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Full Length Research Paper

In vitro evaluation of antioxidant activities of free and bound phenolic compounds from *Posidonia oceanica* (I.) Delile leaves

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The contents of phenolics, flavonoids and proanthocyanidins were determined in FPC and BPC extracted from *Posidonia oceanica* leaves. High phenolic content was obtained in both FPC and BPC (328.00 and 407 mg/g, respectively). Flavonoids were mainly detected in BPC (94.4 mg quercetin equivalent/g). However, the amount of flavonoids in FPC was 2 times lower. The amount of proanthocyanidins was slightly higher in FPC than in BPC (93 versus 73 mg catechin equivalent/g). The highest DPPH and O_2^- scavenging activities (IC_{50} = 10.71 µg/ml and 27.93 µg/ml, respectively) and highest reducing power (IC_{50} = 9.4 µg/ml) were found in BPC. Inhibition of lipid peroxidation was detected in FPC, as measured by the ferric thiocyanate (IC_{50} = 2.08 µg/ml) and the β-carotene-linoleic acid (IC_{50} = 33.33 µg/ml) assays. Noteworthy, we observed low Fe^{++}–chelation and OH-scavenging activity in both FPC and BPC extracts. These findings indicated that, *P. oceanica* leaves could be used as a natural preservative ingredient in food and/or added to fish and marine products to prevent lipid peroxidation.

Key words: *Posidonia oceanica*, natural antioxidant, phenolic compounds, Mediterranean Sea.

INTRODUCTION

Reactive oxygen species (ROS) produced by ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have numerous pathological effects, causing lipid peroxidation, protein oxidation, DNA damage and cellular degeneration related to cardiovascular and inflammatory diseases, aging, cancer and a variety of other disorders (Knight, 1995). ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH). In cellular oxidation reactions, the superoxide radical is initially formed and its effects can be further magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Among ROS, the hydroxyl radical (OH) is the most harmful because it oxidizes all classes of biological macromolecules (Hsu, 2006).

In recent years, the search for novel types of antioxidants from several plant sources has received considerable attention. The protective effects of antioxidants with reference to health are derived from their ability to (1) scavenge free radicals by acting as hydrogen/electron donors or by directly reacting with them; (2) chelate transition-metal ions (thus, preventing the formation of free radicals as a result of the Fenton reaction); (3) inhibit free radical-producing enzymes such as cyclooxygenase, lipoxygenase and NADPH oxidases or increase the expression of antioxidant enzymes, such as superoxide

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Abbreviations: As, Ascorbic acid; **BHT,** butylated hydroxytoluene; **BPC,** bound phenolic compounds; **CAT,** catalase; **CE,** catechin; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **FPC,** free phenolic compounds; **GAE,** gallic acid; **GPX,** glutathione peroxidise; **NADH,** nicotinamide adenine dinucleotide-reduced; **NBT,** nitro blue tetrazolium; **PMS,** phenazine methosulfate; **QE,** quercetin; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase.

dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Ozsoy et al., 2008; Michalak, 2006).

Posidonia oceanica (L.) Delile is a marine phanerogam endemic to Mediterranean Sea where it forms vast meadows from the water surface to depths up to 40 m and it occupies an area of approximately 50 km^2 (Maserti et al., 1991). Meadows are highly productive ecosystems because they produce high amounts of oxygen and organic compounds, sustain complex food webs and act as a nursery/refuge for several species. They also play a crucial role in coastal preservation by stabilizing sediments and reducing hydrodynamic effects (Pirc, 1985). Due to its wide distribution throughout the Mediterranean basin and its ability to react to environmental changes, *P. oceanica* is used as a bioindicator of ecosystem stresses, such as anthropogenic pressures, interspecific competition and metal pollution (Pergent-Martini, 1998).

This phanerogam synthesizes various compounds, such as amino acids (Augier and Santimone, 1979), metallothioneins (Cozza et al., 2006), carbohydrates (Invers et al., 2004), fatty acids (Viso et al., 1993) and sterols (Sica et al., 1984). *P. oceanica* is also able to produce polyphenolic compounds playing an important role in the protection of plants against competitors, predators and pathogens (Cuny et al., 1995). In addition, they contain high level of antioxidant enzymes, such as SOD, CAT and GPX (Ferrat et al., 2002).

P. oceanica extract was shown to be active against various selected bacteria, dermatophytes and yeasts (Bernard et al., 1988) besides its antileishmanial activity (Orhan et al., 2006). More recently, antidiabetic and vasoprotective properties of *P. oceanica* extract were evaluated *in vivo* (Gokce and Haznedaroglu, 2008). *P. oceanica* extract exerted a protective and dose-dependent effect on alloxan-induced drop in antioxidant activities**.**

The aim of the present study is to screen *P. oceanica* for the presence of polyphenolic and antioxidants compounds that could be used as food additives and preservatives for fisheries-derived products. FPC and BPC as well as total phenolic content from *P. oceanica* leaves were evaluated and assayed *in vitro* for their ROS–scavenging activities and their ability to inhibit lipid peroxidation.

