Antioxidant and anticoagulant activities of polysaccharides isolated from the skin of ray *Raja montagui*

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Abstract: In this study, polysaccharides were extracted from ray (*Raja montagui*) skin and screened for their anticoagulant and antioxidant activities. The crude polysaccharide was obtained from the ray skin by enzymatic digestion (papain) followed by several purification steps (precipitation, dialysis…). Physico-chemical characterization revealed two negatively charged populations having different molecular weights, with the occurrence of acetylated hexosamines and uronic acids. Then, the analysis of the crude polysaccharide by anion exchangechromatography led to a highly sulfated fraction (25%) and an unsulfated fraction attributed to dermatan sulfate and hyaluronic acid, respectively. Preliminary tests of antioxidant and anticoagulant activities indicated that the unsulfated fraction exhibited a significant antioxidant potential at high concentrations, while the sulfated fraction showed an important prolongation (concentration dependent) of clotting time. Overall, obtained data suggests that *Raja montagui* skin is a good source of bioactive sulfated and unsulfated polysaccharides which could be exploited in wide range of biotechnological applications.

Keywords: *Raja montagui*, polysaccharide, dermatan sulfate, antioxidant activity, anticoagulant activity

INTRODUCTION

Glycosaminoglycans (GAGs) are heteropoly-saccharides composed of repeating disaccharide units, an uronic acid residue (D-glucuronic acid or iduronic acid) and amino sugar (D-glucosamine or D-galactosamine) [1]. GAGs are generally grouped into four groups: heparan sulfate/heparin, hyaluronic acid or hyaluronan, keratan sulfate and chondroitin sulfate/dermatan sulfate [2-3]. There are often found attached to a protein core called a proteoglycan localized on cell surfaces and especially in the extracellular matrix [4]. They play numerous biological roles in organism by interacting with a wide variety of proteins, including growth factors and chemokines, which regulate important physiological processes [5, 6].

Several studies have shown antioxidant properties of GAGs, mainly for hyaluronic acid and chondroitin Sulfate [7]. Recently, Abdelhedi and coworkers demonstrated the antioxidant role of sulfated polysaccharides isolated from smooth hound [8]. Another study isolated hyaluronic acid from marine origin (bivalve mollusk) and showed its antioxidant activity [9].

Hyaluronic acid is a linear polyelectrolyte based on b1-4-D-glucuronic acid (GlcA) and b1-3 *N*-acetyl-D-glucosamine (Glc-*N*Ac) repeat units [10]. It is widely distributed in vertebrates (mainly humans) tissues and body fluids as well as invertebrates. It is distinct from other GAGs by the non-existence of sulfated groups and lack of covalently linked peptide in their structure [9].

Dermatan sulfate (DS) is a sulfated GAGs, formed by a hexosamine, *N*-acetyl galactosamine (GalNAc) and L-iduronic acid (IdoA) joined by β1,4 or 1,3 linkages, respectively [11]. It is a

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bioactive polymer endowed with many biological and pharmacological properties [3] as it plays an important role in coagulation, cardiovascular disease, infection and wound repair by interacting with a large variety of molecules like coagulation, growth and pathogen virulence factors, cytokines and chemokines [3,12].

DS is isolated from different mammal's tissues, like porcine and bovine intestinal mucosa and porcine skin [4] but also from marine vertebrate's skin such as rays [11], eel [4] and shark [13] and from marine invertebrates like ascidians [14] and sea cucumbers [15]. In this context, Raja *montagui* commonly named spotted ray is a cartilaginous fish of the Rajidae family, found in the inshore waters and Mediterranean seas like Tunisia, Greece, France, Spain and Algeria… Because of its small body size (up to 80 cm total length), it is common in landings from fisheries and trawl surveys [16].

Only Ben Mansour and coworkers, in previous studies, isolated DS from *Raja montagui* skin. They showed its anticoagulant activity and detailed the mechanism of action [17].

The aim of this work is to isolate, from the skin of *raja montagui*, not only the DS but also the totality of the crude polysaccharide fraction and its components; secondly, to investigate for the first time, the antioxidant activity of the DS and the biological activities (anticoagulant and antioxidant) of the Hyaluronic acid extracted from *Raja montagui* skin.

