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Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*

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Abstract

The potential antiproliferative and antiradical activities of an enzymatic extract of *Ecklonia cava* together with its crude polysaccharide (CpoF) and crude polyphenolic fractions (CphF) were evaluated in vitro. Tested extracts showed strong selective cell proliferation inhibition on all cancer cell lines tested, especially CphF extract, containing high polyphenol amount, showed 5.1 μg/ml of IC_{50} value on murine colon cancer (CT-26) cell line. According to the nuclear staining experiment, antiproliferative effect of CphF was associated with apoptotic cell demise in CT-26. In addition, The CphF at 5 μg/ml scavenged 70% of DPPH radical, which is much higher than those of BHA and BHT at same concentration. Further more CphF exhibited interesting antiradical properties, expressed by its capacity to scavenge superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). In reducing power assay, CphF extract at 5 μg/ml was found to be as high as that of BHT at same concentration. Also, in total antioxidant assay the effect of CphF at 50 μg/ml was equivalent or slightly higher than those of commercial counterparts at 5 μg/ml concentration. Taken together, the CphF may be a promising alternative to synthetic substances as natural compound with high antiproliferative and antiradical activity.

Keywords: *Ecklonia cava*; Enzymatic hydrolysate; Antiproliferative activity; Antiradical activity; Apoptosis

1. Introduction

Seaweeds as well-balanced, harmless, natural sources with a high degree of bioavailability of trace elements are strongly advised for fast grown children and pregnant women (Booth, 1964). In contrast to their use as a source of food, marine algae are widely used in the life science as the source of compounds with diverse structural forms and biological activities. Over the years marine algal species offer the biological diversity for sampling in discovery-phase of new drug development (Munro et al., 1987, 1999). Therefore, it is clearly documented that, pre-clinical pharmacological research with new marine compounds continued to be extremely active in resent history (Mayer and Gustafson, 2003).

The formation of cancer cell in human body can be directly induced by free radicals. Further more, ionizing radiation, which causes free radicals, is well documented as a carcinogen. Therefore, radical scavenging compounds such as vegetables and fruits can indirectly reduce cancer formation in human body. It has been reported that, increased consumption of fruits and vegetables, which are rich with free radical scavenging activity, leads up to a doubling of protection against many common types of cancer formations (Nandita and Rajini, 2004; Chu et al., 2002; Cooke et al., 2002).

Recent years, several algal species also have been reported to prevent oxidative damage by scavenging free

Abbreviations: ABTS, 2,2-azinobis (3-ethylbenzthiazolin)-6-sulfonic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CphF, crude phenolic fraction; CpoF, crude polysaccharide fraction; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl 1-2-pricrylhydrazyl; EDTA, ethylenediamine tetraacetic acid; MTT, 3-[4,5-dimethylthiazole-2-yl]–2,5-diphenyltetrazoliumbromide; NBT, nitro blue tetrazolium salt; OE, original extract; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.

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radicals and active oxygen and hence able to prevent the occurrence of cancer cell formation. Therefore, algal species as alternative materials to extract natural antioxidative compounds have attracted much attention of biomedical scientists. There are some evidences that seaweeds contain compounds with a relatively high antioxidant and antiproliferative activity. Therefore, extraction of bioactive natural compounds from seaweeds is desired, but little has happened in this area to systematically study their potentiality. Polyphenols in marine brown algae are called phlorotannins and known to act as potential antioxidants. Phlorotannins are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and synthesized in the acetate–malonate pathway in marine alga (Ragan and Glombitza, 1986). Furthermore, sulfated polysaccharides isolated from marine alga also have been shown to exert radical scavenging activities in vitro and in vivo.

However, biochemical scientists have several techniques to extract bio-active compounds from algal biomass. As one of the techniques, enzymatic hydrolysis of algal biomass gains more advantages over other conventional techniques. Enzymes can convert water-insoluble materials into water soluble materials, also this method do not adapt any toxic chemicals. Interestingly, this technique gains high bioactive compound yield and shows enhanced biological activity in comparison with water and organic extract counterparts (Heo et al., 2005).

In this study, *Ecklonia cava*, which was collected along Jeju Island coast of Korea, was enzymatically hydrolyzed using AMG 300L and after ethanol precipitation (Matsubara et al., 2000; Kuda et al., 2002) the crude polysaccharide (CpoF) and crude polyphenolic fractions (CphF) were evaluated for their suppressive effect on tumor cell growth, antioxidant and radical scavenging activities.

2. Materials and methods

2.1. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), trichloroacetic acid (TCA), naphthylethylene diamine dihydrochloride, xanthine, xanthine oxidase from butter milk, nitro blue tetrazolium salt (NBT), butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), disodium salt (ferrozine), ferric chloride ($FeCl_3 \cdot 6H_2O$), potassium ferricyanide [$K_3Fe(CN)_6$], ferrous chloride ($FeCl_2 \cdot 4H_2O$), $FeSO_4 \cdot 7H_2O$, Folin–Ciocalteu reagent, linoleic acid and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma Co. (St. Louis, USA). RPMI-1640, fetal bovine serum (FBS), phosphate buffer saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). 2,2-Azinobis(3-ethylbenzthiazolin)-6-sulfonic acid (ABTS), ethylenediamine tetraacetic acid (EDTA), peroxidase and 2-deoxyribose were purchased from Fluka Chemie (Buchs, Switzerland). AMG 300L (an exol, 4-alpha-D-glucosidase) was purchased from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). All the other chemicals used in this study were 90% or grater purity.

