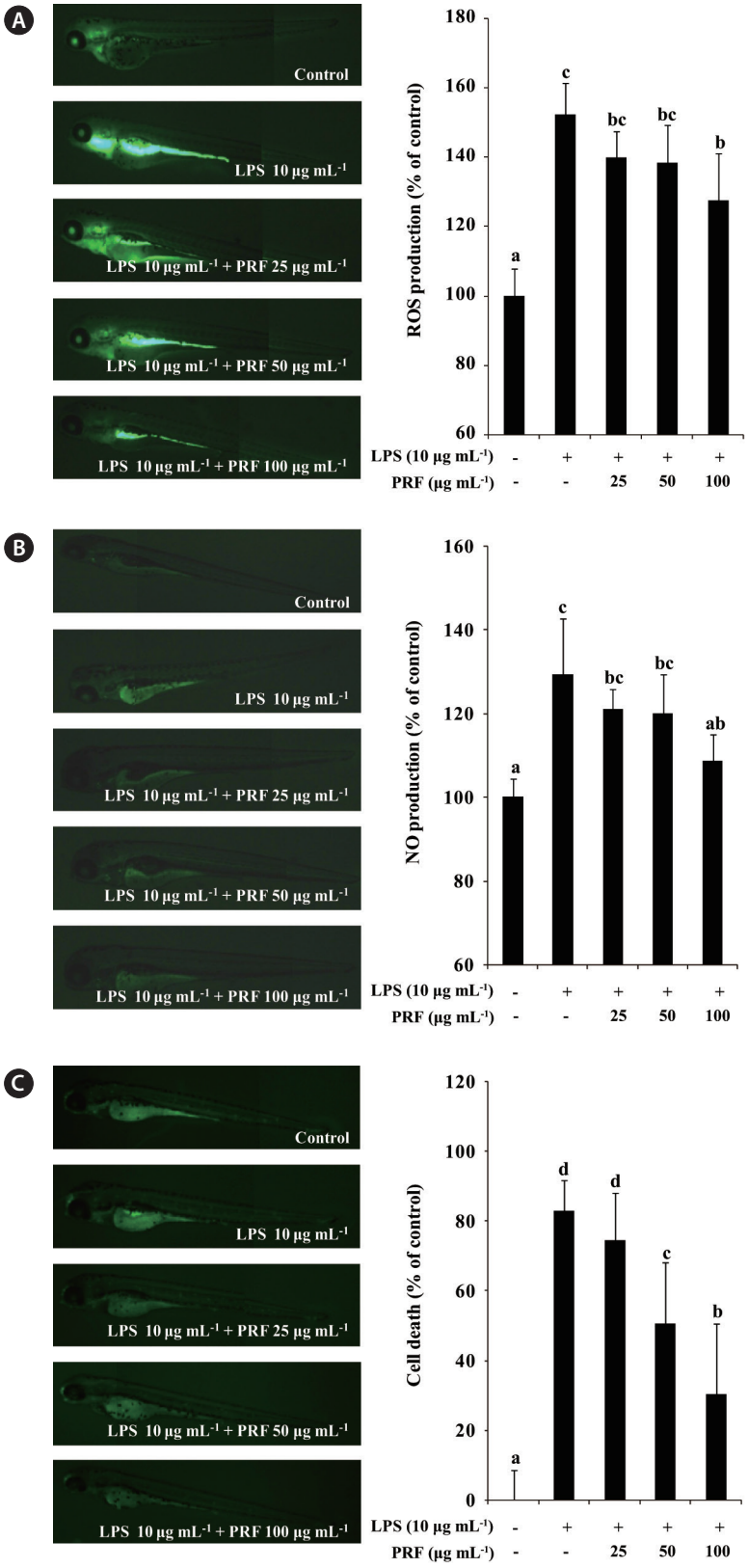

Fig. 5. Reactive oxygen species (ROS) and nitric oxide (NO) production as well as cell death in zebrafish. The zebrafish embryos were exposed to lipopolysaccharide (LPS) (10 μg mL^{-1}) and treated with polyphenol-rich fraction (PRF; 25, 50, and 100 μg mL^{-1}). (A) ROS level. (B) NO level. (C) Cell death. Assessments were measured by image analysis and fluorescence microscope. The fluorescence intensity of individual zebrafish was quantified using image J program. Experiments were performed in triplicate and the data are expressed as the mean ± standard error. $^{a-d}$Values with different alphabets are significantly different $p < 0.05$ as analyzed by Duncan's multiple range test.

simply assess anti-inflammatory activity on tail-cutting-induced, and LPS-stimulated inflammation *in vivo* (Park and Cho 2011, Lee et al. 2013*b*). Furthermore, an inflammatory mechanism has been associated with ROS generation in macrophages and neutrophils (Conforti et al. 2008). Increased ROS generation can damage biological molecules and lead to cells or tissue injury (Rezaie et al. 2007, Ziegler et al. 2011) as well as induces inflammation by increasing the level of cytokines such as IL-1β, IL-6, and TNF-α (Geronikaki and Gavalas 2006). Furthermore, antioxidant polyphenol compounds inhibit nuclear facor-κB (NF-κB) activation and block the expression of NF-κB-dependent cytokines, iNOS, and COX-2 genes (Ahn et al. 2005, Su et al. 2011). Based on these studies, ROS and NO generation as well as cell death were assessed in LPS-induced inflammation zebrafish model. In the present study, ROS and NO production in zebrafish were significantly increased with the LPS treatment. However, PRF-treatment significantly decreased the LPS-induced inflammation in the zebrafish. Cell death was also significantly reduced in PRF treated zebrafish.

In summary, our results demonstrate that PRF inhibited the production of NO, and the expression of iNOS and COX-2 in LPS-induced RAW264.7 cells. Moreover, PRF suppressed ROS and NO generation as well as cell death in LPS-stimulated inflammation zebrafish model. Therefore, we suggest that EPB discarded during seaweed processing should be utilized as potential anti-inflammatory agents for the treatment of inflammatory diseases.

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