



Sulfated polysaccharides from *Loligo vulgaris* skin: Potential biological activities and partial purification



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ABSTRACT

The characteristics, biological properties, and purification of sulfated polysaccharides extracted from squid (*Loligo vulgaris*) skin were investigated. Their chemical and physical characteristics were determined using X-ray diffraction and infrared spectroscopic analysis. Sulfated polysaccharides from squid skin (SPSS) contained 85.06% sugar, 2.54% protein, 1.87% ash, 8.07% sulfate, and 1.72% uronic acid. The antioxidant properties of SPSS were investigated based on DPPH radical-scavenging capacity ($IC_{50} = 19.42 \text{ mg mL}^{-1}$), hydrogen peroxide-scavenging activity ($IC_{50} = 0.91 \text{ mg mL}^{-1}$), and β -carotene bleaching inhibition ($IC_{50} = 2.79 \text{ mg mL}^{-1}$) assays. ACE-inhibitory activity of SPSS was also investigated ($IC_{50} = 0.14 \text{ mg mL}^{-1}$). Further antimicrobial activity assays indicated that SPSS exhibited marked inhibitory activity against the bacterial and fungal strains tested. Those polysaccharides did not display hemolytic activity towards bovine erythrocytes. Fractionation by DEAE-cellulose column chromatography showed three major absorbance peaks. Results of this study suggest that sulfated polysaccharides from squid skin are attractive sources of polysaccharides and promising candidates for future application as dietary ingredients.

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1. Introduction

The fish industry is a major contributor to socio-economic growth in several countries around the world. This sector is, however, known to generate large amounts of waste by-products, which creates troublesome health, environmental, and waste disposal problems. The fish processing waste after filleting is estimated to accounts for about 75% of the total fish weight, and more than 30% of the original raw material remains as waste residues in the form of skins and bones during the preparation of fish fillets [1]. These residues have traditionally been discarded as waste and low value by-products or used as animal feed and fertilizers. Recent research has, however, indicated that those waste by-products can be effectively used to produce a wide range of useful value-added products.

The interest in the improved utilization of by-products has for several reasons, including the possibilities to produce more food from limited resources and to search for viable solutions to

several economic and environmental problems, increased in the last few decades [2]. In fact, the utilization of processing by-products is more important for the economic viability of the aquatic foods industry than most other food processing industries [3]. In addition to the direct use of by-products as human food, much attention has been given to the transformation of this biomass into isolated functional or biologically active components, so-called value-added products, for use in the cosmetic, nutraceutical, and pharmaceutical industries [4].

Several studies have been performed to investigate the potential use of undervalued seafood sources and processing by-products of no or low market value to produce products with potential bioactivity, including antioxidant [5], antidiabetic [6], antimicrobial [7], anti-proliferative [8], and angiotensin-I converting enzyme (ACE) inhibitory activities [9]. Of particular interest, the literature indicates that sulfated polysaccharides, a heterogeneous group of complex macromolecules, are a promising source of various biological activities. Those anionic polymers are widespread in nature, occurring in a wide variety of organisms, including marine invertebrates [10–14]. The literature indicates those polysaccharides are structurally diverse and heterogeneous, particularly in terms of chemical properties, charge density, molecular mass, and

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biological activities. The production of a standardized commercial products based on the constituents of those anionic polymers has often been considered challenging. This is in part due to the dependence of their structural and biological activities on the species, location, time of harvest, and the amount and position of sulfate groups, which in some cases form specific oligosaccharide sequences along the macromolecular backbone [15].

The squid *Loligo vulgaris* (Lamarck, 1798) is a large squid belonging to the family Loliginidae. It is found throughout the Mediterranean and in the eastern Atlantic Ocean from the North Sea to the Gulf of Guinea. The species is extensively exploited by commercial fisheries [2]. Considering the important and promising applications of sulfated polysaccharides, the present study aimed to investigate the extraction, chemical characterization, and partial purification of sulfated polysaccharides from squid (*L. vulgaris*) skin and evaluate their antihypertensive, antimicrobial, and antioxidant activities.

2. Materials and Methods

2.1. Reagents

The chemicals and solvents used in the present study were purchased at the analytical grade or highest level of purity available. Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was obtained from Novozymes® (Bagsvaerd, Denmark). The chemicals used in the experimental assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), angiotensin I-converting enzyme from rabbit lung, the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL), β -carotene, and L-ascorbic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diethylaminoethyl (DEAE)-cellulose were supplied by Pharmacia (Uppsala, Sweden). All other chemicals and reagents were used at the analytical grade, and all solutions were freshly prepared in distilled water.

2.2. Preparation of squid skin

Squid by-products were obtained from a local marine processing industry (CALEMBO) in Sfax, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. Upon arrival, the samples were washed twice with water to eliminate dark ink, which consists of a suspension of melanin granules in a viscous colorless medium. The squid by-products were separated, and only squid outer skin was collected and stored in sealed plastic bags at -20°C until further use for sulfated polysaccharides extraction and analysis.

2.3. Polysaccharide extraction

Sulfated polysaccharides were extracted according to a slightly modified version of the method described by Ben Mansour et al. [16]. In brief, the squid skins were cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). An amount of 5 g of sample was dissolved in 250 mL sodium acetate (0.1 M), EDTA (5 mM), and cystein (5 mM) pH = 6. Alcalase® was added, and the mixture was kept for 24 h at 50°C . The mixture was then left to cool down at room temperature and filtered. The residue was washed with distilled water and filtered again. The filtrates were mixed, and polysaccharides were precipitated with cetylpyridinium chloride (w/v). The mixture was left 24 h at room temperature and centrifuged for 30 min at 6720 g at 4°C using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Germany). The pellet was dissolved in a 200 mL NaCl solution in ethanol (100:15, v/v), and ethanol (700 mL) was added. The

polysaccharide containing solution was left for 24 h at 4°C and then centrifuged for 30 min at 5000 rpm and 4°C . The pellet was washed twice with ethanol 80% and then once with absolute ethanol. The pellet was then redissolved in desionized water and lyophilized in a freeze dryer (CHRIST, ALPHA 1-2 LD plus, Germany). The dry matter was referred to as “Sulfated polysaccharides from squid skin” (SPSS).