MATERIALS AND METHODS

Plant material

P. oceanica samples were collected from the northern Tunisian coast (60 km from Tunisia) in November 2007 and identified at the Biotechnology Center of Borj Cedria, where a voucher specimen is preserved.

Preparation of samples

The leaves of *P. oceanica* were lyophilized and used for the extraction of FPC and BPC. Extraction of soluble FPC was conducted as reported previously by Chu et al. (2002) with minor modifications Dry powder (2 g) was solubilized in methanol-water (80:20, v/v), sonicated and homogenized at room temperature for 1 h 30 min. The solution was filtered through Whatman filter paper using a Buchner funnel under vacuum. The filtrate was then evaporated using a rotary evaporator (Bibby RE-200B) under vacuum at 40°C to obtain the FPC extract.

BPC was extracted according to the method of Krygier et al. (1982) with minor modifications. Briefly, residues retained by the filter paper were dried and hydrolyzed with 4 M NaOH at room temperature under shaking. The mixture was acidified to pH 2 with concentrated HCl, extracted four times with ethyl acetate, pooled and evaporated at 40°C to dryness under vacuum to yield BPC extract. The yield of extraction was calculated as follows:

$$(DWe/DWs) \times 100 \quad (1)$$

Where, DWe is the dry weight of sample extract after evaporation of solvent and DWs, the weight of the dry powder.

Determination of total phenolics, flavonoids and condensed tannins

Total content of phenolic compounds of *P. oceanica* extracts was determined by the Folin-Ciocalteau method (Singleton and Rossi, 1965) using gallic acid as standard. Test sample (50 µl) was mixed with 125 µl of 0.2 M Folin-Ciocalteau reagent, 125 µl of 16% (w/v) Na_2CO_3 and 700 µl of bidistilled water. After 1 h incubation at room temperature, the absorbance was measured at 760 nm (UV-visible spectrophotometer Cary 100 Conc, Varian). Results are expressed as mg gallic acid equivalent (GAE) per g extract. Total flavonoid content was determined according to the method of Dewanto et al. (2002) with minor modifications, using quercetin as standard. Sample (20 µl) was mixed with 30 µl of 5% (w/v) $NaNO_2$ solution for 6 min, before addition of 60 µl of freshly prepared 10% (w/v) $AlCl_3$. $6H_2O$. After 5 min, 200 µl of 1 M NaOH and 690 µl of bidistilled water were added and absorbance determined at 510 nm. Results are expressed as mg quercetin equivalent (QE) per g extract. Proanthocyanidins were measured according to the vanillin assay described by Sun et al. (1998) using catechin as standard. Sample (30 µl) was mixed with 540 µl 4% (w/v) methanol-vanillin solution and 43 µl of concentrated H_2SO_4. After 15 min, the absorbance was measured at 500 nm. Results are expressed as mg catechin equivalent (CE) per g extract and assays done in triplicate.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The DPPH free radical–scavenging activity of *P. oceanica* extracts was evaluated according to Hsu (2006), with minor modifications. Various extract concentrations of 1, 5, 10, 50, 100, 200 and 300 µg/ml were prepared in methanol. A 50 µl aliquot was mixed with 200 µl of 0.1 M DPPH dissolved in methanol and 100 µl of Tris-HCl buffer (50 mM, pH 7.4) at room temperature for 30 min. The decrease in DPPH radical was measured by recording the absorbance at 517 nm with L-ascorbic acid (As) as positive control. Assays were performed in triplicate. The inhibition ratio was calculated from the following equation:

$$\text{Scavenging activity (\%)} = ((A_0 - A_1)/A_0) \times 100 \quad (2)$$

Where, A_0 is the absorbance of the blank and A_1, the absorbance of the extract or of L-ascorbic acid. For each sample, antioxidant activity was expressed by the concentration required to inhibit DPPH radical formation by 50% (IC_{50}).

Measurement of reducing power

The reducing power of crude extracts was determined according to Oyaizu (1986). 50 µl of various concentrations of the sample (5, 10, 50, 100, 200, 300 and 600 µg/ml) was mixed with 50 µl of phosphate buffer (0.2 M, pH 6.6) and 50 µl of 1% (w/v) $K_3Fe(CN)_6$ and the mixture incubated at 50°C for 20 min. After cooling, the reaction was stopped by the addition of 50 µl of 10% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 650 x g for 10 min. From the upper layer, 200 µl were mixed with 200 µl bi-distilled water and 40 µl of 0.1% (w/v) $FeCl_3$ and incubated for 10 min at room temperature and absorbance measured at 700 nm. The reducing power of vitamin C was used as positive control. Increased absorbance indicated stronger reducing power. Assays were performed in triplicate.

