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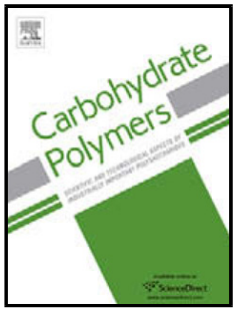
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Isolation, Purification and Structural Characterestics of Chondroitin Sulfate from Smooth hound Cartilage: *In vitro* Anticoagulant and Antiproliferative Properties

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Research Highlights

- The chondroitin sulfate was extracted and purified from Smooth hound cartilage (CSSH).
- The physicochemical and Structural characteristics of CSSH were determined by acetate cellulose electrophoresis, FTIR, ^{13}C NMR and SAX-HPLC.
- CSSH has high sulfate amount and a relatively average molecular mass.
- CSSH showed strong anticoagulant and antiproliferative properties.

Abstract

Chondroitin sulfate was extracted from the cartilage of smooth hound (CSSH) and then purified by anion exchange chromatography. The structual characteristic of CSSH was evaluated by acetate cellulose electrophoresis, FTIR, ^{13}C NMR and SAX-HPLC. Molecular weight of CSSH was average 68.78 KDa. Disaccharide analysis indicated that CSSH was predominately composed of monosulfated disaccharides in position 6 and 4 of the N-acetylgalactosamine (45.34% and 32.49%, respectively).

CSSH was tested for *in vitro* anticoagulant activity using the three classical coagulation assays (activated partial thromboplastin time (aPTT), prothrombine time (TT) and thrombin time (PT) tests). The finding showed that CSSH prolonged significatively ($p < 0.05$), aPTT, TT and PT about 1.4, 3.44 and 1.21 fold, respectively, greater than that of the negative control at a concentration of 100 µg/ml. The CSSH caused a significant antiproliferative activity against HCT116 cell, which was 79% of cell proliferation inhibition at the concentration of 1000 µg/ml. Further, CSSH presented no toxicity against the normal cells and no hemolysis towards bovine erythrocytes for all concentrations tested.

CSSH demonstrated hopeful antiproliferative and anticoagulant potential, which may be used as a novel and effective drug.

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Keywords: Chondroitin sulfate, Smooth hound cartilage, Structural characterization, anticoagulant activity, Antiproliferative activity.

1. Introduction

Chondroitin sulfate (CS) constitue an acidic carbohydrate that belong to the class of glycosaminoglycans (GAGs) (Lauder, 2009). It is highly sulfated, complex and linear polysaccharide that had an important role in various biological processes. Their structure is composed of a repeated disaccharide unit containing N-acetyl-β-D-galactosamine and β-D-glucuronic acid units, which was sulfated in the carbon 6 (CS-C, more common), 4 (CS-A) at N-acetyl galactosamine, both 4 and 6 (CS-E) as well as positions 6 of GalNAc and 2 of GlcA (CS-D) (Malavaki, Mizumoto, Karamanos, & Sugahara, 2008).

CS is largely isolated from animal sources mostly involving cartilages as raw material derived from terrestrial animals such as porcine, avian and bovine (Volpi, 2007, 2009), or from certain marine organisms such as shark (Sugahara, Nadanaka, Takeda, & Kojima, 1996), squid (Cássaro & Dietrich, 1977), skate (Lignot, Lahogue, & Bourseau, 2003), sturgeon (Maccari, Ferrarini, & Volpi, 2010; Zhao et al., 2013) and bony fishes (Maccari, Galeotti, & Volpi, 2015). However, it is more safty to use the marine origin because there are several health effects from terrestrial animal diseases such as Mad Cow disease, hog cholera and foot-and-mouth disease (Volpi, 2006).

Due to its negative charge, disaccharide unit heterogeneity and the number and the position of sulfates on disaccharide units forming the CS polymer are the main factors influencing its biological activities. Indeed, CS is accountable for water retention in the cartilage. Physiologically, CS rise hyaluronan output by human synovial cells to maintain viscosity in the

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synovial fluid (David-Raoudi, Deschrevel, Leclercq, Galéra, Boumediene, & Pujol, 2009). In addition, CS has been involved in diverse physiological events including organogenesis, morphogenesis, cytokinesis and central nervous system development (Mikami & Kitagawa, 2013; Volpi, 2014). Furthermore, the anticoagulant activity is among the most widely studied properties of CS chains isolated from marine sources (Gui et al., 2015; Ben Mansour, Balti, Ollivier, Ben Jannet, Chaubet, & Maaroufi, 2017). In addition to their anticoagulant activity, CS has been shown to have significant anti-inflammatory, antioxidant and neuroprotective activities (Egea, Carcia, Verges, Montell, & Lopez, 2010; Mou, Li, Qi, & Yang, 2018).