2.4. Determination of chemical composition

Moisture and ash contents were determined according to the AOAC standard methods 930.15 and 942.05, respectively [17]. Total nitrogen content was determined using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Crude fat was determined gravimetrically after the Soxhlet extraction of dried samples with hexane. Total carbohydrates were determined by the phenol-sulphuric acid method [18]. Total uronic acid content was quantified colorimetrically according to the method described by Bitter and Muir [19] using galacturonic acid as a standard. Water activity (A_w) was measured at 25°C by a NOVASINA aw Sprint TH-500 apparatus (Novasina, Pfäffikon, Switzerland). All measurements were performed in triplicates.

2.5. Determination sulfate content in polysaccharides

The determination of sulfate content in the polysaccharides was carried out by liquid-Ion Chromatography (HPLC) on a Metrohm chromatograph equipped with columns CI SUPER-SEP using acetonitrile and phthalic acid as eluent. The test precision of the instrument was about $\pm 2\%$.

2.6. Determination of color

The samples were placed between two steel dishes with a hole of 5.7 cm diameter. The color of the samples was determined with a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale ($C/2^{\circ}$), where L^* , a^* , and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate was used as a reference. The results were the average of five measurements taken at ambient temperature and at different points on the samples.

2.7. Infra-red spectroscopic analysis

The absorption spectra of the samples were obtained using FTIR spectroscopy (Analect Instruments fx-6 160). The FTIR spectra of the prepared materials were recorded between 450 and 4000 cm^{-1} in a NICOET spectrometer. The transmission spectra of the samples were recorded using the KBr pallet containing 0.1% of sample.

2.8. X-ray diffraction (XRD) of polysaccharides

The X-ray diffraction pattern of sulfated polysaccharides was recorded at room temperature on an X-ray diffractometer (D8 advance, Bruker, Germany). The data were collected in the 2θ range $2-70^{\circ}$ with a step size of 0.02° and a counting time of 5 s/step.

2.9. Determination of antioxidant activity

2.9.1. DPPH free radical-scavenging activity

The DPPH free radical-scavenging activity of the sulfated polysaccharides extracted from squid skin (SPSS) was determined as described by Bersuder et al. [20]. A volume of $500\text{ }\mu\text{L}$ of each sample at different concentrations ($10-50\text{ mg mL}^{-1}$) was added to $375\text{ }\mu\text{L}$ of 99% ethanol and $125\text{ }\mu\text{L}$ of DPPH solution (0.02% in ethanol) as a free radical source. The mixtures were shaken and then

incubated for 60 min in the dark at room temperature. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, China). In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as a positive control. DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100,$$

where A_{blank} refers to the absorbance of the reaction containing all reagents except when distilled water was used instead of the sample, and A_{sample} to absorbance in the presence of the sample. The experiment was carried out in triplicate.

2.9.2. Hydrogen peroxide-scavenging activity assay

Hydrogen peroxide-scavenging activity assay was measured by replacement titration [21]. A solution of 1.0 mL of 0.1 mM H_2O_2 and 1.0 mL of various concentrations of sulfated polysaccharides were mixed, followed by 2 drops of 3% ammonium molybdate, 10 mL of H_2SO_4 (2 M), and 7.0 mL of KI (1.8 M). The mixed solution was titrated with $\text{Na}_2\text{S}_2\text{O}_3$ (5.09 mM) until the disappearance of yellow color. BHA was used as a positive control. The relative activities of the test compounds to scavenge hydrogen peroxide were expressed as percentage (%) of the titer volume change:

$$\text{Scavenging activity} = \left[\frac{(V_{\text{control}} - V_{\text{sample}})}{V_{\text{control}}} \times 100 \right].$$

2.9.3. The β -carotene bleaching method

The ability of the sample to prevent the bleaching of β -carotene was assessed as described by Koleva et al. [22]. A stock solution of β -carotene/linoleic acid mixture was 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 completely evaporated under vacuum at 40 °C in a rotatory evaporator (Rotary evaporator, Heidolph, Germany), and then 100 mL of bi-distilled water was added. The resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β -carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 mL of each sample at different concentrations of polysaccharides (1–8 mg mL^{-1}). Following incubation for 2 h at 50 °C, the absorbance of each sample was measured spectrophotometrically (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, China) at 470 nm. BHA was used as a positive standard. A control consisting of 0.5 mL of distilled water instead of sample was also used. The antioxidant activity of the sample was evaluated in terms of β -carotene bleaching using the following formula:

$$A(\%) = \left[1 - \frac{(A_0 - A_t)}{(A'_0 - A'_t)} \right] \times 100,$$

where A_0 and A'_0 refer to the absorbances of the sample and the control measured at time zero, respectively, and A_t and A'_t to the absorbances of the sample and the control measured after incubation for 2 h, respectively. The same procedure was repeated with BHA as a positive control.

2.10. Angiotensin-I-converting enzyme inhibitory activity

The angiotensin-I-converting enzyme (ACE) inhibitory activity was measured in triplicate as reported by Nakamura et al. [23]. A sample solution (80 μL) containing different concentrations (0.2–1 mg mL^{-1}) of sulfated polysaccharides was mixed with

200 μL of 5 mM HHL, and then preincubated for 3 min at 37 °C. The polysaccharides and HHL were prepared in 100 mM borate buffer (pH 8.3) containing 300 mM NaCl. The reactions were then initiated by adding 20 μL of 0.1 U mL^{-1} ACE from rabbit lung prepared in the same buffer. After incubation for 30 min at 37 °C, the enzyme reactions were stopped by the addition of 250 μL of 0.05 M HCl. The liberated hippuric acid (HA) was extracted with ethyl acetate (1.7 mL) and then evaporated at 90 °C for 10 min by rotary evaporation under reduced pressure (Rotary evaporator, Heidolph, Germany). The residue was dissolved in 1 mL of distilled water, and the absorbance of the extract at 228 nm was determined using a UV-visible spectrophotometer (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, China). The average value from three determinations at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE inhibition (\%)} = \left[\frac{B - A}{B - C} \right] \times 100,$$

where A refer to the absorbance of HA generated in the presence of ACE inhibitor, B to the absorbance of HA generated without ACE inhibitors (100 mM borate buffer pH 8.3 was used instead of sulfated polysaccharides), and C to the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay).

