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Comparison of *Ecklonia cava*, *Ecklonia stolonifera* and *Eisenia bicyclis* for phlorotannin extraction

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Abstract

Phlorotannins are polyphenols of marine algae, particularly brown seaweed, having multiple biological activities. A reverse phase-high performance liquid chromatography method was developed for rapid and routine quantification of two major phlorotannins, dieckol and phlorofucofuroeckol-A (PFE-A), from boiling water- and organic solvent-extracts of brown seaweeds *Ecklonia cava*, *E. stolonifera* and *Eisenia bicyclis*. The regression equations for dieckol and PFE-A were as follows: the concentration (mg ml-1) = 16.56 × peak height (cm) + 0.44, and the concentration = 20.60 × peak height (cm) + 0.11, with correlation coefficients of 0.996 and 0.999, respectively. Compared to organic solvent extraction, the recovery yield of dieckol from boiling water extracts of *E. cava*, *E. stolonifera* and *E. bicyclis* was 86%, 93%, and 98%, respectively. The recovery yield of PFE-A was 74%, 86% and 62%, respectively. Antioxidant activity was detected in each *E. bicyclis* water extract (91%), followed by *E. stolonifera* (90%) and *E. cava* (74%). Dieckol and PFE-A showed almost 9- and 7-fold stronger antioxidant activity than the standard butylhydroxytoluene, and 6- and 4-fold greater than L-ascorbic acid in molar concentration, respectively.

Key words

Dieckol, *Ecklonia cava*, *Ecklonia stolonifera*, *Eisenia bicyclis*, Phlorofucofuroeckol-A

Introduction

Addition of antioxidative and functional ingredients needs to be implemented to maintain the prime quality and longer storage time of some foods. Reactive oxygen species (ROS) are produced by all aerobic organisms and are known to cause oxidative modifications of DNA, proteins, lipids and small cellular molecules (Ames, 1983). ROS are associated with tissue damage and are the prime contributing factors in various diseases, such as inflammation, cancers, hypertension, diabetes, and the degenerative process associated with aging (Stadtman, 1992; Wiseman and Halliwell, 1996). Antioxidants are important inhibitors for lipid peroxidation for food production and act as a defence mechanism of living cells against ROS-mediated oxidative damage (Carlos *et al*., 2008). Most of the synthetic antioxidants have been reported to be toxic, carcinogenic and responsible for liver damage (Ito *et al*., 1986; Safer, 1999). Much attention of consumers and food producers is focusing on natural materials to replace synthetic antioxidants. Marine algal polyphenols, known as phlorotannins, are only found within brown algae and are synthesized via the acetate-malonate pathway by polymerization of phloroglucinol (1,3,5-tri hydroxybenzene) (Ragan and Glombitza, 1986). Phlorotannins purified from several brown seaweeds have been reported to possess strong antioxidant activity which may be associated with their unique molecular skeleton (Ahn *et al*., 2007). Phlorotannins including dieckol and phlorofucofuroeckol-A (PFE-A) are known to have potent biological effects, such as antioxidant and anti-inflammatory activities (Kim *et al*., 2009), acetyl cholinesterase inhibitory activity (Myung *et al*., 2005; Yoon *et al*., 2008),

tyrosinase inhibitory activity (Heo *et al*., 2009), and α-glycosidase and α-amylase inhibitory activities (Lee *et al*., 2010).

Phlorotannin level can be determined as total quantity of whole polyphenolic compound group by colorimetric method using phloroglucinol as a standard agent (Stern *et al*., 1998). Total phlorotannins consist of a complex set of phloroglucinol oligomers. Accordingly, reverse phase-high performance liquid chromatography (RP-HPLC) may be a suitable tool for quantitative and qualitative analyses of specific phlorotannins (Goo *et al*., 2010). We compared distribution of phlorotannins in *Ecklonia cava* and different pretreatments for its extraction (Chowdhury *et al*., 2011). To our knowledge, there is no report of comparative determination of phlorotannins from three major phlorotannin producers of brown seaweeds. In this study, we validated the RP-HPLC method and determined amounts of dieckol and PFE-A, which are common phlorotannic components found abundantly in brown seaweeds *E. cava*, *E. stolonifera* and *Eisenia bicyclis*, from hot water and organic solvent extracts. To assess their suitability for use as a polyphenol-rich functional food ingredient, the antioxidant capacity of the brown seaweed water extracts was also determined.