Superoxide anion radical-scavenging activity

The superoxide anion radical (O_2^-) scavenging activity was assayed by the method of Nishikimi et al. (1972) with minor modifications. The reaction mixture, containing test samples (final concentrations were 20, 40, 60, 80, 100, 120, 140 and 160 µg/ml, respectively), 30 µM phenazine methosulfate (PMS), 338 µM reduced-nicotinamide adenine dinucleotide (NADH) and 72 µM nitro blue tetrazolium (NBT) in phosphate buffer (0.1 M, pH 7.4), was incubated at room temperature for 5 min and absorbance measured at 560 nm. A synthetic antioxidant, butylated hydroxytoluene (BHT), was used as positive control. Assays were performed in triplicate. The inhibition ratio was calculated according to the equation:

$$\text{Scavenging activity (\%)} = ((A_0 - A_1)/A_0) \times 100 \quad (3)$$

Where, A_0 is the absorbance of the blank and A_1, the absorbance of the extract or BHT.

Ferrous ion chelating activity assay

The iron-chelating ability of *P. oceanica* extracts was determined according to Dinis et al. (1994). A 300 µl aliquot of each extract (final concentrations ranging from 0.1 to 4 mg/ml) was mixed with 25 µl of 2 mM ferrous chloride tetrahydrate ($FeCl_2.4H_2O$). The reaction was initiated by adding 50 µl of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as positive control and the chelating activity towards ferrous ion was calculated using the following equation:

$$\text{Chelating effect (\%)} = ((A_0 - A_1)/ A_0) \times 100 \quad (4)$$

Where, A_0 and A_1 are the absorbances of the control and the sample, respectively. Assays were carried out in triplicate.

Hydroxyl radical-scavenging activity

The hydroxyl (OH) radical–scavenging activity of *P. oceanica* extracts was measured according to Halliwell et al. (1987). Samples (final concentrations of 50, 100, 250, 500 and 1000 µg/ml) were added to the reaction mixture containing 100 µl of 30 mM deoxyribose, 100 µl of 1 mM $FeCl_3$, 100 µl of 1.04 mM EDTA, 100 µl of 1 mM ascorbic acid and 100 µl of 10 mM H_2O_2 in phosphate buffer (20 mM, pH 7.4) in 1 ml final volume. Incubation was carried out for 1 h at 37°C and the reaction stopped by addition of 1 ml 2.8% (w/v) TCA and 1 ml 1% (w/v) thiobarbituric acid. The mixture was boiled for 10 min, cooled and absorbance measured at 532 nm. BHT was used as positive control. Methanol was used as blank and the sample solution without deoxyribose as sample blank. The inhibition ratio was calculated from the following equation:

$$\text{Scavenging activity (\%)} = ((A_0 - (A_1 - A_2)/A_0) \times 100 \quad (5)$$

Where, A_0, A_1, and A_2 are the absorbances of the blank, extract (or BHT) and the sample blank, respectively at 532 nm.

Antioxidant activity in a linoleic acid system

The antioxidant capacity of the extracts with reference to inhibition of linoleic acid peroxidation was assayed using the ferric thiocyanate (FTC) method (Yen et al., 2003). A linoleic acid emulsion was prepared with 0.2804 g linoleic acid and 0.2804 g tween 20 in 50 ml of phosphate buffer (0.2 M, pH 7). A reaction solution, containing 125 µl of extract sample (final concentrations of 1, 10, 50, 100, 200 and 300 µg/ml), 625 µl of linoleic acid emulsion and 500 µl of phosphate buffer (0.2 M, pH 7) was prepared. The reaction mixture was incubated at 37°C in the dark to accelerate the oxidation process and samples were withdrawn every 24 h, up to 5 days, for evaluation of their oxidative capacity. 2 ml methanol, 20 µl of 30% (w/v) ammonium thiocyanate, 20 µl of sample and 20 µl of $FeCl_2$ (20 mM dissolved in 3.5% (v/v) HCl) were added successively. The peroxide value was determined by measuring the absorbance at 500 nm. The inhibiting effect of BHT on linoleic acid peroxidation was also assayed within the same range of concentrations. The reaction solution without plant extracts or BHT was used as blank. Assays were carried out in triplicate. The percentage of inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = ((A_0 - A_1) / A_0) \times 100 \quad (6)$$

Where, A_0 and A_1 are the absorbances of the blank and extract (or BHT), respectively at 500 nm.

Antioxidant activity by β-carotene-linoleic acid assay

The antioxidant activity of *P. oceanica* extracts was evaluated by the β-carotene bleaching method (Mi-Yae et al., 2003). A β-carotene solution was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform. 40 mg of linoleic acid and 400 mg of tween 40 were mixed with 4 ml of β-carotene solution. After removal of chloroform at 40°C under vacuum, 100 ml of oxygenated water was added and the mixture vigorously shaken. A 3 ml aliquot was mixed with 250 µl of phenolic extract (final concentrations of 1, 20, 40, 60, 80 and 120 µg/ml) and the mixture was disposed in a water bath at 50°C for 2 h. After cooling, the absorbance was measured at 470 nm at t = 0 and after 2 h of incubation with BHT as positive control. Assays were performed in triplicate. The antioxidant activity of the extract was evaluated by the photo-oxidation of β-carotene using the following formula:

$$\text{Antioxidant activity} = (A_1/A_0) \times 100 \quad (7)$$

Where, A_1 is the absorbance of β-carotene after 2 h of incubation and A_0, the absorbance of β-carotene at t = 0.