The dysfunction of the blood circulatory system is the leading cause of mortality in developing countries around the world (WHO Report, 2003). In fact, many drugs are used for preventing and treating bleeding, which have focused on inhibition of thrombin generation and blocking its activity (Pawlaczyk et al., 2011; Łopaciuk, 2002). Actually, anticoagulants from animal origin, in particular heparin are mostly engaged preventing human body from thromboembolic diseases (Kreutz, 2014).

Despite the beneficial effects of heparins with thromboembolic diseases, their use is limited because its problems on humans healths (thrombocytopenia and thrombosis syndrome, hemorrhagic complications) (Warkentin, 2006) and animal diseases (Mad Cow disease). Therefore, attention has recently been wear of the development of novel anticoagulants agents from natural sources more safety and potency, in particular sulfated polysaccharide (Maas, Gracher, Sassaki, Gorin, Iacomini, & Cipriani, 2012;). Currently, studies suggest that CS can inhibit the formation of a blood clot and preclude vein hardening (Sugahara, Mikami, Uyama, Mizuguchi, Nomura, & Kitagawa, 2003; Gui et al., 2015). In order to develop and extract anticoagulants from a safer source, seafood processing by-products is considered as a good source.

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Furthermore, the global awareness of cancer as the second largest cause of death in people of various ages. In Tunisia, Colon cancer is the second leading cause cancer death in both men and women (OMS, 2014). The high mortality of this disease is related to the advanced stage of colon cancer. A large part of the intervention in cancer cases implies chemotherapy and surgery that goals to eliminate cancer tissues (Dong et al., 2011), but this drug exhibit serious side effects. In this reason, considerable attention has recently carried of the development of natural antitumoral drug such as marine CS. Thus, these polymers have been reported to induce apoptosis in several cancer lines and rouse immune system cells to induce tumor cell death (Aisa et al., 2005; Sayari et al., 2016; Krichen et al., 2017).

The smooth hound (*Mustelus mustelus*) is a hound shark of the family Trikidae, found on the continental shelves of the eastern Atlantic Ocean, from the British Isles and France to South Africa, and in the Mediterranean Sea. Smooth hound, is relatively important in the fish-catches of Tunisia, and is utilised for human consumption. Head, skin and cartilage are considered as by products. These by-products are commonly recognized as low value resources with negligible market value. Additionally, their inappropriate disposal is a major cause of environmental pollution. Recently, a few studies have investigated CS from marines cartilage or bone (Im, Park, & Kim, 2010; Maccari et al., 2010, Maccari et al., 2015). However, no study has been conducted to characterize the chemical composition and biological activities of CS from smooth hound cartilage.

This work was carried out aiming to extract and purify chondroitin sulfate for the first time from smooth hound cartilage (CSSH). These CS were structurally characterized and their anticoagulant and antiproliferative effects were also investigated.

2. Materials and methods

2.1. Reagents

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All solvents and chemicals were of analytical grade and used as received from commercial sources. Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was purshased from Novozymes® (Bagsvaerd, Denmark) (11.44×10^4 U/ml) using casein as a substrate. GAGs standard, Heparan sulfate (HS) from bovine kidney, Dermatan sulfate (DS) from porcine intestinal mucosa and Chondroitin sulafate (CS) from bovine trachea, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5–2 units/mg were from Sigma–Aldrich. Unsaturated chondro/dermato disaccharides [ΔDi0S (ΔUA-[1→3]-GalNAc), ΔDi2S (ΔUA-2S-[1→3]-GalNAc), ΔDi6S (ΔUA-[1→3]-GalNAc-6S), ΔDi4S (ΔUA-[1→3]-GalNAc-4s), ΔDi2,6diS (ΔDi-dis D, ΔUA-2S-[1→3] GalNAc-6S), ΔDi2,4diS (ΔDi-dis B, ΔUA-2S-[1→3]-GalNAc- 4S), 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. Diethylaminoethyl (DEAE)-cellulose obtained from Pharmacia (Uppsala, Sweden). 2,5-diphenylte- trazolium bromide (MTT), cell culture medium (RPMI1640), phosphate buffer saline (PBS), foetal calf serum (FCS), penicillin, trypsin-EDTA and streptomycin mixture and L-glutamine (200 mM) were obtained from GIBCO-BCL (UK).

Smooth hound by-products were collected from the local fish market of Sfax, Tunisia. The cartilage samples were packed in polyethylene bags, placed in ice and transported to the research laboratory within 30 min. The cartilaginous material was then milled and homogenized using a Moulinex R62 homogenizer and stored at −20 °C.