Materials and Methods

Algal materials and reagents : Leafy thalli of brown seaweeds *E. cava* and *E. stolonifera* were collected from the coast of Busan, Korea, and those of *E. bicyclis* were collected from Ulleung, Korea, between June 2010 and December 2012. Voucher specimens were deposited in our laboratory (Y.K. Hong). Epiphytes and salts were removed by washing with fresh water followed by sonication for 1 min. Then, the seaweed tissues were dried completely for one week at room temperature and ground to make powder using a coffee grinder. The powdered samples were then stored at -20°C until further use. All solvents used in this study were of high-purity spectroscopic grade solvents (J.T. Baker, Avantor Performance Materials, NJ, USA). DPPH (2, 2-diphenyl-1-picrylhydrazyl), L-ascorbic acid and butylhydroxy toluene (BHT) were purchased from Sigma (Sigma-Aldrich, St. Luis, MO, USA).

Boiling water extraction of phlorotannin : One gram of each powdered algal samples each was suspended in 100 ml of boiling distilled water in a 250 ml beaker. The sample was then stirred at 350 rpm with a magnetic stirring hot plate (Cimarac SP131320, Barnstead International, Dubuque, IA, USA) at 100°C for 5 min. After removal of seaweed debris using defatted cotton filter, the water extract was evaporated at 80°C and dried under nitrogen generator (G 4510E; Dominic Hunter Ltd., Dukesway, England) to prevent oxidation of polyphenolics. The residue was weighed and stored at -20°C until use. Before HPLC injection, the residue was re-dissolved in distilled water (200 mg ml^{-1}) and filtered through 0.45 µm syringe filter.

Organic solvent extraction of phlorotannin: Phlorotannins were extracted from algal powder according to the method of Chowdhury *et al*. (2011) with some modifications. Briefly, algal powder (1 g) was shaken with methanol (4 ml) at room temperature for 2 hr. Chloroform (8 ml) was added, and the mixture was shaken for 5 min. Then, the mixture was partitioned to upper and lower layers by addition of distilled water (3 ml). The upper water fraction was extracted with diethyl ether (6 ml) and evaporated under nitrogen generator. The residue was dissolved in 100% methanol (1 mg ml^{-1}) and stored at -20°C until use.

RP-HPLC analysis of extracts: Each 100 µl aliquot of extract was separated on a 5 µm Alltima C_{18} column (250 × 10 mm) (Alltima; Alltech, Deerfield, IL, USA) with an Alltima C_{18} 5µm (7.4 mm × 4.6 mm) guard column. The isolation was performed on a Waters 600 gradient liquid chromatograph (Waters, Milford, MA, USA), and monitored at 290 nm. The linear gradient solvent system consisted of water and 100% methanol. The gradient was made from 30% to 100% methanol over 40 min and with isocratic 100% methanol over 10 min, at a flow rate of 1.0 ml min^{-1}.

Analytical methods: Phlorotannins dieckol and PFE-A were analyzed on a JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan), using methanol-d_4 (CD_3OD) for the ^{1}H- and ^{13}C-NMR spectra. High-resolution fast atom bombardment mass spectrometry (HR-FABMS) data were obtained using a JMS-700 spectrometer (JEOL).

Method validation and quantification : To measure dieckol and PFE-A levels in *E. cava*, *E. stolonifera* and *E. bicyclis*, a 100 µl aliquot of extract was subjected to RP-HPLC. The quantity of each compound from both extracts of three seaweeds was assessed by measuring their dimensions of RP-HPLC peaks, using standard curve of each pure compound. Validation of HPLC quantification was performed for accuracy (Chowdhury *et al*., 2011; Snyder *et al*., 1997).

