



## Protective effects of extracts from six local strains of *Pyropia yezoensis* against oxidative damage *in vitro* and in zebrafish model

Yu-Lin Dai<sup>1,2</sup>, Gwang Hoon Kim<sup>3</sup>, Min-Cheol Kang<sup>4</sup> and You-Jin Jeon<sup>2,5,\*</sup>

<sup>1</sup>Jilin Ginseng Academy, Changchun University of Chinese Medicine, Changchun 130117, China

<sup>2</sup>Department of Marine Life Science, Jeju National University, Jeju 63243, Korea

<sup>3</sup>Department of Biology, Kongju National University, Kongju 32588, Korea

<sup>4</sup>Research group of Food Processing, Research Division of Strategic Food Technology, Korea Food Research Institute (KFRI), Wanju 55365, Korea

<sup>5</sup>Marine Science Institute, Jeju National University, Jeju 63333, Korea

*Pyropia yezoensis* has been used as functional food in East Asia, especially in Korea and Japan, for more than five hundred years. This study aims to evaluate the antioxidant effect of polyphenols and proteins-rich extracts from *P. yezoensis* (PPPs) against 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative cell damage. Among six Korean local strains obtained from Jinhae (JiH), Haenam (HN), Jangheung (JaH), Jindo (JD), Wando (WD), and Sinan (SA) areas, the extracts of *P. yezoensis* from SA and JD are relatively higher in polyphenols and proteins contents. SA showed the lowest IC<sub>50</sub> scavenging activities against 1,1-diphenyl-2-picryl-hydrazyl and alkyl radicals and displayed protective effects against reactive oxygen species (ROS) in AAPH-induced Vero cells. Especially, the PPPs extracts from SA and JD showed protective activities against AAPH-induced apoptosis, as observed by nuclear staining with Hoechst 33342. Furthermore, *in vivo* studies of the SA extract in zebrafish showed significantly reduced ROS generation, lipid peroxidation, and cell damage. This is the first study, to our knowledge, to evaluate the antioxidant bioactivity of PPP in the Korean Peninsula using a zebrafish model. Due to SA and JD both located in the west coast of Korea, we deduced that the chemical content of the different PPP extracts was mildly influenced by their geographic location, and this alga has potential of protective activity against AAPH-induced ROS both *in vitro* and *in vivo*.

**Key Words:** antioxidant effect; Gim; polyphenols; proteins; *Pyropia yezoensis*; zebrafish

**Abbreviations:** AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; DPPP, diphenyl-1-pyrenylphosphine; FBS, fetal bovine serum; HN, Haenam; JaH, Jangheung; JD, Jindo; JiH, Jinhae; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PPP, polyphenols and proteins-rich extracts from *P. yezoensis*; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute medium; SA, Sinan; WD, Wando



This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received October 30, 2019, Accepted May 14, 2020

\*Corresponding Author

E-mail: youjinj@jejunu.ac.kr

Tel: +82-64-754-3475, Fax: +82-64-756-3493

## INTRODUCTION

Environmental stresses such as chemical agent and ultraviolet rays can disrupt the homeostasis between the antioxidant systems which plays an essential role in maintaining cellular balance (Sgherri et al. 2017). Reactive oxygen species (ROS) contain oxygen ions and peroxides and are key factors in antioxidant system (Choudhury et al. 2017). Excessed ROS production leads to the generation of superoxide anion radicals and hydrogen peroxide, and may underlie the pathology of diseases in human (Seifried et al. 2007). It has been proven that diseases such as inflammation and cancer often result from the oxidative damage of lipids, proteins, and even nucleic acids, leading to several physiological consequences (Diebold and Chandel 2016).

Over the last decades, polyphenols and proteins are reported to be effective nature antioxidants (Köksal et al. 2017, Ding et al. 2019). Polyphenols play as an antioxidant in human body; one of the main mechanisms of antioxidative activity in polyphenols is the H atom transfer, the single electron transfer and the metals chelation (Leopoldini et al. 2011). The mechanisms include the following two actions, one is non-specific actions based on chemical features which are more common to most polyphenols. Another one is specific mechanism based on particular structural and conformational characteristics which were identified by selected polyphenols (Fraga et al. 2010). This mechanism is associated with interactions of iron (Perron and Brumaghim 2009).

Brown algae are abundant in polyphenols while green and red algae have less. Bioactivities of *Pyropia yezoensis* are mostly related to proteins or peptides (Cian et al. 2015). In the study, the polyphenol contents of *P. yezoensis* were investigated in association with protein contents for antioxidant effects. In particular, polyphenols and proteins from marine resources have increased free radical scavenging activity and have been shown to inhibit the effects of lipid peroxidation (Cian et al. 2013). Compared to the synthetic drug for antioxidant, marine product becomes more flexible to obtain (Hamann 2007, Dai et al. 2020b). Thus, the exploration and screening the marine antioxidants is necessary to investigate.

*P. yezoensis* (Gim in Korean) is a popular seafood that is traditionally used as a marine vegetable along the East Asian coast (Kim et al. 2014b). It has been cultivated abundantly in Korea and is used to roll rice and other ingredients constituting the traditional Korean dish, gim-bap (Aoki and Kamei 2006). *Pyropia* species are rich in different nutrients, including polysaccharides, polyphe-

nols, sterols, glycoprotein, and lipids. They have several beneficial effects, such as antioxidant, anticancer, anti-inflammatory, and anti-fatigue activity and protective effects against ultraviolet rays-induced aging (Kwon and Nam 2006, Ryu et al. 2014, Lee et al. 2015, Choi et al. 2016, Hwang et al. 2018, Kim et al. 2018c). The antioxidant property of the acetone / water extract of *Porphyra columbina* was evaluated, and its polyphenol and peptide content showed high angiotensin-converting-enzyme inhibitory activity (Cian et al. 2013). We hypothesized that the polyphenols and protein-rich extracts from *P. yezoensis* (PPP) might exert antioxidant properties. In this study, we evaluate the protective properties of PPP against 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced lipid peroxidation in Vero cells and a zebrafish model, and find the best local strain of Korean from the six major locations for Gim aquaculture in Korea.

