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Characterization and assessment of antioxidant and antibacterial activities of sulfated polysaccharides extracted from cuttlefish skin and muscle

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ABSTRACT

In this study, novel polysaccharides extracted from cuttlefish skin (CSP) and muscle (CMP), by precipitation with cetylpyridinium, were characterized and their antioxidant and antibacterial activities were investigated. CMP showed the highest amounts of sulfated groups (6.6%), uronic acids (9.2%) and proteins (3.7%). Infrared spectroscopic analysis indicated the presence of sulfonyl (O═S═O) and acetyl (CH_3CO^-) groups for both CSP and CMP. In addition, CSP showed the presence of glucuronic acid (GlcA) and galacturonic acid (GalA) as major components, while CMP showed highest amount of GalA in its monosaccharide composition. Sulfated polysaccharides were found to display important antibacterial activity against several Gram+ and Gram− bacteria. In addition, they exhibited strong antioxidant activities as showed by various *in vitro* tests. Fractionation of cuttlefish polysaccharides, by DEAE-cellulose column showed one peak during the buffer elution phase and three major fractions for CMP and two peaks for CSP during the linear gradient of NaCl. The last eluting sulfated fraction from each sample, characterized by the highest negative charge, was found to exhibit the best antioxidant and antibacterial activities. The obtained results demonstrated that cuttlefish polysaccharides and their fractions could serve as natural antioxidant and antibacterial agents.

1. Introduction

Fish industry is a major contributor to the socio-economic growth in several countries all over the word. However, this sector leads to generate large amounts of by-products, which creates numerous health and environmental problems. Besides the direct use of these by-products for animal food, much attention has been given for their transformation into more interesting molecules, called value-added products. For instance, cuttlefish (*Sepia officinalis*), one of the most industrially transformed fish species in Tunisia, gave rise to several types of byproducts, where the skin (9% of total body weight of the fish) could be a source of many interesting molecules such as collagen, peptides, polysaccharides, *etc.*

Polysaccharides are a heterogeneous group of complex macromolecules that are widely found in animals, fish, plants, and microorganisms. In fact, they have been readily extracted from algae [1], sponges [2], and viscera [3,4]. Polysaccharides have been extensively studied due to their numerous biological activities including anti-coagulant [5], immune enhancing, hypoglycemic [6], antioxidant [1] and antibacterial [7] activities. In addition, some acetylated [8], sulfated [9], and selenylated [10] derivatives of polysaccharides exhibited significant antioxidant activities, higher than those of the native polysaccharide. Particularly, sulfation is a simple, powerful and effective way to give increased biological activity to the polysaccharide molecules [5,11].

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various diseases such as cancer, Alzheimer and atherosclerosis [12,13]. In fact, reactive oxygen species and free radicals are powerful oxidants that can react with most biological molecules such as proteins, lipids and carbohydrates causing their damage and in turn, generating several health problems. Although biological organisms possess native antioxidant defense involved in their protection against oxidative damage, increased exposure to free radicals results in more complex health problems [9]. So, it is crucial to find exogenous natural derived-antioxidants that may improve the natural defense against oxidative damage. Particularly, sulfated polysaccharides, from marine source constitute a class of bioactive compounds gifted with a great potential against free radicals.

Thus, in the present study, sulfated polysaccharides were extracted from the skin and the muscle of cuttlefish and their infrared

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characterization and monosaccharide composition were investigated. Furthermore, the antioxidant and antibacterial activities of the crude samples and their DEAE-cellulose fractions were assessed.

2. Materials and methods

2.1. Cuttlefish polysaccharides (CPs) extraction

Cuttlefish (*Sepia officinalis*) skin and muscle were obtained from the local fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice and transported to the laboratory within 30 min. Upon arrival, they were immediately rinsed with tap water to remove contaminants and then placed in sealed plastic bags at −20 °C.

Two crude CPs were extracted from cuttlefish skin and muscle by precipitation with cetylpyridinium chloride (CPC) [14]. Both polysaccharides were extracted as follows: 100 g of skin or muscle were ground and suspended in 500 ml of distilled water. The endogenous enzymes were inactivated by heating the mixtures for 20 min at 95 °C. Then, the pH was adjusted to 10.0 and 100 mg of Purafect® (alkaline crude proteases) were added and the mixtures were left for 12 h at 50 °C. After enzymatic deproteinization, the samples were left to cool at room temperature and filtered under vacuum. The residues were washed with distilled water and filtered again. The filtrates were mixed and polysaccharides were then precipitated by adding 10% (w/v) of CPC. The mixtures were kept for 24 h at room temperature. The precipitates formed were collected by centrifugation at 8000 rpm for 30 min at 4 °C and then re-suspended in 50 mM NaCl solution in ethanol (100:15, v/v) for 24 h at 4 °C. Finally, the pellets, obtained after centrifugation at 8000 rpm for 30 min at 4 °C, were washed with 80% ethanol, and then with absolute ethanol. Pellets were re-dissolved in distilled water and finally lyophilized using a freeze-drier (Bioblock Scientific Christ ALPHA 1-2, IllKirch-Cedex, France). Crude sulfated polysaccharides from cuttlefish skin and muscle were referred to CSP and CMP, respectively.

2.2. Chemical composition of CSP and CMP

Protein content of fresh skin and muscle was determined by the Kjeldahl method, AOAC number 984.13 [15]. The protein content of CSP and CMP was estimated according to the method of Lowry [16]. Total carbohydrates were determined by the phenol-sulfuric acid method and quantified by the OD recorded at 490 nm [17]. The content of sulfate group was determined according to the method of barium chloride–gelatin [18]. Total uronic acid content was quantified using the method described by Bitter et al. [19] and the glucuronic acid was used as standard.