Determination of antioxidant activity : The radical scavenging activities of three seaweed extracts and purified phlorotannins were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Blois, 1958) with some modification. Briefly, one ml of 0.2 mM DPPH prepared freshly in ethanol was added to one ml of seaweed extracts or purified compounds at various concentrations (5 to 200 µg ml^{-1}). The mixture was vortexed and incubated at 25°C for 30 min in dark, and the absorbance at 517 nm was measured. Lower absorbance indicates higher free radical scavenging activity. The percentage of scavenging activity was calculated using the following formula: scavenging activity (%) = $[1 – (A_1–A_2)/A_0] \times 100\%$, where A_0 is the absorbance of the control (water instead of test sample solution), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample with water instead of DPPH. The IC_{50} value, which is the concentration of test material that reduce free radical concentration by 50%, was calculated as µg ml^{-1} and µM using a dose-response curve.

Statistical analysis : All data were compiled as mean ± SE of at least three independent determinations. Statistical comparisons of mean values were performed with an analysis of variance (ANOVA), followed by Duncan's multiple test using SPSS software version 16 (SPSS Inc. Chicago, IL). *P* values <0.05 were considered statistically significant.

Results and Discussion

To study the structures of major phlorotannins from both boiling water and organic solvent extracts, the peaks of major phlorotannins with retention times of 32 and 40 min in RP-HPLC were detected and isolated. The structures (Fig. 1) of isolated compounds were identified to be identical to the spectral data of dieckol and PFE-A (Kim *et al*., 2009; Myung *et al*., 2005), and confirmed as follows:

Dieckol: light brown powder, FABMS *m/z* (%) found: 742.0811 (M^+; bp), 743.0880 $[M+1]^+$; (64), 741.0726 $[M-1]^+$; (18), calculated for $C_{36}H_{22}O_{18}$: 742.0806; ^{1}H-NMR (400 MHz, CD_3OD) δ :6.14 (1H, s, H-3"), 6.12 (1H, s, H-3), 6.08 (2H, s, H-2"', H-6), 6.06 (1H, d, *J* = 2.72 Hz, H-8), 6.04 (1H, d, *J* = 3.08 Hz, H-6"'), 5.98 (1H, d, *J* = 2.72 Hz, H-6"), 5.95 (1H, d, *J* = 2.72 Hz, H-8"), 5.92 (3H, s, H-2',4',6'); ^{13}C-NMR (100 MHz, CD_3OD) δ :161.9 (C-1'), 160.1 (C-3',5'), 157.8 (C-7), 155.9 (C-7"), 154.5 (C-4a, 4a"), 152.4 (C-3"',5"'), 147.4 (C-9a"), 147.3 (C-2", C-1"), 147.2 (C-2), 146.9 (C-9, 9"), 144.3 (C-5a"), 144.1 (C-5a, 4), 143.4 (C-4"), 143.3 (C-4"'), 138.6 (C-10a), 138.5 (C-10a"), 126.1 (C-1"'), 125.5 (C-9a), 124.5 (C-1), 99.8 (C-8"), 99.6 (C-8), 99.4 (C-3), 99.3 (C-3"), 97.6 (C-4'), 96.1 (C-2"',6"'), 95.8 (C-6"), 95.7 (C-6'), 95.3 (C-2',6).

PFE-A : light brown powder, FABMS *m/z* (%) found: 603.0779 (M^+; bp), 602.0693 $[M+1]^+$; (70), calculated for $C_{30}H_{18}O_{14}$: 602.0697; ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.64 (1H, s, H-9), 6.41 (1H, s, H-13), 6.27 (1H, s, H-3), 5.97 (2H, d, *J* =2.04 Hz, H-2", H-6"), 5.94 (1H, m, H-4'), 5.92 (1H, m, H-4"), 5.89 (2H, d, *J* = 2.04 Hz, H-2', H-6'), ^{13}C-NMR (100 MHz, CD_3OD) δ:161.6 (C-1), 161.5 (C-1"), 160.0 (C-5"), 159.9 (C-3', C-5', C-3"), 152.9 (C-12a), 151.5 (C-10), 150.9 (C-11a), 148.1 (C-2, C-8), 145.7 (C-14), 143.7 (C-4), 138.1 (C-15a), 135.1 (C-5a), 127.9 (C-14a), 124.8 (C-4a), 124.5 (C-1'), 122.1 (C-11), 105.1 (C-6, C-7), 99.8 (C-9), 99.2 (C-3), 97.6 (C-4"), 97.4 (C-4'), 96.0 (C-13), 95.2 (C-2", C-6"), 95.2 (C-2', C-6').