2.2. **Extraction and purification of Chondroitin sulfate**

Chondroitin sulfate was isolated according to a little modified version of the method described by Ben Mansour et al., (2009). Five grams of cartilaginous material was dissolved in 250 mL sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH 6. Enzymatic hydrolysis was

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realized by the addition of Alcalase® and the mixture was kept for 24 h at 50 °C. Then, the mixture was filtered. The filtrates were precipitated with cetylpyridinium chloride 0.5 % (w/v) for 24 h at room temperature. Thereafter, the mixture was centrifuged for 30 min at 5869 g/min and 4 °C. The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and disbanded in 200 mL NaCl solution (2M) in ethanol (100:15, v/v). Then, two volumes of absolute ethanol were added. The polysaccharide containing solution was left for 24 h at 4 °C and then centrifuged for 30 min at 5869 g/min and 4 °C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. Finally, the pellet was redissolved in distilled water and lyophilized in a freeze dryer.

The lyophilized CS was dissolved in distilled water and then applied to a column (2 cm × 6 cm) packed with DEAE-cellulose anion-exchange resin equilibrated with NaCl 50 mM. CS was eluted with a linear gradient of NaCl from 50 mM to 2 M from 0 to 150 min at a flow of 1 ml/min. Then, absolute ethanol was added (2V/V) to the collected fractions corresponding to single species of GAGs. GAGs were precipitated at 4 °C for 24 h. After centrifugation at 10,000 g for 10 min, the pellet was dried at 50°C and then solubilized in distilled water.

2.3.Chemical composition analysis

Total sugar was measured by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total uronic acid content was assessed as described by Cesaretti, Luppi, Maccari, &Volpi (2003). Sulfate content in CS was carried by liquid-Ion Chromatography (HPLC) on a Metrohm chromatograph equipped with columns CI SUPER-SEP using phthalic acid and acetonitric as eluent. All measurements were carried out in triplicate.

2.4.UV spectroscopy analysis

The solution of CS at a concentration of 1 mg mL^{-1} was used for UV measurement in the wavelength range from190 to 700 nm.

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2.5.Cellulose acetate electrophoresis

Thirty five microlitre of the GAGs solution standard containing Chondroitin Sulfate (CS) from bovine trachea, Dermatan sulfate (DS) from porcine intestinal mucosa, and Heparan sulfate (HS) from bovine kidney and thirty five microlitre of chondroitin sulfate from smooth hound cartilage (CSSH) were filed at the origin (10 mm from the cathode side) of a cellulose acetate strips. The cellulose acetate electrophoresis was performed in Zn-acetate 0.1 M, buffer pH 6 and run at 60 V, for 2 h. Then, the cellulose acetate strip was stained by alcian blue (Wegrowski & Maquart, 2001).

2.6. CS molecular weight determination

The molecular weight of CSSH was determined by PAGE according to Edens, al-Hakim, Weiler, Rethwisch, Fareed, & Linhardt (1982). 15 µl of the purified CS determined by uronic acid assay were layered on the gel. The related calibration curve was constructed by using oligosaccharide standards of known molecular masses prepared from CS (Buzzega, Maccari, & Volpi, 2010). 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 weight 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 weights values.

2.7.Infra-Red and NMR Spectroscopic Analysis

The absorption spectra of the samples were obtained using Agilent Cary 630 FTIR. All spectra were scanned in the range between 650 and 4000 cm^{-1}.

The ^{13}C NMR spectrum of CSSH was recorded at 298.1 k on a Bruker ASX300, equipped with a 5 mm diameter tunable probe, with BRUKER software. Thirty milligrams of sample was suspended in 1 ml D_2O at a high level of deuteration (99.997%) to avoid the presence of

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relatively high water. The spectra were registered at a temperature of 25-28°C and at 100.62 MHz, unless specified. ^{13}C chemical shifts (d, ppm) are quoted with respect to external sodium 4, 4-dimethyl-4-silapentane- 1-sulfonate (0.0 ppm).

2.8.Enzymatic treatments and disaccharide composition evaluation

After treatment of the purified CSSH with chondroitinase ABC, the generated unsaturated disaccharides were separated and quantified by anion-exchange (SAX) by means of HPLC equipment from Jasco equipped with a150 mm × 4.6 mm stainless-steel column spherisorb 5-SAX (5 µm, trimethylammoniopropyl groups $Si\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N^+(CH_3)_3$ 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. *In vitro* Anticoagulant activity of CS