2.3. Monosaccharide composition of CSP and CMP

For the determination of the monosaccharide composition, 5 mg of each crude polysaccharide were hydrolyzed with 2 M trifluoroacetic acid for 2 h at 121 °C and released monosaccharides were separated by high-performance anion-exchange chromatography on a Dionex Carbopac column with integrated amperometry detection. The separated monosaccharides were quantified using external calibration with an equimolar mixture of five monosaccharide standards (glucuronic acid, galacturonic acid, mannose, rhamnose and xylose).

2.4. Fourier transform infrared (FT-IR) spectroscopy analysis

The IR spectroscopic measurements were performed using a Nicolet FTIR spectrometer equipped with an attenuated total reflection (ATR) accessory. A mass of 2 mg of each sample was mixed with 100 mg of dried potassium bromide (KBr) and then compressed to prepare discs of 3 mm diameter. These discs were subjected to IR-spectrometry. The absorbance was read between 500 and 4000 cm^{-1}. The background noise was corrected using the data for pure KBr.

2.5. Fractionation of crude CPs by DEAE-cellulose chromatography

Crude sulfated polysaccharides (1 g) were dissolved in 10 ml of sodium acetate buffer (0.5 M, pH 6.0) and then applied onto a DEAE-cellulose column (1 cm × 15 cm) pre-equilibrated with sodium acetate buffer (0.5 M, pH 6.0). Elutions were first performed by sodium acetate (0.5 M, pH 6.0) and then by a linear gradient of 0.1–2 M NaCl solution with a flow rate of 0.45 ml/min. Fractions of 4 ml were collected. The content of carbohydrates was determined in each fraction. Peaks referring to the different polysaccharide fractions were pooled and then lyophilized.

2.6. Evaluation of antioxidant activities

2.6.1. Ferric reducing power

The ability of polysaccharides to reduce iron III was determined according to the method of Yıldırım et al. [20]. A sample (0.5 ml) of each polysaccharide, at various concentrations, or each DEAE-cellulose fraction (200 μg/ml, w/v) was mixed with 1.25 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of 1% potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50 °C. After incubation, 0.5 ml of 10% trichloroacetic acid was added and the reaction mixtures were then centrifuged for 10 min at 3000 rpm. Finally, 1.25 ml of the supernatant solution from each sample mixture was mixed with 1.25 ml of distilled water and 0.25 ml of 0.1% (w/v) ferric chloride. The absorbance of the resulting solutions was measured at 700 nm after 10 min of incubation. The blank tubes were conducted in the same manner, except that distilled water was used instead of ferric chloride solution to determine the absorbance of the sample. Three replicates were done for each test sample.

2.6.2. Antioxidant assay using the β-carotene bleaching method

The prevention of β-carotene from bleaching was determined according to the method of Koleva et al. [21]. First, the emulsion of β-carotene/linoleic acid was freshly prepared by dissolving 0.5 mg of β-carotene, 25 μl of linoleic acid and 200 μl of Tween 40 in 1 ml of chloroform. The chloroform was then completely evaporated under vacuum in a rotatory evaporator at 50 °C; then 100 ml of distilled water were added and the resulting mixture was vigorously stirred. Thereafter, 2.5 ml of the β-carotene/linoleic acid emulsion was transferred to test tubes containing 0.5 ml of each polysaccharide solution. Control tubes were prepared in the same conditions by adding 0.5 ml of water to the emulsion. The absorbance of every test tube was measured at 470 nm twice, before and after incubation for 1 to 2 h at 50 °C. Butylated hydroxyanisole (BHA) was used as a positive standard. Tests were carried out in triplicate and the antioxidant activity was evaluated in terms of β-carotene bleaching using the following equation:

$$\text{Antioxidant activity } (\%) = \left[1-(OD_0-OD_t)/(OD_0'-OD_t')\right] \times 100,$$

where OD_0 and OD_t are the absorbances of the test sample measured before and after incubation, respectively; and OD'_0 and OD'_t are the absorbances of the control measured before and after incubation, respectively.

2.6.3. Metal chelating activity

The iron chelating activity of the different samples was estimated according to the following method. Briefly, 100 μl of each sample (CP and its fractions), prepared at different concentrations in distilled water, were added to 50 μl of 2 mM $FeCl_2$ and 450 μl of distilled water. The mixtures were incubated at room temperature for 3 min and the reaction was initiated by the addition of 200 μl of 5 mM of ferrozine solution. Then, the mixtures were shaken vigorously and left to stand at room

temperature for 10 min. Control tubes were prepared by the same manner, substituting the polysaccharide with water. EDTA was used as positive control. The absorbance of solutions was measured at 562 nm, and the chelating activity (%) was calculated as follows:

$$\text{Metal chelating activity } (\%) = [(OD_C + OD_B - OD_S)/OD_C] \times 100,$$

where OD_C, OD_B and OD_S represent the absorbance of the control, the blank and the sample reaction tubes, respectively. The test was carried out in triplicate. The metal chelating ability of the different DEAE-cellulose fractions was measured at 100 μg/ml.