2.2. Plant material and extraction

Marine brown alga *E. cava* was collected close to the shores of Jeju Island in Korea during March and October 2004. Salt, sand and epiphytes were removed using tap water. Then, seaweed samples were rinsed carefully with fresh water and freeze-dried. Dried alga sample was ground (MFC SI mill, Janke and Kunkel Ika-Wreck, Staufen, Germany) and sifted through a 50-mesh standard testing sieve. One hundred gram of alga sample was homogenized with water (2 l), and mixed with 1 ml of AMG enzyme. The enzymatic hydrolytic reaction (pH 4.5) was performed for 12 h to achieve optimum degree of the hydrolysis of the plant material at 60 °C. Following digestion, the digest was boiled for 10 min at 100 °C to inactivate the enzymes. Then, sample was clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. This extract was adjusted to pH 7.0 hereafter and designated to as original extract (OE). Then, combined OE was (240 ml) mixed well with 480 ml of 99.5% ethanol. After the mixture was allowed to stand for 30 min at room temperature, crude polysaccharides were collected by centrifugation at 10,000*g* for 20 min at 4 °C (Matsubara et al., 2000; Kuda et al., 2002). Hereafter, the collected precipitate was referred to as crude polysaccharide fraction (CpoF) and the resulted supernatant was referred to as crude phenolic fraction (CphF). CpoF and CphF were concentrated separately under vacuum at 40 °C and removed all ethanol, and then samples were dissolved in water for further experiments.

2.3. Cell culture

The murine colon cancer cell line (CT-26), human leukemia cell line (THP-1), mouse melanoma cell line (B-16) human leukemia cell line (U-937), and chinese hamster fibroblast cell line/normal cell line (V79-4) were maintained in RPMI 1640 or DMEM medium containing 10% (v/v) heat inactivated fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C under 5% CO_2 in the air.

2.3.1. MTT assay

In this study, cancer cell growth inhibition activity was measured by using MTT assay (Mossman, 1983; Carmichael et al., 1987). Tumor cells were seeded in a 96-well plate at the concentration of 2×10^4 cells/ml using RPMI. After 16 h (at 37 °C, in a humidified atmosphere of 5% CO_2), all algal extracts were treated to the wells at a concentration range from 5 to 100 μg/ml. The cells were then further incubated for an additional 72 h at 37 °C. MTT stock solution (50 μl; 2 mg/ml in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C, the plate was centrifuged at 800*g* for 5 min and the supernatants were aspirated to remove untransformed MTT. The formazan crystals in each well were dissolved in 150 μl of DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm. For treated cells, viability was expressed as a percentage of control cells. All determinations were carried out in triplicate. The IC_{50}, the antiproliferative activity of the tested enzymatic fractions was determined terms of the amount (μg/ml) of the extract necessary for inhibiting 50% of the cell growth.

2.3.2. Nuclear staining with Hoechst 33342

Apoptotic cells are characterized by nuclear condensation of chromatin and/or nuclear fragmentation (Lizard et al., 1997). First, CT-26 cells were seeded in a 96-well plate at the concentration of 1×10^5 cells/ml. After 16 h incubation time, cells were treated with CphF (5 and 25 μg/ml) and further incubated for 12 h. Then, Hoechst 33342, a DNA specific fluorescent dye was added into the culture medium at a final concentration of 10 μg/ml and plate was incubated for another 10 min at 37 °C. The morphological aspect of cell nuclei was observed under a florescent microscope equipped with a CoolSNAP-pro color digital camera.

2.4. Evaluation of radical scavenging ability of E. cava hydrolysate

2.4.1. DPPH radical scavenging assay

The free-radical scavenging activity of the different enzymatic fractions of *E. cava* was measured according to the modified method of Brand-Williams, 1995. Two milliliters of each sample fraction (OE, CpoF and CphF) at 5, 50, 100 and 500 μ/ml were mixed thoroughly with 2 ml of freshly prepared DPPH solution (3×10^{-5} M) dissolved in DMSO. The

reaction mixture was incubated for 1 h and the absorbance was measured at 517 nm using a UV–vis spectrophotometer (Opron 3000 Hanson Tech. Co., Korea).

Scavenging activity was calculated as $[1(A_i - A_j)/A_c] * 100$, where in the DPPH method: A_i is the absorbance of organic solvent extract mixed with DPPH solution; A_j is the absorbance of same organic extract mixed with 2 ml DMSO; A_c is the absorbance of DPPH solution adding 2 ml DMSO.

2.4.2. Superoxide anion scavenging assay

The superoxide anion-scavenging assay was carried out according to the method of Nagai et al. (2003). The reaction mixture consisted of 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM EDTA, 0.02 of ml 0.15% bovine serum albumin, 0.02 ml of 0.75 mM NBT and 0.02 ml of *E. cava* sample. After incubation at 25 °C for 10 min, the reaction was started by adding 6 mU xanthine oxidase and keeping the temperature at 25 °C for 20 min and then, by adding 0.02 ml of 6 mM CuCl, the reaction of the sample was stopped. The absorbance was measured with an enzyme-linked immunosorbent assay (ELISA) reader (Sunrise; Tecan Co., Austria) at 560 nm.

2.4.3. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging assay was carried out according to the method of Muller (1995). *E. cava* sample (80 μl) and 20 μl of hydrogen peroxide (10 mM) were mixed with 0.1 M of phosphate buffer (100 μl, pH 5.0) in a 96-microwell plate and incubated at 37 °C for 5 min. Thereafter, 30 μl of freshly prepared ABTS (1.25 mM) and 30 μl of peroxidase (1 U/ml) were mixed and incubated at 37 °C for 10 min and the absorbance was recorded with an ELISA reader at 405 nm.

2.4.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined according to the method of Chung et al. (1997). The Fenton reaction mixture consisted of 200 μl of $FeSO_4 \cdot 7H_2O$ (10 mM), EDTA (10 mM) and 2-deoxyribose (10 mM). Then, 200 μl of *E. cava* extract and 1 ml of 0.1 M phosphate buffer (pH 7.4) were mixed together and made the total volume of 1.8 ml. Thereafter, 200 μl 10 mM H_2O_2 was added and the reaction mixture was incubated at 37 °C for 4 h. After incubation, 1 ml of 2.8% TCA and 1 ml of 1% TBA were mixed and placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged (5 min, 395*g*) and the absorbance was measured at 532 nm with a UV–vis spectrophotometer.

2.5. Reducing power assay

The determination of the reducing power was conducted according to the method developed by Oyaizu (1986). *E. cava* fractions (2.5 ml) were spiked with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then placed in a 50 °C water bath for 20 min. Then, samples were kept at room temperature and 2.5 ml of 10% trichloroacetic acid was added to the mixture. Finally, 0.125 ml of the mixture mixed with 0.125 ml distilled water and 1 ml of 0.1% ferric chloride and incubated for 10 min. The absorbance of the samples was measured at 700 nm with a UV–vis spectrophotometer.