The effect of CSSH on haemostatic system was estimed by the evaluation of its anticoagulants activities in prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (aPTT), using a semi-automatic line STA (Diagnostica Stago). CSSH was suspended in distilled water. All analyses were carried out in triplicate. aPTT test was performed by mixing 45 µL of normal citrated platelet poor plasma (PPP) with 5 µL various concentrations of CSSH and incubated at 37 °C after addition of 50 µl of aPTT reagent (CK-PREST). The cogulation time was recorded after the addition of 100 µL of 25 mM $CaCl_2$. The control (C) is determined by replacing the 5 µL of CS with distilled water. PT test was carried out by mixing 5 µL of CS at different concentrations with 45 µL of PPP for 3 min at 37 °C. 100 µL of

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Néoplastine® CI was added into the mixture and the PT was saved. TT test was performed by mixing 5 µL of CS at different concentrations with 45 µL of PPP and incubated for 3 min at 37 °C. The coagulation time was recorded after the addition of 100 µL of thrombin.

2.10. Anti-proliferative activity of CSSH

2.10.1. Cell culture and treatment

The tumor cells of human colon carcinoma HCT116 were acquired from the Faculty of pharmacy Chatenay malabry University Paris 11. These tumor cells were cultured in DMEMF-12, supplemented with 1% L-glutamine (200 mM), 10% FBS, streptomycin (100 mg/mL) and 1% of mixture penicillin (100 IU/mL) at 37 °C with 5% CO_2.

The normal lymphocytes cells was taken from heparinized human peripheral blood samples of healthy volunteers by using the Ficoll-Paque gradient density method, as described previously (Bicalho, Gontijo, & Nogueira-Machado, 2016). The human lymphocytes were used for cell culture analysis as a normal control cells.

2.10.2. Determination of cell mortality (MTT assay)

Cytotoxicity of CS was determined as described by Carmichael, De Graff, Gadza, Minna, and Mitchell (1987). This method evaluates the ability of viable HCT 116 and human lymphocytes cells to form MTT formazan by the mitochondrial enzyme succinate dehydrogenase. The measurements mirror the first cellular redox changes (Mosman, 1983).

2.11. Hemolytic activity

The hemolytic activity of CS was evaluated by a little modified version method of Dathe et al. (1996). In brief, five milliliters of bovine blood were centrifuged at 3500 rpm for 10 min to isolate erythrocytes, which were then washed three times with 10 mM sodium phosphate, pH 7.5, containing NaCl 9 g/L (NaCl/Pi). The cell concentration of stock suspension was adjusted to 10^9

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cells/mL. The cell suspension (12 mL), along with varying amounts of stock solution fractions and buffer, were pipetted into Eppendorf tubes to give a final volume of 50 mL. The Eppendorf tubes with 2.5 $\times 10^8$ cells/mL were then incubated at 37 °C for 40 min. After centrifugation (5000 rpm, 5 min), 30 mL of supernatant were diluted in 500 mL 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 check reproducibility.

2.12. Statistical analysis

All data were expressed as the mean standard deviation (SD). Results were analyzed using the SPSS statistic program (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL). Statistical differences between sample treatments were evaluated by one-way analysis of variance, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Extraction, purification and chemical composition of CSSH

Based on the dry weight, the CS from smooth hound cartilage (CSSH) extraction yield of 2.52% was obtained. This yield was similar to fucosylated CS from *Holothuria scabra* (2.89%) (Yang, Wang, Yang, & Lv, 2018) but it was lower than that of CS extracted from shark fin cartilage (15.05%) and ray cartilage (7.49%) (Garnjanagoonchorn, Wongekalak, & Engkagul, 2007). In fact, Armis et al. (1995) demonstrated that generally the extraction yield of polysaccharides is very variable due to several factors, such as seasonal variation, environmental conditions, physiological factors and extraction methods. After proteolytic treatment, CSSH was purified by anion-exchange resin DEAE cellulose. The chemical composition of purified CSSH is shown in Table 1. The analysis of total sugars by the phenol/sulfuric acid method revealed that CSSH had an average percentage of 89.43% ± 1.25. The uronic acid content determined by

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carbazole methods was estimated to be 80.7 % ± 0.42. It was similar to the uronic acid amount previously described for CS from keel cartilage (82.7%) (Srichamroen, Nakano, Pietrasik, Ozimek, & Betti, 2013). However, the uronic acid content was higher than that reported for chondroitin sulfate/dermatan sulfate from grey triggerfish skins (70%) (Krichen et al., 2017).

The sulfate content in CSSH was recorded a value in the order of 21.48 ± 1.2%. It was similar to that previously describes for sulfated glycosaminoglycans from Norway lobster shell (23%) (Sayari et al., 2016). However, it was lower than that described for fucosylated CS from Sea cucumber *Holothuria polii* (43%) (Ben Mansour et al., 2017).