A RP-HPLC analysis was performed to quantitatively evaluate the dieckol and PFE-A levels in *E. cava*, *E. stolonifera* and *E. bicyclis* water and organic solvent extracts (Fig. 2). Phlorotannin levels were quantified from the dimensions of their HPLC peaks using standard curve of each pure compound. For dieckol and PFE-A, the validation data of boiling water extraction also showed high accuracy of 92.64 and 94.02%, respectively, same as data of organic solvent extraction from the previous result (Chowdhury *et al*., 2011). Calibration plots of peak height (× cm) *vs*. pure compound concentration (y mg ml^{-1}) exhibited a straight line. The regression equations for dieckol and PFE-A were y=16.56x+0.44 and y=20.60x+0.11, respectively, and their correlation coefficients (r^2) were 0.992 and 0.998, respectively. The validation process intended to challenge the method and determine the limits of allowed variability under the conditions needed to run the quantification. To assess low-level impurities, a

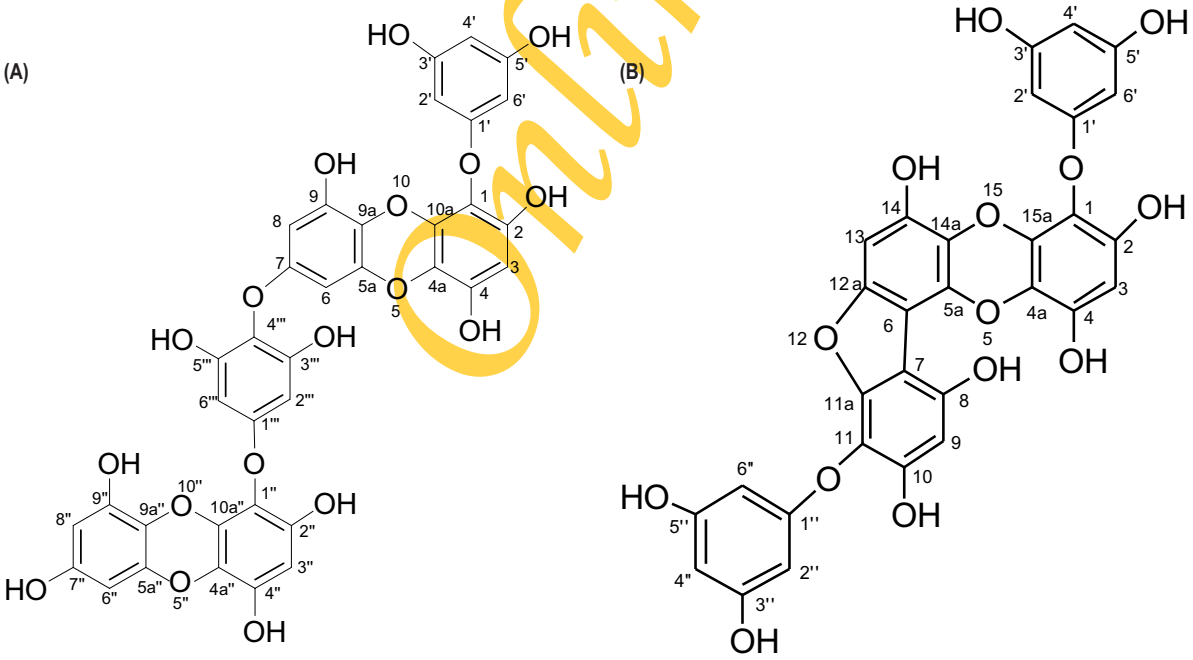

Fig. 1: Structures of dieckol and phlorofucofuroeckol-A isolated from brown seaweeds *Ecklonia cava*, *Ecklonia stolonifera* and *Eisenia bicyclis*