2.6. Antioxidant activity in a hemoglobin-induced linoleic acid system

The antioxidant activity of *E. cava* samples was measured by a photometry assay (Kuo et al., 1999), with little modifications. The sample solution of the alga (0.1 ml) was mixed with 0.025 ml of 0.1 M linoleic acid in ethanol and 0.075 ml of 0.2 M phosphate buffer (pH 7.2). By adding 0.05 ml of hemoglobin (0.08%), the auto-oxidation of the sample was started, and then sample was incubated for 6 min at 37 °C for the reaction. After incubation period, peroxidation of the mixture was stopped by adding 5 ml of 0.6% HCl in ethanol. The peroxidation value of the react mixture (0.2 ml) was measured (at 490 nm) in triplicate using thiocyanate method, after coloring with 0.02 ml of 20 mM $FeCl_2$ and 0.01 ml of 30% ammonium thiocyanate. BHA and BHT were used as positive controls.

2.7. Determination of total polyphenolic content

The polyphenolic compounds were determined according to a protocol similar to that of Chandler and Dodds (1993). *E. cava* extract (1 ml) was mixed with 1 ml of ethanol 95%, 5 ml of distilled water and 0.5 ml of 50% of Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Finally, each sample was mixed thoroughly and placed in dark for 1 h and absorbance was measured at 725 nm with a UV–vis spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic content.

2.8. Determination of total carbohydrate content

The total carbohydrate content of the relevant extracts was determined by the AOAC (1990). Glucose standard curve was used for the calculation of carbohydrate content.

3. Results

3.1. Inhibitory effect of E. cava hydrolysate on the growth of tumor cells

In this study, four tumorigenic cell lines including, murine colon cancer cell line (CT-26), human leukemia cell line (THP-1), mouse melanoma cell line (B-16) and human leukemia cell line (U-937) were chosen to determine the antiproliferative activity of *E. cava* extracts. Also, under same experimental conditions the extracts were evaluated on chinese hamster lung fibroblast cell line (V-79-4) in order to examine its cytotoxicity effect on normal cells.

Cultures of CT-26 cells were treated with increasing concentrations (5–100 μg/ml) of the enzymatic extracts of *E. cava* (Fig. 1A). Treatment with CphF on CT-26 cells resulted a high antiproliferative effect in a concentration-dependant manner compared to those of other two counterparts. As shown in the figure, cell growth inhibition was 70% with the presence of CphF at 50 μg/ml, the activity of this fraction reached a maximal (~77%) at 100 μg/ml concentration. Thus, CphF displayed a strong antiproliferative activity on colon cancer cell with an IC_{50} of ~5.1 μg/ml (Table 1). On CT-26 cell, CpoF and OE exhibited relatively lower activities, however the latter fraction showed ~45% cell growth inhibition at 50 μg/ml concentration. Fig. 1B shows the cell growth inhibitory activity on THP-1 cells by the enzymatic extracts of *E. cava*. On THP-1 cell line, the tested samples caused a low, but similar kind of suppression in a dose-dependant manner as it was observed in CT-26 cell line. After treating THP-1 cell with CphF at 5 μg/ml concentration, there was ~22% cell growth inhibition. However, CpoF and OE extracts at 50 μg/ml showed ~20% and ~17% cell growth inhibition effect respectively. On THP-1 cell line all the extracts tested on this study showed high IC_{50} values (>100 μg/ml). Cell growth inhibitory effects of B-16 cell line with the presence of *E. cava* extracts are shown in Fig. 1C. Of the extracts tested, the CphF of *E. cava* had the highest cell growth inhibition activity with an average IC_{50} value of 29.3 μg/ml (Table 1), however CphF from 5 μg/ml to 50 μg/ml concentrations indicated a dramatic activity enhancement.