Table 1. Chemical caracterization of CS isolated from smooth hound cartilage (CSSH). Measurements are given as mean ± SD from triplicate determinations. Chemical composition of CSSH was determined based on dry matter.

	CSSH
Total sugar (%)	89.43 ± 1.25
Uronic acid (%)	80.7 ± 0.42
Sulfate (%)	21.48 ± 1.2
Yield (%)	2.52 ± 0.07

3.2. Purity analysis, acetate cellulose electrophoresis and molecular weight of CSSH

The purity of CS isolated from smooth hound cartilage was identified by Ultraviolet–visible spectra, in the wavelength range from190 to 700 nm. As presented in Fig. 1A. The CSSH sample was emerged a stronger absorption peak at 200 nm, which showed characteristic of polysaccharides. While, CSSH had no absorbance at 260 and 280 nm indicating the absence nucleic acid and proteins.

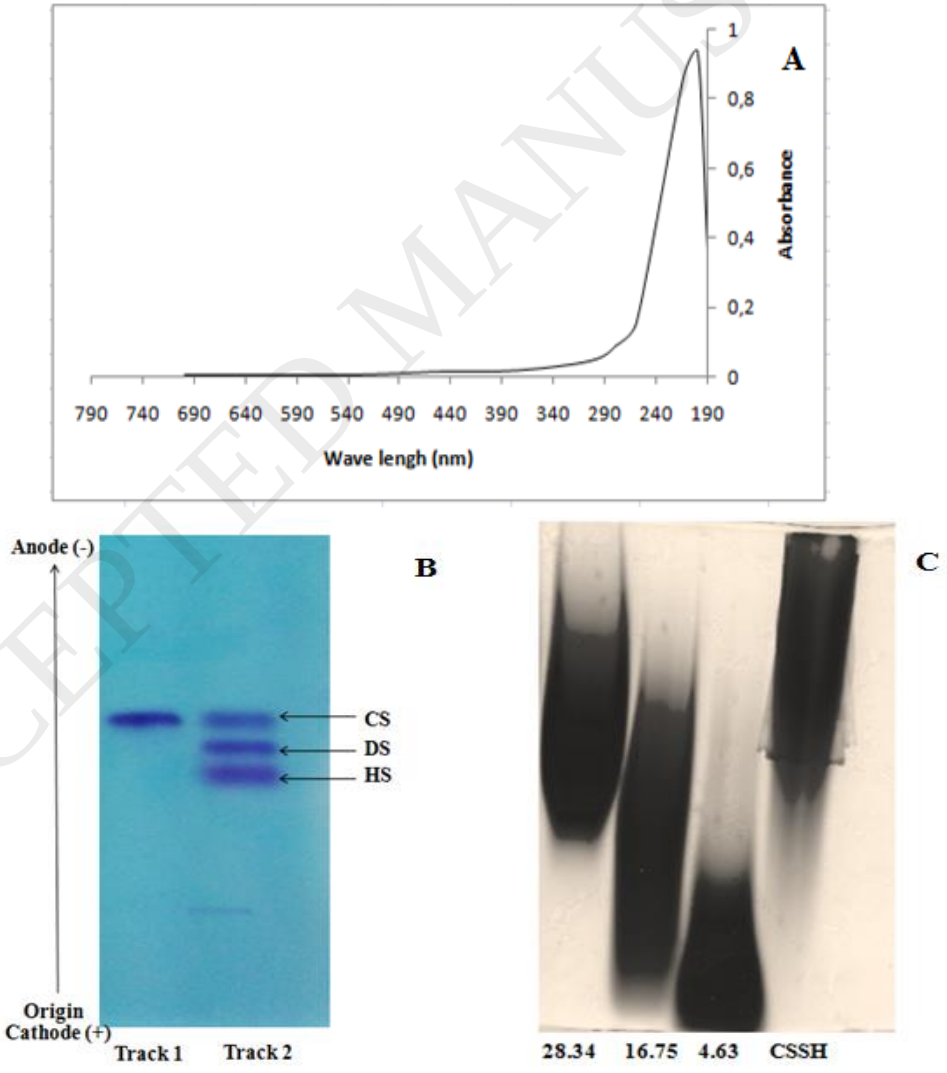
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The acetate cellulose electrophoresis of CSSH revealed the presence of a single band which identified as CS (Fig 1B). In the same contexte, the agarose gel of sulfated glycosaminoglycans from cartilages of bony fishes indicated the presence of CS as a principal polysaccharide (Maccari et al., 2015).