Statistical analysis

Data were expressed by mean ± SD. Statistical analysis was carried out by analysis of variance (ANOVA) and by Student's t-test. p value of 0.05 or less was considered significant.

Table 1. Content of total phenolics, flavonoids and proanthocyanidins in FPC and BPC extracts from *P. oceanica* leaves.

Sample	Yield (%)	Total phenolics (mg GAE/gextract)	Flavonoids (mg QE/g extract)	Proanthocyanidins (mg CE/g extract)
FPC	7.45	328.00 ± 12.20[b]	44.80 ± 1.60[b]	93.00 ± 1.40[a]
BPC	1.25	407.00 ± 18.30[a]	94.40 ± 5.60[a]	70.00 ± 0.50[b]
Total	8.75	735.00 ± 29.50	139.20 ± 7.90	163.00 ± 1.92

The content of total phenolics is expressed as mg gallic acid equivalents (GAE) per g extract; the content of flavonoids is expressed as mg quercetin equivalents (QE) per g extract; the content of proanthocyanidins is expressed as mg catechin equivalents (CE) per g extract.
Values are expressed as mean ± standard deviation (n=3).
Values followed by superscript letters in each column differ significantly (Student's t-test $p < 0.05$).

RESULTS AND DISCUSSION

Total phenolics, flavonoids and proanthocyanidins content

Table 1 reported the yield extract obtained for FPC and BPC isolated from *P. oceanica* leaves. The highest value (7.4%) was observed for FPC, whereas BPC yield was 7 times lower (1.25%). Gokce and Haznedaroglu (2008) obtained a 2% value by using the procedure proposed by Sauvesty and Page (1992) for the extraction of phenolic compounds from *P. oceanica*. They emphasized the dependence towards several factors such as season of harvesting, environment and extraction procedure. Extracts from *P. oceanica* leaves contained high level of total phenolics (735 ± 29.50 mg GAE/g), in which BPC accounted for 407 ± 18.30 mg GAE/g, representing 55.37% of the overall polyphenol content. Variations in phenolic content between FPC and BPC extracts were also observed for other plants. For instance in beer, BPC content was 4 to 6 times higher than that of the corresponding FPC (Szwajgier et al., 2005b). In contrast, BPC accounted for only a minor fraction of the total phenolic content in pak choi, broccoli and carrots (Beveridge et al., 2000).

Flavonoids represent 18.9% of total phenolic content and the highest level (94.4 ± 5.60 mg QE/g) was found in the BPC extract which is 2 times higher than that of the FPC extract. Proanthocyanidins represent 22.17% of total phenolics. FPC extract exhibited the highest content (93 ± 1.40 mg CE/g) when compared with BPC extract, which is 1.3 times lower.

Up till now, only limited studies on proanthocyanidins and flavonoids of *P. oceanica* have been carried out. Thus, four flavonols have been identified in *P. oceanica* leaves: myrcetin, quercetin, isorhamnetin and kaempferol (Cannac et al., 2006) which have also been observed in other terrestrial plant, such as *Pinus halepensis* (Kaundun et al., 1998). Various phenolic compounds from *P. oceanica* leaves infused in 50% ethanol at 40°C and extracted with ethyl acetate have been previously described (Cuny et al., 1995; Agostini et al., 1998). Their level depends mainly on the plant metabolism and on other parameters such as depth, season or location. Cuny et al. (1995) and Agostini et al. (1998) reported ferulic acid as the major compound whereas, Haznedaroglu and Zeybek (2007) found that gentisic acid was mainly represented both in young (156.8 µg/g dry) and mature (395.0 µg/g) leaves.

DPPH radical-scavenging activity

DPPH is a lipophile radical that could be reduced by donation of either hydrogen or electrons. Figure 1 shows the scavenging effect of the phenolic compounds from *P. oceanica* and ascorbic acid on DPPH free radicals. Both BPC and FPC extracts showed a strong ability to quench DPPH radicals. The scavenging effect increased with concentrations up to 30 µg/ml and remained constant between 30 to 250 µg/ml. BPC exhibited the highest DPPH–scavenging activity, as indicated by its lower IC_{50} value (10.71±0.001 µg/ml, Table 2). The antiradical activities of BPC (10.71 ± 0.001 µg/ml) and FPC (17.63l ± 0.09 µg/ml) was respectively 3 and 5 fold lower than that of ascorbic acid (3.53 ± 0.02 µg/ml). However, the DPPH–scavenging capacities of both FPC and BPC extracts of *P. oceanica* leaves are more efficient than found in several marine algae, such as *Avrainvillea longicaulis* (IC_{50} = 1.44 mg/ml), *Padina gymnospora* (IC_{50} = 3.45 mg/ml) and *Chondria baileyana* (IC_{50} = 2.84 mg/ml) (Zubia et al. 2007), which emphasize the relevance of *P. oceanica* leaves as a 125 abundant source of polyphenolic compounds.