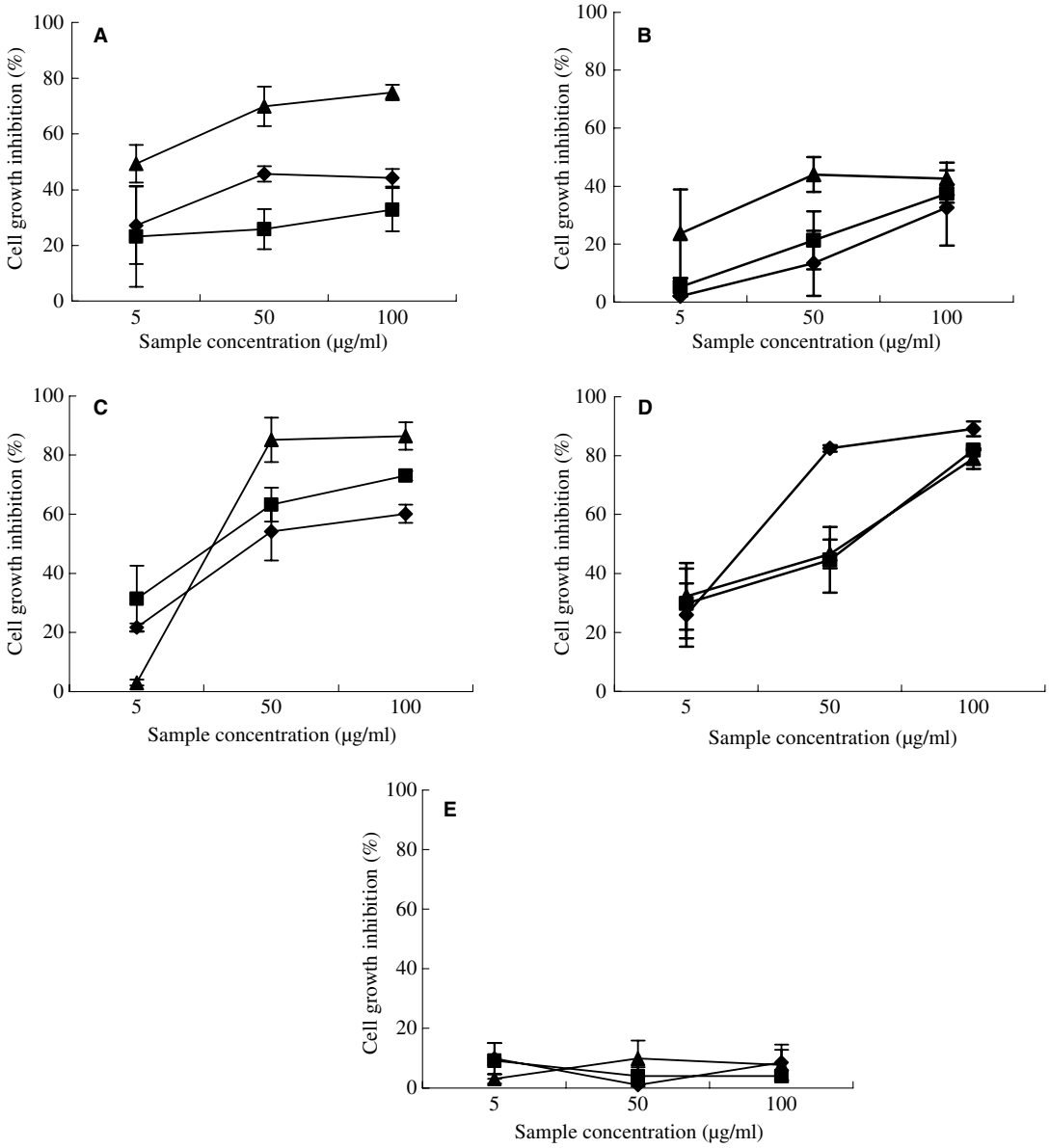

Fig. 1. Antiproliferative effect of enzymatic hydolysate of *E. cava* on cancer cell lines: (A) murine colon cancer cell line (CT-26); (B) human leukemia cell line (THP-1); (C) mouse melanoma cell line (B-16); (D) human leukemia cell line (U-937) and (E) chinese hamster fibroblast cell line/normal cell line (V79-4). Cells were treated with different concentrations of *E. cava* extracts (-◆- OE, -▲- CphF, -■- CpoF) for 3 days, then the cell viability was determined by the MTT assay. The growth inhibitory activity was calculated as % of inhibition compared with the control.

Table 1
Antiproliferative activity by enzymatic hydrolysate of *E. cava*

Treatment (μg/ml)	CT-26	THP-1	B-16	U-937
OE	>100	>100[a]	39	30.2
CphF	5.1	>100	29.3	53.6
CpoF	>100	>100	54	56.0

[a] IC_{50} values are larger than 100 μg/ml.

In addition, CpoF and OE also showed good cell growth inhibition activities on mouse melanoma cell (54 and 39 μg/ml of IC_{50}, respectively) at 100 μg/ml sample concentration. Taken together, on CT-26 cancer cell, THP-1 cell, B-16 cell CphF induced high antiproliferative activity dose-dependently than those of other two counterparts.

As in Fig. 1D, all extracts of *E. cava* hydrolysate showed good cell growth inhibition activities on U-937 cell line. The activities of the extracts dramatically enhanced with increased sample concentrations. On U-937 cell, OE showed the highest cell growth inhibition, especially at 50 μg/ml there was an exceptional activity (~82%) than those of the other extracts at a same concentration. For all extracts, the activities reached a maximal level (~80%) at the highest dosage of 100 μg/ml. CphF and CpoF showed very much similar antiproliferative activity on U-937 cells. The IC_{50} values of OE, CphF and CpoF were 30.2, 53.6 and 56 μg/ml, respectively.

Taken together, these data suggest the ability of enzymatic hydrolysate of *E. cava* and its sub fractions to inhibit

cancer cell proliferation, especially CphF strongly and selectively retard the cancer cell growth. Moreover, as it is shown in Fig. 1E, it is evident that treatment of the all extracts on V79-4 cells show low cell cytotoxicity, hence the results confirm the ability of extracts to protect normal cells. Lower cytotoxicity in normal cells compared to cancer cells is a prerequisite for any chemo-preventive agent. Apoptosis is an ordered and characteristic sequence of structural changes resulting in programmed cell death. In this study, CphF showed 50% cell growth inhibition on CT-26 cells at 5.1 μg/ml concentration. Therefore, the possibility of induction of apotosis by CphF was investigated by observing the apoptotic body formation. The images of this study (Fig. 2) confirm the ability of CphF to induce apoptosis in CT-26 cell line. The extract dose-dependently increased the formation of apoptotic bodies on the cell line. The negative control, without algal extract, has a clear image suggesting that minor or no apoptotic bodies can take place without algal extract.

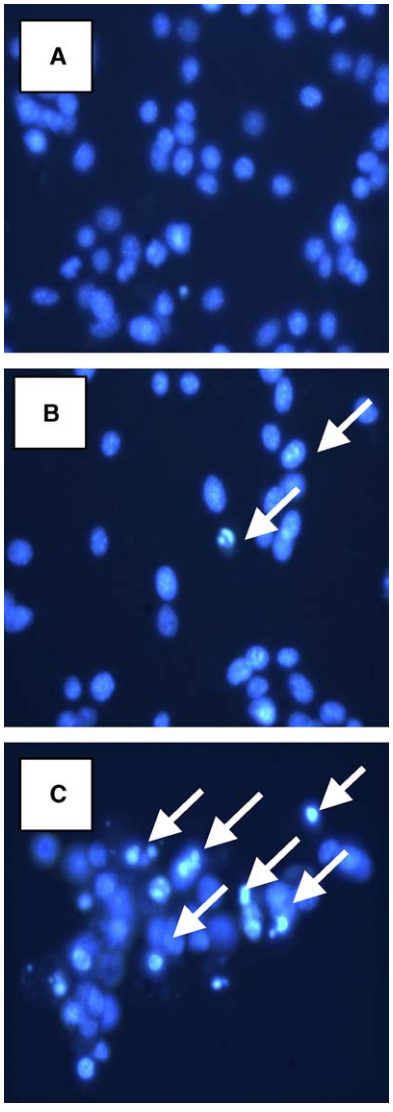

Fig. 2. Effect of CphF on morphological changes in CT-26 cell line. Cells were treated in the absence of (A) or in the presence of (B) 5 μg/ml and (C) 25 μg/ml of crude phenolic extract for 24 h, stained with Hoechst 33342, and observed by fluorescence microscopy. Arrows (B and C) indicate a typical apoptotic cell with apoptotic body.