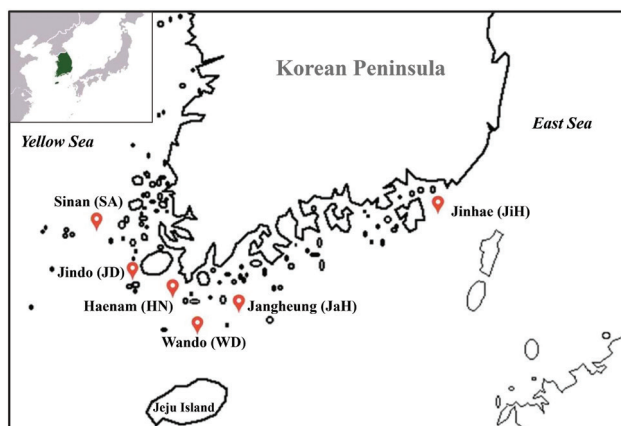
## MATERIALS AND METHODS

### Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), diphenyl-1-pyrenylphosphine (DPPP), dimethyl sulfoxide (DMSO), and Hoechst 33342 were obtained from commercial company (Sigma-Aldrich, St. Louis, MO, USA). All solvents used were of analytical grade.

### Preparation of *Pyropia yezoensis* extracts

From 2015 to 2016, samples of *P. yezoensis* were collected from six areas along the south coast of the Korean Peninsula where major aquaculture farms are located; from Jinhae (JiH), Haenam (HN), Jangheung (JaH), Jindo (JD), Wando (WD), and Sinan (SA) (Fig. 1). Extracts containing the polyphenol- and protein-rich fraction from *P. yezoensis* followed the previous report (Kim et al. 2014b). Samples were cleaned with tap water to remove organic debris, salt, sand, and epiphytes and freeze-dried, maintained at -20°C. The sample of *P. yezoensis* from each area was ground to a powder; 3 g of each powder was shaken in 100 mL of 70% ethanol at 40°C for 24 h. The liquid layer was obtained via filtration, and then the filtrate was concentrated using an evaporator under vacuum pressure at 37°C. The extract after lyophilization was regarded as PPP. PPP was dissolved in DMSO, and then used for further experiments after adjusting the final concentration



**Fig. 1.** Map of the distribution of the six *Pyropia yezoensis*-producing areas in South Korea.

of DMSO in culture medium to <0.1%.

## Chemical analysis

The approximate composition of the six samples from the different areas was evaluated according to the guidelines of the Association of Official Analytical Chemists methods. Therefore, the Kjeldahl method was applied to analyze the protein content, the Soxhlet method was applied to analyze the lipid content, and the muffle furnace (600°C for 6 h) was employed to analyze the ash content (Horwitz 1975). The determination of total polysaccharide and total polyphenol content were analyzed according to colorimetric standard methods (Dubois et al. 1956, Chandler and Dodds 1983).

## Evaluation of antioxidant activities

The antioxidant activity of the extract was analyzed as a measurement of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and alkyl free radical scavenging activities. The electron spin resonance spectrometer (JESFA200; Jeol, Tokyo, Japan) at 298 K was used for analysis. The DPPH and alkyl radical scavenging activities were analyzed according to the protocols followed by Nanjo and Hiramoto, respectively (Hiramoto et al. 1993, Nanjo et al. 1996).

## Cell culture

Vero monkey kidney epithelial cell lines were purchased from Korean Cell Line Bank, Seoul, Korea. Roswell Park Memorial Institute medium (RPMI) and fetal bovine serum (FBS) were purchased from commercial

company (Gibco Inc., Grand Island, NY, USA). Vero cells maintained at 37°C in a 5% CO<sub>2</sub> incubator and cultured in RPMI medium supplemented with 10% (*v/v*) FBS, penicillin, and streptomycin (100 µg mL<sup>-1</sup> each).

## Antioxidant activity against AAPH-induced cell damage

MTT assay was employed to analyze the cell survival rate (%) after AAPH-induced cell death. AAPH is a promising water-soluble chemical for generate ROS (Mosmann 1983). The third passage Vero cells were seeded at a density of 1 × 10<sup>5</sup> cells mL<sup>-1</sup> in 96-well culture plates. After seeding, the cells were treated with different concentrations (12.5, 25, and 50 µg mL<sup>-1</sup>) of PPPs. After 1 h incubation, cells were treated with AAPH (10 mM) for 24 h; untreated cells were used as control. The cell survival rate was evaluated using the MTT assay 24 h after sample treatment.

## Nuclear staining with Hoechst 33342

To identify the protective effect of PPP on the AAPH-induced nuclear morphological changes in Vero cells, the Hoechst 33342 staining protocol was applied (Dai et al. 2019). Vero cells were seeded at a density of 1 × 10<sup>5</sup> cells mL<sup>-1</sup> in 24-well culture plates. Cells were treated with different concentrations of PPPs from SA and JD for 1 h and spiked with 10 mM AAPH solution for 24 h. Then, Hoechst 33342 (stock, 10 mg mL<sup>-1</sup>) was added to each well at a final concentration of 10 µg mL<sup>-1</sup> after 24-h incubation. Cells stained with 25 µL of Hoechst 33342 for 10 min and imaged using a CoolSNAP-Pro color digital fluorescent microscope (Olympus, Tokyo, Japan).

## Origin and maintenance of parental zebrafish

Adult zebrafish were obtained from a commercial dealer (Jeju, Korea) and housed in a 3 L acrylic tank at 28.5°C with a 14 : 10-h light : dark cycle. The fish were fed with live brine shrimp three times daily. Embryos were collected by natural spawning, and the collection time of the embryos was controlled less to than 30 min.

## Waterborne exposure of embryos to PPP and AAPH

The embryos (n = 15) were randomly transferred to each well of 24-well plates and grown in embryo medium between 7 to 9 h post-fertilization (hpf). Then, different

concentrations (12.5, 25, and 50  $\mu\text{g mL}^{-1}$ ) of PPP from SA were added to each well. After incubation for 1 h, 25  $\mu\text{L}$  of 15 mM AAPH solution was added to the wells up to 24 hpf. The embryo medium was substituted with fresh medium.

### Measurement of AAPH-induced intracellular ROS generation and image analysis

DCFH-DA, an oxidation-sensitive fluorescent probe dye, was used to determine the generation of ROS in embryos. At 7-9 hpf, the embryos were treated with different concentrations (12.5, 25, and 50  $\mu\text{g mL}^{-1}$ ) of PPP and incubated. One hour later, 25  $\mu\text{L}$  of 15 mM AAPH solution was added to the plate. After 24 h, the embryo medium was substituted with fresh medium. The embryos were transferred into a 96-well plate, which was subsequently spiked with DCFH-DA solution (20  $\mu\text{g mL}^{-1}$ ) and incubated in the dark at 28.5°C for 1 h. Then, the embryo medium was rinsed off, and embryos were anesthetized before observation. A spectrofluorometer (Perkin-Elmer LS-5B, Vienna, Austria) was used to calculate the fluorescence intensity of each embryo, and the CoolSNAP-Pro color digital fluorescent microscope (Olympus) was used to quantify and image the stained embryos (Dai et al. 2020a).