2.6.4. DPPH radical scavenging activity

The DPPH free radical-scavenging activity of both polysaccharides according to the method of Bersuder et al. [22]. A volume of 500 μl of each polysaccharide at different concentrations was mixed with 375 μl of absolute ethanol and 125 μl of 0.2‰ DPPH. The mixtures were then left to stand at room temperature for 60 min in the dark, and the absorbance was measured at 517 nm using an UV–Visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China). Lower absorbance of the reaction mixture indicated higher DPPH radical-scavenging activity. The control was conducted in the same manner, except that distilled water was used instead of sample. BHA was used as positive control. The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical}-\text{scavenging activity } (\%) = [(A_C - A_S) \times 100]/A_C$$

where A_C and A_S represent the absorbance of the control and the sample reactions, respectively. The test was carried out in triplicate.

2.7. Antibacterial activity

2.7.1. Microbial strains

The antibacterial activities of CSP and CMP were tested against three Gram-positive (*Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698) and *Bacillus cereus* (ATCC 11778)) and four Gram-negative bacteria (*Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 43972), and *Enterobacter* sp.) bacteria strains.

2.7.2. Agar diffusion method

The antibacterial activity assay was performed according to the method described by Valgas et al. [23]. Culture suspensions (200 μl) of the microorganisms (10^6 colony-forming units (CFU/ml) of bacteria cells estimated by absorbance at 600 nm) were inoculated onto the surface of spread on Luria-Bertani (LB) agar, Then, 50 μl of polysaccharide, at 25 and 50 mg/ml dissolved in distilled water, were loaded into wells (6 mm in diameter) punched in the agar layer. Thereafter, the agar plates were kept for 1 h at 4 °C, and then, they were incubated for 24 h at 37 °C. The antimicrobial activity was evaluated by determining the zone of growth inhibition (diameter expressed in millimeters) around the wells.

2.7.3. Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration (MIC), determined as the lowest CP concentration required to totally inhibit the growth of a microorganism, was estimated in sterile 96-well microplates using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent (MTT), an indicator of microorganism viability. First, serial dilutions of the CPs stock solution ranging from 12 μg/ml to 25 mg/ml were prepared in Muller-Hinton broth (MHB). In each well, 100 μl of diluted CP were mixed with 10 μl of cell suspension to a final inoculums concentration of 10^6 CFU/ml. The medium containing the sample alone and medium containing each bacteria without test compounds were used as controls. Then, the plates were incubated at 37 °C for 24 h.

After incubation, a volume of 25 μl MTT (0.5 mg/ml) was added to the wells and incubated at 37 °C for 30 min. The MIC was defined as the lowest concentration of CP that completely inhibited the strain growth after 24 h of incubation (no visible growth).

2.8. Statistical analysis

Statistical analyses were performed with SPSS ver. 17.0, professional edition using ANOVA analysis. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Chemical and monosaccharide compositions of cuttlefish polysaccharides

The yields of polysaccharides extracted and their proximate compositions are shown in Table 1. Cuttlefish skin and muscle possessed high percentage of moisture (82.8% and 78.6%, respectively), followed by protein (14.0% and 11.6%, respectively). The fat and ash contents were <1% for both muscle and skin (data not shown). The presence of ash and lipids at very low content is important for the quality of polysaccharide. Carbohydrates present in the raw material were separated from their protein component by hydrolysis to elaborate crude cuttlefish polysaccharides, after CPC precipitation.

Table 1 showed that the yield of CPs depend on the raw material. In fact, the extraction yield of CSP was only about 1.6%, while the yield of CMP, obtained from muscle, was about 8.6%. Furthermore, regarding the chemical composition of the polysaccharides, protein contents were found to be 0.9% and 3.7% for CSP and CMP, respectively. In addition, the total sugar and sulfate contents obtained in CMP (81.7% and 6.6%, respectively) were higher than those of CSP (68.2% and 5.9%, respectively) ($p < 0.05$). Thus, regarding their degrees of sulfation, both polysaccharides could be bioactive.

Sulfated polysaccharide chains are composed of disaccharide repeating units, called glycosaminoglycanes (GAGs) , which consist of uronic acid (D-glucuronic acid or L-iduronic acid) and amino sugar (D-galactosamine or D-glucosamine). CMP showed uronic acid content (9.2%) higher than that of CSP (7.2%). In fact, the differences observed in the uronic acid content of both CPs could presumably be ascribed to the original compositions of raw materials. Maciel et al. [24] reported similar sulfate amounts for polysaccharides extracted from *Gracilaria birdiae* (6.4%). Moreover, Wijesinghe and Jeon [25] reported that the prominent biological activities of the sulfated polysaccharides are probably due to the presence of sulfate groups in varying amounts. In addition, positions of the sulfated groups along the macromolecular chain also play a significant role in their functional properties.

Monosaccharide analysis indicated that the CPs contained various types of sugars (Table 1). Both CPs showed the presence of glucuronic acid (GlcA) and galacturonic acid (GalA) as major components, and CMP showed the highest amount of GalA. Suh and Matthew [26] reported that glycosaminoglycanes (GAGs), composed of *N*-acetylglucosamine or *N*-acetylgalactosamine along with a uronic sugar (glucuronic acid or galacturonic acid), are known for their various bioactivities. This is in agreement with the findings of Abdelhedi et al. [27] who extracted sulfated polysaccharides from the smooth hound wastes, and demonstrated the presence of GlcA, GalA and *N*-acetylated galactosamine (GalNAc) in the monosaccharide composition. In addition, CPs from cuttlefish skin and muscle also contained other monosaccharides like rhamnose, xylose, and mannose. It may, therefore, be concluded that the nature of the raw material used is a determining factor for the monosaccharide composition. In addition, it is important to note that chemical composition, sulfated degree and structure are very dependent on the first matter origin [28].