RESULTS AND DISCUSSION

1. Isolation and identification of the crude polysaccharide from ray skin

Alcohol insoluble substances (AIS) were firstly isolated from *Raja montagui* skin using absolute ethanol and acetone with an approximately yield of 91%. Then, the crude polysaccharide CPR was obtained from AIS by enzymatic digestion followed by several purification steps (CCP, ethanol, dialysis…) and represented 1.03% of the dried skin. This yield (1.03%) was close to those found in other studies done on the skins of *Raja radula* (1%) [18], *Scyliorhinus canicula* (1%) [19] and ascidians (1%) [20].

The preliminary identification of the crude polysaccharide (CPR) required the use of several techniques allowing the evaluation of the number of populations and the estimation of the molecular masses (cellulose acetate cell electrophoresis and size exclusion chromatography), as well as a preliminary study on the chemical structure of CPR (FTIR). Indeed, CPR analysis by size exclusion chromatography (Figure 1) revealed the existence of two populations of different sizes and masses. The first population constituted the majority of the polysaccharide fraction due to its high intensity of refractive index signal (RI).

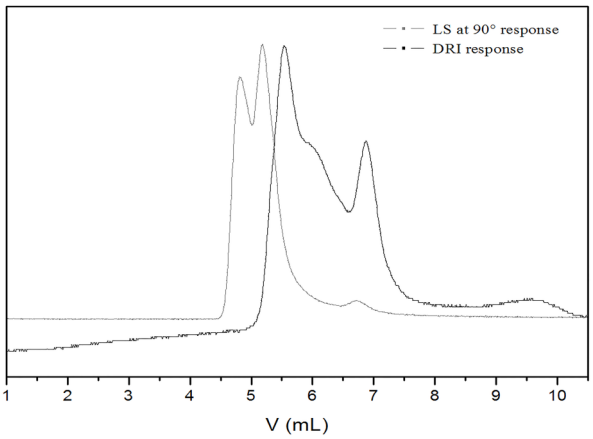

Figure 1: Molecular weights distribution versus elution volume for CPR sample (Black line: Differential refractive index; grey line: Light scattering at 90°) in 0.1 M $LiNO_3$ aqueous solution

Furthermore, electrophoresis analysis showed that CPR included two populations of different charges and masses and suggested that these two populations have structures very close to those of GAGs. The electrophoretic profile (Figure 2) showed two bands: a first band A, which was devoid of sulfate groups, migrated at the same level as hyaluronic acid, whereas the second band

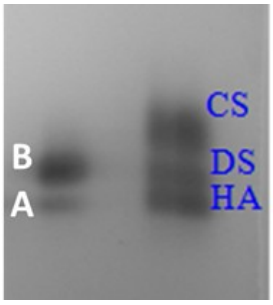

Figure 2. Acetate cellulose electrophoresis of crude polysaccharide from ray *Raja montagui* skin CPR (track 1), standard GAGs (chondroitin sulfate (CS), dermatan sulfate (DS), and hyaluronic acid (HA)) (track 2).

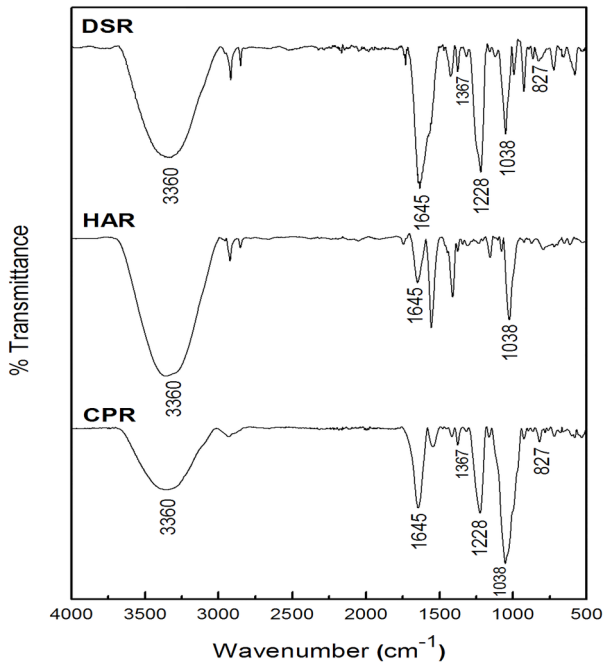

Figure 3. FT-IR spectrum of CPR, HAR and DSR

B (rich in sulfate groups) migrated at the same level as dermatan sulfate.