### Measurement of oxidative stress-induced cell death in zebrafish embryos

Acridine orange preferentially stains necrotic or late apoptotic cells because acridine orange can disturb plasma membrane permeability. At 4 hpf, the embryos were exposed to different concentrations (12.5, 25, and 50  $\mu\text{g mL}^{-1}$ ) of PPP from SA. One hour later, 25  $\mu\text{L}$  of 15 mM AAPH solution was added to each plate. The em-

bryos were maintained until 2 dpf, and the medium was replaced with fresh medium every 24 h. The treated embryos were transferred into a 96-well plate, and acridine orange stain was added at a concentration of 7  $\mu\text{g mL}^{-1}$ ; the plate was incubated for 30 min in the dark at 28.5°C. Before visualization, the embryo medium was replaced with fresh medium and the embryos were anesthetized. The fluorescence intensity and images of embryo were quantified and observed, followed by the protocol of “measurement of AAPH-induced intracellular ROS generation and image analysis.”

### Statistical analysis

All assays involved three independent experiments. Values are expressed as the mean  $\pm$  standard error (SE), and one-way ANOVA (SPSS statistical software; IBM Corp., Armonk, NY, USA) was used to analyze the mean values. Student's t-test was used for the analysis of the means of parameters showing significant difference.

## RESULTS

### Chemical composition of PPP obtained from six locations

*P. yezoensis* samples were collected from six different locations across South Korea, and their chemical compositions were determined. As shown in Table 1, the extract from samples obtained in WD showed the highest ash content, followed by that from JD. The highest polyphenol and carbohydrate content were observed in the samples from SA and JD. The samples from SA and JD had the highest total sterol content, followed by the others. The highest protein content was observed in samples from

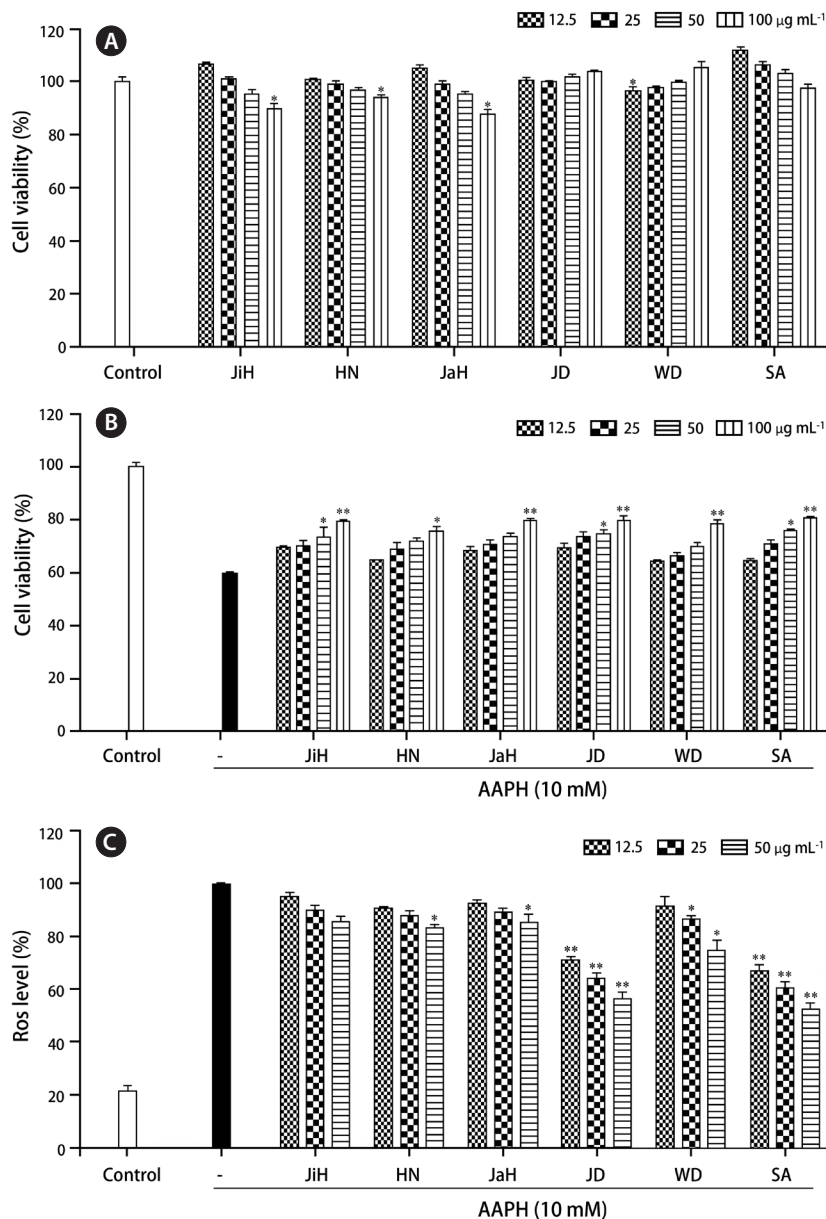
**Table 1.** Proximate composition of the polyphenols and protein-rich fraction from six samples of *Pyropia yezoensis* obtained from different locations

Content (%)	JiH	HN	JaH	JD	WD	SA
Yield	14.80 $\pm$ 0.20	11.80 $\pm$ 0.20	10.20 $\pm$ 0.10	20.00 $\pm$ 0.20	16.20 $\pm$ 0.30	14.70 $\pm$ 0.20
Polyphenol	1.31 $\pm$ 0.21	2.76 $\pm$ 0.31	2.22 $\pm$ 0.10	4.92 $\pm$ 0.10	2.31 $\pm$ 0.31	6.31 $\pm$ 0.21
Carbohydrate	26.31 $\pm$ 2.93	24.40 $\pm$ 3.92	23.28 $\pm$ 2.88	28.22 $\pm$ 2.92	28.15 $\pm$ 2.97	29.67 $\pm$ 2.90
Protein	25.86 $\pm$ 3.67	28.15 $\pm$ 2.34	20.78 $\pm$ 3.67	26.82 $\pm$ 4.22	21.57 $\pm$ 3.44	28.33 $\pm$ 3.67
Total sterols	0.27 $\pm$ 0.01	0.31 $\pm$ 0.01	0.22 $\pm$ 0.01	0.33 $\pm$ 0.01	0.21 $\pm$ 0.01	0.34 $\pm$ 0.01
Lipid	3.27 $\pm$ 0.01	2.95 $\pm$ 0.01	4.06 $\pm$ 0.01	4.10 $\pm$ 0.01	3.51 $\pm$ 0.01	3.22 $\pm$ 0.01
Ash <sup>a</sup>	11.50 $\pm$ 0.01	11.80 $\pm$ 0.01	10.00 $\pm$ 0.01	13.20 $\pm$ 0.01	14.40 $\pm$ 0.01	10.00 $\pm$ 0.01
Moister <sup>a</sup>	5.36 $\pm$ 0.10	5.54 $\pm$ 0.10	5.04 $\pm$ 0.10	5.85 $\pm$ 0.10	5.53 $\pm$ 0.10	5.25 $\pm$ 0.10

JiH, Jinhae; HN, Haenam; JaH, Jangheung; JD, Jindo; WD, Wando; SA, Sinan.