Table 1
Chemical and monosaccharide compositions of CSP and CMP extracted from cuttlefish skin and muscle.

	CSP	CMP
Total sugars (%)*	68.2 ± 0.5[b]	81.7 ± 1.0[a]
Uronic acid (%)*	7.2 ± 0.0[b]	9.2 ± 0.0[a]
Total proteins (%)*	0.9 ± 0.1[b]	3.7 ± 0.1[a]
Sulfated groups (%)*	5.9 ± 0.2 [a]	6.6 ± 0.1 [a]
Yield (%)*	1.6 ± 0.1[b]	8.6 ± 0.2[a]
Monosaccharide composition (mol %)		
Rhamnose	8.0	9.0
Xylose	11.0	12.7
Mannose	10.5	15.3
GlcA	38.3	39.4
GalA	32.2	23.6

GlcA (glucuronic acid) and GalA (galacturonic acid). Data are calculated based on wet weights. [a,b] Different letters in the same line indicate significant differences ($p < 0.05$).
* % of dry weight.

3.2. FT-IR spectra analysis of CPs

FT-IR spectroscopy was performed in the 4000–500 cm^{-1} region to further characterize both polysaccharides. The FTIR spectra of CPs are shown in Fig. 1. Significant differences between spectra of both CPs were observed. The spectra of CPs showed major bands at approximately 3285, 1614, and 1535 cm^{-1}, corresponding to amides A, amide-I and amide-II, respectively. Mansour et al. [5] reported a similar spectrum for polysaccharide extracted from *Raja radula* skin, where amide A, amide I, and amide II peaks were found at the wavelengths of 3450, 1639, and 1556 cm^{-1}, respectively. In addition, as shown in Fig. 1, absorption band observed at 2847 cm^{-1}, could be attributed to the stretching of C—H [29], typical bands for alkyl functionality of a polysaccharide. Uronic acids were evidenced by the two absorbance bands at 1383 and 1457 cm^{-1} (O—C═O bending). In this range, peaks were more strong and broad in CSP than in CMP.

The results of the FTIR analysis confirmed the presence of sulfated groups in the CPs. In fact, for both CPs important peak was located at 1244 cm^{-1}, which correspond to the stretching vibration of the sulfate groups (S═O). Moreover, pyranose units, C—O—C group and C—O—S bindings were consistent with absorption bands at 1051 cm^{-1}, 955 cm^{-1} and 680 cm^{-1}, respectively. The detection of peak at 934 cm^{-1} is probably related to the presence 3,6-anhydrogalactose in both samples [30]. As it could be seen in Fig. 1, all sulfated bindings were stronger and broader in muscle polysaccharide spectra compared to that of the skin polysaccharide, which is consistent with the physicochemical analyses. From their results, it could be concluded that the CMP was more sulfated than the CSP. The infrared spectrua for both polysaccharides revealed similar characteristics to those extracted from smooth hound viscera using different methods [27].

3.3. Evaluation of antioxidant activity of CPs

3.3.1. Reducing power

The reducing power results, of both crude polysaccharides at different concentrations, are reported in Fig. 2a. It was clear that the reducing power of both samples, as well as BHA, increased with increasing concentrations. Both polysaccharides exhibited similar reducing power activity. At low concentrations (<300 μg/ml), CPs showed lower activity than BHA, whereas, above 300 μg/ml, BHA and CPs showed similar potential and they reach the maximal OD value of 3. The results indicated that both CPs can act as an effective electron donor and react with free radicals to convert them to more stable products and thereby terminate the radical chain reaction. Similar results were reported by Abdelhedi et al. [27] and Arima et al. [4] for sulfated polysaccharides from smooth hound viscera, Atlantic mackerel, Japanese jack mackerel, pacific bluefin tuna and yellowfin sole, respectively.

3.3.2. β-Carotene bleaching inhibition

The antioxidant assay using the discoloration of β-carotene is widely used to measure the antioxidant activity of bioactive molecules, because β-carotene is extremely sensitive to linoleic acid free radicals-mediated oxidation. The antioxidant activities of both polysaccharides determined by β-carotene bleaching capacity are reported in Fig. 2b. Both CPs protected β-carotene from bleaching in a dose-dependent manner, and CSP showed higher antioxidant effect than did CMP. In fact, at 1 mg/ml, CSP displayed an important antioxidant activity estimated at 93%, while at the same concentration, the CMP activity was about 64%. However, both CPs showed lower inhibition of β-carotene bleaching than did BHA. Similar finding was reported by Abdelhedi et al. [27] for sulfated CPs from smooth hound viscera. The obtained results demonstrated that CPs prevent β-carotene from bleaching by potentially donating hydrogen atoms to peroxyl radicals of linoleic acid.