3.2. Assessment of *E. cava* extracts on antioxidant and radical scavenging activities

3.2.1. DPPH radical scavenging activity

DPPH is the choice of many scientists to evaluate the free radical scavenging activity of natural compounds (Shimada et al., 1992). Table 2 shows the decrease in the concentration of DPPH radical due to the scavenging ability of the enzymatic extracts of *E. cava* and the commercial standards. In this study we used BHA and BHT as commercial standards. According to the results, CphF at 5 μg/ml showed an excellent DPPH radical scavenging activity (70%) among all the tested samples and the activity increased with increasing concentrations. The CpoF and OE also showed good DPPH radical scavenging activities, the activities of the both extracts reached to its maximum level at 50 μg/ml sample concentration. However, the scavenging effects of the extracts decreased in the order of CphF > OE > CpoF, respectively. Under same experimental conditions, positive control counterparts, BHA and BHT at 5 μg/ml showed 60% and 68% DPPH radical scavenging activities, respectively.

3.2.2. Superoxide anion scavenging activity

Superoxide anion scavenging results with the presence of the *E. cava* extracts and commercial antioxidants are exhibited in Table 2. All samples treated in this experiment showed considerable scavenging abilities over superoxide

Table 2
Reactive oxygen radical species scavenging activity of enzymatic hydrolysate of *E. cava*

Treatment	% Scavenging ability			
	DPPH	O_2^-	H_2O_2	HO·
OE (μg/ml)				
5	50.3 ± 0.3	10.3 ± 1.3	10.2 ± 0.9	10.2 ± 0.9
50	87.2 ± 0.8	18.1 ± 1.5	10.2 ± 0.4	13.6 ± 1.6
100	83.2 ± 0.7	31.2 ± 0.8	12.8 ± 1.0	16.2 ± 0.6
500	82.6 ± 1.7	40.3 ± 1.3	35.6 ± 1.3	20.3 ± 0.7
CpoF (μg/ml)				
5	50.3 ± 0.3	10.6 ± 0.8	–	–[a]
50	81.3 ± 0.6	22.4 ± 0.7	13.1 ± 0.9	10.8 ± 0.7
100	73.7 ± 0.9	28.1 ± 0.5	16.2 ± 0.4	13.2 ± 0.7
500	71.7 ± 0.8	31.5 ± 0.7	23.0 ± 0.8	15.2 ± 0.5
CphF (μg/ml)				
5	70.1 ± 0.8	31.3 ± 1.2	17.0 ± 0.9	10.2 ± 0.6
50	92.0 ± 0.6	32.6 ± 0.3	21.7 ± 1.2	17.8 ± 1.3
100	93.7 ± 0.7	50.4 ± 0.8	28.6 ± 1.3	28.3 ± 1.5
500	96.5 ± 0.9	65.2 ± 1.6	67.3 ± 0.8	31.2 ± 0.7
BHA (5 μg/ml)	60.2 ± 0.5	50.1 ± 0.8	42.1 ± 0.8	42.1 ± 0.2
BHT (5 μg/ml)	68.3 ± 0.6	51.0 ± 0.6	38.5 ± 0.7	35.1 ± 1.3

Scavenging ability are mean values of three determinants.
[a] Means no activity.

anion. Moreover, it was clear that, the effect of the extracts on the reduction on superoxide anion was dose-dependant upon concentrations. Of the tested extracts, addition of the CphF at 500 μg/ml showed the highest superoxide anion scavenging ability (~65%). Over superoxide radical, the OE and CpoF at 500 μg/ml showed ~40% and 31% scavenging effect, respectively. In this assay, both BHA and BHT at 5 μg/ml showed ~50% superoxide anion scavenging ability. Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, therefore it indirectly initiate lipid peroxidation. Apart from that, the presence of superoxide anion can magnify the cellular damage because it produces other kinds of free radicals and oxidizing agents. In fact, as a natural source the capacity of enzymatic hydrolysate of *E. cava* to scavenge O_2^- will be useful in pharmaceutical industry.

3.2.3. Hydrogen peroxide scavenging activity

In this study, all the extracts of *E. cava* showed considerable hydrogen peroxide scavenging activities. Specially, CphF at 500 μg/ml exhibited ~67% scavenging activity (Table 2). CpoF and OE showed relatively low hydrogen peroxide scavenging activities, however the activity increased with the sample concentration. As commercial counterparts, BHA and BHT showed ~42% and ~38% scavenging activities at 5 μg/ml under the same experimental condition.

3.2.4. Hydroxyl radical ($HO^{\bullet}$) scavenging activity

All the extracts of *E. cava* showed a considerable scavenging ability on hydroxyl radical and the ability was concentration-dependant (Table 2). However, CphF at 500 μg/ml showed 31.2% scavenging activity whereas those from OE and CpoF at the same concentration showed 20% and 15% scavenging ability consequently. For this assay BHA and BHT showed 42% and 35% radical scavenging effect at 5 μg/ml concentration. In fact, the inhibitory ability of *E. cava* was inferior to those of the commercial counterparts such as BHA and BHT.

3.3. Reducing power ability

In this assay, reducing power ability of the tested *E. cava* extracts steadily increased with increasing sample concentration (Fig. 3). Especially, CphF at 5 μg/ml showed slightly higher activity than that of BHT. The other two counterparts of *E. cava* sowed almost similar activities.