Values are presented as mean  $\pm$  standard deviation (n = 3).

<sup>a</sup>Determined from origin sample.



**Fig. 2.** Evaluation of the six samples for their toxicities and intracellular ROS scavenging activities against AAPH-induced oxidative stress in Vero cells. (A) Assessment of toxicities of the samples. (B) Protective activities of PPPs against AAPH-induced cytotoxicities. (C) Scavenging activity of PPPs on AAPH-induced intracellular ROS. ROS, reactive oxygen species; AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; PPP, polyphenols and proteins-rich extracts from *Pyropia yezoensis*; JiH, Jinhae; HN, Haenam; JaH, Jangheung; JD, Jindo; WD, Wando; SA, Sinan. Results represent the percentage (%) of cell viability and intracellular ROS levels. All assays involved three independent experiments and the data are expressed as mean  $\pm$  standard error. \* $p < 0.05$ , \*\* $p < 0.01$ .

**Table 2.** Scavenging activities of the 70% ethanol extracts of *Pyropia yezoensis* against DPPH and alkyl radicals

IC <sub>50</sub> (mg mL <sup>-1</sup> )	JiH	HN	JaH	JD	WD	SA
DPPH <sup>a</sup>	1.15 $\pm$ 0.10	0.90 $\pm$ 0.03	0.73 $\pm$ 0.04	0.26 $\pm$ 0.02	0.26 $\pm$ 0.10	0.05 $\pm$ 0.01
Alkyl <sup>b</sup>	0.67 $\pm$ 0.02	0.16 $\pm$ 0.03	0.75 $\pm$ 0.03	0.38 $\pm$ 0.03	0.53 $\pm$ 0.02	0.19 $\pm$ 0.02

DPPH, 1,1-diphenyl-2-picryl-hydrazyl; JiH, Jinhae; HN, Haenam; JaH, Jangheung; JD, Jindo; WD, Wando; SA, Sinan.

<sup>a</sup>DPPH radical scavenging activity.

<sup>b</sup>Alkyl radical scavenging activity.

SA and HN. The highest extract yield was shown by the sample from JD at  $20 \pm 0.2\%$  followed by that from WD at  $16.2 \pm 0.3\%$  yield. The moisture content slightly varied among the samples. Interestingly, SA and JD are both located towards the western coast of Korea and, among the six samples, the samples from these areas showed relatively high polyphenol, carbohydrate, total sterol, and protein content. Factors such as sample condition, water temperature, and the intensity of sunlight may have an influence on the content of polyphenols and other active compounds, which could be investigated further in future.

### Radical scavenging activities of PPP

PPP from SA showed the highest DPPH and alkyl radical scavenging activities, with  $IC_{50}$  values of  $0.05 \pm 0.01$  and  $0.19 \pm 0.02 \mu\text{g mL}^{-1}$ , respectively (Table 2). PPP from HN showed the second highest alkyl radical scavenging activities with  $IC_{50}$  values of  $0.16 \pm 0.03 \mu\text{g mL}^{-1}$  among these six PPPs.

### Antioxidant activity of PPP against AAPH-stimulated damage in Vero cells

The cytotoxicity of PPP from the *P. yezoensis* samples collected in the six different areas was determined on Vero cells using the MTT assay. Vero cells are one of the most commonly and promising used mammalian kidney cells, which become the best cell line for antioxidant effect *in vitro* (Ammerman et al. 2008). No cytotoxic effects of the six *P. yezoensis* were observed (Fig. 2A). Subsequently, we analyzed the protective effects of the six *P. yezoensis* samples against AAPH-induced cell damage using the MTT assay. Upon exposure of AAPH to Vero cells, pretreated with 12.5 to  $100 \mu\text{g mL}^{-1}$  concentrations of PPP, the cell viabilities indicated higher than AAPH-treated group (Fig. 2B). This result demonstrated PPPs have the protective effect in AAPH-treated Vero cells. ROS production was assessed in the Vero cells pretreated with PPPs followed by exposed to AAPH. Significant decreases in the ROS level were observed in the treated samples with JD and SA compared to the untreated AAPH-stimulated control; this indicated the strong scavenging properties of PPP (Fig. 2C).

### Apoptotic body formation in Vero cells upon treatment with PPPs from SA and JD

Since PPPs from SA and JD significantly decreased ROS

production amongst the six samples, SA and JD were selected for further investigation. The nuclear morphology of Vero cells, pretreated with PPPs and exposed to AAPH, was examined by Hoechst 33342 staining followed by fluorescence microscopy. Apoptotic body formation was seen in the AAPH-treated cells; these results are shown in Fig. 3. Cells pretreated with SA and JD showed decreases in the amount of apoptotic bodies in a dose-dependent manner.