3.3.3. Ferrous ion-chelating ions

In biological systems, Fe^{2+} can interact with hydrogen peroxide (H_2O_2) in Fenton reaction to produce the reactive oxygen species leading to the acceleration of oxidation process [31]. In ferrous chelating

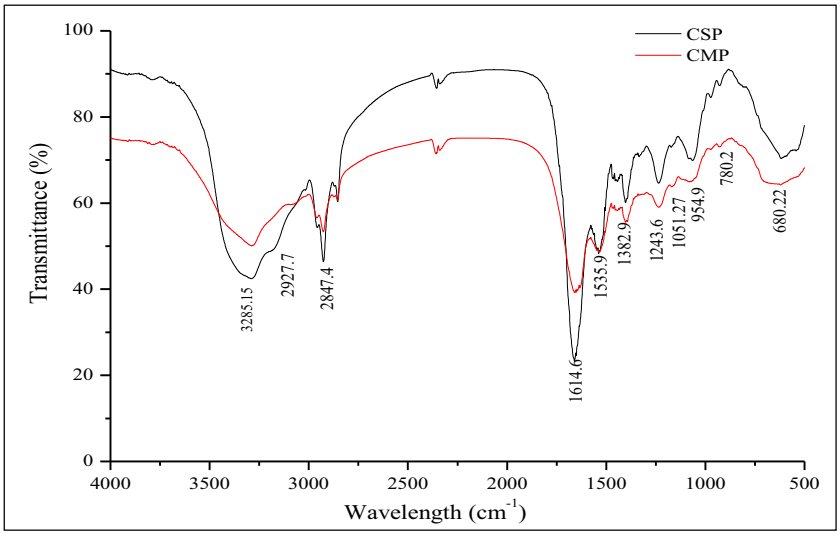

Fig. 1. Infrared spectra of CMP (red) and CSP (black).

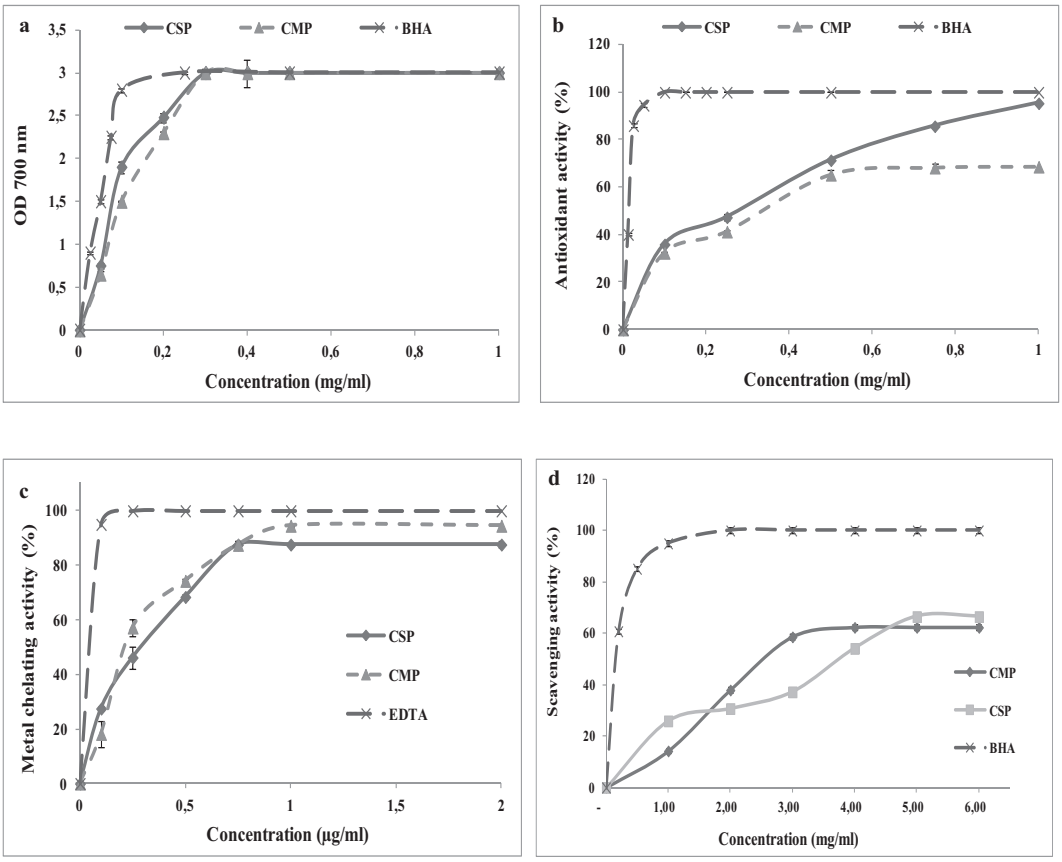

Fig. 2. Antioxidant properties of CMP and CSP at different concentrations; (a) Reducing power; (b) β-carotene bleaching inhibition; (c) Metal chelating activity; (d) Scavenging activity. BHA and EDTA were used as positive controls. Each value is the mean of triplicate measurements.

test, the antioxidant capacity of the reactive substance is evaluated by the disruption of the Fe^{2+}-ferrozine complex, which result in the decrease in the intensity of the purple colored complex [32]. The chelating ability of CPs and EDTA, used as reference chelating agent, at different concentrations, is presented in Fig. 2c. As shown, both CPs exhibited excellent chelating activities which increased in a dose-dependent manner, to reach their maximum at 1 mg/ml (>90%). However, whatever the concentration tested, EDTA showed higher potentiality than CPs. The IC_{50} of Fe^{2+} ions chelating ability of CMP and CSP were of 250 and 367 μg/ml, respectively. This difference could be due to the proposed higher sulfation of CMP, as compared to CSP. Indeed, Mansour et al. [5] reported that the sulfation of polysaccharides increased their chelating potentialities. In the same context, Abdelhedi et al. [27] found that sulfated polysaccharides, with 10% of sulfate content, possessed an effective Fe^{2+} chelating activity (IC_{50} = 25 μg/ml). Also, Chang et al. [33] illustrated that the presence of galacturonic acid in polysaccharide is essential for the ability of chelating ferrous ions. These findings indicated that CPs can exhibit antioxidant activity by capturing iron ions.