The IC_{50} value, defined as the concentration of sulfated polysaccharides (mg/mL) required to inhibit 50% of ACE activity, was calculated for each sample using non-linear regression from a plot of percentage ACE inhibition versus sample concentrations.

2.11. Antimicrobial activity

2.11.1. Microbial strains

The antibacterial activities of the sulfated polysaccharides extracted from squid skin (SPSS) were tested against 6 strains of bacteria, namely three Gram-negative (*Salmonella enterica* ATCC 43972, *Escherichia coli* ATCC 25922, and *Enterobacter* sp) and three Gram-positive (*Listeria monocytogenes* ATCC 43251, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* (isolated from food products)) bacteria. Antifungal activities were tested using *Fusarium solani*, *Botrytis cinerea*, and *Alternaria solani*. Those fungal strains were kindly provided by the microbial collection of the Centre of Biotechnology of Sfax-Tunisia.

2.11.2. Agar diffusion method

The antimicrobial activity was determined by the method of Berghe and Vlietinck [24]. A culture suspension (200 μL) of the tested microorganisms (10^6 colony forming units (cfu) mL^{-1} of bacteria cells and 10^8 spores mL^{-1} of fungal strains) was spread on a Mueller–Hinton broth and potato dextrose agar media, respectively. The sulfated polysaccharides (20 and 50 mg mL^{-1}) were dissolved in distilled water and added (20 μL) to wells punched in the agarose layer, allowed to diffuse in the layer, and incubated in a humidified close container for 3 h at 4 °C. At the end of incubation time (24 h at 37 °C for bacteria strains or 72 h at 30 °C for fungal strains), antibacterial activity was measured as the diameter of the clear zone of growth inhibition and compared to a positive control (ampicilline) and a negative control (distilled water) dissolved in Petri plates. All tests were carried out for three sample replications, and the results were averaged.

2.11.3. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the sulfated polysaccharides was determined by liquid growth inhibition assay in a microtiter plate assay system [25]. MIC was the lowest concentration of the polysaccharides that completely inhibited the strain growth after 24 h of incubation. A total of 100 mL of cell

suspension were distributed in a microtiter plate well, and 100 μL of the sulfated polysaccharides were added. Cells were enumerated on nutrient agar medium before and after 24 h of incubation at 30 or 37 °C.

2.11.4. Antibacterial activity in liquid medium

Antibacterial activity was determined as described by Hammami et al. [26]. The antibacterial activities of the SPSS in liquid media were tested against *S. enterica*, *L. monocytogenes*, *S. aureus*, and *Enterobacter* sp. The strains were inoculated in Mueller–Hinton broth media. The precultures were performed on a rotary shaker (SI-300R, Lab Companion, Korea) for 24 h (200 rpm) at 37 °C, in 250 mL Erlenmeyer flasks with a working volume of 25 mL. After incubation, the cell concentration was around 10^6 CFU mL⁻¹. Cultures were prepared by inoculating 1 mL of preculture in 25 mL of sterile Mueller–Hinton broth. After that, 1 mL (20 and 50 mg mL⁻¹) of sulfated polysaccharides was added, and the mixture was incubated for 1 h at 4 °C. A flask that did not contain sulfated polysaccharides was used as a control. After incubation at 4 °C, the Erlenmeyer flasks were incubated for 10 h at 37 °C (200 rpm), and absorbance at 595 nm was measured every 60 min using a UV-visible spectrophotometer (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, China).

2.12. Hemolytic activity

The hemolytic activity of the sulfated polysaccharides was determined using a slightly modified version of the methods described by Dathe et al. [27]. In brief, 5 mL of bovine blood was centrifuged at 3500 rpm for 10 min to isolate erythrocytes, which was then washed three times with 10 mM sodium phosphate, pH 7.5, containing NaCl 9 g L⁻¹ (NaCl/Pi). The cell concentration stock suspension was adjusted to 10^9 cells mL⁻¹. The cell suspension (12 μL), along with varying amounts of sample stock solutions and the buffer, were pipetted into Eppendorf tubes to give a final volume of 50 μL . The Eppendorf tubes with 2.5×10^8 cells mL⁻¹ were then incubated for 40 min at 37 °C. After centrifugation (6720 g, 5 min), 30 μL of the supernatant was diluted in 500 μL water. The absorbance of the diluted solution was measured at 420 nm. The absorbance obtained after treating erythrocytes with only NaCl/Pi and SDS (0.2%) was taken as 0 and 100%, respectively. The experiments were repeated three times to verify reproducibility.

2.13. Fractionation by anion-exchange chromatography

The lyophilized sulfated polysaccharides were suspended in distilled water and then fractionated by anion-exchange chromatography on a DEAE-cellulose column (2.25 \times 10 cm) pre-equilibrated with sodium acetate buffer (0.1 M), pH 6.5. The fractions (4 mL) were prepared in stepwise elution and a step-wise gradient of 0.1, 1, and 2.0 mol L⁻¹ NaCl at a flow rate of 50 mL h⁻¹. The elution was detected by the phenol–sulfuric acid method [18]. The major fraction was pooled, concentrated, desalted, and freeze dried in a freeze dryer (CHRIST, ALPHA 1-2 LD plus, Germany).

2.14. Statistical analysis

All data were submitted to Analysis of Variance (ANOVA), and differences between means were evaluated by the Duncan's Multiple Range Test. The data were analyzed using the Statistical Package for the Social Sciences version 10.0 (Chicago, Illinois, USA). Differences were considered significant at $P < 0.05$.