Reducing power measurement

The reducing power of BPC and FPC extracts was dose-dependent within the concentration range of 0 to 250 µg/ml (Figure 2). Both BPC and FPC extracts showed a strong reducing power reaching 3.41 and 2.23 µg/ml, respectively, when tested at 227 µg/ml. Moreover, the BPC and FPC extracts showed high EC50 values of 9.40 ± 0.01 and 8.73 ± 1.06 µg/ml, respectively, when compared with ascorbic acid (1.58 ± 0.09 µg/ml; Table 2).

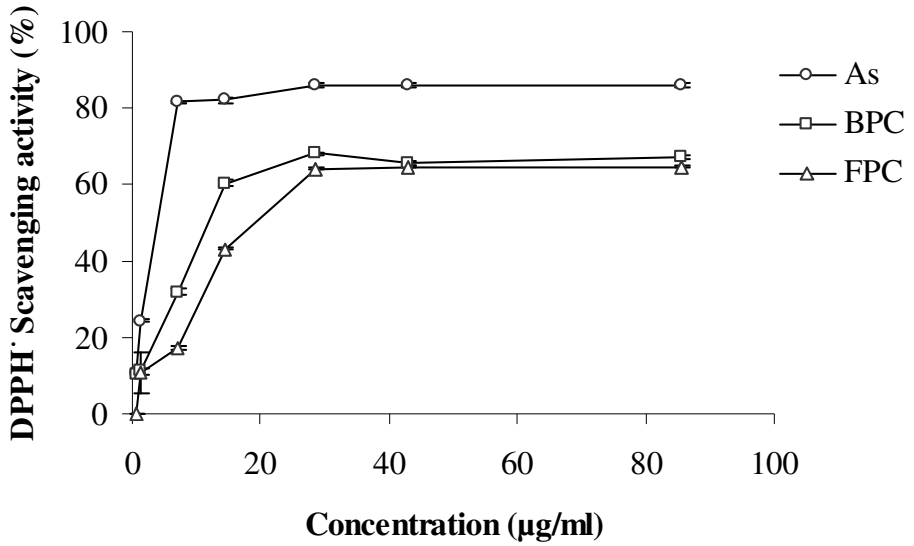

Figure 1. Scavenging effect of FPC and BPC extracts of *P. oceanica* on DPPH radicals. Each value is expressed as the mean ± standard deviation (n = 3).

Table 2. Comparison of the IC_{50} values of BPC and FPC extracts with standard antioxidants.

IC50 (µg/ml)	Samples			
	FPC	BPC	As	BHT
DPPH· scavenging activity	17.63 ± 0.09^{c}	10.71 ± 0.001^{b}	3.53 ± 0.02^{a}	
Reducing power	8.73 ± 1.06^{b}	9.40 ± 0.01^{b}	1.58 ± 0.09^{a}	
O_2^{-}· scavenging activity	66.80 ± 0.50^{c}	27.93 ± 1.85^{b}		11.20 ± 0.21^{a}
OH· scavenging activity	404 ± 0.47^{c}	276 ± 0.40^{b}		12 ± 0.79^{a}
Linoleic acid peroxidation*	2.08 ± 0.17^{b}	2.97 ± 0.30^{c}		0.91 ± 0.11^{a}
β-carotene bleaching	33.33 ± 0.71^{a}	54.73 ± 0.85^{b}		62.93 ± 0.70^{c}

IC_{50} value is the effective concentration at which DPPH, O_2^{-} and OH, radicals were scavenged, linoleic acid peroxidation and β-carotene bleaching inhibited by 50% or the absorbance was 0.5 at 700 nm for reducing power.
*Measured at 72 h.
Values are expressed as mean ± standard deviation (n = 3).
Values followed by superscript letters in each line differ significantly (Student's t-test $p < 0.05$).

The reducing properties of plant extracts are generally associated with the presence of reductones (Pin-Der-Duh, 1998) which have been shown to exert an antioxidant action by breaking the free-radical chain through donation of a hydrogen atom (Gordon, 1990). Reductones are also reported to react with various precursors of peroxides, thus, preventing their generation.

Superoxide anion radical-scavenging activity

The superoxide radical–scavenging capacity of the phenolic compounds of *P. oceanica* extracts was measured by the PMS–NADH superoxide generating system. Both extracts exhibited a significant scavenging activity on superoxide radicals in a dose-dependent manner (Figure 3), although, the scavenging effect of BPC extract was higher than that of FPC whatever the concentration (20 to 160 µg/ml). Moreover, no difference ($p > 0.1$) was observed between the superoxide radical–scavenging capacity of the BPC extract and BHT for concentrations up to 100 µg/ml. Although, superoxide is a relatively weak oxidant, it decomposes to more harmful species such as singlet oxygen and hydroxyl radicals, which initiate lipid peroxidation. Furthermore, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Qi et al., 2005). In the present study, BPC and FPC effectively scavenged superoxide with IC50 values of 27.93 ± 1.85 and 66.80 ± 0.50 µg/ml, respectively (Table 2). These values are respectively 3 and 6 times higher than found for BHT (11.20 ± 0.21 µg/ml). The strong antioxidant activity of the BPC extract might be related to its high flavonoid content. In this regard, Robak and Gryglewski (1988) showed that the antioxidant properties of flavonoids mainly beared on their scavenging property