3.3.4. DPPH free radical-scavenging activity

DPPH free radical-scavenging is a primary mechanism by which antioxidants inhibit oxidative processes. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance is reduced. The DPPH radical scavenging activities of CPs, as well as BHA, at various concentrations, are reported in Fig. 2d. CMP and CSP were able to scavenge DPPH radical at a dose-dependent manner. CMP and CSP exhibited great antioxidant activity with a maximal potential reached at 3 and 5 mg/ml, respectively. Interestingly, both CPs displayed higher activity than polysaccharides from *Sepia aculeate* cuttlebone [34], which showed scavenging ability against DPPH radical of 36.27% at 10 mg/ml.

3.4. Evaluation of the antibacterial activity of CPs

The antimicrobial activity of CPs was assessed at 25 and 50 mg/ml using the agar diffusion method. Table 2 shows the antimicrobial activity of CPs measured by the inhibitory zone against several Gram-negative (*E. coli*, *K. pneumoniae*, *S. enterica*, and *Enterobacter* sp.,) and Gram-positive bacteria (*S. aureus*, *M. luteus* and *B. cereus*). Data showed that at the same concentration, CMP and CSP exhibited similar activity. Interestingly, *M. luteus* and *E. coli* were the most sensitive bacteria to both CPs. Indeed, inhibition zones with diameters superior to 4 cm were observed with *M. luteus* at 25 mg/ml of CPs. Further, inhibition zones with diameter of 24.2 mm for CMP and 24.5 mm for CSP were

Table 2
Antibacterial activity of CSP and CMP at different concentrations.

		CSP		CMP	
		25 mg/ml	50 mg/ml	25 mg/ml	50 mg/ml
Gram−	*E. coli*	24.0 ± 0.0^{a}	24.5 ± 0.7^{a}	24.0 ± 0.0^{a}	24.2 ± 1.1^{a}
	K. pneumoniae	19.0 ± 1.4^{a}	21.5 ± 0.7^{a}	19.5 ± 2.1^{a}	22.0 ± 1.4^{a}
	S. enterica	18.5 ± 0.7^{a}	18.5 ± 0.7^{a}	19.0 ± 0.0^{a}	18.5 ± 0.2^{a}
	Enterobacter sp.	17.2 ± 1.1^{b}	22.7 ± 1.1^{a}	17.7 ± 1.1^{b}	17.5 ± 0.0^{b}
Gram+	*M. luteus*	42.5 ± 0.70^{a}	44.5 ± 2.1^{a}	44.5 ± 2.1^{a}	43.0 ± 0.0^{a}
	S. aureus	17.5 ± 0.70^{a}	17.7 ± 0.3^{a}	18.0 ± 0.0^{a}	17.5 ± 0.3^{a}
	B. cereus	–	11.5 ± 1.0^{b}	–	19.0 ± 0.5^{a}

–: not detected. Data are expressed in mm and given in mean ± SD. CSP and CMP represent cuttlefish sulfated polysaccharides prepared from skin and muscle, respectively. [a,b]Different letters in the same line within different CPs indicate significant differences ($p < 0.05$).

Table 3
Minimal Inhibitory Concentration (MIC) (mg/ml) of CSP and CMP degradation products against Gram-negative and positive bacteria after 24 h incubation at 37 °C.

		CSP	CMP
Gram−	*E. coli*	3.12	1.56
	K. pneumoniae	12.50	3.12
	S. enterica	6.25	0.78
	Enterobacter sp.	12.5	3.125
Gram+	*M. luteus*	12.50	3.12
	S. aureus	6.25	6.25
	B. cereus	6.25	6.25

recorded for the *E. coli* strain. However, *B. cereus* was the most resistant strain and the highest inhibition zone diameter was of 19 mm for CMP at 50 mg/ml. In addition, all the tested Gram-negative bacteria were more inhibited than the Gram-positive ones, which is in agreement with the results of Shanmugam et al. [35], who reported that the polysaccharide extracted from cuttlebone possessed the best inhibitory effects against Gram-negative bacteria, including *Pseudomonas aeruginosa* and *S. typhi*.

The MIC values for both CPs (Table 3), revealed that the CP extracted from the skin was more effective than that obtained from the muscle. In fact, CMP showed the lowest MICs, compared to those of CSP. Of particular, the lowest MIC values were obtained for *S. enterica* and *E. coli* (0.78 and 1.56 mg/ml, respectively, for CMP and 6.25 and 3.125 mg/ml, respectively, for CSP). These data indicated also that cuttlefish polysaccharides demonstrated antibacterial activity more potent against Gram-negative bacteria. However, similar MIC results were obtained for *S. aureus* and *B. cereus* (6.25 mg/ml). CPs may, therefore, be considered as natural preservatives against food-borne pathogens, useful in food production industries and for protecting human health. In this context, polysaccharides from mushrooms, actinomycetes and alga were tested for their antibacterial activities on food-borne pathogenic microorganisms [36,37]. In fact, He et al. [36] reported that sulfated polysaccharides disrupted the cytoplasmic membranes that led to the dissolution of the proteins and leakages of essential molecules in bacteria which resulted in cell death. Some reports indicated that the cell walls and membranes of *E. coli* could be disrupted by polysaccharides which induced the rapid increase of the water-soluble intracellular proteins of this bacterium [38]. However, the exact antibacterial mechanism of polysaccharides is still unclear.