Table 1

Proximate composition of squid skin and the sulfated polysaccharides (SPSS). Physico-chemical composition was calculated basis on the dry mater. Values represent averages \pm standard deviations for triplicate experiments.

| Composition | Squid skin | SPSS |
|-----------------|------------------|------------------|
| Total sugar (%) | – | 85.06 \pm 2.40 |
| Sulfate (%) | – | 8.07 \pm 0.13 |
| Protein (%) | 22.96 \pm 3.11 | 2.54 \pm 0.48 |
| Fat (%) | 0.73 \pm 0.08 | 0.14 \pm 0.04 |
| Ash (%) | 3.74 \pm 0.39 | 1.87 \pm 0.25 |
| Uronic acid (%) | – | 1.72 \pm 0.06 |
| Aw | – | 0.51 \pm 0.02 |
| Yield % (v) | – | 21.14 \pm 1.27 |
| Color | | |
| L* | – | 50.74 \pm 0.13 |
| a* | – | 7.12 \pm 0.01 |
| b* | – | –1.77 \pm 0.01 |

^vCalculated based on wet weight.

3. Results and discussion

3.1. Sulfated polysaccharides extractions and chemical analyses

Based on the wet weight, a sulfated polysaccharides extraction yield of 21.14% was obtained. This yield was similar to those previously obtained for *Gracilaria cervicornis* and *Gracilaria cornea* as reported by Marinho-Soriano [28] and Melo et al. [29], respectively. The proximate composition of both fresh squid skin and SPSS are shown in Table 1. Squid skin showed high protein content (22.96%) and an ash content of 3.74%. The fat content of squid skin was lower than 1%. The sugar content in SPSS was very high (85.06%). The high sugar content was presumably due to the efficiency of the extraction protocol. The sugar content was similar to the one obtained by Souza et al. [12] for sulfated polysaccharides from red seaweed and higher than those reported from *Sphacelaria indica* [30] and the skin of *Raja radula* [16]. The lower protein (2.54%), fat (0.14%), and ash (1.87%) contents suggest the efficient removal of lipids, proteins, and minerals from the skin material. The sulfate content was found to be 8.07 \pm 0.13%, a value that was significantly lower than that reported for sulfated polysaccharides extracted from abalone [14] but higher than that reported from brown marine algae [31]. It may, therefore, be concluded that origin, nature of first matter, and type of extraction are responsible for the amount of sulfate. Sulfated polysaccharide chains have been previously reported to contain disaccharide repeating units, called disaccharide repeating regions, which consist of uronic acid and amino sugar. The uronic acid content of SPSS (1.72 \pm 0.06%) was lower than that reported by Bandyopadhyay et al. [30].

The color values of sulfated polysaccharides extracted from squid skin are also shown in Table 1. Color influences the overall acceptability of products. SPSS was light ($L^* = 50.74$) and a mixture of blue and magenta ($b^* = -1.77$ and $a^* = 7.12$). The sulfated polysaccharides showed low water activity (0.511), which is known to prevent bacteria, mould and yeast development [32].

3.2. FT-IR spectroscopy and X-ray diffraction

Fourier transform infra-red (FT-IR) spectroscopy was performed in the 4000–450 cm⁻¹ region to further characterize and corroborate the data so far obtained for the polysaccharides present in SPSS. As shown in Fig. 1, the peak observed at 1387 cm⁻¹ derived from the bending vibration of the stretching vibration of the ester sulfate groups (S=O) of sulfate. The IR absorption bands at 1387 cm⁻¹ were assigned based on the principle originally published by Orr [33] and by Lloyd et al. [34]. Melo et al. [29] also reported the presence of a band at 1370 cm⁻¹ when investigating the extraction of polysaccharides from *Gracilaria birdiae*. Furthermore, signals at 1630 cm⁻¹

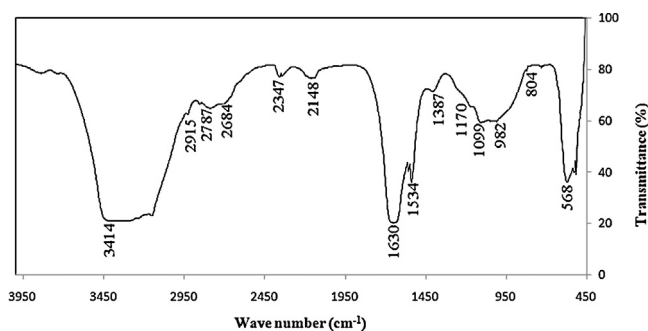


Fig. 1. FT-IR spectrometry of sulfated polysaccharides extracted from squid skin. (The test was carried out in duplicate).

were attributed to the asymmetric stretch vibration of COO^- of uronic acids, and the region around 1099 cm^{-1} was equivalent to the skeleton of galactans specific band. The region around 980 cm^{-1} could be attributed to the $\text{C}-\text{O}-\text{C}$ group, and the absorption around 804 cm^{-1} indicated the presence of sulfate groups [14]. The band at 3414 cm^{-1} derived from the stretching vibration of $\text{O}-\text{H}$, and the signal at 2915 cm^{-1} could be attributed to the stretching vibration of $\text{C}-\text{H}$ [35].

X-ray diffraction is a method used for the analysis of the crystalline structure of materials [36]. The X-ray diffraction patterns recorded for SPSS were between 10 and 60 of 2θ (Data not shown). The results suggested that the sulfated polysaccharides of squid skin were crystalline. Han et al. [37] reported that the sulfated polysaccharides produced by the cyanobacteria "*Nostoc flagelliforme*" were non-crystalline.

3.3. Determination of antioxidant activity

3.3.1. Free radical-scavenging activity

DPPH is a free radical compound widely used for determining the free radical-scavenging ability of samples [38,39]. This method allows for the determination of the antiradical activity of samples by measuring the decrease in absorbance at 517 nm of the DPPH radical caused by the scavenging of the hydroxyl radical through hydrogen donation. The DPPH radical-scavenging capacities of SPSS and BHA (used as positive control) are shown in Fig. 2. A. The results revealed that the sulfated polysaccharides had unnoticeable effects on the inhibition of those radical formations with a dose-effect relationship. Similar results were reported by Shao et al. [40] for sulfated polysaccharides extracted from algae. The DPPH scavenging activities displayed by SPSS and BHA at a concentration of 50 mg mL^{-1} were 83.8 and 97.48%, respectively. The sulfated polysaccharides from squid skin were a strong radical scavenger with an inhibition concentration (IC_{50}) of 19.42 mg mL^{-1} . Souza et al. [12] have previously reported that the sulfated polysaccharide from the red seaweed (*Gracilaria birdiae*) had a noticeable effect on inhibiting the formation of those radicals. The results obtained suggest that SPSS were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

3.3.2. Hydrogen peroxide-scavenging activity

The measurement of H_2O_2 -scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2 [41]. Hydrogen peroxide can cross membranes and may slowly oxidize a number of compounds. As shown in Fig. 2. B, the hydrogen peroxide-scavenging activity of SPSS and BHA increased with increasing concentration. SPSS had an important antioxidant activity at all the concentrations tested. The antioxidant activity displayed by BHA was significantly

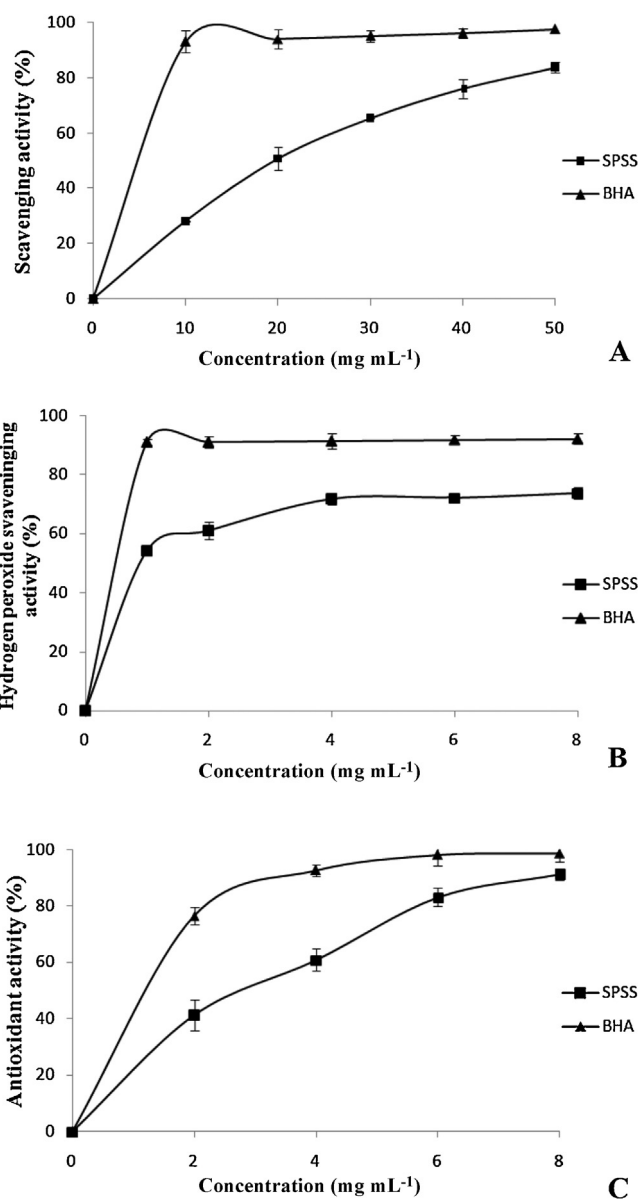


Fig. 2. Antioxidant activities of SPSS at different concentrations. DPPH free radical-scavenging activities (A), hydrogen peroxide-scavenging activities (B) and inhibition of β -carotene bleaching (C). Values are means of three replications \pm SD.

($P < 0.05$) higher than that of SPSS at all concentrations tested. At 1 mg mL^{-1} , the hydrogen peroxide-scavenging capacity of SPSS was 54.17% whereas that of BHA was 89.6%. At the same concentration, Abu et al. [13] reported that the sulfated polysaccharide isolated from *Ascophyllum nodosum* toward hydrogen peroxide displayed no significant scavenging effects. The IC_{50} value recorded for SPSS was 0.87 mg mL^{-1} , which was lower than the value previously reported for sulfated polysaccharides from *Sargassum tenerrimum* [31].

3.3.3. β -carotene–linoleic acid assay

Antioxidant assays using the discoloration of β -carotene are widely employed to measure the antioxidant activity of compounds. In the present study, the presence of SPSS hindered the extent of β -carotene bleaching by neutralizing the formed linoleic hydroperoxyl radicals [42]. The antioxidant activity of the sulfated polysaccharides from squid skin determined in terms of percent inhibition in the β -carotene–linoleic acid system is presented in Fig. 2. C. The antioxidant activity of sulfated polysaccharides from

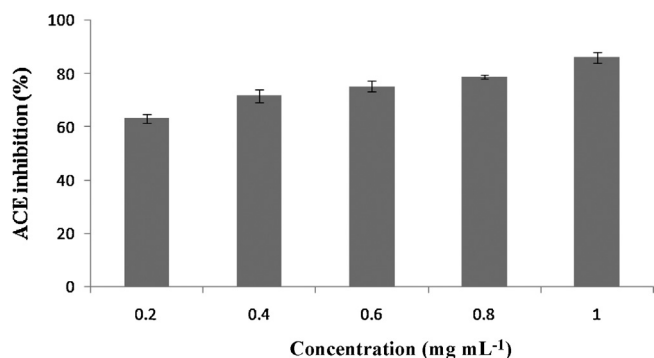


Fig. 3. ACE-inhibitory activity of sulfated polysaccharides at different concentration. Values are given as mean \pm SD from triplicate determinations.

L. vulgaris skin increased with the increase of SPSS concentration. Sulfated polysaccharides from squid skin showed a strong antioxidant activity, with an IC_{50} of 2.79 mg mL^{-1} . Similar results were reported by Chen et al. [43] for sulfated polysaccharides from *Ganoderma atrum*. BHA displayed a significantly ($P < 0.05$) higher antioxidant activity than that of SPSS at all concentrations tested. At 8 mg mL^{-1} , the antioxidant capacity of SPSS was 54.17% whereas that of BHA was 89.6%. These antioxidant activity results provided strong support for the promising candidacy of SPSS for use as a new source of natural additives. Sulfated polysaccharides from squid skin (SPSS) with proper substitution could be used as natural antioxidants to inhibit lipid peroxidation during food processing and preservation.

3.4. Angiotensin-I-converting enzyme inhibitory activity

The inhibition of ACE by antihypertensive agents is a promising strategy for hypertension management. In fact, recent research shows that ACE inhibition may be considered as a useful therapeutic approach for the treatment of high blood pressure [44]. Since synthetic ACE inhibitors may cause adverse side effects, biomolecules may offer natural and cost-effective alternatives ACE inhibitors for the treatment and prevention of hypertension. The sulfated polysaccharides extracted from squid skin were evaluated in terms of ACE-inhibitory activity. Fig. 3 shows that ACE-inhibitory activity was concentration-dependent, increasing with the increase of SPSS concentration. SPSS was also noted to exhibit strong ACE-inhibitory activity, with the highest levels (86.3%) being observed at a concentration of 1 mg mL^{-1} . The IC_{50} value for the ACE inhibition of SPSS was 0.14 mg mL^{-1} .

Results presented in this work suggest that sulfated polysaccharides from squid skin could enhance the biological properties of functional foods and an attractive ingredient for future application in nutraceuticals against hypertension.

3.5. Antimicrobial activity

Spurred by the proliferation of microbial strains that exhibit resistance to conventional antibiotics, the last few decades have seen a flurry of research activities seeking to develop novel therapeutic antibiotics from natural origins. In line with this search, the antibacterial activity of the sulfated polysaccharides was tested against six bacteria (*E. coli*, *S. enterica*, *E. sp.*, *L. monocytogenes*, *S. aureus*, and *B. cereus*). Antibacterial activity was assessed by evaluating the diameter of the clear zone of growth inhibition and the determination of MIC values ($\text{mg of SPSS mL}^{-1}$). As shown in Table 2, SPSS showed varying degrees of antibacterial activity against the tested strains. No antibacterial activity was, however, detected for *E. coli* and *B. cereus*. The inhibition zones and

Table 2

Antibacterial and antifungal activities of sulfated polysaccharides extracted from squid skin. Values represent averages \pm standard deviations for triplicate experiments.

| Tested microorganisms | Inhibition zone diameter (mm) | | MIC (mg mL^{-1}) |
|-------------------------------|-------------------------------|--------------------------|-----------------------------|
| | 20 mg mL^{-1} | 50 mg mL^{-1} | |
| Bacteria | | | |
| <i>Salmonella enterica</i> | – | ++ | 25 |
| <i>Escherichia coli</i> | – | – | ND |
| <i>Enterobacter sp.</i> | ++ | +++ | 12.5 |
| <i>Listeria monocytogenes</i> | – | ++ | 50 |
| <i>Staphylococcus aureus</i> | + | ++ | 12.5 |
| <i>Bacillus cereus</i> | – | – | ND |
| | 100 mg mL^{-1} | 150 mg mL^{-1} | |
| Fungi | | | |
| <i>Fusarium solani</i> | ++ | ++ | ND |
| <i>Botrytis cinerea</i> | – | ++ | ND |
| <i>Alternaria solani</i> | ++ | +++ | ND |

Inhibition zones: +++: $> 15 \text{ mm}$; ++: $5\text{--}15 \text{ mm}$, + $< 5 \text{ cm}$, –: no activity and ND: not determined.

MIC values of microbial strains were in the range of $6\text{--}24 \text{ mm}$ and $12.5\text{--}50 \text{ mg mL}^{-1}$, respectively. The results showed that SPSS inhibited both Gram-positive and Gram-negative bacteria, though Gram-negative bacteria were noted to be more sensitive to SPSS than Gram-positive bacteria. This result was similar to the findings previously reported by Hellio et al. [45] who found that sulfated polysaccharides extracted from macroalgae inhibited both of Gram-positive and Gram-negative bacteria. The antibacterial activity of SPSS increased with the increase of sulfated polysaccharides concentration. For the strains tested, the antibacterial activity of SPSS at a concentration of 50 mg mL^{-1} was significantly higher ($P < 0.05$) than that at a concentration of 20 mg/mL . In fact, *Enterobacter sp.* was the most susceptible bacterium for this sulfated polysaccharide (Clear zone = 24 mm at 50 mg mL^{-1} , MIC = 12.5 mg mL^{-1}). Vijayabaskar, et al. [46] reported that the most susceptible bacterium for sulfated polysaccharides from *Sargassum swartzii* was *B. subtilis*.

The antibacterial activity of the sulfated polysaccharides from squid skin against *S. enterica*, *E. sp.*, *L. monocytogenes*, and *S. aureus*, was also investigated in Erlenmeyer flasks containing liquid broth media (Fig. 4). The results obtained for pathogenic bacterial growth kinetics showed that antibacterial properties of SPSS were confirmed in liquid media after the addition of SPSS at a concentration of 20 and 50 mg mL^{-1} . Bacterial growths were inhibited in the presence of sulfated polysaccharides for all the bacterial strains tested as compared to their controls without sulfated polysaccharides.

Furthermore, the antifungal activity of sulfated polysaccharides from squid skin was tested using three fungal strains (*F. solani*, *B. cinerea*, and *A. solani*), and the results revealed that they exhibited markee inhibitory activity against the three strains (Table 2). SPSS exhibited the best inhibitory activity against *Alternaria solani*. At 150 mg mL^{-1} , the inhibition zones values of *F. solani*, *B. cinerea*, and *A. solani* were 12 , 11 , and 23 mm , respectively.

3.6. Hemolytic activity of SPSS

The hemolytic activity of sulfated polysaccharides was tested on bovine erythrocytes (data not shown). Several concentrations for samples were tested ($10\text{--}150 \text{ mg mL}^{-1}$). For all concentrations, no hemolysis was observed. These results show that sulfated polysaccharides from squid skin would be non-toxic even if used at high concentrations.

3.7. Partial purification of the sulfated polysaccharides

The sulfated polysaccharides were fractionated using a Diethylaminoethyl-cellulose chromatography. SPSS were applied

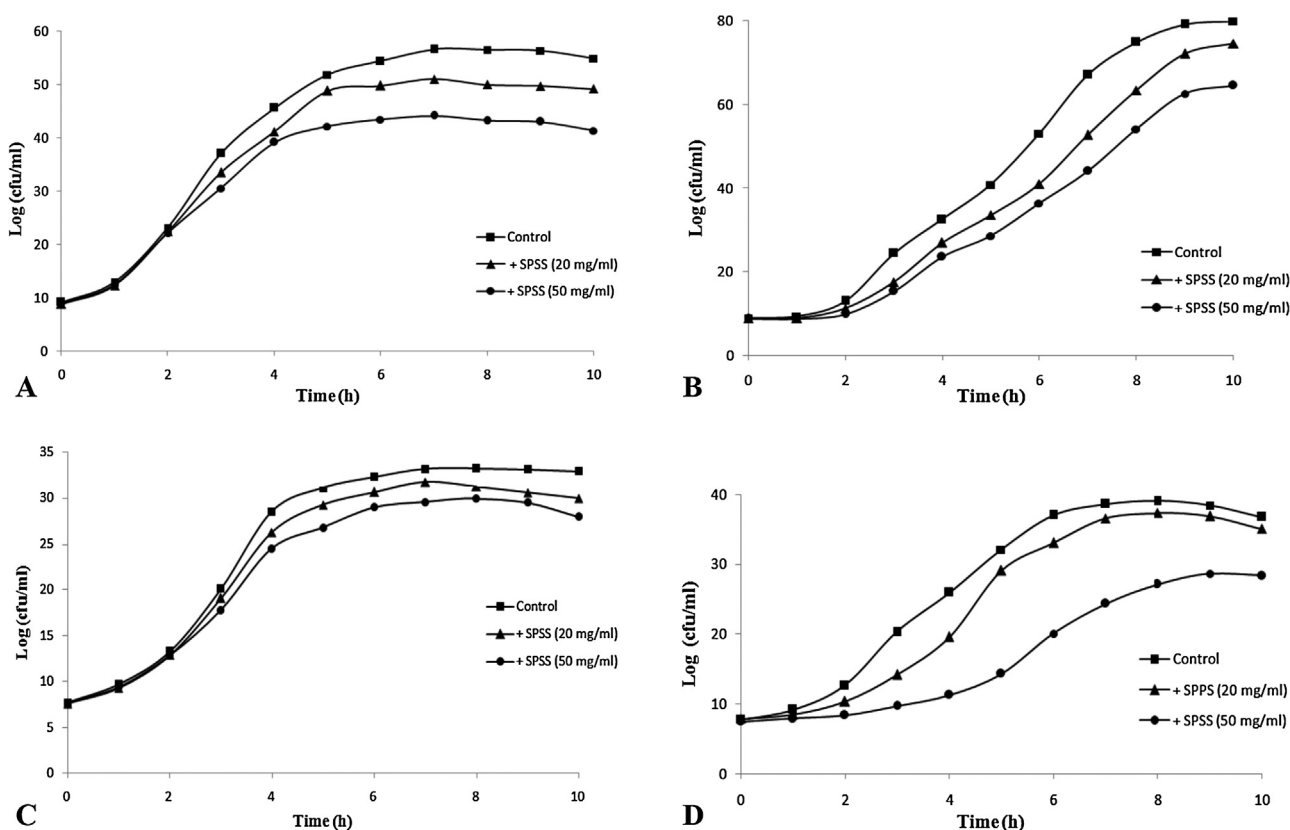


Fig. 4. Determination of the antibacterial activity of SPSS in liquid medium. Activity against *S. enterica* (A), *Enterobacter sp* (B), *L. monocytogenes* (C), and *S. aureus* (D) compared with positive bacterial controls without SPSS.

to a DEAE-cellulose column equilibrated with sodium acetate buffer beforehand. After being washed with the same buffer, the sulfated polysaccharides were eluted with a step-wise gradient of sodium chloride (0.1, 1, and 2 M). The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method. As illustrated in Fig. 5. A, there are three major absorbance peaks (F_1 , F_2 , and F_3). The fractions associated with each peak were collected, desalted, and concentrated by lyophilisation. The yields of the three F_1 , F_2 , and F_3 fractions were 27, 10, and 41%, respectively.

In order to have a more precise characterization of the three DEAE-cellulose fractions of the sulfated polysaccharides, FTIR spectra were acquired in the $4000\text{--}400\text{ cm}^{-1}$ region (Fig. 5. B). The major difference was due to the sulfate ester and carbohydrate bands in the infrared spectra. All fractions showed two bands in the $4000\text{--}2000\text{ cm}^{-1}$ region: a broadly intense stretched peak at around 3412 cm^{-1} , and a weak band at around 2917 cm^{-1} , which

are characteristic absorption bands of hydroxyl groups and C–H bonds in the polysaccharide molecule, respectively [47]. The fractions also showed specific bands in the $1200\text{--}1000\text{ cm}^{-1}$ region due to ring vibrations overlapped with the stretching vibrations of C–OH side groups [48]. The broad absorption band observed at $1200\text{--}1400\text{ cm}^{-1}$, and associated to the ester sulfate group, is common to all sulfated polysaccharides [49]. This absorption band appeared in all fractions.