Deponding on the origin of raw material, GAGs may own different molecular weights and polydispersities depending on the source. The molecular weight of CSSH was calculated using PAGE analysis and a serie of chondroitin sulfate standards with known molecular weights. Calibrated curve of chondroitin sulfate fractions was given as follow logMw= -1.9058x+5.3669. As shown in Fig 1C. CSSH have a molecular weight of around 68.78 KDa. This molecular weight was similar to that reported for fucosylated CS from *Holothuria scabra* (69.1KDa) and for CS from shark cartilage (68.22 KDa) (Yang et al., 2018; Buzzega et al., 2010). Furthermore, Wang, Chang, Wu, Xu, & Xue (2018) reported that fucosylated CS from *Apostichopus japonicus* has a molecular weight of around 56.2 KDa, lower than that observed for CSSH. These results indicate that the disaccharide composition, the charge density in the polymer and the molecular weights were influenced by the raw material source. Indeed, CS isolated from 'terrestrial' origins, such as bovine, porcine and avian cartilages have in general molecular weight values ranged from 13 to 26 KDa (Volpi, 2007; 2009), lower than that observed for CS from smooth hound cartilage. In the same context, Volpi (2007; 2009) reported that cartilaginous fishes CS, such as raja and shark had molecular weights ranged from 50 to 70 KDa. On contrary, bony fishes CS have molecular mass values between 13.46 and 48.68 KDa (Maccari et al., 2015). As a consequence, CS from cartilage sources had molecular weight and structural characterization different to CS from bones and terrestrial sources.

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Fig 1. (A) UV spectrum of the purified CS from smooth hound cartilage (CSSH). (B) Acetate cellulose electrophoresis of chondroitin sulfate from smooth hound (CSSH). Purified chondroitin sulfate from smooth hound (Track 1), Standard GAGs (Heparan sulfate (HS), Dermatan sulfate (DS) and Chondroitin sulfate (CS)) (Track 2). The direction of electrophoretic migration was indicated by the arrow (C) PAGE analysis of CSSH. The calibration curve was constructed by using chondroitin sulfate standards of known molecular weights (4.63 KDa, 16.75 KDa and 28.34 KDa).



3.3.Infra-Red and NMR Spectroscopic Analysis

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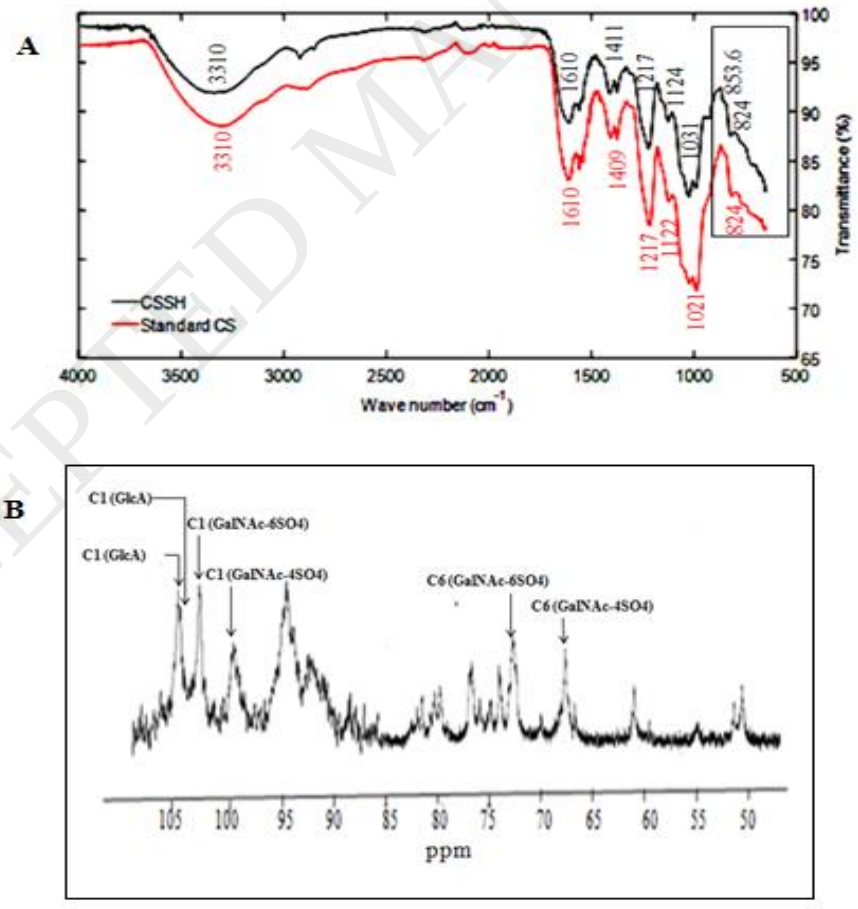
The type of chondroitin sulfate from smooth hound cartilage (CSSH) was identified by FTIR spectroscopy using chondroitin-6-sulfate as standard (Fig. 2A). Comparison of spectra of CSSH and standard CS indicated the presence of peaks at the same wave number (824 cm^{-1}). Moreover, the presence of sulfate group in CSSH was detected also at the wave number 853.6 cm^{-1}. In this context, Uchisawa, Okuzaki, Ichita, & Matsue (2001) reported that peaks of chondroitin-4-sulfate and chondroitin-6-sulfate were detected at 854.5 cm^{-1} and 823.7 cm^{-1}, respectively. Therefore, the spectrum of CSSH indicated that this cartilage sample has both chondroitin 4-sulfate and chondroitin 6-sulfate. In the same context, Garnjanagoonchorn et al. (2007) demonstrated that the spectrum of CS from shark fin cartilage showed two peaks at 824 cm^{-1} and 857 cm^{-1}, indicating that it has both chondroitin-4-sulfate and chondroitin-6-sulfate. Furthermore, the absorbance band detected at 1411 cm^{-1} (CSSH) and 1409 cm^{-1} (Standard CS), is characteristic of S = O stretching. The strong bands at 1610 cm^{-1} for both CSSH and standard CS showed the presence of uronic acid (Santhiya, Subramanian, & Natarajan, 2002). Moreover, bands observed at around 1031 cm^{-1} (CSSH) and 1021 cm^{-1} (standard CS) were attributed to the C–O–C ring vibrations. The strong bands at 3310 cm^{-1} for both CSSH and standard CS indicated the stretching of the hydroxyl groups.