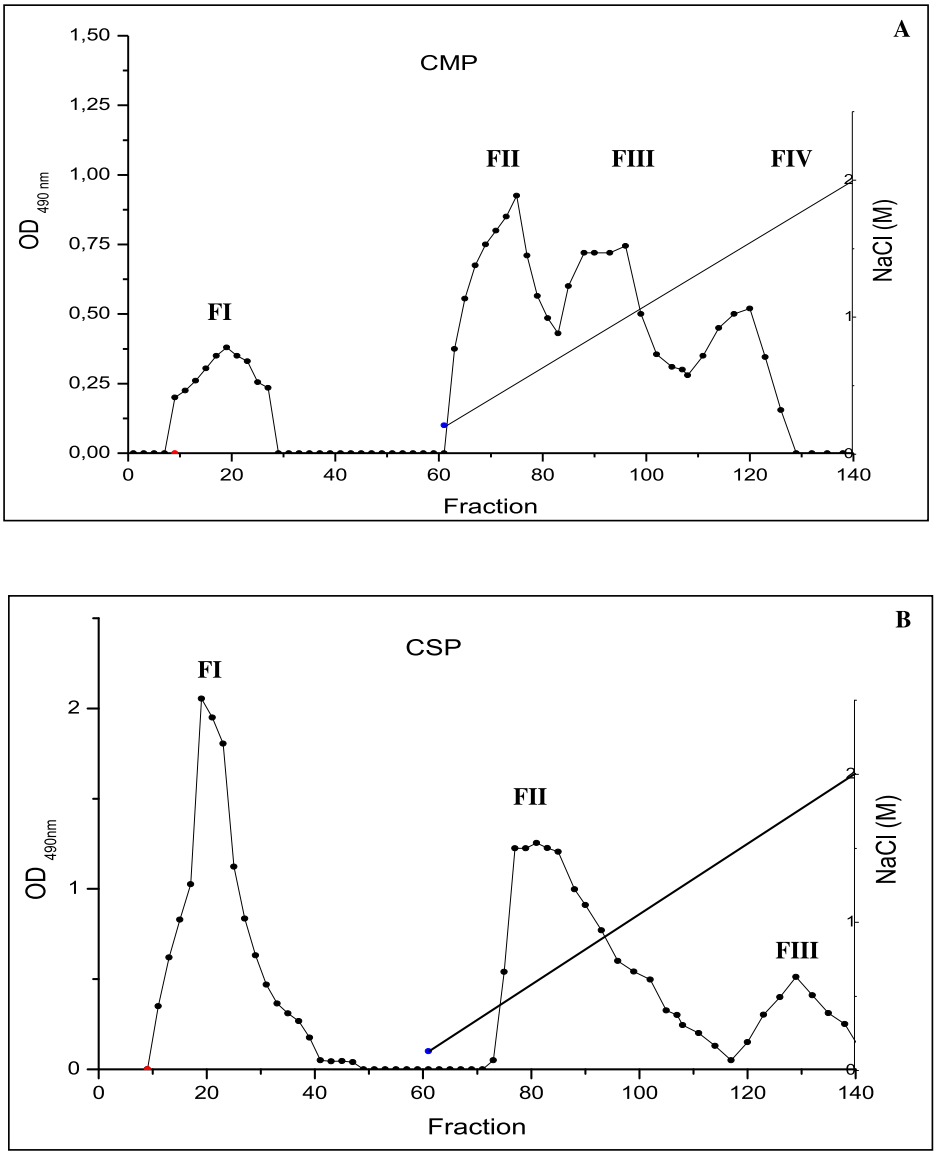

Fig. 3. Elution profiles of sulfated polysaccharides of CMP (A) and CSP (B) separated by DEAE-cellulose anion-exchange chromatography.

3.5. Fractionation and evaluation of biological activities of CPs fractions

Cuttlefish crude polysaccharides were fractionated by anion-exchange chromatography on DEAE-cellulose column and the carbohydrate content in the collected fractions was determined. As shown in Fig. 3, four and three peaks of polysaccharides were eluted from CMP and CSP, respectively, with the presence of a non-sulfated high molecular weight polymer dominating the earlier eluting fraction (FI), in each sample. Negatively-charged fractions, present in the gradient of NaCl, were noted FII, FIII and FIV. These fractions, eluted with different concentrations of NaCl, revealed significant differences between their charge density. Moreover, particularly for CMP, the overall intensity of the negative charged polysaccharides peaks was higher than that of the positive one, which indicated that this polysaccharide contained more sulfated groups.

The antioxidant activities of the obtained fractions from each CP are illustrated in Fig. 4. Results showed that all the fractions displayed interesting antioxidant activities, which increased with the negative charge increase. In fact, except the reducing power of CSP-FI, the first chromatography eluting fraction from each CP displayed the lowest antioxidant activity. However, FIII from CSP and FIV from CMP showed the highest reducing power (OD of 1.48 and 1.27, respectively) at 0.2 mg/ml, and ferrous chelating ability (72% and 96%, respectively) at 0.1 mg/ml. Therefore, the presence of sulfated groups appeared to be essential in demonstrating the chelating effect or the electron-donating ability of polysaccharides. In this context, Rodrigues et al. [39] demonstrated that, among the four DEAE-cellulose fractions of sulfated polysaccharides from *Halymenia floresia*, FIII had the highest anticoagulant activity. In another study, Fan et al. [40] fractionated four polysaccharide fractions from the leaves of *Ilex latifolia* by DEAE-cellulose chromatography and the last one, showing the highest contents of sulfuric (3.7%) and uronic acid (23.2%), displayed the highest radical scavenging and ferrous chelating activities, meanwhile, its activity was lower than that of the crude polysaccharide.