3.4. Antioxidant activity in a hemoglobin-induced linoleic acid system

In this study, the total antioxidant efficacy of the *E. cava* extracts was evaluated on hemoglobin-mediated linoleic acid system. The antioxidant activity results for linoleic acid treated with *E. cava* extracts are shown in Fig. 4. The addition of algal extracts to the mixture delayed lipid peroxidation dose-dependently. However, among additives

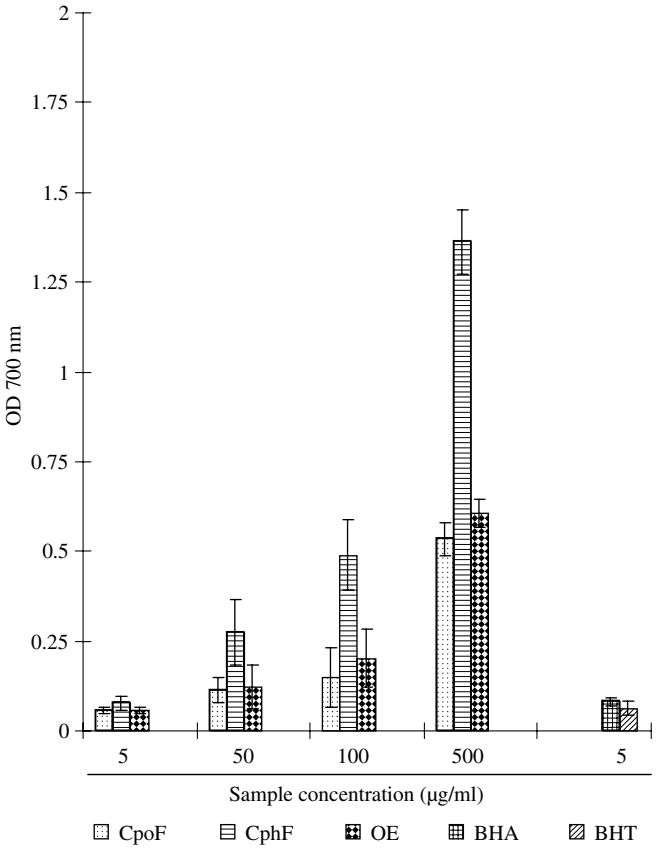

Fig. 3. Effect of enzymatic hydrolysate of *E. cava* and commercial antioxidants on ferrous reducing power assay. Data are presented as the mean SD of results from three independent experiments.

CphF at 500 μg/ml was the most effective in retarding lipid oxidation, meanwhile at the same concentration OE and CpoF showed >40% antioxidant activity under same experimental condition. Albeit, antioxidant activities of OE and CpoF increased with the sample concentration a prominent activity enhancement was not observed as it was observed from CphF. At 5 μg/ml, commercial counterparts, BHA and BHT showed 26% and 18% total antioxidant activity in this assay, almost similar results obtained from CphF and OE at 50 μg/ml concentration. Taken together, our findings suggest that CphF obtained after ethanol precipitation showed a strong antioxidant activity in this assay, this may be due to its high polyphenolic content.

3.5. Total phenolic assay

The total phenolic content of *E. cava* extracts was measured according to Folin–Ciocalteu method. The Folin–Ciocalteu regent determines total phenols, producing blue colour by reducing yellow heteropolyphosphomolybdatetungstate anions. The phenolic contents of OE, CpoF and CphF were 301, 241 and 901 μg/100 ml, respectively, at 500 μg/ml concentration (Fig. 5). In fact, the phenolic content of the present study extracts decreased in order of CphF > OE > CpoF. Previously it has been observed a

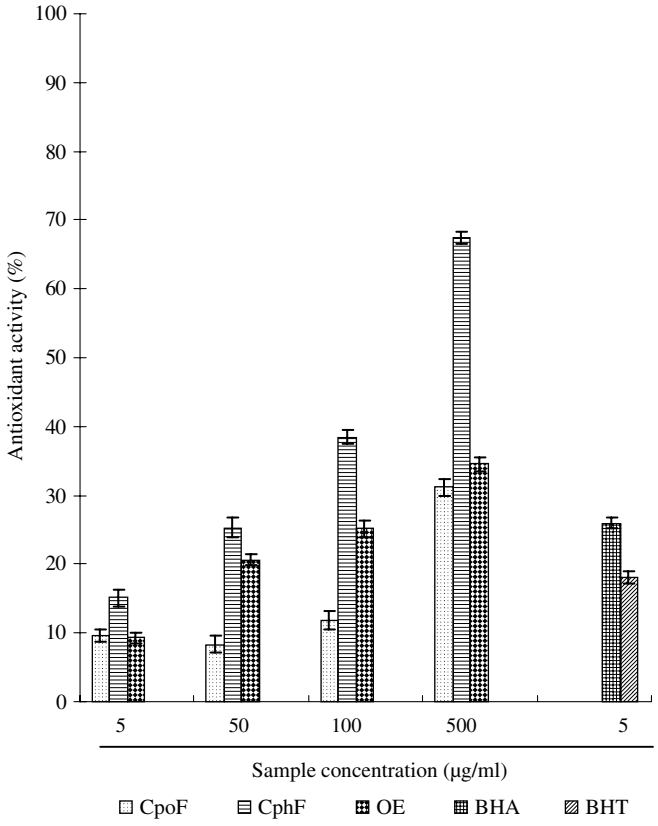

Fig. 4. Antioxidant activity of enzymatic hydrolysate of *E. cava* and commercial antioxidants against linoleic acid peroxidation induced by hemoglobin. The peroxide value was measured in triplicate by thiocyanate method.

high positive relationship between total polyphenolic content and DPPH radical scavenging activity (Oki et al., 2002; Siriwardhana et al., 2003). Similar phenomena were also observed in the present study since the scavenging efficacy decreased in a similar manner with polyphenolic content of the samples.

4. Discussion

Jeju Island is located in the southwest sea of the Korean peninsula and is highlighted for its uniqueness. Especially in the coastal area of this island the seawater level fluctuate rapidly. Therefore, the algal species present along the shoes of Jeju Island may require high endogenous antioxidant protection as an adaptative response to this especial environment. Recently it has been reported several biologically important seaweed species from Jeju Island (Athukorala et al., 2003, 2005; Siriwardhana et al., 2003; Heo et al., 2005; Karawita et al., 2005). But due to some of limitations, it is hard to purify exactly biologically active compounds. Some of the biggest hurdles include the low concentration, instability and difficulty in separation and detection of these bioactive compounds. As an alternative technique, here we used in-expensive carbohydrate digestive enzyme to digest algal bio-mass to extract marine plant

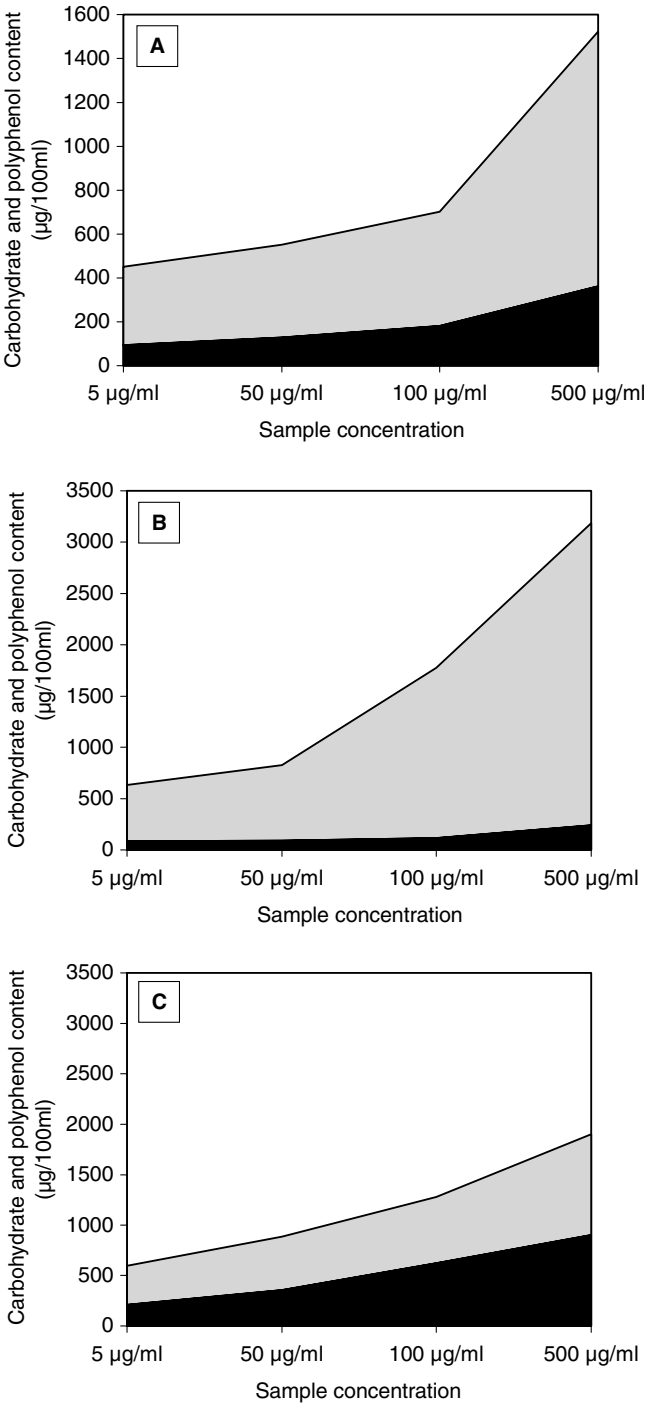

Fig. 5. The total carbohydrate and polyphenolic compound amount of the enzymatic hydrolusate of *E. cava* (A) OE; (B) CpoF; (C) CphF. Carbohydrate content ▭, Phenolic content ■. Glucose standard curve was used for the calculation of carbohydrate content while a gallic acid standard curve was obtained for the calculation of phenolic content.

photochemical materials. As previously mentioned, this enzymatic extraction possesses much more advantages than conventional techniques. According to our lab previous experiments, several brown algal species were enzymatically digested with several carbohydrates and proteases to investigate their potential bioactivities. The degree of

enzymatic hydrolysis was different with the treated enzymes, especially, in that study AMG extract of *E. cava* showed the highest extraction yield (41.52%) among the tested enzymatic hydrolysates (Heo et al., 2003). AMG is able to digest 1, 4 and 1, 6-α linkages of the plant cell wall materials. The rate of hydrolysis depends on the type of linkage and on chain length. Especially, AMG hydrolysis 1, 4-α linkages more easily than 1,6-α linkages. Therefore the digestion of alga by enzymes increase the extractable polysaccharide and meantime release polysaccharide attached polyphenolic compounds to the medium.

According to our preliminary studies, polysaccharide and polyphenolic compounds are highly possible bio-active principals present in *E. cava*. Therefore, in this study, original AMG enzymatic digest was subjected to ethanol precipitation and the resulted precipitate (CpoF) and supernatant (CphF) together with original extract were subjected to evaluate their potential antiproliferative and antiradical effects. Activities of the three fractions were compared with that of commercial counterparts (BHA and BHT).

Especially, the present experiment data suggest that, components within the CphF may have inherent properties that suppress cancer cell proliferation and this may be due to its high amount of polyphenol content. It has been shown that phenolic compounds including phlorotannins can induce oxidative stress in cancer cells and that some or many of their effects seen in vitro may be due to induction of such stress (Reddy et al., 2003). In this study, as a natural antiproliferative agent CopF showed 5.1 μg/ml of IC_{50} value on CT-26 cell, and it dose-dependently enhanced the apoptotic body formation. Therefore the results of the present study strongly suggest that the suppressive effect of CphF on CT-26 cell proliferation is related to apoptotic body formation. Apoptosis gives an idea about the effectiveness of anticancer therapy; some anticancer drugs were reported to show their anticancer activity by inducing apoptosis of cancer cells (Kamesaki, 1998). Apoptosis body formation can be taken place as a result of DNA damage and protein damage. While DNA damage initiate death signaling, protein damage can distort the cell redox homeostasis, which facilitates apoptosis execution. However, the exact underlying mechanisms of the antitumor activity of algae are varied as the chemistry of the various secondary metabolites involved. Such selective antiproliferative activity will may expand future studies. Actually, this is not surprise matter, even in 1534 BC there were records regarding algal treatment for breast cancer patients (Teas, 1981). *P. tenata*, a red alga has been reported for its high anticarciogenic effect. The extracts of this algal species can reduce intestinal tumor incidence in rats (Yamamoto et al., 1986). Also, carotine, lutein and chlorophyll related compounds isolated from algal species have been reported to show strong antimutagenic activity in vitro and in vivo (Okai et al., 1996a). Pheophytin, isolated from *E. prolifera* has been reported to show potent suppressive effect against chemically induced mouse skin tumorigenesis through suppression at initiation and promotional phases (Okaj et al., 1999). Meanwhile, chlorophyll-*a* and chlorophyllin-*a* also have exhibited significant suppression against the induction of ornithine decarboxylase in mouse skin fibroblasts caused by a tumor promoter using in vitro cell culture experiments (Okai et al., 1996b).