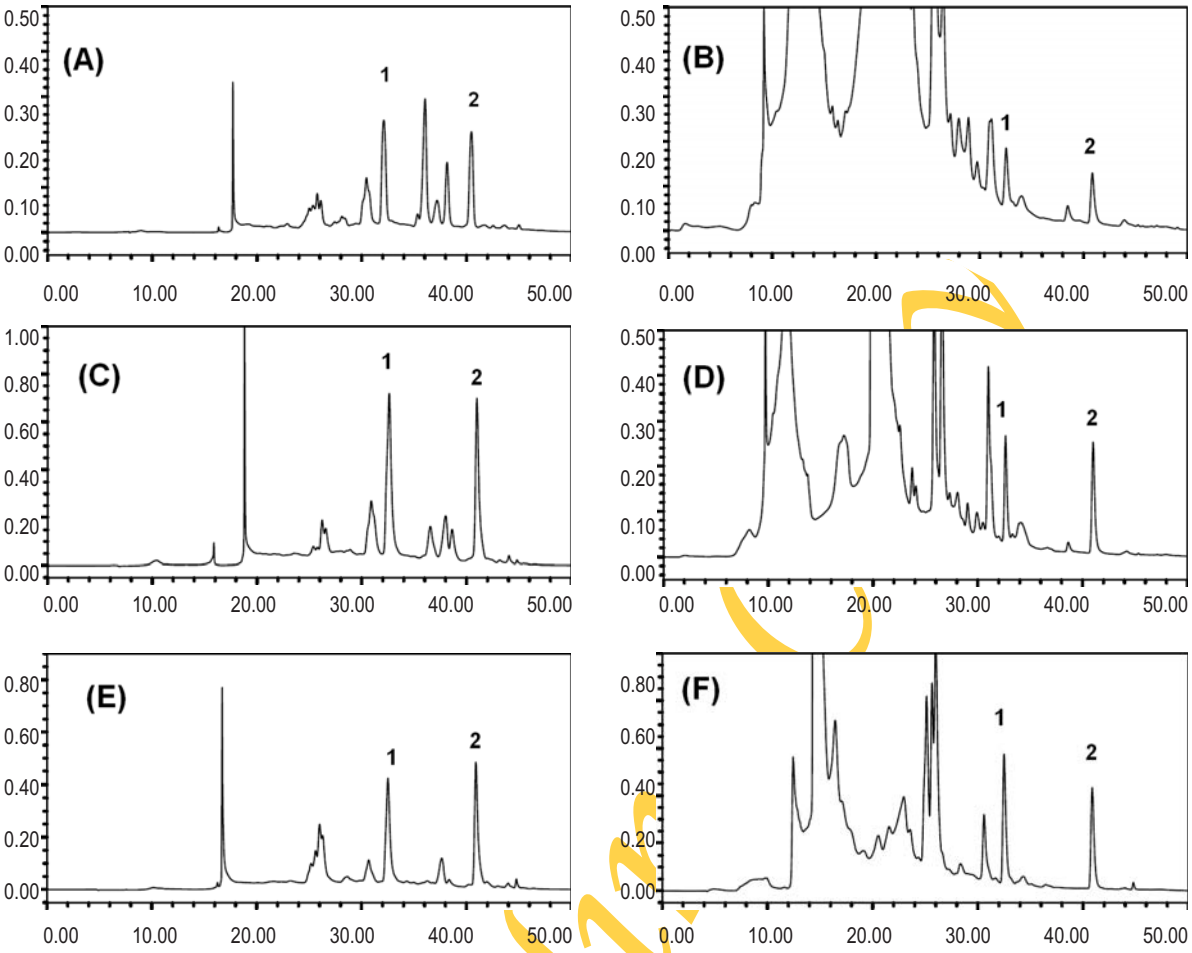

Fig. 2 : RP-HPLC chromatogram patterns of *Ecklonia cava* by organic solvent (A) and boiling water (B) extraction; *Ecklonia stolonifera* by organic solvent (C) and boiling water (D) extraction; and *Eisenia bicyclis* by organic solvent (E) and boiling water (F) extraction. Peak 1 represents dieckol and peak 2 represents phlorofucofuroeckol-A. HPLC conditions are described in the Material and Methods section