The structural features of CSSH were further elucidated by ^{13}C NMR spectral analysis. Examination of the 50–70 and 100–110 ppm regions indicated a high content of chondroitin sulfated in position 4 and/or 6 of the GalNAc (Figure 2B) (Maccari et al., 2010). The signals at 102.85 and 104.34 were attributed to the C1 of GalNAc-6SO4 and to the C1 of the GlcA, respectively, and the signals at 99.11 and 103.93 were attributed to the C1 of GalNAc-4SO4 and to the C1of GlcA, respectively. These results were similar to those described for sturgeon CS (Maccari et al., 2010). In the same context, Mou et al. (2018) reported that the signals at 103.8 and 99.8 were attributed to the C1 of GlcA and to the C1 of GalNAc in the spectrum of

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fucosulated CS from Sea cucumber. Moreover, the purified CSSH showed the occurrence of two signals at 67.67 and 72.67 corresponding to C6 of GalNAc-6SO_4 and C6 of GalNAc-4SO_4. Coppa et al. (2012) reported that the signals detected at 70.5 and 64.1 in the spectrum of CS from Italian cheese Parmigiano-Reggiano which indicated that C6 of GalNAc residues were sulfated in position 6 and 4, respectively. Therefore, the ^{13}C NMR spectrum of CSSH confirmed that this polymer contains high content of chondroitin sulfated in both positions 4 and 6 of the GalNAc.

Fig 2. **(A)** FT-IR spectrometry of CS purified from smooth hound (CSSH) and standard chondroitin-6-sulfate. **(B)** ^{13}C NMR analysis of CS from smooth hound (CSSH). The signals for characteristic structural features of CSSH were noted.



3.4. SAX-HPLC analysis

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In order to obtain more accurate structure information about the purified CS from smooth hound cartilage, SAX-HPLC analysis was studied. CSSH was subjected to specific treatment with chondroitinase ABC.

As shown in Table 2 and Fig 3, chondroitinase ABC produced different percentages of unsaturated disaccharides from CSSH. Obviously, CSSH contained 2.92% of nonsulfated disaccharide ΔDi0S. CSSH showed abundant ΔDi6S (45.34%) and ΔDi4S (32.49%). In the same contexte, the monosulfated disaccharides ΔDi6S and ΔDi4S showed in shark cartilage CS were 40.8% and 34.9%, respectively (Im, Park, & Kim, 2010). The disulfated disaccharides were observed in CSSH in minor percentages than monosulfated disaccharides. Interestingly, The disulfated disaccharide ΔDi2,4 diS and ΔDi4,6 diS were presented in little percentage in CSSH (0.87% and 0.99%, respectively), while the disulfated disaccharide ΔDi2,6 diS were presented in high percentage (17.36%). This is confimed the purity of CSSH. Moreover, SAX-HPLC analysis of CSSH showed that this polymer hasn't trisulfated disaccharides. Maccari et al. (2010) and Gui et al. (2015) demonstrated that no disulfated and trisulfated disaccharides were shown in sturgeon bone CS and sturgeon skull CS, respectively. The charge density was influenced by the presnce of disulfated and trisulfated disaccharides in the polymers. In fact, the overall charge density of CSSH was calculated to be 1.16. This value was similar to that reported for CS from tuna and codofish cartilages (Maccari et al., 2015) and higher than that reported for CS from sturgeon backbone (Gui et al., 2015).

