Purification, compositional analysis, and anticoagulant capacity of chondroitin sulfate/dermatan sulfate from bone of corb (Sciaena umbra)

Hajer Bougatef, Fatma Krichen, Federica Capitani, Ikram Ben Amor, Jalel Gargouri, Francesca Maccari, Veronica Mantovani, Fabio Galeotti, Nicola Volpi, Ali Bougatef, Assaâd Sila

PII: S0141-8130(18)34370-8
DOI: https://doi.org/10.1016/j.ijbiomac.2019.05.036
Reference: BIOMAC 12321

To appear in: *International Journal of Biological Macromolecules*

Received date: 19 August 2018
Revised date: 2 May 2019
Accepted date: 5 May 2019

Please cite this article as: H. Bougatef, F. Krichen, F. Capitani, et al., Purification, compositional analysis, and anticoagulant capacity of chondroitin sulfate/dermatan sulfate from bone of corb (Sciaena umbra), International Journal of Biological Macromolecules, https://doi.org/10.1016/j.ijbiomac.2019.05.036

Purification, compositional analysis, and anticoagulant capacity of chondroitin sulfate/dermatan sulfate from bone of corb (*Sciaena umbra*)

Hajer BOUGATEF[1], Fatma KRICHEN[1], Federica CAPITANI[2], Ikram BEN AMOR[3], Jalel GARGOURI[3], Francesca MACCARI[2], Veronica MANTOVANI[4], Fabio GALEOTTI[2], Nicola VOLPI[2], Ali BOUGATEF[1], Assaâd SILA[1,5,*]

[1] *Laboratory for the Improvement of Plants and Valorization of Agroresources, National School of Engineering of Sfax (ENIS), University of Sfax, Sfax 3038, Tunisia.*

[2] *Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy.*

[3] *Regional Centre for Blood Transfusion Sfax, El-Ain Road Km 0.5, P.C. 3003 Sfax, Tunisia.*

[4] *Clinical and Experimental Medicine PhD Program, University of Modena and Reggio Emilia, Modena, Italy.*

[5] *Department of Life Sciences, Faculty of Sciences of Gafsa, University of Gafsa, 2100 Gafsa, Tunisia.*

*Corresponding author: Tel.: + 216 74 674 354; Fax: + 216 74 275 595;

E-mail address: assaadsila@gmail.com

Abstract

Chondroitin sulfate/dermatan sulfate (CS/DS) were isolated and purified for the first time from the bone of corb (*Sciaena umbra*) (CBG) and their chemical composition and anticoagulant activity were assessed. Infrared spectrum and agarose-gel electrophoresis for extracted CS/DS were also investigated. The results showed that the purified CS/DS obtained at a yield of 10% contains about 31.28% sulfate and an average molecular mass of 23.35 KDa. Disaccharide analysis indicated that CBG was composed of monosulfated disaccharides in position 6 and 4 of the N-acetylgalactosamine (8.6% and 40.0%, respectively) and disulfated disaccharides in different percentages. The charge density was 1.4 and the ratio of 4:6 sulfated residues was equal to 4.64. Chondroitinase AC showed that the purified CS/DS contained mainly 74% CS and 26% DS. Moreover, the new CS/DS extracted from bone of corb showed a strong anticoagulant effect through activated partial thrombosis time (aPTT), thrombin time (TT) and prothrombin time (PT). In fact, CBG prolonged significatively ($p < 0.05$), aPTT and PT about 2.62 and 1.26 fold, respectively, greater than that of the negative control at a concentration of 1000 μg/mL. However, TT assay of CBG was prolonged 3.53 fold compared with the control at 100 μg/mL. The purified CS/DS displayed a promising anticoagulant potential, which may be used as a novel and soothing drug.

Keywords: Fish bone; chondroitin sulfate / dermatan sulfate; anticoagulant effect.

1. Introduction

Marine environment provides a diverse range of bioactive compounds. The great chemical and biological variety observed in the marine environment makes the ocean an enormous source of new bioactive substances which can be employed in many applications. These bioactive molecules can be proteins, peptides, fatty acids, polyphenols, enzymes, probiotics and polysaccharides. Sulfated polysaccharides with marine origin constitute one of these biochemical compounds that have already proved to have different chemical propreties and biological activities [1]. Their propreties are mainly due to their complex monosaccharide composition, various sulfation patterns, different linkage and specific sequences on their backbone structure [2].

The glycosaminoglycans (GAGs) are among the most studied group of these anionic polymers. These polysaccharides are composed of disaccharide repeating units including hexosamine residues (N-acetylgalactosamine (GalNAc) or N-acetylglucosamine) and either hexuronic acid or galactose units that are substituted by sulfate esters in various positions or amount. GAGs are very heterogeneous polysaccharides in terms of relative molecular mass, charge density, and physicochemical properties. They are generally divided into 2 groups: galactosaminoglycans (chondroitin sulfate and dermatan sulfate) and glucosaminoglycans (heparan sulfate (HS), heparin, keratan sulfate and hyaluronic acid) [3].

In recent years, the interest in natural glycosaminoglycans, in relation to their therapeutic properties, has increased considerably. Scientific research in various specialties has been developed for the extraction and purification of these compounds from several natural substances. In fact, GAGs can be isolated from animal sources by extraction and purification processes using cartilages as raw material derived from terrestrial animals such as bovine, porcine and avian [4, 5]. However, numerous studies have confirmed that GAGs, purified from land animal tissues, are

suspected to induce pathological and toxic effects, such as mad-cow disease, foot-and-mouth disease, and hog cholera.

The occurrence of GAGs in marine organisms, extracted from cartilages as raw material, has been widely reported by several authors [6, 7, 8, 9, 10]. Most of these studies indicated the presence of glycosaminoglycans with heterogenous structures and anticoagulant activities. Generally, the coagulation process is complex in the human body, and the key step for coagulation formation is to transform fibrinogen into fibrin, which is catalyzed by thrombin. In fact, blood coagulation might be prevented by glycosaminoglycans, by interaction with the serine-proteinases of the coagulation system and their physiological inhibitors antithrombin (AT) and heparin cofactor II (HCII). Heparin is a well known as anticoagulant drug and it is widely used in medical practice [11], whereas the clinical use of this drug is limited bythe risk of excessive bleeding and heparin induced thrombocytopenia [12]. Consequently, considerable efforts have been made in recent years to create safer anticoagulants without such hemorrhagic complications.

The objective of this work was to characterize the chemical structure of sulfated glycosaminoglucans extracted from the bone of corb (*Sciaena umbra*) (CBG). In addition, *in vitro* anticoagulant activity of the new GAGs was investigated.

2. Materials and methods

2.1 Reagents

All chemicals and solvents were of analytical grade and used as received from commercial sources. Alcalase® 2.41 serine-protease from *Bacillus licheniformis* was purchased from Novozymes® (Bagsvaerd, Denmark). GAGs standard, heparan sulfate from bovine kidney, chondroitin sulfate from bovine trachea and dermatan sulfate from porcine intestinal mucosa

were from Sigma–Aldrich (St. Louis, MO, USA). Chondroitinase ABC, chondroitin ABC lyase, from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5-2 units/mg, and chondroitinase AC, chondroitin AC lyase, from *Flavobacterium heparinum* (EC 4.2.2.5), specific activity of 0.5-1.5 units/mg, were from Sigma–Aldrich. Unsaturated chondro/dermato disaccharides [ΔDi0S (ΔUA-[1→3]- GalNAc), ΔDi4S (ΔUA-[1→3]-GalNAc-4S), ΔDi6S (ΔUA-[1→3]-GalNAc-6S), ΔDi2S (ΔUA- 2S-[1→3]-GalNAc), ΔDi2,4diS (ΔDi-diS B, ΔUA-2S-[1→3]-GalNAc- 4S), ΔDi2,6diS (ΔDi-diS D, ΔUA-2S-[1→3] GalNAc-6S), ΔDi4,6diS (ΔDi-diS E, ΔUA-[1→3]-GalNAc-4,6diS), and ΔDi2,4,6triS (ΔDitriS, ΔUA-2S-[1→3]-GalNAc-4S,6S)] were from Seikagaku Corporation (Tokyo City, Japan) and Sigma–Aldrich. Stains-All (3,3'-dimethyl-9-methyl-4,5,4'5'-dibenzothiacarbocyanine) was from Sigma–Aldrich. QAE Sephadex® A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade.

2.2 Raw material and preparation of bone from corb

Brown meager or corb (*Sciaena umbra*) were freshly purchased from the local fish market of Sfax, Tunisia. The biological materials were placed in ice and transported to the laboratory. Upon arrival, the samples were washed twice with water and separated. The bone of corb was collected and then stored in sealed plastic bags at -20 °C until use for the extraction and analysis of GAGs.

2.3 Extraction of GAGs from bone of corb

GAGs were isolated according to a slightly modified version of the method of Ben Mansour et al. [13]. Firstly, the fish bone was cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). Five grams of sample was suspended in 250 mL sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH 8. Enzymatic hydrolysis was achieved by the addition of Alcalase® (500 U/g), and the mixture was kept for 24

h at 50 °C. After the required digestion time, the mixture was left to cool down at room temperature and then filtered. The residue was washed with distilled water and filtered again. GAGs in the supernatant were precipitated with cetylpyridinium chloride 2% during 24 h at room temperature and then separated using centrifugation (Hettich-Rotina-380-380R) for 30 min at 5869 g and 4 °C. The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and then blended with 200 mL NaCl solution (2M) in ethanol (100:15, v/v).

After that, an amount of 700 mL ethanol was added. The mixture of GAGs was left for 24 h at 4 °C and then centrifuged for 30 min at 5869 g and 4 °C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. Finally, the pellet was redissolved in desionized water and freeze-dried using freeze-dryer (CHRIST, ALPHA 1–2 LD plus, Germany).

The recovery yield of crude glycosaminoglycans from bone of corbwas calculated as follows: **Yield (%) = (weight of dried crude GAGs (g) / weight of dry corb bone (g)) x 100**

2.4 Purification of fish bone GAGs

The crude GAGs were dissolved in 5 mL of 10 mM NaCl. After centrifugation at 4000 rpm for 10 min, the supernatant was applied to a column (1.5 cm × 3 cm) packed with QAE-Sephadex® A-25 anion-exchange resin equilibrated in NaCl 10 mM. GAGs were eluted with a linear gradient of NaCl from 10 mM to 2.5 M from 0 to 150 min using low-pressure liquid chromatography (Biologic LP chromatography system from BioRad) at a flow of 1 mL/min. Two volumes of ethanol were added to the collected fractions corresponding to single species of GAGs evaluated by uronic acid assay [14] and agarose-gel electrophoresis [15, 16], and GAGs precipitated at 4 °C. After centrifugation at 4000 rpm for 10 min, the pellet was dried at 40 °C and solubilized in distilled water.

The recovery yield of purified CS/DS from bone of corb was calculated as follows:

Yield (%) = (weight of dried CS/DS (g) / weight of dry crude GAGs (g)) x 100

2.5 Physicochemical characterization

Neutral hexoses were measured by the phenol-sulfuric acid method using glucose as a standard [17]. The sulfate content was performed using volumetric method (described by Archer [18] and elaborated by White [19]). The method employs titration with lead nitrate as the titrant and dithizone as the indicator. Hexuronic acid content was assessed by carbazole reaction using glucuronic acid as the standard [14]. The color was determined with a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale (C/2°), where L^*, a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate with reflectance values of L^* = 93.68, a^* = - 0.69, b^* = - 0.88, was used as reference.

2.6 Agarose-gel electrophoresis

Agarose-gel electrophoresis in barium acetate/1,2-diaminopropane was performed as reported by Volpi [15] and Volpi et al. [16]. A Pharmacia Multiphor II (from Pharmacia LKB Biotechnology, Uppsala, Sweden) electrophoretic cell instrument was used. Agarose gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8. 10 μg ofthe purified GAGs extracted from the bone of corb were applied to agarose gels and run in 0.05 M 1, 2-diaminopropane (pH 9.0) for 240 min at 100 mA. After electrophoresis, GAGs were fixed in the gel with 0.1% N-cetyl- N,N,N-trimethyl ammonium bromide in water for at least 6 h, dried and stained with 0.2% toluidine blue in ethanol– acetic acid–water (100:2:98) for 30 min. Then destained with ethanol– acetic acid–water (100:2:98). The gel is also stained with the Stains-All, 12.5 mg/250 mL ethanol–water 50:50 overnight in the dark and destained with water.

2.7 Ultraviolet (UV) and infra-red (IR) spectra

The purified CS/DS from corb bone was applied to ultraviolet spectrum (UV) and infrared spectrum (IR) analysis. Fourier transform infra-red spectrum was registered by Agilent Cary 630 FTIR in the range between 650 and 4000 cm^{-1} using a KBr pellet containing 0.1% of sample. UV spectrum was recorded by scanning the purified CS/DS solution (1 mg/mL) in UV-visible spectrophotometer (UV mini-1240, Shimadzu, China) with wavelength range of 190-700 nm using distilled water as control.

2.8 Enzymatic treatement and disaccharide composition evaluation

After treatment of the purified CS/DS samples with chondroitinase ABC or chondroitinase AC (0.5 U/mg sample, pH 8.0, for 24 h and at 37 °C), the generated unsaturated disaccharides were separated and quantified by anion-exchange (SAX) by means of HPLC using Jasco chromatograph equipped with a 150 mm × 4.6 mm stainless-steel column spherisorb 5-SAX (5 µm, trimethylammoniopropyl groups $Si\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N^+(CH3)^3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, U.K.) and detection at 232 nm. Isocratic separation was performed using 50 mM NaCl pH 4.00 for 5 min followed by a linear gradient from 5 to 60 min of 50 mM NaCl to 1.2 M NaCl pH 4.00, at a flow rate of 1.2 mL/min. Authentic unsaturated standard disaccharides were used for qualitative and quantitative purposes.

2.9 Molecular weight determination

The molecular weight of CS/DS obtained from the bone of CBG was determined by PAGE according to Edens et al. [20].

Approximately, 15 µg of the purified CS/DS determined by uronic acid assay [14] were layered on the gel. The related calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS and having masses of 28.34 KDa, 16.75

KDa and 4.63 KDa [21]. After a run of 40 min at 100 V, the gel was stained with toluidine blue (0.1% in acetic acid 1%) for 30 min followed by destaining in 1% acetic acid. Molecular mass evaluation was performed by densitometric acquisition of bands and comparison of their migration times on the calibration curve constructed by plotting retention times of standards against their logarithm of molecular mass values.

2.10 Anticoagulant activity

The anticoagulant activity of the purified CS/DS was evaluated by the classical coagulation assays: activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombine time (PT), using a semi-automatic line STA (DiagnosticaStago, Asnières, France). The sample was dissolved in physiological serum. All analyses were carried out in triplicate and mean values were taken. Heparin sodium Choay (Sanofi-Aventis, France) was used as reference.

2.10.1 aPTT assay

The aPTT assay was estimated by mixing normal citrated platelet poor plasma (PPP) (45 µl) with 5 µl of the purified CS/DS at different concentrations and incubated for 3 min at 37 °C. 50 mL of aPTT reagent (CK-PREST) were added and the mixture was incubated for 3 min at 37 °C. The clotting time was immediately saved after the addition of 100 µl of 25 mM $CaCl_2$. The clotting time is expressed in seconds and as ratio, with the average value of a normal subject less than 1.2. The enzyme activity control (C) is measured by replacing the 5 µl of glycosaminoglucans with physiological serum.

2.10.2 PT assay

The prothrombin time assay was determined by mixing 5 µl of CS/DS at different concentrations with 45 µl of PPP. The mixture was incubated for 3 min at 37 °C. The clotting

time was measured after the addition of 100 µl of Neoplastine® CI (DIAGNOSTICA-STAGO). The prothrombin time value is expressed in seconds.