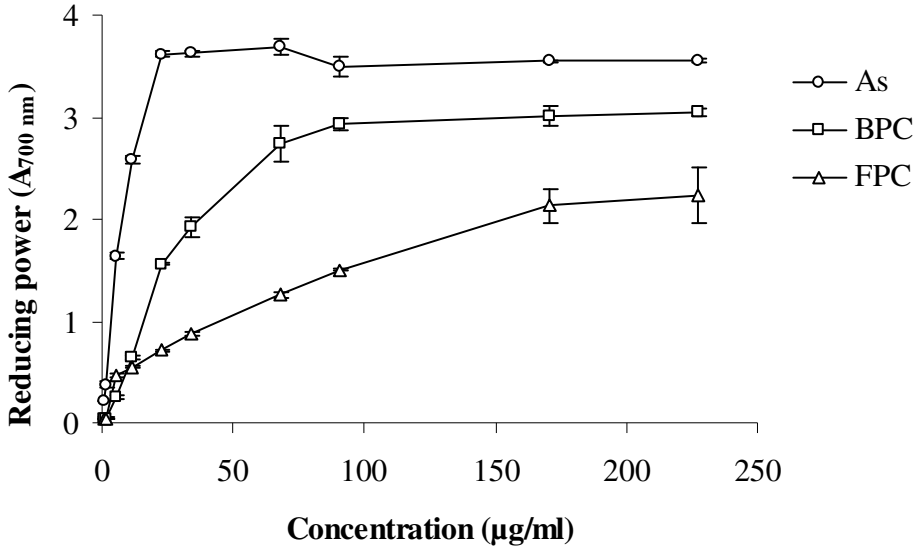

Figure 2. Reducing power of FPC and BPC extracts of *P. oceanica*. Each value is expressed as the mean ± standard deviation (n = 3).

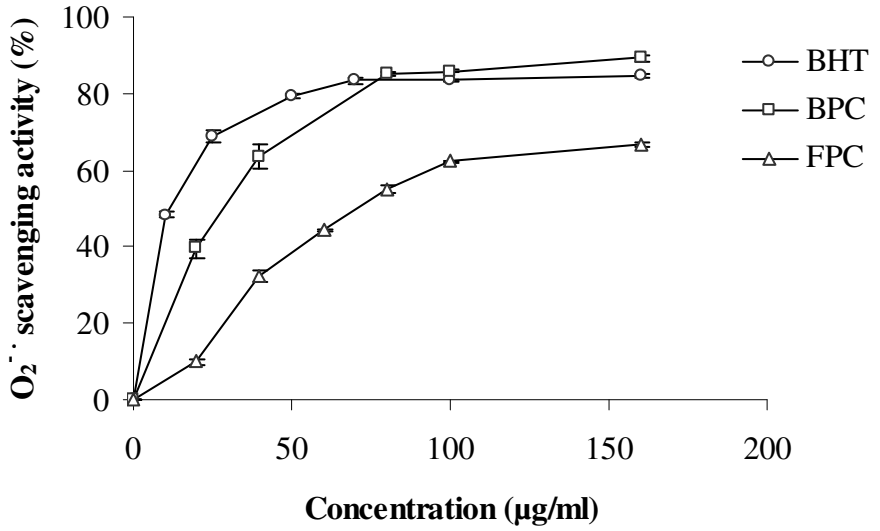

Figure 3. Superoxide anion scavenging activity of FPC and BPC extracts of *P. oceanica*. Each value is expressed as the mean ± standard deviation (n = 3).

towards superoxide anion. The IC50 of the BPC extract was close to that of marine algae *Laminaria japonica* which exhibited an IC50 value of 26.6 µg/ml (Wang et al., 2008).

Ferrous ion chelating activity

Iron (II)-chelators are important antioxidative agents as they retard metal-catalyzed oxidation (Kehrer, 2000). They also afford protection against oxidative damage by removing iron (II) which participates in Fenton-type reaction. Thus, minimizing iron (II) may afford protection against oxidative damage by inhibiting the production of harmful ROS and lipid peroxidation. The iron (II) chelating capacity of the FPC and BPC extracts was measured using the ferrozine method and data are summarized in Figure 4. At concentrations below 1.5 mg/ml, the samples showed dose-dependent chelating effect reaching 33.27 and 45.22% for FPC and BPC, respectively. At concentrations above 2 mg/ml, the antioxidant ability of FPC and BPC declined reaching a lower level than EDTA used as positive control. Previous studies showed that, the chelating effect of *L. japonica* was also independent from concentration (Wang et al., 2008). According to Chew et al. (2008), *Caulerpa racemosa* and *Kappaphycus alvarezzi*

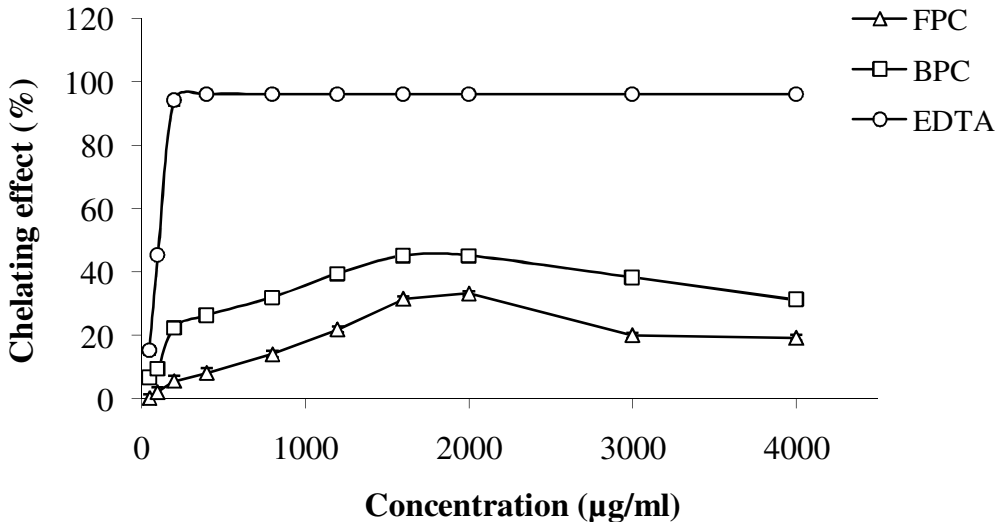

Figure 4. Fe^{++} chelating activity of FPC and BPC extracts of *P. oceanica*. Each value is expressed as the mean ± standard deviation (n = 3).

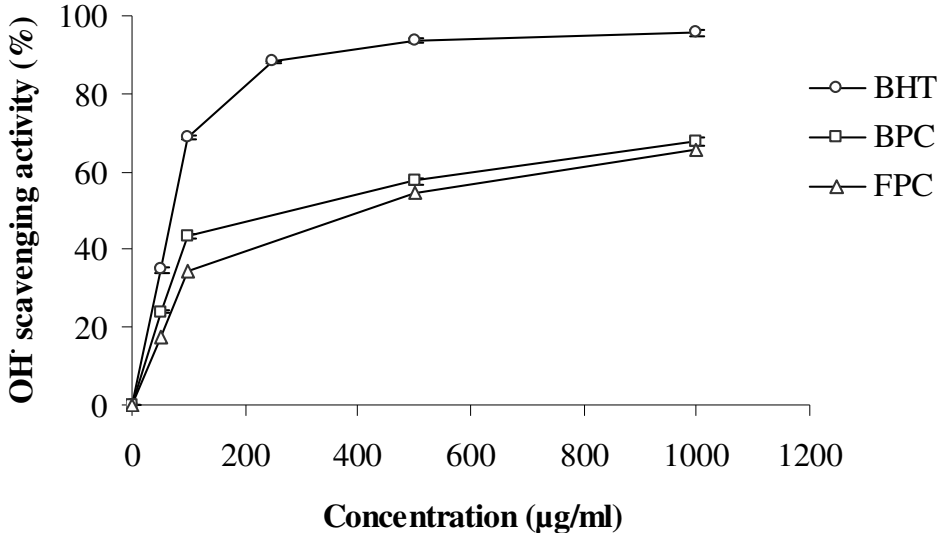

Figure 5. Hydroxyl scavenging activity of FPC and BPC extracts of *P. oceanica*. Each value is expressed as the mean ± standard deviation (n = 3).

also had a moderate chelating ability, which did not exceed 30% at 7 mg/ml. It is generally recognized that the metal-chelating ability of polyphenols is related to the presence of orthodihydroxy polyphenols, bearing either catechol or galloyl groups (Khokhar and Apenten, 2003; Moran et al., 1997).

Hydroxyl radical-scavenging activity

The OH radical is the most toxic ROS as it can damage almost all vital macromolecules. The scavenging ability of FPC and BPC extracts towards OH radicals, shown in Figure 5, is dose-dependent, reaching 65.49 and 67.58%, respectively at 1 mg/ml. The IC_{50} values for FPC and BPC extracts were 404 ± 0.47 and 276 ± 0.40 µg/ml, respectively ($p < 0.05$; Table 2). BHT, used as positive control, was highly effective in quenching the OH radical (IC_{50} = 12 ± 0.79 µg/ml). Several studies used the deoxy-ribose system to assess the biological activity of various natural plant-derived biomolecules. Smith et al. (1992) reported that molecules which are able to chelate iron might have scavenging ability on OH radicals. In the present study, as both FPC and BPC had weak ferrous ion-chelating power and low hydroxyl–scavenging activity, their use in preventing DNA damage is unlikely.

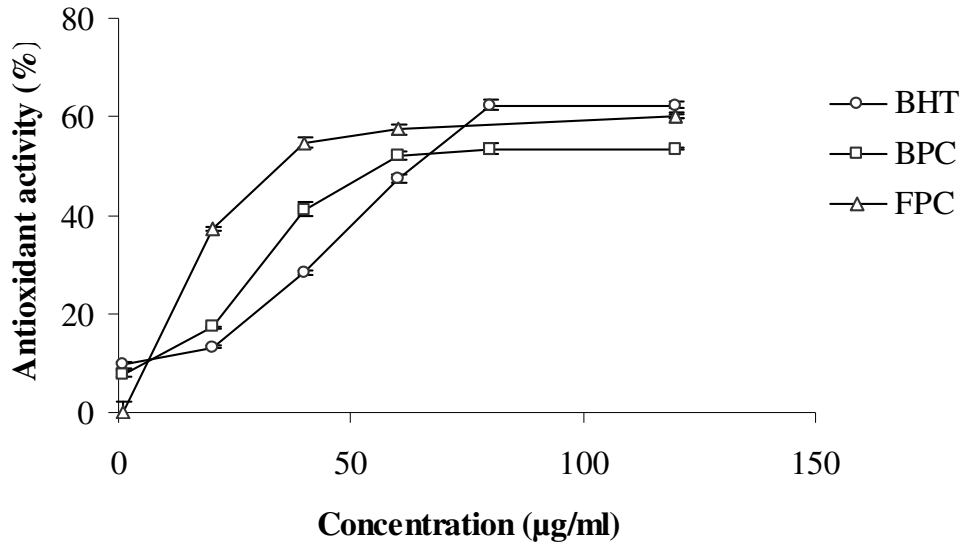

Figure 6. Antioxidant activity of FPC and BPC of *P. oceanica*, determined by the β-carotene bleaching method. Each value is expressed as the mean ± standard deviation (n = 3).

Antioxidant activity in a linoleic acid system

Lipid peroxidation occurring in food products deteriorates their quality, resulting in rancidity, unacceptable taste and shorter shelf life. The inhibitory effect of FPC and BPC extracts (1 to 300 µg/ml) on the peroxidation of linoleic acid was determined and compared with BHT. Both extracts reduced the peroxidation level indicating the effectiveness of the inhibition of linoleic acid peroxidation by FPC and BPC extracts, among which FPC extract exhibited the highest antioxidant activity. Thus, at 10 µg/ml, FPC and BPC extracts showed 80 and 75% inhibition of lipid peroxidation after 72 h of incubation (data not shown) whereas, BHT exhibited 89% inhibition. In addition, FPC and BPC extracts showed higher IC_{50} values of 2.08 ± 0.17 and 2.97 ± 0.30 µg/ml, respectively, when compared with BHT (0.91 ± 0.11 µg/ml) (Table 2). These data suggest that, the antioxidant compounds from FPC extract are more effective as chain-breaking molecules, rather than as reductors, whereas those from BPC extract have good reductive and chain-breaking capacities. These results are in agreement with those of marine alga *Lobophora variegate* previously reported by Zubia et al. (2007).

Antioxidant activity by β-carotene-linoleic acid assay

In the β-carotene bleaching assay, oxidation of linoleic acid produces hydroperoxides which attack β-carotene molecules and cause a rapid discoloration of the solution whereas; antioxidants prevent β-carotene bleaching. The effect of BPC and FPC extracts is shown in Figure 6. The antioxidant activity of FPC and BPC extracts increased versus doses. At concentrations below 80 µg/ml, the antioxidant activities of both extracts were higher than BHT. FPC extract showed the highest antioxidant activity with an IC_{50} of 33.33 ± 0.71 µg/ml when compared with BPC and BHT, (54.73 ± 0.85 and 62.93 ± 0.70 µg/ml, respecttively) (Table 2). The strongest antioxidant activity of FPC, mainly containing non polar compounds, is reminiscent of the “polar paradox” phenomenon in which non polar antioxidants show antioxidative activity in emulsion because they concentrate at the lipid/air interface, thus, ensuring a high protection of the emulsion (Porter, 1993). These results obtained *in vitro* are in accordance with recent *in vivo* data showing that *P. oceanica* extracts decrease lipid peroxidation and restore antioxidant enzymes in alloxan-diabetic rats (Gokce and Haznedaroglu, 2008). The present results also indicated that, both FPC and BPC extract exhibited antioxidant activities. Prior studies have shown a higher antioxidant potential of BPC when compared with free phenolics (Porter, 1993). Consequently, the extraction of bound phenolics should be optimized. Most studies have determined the BPC content using alkaline hydrolysis, divided into rapid hydrolysis (from 1 to 4 to 6 h) and long term hydrolysis (more than 16 h) (Maillard and Berset, 1995). Others reported the extraction of bound phenolics using acid hydrolysis (Yu et al., 2001). In contrast, Cannac et al. (2007) showed that different prehandling methods, such as chilling and freeze-drying of *P. oceanica* leaves, considerably altered the amount of flavonoids but not their profile.

Conclusion

In this study, high reducing power as well as strong DPPH and O_2^- scavenging activities were found in FPC

and BPC extracts from *P. oceanica* leaves. Both extracts showed low OH radical scavenging activity. The analyses of lipid-peroxidation inhibitory effect indicated that FPC and BPC exhibited powerful antioxidant activities. Thus, phenolic compounds from *P. oceanica* leaves should be considered as an important source of phenolic compounds that could be used as food preservatives, especially in the prevention of lipid peroxidation that causes food spoilage.

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