### Toxicity of AAPH and PPP treatment in zebrafish embryos

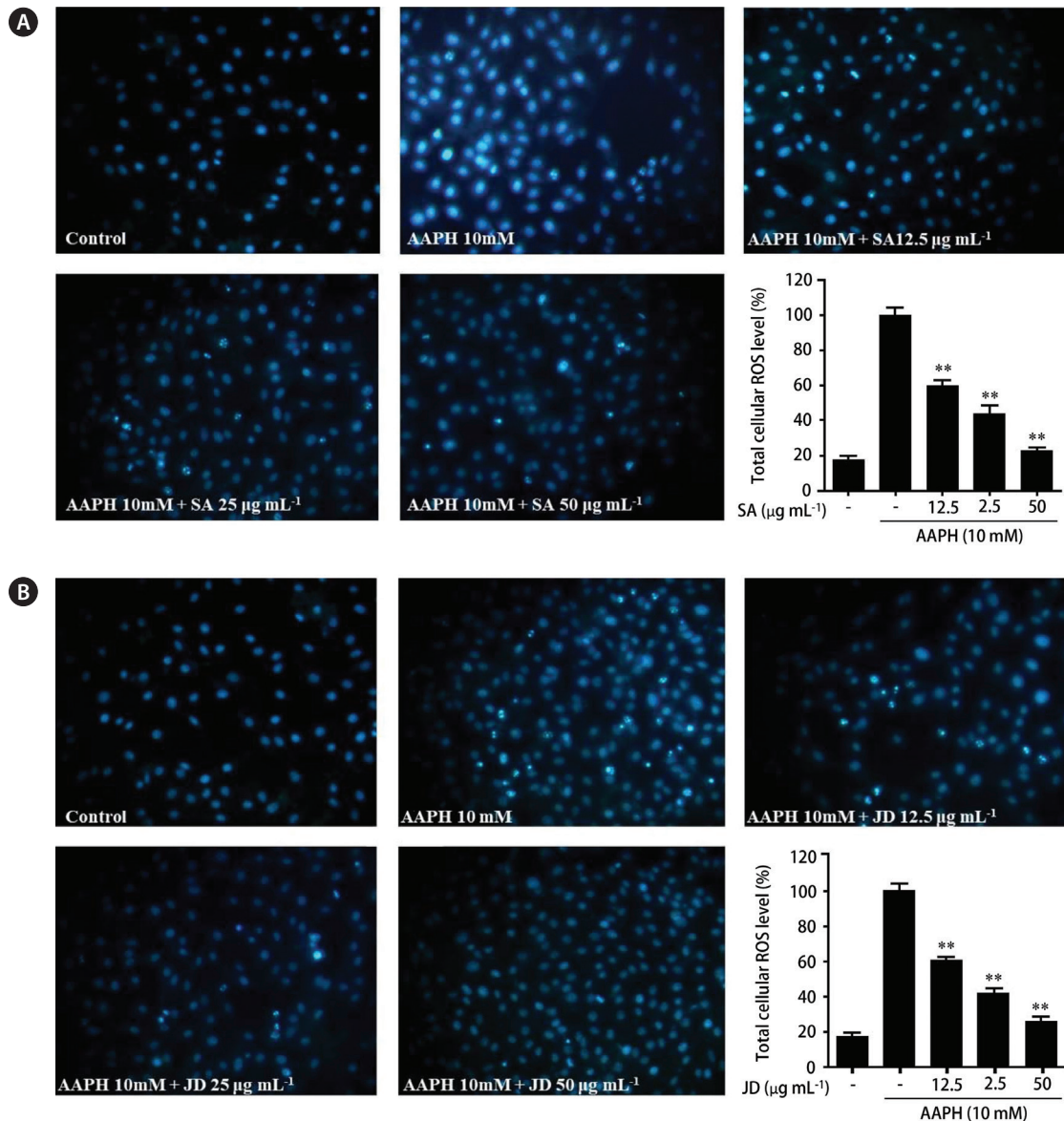
PPP from SA showed minimal toxicity compared to the control (data not shown). Therefore, the viability of zebrafish embryos, treated with PPP from SA and exposed to AAPH, was evaluated; these results are shown in Fig. 4. The AAPH-induced embryos showed lower viability compared to the control, whereas the embryos pretreated with SA showed a survival rate of 50-80% post-AAPH treatment.

### Protective effect of PPP from SA against AAPH-induced oxidative stress and cell death in zebrafish

The fluorescence intensity of acridine orange, a nucleic acid-specific metachromatic dye, was used to determine cell death and evaluate the protective effect of PPP from SA against AAPH-stimulated cell death in the zebrafish model. Cell death in AAPH-treated zebrafish embryos was 232.9% increase compared than untreated embryos; however, the pretreatment with different concentrations of SA significantly decreased cell deaths (Fig. 5).

The antioxidative activity of SA on ROS production in AAPH-induced zebrafish embryos was observed using the DCFH-DA detection assay. The embryos pretreated with PPP from SA showed a strong inhibitory effect on ROS production. The control group (untreated zebrafish embryos) exhibited low fluorescence intensity, whereas AAPH treatment led to a significant increase in fluorescence. This suggested that ROS production in the zebrafish embryos increased upon stimulation with AAPH.

DPPP is widely used to evaluate lipid peroxidation in zebrafish. The untreated embryos showed lower fluorescence, whereas the treatment with AAPH resulted in higher fluorescence than the control, indicating that AAPH treatment induced lipid peroxidation. However, the pretreatment with SA decreased the fluorescence intensity of AAPH-induced embryos suggesting that the



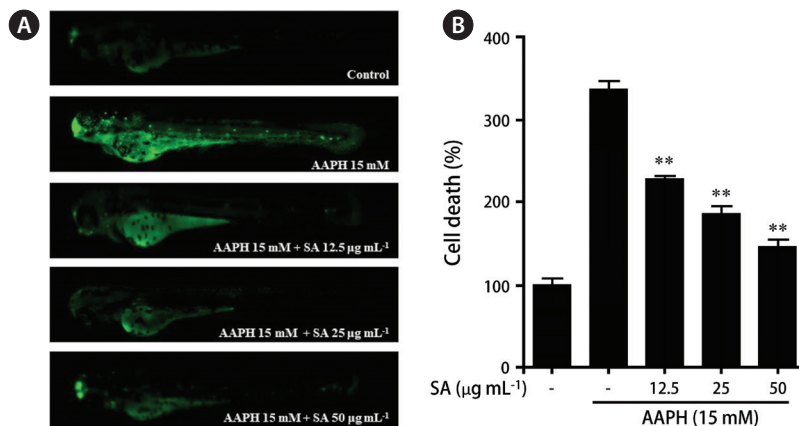
**Fig. 3.** Effects of Sinan (SA) (A) and Jindo (JD) (B) on cellular ROS levels and apoptotic body formation induced by AAPH. Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and cellular ROS levels were evaluated by DCFH-DA analysis. ROS, reactive oxygen species; AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate. All assays involved three independent experiments and the data are expressed as mean  $\pm$  standard error. \*\* $p < 0.01$ .

treatment affords protection against lipid peroxidation (Fig. 6). Collectively, these results demonstrate that PPP from SA could be a potential natural antioxidant.