As it has been previously reported, the total carbohydrate content of the original *E. cava* sample is 68.42% (Heo et al., 2003). In this study, CpoF separated from AMG hydrolysate of *E. cava* showed a potential antiproliferative activity. This fraction mainly constituted with polysaccharides (Fig. 5B). Recently, we isolated fucoidan as the active principal of CpoF for its antiproliferative activity. The highly sulfated (0.92 sulfate/total sugar) polysaccharide was mainly composed with fucose and small amount of galactose (unpublished data). The ability of algal polysaccharides to induce tumor cell proliferation has been well documented. Moreover, polysaccharides isolated from Japanese seaweeds like *Laminaria japonica*, *Undaria pinnatifida*, *Eisenia bicyclis* and *Hijikia fusiforme* were identified as potential anti-genotoxic substances (Okai et al., 1993; Okai and Higashi-Okai, 1994).

The positive correlation between polyphenolic content of alga and its antioxidant activity is well documented (Yen and Duh, 1993; Siriwardhana et al., 2003; Karawita et al., 2005). Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. In this study also CphF showed a remarkable antioxidant activity, this might be due to its high polyphenolic content. The CphF at 5 μg/ml showed higher DPPH radical and reducing power activity, which is much higher than those of BHA and BHT at same concentration.

Phloroglucin one of tannins isolated from algae showed high antioxidative activity (Yamada, 2000). Furthermore, pheophytin is the main antioxidative compound present in *Enteromopha* species (Nishibori and Namiki, 1988). Phlorotannins from brown algae *E. kurome* and *Eiseuia bicyclis* were showed antiplasmin, hyaluronidase and antioxidant activities (Nagayama et al., 1989). In addition, phlorotannins deter feeding or inhibit growth of a variety of marine herbivores as chemical defenses (Targett et al., 1995). The crude phlorotannins isolated from *E. kurome* were composed of phloroglucinol (2%), eckol (9%), phlorofucofuroeckol A (28%), dieckol (24%), 8,8 V-7 biekcol (7%) and others (30%); it was determined by high performance liquid chromatography (Nagayama et al., 2003). Therefore, similar kind of compound/compounds may be associated with the present experiment results. Those kinds of phenolic compounds show antioxidant activity due to their redox properties, which play an important role in absorbing and neutralizing free radicals quenching singlet and triple oxygen or decomposing peroxide.

Although, CpoF was less effective in most of the tested assays the fraction could considerably control cancer cell proliferation and radical production in vitro. Recently, a considerable number of scientists have been reported

antioxidant and anticancer activities with sulfated polysaccharides isolated from marine alga (Rupérez et al., 2002). Specially, sulfated polysaccharides from *Fucus vesiculosus*, *Laminaria japonica* and *Ecklonia kurome* were demonstrated to have good antioxidant activity (Hu et al., 2001). Furthermore, porphyran, a sulfated polysaccharide isolated from porphyra (Rhodophyta) have been reported to delay aging process in mice by enhancing the amount of antioxidative enzymes and thereby reduce the risk of lipid peroxidation (Zhang et al., 2003). Polysaccharides promote antioxidant activity because polysaccharides exhibited the grater ease of abstraction of the anomeric hydrogen from the internal monosaccharide units.

Therefore, algal polysaccharides of *E. cava* are also may be a possible active principle for its high antioxidant activity. Therefore both CphF and CpoF fractions are separately being investigated to isolate active compounds and due to its high activity these compounds may be useful to replace commercial antioxidants.

In total antioxidant assay the effect of CphF at 50 μg/ml was equivalent or slightly higher than those of commercial counterparts at 5 μg/ml concentration. Algal polyphenolic compounds are effective antioxidants to delay oil rancidity. Polyphenols easily transfer a hydrogen atom to lipid peroxil cycle and form the aryloxyl, which being incapable of acting as a chain carrier, couples with another radical thus quenching the radical process (Ruberto et al., 2001). In accordance with the result, Yan, 1996 reported phlorotanin isolated from *Sargassum kjellmanianum* could protect fish oil from rancidity.

Even though, commercial compounds like BHA, BHT and related derivatives are most frequently use as synthetic antioxidants to retard lipid oxidation. However BHT do not show a good antioxidative efficacy in fish oils (Ke et al., 1977), moreover BHT and BHA show bad side effects (lesion formation, hemorrhage etc) at high doses in mice and severe enough to cause death in some strains of mice (Shahidi and Wanasundhara, 1992). Also, it has been suggested that α-tocopherol, a natural antioxidant may act as a pro-oxidant at high dosages (Young and Min, 1990), and especially α-tocopherol is very weak in heat. Therefore, as an alternative the CphF have several advantages over commercial counterparts.

The active compounds of the enzymatic hydrolysate of *E. cava* seem to be much thermal stable. Also, the activities of the enzymatic hydrolysate were stable even after heating 10 min at 100 °C. Therefore, thermal stability of the hydrolysate provides some added advantages for its use in different food formulations, in where high cooking temperature adept during the processing. Taken together, the crude enzymatic fractions showed strong antioxidant activities compared to those of BHA and BHT. Also, tested enzymatic extracts showed high antiproliferative ability in vitro. The systematically performed in vitro assays of this study reveal that the tested enzymatic hydrolysate may find in therapy as agent with high pharmaceutical value. Therefore both CphF and CpoF are separately being investigated to purify relevant biological active compounds.

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