Fig.3. SAX-HPLC analysis of the unsaturated disaccharides produced by hydrolysis with chondroitin ABC lyase of chondroitin sulfate isolated from smooth hound cartilage. ΔDi0S (ΔUA-GalNAc), ΔDi6S (ΔUA-GalNAc6S), ΔDi4S (ΔUA-GalNAc4S), ΔDi2, 6diS (ΔUA2S-GalNAc6S) ΔDi4, 6diS (ΔUA GalNAc4, 6diS), ΔDi2,4diS (ΔUA2S-GalNAc4S).

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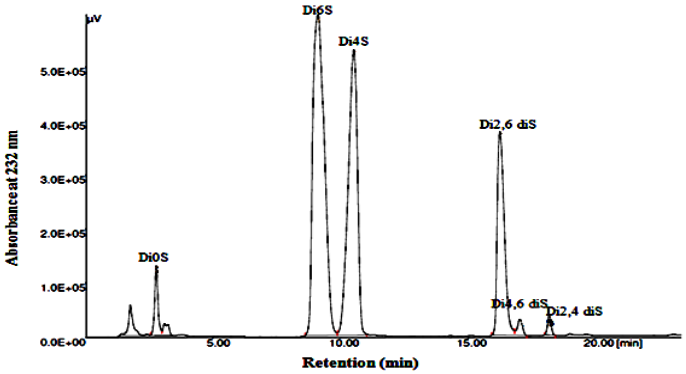
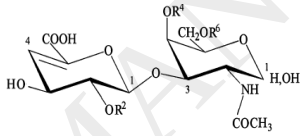


Table.2. Disaccharide composition, charge density and molecular weight values of CS isolated from smooth hound cartilage



	CSSH
ΔDi0S (ΔUA-GalNAc) (%)	2.926
ΔDi6S (ΔUA-GalNAc 6S) (%)	45.349
ΔDi4S (ΔUA-GalNAc 4S) (%)	32.498
ΔDi2, 6S (ΔUA2S-GalNAc 6S) (%)	17.363
ΔDi4, 6S (ΔUA 4S-GalNAc 6S) (%)	0.991
ΔDi2, 4S (ΔUA2S-GalNAc 4S) (%)	0.873
4S/6S	0.72
Charge density	1.16
Molecular weight (kDa)	68.78

The scheme showed the CS unsaturated disaccharides produced by hydrolysis of CS with chondroitinase lyases. Δ UA, 4,5-unsaturated uronic acid; S, sulfate group; GalNAc, N-acetyl-galactosamine. The percentage of each disaccharide was identified by purified standards and reported as weight percent. Charge density was calculated by considering the number of sulfated groups per disaccharide unit.

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Various investigations have reported that the degree and position of sulfation, inside the chondroitin sulfate chains, possess different proprieties and biological activities. Indeed, the analysis by SAX-HPLC proved that the 6-sulfated disaccharide content in CSSH is higher than sulfated disaccharide in position 4 producing a 4S/6S ratio of 0.72 quite similar to the 4S/6S ratio content previously reported by Im et al. (2010) for CS purified from shark cartilage.

3.5. Biological functions of CSSH

Depending to sulfate group placing, CS was classified in five types (CS-A, CS-C, CS-E, CS-D and CS-B) (Malavaki et al., 2008). Moreover, the number and position of sulfate on the disaccharides influenced on the diversity of biological functions of CS. Thus, the present work was undertaken to evaluate the anticoagulant and antiproliferative properties of CSSH.

3.5.1. Anticoagulant activity

The anticoagulant activity is commonly estimated by the classical coagulation tests including aPTT, PT and TT (Xiang, Wang, Qin, Xiang, Su, & Zhao, 2015). Indeed, aPTT and PT is indicated to the intrinsic coagulation pathways and extrinsic pathways, respectively, while TT is considered as an indicator of the amount and coagulation activity of fibrinogen in plasma in the common phase of coagulation process (Ye, Xu, & Li, 2012).

As shown in Fig. 4A, the anticoagulant activity of CSSH prolonged aPTT in a concentration dependent. In fact, the results indicated that the addition of 500 µg/mL of CSSH caused a significant ($p < 0.05$) prolongation of the coagulation time, which was about 2.14 times greater than that of the negative control. The significant effect of CSSH on aPTT test suggested an inhibition of the intrinsic pathways of blood clots. Therefore, CSSH could be a promising antithrombotic agent. However, Majdoub, Mansour, Chaubet, