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Polysaccharides from *Padina pavonia*: Chemical structural and antioxidant activity

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Abstract: The exopolysaccharide was obtained from *Padina pavonia* by extraction. The polysaccharides were purified by gel permeation chromatography on Sephacryl S-200 column, when three polysaccharide fractions termed P-1, P-2 and P-3 were obtained. The fractions P-1, P-2 and P-3 were purification on the gel permeation chromatography (GPC) column. The average molecular weight of P-1, P-2 and P-3 were determined as 3.3x104, 5.7x104 and $2.5x10^4$ Da, respectively. The monosaccharide of P-1 and P-3 hydrolysate was measured by HPLC, where in rhamnose, fucose, xylose, mannose, glucose, and galactose were identified in the hydrolysate and their ratios were, tr: 35:8.0: 2.0: 5.0: 1.0 and tr: 36: 7.0: 0.0: 5.2: 1.0, respectively. Whoever, P-2 was containing uronic acid as a mannouronic acid and glucouronic acid ad molar ratio of 1.1: 1.0, respectively. The antioxidant activity of the P-1, P-2 and P-3 was evaluated in-vitro by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (RSA). P-3 fraction showed the highest antioxidant activity among the three fractions, with an IC_{50} value of 180 μg ml^{-1}.

Key words: *Padina pavonia*; polysaccharide; purification; structure features; GC-MS, IR, DPPH; antioxidant activity.

INTRODUCTION

Recently, there has been increasing interest in the screening of bioactive compounds from natural sources such as marine organisms. Brown seaweeds are known to be not only as very important resources of food, feed and energy but as also rich sources of structurally diverse polysaccharides with valuable biological activities. Brown seaweeds produce a variety of polysaccharides including alginic acid, laminarans, and sulfated polysaccharides (Vishchuk, *et al*., 2011). While marine algae have traditionally formed part of the oriental diet, especially in Asian-pacific region; their major use in Western countries has traditionally concentrated on the extraction of compounds used by pharmaceutical, cosmetics, and food industries (Ordonez, *et al*., 2010). Nowadays, the field of marine natural products becomes more sophisticated. Seaweeds produce a variety of biologically active components with different structures and interesting functional properties (Amarowicz, *et al*., 2004; Choi *et al*., 2002; Kong, *et al*., 2009; Kim & Bae, 2010; Shibata, *et al*., 2008). The bioactive components of seaweeds include polyphenols, peptides, polysaccharides, etc. Many of these active compounds found to be useful functional ingredients with numerous health benefits. Brown seaweeds belong to a very large group and it is the second most abundant group of seaweeds (Davis, *et al*., 2003; Mestechkina & Shcherbukhin, 2010; Reddy & Urban, 2009). Most brown seaweeds contain the pigment fucoxanthin, which is responsible for the distinctive greenish-brown colour that gives them their name. Brown seaweeds also produce a range of active components including unique secondary metabolites such as phlorotannins and many of which have specific biological activities that give possibilities for their economic utilization. Polysaccharides produced by seaweeds expand the economically important and global industries. Significant amounts of seaweed derived polysaccharides are used in food, pharmaceuticals and other products for human consumption. Thus, the global seaweed polysaccharide industry operates in a highly regulated environment (Renn, 1997). Over the last decade, bioactive sulfated polysaccharides isolated from brown seaweeds have attracted much attention in the fields of pharmacology and biochemistry. Functional polysaccharides such as fucans and alginic acid derivatives produced by brown seaweeds are known to exhibit different biological properties including anticoagulant, anti-inflammatory, antiviral and antitumoral activities (Boisson-Vidal *et al*., 1995; Costa *et al*., 2010; Lee, *et al*., 2008). In the recent years, sulfated polysaccharides, fucoidans have been isolated from different brown algal species such as *Ecklonia cava*, *Ascophylum nodusum,* and *Undaria pinnatifida* (Athukorala, *et al*., 2006; Matou, *et al*., 2002). This gives brown seaweeds great value as potential sources of fucoidans for the development of health promoting natural products. Therefore, as will be discussed in this review, the functional properties of fucoidans from brown seaweeds could be contributed to the development of pharmaceuticals, cosmeceuticals and functional foods. Taken together, this communication focuses on recent efforts in discovering the biological aspects associated with brown algal fucoidans. In addition, some important perspectives on the potential industrial uses of fucoidans for the development of functional ingredients are also discussed and summarized. The present study reports isolation and chemical characterization of polysaccharides present in the *Padina*

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pavonica. Using chemical, chromatographic and spectroscopy methods we have been able to deduce structural features of polysaccharides and biological activity.

MATERIALS AND METHODS

Algae sources:

The brown alga, *Padina pavonia*, collected periodically, during (2010) from red sea (Saudi Arabia). After collection, the seaweeds were thoroughly washed with running water to remove foreign substances, spread air dried and finally ground (Asker *et al*., 2007). Algal powder was depigmented using sequential extraction with petroleum ether and acetone in a Soxhlet apparatus. The unextracted material was placed in a beaker and air dried to yield depigmented algal powder **(DAP).**

Extraction by hydrochloric acid:

Extraction of depigmented algal powder **(DAP)** (5 g) with 0.1 M HCl (1:100, w: v) were conducted at room temperature for 24 h under constant stirring (twice). Separation of the residue from the extract was performed by filtration through a glass filter (G2). The residue was washed with additional distilled water and the wash was collected to maximize polysaccharide recovery. The extracts were combined, dialyzed extensively against distilled water and lyophilized to yield the acid extracted polymer, named P-1.

Extraction by sodium carbonate:

The acid insoluble residue was extracted with 2% Na_2CO_3 (1:100 w: v) at 45–50 ºC for 5 h under constant stirring (twice). The combined extract was carefully acidified with HCl to pH, 1 and the precipitate formed was collected by centrifugation at 5000 rpm for 20 min, washed with water and then dissolved by careful addition of NaOH. The alkaline solution was dialyzed, concentrated and diluted with 4 M $CaCl_2$ solution to make a final concentration of 2% $CaCl_2$. The precipitate formed at this stage was isolated by centrifugation, washed with water and treated with 0.1 M HCl (4 × 50 ml, stirring at room temperature for 2 h). Then it was dissolved in NaOH, dialyzed and finally lyophilized to yield sodium alginate P-2. The supernatant after precipitation with HCl was dialysed and lyophilized separately to give P-3.

Gel permeation chromatography (GPC)):

Each fraction was subjected to gel permeation chromatography on a column (2.5 x 60 cm) of Sephacryl S-200, which was pre-calibrated with standard dextrans (Fluka Chemical Co., Busch, Switzerland), of known molecular weights. The void volume was determined using dextran blue. The column was equilibrated and later eluted with 0.1 M NaCl. An aqueous solution of sample (50 mg) was dissolved in 2.0 ml of 0.1 M NaCl and loaded on to the column bed. Fractions (5.0 ml) were collected and tested for the total carbohydrates by the phenol-H_2SO_4 reagent (Dubois, *et al*., 1956).

Molecular weight determination:

The average molecular weights of P-I, P-2 and P-3 were determined by a gel permeation chromatography technique (GPC). Standard dextrans (40, 500, 700 and 2000 KDa, Fluka Chemical Co., Bush, Switzerland) were passed through a (2.5 x 60 cm) Sephacryl S-200 column, and then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volumes of P-I, P-2 and P-3 were plotted on the same graph, and the molecular weights were determined (Mozzi, *et al*., 1996).

Sugar analysis:

Total sugars and uronic acids were determined by the phenol-H_2SO_4 reagent (Dubois, *et al*., 1956) and *m*-hydroxydiphenyl (Ahmed & Labavitch, 1977), respectively. The polysaccharides P-I, P-2 and P-3 were hydrolyzed with 2 M trifluoroacetic acid in a sealed tubes. Hydrolysis was carried out at 100ºC for 2 h. After the hydrolysis, the acid was removed by flash evaporation on a water bath at a temperature of 40ºC and co-distilled with water (5 ml x 3) (Sudhamani, *et al*., 2004). The purified hydrolyzates (20 µl) were analysis by HLPC according to El-Sayed, *et al*., (2005).

Sulfate analysis:

Sulfate-free polysaccharide, (**P-3**) were achieved by treatment of the polysaccharide with 2% MeOH/HCl (20 ml) for 72 h at 25ºC and then dialyzed against deionized water followed by freeze drying Hasui, *et al*. (1996).

Fourier infra red spectroscopy (FT-IR):

The polysaccharide fractions (P-I, P-2 and P-3) fractions were characterized using a Fourier transform infrared (FT-IR) in Burcker Vector 22-spectrophotometer. The dried polysaccharides were ground with KBr

powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 400-4000 cm^{-1} (Bruhn *et al*., 1996).

Methylation analysis:

The polysaccharide P-2 and P-3 were methylated separately using the method of Ciucanu & Kerek (1984). The methylated products were isolated by partition between chloroform and water (5:1, v/v). The products were then hydrolyzed with 2 M trifluoroacetic acid at 105ºC for 2 h., and excess of acid was evaporated by co-distillation with distilled water. The hydrolyzed products were then reduced with $NaBH_4$ (20 mg) and acetylated with pyridine-acetic acid (Guilherme, *et al*., 2005). The alditol acetate of the methylated sugars was analyzed by GC-MS Finnigan SSQ-7000 (Morelte *et al*. 2005). A temperature programming of 60-280 ºC increased by 4º C min^{-1} was maintained for the analysis. Ionization potential was 70ev and mass range (m/z) was 40−4000 amu. Helium was the carrier gas used. Qualitative and quantitative identification of the methylated sugars by comparing retention time and mass fragmentation patterns with those of the available authentic data base were performed.

Radical scavenging activity (RSA) of P-1 and P-3:

The RSA of the P-1 and P-3 were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test according to the method of Shimada *et al*. (1992) with some modifications. Two mL of DPPH solution in 95% ethanol (10^{-4} M) was added with 1 mL of the samples of different addition quantity (50-400 μg) in water and 2 mL of 95% ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

Scavenging ability (%) = $[(A_{517}$ of control – A_{517} of sample) / A_{517} of control] × 100

The EC_{50} value is the effective concentration (μg/mL) of P-1 and P-3 at which the DPPH radicals were scavenged by 50 %.

RESULTS AND DISCUSSION

The central goal this study was to determine the classes of polysaccharides present in brown alga *Padina pavonia*. For this sugar composition of depigmented algal powder **(DAP)** was determined. It contained 42% sugars of which about seventh were uronic acids. The main neutral sugar was fucose in addition other sugar including glucose, galactose and xylose. To achieve these goals, sequential extraction of polysaccharides was carried out Karmakar *et al*. (2010). This procedure was based on the different solubility of polysaccharides from brown seaweeds.

3.1. Chemical characterization:

3.1.1. Sugar composition:

The HCl extracted material (named as P-1) contains protein (14%; w/w) and other polymers. The Na_2CO^3 extract, after removal of alginic acid by sequential precipitation with HCl and $CaCl_2$, was dialyzed and lyophilized to give a fucoidan (P-3). Sugar compositional analysis revealed that P-3 consist mainly of fucose as the major neutral sugar together with smaller amount of galactose and xylose units (Table 1). The uronic content of this fucoidan fraction (7%; w/w) was higher than that of the water extracted fucoidan (Karmakar *et al*., 2009), but the former contained lesser amount of sulfate. Sodium alginates form insoluble precipitates at acidic pH and with calcium salts, but they are stable in solution between pH 6 and 10. The fractions P-1, P-2 and P-3 were purification on the gel permeation chromatography (GPC) column. The average molecular weight of P-1, P-2 and P-3 were determined as $3.3x10^4$, $5.7x10^4$ and $2.5x10^4$ Da, respectively by GPC (Figure 1). Particularly, the molecular weight of the fucoidan of present study is lower than that of water extracted one (Karmakar *et al*., 2009). But the extraction conditions used in these studies are different. The polymer was extracted with $_{Na2CO3}$, whereas in the earlier study water was used as extracting solvent. The monosaccharide of P-1 and P-3 hydrolysate was measured by HPLC, wherein rhamnose, fucose, xylose, mannose, glucose, and galactose were identified in the hydrolysate and their ratios were, tr: 35:8.0: 2.0: 5.0: 1.0 and tr: 36: 7.0: 0.0: 5.2: 1.0, respectively. Whoever, P-2 was containing uronic acid as a mannouronic acid and glucouronic acid ad molar ratio of 1.1: 1.0, respectively.

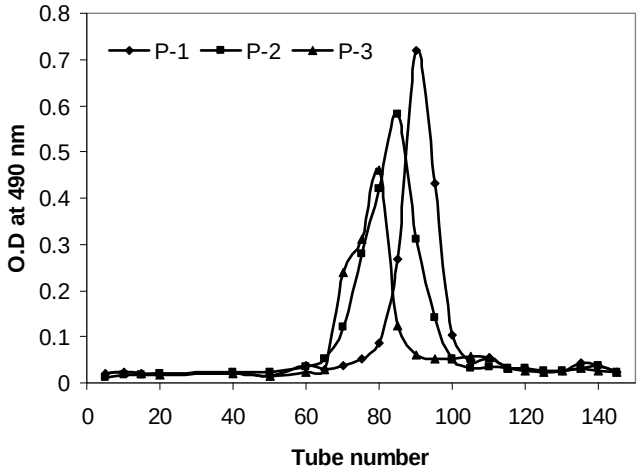

Fig. 1: The gel permeation chromatography of polysaccharide fraction P-1, P-2 and P-3 on Sephacryl S-200

Table1: Sugar composition (molar ratio) of fractions obtained from *Padina pavonia.*

	Yield of (% DAP)	Molar ratio							
		Manouronic	Glucouronic	Rhamnose	Fucose	Xylose	Mannose	Glucose	Galactose
DAP		3.4	3.0	1.0	18	5.0	21	2.0	3.0
P-1	3.8	0.0	0.0	tr	35	8.0	2.0	5.0	1.0
P-2	4.7	1.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0
P-3	2.6	0.0	0.0	tr	36	7.0	0.0	5.2	1.0

3.1.2. Fourier transform infra red (FT-IR):

The FT-IR spectrum of this fraction contains band at 3400 cm^{-1} (OH stretching), 2925 cm^{-1} (CH stretching), 1675 and 1420 cm^{-1} (COO^{-} stretching) characteristic of alginate (Figure 2). Moreover two bands at approximately 1100 and 1025 cm^{-1} responsible for mannuronic (M) and guluronic (G) units respectively, were observed (Stacey & Barker 1960; Brck-Ncely, 1957, Pereira *et al*., 2003).

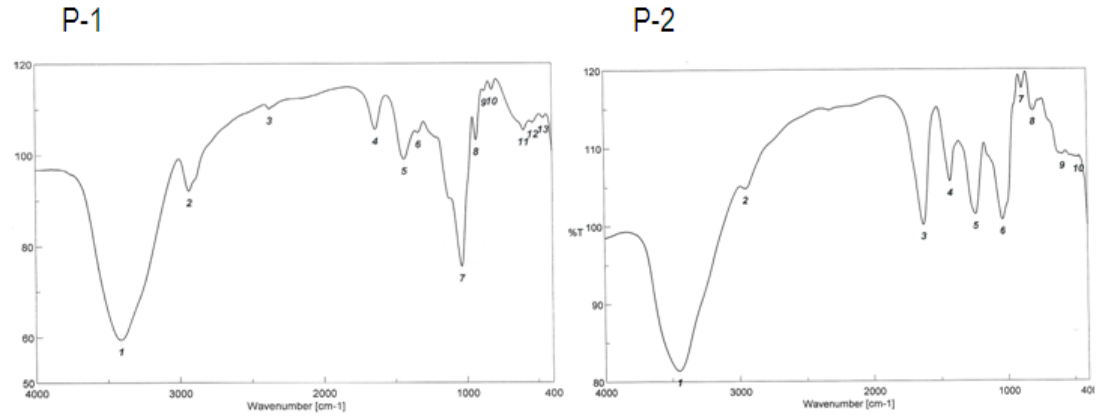

Fig. 2: Infrared spectrum of polysaccharide fraction P-1 and P-3.