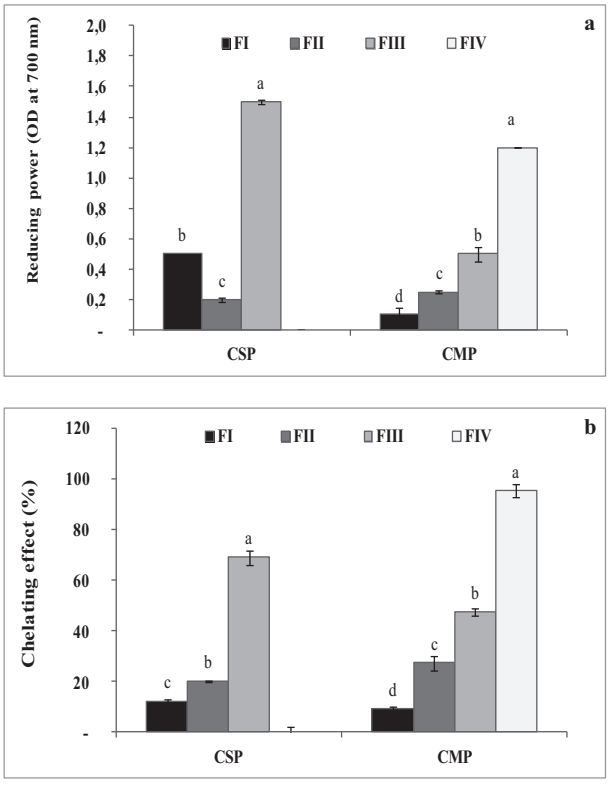

Fig. 4. Antioxidant activities of CMP and CSP fractions (FI, FII, FIII and FIV): (a) reducing power assay and (b) metal chelating activity. Data are given in mean ± SD. The reducing power and the metal chelating ability of the different fractions were measured at 0.2 mg/ml and 0.1 mg/ml, respectively. Different letters in the same sample within different fractions indicate significant differences ($p < 0.05$).

Table 4
Antibacterial activity of CSP and CMP negatively-charged fractions at 10 mg/ml.

		CSP		CMP		
		FII	FIII	FII	FIII	FIV
Gram−	*E. coli*	20.0 ± 0.0^b	24.5 ± 0.5^a	–	–	24.0 ± 1.0^a
	K. pneumoniae	17.0 ± 1.4^b	21.5 ± 0.5^a	–	–	19.3 ± 1.4^a
	S. enterica	18.5 ± 0.7^a	18.5 ± 0.5^a	–	–	18.5 ± 0.7^a
	Enterobacter sp.	17.0 ± 1.1^b	22.5 ± 1.0^a	–	–	17.0 ± 0.0^b
Gram+	*M. luteus*	42.5 ± 0.5^a	44.5 ± 2.0^a	–	–	43.0 ± 0.0^a
	S. aureus	17.5 ± 0.2^a	17.5 ± 0.5^a	–	–	17.5 ± 0.3^a
	B. cereus	–	11.5 ± 1.0^b	–	–	19.0 ± 0.5^a

CSP and CMP represent cuttlefish sulfated polysaccharides prepared from skin and muscle, respectively. –: not detected. Data are expressed in mm and given in mean ± SD.

Moreover, the antibacterial activity of the different fractions was tested against *E. coli*, *K. pneumoniae*, *S. enterica*, *Enterobacter* sp., *M. luteus*, *S. aureus*, and *B. cereus*. The inhibition of bacteria growth results are illustrated in Table 4. Data showed that only negative fractions (FII and FIII of CSP and FIV of CMP) exhibited antibacterial activity against all the tested strains, expect CSP-FII that was ineffective against *B. cereus* at 10 mg/ml. Nevertheless, CSP-FIII and CMP-FIV were more effective against *M. luteus*, *K. pneumoniae* and *Enterobacter* sp. The strong antibacterial activity obtained in these fractions is likely due to their higher sulfate content, compared to the earlier eluted ones. In fact, Li and Shah [41] demonstrated that sulfation could be an effective modification to improve the bioactivity of bacterial *exo*-polysaccharides, by improving their ability to disrupt cell cytoplasmic membranes.

In this context, Han et al. [42] demonstrated that only the purified fraction eluted within the highest concentration of NaCl displayed high antibacterial activity against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. Additionally, Abdelhedi et al. [27] have shown that the most negative fractions (FIII and FIV) obtained from smooth hound viscera sulfated polysaccharides exhibited the best antibacterial activity against *S. typhi*.

4. Conclusion

The present study demonstrated the efficiency of CPC precipitation on the extraction of polysaccharides from cuttlefish skin and muscle CMP, with high sulfate and uronic acid contents. CSP and CMP showed high antioxidant potential and could inhibit the radicals' chain reaction propagation, either by the donation hydrogen/electron to free radicals, or by chelating iron involved in the Fenton reaction. They were able, furthermore, to inhibit the bacterial growth of various Gram-positive and Gram-negative bacteria. These biological activities were mainly governed by the negative polysaccharide fractions, as revealed by the DEAE fractionation results. All over, the present study findings demonstrated that cuttlefish skin and muscle are promising sources of natural-derived antioxidant and antibacterial agents that might be applied in food and non-food stuffs.

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