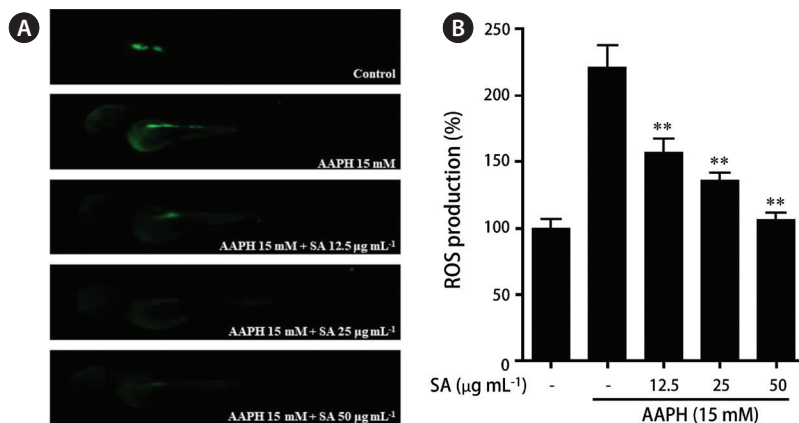
## DISCUSSION

The environmental factors affect the quality of seaweeds in various ways. The antioxidant and anti-inflammatory activities of methanol extracts of were evalu-

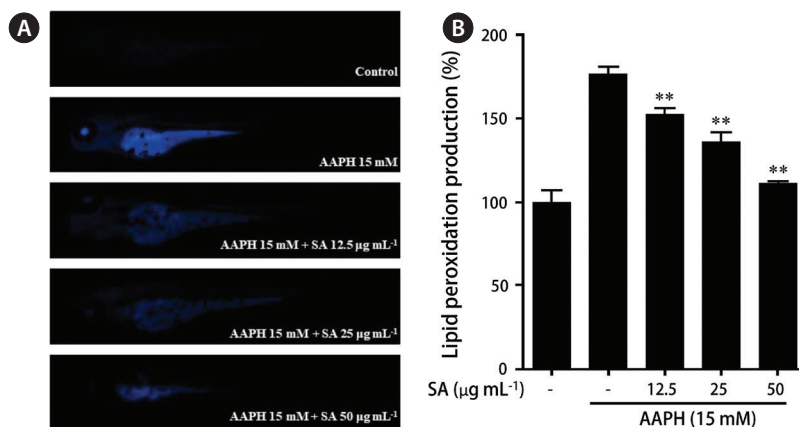
ated in brown seaweed, *Undaria pinnatifida* using local strains collected from southeastern sea shores in Korea (Hwang et al. 2014). The different antioxidant and anti-inflammatory activities of the seaweeds in the three areas indicated differences chemical compositions and different inhabited areas (Sanjeewa et al. 2019). The climates of the eastern and western parts of Korean Peninsula are influenced by ocean currents (Tanaka et al. 2012, Kim et al. 2018b). Therefore, we believe that locations strongly influence the quality of Gim. In this study, we collected



**Fig. 4.** Protective effects of Sinan (SA) against AAPH-induced cell death in zebrafish embryo model. (A) Microscopic fluorescence images of the embryos stained with acridine orange. (B) The relative fluorescence intensities indicate the cell death in the embryos. AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride. All assays involved three independent experiments and the data are expressed as mean ± standard error. \*\*p < 0.01.



**Fig. 5.** Protective effects of Sinan (SA) against AAPH-induced ROS production in zebrafish embryo model. (A) Microscopic fluorescence images of the embryos stained with DCFH-DA. (B) The relative fluorescence intensities indicate the levels of ROS in the embryos. AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; ROS, reactive oxygen species; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate. All assays involved three independent experiments and the data are expressed as mean ± standard error. \*\*p < 0.01.



**Fig. 6.** Protective effects of Sinan (SA) against AAPH-induced lipid peroxidation in zebrafish embryo model. (A) Microscopic fluorescence images of the embryos stained with DPPP. (B) The relative fluorescence intensities indicate the lipid peroxidation level in the embryos stained with DPPP. AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; DPPP, diphenyl-1-pyrenylphosphine. All assays involved three independent experiments and the data are expressed as mean ± standard error. \*\*p < 0.01.



*P. yezoensis* from six regions where major *Pyropia* aquafarms locates screened their antioxidant effects both *in vitro* and *in vivo*.

Environmental stressors such as cold shock and UV-rays as well as biotic stressors like viral infection cause the accumulation of ROS, leading to lipid peroxidation, cell damage, and death. AAPH is a water-soluble compound that has long been known to be a free radical generator (Niki 1990). In this study, we used AAPH to induce oxidative stress in Vero cells and zebrafish embryos. Typically polyphenols are rich in brown seaweed (Namvar et al. 2013), and they exhibit strong antioxidant activity against DPPH, hydroxyl, and peroxy radicals (Kang et al. 2012). There have been very few studies on the extracts of red seaweed and their bioactivity and polyphenol content, particularly because of the lower levels of these compounds in these seaweeds than brown seaweeds. Hence, the content and antioxidant effects of polyphenols extracted from the red seaweed *P. yezoensis*, collected from different areas, were studied. Our results demonstrated that the content of polyphenols and protein in the 70% ethanol extract of *P. yezoensis* was relatively higher than that of other compounds. Among the samples collected from the six areas, those from HN and SA exhibited the strongest antioxidant effects against alkyl radicals, with recorded  $IC_{50}$  values of  $0.16 \pm 0.03$  and  $0.19 \pm 0.02$  mg mL<sup>-1</sup>, respectively. SA and JD exhibited the strongest antioxidant effects against DPPH radicals, with recorded  $IC_{50}$  values of  $0.05 \pm 0.01$  and  $0.26 \pm 0.02$  mg mL<sup>-1</sup>, respectively. Additionally, the total sterol contents of the extracts from SA and JD were higher than those of the other samples, at  $0.34 \pm 0.01$  and  $0.33 \pm 0.01\%$ , respectively. The sterol fraction from *Porphyra dentata* has been reported to possess anticancer effects *in vitro* and *in vivo* (Kazłowska et al. 2013). Hence, we hypothesize that the sterol content in *P. yezoensis* may also contribute to its antioxidant activity. The antioxidant effect of PPP against AAPH-induced cell death was evaluated using Vero cells. The PPPs from SA and JD were found to decrease the cell death and ROS production in a dose-dependent manner in AAPH-induced Vero cells; this clearly exhibited their protective effects against oxidative damage. Furthermore, treatment with PPPs from these samples also decreased the formation of apoptotic bodies in AAPH-stimulated Vero cells in a dose-dependent manner. These results confirm our hypothesis that PPPs from SA and JD show strong antioxidant properties. All the results confirmed our hypothesis that the antioxidant effect of SA and JD in Vero cells was exerted via anti-apoptosis manner, which was mediated by a signaling cascade.

In pharmacological studies, the zebrafish, as an *in vivo* model, has unique advantages, such as large clutch size, transparent embryos, low cost, and easy handling. Hence, it is routinely used for screening in predictive toxicology (Howe et al. 2013). In the zebrafish model, lipid peroxidation was evaluated using a fluorescent probe DPPH, which shows diminished fluorescence under peroxidized conditions. Cell death was observed in zebrafish embryos by acridine orange staining, a nucleic acid-specific metachromatic dye (Kim et al. 2014a). We have also validated our hypothesis using zebrafish embryos as an *in vivo* model system. The PPP from SA inhibited the AAPH-simulated cell death and ROS generation in zebrafish. Lipid peroxidation is a well-characterized toxicological phenomenon that is most commonly initiated by ROS. The free radical generator and AAPH can stimulate lipid peroxidation in cells leading to cell death (Yokozawa et al. 1997). Recent studies reported that stimulator-induced lipid peroxidation in zebrafish embryos was decreased by the polyphenol-rich fraction obtained from seaweeds (Kim et al. 2014c). It was proven that PPP from Korean Gim (SA) has a strong protective effect against lipid peroxidation and cell death in AAPH-treated zebrafish embryos. Lipid peroxidation is a chain reaction in which the reaction of lipids with a free radical generates lipid hydroperoxide and peroxy-fatty acid radicals; these subsequently attack different biological molecules resulting in a sustained reaction that can induce physiochemical alterations, ultimately leading to cell death (Miki et al. 1987). Hence, PPP from SA exerted protective effects against the damage caused by lipid peroxidation.

In this study, subsequently, the free radical scavenging and protective activities of PPP on AAPH-stimulated oxidative stress using cells and zebrafish embryos were evaluated. PPP extracts from *P. yezoensis* obtained from six locations were analyzed. Of these, SA and JD contained relatively higher contents of polyphenols and protein and showed the strongest radical scavenging activity against AAPH and the protective activity of PPP against AAPH-simulated intracellular ROS production and cell death of Vero cells. Finally, it was found that Korean Gim from SA and JD strongly inhibited AAPH-simulated cell death, ROS generation, and lipid peroxidation in zebrafish.

Thus, our studies using Vero cells and zebrafish embryos have demonstrated the antioxidant, free radical scavenging, and protective activities of the polyphenol and protein-rich extract of *P. yezoensis*. The product from SA and JD, which located southwestern coast of Korea, showed the strongest antioxidant activity partly because

of the environmental factors. These results suggest that the geographical location of algae could be a major determinant in its chemical content and activities even when the same species was used for culture.

## ACKNOWLEDGEMENTS

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Golden Seed Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (213008-05-2-SB810) and National Research Foundation of Korea Grant (NRF-2015M1A5A1041804) awarded to GHK.

## REFERENCES

- Ammerman, N. C., Beie-Sexton, M. & Azad, A. F. 2008. Growth and maintenance of Vero cell lines. *Curr. Protoc. Microbiol.* 11:A.4E.1-A.4E.7.
- Aoki, Y. & Kamei, Y. 2006. Preparation of recombinant polysaccharide-degrading enzymes from the marine bacterium, *Pseudomonas* sp. ND137 for the production of protoplasts of *Porphyra yezoensis*. *Eur. J. Phycol.* 41:321-328.
- Chandler, S. F. & Dodds, J. H. 1983. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of *Solanum laciniatum*. *Plant Cell Rep.* 2:205-208.
- Choi, J. -W., Kim, I. -H., Kim, Y. -M., Lee, M. -K. & Nam, T. -J. 2016. *Pyropia yezoensis* glycoprotein regulates antioxidant status and prevents hepatotoxicity in a rat model of D-galactosamine/lipopolysaccharide-induced acute liver failure. *Mol. Med. Rep.* 13:3110-3114.
- Choudhury, F. K., Rivero, R. M., Blumwald, E. & Mittler, R. 2017. Reactive oxygen species, abiotic stress and stress combination. *Plant J.* 90:856-867.
- Cian, R. E., Alaiz, M., Vioque, J. & Drago, S. R. 2013. Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake. *J. Appl. Phycol.* 25:1197-1206.
- Cian, R. E., Garzón, A. G., Ancona, D. B., Guerrero, L. C. & Drago, S. R. 2015. Hydrolyzates from *Pyropia columbina* seaweed have antiplatelet aggregation, antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion. *LWT-Food Sci. Technol.* 64:881-888.
- Dai, Y. -L., Jiang, Y. -F., Lee, H. G., Jeon, Y. -J. & Kang, M. -C. 2019. Characterization and screening of anti-tumor activity of fucoidan from acid-processed hijiki (*Hizikia fusiforme*). *Int. J. Biol. Macromol.* 139:170-180.
- Dai, Y. -L., Jiang, Y. -F., Lu, Y. -A., Kang, M. -C. & Jeon, Y. -J. 2020a. Fucoidan from acid-processed *Hizikia fusiforme* attenuates oxidative damage and regulate apoptosis. *Int. J. Biol. Macromol.* <https://doi.org/10.1016/j.ijbiomac.2020.05.143>.
- Dai, Y. -L., Kim, E. -A., Luo, H. -M., Jiang, Y. -F., Oh, J. -Y., Heo, S. -J. & Jeon, Y. -J. 2020b. Characterization and anti-tumor activity of saponin-rich fractions of South Korean sea cucumbers (*Apostichopus japonicus*). *J. Food Sci. Technol.* 57:2283-2292.
- Diebold, L. & Chandel, N. S. 2016. Mitochondrial ROS regulation of proliferating cells. *Free Radic. Biol. Med.* 100:86-93.
- Ding, Y., Kim, S. H., Lee, J. J., Hong, J. T., Kim, E. A., Kang, D. H., Heo, S. J. & Lee, S. H. 2019. Anti-melanogenesis activity of *Ecklonia cava* extract cultured in tanks with magma seawater of Jeju Island. *Algae* 34:177-185.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350-356.
- Fraga, C. G., Galleano, M., Verstraeten, S. V. & Oteiza, P. I. 2010. Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol. Aspects Med.* 31:435-445.
- Hamann, M. T. 2007. The manzamines as an example of the unique structural classes available for the discovery and optimization of infectious disease controls based on marine natural products. *Curr. Pharm. Design* 13:653-660.
- Hiramoto, K., Johkoh, H., Sako, K. -I. & Kikugawa, K. 1993. DNA breaking activity of the carbon-centered radical generated from 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). *Free Radic. Res. Commun.* 19:323-332.
- Horwitz, W. 1975. *Official methods of analysis*. Association of Official Analytical Chemists, Washington, DC, 1094 pp.
- Howe, K., Clark, M. D., Torroja, C. E., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. -J., White, S., Chow, W., Kilian, B., Quintais, L. T., Guerra-Assunção, J. A., Zhou, Y., Gu, Y., Yen, J., Vogel, J. -H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S. F., Laird, G. K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold,