The biological activities of the DEAE-cellulose fractions of the sulfated polysaccharides (F_1 , F_2 , and F_3) were also investigated. As shown in Table 3, no hemolysis was observed for all fractions. The results indicated that F_1 , F_2 , and F_3 would be non-toxic even when used at a high concentration (50 mg mL^{-1}). The F_2 fraction exhibited the highest free radical-scavenging activity of 82.2% at a concentration of 10 mg mL^{-1} . No scavenging capacity was observed for fraction F_3 . The antihypertensive activity of the SPSS fractions

Table 3

Biological activities of sulfated polysaccharides fractions (F_1 , F_2 , and F_3). Values represent averages \pm standard deviations for triplicate experiments. Different superscripts in the same row indicate the significant differences ($P < 0.05$).

| Biological activities | SPSS fractions | | |
|---|--------------------|--------------------|--------------------|
| | F_1 | F_2 | F_3 |
| DPPH free radical-scavenging activity at 10 mg mL^{-1} (%) | 11.85 ± 0.23^a | 82.2 ± 2.05^b | – |
| Antihypertensive activity at 0.2 mg mL^{-1} (%) | – | 17.98 ± 1.73^a | 87.75 ± 1.96^a |
| Hemolytic activity at 50 mg mL^{-1} (%) | – | – | – |
| Antibacterial activity at 20 mg mL^{-1} : inhibition zone (mm) | | | |
| <i>Salmonella enterica</i> | – | 8 ± 0.5^a | 11 ± 1^a |
| <i>Enterobacter sp</i> | – | 9 ± 2^a | 16 ± 1.5^a |
| <i>Listeria monocytogenes</i> | – | 8 ± 1^a | 10 ± 2^b |
| <i>Staphylococcus aureus</i> | 6 ± 0.5^a | – | 13 ± 3^b |
| Antifungal activity at 50 mg mL^{-1} : inhibition zone (mm) | | | |
| <i>Fusarium solani</i> | – | 6 ± 1^a | 8 ± 1.5^a |
| <i>Botrytis cinerea</i> | – | – | 7 ± 2 |
| <i>Alternaria solani</i> | 6 ± 0.5^a | 10 ± 2.5^b | 15 ± 1^c |

–: No activity

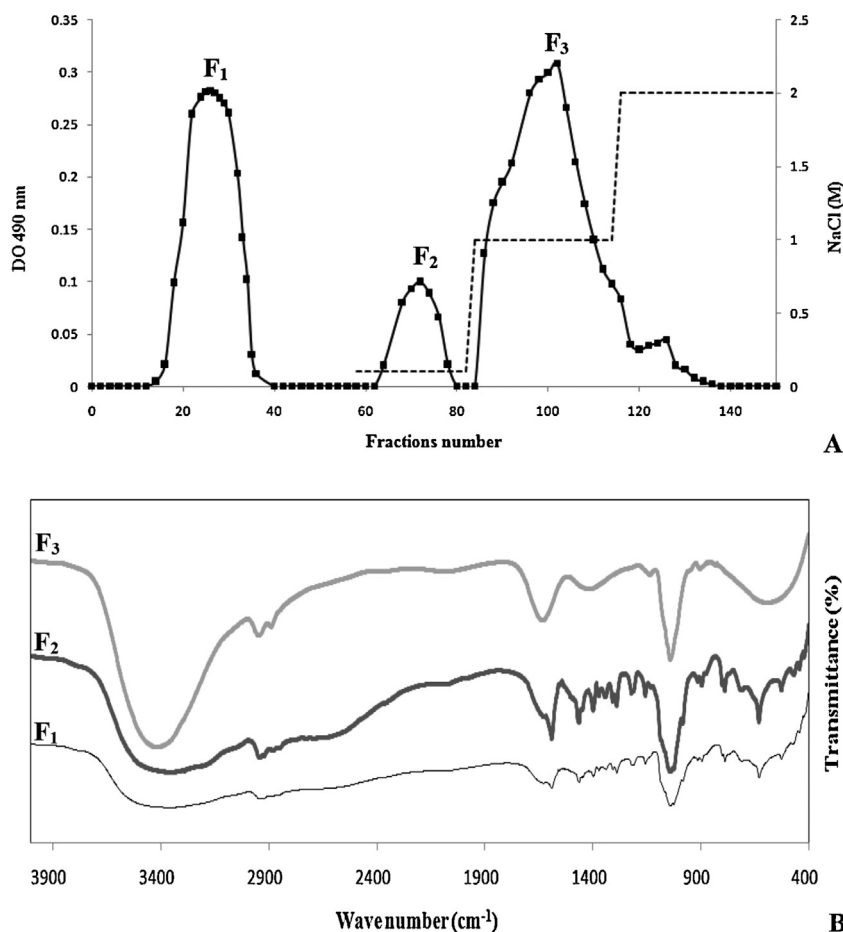


Fig. 5. (A) Elution profile of sulfated polysaccharide separated by DEAE-cellulose anion-exchange chromatography. (B) The FT-IR spectrometry of SPSS fractions F₁, F₂, and F₃.

was also studied. At 0.1 mg mL⁻¹, the angiotensin-I converting enzyme inhibitory activity displayed by fraction F₃ (87.75%) was significantly ($P < 0.05$) higher than those exhibited by fraction F₂ (17.98%) and fraction F₁ (No activity). For antimicrobial activity, and among the three DEAE-cellulose fractions, fraction F₃ was noted to exhibit a remarkable inhibitory activity against all the microbial (bacterial and fungal) strains tested. The inhibition zone (Table 3) of the F₃ fraction against *S. enteric*, *S. aureus*, *F. solani*, and *A. solani* were 11, 13, 8, and 15 mm, respectively. This fraction was strongly protective against pathogenic microbial growth. The lower antimicrobial activity was observed for fraction F₁. The latter fraction exhibited antimicrobial activity only against *S. aureus* and *A. solani*.

4. Conclusion

This study was undertaken to investigate the antioxidant, antimicrobial, and angiotensin I-converting inhibitory activities of sulfated polysaccharides extracted from squid skin. The findings revealed that SPSS exhibited high antioxidant, anti-ACE, and antimicrobial activities. Although SPSS was less effective than the positive controls, polysaccharides from fish in general are considered as safe products and are, therefore, not subject to restricted use in foods. The SPSS samples were fractionated by DEAE-cellulose column, and three major absorbance peaks were observed. Overall, the results presented in this work indicate that SPSS offer a promising source for natural antioxidants, ACE-inhibitory biomolecules, and natural antimicrobial agents that could be used to enhance the

biological properties of functional foods and prevent the oxidation reaction problems associated with food processing.

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