precision of 5–10% is usually accepted (Snyder *et al*., 1997), which was higher than our precision range. Thus, quantification of dieckol and PFE-A levels in boiling water and organic solvent extracts of *E. cava*, *E. stolonifera* and *E. bicyclis* using RP-HPLC was reliable and rapid, and did not depend on the degree of multiplicity of seaweed extracts.

After hot water extraction, the highest amount of extract including water-soluble substances was measured in *E. bicyclis* (417.88 mg g^{-1} dry tissue). Water extracts of 346.89 mg g^{-1} and 395.00 mg g^{-1} were obtained from *E. cava* and *E. stolonifera*, respectively. After organic solvent extraction, the highest amount of extract was detected in *E. bicyclis* (3.37 mg g^{-1} dry tissue). Solvent extracts of 2.93 and 2.93 mg g^{-1} were detected in *E. cava* and *E. stolonifera*, respectively. To determine dieckol and PFE-A levels in *E. cava*, *E. stolonifera*, and *E. bicyclis* tissues, boiling water and organic solvent extractions were performed. Using boiling water extraction, the highest amount of dieckol was obtained from *E. stolonifera* (1.42 mg g^{-1} dry tissue) (Fig. 3A). Using organic solvent extraction, the highest amount of dieckol was obtained from *E. cava* and *E. stolonifera* (each 1.52 mg g^{-1} dry tissue) and the lowest from *E. bicyclis* (1.33 mg g^{-1} dry tissue). Using boiling water extraction, the highest amount was obtained from *E. stolonifera* (1.00 mg g^{-1} dry tissue) (Fig. 3B). In contrast, using organic solvent extraction, the highest amount of PFE-A was obtained from *E. bicyclis* (1.30 mg g^{-1} dry tissue) and the lowest from *E. cava* (0.93 mg g^{-1} dry tissue). An ethyl acetate extract of *E. stolonifera* yielded dieckol and PFE-A levels of 30.1 and 7.7 mg g^{-1} crude extract, respectively (Goo *et al*., 2010). On considering tissue dry weight, the ethyl acetate extract contained dieckol and PFE-A of 0.3 mg and 0.07 mg g^{-1} dry tissue, respectively, which were lower than those obtained from boiling

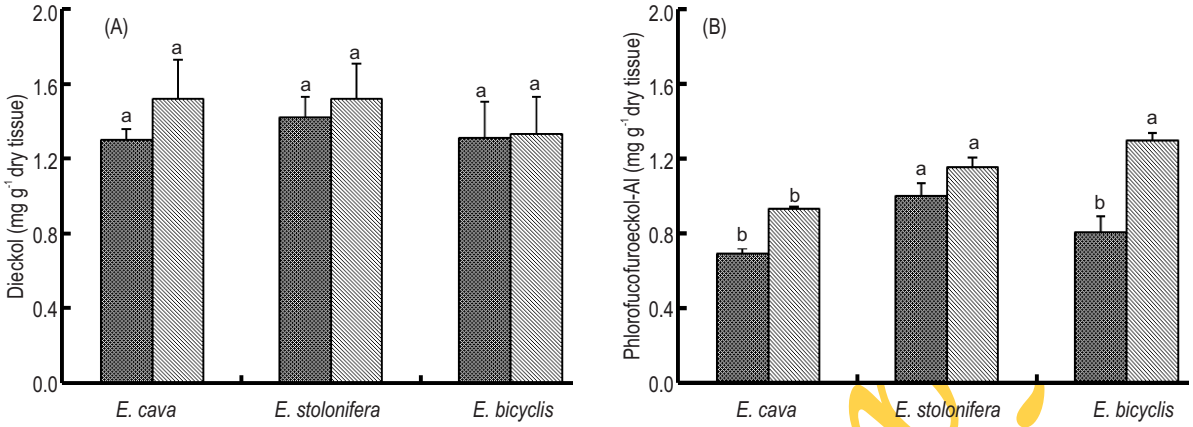

Fig. 3: Comparison of organic solvent (▒) and boiling water (▓) extraction of dieckol (A) and phlorofucofuroeckol-A (B) from the brown seaweeds *Ecklonia cava*, *Ecklonia stolonifera* and *Eisenia bicyclis*. Values are expressed per 1 g dry tissue and as mean ± SE ($n \geq 3$). Letters of a and b indicate significant differences by Duncan's multiple test ($p < 0.05$)