Polysaccharides representing the first band (A) will be called, throughout this work, HAR for "Hyaluronic Acid from *Raja montagui*"; the second type (band B) will be called DSR for "Dermatan Sulfate from *Raja montagui*".

The IRTF spectrum of CPR (Figure 3) compared to those of DSR an HAR, displayed the characteristic bands of sulfated and carboxylated polysaccharides. Firstly, sulfate groups were ensured by the presence of two absorption bands centered around 1228 cm^{-1} and 827 cm^{-1} corresponding to the bonds S=O and C-O-S, respectively. Moreover, the band at 1645 cm^{-1} (elongation of C=O bonds) and the two medium size bands centered at 1367 and 1473 cm^{-1} (elongation of O-C=O) indicated the presence of uronic acids. Finally, thin and strong absorption bands centered around 1038 cm^{-1} and 1165 cm^{-1} correspond to the cyclic vibrations of the C-O-C, C -OH and C-C bonds, which suggested the presence of the glycosidic cycles [15,18].

2. Anion-exchange chromatography

Besides the release of polysaccharides from the protein nuclei, the used extraction method (enzymatic digestion) has a selective character which ensures the isolation of, only, anionic macromolecules. The fractionation of the crude polysaccharide CPR was carried out by anion-exchange chromatography on DEAE-cellulose which ensures the separation of different components as a function of their charges.

The obtained chromatogram (Figure 4) shows the occurrence of two populations eluted by two steps of ionic strength of 0.5 and 1.5 M, respectively for the non-sulfated (Peak 1) and sulfated (Peak 2) fractions. This outcome confirmed the previous results which assert the existence of two GAGs in the crude fraction CPR (HAR and DSR). Moreover, the amplitude of peak 2 (DSR) was greater than that of peak 1 (HAR), which indicated that the fraction 2 was predominant in the crude extract.

By comparing the FTIR spectra of the two eluted fractions by anion exchange chromatography with that of CPR (Figure 3), we noticed that uronic acids was present in both fractions, whereas the vibrations corresponding to the acetyl and sulfate residues were evidenced only in the spectrum of fraction 2 (DSR).

Fractionation yields, macromolecular characteristics (M_w, M_n, and Đ) and sulfate content for the various fractions were summarized in Table I.

Size exclusion chromatography showed that average molecular weight (M_w) of HAR (hyaluronic acid) was in the order of 773150 g/mol, whereas M_w was in the order of 39000 g/mol for DSR

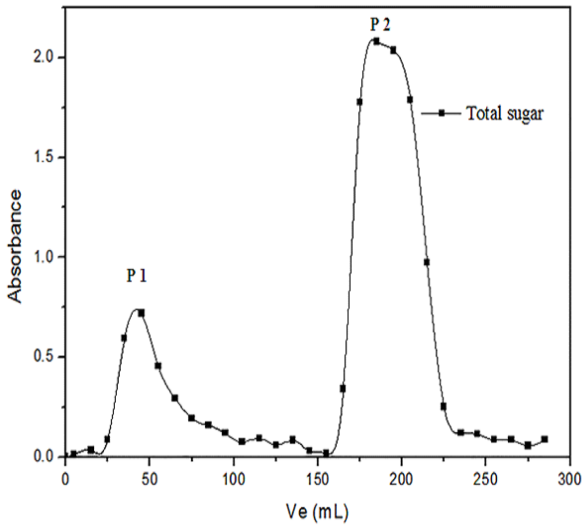

Figure 4. Anion-exchange chromatography of the crude polysaccharide on DEAE-cellulose

Table I. Fractionation yields, macromolecular characteristics and sulfate content of the two fractions eluted by anion exchange chromatography

Extract	Fraction	Yield (%)	M_W (g/mol)	M_n (g/mol)	Đ (M_W/ M_n)	SO_4^{2-} (%)
CPR	1 (HAR)	30	773150	211790	3.65	-
	2 (DSR)	70	39000	31400	1.24	25

(dermatan sulfate). Otherwise, the turbidimetric assay asserted all previous results by indicating that the fraction HAR is unsulfated while the fraction DSR contains 25% of sulfate groups.

These obtained results were in agreement with those found by *Sakai et al.* [4] which reported that a dermatan sulfate, having a molecular weight of 14000 g/mol, was extracted from the eel (*Anguilla japonica*) skin, whereas *Souza et al.* [21] have extracted a dermatan sulfate from the eel (*Electrophorus electricus*) skin with a molecular weight of about 40000 g/mol. Furthermore, dermatan sulfate extracted from porcine intestinal mucosa and porcine skins have molecular weights (M_w) of 21000 and 19000 g/mol, respectively [19]. These data indicate that the molecular weight of these polysaccharides varies according to species and tissue origin.

3. Antioxidant activity

The antioxidant capacities of different fractions (CPR, HAR and DSR) were evaluated by two *in vitro* assays considering ascorbic acid (AA) as reference. The obtained results for different tests are illustrated in Figure 5.

In fact, the stability of DPPH radical makes the DPPH radical scavenging assay the simplest and the most used method for evaluating the *in vitro* antioxidant activity [22]. From data in Figure 5 A, we notice that at low concentration (0.5 mg/mL) the sulfated fraction DSR was found to be relatively more active. At the concentration of 1 mg/mL, HAR and DSR displayed comparable antioxidant capacity, the inhibition percentages of CPR, HAR, DSR and AA were 16.70%, 31.39%, 32.07% and 90.16%, respectively. At the concentrations of 2 and 4 mg/mL, the antioxidant potential became more significant for the unsulfated fraction HAR. This potential reached 61.35% against 97.62% for ascorbic acid at a concentration of 4 mg/mL.

These findings are more significant than that found by et Krichen al. indicating that two sulfated polysaccharides extracted from grey triggerfish (*B. capriscus*) and smooth hound (*M. mustelus*) skins exhibited about 45% and 30%, respectively, on DPPH radical-scavenging activity at a concentration of 50 mg/mL [23]. Otherwise, the same authors showed in earlier study that a sulfated polysaccharide from *Loligo vulgaris* skin scavenges only 10% of DPPH radicals at a concentration of 4 mg/mL [24].

Generally, it has been reported that tissue and cell injuries are enormously evaluated by lipid peroxidation which is one the most manifestation of oxidative stress [25]. The obtained results (Figure 5.B) showed that the highest inhibitory effect was got for the sulfated fraction DSR but still less important than that of ascorbic acid at the concentration of 1 and 2 mg/mL. At a concentration of 2 mg/mL, the inhibition percentages were 37.04%, 55.78%, 53.21% and 96.28% for CPR, DSR, HAR and AA, respectively. Beyond 2 mg/mL, the unsulfated fraction HAR exhibited the most important antioxidant activity, which increases to 70% at a concentration of 4 mg/mL.

During this study, the antioxidant potentials of different ray skin GAGs (CPR, DSR and HAR) were compared against that of ascorbic acid (AA). The obtained results showed that, at the concentrations less than 2 mg/mL the sulfated GAGs (DSR) had a higher activity than the unsulfated GAGs (HAR) and the crude polysaccharide CRP, whereas HAR exhibited the highest activity at the highest concentration used (4 mg/mL). Maybe, this could be due to the self-aggregation of dermatan sulfate in high concentration.

The antioxidant activity of DSR and HAR could be explained by the presence in theirs structures of carboxylic group (HAR and DSR) and sulfates

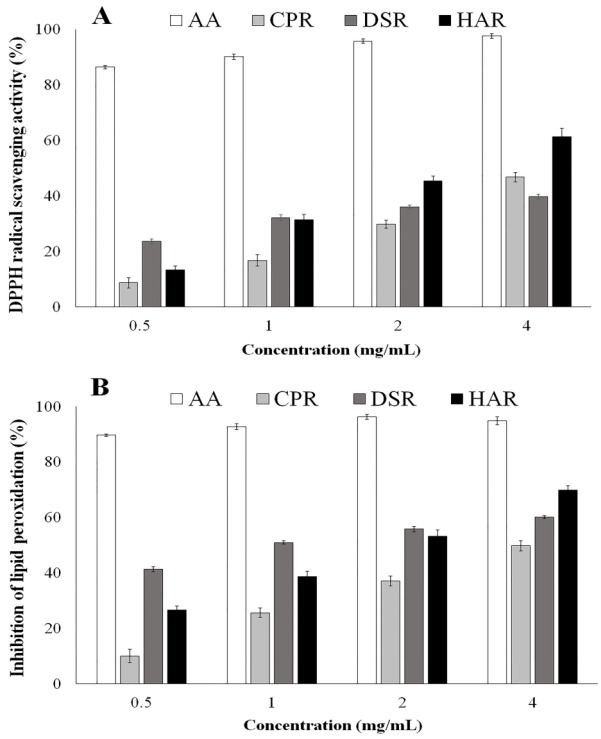

Figure 5. DPPH radical-scavenging capacity (A) and inhibition of lipid peroxidation (B) of CPR, DSR and HAR isolated from *Raja montagui* skin
Acid ascorbic (AA) was used as standard.
Each value is presented as mean ± SD (n = 3).

groups (DSR) which may act as hydrogen donor to DPPH free radical. Indeed, DPPH free radical receives an electron or hydrogen radical (H•) from the antioxidant compounds and becomes stable in its reduced form. Therefore, the antioxidant activity of a compound can be expressed as its ability in scavenging the DPPH• free radical [26, 27]. In addition, DSR and HAR may act as metal chelating agents and inhibit lipid peroxidation by chelating transition metal ions like Cu^{2+} or Fe^{2+}.
In fact, transition metal ions like Cu^{2+} and Fe^{2+} are implicated in the initiation of Haber–Weis and Fenton reactions [7].
Several authors reported the important role played by carboxylic and sulfates groups as metal chelators. Indeed, metals ions like Cu^{2+} or Fe^{2+} plays critical roles in REDOX systems. They are responsible of the initiation of Fenton's reaction to generate coordinative complex [7, 28].

4. Anticoagulant activity

The anticoagulant activity of the crude polysaccharide CPR and the two obtained fractions by anion exchange chromatography (DSR and HAR) was evaluated by activated partial thromboplastin time (APTT) and Thrombin time (TT) assays, compared with that of unfractionated heparin (UFH) used as reference. The obtained results (Figure 6) show a prolongation (concentration dependent) of the clotting time for unfractionated heparin, DSR and CPR in both tests. However, concerning DSR and CPR, this prolongation stills less important than heparin. Moreover, no prolongation of the coagulation time was produced by the unsulfated fraction HAR. This indicates that the observed anticoagulant activity for CPR is evidently due to the sulfated polysaccharide DSR. This activity still remains lower than that of heparin, but higher than that of dermatan sulfate of mammals [29]. In fact, it has also been previously reported that dermatan sulfate with a high sulfate content (dermatan polysulfates) were isolated from myxin and shark skins and exhibited significant anticoagulant potential. This latter was mainly attributed to their high content on sulfate groups [30]. In this context, it can be noted

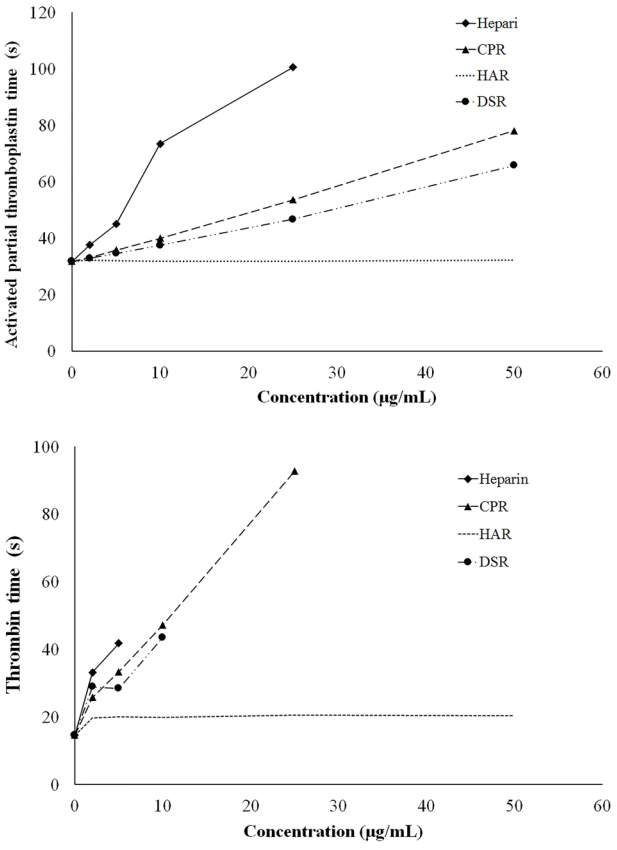

Figure 6. Anticoagulant activity of CPR, HAR, DSR and Heparin measured by APTT **(A)** and TT **(B)** assays using unfractionated heparin as standard

that dermatan sulfate from different sources (marine animals or chemically over sulfated) showed high anticoagulant activity mediated by the two serpins HCII and AT [31]. On the basis of these data, we can explain the relatively high anticoagulant potential observed for the sulfated polysaccharide (DSR) by its high content of sulfate groups (25%).

EXPERIMENTAL

1. Materials

Raja montagui (Fowler, 1910) commonly named spotted ray is a cartilaginous fish of the Rajidae family found in the inshore waters and Mediterranean seas. Because of its small body size (up to 80 cm total length), it is common in landings from fisheries and trawl surveys [32].

Specimens of *Raja montagui* were caught by commercial trawl in the seas surrounding Monastir on the Eastern shore of Tunisia (35° 46' N, 10° 49' E) during spring (May 2013). Their skins were collected from the local fish market of Monastir, cleaned from pulpit and thorns, washed with water then sun dried.

2. Polysaccharides extraction

Dried ray skin was cut into small pieces, grinded with a mechanical grinder and then extracted by organic solvent (ethanol and acetone) to obtain an alcohol insoluble substance (AIS). After that, the crude polysaccharide (CPR) was obtained from AIS by enzymatic digestion with papain followed by several purification steps consisting of precipitation with cetylpyridinium chloride and ethanol, dialysis and lyophilization [18].

3. Anion exchange chromatography

The crude polysaccharide CPR (200 mg) was suspended in 4 ml sodium acetate 0.5 M (pH 6) and fractionated with anion-exchange chromatography on DEAE-cellulose column (1x15 cm) in two steps by sodium acetate 0.5 M (pH6) and sodium acetate 0.5 M, NaCl 1 M (pH6), respectively. Samples of the fractions were assayed by the phenol-sulfuric method for total hexoses. Finally, fractions corresponding to each population were pooled, dialyzed against deionized water and lyophilized [31].

4. Physicochemical characterization

Total hexoses were estimated by the phenyl-sulfuric assay [33]. Electrophoresis was carried out as described by Ben Mansour *et al.* on acetate cellulose strips (*Sartorius*) in Zn-acetate buffer at 200 V, and polysaccharides were stained by alcian blue [11]. Infrared spectra were obtained using a Fourier transform infrared spectrophotometer *PerkinElmer Spectrum Two ATR-FTIR*, over thewave number range between 4000 and 50 cm^{-1} [34]. Sulfate content was determined by turbidimetry according to Dodgson & Price [35]. Molecular weight determination was performed using size exclusionchromatography (SEC) with on line three detectors: multianglelight scattering (MALS) (DAWN HELEOS II, Wyatt Technology, Santa Barbara, CA), viscometer detector (VD) (Viscostar II, Wyatt Technology) and differential refractive index (DRI) (RID 10A, Shimadzu, Kyoto, Japan) [36].

5. Antioxidant activity

5.1. DPPH radical Scavenging Assay: The free radical scavenging ability of extracted samples was evaluated as described by Qiao *et al.* [37]. 1 mL of each solution was incubated with 0.2 mL of DPPH (1,1-Diphenyl-2-picryl-hydrazil) methanolic solution (400 µM) and 2 mL of water for 30 min at room temperature. The absorbance of each mixture was measured at 517 nm and the DPPH inhibitory percentage was determined using the following equation (1) considering Ascorbic acid (AA) as standard:

$$\text{DPPH radical scavenging activity (\%)} = (1- Abs_{sample}/Abs_{control}) \times 100 \quad \textbf{(1)}$$

5.2. Linoleic acid peroxidation with TBARS assay*:* The reaction mixture was prepared by adding 500 µL of linoleic acid (20 mM), 100 µL of $FeSO_4.7H_2O$ (4 mM) and 500 µL of TrisHCl (100 mM, pH 7.5) to a varying concentration of each sample. Thereafter, 100 µL of ascorbic acid (2 mM) were added to initiate the linoleic acid peroxidation and the mixture was then incubated at 37 °C for 30 min. The peroxidation was achieved by the addition of 10% trichloroacetic acid (2 mL). Then, 1 mL of the mixture was mixed with 1 mL of thiobarbituric acid (1% in 50 mM NaOH) and left under heating at 95 °C for 10 min. The solutions were centrifuged for 10 min at 3500/g and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was measured at 532 nm. Ascorbic acid was used as standard [38]. The percentage of antioxidant

activity was estimated accordingto the following equation (2):

$$\text{Linoleic acid peroxidation inhibition (\%)} = [(Ac-As) / (Ac-An)] \times 100 \quad \textbf{(2)}$$

Ac = Absorbance of control (without extract)
As = Absorbance of extract
An = Absorbance of blank (without extract and $FeSO_4.7H_2O$)

6. Anticoagulant activity

Coagulation tests were carried out at various concentrations of different samples (CRP, DSR and HAR) diluted in a pool of frozen normal plasma. Activated partial thromboplastin time (APTT) and thrombin time (TT) were evaluated using Platelin LS reagent (Trinity Biotech PLC, Cowicklow, Ireland) and bovine thrombin 1.5 NIH/mL (Dade Behring, Marburg, Germany), respectively, on a *STAR* analyzer (DiagnosticaStago, Asnières, France) [19]. Unfractionated heparin (UFH) was used as reference.

7. Data Treatment

Each value in all figures was carried in triplicate and presented as mean ± standard deviation (SD; $n = 3$). All experiments were carried out in triplicate and the results were expressed as mean values ± SD ($n = 3$). Antioxidant activity measurements were compared in terms of mean values.

CONCLUSION

In this study, three polysaccharide fractions (CPR, HAR, DSR) from *Raja montagui* skin were extracted, characterized and evaluated for their antioxidant and anticoagulant activities.

Initially, a crude polysaccharide (CPR) was extracted from ray skin by enzymatic digestion with papain. CPR was then fractionated by anion-exchange chromatography on DEAE-cellulose and the different obtained fractions were characterized by different techniques (cellulose acetate electrophoresis, size exclusion chromatography, infrared spectroscopy ...). Results indicated the presence of two negatively charged populations consisting of uronic acids and acetylated hexosamines with different Molecular weights (M_w) (39000 and 773150 g/mol). These data suggested that the two populations correspond to dermatan sulfate (DSR) and hyaluronic acid (HAR). Then, all fractions were screened for their antioxidant (DPPH radical scavenging andlipid peroxidation inhibition) and anticoagulant (APTT and TT) activities. The results showed that the sulfated fraction DSR exhibited the most significant anticoagulant potential and the highest antioxidant capacity at low concentrations. On the other hand, the unsulfated fraction HAR showed the most important antioxidant potential at high concentration, without showing any anticoagulant potential.

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