3.1.3. Linkages analyses:

The fully methylated P-3 and dP-3 were hydrolyzed with acid, converted into alditol acetates, and analyzed by GC–MS. As summarized in Table (2), P-3 and dP-3 showed the present of three derivatives, namely 3,4-di-O-methyl-xylose; 4-O-methyl-fcuose, 4-O-methyl-galactose at molar ratio of 1.0: 1.3: 1.8, respectively for P-3. On the other hand, present seven derivatives, namely 3,4-tri-O-methyl-xylose; 3,4-di-O-methyl-xylose; 2,3,4-tri-O-methyl-fucose; 2,4-di-O-methyl-fucose; 3,4-di-O-methyl-fucose; 4-O-methyl-fcuose; 4-O-methyl-galactose at molar ratio 2.7: 1.2: 6.2: 3.3: 1.0: 5.1: 2.4, respectively for dP-3. The methylation analysis of the P-3 and desulfated fucoidan (dP-3) suggest that it contains (1→2)- and (1→3)-linked fucopyranosyl residues. This data is in accordance to the result obtained from the water extracted fucoidan from the same alga (Karmakar *et al*., 2009). On the other hand, gave different partially methylated derivatives indicating that this polymer is highly sulfated (Table 2). The composition of the methylated derivatives remains same even after one more two rounds

of methylation. It should, conversely, be renowned that complete methylation of the sulfated polysaccharide, because of the steric obstruction by the sulfate esters, is very difficult (Pereira, *et al*., 1999).

Table 3: GC-MS results of methylation analysis of P-3 and dP-3

Methylation product	Molar ratios	
	P-3	dP-3
2,3,4-tri-*O*-methyl-xylose	0.0	2.7
3,4-di-*O*-methyl-xylose	1.0	1.2
2,3,4-tri-*O*-methyl-fucose	1.3	6.2
2,4-di-*O*-methyl-fucose	0.0	3.3
3,4-di-*O*-methyl-fucose	0.0	1.0
4-*O*-methyl-fucose	0.0	5.1
4-*O*-methyl-galactose	1.8	2.4

3.2. Radical scavenging activity (RSA) of P-1 and P-3:

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Lee, *et al*., 2003). In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-colored diphenylpricrylhydrazine. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their hydrogen-donating ability. The DPPH radical-scavenging activities of P-1, P-2 and P-3 were determined (Shimada *et al*., 1992). The scavenging impacts of different fractions P-1, P-2 and P-3 on DPPH are shown in Figure (3). P-1 and P-3 exhibited the highest inhibition effect with an IC_{50} value of 170 and 180µg/ml, respectively. Under the same conditions, vitamin C, a free radical scavenger, showed a slightly weaker effect on the hydroxyl radicals, with an EC_{50} value of 180 µg/ml. The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular weight, chemical structure, linkage, substitution groups, even the extraction and isolation methods (Chen *et al*., 2008).

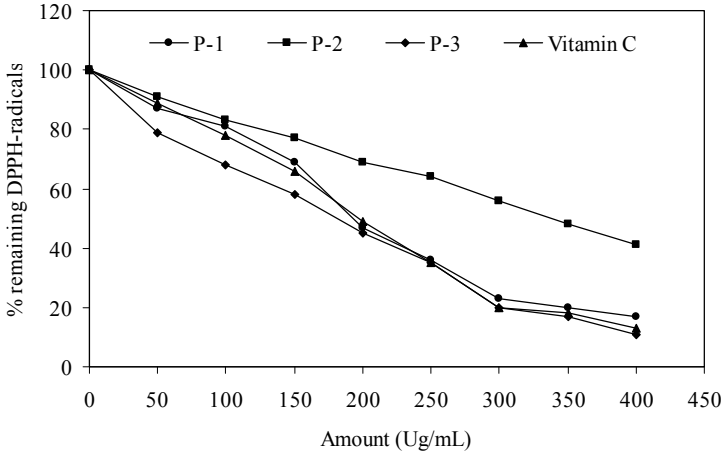

Fig. 3: Scavenging effect of fraction P-1, P-2, P-3 and vitamin C during DPPH test by changes in absorbance at 517 nm.

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