2.10.3 TT assay

For the TT assay, 10 µl of CS/DS at different concentrations were incubated with 90 µl of PPP for 3 min at 37°C. The clotting time was determined after the addition of 100 µl of thrombin (80 NIH). The Thrombin time value is expressed in seconds.

2.11 Statistical analysis

All experiments were carried out in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple RangeTest. The SPSS software package (SPSS, Chicago, IL) was used for data analysis.

3. Results and discussion

3.1 Extraction and purification of CS/DS from bone of *Sciaena umbra*

GAGs were isolated from the bone of corb (CBG) by digestion with Alcalase®, which is an endo-protease of serine types that is characterized by its broad substrate specificity. The deproteinization step was followed by cetylpyridinium chloride treatement to precipitate the sulfated components, which were then transformed into a water-soluble sodium salts by stirring with NaCl in ethanol. After extraction by proteolytic treatement, the crude GAGs extracts was subjected to anion-exchange resin and eluted with a linear NaCl gradient of increasing molarity.

3.2 Chemical characterization and agarose-gel electrophoresis

The proximate compositions of glycosaminoglycans obtained from the bone of corb (CBG) are summarized in Table 1. The yields of crude glycosaminoglycans and the purified CS/DS were about 8.64 ± 0.84% and 10 ± 0.01%, respectively, based on the dry weight. This obtained

recovery yield of crude GAGs was higher than those previously reported from smooth hound cartilage [9] (2.52 ± 0.07%) but lower than that of CS extracted from shark fin cartilage (15.05%) [22]. The net yield may vary due to species variation and extraction method. Armis [23] showed that, in general the yield of extraction of polysaccharides is very variable due to several factors, such as environmental conditions, seasonal variation, physiological factors and extraction methods.

The analysis of total sugars by the phenol/sulfuric acid method indicated that CBG had a high percentage about 49.37 ± 1.05%. Furthermore, the quantitative analyses of uronic acid by carbazole method showed that CBG contained 46.02 ± 0.46%, which is higher than that reported by Maccari et al. [24].The sulfate content was 31.28 ± 0.87%, indicating an average polyanionic structure for CBG. This value is higher than that reported by Krichen et al. [25]. It was, however, lower than that previously obtained by Ben Mansour et al. [26]. The amount of sulfate is affected by many factors such as the marine origin, nature of the raw material, and the type of extraction [25]. The instrumental color measurement (expressed in terms of L^*, a^* and b^*) of glycosaminoglycans extracted from corb bone is also presented in Table 1. The lightness (L^*) value of CBG was 69.44 ± 0.05 and indicated a light color. Similar results were obtained by Sayari et al. [27] for sulfated GAGs from Norway lobster. The finding also showed that CBG displayed relatively low yellowness degree (b^*= 15.76 ± 0.06) and a low redness (a^*=5.01 ± 0.01). The color of glycosaminoglycans is an important aesthetic property, depending on the application for which GAGs is intended. Meanwhile, the color did not affect functional properties of GAGs.

Agarose-gel electrophoresis is a highly effective method for separating and identifying GAGs in mixtures.This approach has been applied for different aims, such as to separate GAGs extracted from tissues, organs, biological fluids of invertebrates and vertebrates, to evaluate

polyanions used as drugs both qualitatively and quantitatively and to control a purification step of a single GAGs species. Furthemore, the electrophoretic migration of sulfated polysaccharides in 1, 2- diaminopropane/acetate depends on the structure of polysaccharide, which forms a complex with the diamino buffer [28].

In this context, agarose-gel electrophores is confirmed the presence of a mixture of GAGs which possessed a migration behaviour intermediate between CS and DS (Fig.1). Previous results reported by Maccari et al. [8] confirmed the presence of CS as a principal polysaccharide in cartilages of bony fishes by using agarose-gelelectrophoresis.

3.3 Ultraviolet (UV) and infra-red (IR) spectra

The Ultraviolet–visible spectra of the purified CS/DS in the range of 190-700 nm, was investigated (Fig. 2a). CBG had very small absorbance at 260 nm, while at 280 nm CBG had an extremely weak absorbance which could hardly be observed, indicating very small amounts of proteins content. The largest absorbance peak of CBG was at 210-215 nm, which showed polysaccharidic structures.

The FT-IR spectrum of the purified CS/DS extracted from bone of corb was performed in the 4000-650 cm^{-1} region (Fig. 2b). The obtained spectrum displayed essentially the same absorption bands which are characteristic for sulfated polysaccharides [29, 30]. The bands at 3313 and 1660 cm^{-1} were corresponded to O-H ring vibrations [31]. Thin and strong absorbance band at 1051 cm^{-1} assigned to C-O stretching vibrations of pyranose rings. The bands at 2924 cm^{-1} and 2855 cm^{-1} were assigned to the C-H stretching bending vibration. Furthermore, the band measured at 1410 was attributed to the bond stretching vibration of uronic acid (O–C=O bending). The presence of sulfates was confirmed by the absorbance band at 1244 cm^{-1} assigned to the S=O stretching band and the band at 726 cm^{-1} which was specific to CO-S stretching

vibration of sulfate. Similar results were previously reported by Sila et al. [32] and Ben Abdallah Kolsi et al. [33].

3.4 Enzymatic treatments and constitutive disaccharide evaluation of CS/DS

To characterize the structure of GAGs obtained from corb bone, the purified CS/DS was subjected to treatment with two bacterial lyases: Chondroitinase ABC, from *Proteus vulgaris*, is non specific and cleaves all forms of CS in a predominately endolytic pattern. Chondroitin lyase AC, from *Flavobacterium heparinum*, acts on CS-A and CS-C in a random endolytic action pattern. The unsaturated disaccharides produced were also quantitatively and qualitatively evaluated by strong anion-exchange SAX-HPLC (Fig. 3).

As illustrated in table 2, chondroitinase ABC produced several unsaturated disaccharides in different percentages from CBG. The nonsulfated disaccharide ΔDi0S of CBG was 2.8%, while monosulfated disaccharides ΔDi6S and ΔDi4S were estimated to be 8.63% and 40.07%, respectively. The ratio 4S/6S was about 4.64. Maccari et al. [8] reported that CS from bony fishes have 4S/6S ratio ranging from 0.45 to 2.23. Furthemore, disulfated disaccharides were observed in the purified CS/DS with different percentages. In particular, the disulfated species ΔDi2, 6 diS and ΔDi4, 6diS were about 4.3% and 7.24%, respectively. In addition, the disulfated disaccharide ΔDi2, 4diS of CBG was evaluated to be 36.91%, while the obtained data revealed the absence of trisulfated disaccharides species ΔDi2,4,6 triS.

It was reported that CS from sturgeon backbone was poor in disulfated and trisulfated disaccharides [34]. Interestingly, the presence of disulfated disaccharides produced a great overall charge density of CBG which was 1.4. This value was found to be generally higher than mammalian CS [4].

The determination of the disaccharide pattern of the purified CS/DS by means of SAX-HPLC after treatement with this highly specific chondroitinase AC allowed quantitative analysis

of the main non sulfated and sulfated disaccharides just belonging to CS backbone. In fact, the CS component of CBG is composed of 2.6% of the nonsulfated disaccharide, 5.3% of the sulfated disaccharides in position 6 (ΔDi6S) and a high amount (69.0%) of the 4-sulfated disaccharide (ΔDi4S) (Table 2). In addition, the disulfated disaccharides ΔDi2, 6 diS and ΔDi4,6 diS were about 11.5% and 2.8%, respectively, while disulfated disaccharide ΔDi2,4 diS was evaluated to be 8.82%. Finally, the obtained data also revealed the absence of trisulfated disaccharides in the CS belonging to CBG.

Furthemore, by using chondroitinase ABC (able to act simultaneously on CS and DS) and AC (specific for CS), we were able to calculate the GlcUA/IdoUA ratio. Therefore, our results suggest that the percentage of CS and DS recovered in this polymer was 74% and 26%, respectively. In this context, it was reported that the content of the CS/DS hybrid chain are higher in cartilaginous fishes than in bony fishes [35]. These findings are in agreement with other reports which confirms that the most relevant structural characteristic common to CS/DS purified from the different bony fishes is the presence of disulfated disaccharides in various percentages and types. Moreover, the highly sulfated disaccharides sequences inside the CS/DS backbone are essential for the anticoagulant activity [36].

3.5 CS/DS molecular mass determination

The molecular mass measurements performed by using a specific gradient PAGE showed a single glycosaminoglycans population (Fig. 4), comfirming the above results obtained by electrophoresis analysis. Using a series of standard chondroitin sulfate with defined molecular weight, a calibrated curve was given as follow logMw = -1.9058x + 5.3669 (r = -0.99887). In fact, based on the calibration curve, CBG indicate an average molecular mass of 23.35 KDa. The obtained molecular weight of CBG is higher than that previously obtained from *Achatina fulica* [3], but lower than that obtained by Arima et al. [37] for the purified CS/DS from yellowfin sole.

Recent studies have indicated that CS/DS have various molecular masses and polydispersities depending on the sources. Moreover, Volpi [4, 5] confirmed that CS from cartilaginous fishes such as raja and shark possesses a greater molecular weight values (70 and 50 KDa). As a consequence, CS/DS from bones shows variable characteristics as opposed to those isolated from cartilaginous fishes.

3.6 *In vitro* anticoagulant activity

The anticoagulant activity of the new CS/DS was assessed by measuring aPTT, PT and TT. aPTT is related to the intrinsic coagulation phase in plasma and used to evaluate the coagulation factors such as II, VIII, IX, X, XI, and XII, while PT is used to characterize the extrinsic coagulation factors. TT is a simple screening test for the fibrin polymerization process which measures the formation time of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample [38, 39].

The anticoagulant activity of CBG, with the aPTT test, was represented in Fig. 5a. This assay indicated that CBG prolonged aPTT in a concentration-dependent manner. The addition of 1000 µg/mL of CBG in the reaction medium generated a significant ($p < 0.05$) prolongation of the clotting time (about 2.62 times greater than that of the control). Therefore, the significant effect of CBG on aPTT suggested an inhibition of the intrinsic and/or the common coagulation pathways. The obtained results showed that the addition of 7 µg/mL of heparin, used as positive control, caused a significant prolongation of aPTT. At this concentration the clotting time of heparin was 120 s which can induce a high risk of bleeding. The anticoagulant activity of CBG was also studied by their capacity to prolong the Thrombin time (TT). The measurement of TT allows an overall evaluation of fibrin formation step, during which, under the action of thrombin, a component of blood fibrinogen is transformed into fibrin. As presented in Fig. 5b, CBG was able to prolong TT in a concentration dependent manner. Interestingly, CBG prolonged TT about

3.53 times greater than that of the control at a concentration of 100 µg/mL. However, this effect was lower than that of heparin used at 7 µg/mL. In fact, at this concentration, the clotting time of heparin was higher than 120 s. The prolongation of Thrombin time revealed the inhibition of thrombin activity or fibrin polymerization as thrombin inhibition-dependent clotting time. In addition, the anticoagulant effect of CBG was assessed by the Prothrombin time (PT) assay. This test is often used to detect bleeding disorders. As illustrated in Fig. 5c, at concentrations of 1000 µg/mL, PT determined in the presence of CBG, was significantly ($p < 0.05$) higher than the control clotting time. In fact, the prolongation of the clotting time was 17.35 s for CBG while that of the control was 13.7 s. Consequently, prolongation of PT suggests inhibition of the extrinsic pathway of blood clots.

These results suggest that CBG isolated from the bone of corb possess a higher anticoagulant effect both in the intrinsic and extrinsic blood coagulation pathways. In this context sulfated polysaccharides, from different sources, such as pumpkin [40], Sea cucumbers [41], *Achatina fulica* [3] and *Gentiana scabra* [42] exhibited a strong anticoagulant effect. According to several studies, the anticoagulant effect of sulfated polysaccharides might be due to numerous structural parameters such as, the sulfate content, the degree of sulfation, charge density, sugar composition, the presence of branches and the sulfation position. In this context, the elevated sulfate content in the purified CS/DS was probably responsible for the anticoagulant effect observed. More recently, Liang et al. [40] reported that the anticoagulant activityof sulfated polysaccahrides improves with increasing of DS and molecular weight. Furthemore, Linhardt et al. [43] demonstrated that two disulfated disaccharides species, ΔDi2,4diS and ΔDi4,6diS, were essentially identified as DS having the ability to activate cofactor II. These works were confirmed by results obtained with enzymatic treatment using specific lyases. Moreover, even though the anticoagulant effect of heparin is significantly better than that of CS and DS, its venous

antithrombotic activity seemed to be significantly lower. Further, Volpi [44] reported that the hemorrhagic propreties of CS and DS isolated from natural sources and used as antithrombotic agent, were effectively decreased when compared to heparin.

4. Conclusion

In the present study, it is concluded that CS/DS isolated from bone of corb (*Sciaena umbra*) was composed of high percentages of monosulfated and disulfated disaccharides with relatively low molecular weight of 23.35 KDa. This purified CS/DS was clearly found to possess 74% CS and 26% DS. Moreover, CS/DS showed stronger anticoagulant activities through activated partial thrombosis time (aPTT), thrombin time (TT) and prothrombin time (PT), which might be attributed to a higher percentage of DS. In particular, the occurrence of disulfated disaccharide in positions 2 and 4 (ΔDi2,4diS) and / or 4 and 6 (ΔDi4,6diS) were required for thrombin inhibition by activating cofactor II. Results suggested also that strong anticoagulant activities could be obtained when molecular weight maintained in a moderate range. Obviously, CS/DS functions and possible biological activities were significantly related to their specific structures and properties as well as distinctive oligosaccharide sequences. On the basis of the data collected, it is reasonable to assume that CS/DS isolated from corb bone might be potentially useful for scientific and pharmacological applications.

Acknowledgement

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

References

1. P. Laurienzo, An overview. Mar. Drugs, 8(2010) 2435-2465.
2. K. Senni, J. Pereira, F. Gueniche, C. Delbarre-Ladrat, C. Sinquin, J. Ratiskol, G. Godeau, A.M. Fisher, D. Helley, S. Colliec-Jouault, Mar. Drugs, 9 (2011) 1664-1681.

3. J. Liu, L. Zhou, Z. He, N. Gao, F. Shang, J. Xu, Z. Li, Z. Yang, M. Wu, J. Zhao, Carbohydr.Polym. 181 (2018) 433-441.
4. N. Volpi, J. Pharma. Sci. 96 (2007) 3168-3180.
5. N. Volpi, J. Pharm. Biomed. Anal. 49 (2009) 868–871.
6. T. Zhao, Y. Zhou, G. Mao, Y. Zou, J. Zhao, S. Bai, L. Yang, X. Wu, J. Sci. Food Agric. 93 (2013) 1633-1640.
7. K. Higashi, Y. Okamoto, A. Mukuno, J. Wakai, S. Hosoyama, R.J. Linhardt, T. Toida, Carbohyd. Polym. 134 (2015) 557-565.
8. F. Maccari, F. Galeotti, N. Volpi, Carbohydr. Polym. 129 (2015) 143-147.
9. F. Krichen, H. Bougatef, N. Sayari, F. Capitani, I. Ben Amor, I. Koubaa, F. Maccari, V. Mantovani, F. Galeotti, N. Volpi, A. Bougatef, Carbohyd. Polym. 197 (2018) 451-459.
10. Suzuki S, J. Biol. Chem. 235 (1960) 3580-3588.
11. I. Capila, R.J. Linhardt, Heparin-protein interactions. Angew. Chem. Int. Ed, 41 (2002) 390-412.
12. T.E. Warkentin, HIT: lessons learned. Pathophysiology of Haemostasis and Thrombosis, 35 (2016) 50-57.
13. M. Ben Mansour, M. Dhahri, I. Bertholon, V. Ollivier, I. Bataille, N. Ajzenberg, M. Hassine, M. Jandrot-Perrus, F. Chaubet, R.M. Maaroufi, Thromb. Res. 123 (2009) 887-894.
14. M. Cesaretti, E. Luppi, F. Maccari, N. Volpi, Carbohydr. Polym. 54 (2003) 59-61.
15. N. Volpi, Carbohydr. Res. 247 (1993) 263-278.
16. N. Volpi, F. Maccari, Electrophoresis 23 (2002) 4060–4066.
17. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F.Smith, Anal. Chem. 28 (1956) 350-356.
18. Archer, E. E., Andyst, 82, (1957) 208.

19. White, D. C., Mikrochim. Acta, (1959) 254.

20. R.E. Edens, A. al-Hakim, J.M. Weiler, D.G. Rethwisch, J. Fareed, R.J. Linhardt, J. Pharmac. Sci. 81 (1982) 823-827.

21. D. Buzzega, F. Maccari, N. Volpi, J. Pharm. Biomed. Anal. 51 (2010) 969-972.

22. W.Garnjanagoonchorn, L. Wongekalak, A. Engkagul, Chem. Eng.Process, 46 (2007) 465-471.

23. R. Armis, J. Appl. Phycol. 7 (1995) 231-243.

24. F. Maccari, F. Ferrarini, N. Volpi, Carbohydr. Res. 345 (2010) 1575-1580.

25. F. Krichen, W. Karoud, A. Sila, B. E. Abdelmalek, R. Ghorbel, S. Ellouz-Chaabouni, A. Bougatef, Int. J. Biol. Macromol. 75 (2015) 283-289.

26. M. Ben Mansour, R. Balti, V. Ollivier, H. Ben Jannet, F. Chaubet, R.M. Maaroufi, Carbohydr. Polym. 174 (2017) 760-771.

27. N. Sayari, R. Balti, M. Ben Mansour, I. Ben Amor, I. Graiet, J. Gargouri, A. Bougatef, Biomed. Pharmacother. 80 (2016) 322-330.

28. C.P. Dietrich, S.M.C. Dietrich, Anal.Bioch. 70(1976) 645-647.

29. M. Ben mansour, M. Dhahri, M, Hassine, N, Ajzenberg, L. Venisse, V. Ollivier. F.Chaubel, M. Jandort-Perrus, R.M. Maaroufi, Comp.Bioch. Physiol, 156 (2010) 206-215.

30. P. Sabboural, F. Chaubet, F. Rouzet, F. Al-Shoukr, R. Ben Azzouna, N. Bouchemal, L. Picton, L. Louedec, M. Maire, L. Rolland, G. Potier, D. Le Guludec, D. Letourneur, C. Chauvierre, Mar. Drugs, 12 (2014) 4851-4867.

31. S.C. Wang, S.W.A. Bligh, S.S. Shi, Z.T. Wang, Z.B. Hua, J. Crowder, C. Brandford-White, C.Vella, Int. J. Biol. Macromol. 41 (2007) 369-375.

32. A. Sila, H. Bougatef, F. Capitani, F. Krichen, V. Mantovani, I. Ben Amor, F. Galeotti, F. Maccari, N. Nedjar, N. Volpi, A. Bougatef, Int. J. Biol. Macromol. 115 (2018) 891-899.

33. R. Ben Abdallah Kolsi, H. Ben Salah, N. Jardak, R.Chaaben, I. Jribi, A.ElFeki, T. Rebai, K. Jamoussi, N.Allouche, C. Blecker, H. Belghith,K.Belghith,Carbohyd. Polym, 170 (2017) 148-159.

34. M. Guia, J. Songa, L. Zhanga, S. Wang, R. Wua, C. Ma, P. Li, Carbohyd. Polym, 123 (2015) 454-460.

35. N. Takeda, S.Horai, J.Tamura, Carbohydr. Res. 424 (2016) 54-58.

36. S. G. Chen, G. Li, N. Wu, X. Guo, N. Liao, X. Ye, D. Liu, C. Xue, W. Chai, Bioch. Biophys. Acta. 1830 (2013) 3054-3066.

37. K.Arima, H.Fujita, R.Toita, A.Okada, N.Tsutsumishita-Nakai, N.Takeda, Y.Nakao, H. Wang, M.Kawano, K.Matsushita, H.Tanaka, S.Morimoto, A.Nakamura, M.Kitagaki, Y.Hieda, R.Hatto, A.Watanabe, T.Yumura, …J.Tamura, Carbohydr. Res. 366 (2013) 25-32.

38. P.M. Ekanayake, C. Nikapitiya, M. De Zoysa, I. Whang, S.J. Kim, J. Lee, Eur Food Res Technol. 277 (2008) 897-903.

39. L. Ye, L. Xu, J. Li, Carbohyd. Polym. 87 (2012) 2052-2057.

40. L. Liang, L. Ao, T. Ma, Y. Ni, X. Liao, X. Hu, Y. Song, Int. J. Biol. Macromol. 106 (2018) 447-455.

41. J. Li, S. Li, L. Yan, T. Ding, R J.Linhardt, Y. Yu, X. Liu, D. Liu, X. Ye, S. Chen, Eur J Med Chem, 193 (2017) 191-200.

42. W. Cai, H. Xu, L. Xie, J. Sun, T. Sun, X. Wu, Q. Fu, Carbohyd. Polym. 140 (2016) 308-313.

43. R. J. Linhardt, A. Al-Hakim, S. Y.Liu, Y. S. Kim, J. Fareed, Seminars in Thrombosis and Hemostasis, 17 (1991) 15–22.

44. N. Volpi, Carbohy. Polym. 82 (2010) 233-239.

Figure caption

Figure1. Agarose-gel electrophoresis stained with toluidine blue and stain all of the purified CS/DS from the bones of corb (CBG).

Figure2. Ultraviolet-visible spectrum in the wave length range from 190 to 700 nm (a) and Fourier transforms infra-red spectrum in the wave number range from 650 to 4000 cm-1 (b) of the purified CS/DS from bone of corb.

Figure3. SAX-HPLC separation of the unsaturated disaccharides produced by chondroitin sulfate/ dermatan sulfate purified from fish bone and treated with chondroitinase AC (a) and chondroitinase ABC (b). ΔDi0S (ΔUA-GalNAc), ΔDi6S (ΔUA-GalNAc 6S), ΔDi4S(ΔUA-GalNAc 4S), ΔDi2, 6diS (ΔUA2S-GalNAc 6S) ΔDi4, 6diS (ΔUA GalNAc4, 6diS),ΔDi2, 4diS (ΔUA2S-GalNAc4S). The identity of disaccharide species was assured by coelution with purified standards (Seikagaku Co./Sigma–Aldrich).

Figure4. PAGE analysis of chondroitin sulfate/dermatan sulfate GAGs purified from the bone of CBG. The calibration curve was constructed by using chondroitin sulfate standards of known molecular mass prepared from chondroitin sulfate and having masses of 28.640 KDa, 16.750 KDa and 4.630 KDa.

Figure5. Anticoagulant activity of the purified CS/DS extracted from the bone of corb (CSG) at different concentrations evaluated by the measurement of (**a**) the activated partial thromboplastin time (aPTT), (**b**) thrombin time (TT) and (**c**) prothrombin time (PT).

Table 1. Chemical composition of glycosaminoglycans extracted from corb (*Sciaena umbra*) bone (CBG). Values are given as mean ± SD from triplicate determinations. Physico-chemical composition was calculated based on dry matter.

Composition (%)	CBG
Sulfate	31.28 ± 0.87
Total sugars	49.37 ± 1.05
Uronic acid	46.02 ± 0.46
Color	
*L**	69.44 ± 0.05
*a**	5.01 ± 0.01
*b**	15.76 ± 0.06
Yield of crude GAGs	8.64± 0.84
Yield of purified CS/DS	10 ± 0.01

Table 2. Amount, disaccharide composition and charge density values of CS/DS purified from fish bone. Δ UA, 4,5-unsaturated uronic acid; GalNAc, N-acetyl-galactosamine; S, sulfate group. The percentage of each identified disaccharide was determined by purified standards (Seikagaku Co./Sigma–Aldrich) and reported as weight percent. Charge density was calculated by considering the number of sulfated groups per disaccharide unit.

	Chondroitinase AC	**Chondroitinase ABC**
ΔDi0S (ΔUA-GalNAc) (%)	2.6	2.8
ΔDi6S (ΔUA-GalNAc 6S) (%)	5.3	8.6
ΔDi4S (ΔUA-GalNAc 4S) (%)	69	40
ΔDi2, 6S (ΔUA2S-GalNAc 6S) (%)	11.4	4.3
ΔDi4, 6S (ΔUA 4S-GalNAc 6S) (%)	2.7	7.2
ΔDi2, 4S (ΔUA2S-GalNAc 4S) (%)	8.8	36.9
Charge density	1.2	1.4

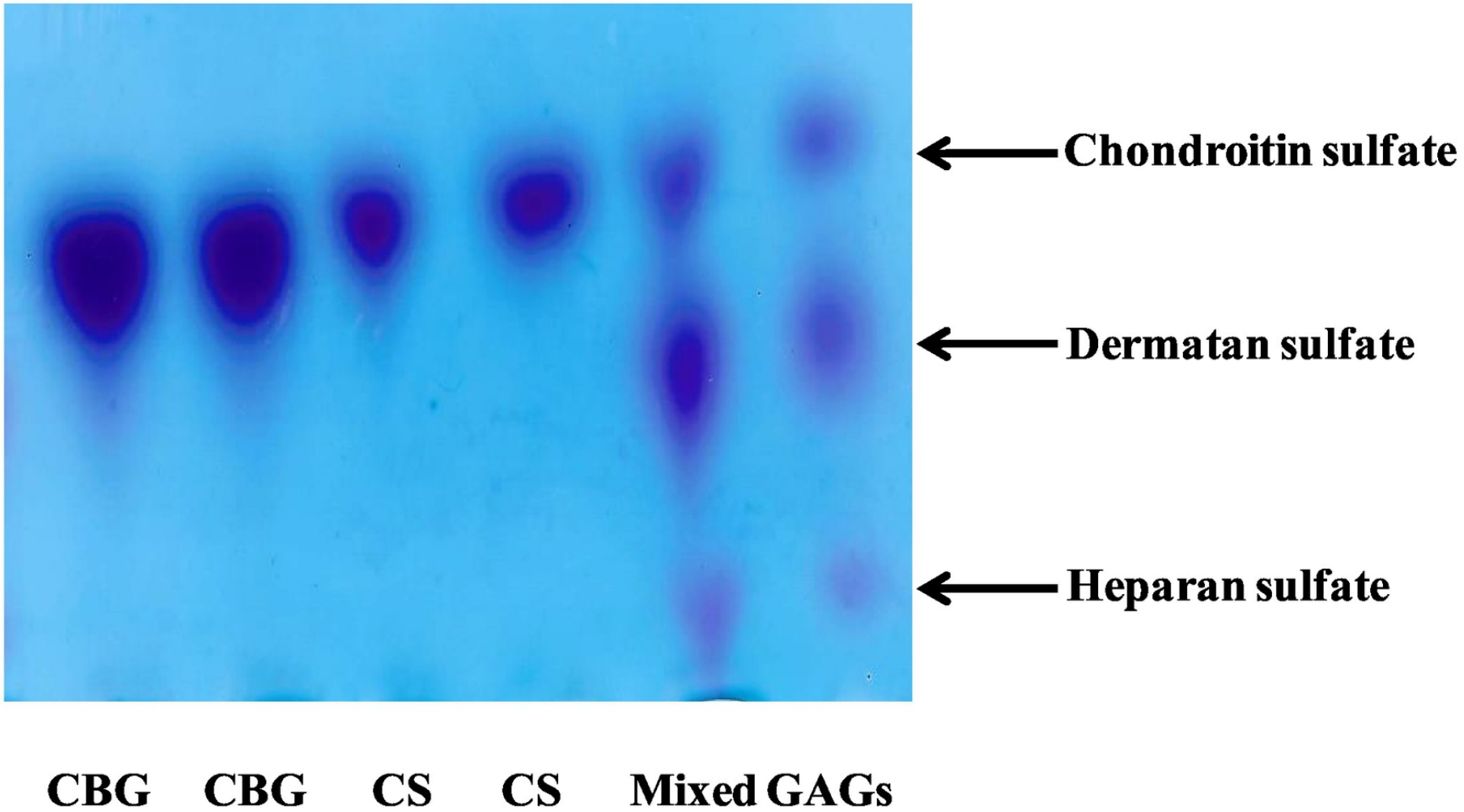
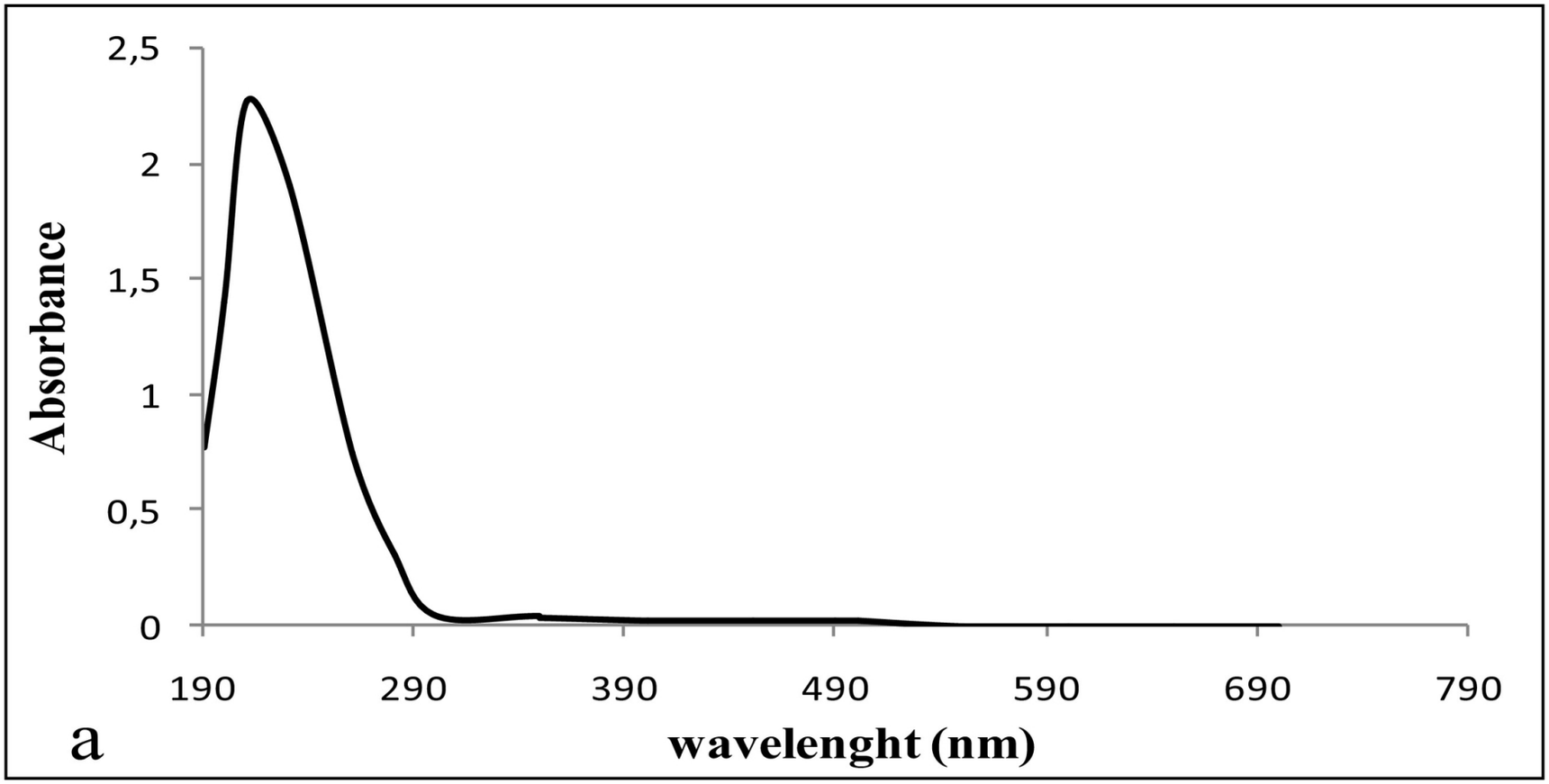

Chondroitin sulfate
Dermatan sulfate
Heparan sulfate
CBG
CBG
CS
CS
Mixed GAGs

Figure 1


2,5
2
1,5
1
0,5
0
Absorbance
190
290
390
490
590
690
790
wavelenght (nm)
a

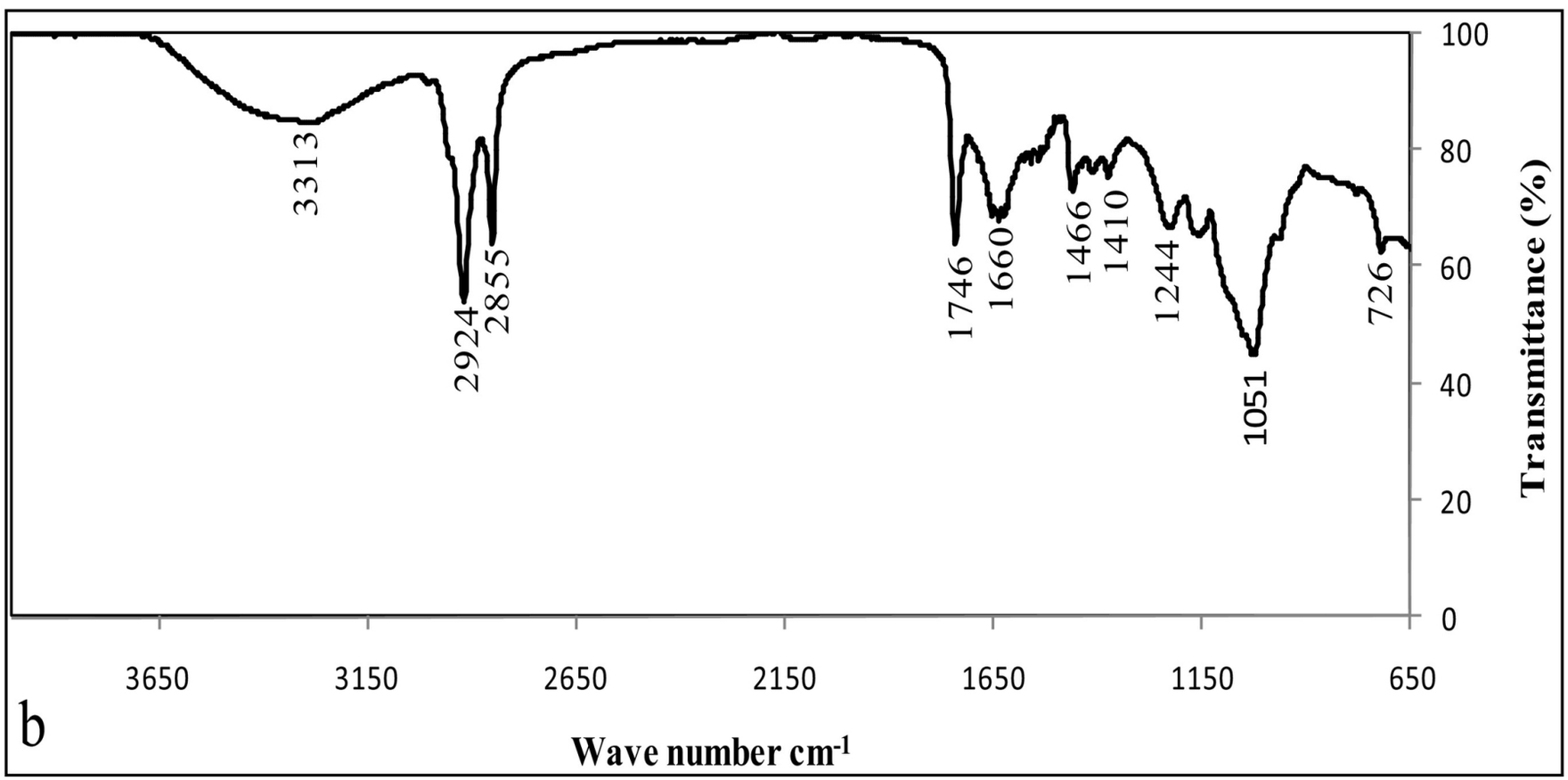

3313
2924
2855
1746
1660
1466
1410
1244
1051
726
100
80
60
40
20
0
Transmittance (%)
3650
3150
2650
2150
1650
1150
650
Wave number cm-1
b

Figure 2

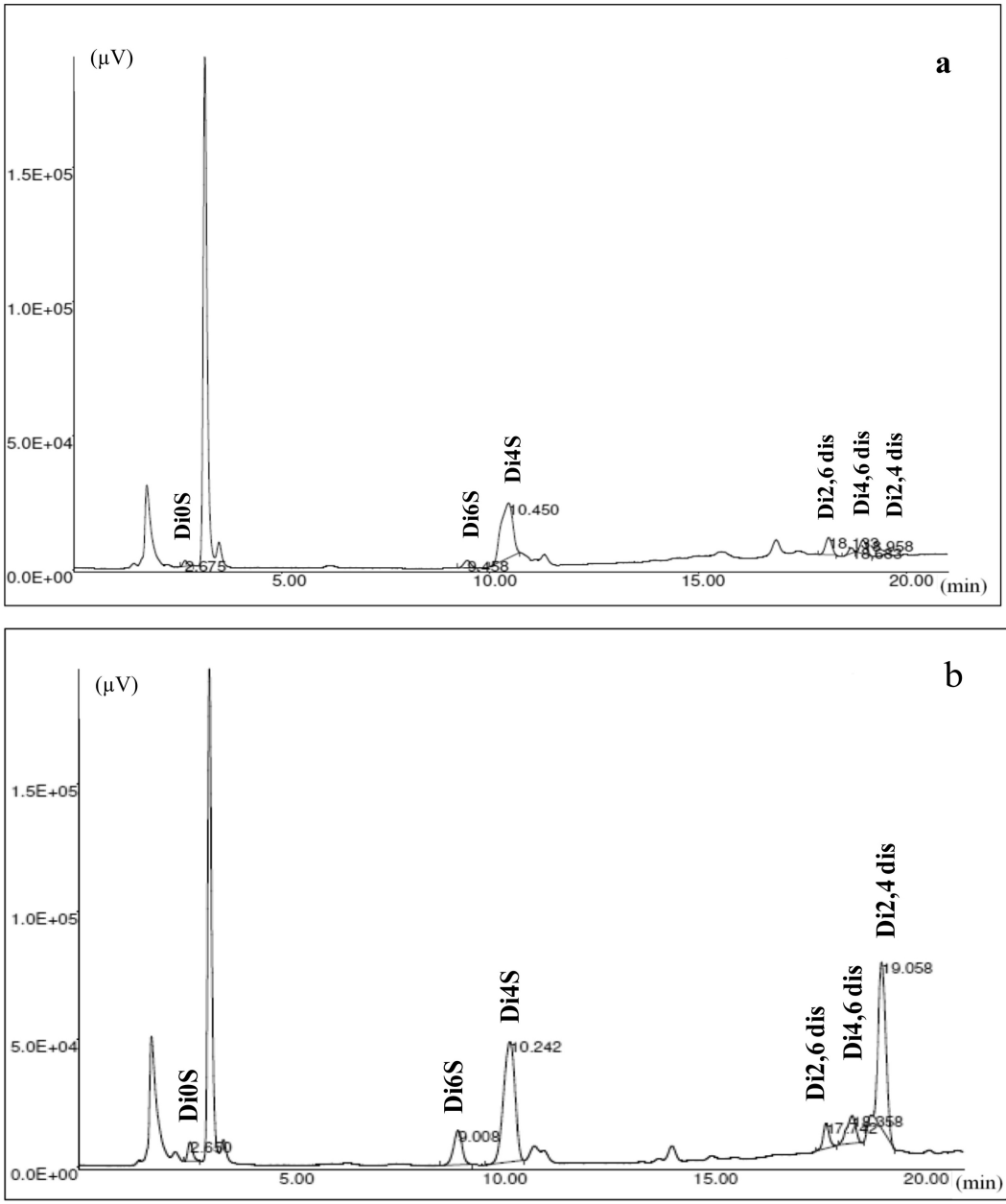

a
(μV)
1.5E+05
1.0E+05
5.0E+04
0.0E+00
Di0S
Di6S
Di4S
Di2,6 dis
Di4,6 dis
Di2,4 dis
10.450
5.00
10.00
15.00
20.00
(min)
b
(μV)
1.5E+05
1.0E+05
5.0E+04
0.0E+00
Di0S
Di6S
Di4S
Di2,6 dis
Di4,6 dis
Di2,4 dis
10.242
19.058
5.00
10.00
15.00
20.00
(min)

Figure 3

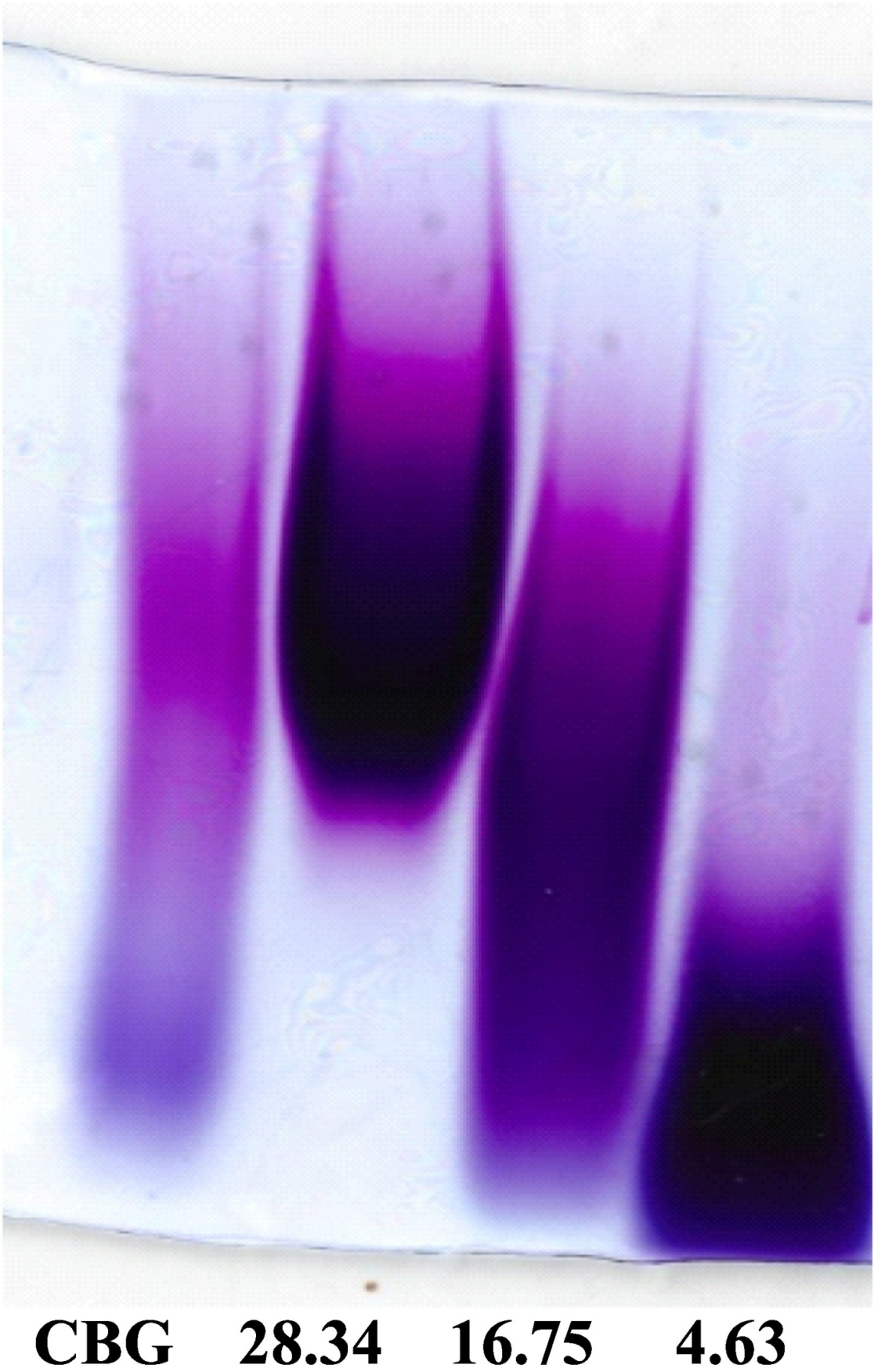
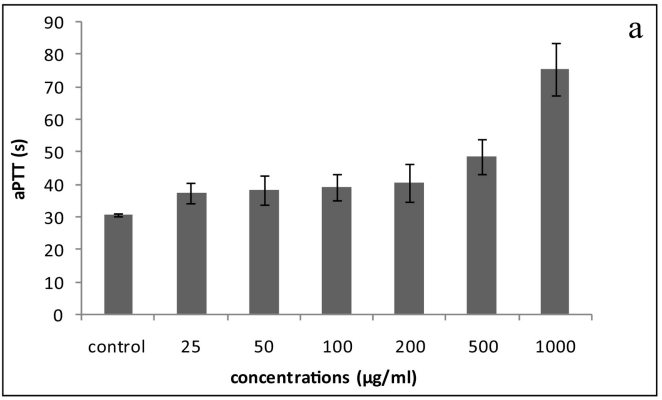
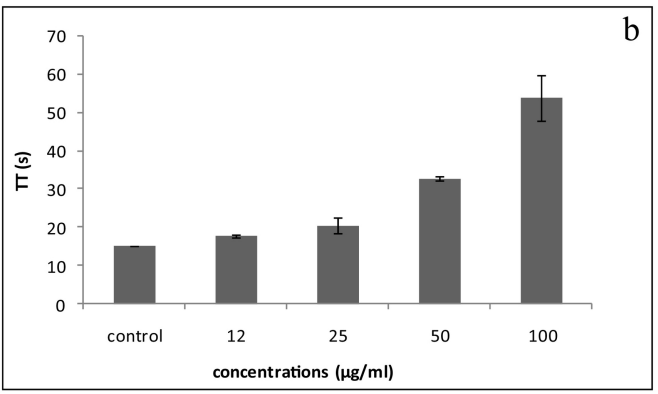


Figure 4


a
aPTT (s)
90
80
70
60
50
40
30
20
10
0
control
25
50
100
200
500
1000
concentrations (µg/ml)


b
TT (s)
70
60
50
40
30
20
10
0
control
12
25
50
100
concentrations (µg/ml)

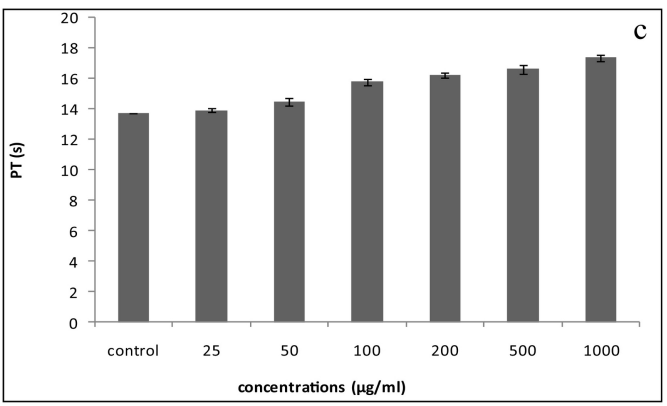

c
PT (s)
20
18
16
14
12
10
8
6
4
2
0
control
25
50
100
200
500
1000
concentrations (µg/ml)

Figure 5