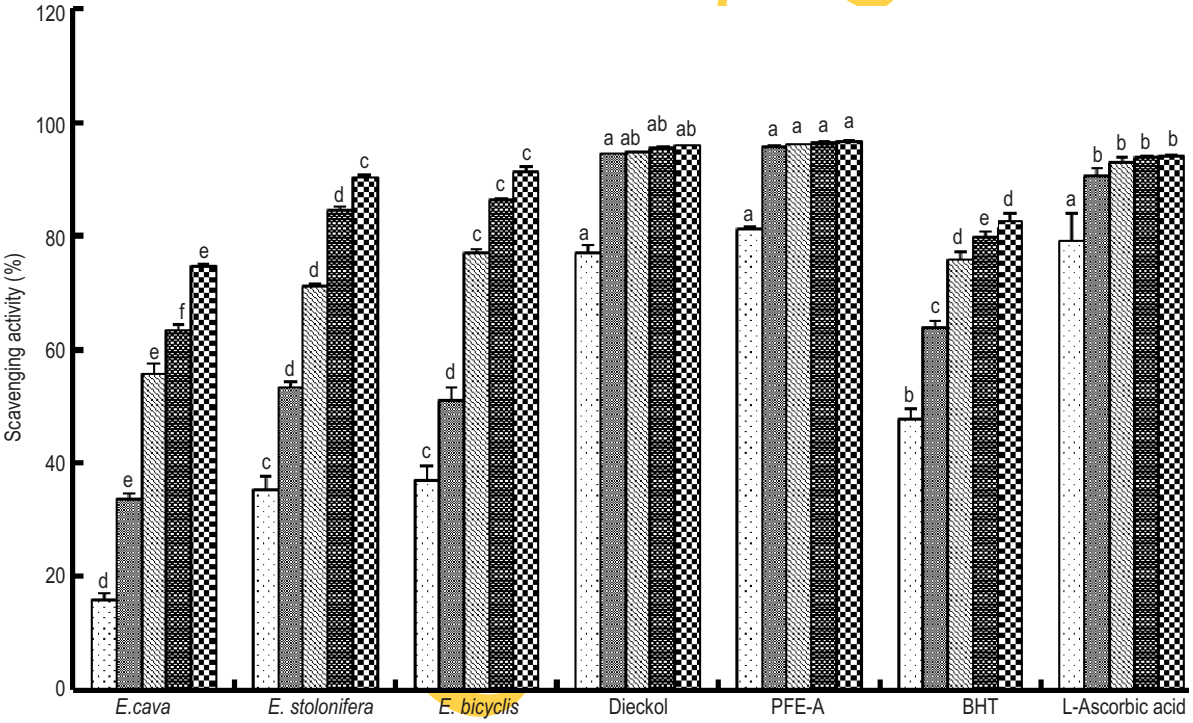

Fig. 4: DPPH free radical scavenging activity of *Ecklonia cava*, *Ecklonia stolonifera* and *Eisenia bicyclis* water extracts and pure dieckol and phlorofucofuroeckol-A compared with standards of butylated hydroxytoluene (BHT) and L-ascorbic acid. Seaweed extracts or pure compounds of 25 µg ml^{-1} (▒), 50 µg ml^{-1} (▒), 100 µg ml^{-1} (▒), 150 µg ml^{-1} (▓), and 200 µg ml^{-1} (▒) were reacted with 1 ml of 0.2 mM DPPH. Values are expressed as mean ± SE ($n \geq 3$). Letters of a, b, c, and d indicate significant differences by Duncan's multiple test ($p < 0.05$)

water extract (in our observation). Intense heat of hot water may facilitate the release of cell wall or cell wall bound phenolics due to breakdown of cellular constituents (Lim and Murtijaya, 2007), resulting in high phlorotannin levels. Organic solvent extraction is the most common method of phlorotannin extraction. Dieckol yield by boiling water extraction was 86%, 93% and 98% of organic solvent extraction from *E. cava*, *E. stolonifera* and *E. bicyclis*, respectively. For PFE-A, the yield from *E. cava*, *E. stolonifera* and *E. bicyclis* was 74%, 86% and 62%, respectively. Thus, boiling water extraction yielded fair amount of

phlorotannins comparing to the organic solvent extraction, and is a safe and cost effective method.

DPPH is a free radical compound that is widely used to evaluate free radical scavenging activity of natural antioxidants. The degree of reduction in absorbance is indication of radical scavenging power of the antioxidant compound. Fig. 4 shows decrease in DPPH free radical concentration due to the scavenging ability or capacity of boiling water extracts from three seaweed extracts and two purified phlorotannins as compared to the standard commercial antioxidants. Among boiling water extracts, those from *E. bicyclis* and *E. stolonifera* exhibited greater scavenging activity than BHT at higher concentrations and weaker activity at lower concentrations. The highest scavenging activity in 200 µg ml^{-1} concentration was observed in *E. bicyclis* (91%), followed by *E. stolonifera* (90%) and *E. cava* (74%), respectively, in comparison with BHT (82%) and L-ascorbic acid (94%). Thus, the scavenging effect of seaweed boiling water extracts was comparable with the standard antioxidants. Heat pre-treatment significantly enhanced the overall antioxidant capacities of three Irish brown seaweeds (Rajauria *et al*., 2010). Purified dieckol and PFE-A exhibited stronger antioxidant capacities than BHT and L-ascorbic acid. The dieckol concentration producing 50% inhibition (IC_{50}) was 10.57 µg ml^{-1} or 14.24 µM for DPPH free radical scavenging activity (Table 1). The PFE-A IC_{50} concentration was 11.15 µg ml^{-1} or 18.51 µM. Thus, dieckol and PFE-A showed almost nine- and seven-fold stronger antioxidant activity than standard BHT (127.85 µM) and six- and four-fold greater than L-ascorbic acid (78.74 µM) in molar concentration, respectively.

Brown seaweed boiling water extracts exhibit water soluble and heat stable antioxidant capacities. Dieckol and PFE-A were isolated from boiling water extracts of three brown seaweeds and quantified by RP-HPLC. Recovery of dieckol and PFE-A from *E. stolonifera* boiling water extract was approximately 90%, compared to organic solvent extraction. The seaweeds *E. cava*, *E. stolonifera* and *E. bicyclis* are consumed as food materials. Therefore, the boiling water extracts or pure compounds isolated from those seaweeds could be promising for the use in the functional food industry as a natural additive in situations where water solubility, heat stability, and high antioxidant activity are prerequisites.

Table 1 : IC_{50} values of seaweed boiling water extracts, dieckol and phlorofucofuroeckol-A for DPPH free radical scavenging activity

	IC_{50} (µg ml^{-1})	IC_{50} (µM)
Ecklonia cava extract	87.49 ± 3.28^{a}	ND
Ecklonia stolonifera extract	45.37 ± 1.32^{b}	ND
Eisenia bicyclis extract	48.68 ± 3.78^{b}	ND
Dieckol	10.57 ± 0.30^{d}	14.24 ± 0.41^{c}
Phlorofucofuroeckol-A	11.15 ± 0.18^{d}	18.51 ± 0.31^{c}
BHT	28.14 ± 0.32^{c}	127.85 ± 1.46^{a}
L-Ascorbic acid	13.87 ± 0.32^{d}	78.74 ± 1.79^{b}

Values are mean ± SE. Letters of a, b, c, and d indicate significant differences by Duncan's multiple test at $p < 0.05$. ND = not determined

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