- G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J. D., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C. M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S. C., Carter, N. P., Harrow, J., Ning, Z., Herrero, J., Searle, S. M. J., Enright, A., Geisler, R., Plasterk, R. H. A., Lee, C., Westerfield, M., de Jong, P. J., Zon, L. I., Postlethwait, J. H., Nüsslein-Volhard, C., Hubbard, T. J. P., Crollius, H. R., Rogers, J. & Stemple, D. L. 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496:498-503.
- Hwang, I. K., Kim, S. O., Hwang, M. S., Park, E. J., Ha, D. S., Lee, S. R., Hwang, I. K., Kim, S. O., Hwang, M. S., Park, E. J. & Ha, D. S. 2018. Intraspecific variation of gene structure in the mitochondrial large subunit ribosomal RNA and cytochrome c oxidase subunit 1 of *Pyropia yezoensis* (Bangiales, Rhodophyta). *Algae* 33:49-54.
- Hwang, J. -H., Oh, Y. -S. & Lim, S. -B. 2014. Anti-inflammatory activities of some brown marine algae in LPS-stimulated RAW 264.7 cells. *Food Sci. Biotechnol.* 23:865-871.
- Kang, M. -C., Kim, E. -A., Kang, S. -M., Wijesinghe, W. A. J. P., Yang, X., Kang, N. & Jeon, Y. -J. 2012. Thermostability of a marine polyphenolic antioxidant dieckol, derived from the brown seaweed *Ecklonia cava*. *Algae* 27:205-213.
- Kazłowska, K., Lin, H. -T. V., Chang, S. -H. & Tsai, G. -J. 2013. *In vitro* and *in vivo* anticancer effects of sterol fraction from red algae *Porphyra dentata*. *Evid. Based Complement. Alternat. Med.* 2013:493869.
- Kim, E. -A., Lee, S. -H., Ko, C. -I., Cha, S. -H., Kang, M. -C., Kang, S. -M., Ko, S. -C., Lee, W. -W., Ko, J. -Y., Lee, J. -H., Kang, N., Oh, J. -Y., Ahn, G., Jee, Y. H. & Jeon, Y. -J. 2014a. Protective effect of fucoidan against AAPH-induced oxidative stress in zebrafish model. *Carbohydr. Polym.* 102:185-191.
- Kim, G. H., Moon, K. -H., Kim, J. -Y., Shim, J. & Klochkova, T. A. 2014b. A reevaluation of algal diseases in Korean *Pyropia* (*Porphyra*) sea farms and their economic impact. *Algae* 29:249-265.
- Kim, H. -S., Sanjeeva, K. K., Fernando, I. P. S., Ryu, B., Yang, H. -W., Ahn, G., Kang, M. C., Heo, S. -J., Je, J. -G. & Jeon, Y. -J. 2018a. A comparative study of *Sargassum horneri* Korea and China strains collected along the coast of Jeju Island South Korea: its components and bioactive properties. *Algae* 33:341-349.
- Kim, J., Yoon, M., Yang, H., Jo, J., Han, D., Jeon, Y. -J. & Cho, S. 2014c. Enrichment and purification of marine polyphenol phlorotannins using macroporous adsorption resins. *Food Chem.* 162:135-142.
- Kim, S. -M., Choi, H. -G., Hwang, M. -S. & Kim, H. -S. 2018b. Biogeographic pattern of four endemic *Pyropia* from the east coast of Korea, including a new species, *Pyropia retorta* (Bangiaceae, Rhodophyta). *Algae* 33:55-68.
- Kim, S. -Y., Kim, E. -A., Kang, M. -C., Lee, J. -H., Yang, H. -W., Lee, J. -S., Lim, T. I. & Jeon, Y. -J. 2014c. 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. *Algae* 29:165-174.
- Köksal, E., Bursal, E., Gülçin, İ., Korkmaz, M., Çağlayan, C., Gören, A. C. & Alwasel, S. H. 2017. Antioxidant activity and polyphenol content of Turkish thyme (*Thymus vulgaris*) monitored by liquid chromatography and tandem mass spectrometry. *Int. J. Food Prop.* 20:514-525.
- Kwon, M. -J. & Nam, T. -J. 2006. Porphyran induces apoptosis related signal pathway in AGS gastric cancer cell lines. *Life Sci.* 79:1956-1962.
- Lee, H. -A., Kim, I. -H. & Nam, T. -J. 2015. Bioactive peptide from *Pyropia yezoensis* and its anti-inflammatory activities. *Int. J. Mol. Med.* 36:1701-1706.
- Leopoldini, M., Russo, N. & Toscano, M. 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chem.* 125:288-306.
- Miki, M., Tamai, H., Mino, M., Yamamoto, Y. & Niki, E. 1987. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by  $\alpha$ -tocopherol. *Arch. Biochem. Biophys.* 258:373-380.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-63.
- Namvar, F., Mohamad, R., Baharara, J., Zafar-Balanejad, S., Fargahi, F. & Rahman, H. S. 2013. Antioxidant, antiproliferative, and antiangiogenesis effects of polyphenol-

- rich seaweed (*Sargassum muticum*). *BioMed Res. Int.* 2013:604787.
- 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 Radic. Biol. Med.* 21:895-902.
- Niki, E. 1990. [3] Free radical initiators as source of water- or lipid-soluble peroxy radicals. *Methods Enzymol.* 186:100-108.
- Perron, N. R. & Brumaghim, J. L. 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem. Biophys.* 53:75-100.
- Ryu, J., Park, S. -J., Kim, I. -H., Choi, Y. H. & Nam, T. -J. 2014. Protective effect of porphyra-334 on UVA-induced photoaging in human skin fibroblasts. *Int. J. Mol. Med.* 34:796-803.
- Sanjeeva, K. K., Fernando, I. P. S., Kim, S. Y., Kim, W. S., Ahn, G., Jee, Y. & Jeon, Y. -J. 2019. *Ecklonia cava* (Laminariales) and *Sargassum horneri* (Fucales) synergistically inhibit the lipopolysaccharide-induced inflammation via blocking NF- $\kappa$ B and MAPK pathways. *Algae* 34:45-56.
- Seifried, H. E., Anderson, D. E., Fisher, E. I. & Milner, J. A. 2007. A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.* 18:567-579.
- Sgherri, C., Pinzino, C. & Quartacci, M. F. 2017. Reactive oxygen species and photosynthetic functioning: past and present. *In* Singh, V. P., Singh, S., Tripathi, D. K., Prasa, S. M. & Chauhan, D. K. (Eds.) *Rrevisiting the Role of Reactive Oxygen Species (ROS) in Plants: ROS Boon or Bane for Plants?* John Wiley & Sons, Hoboken, NJ, pp. 137-155.
- Tanaka, K., Taino, S., Haraguchi, H., Prendergast, G. & Hiraoka, M. 2012. Warming off southwestern Japan linked to distributional shifts of subtidal canopy-forming seaweeds. *Ecol. Evol.* 2:2854-2865.
- Yokozawa, T., Dong, E., Liu, Z. W. & Shimizu, M. 1997. Antioxidative activity of flavones and flavonols *in vitro*. *Phytother. Res.* 11:446-449.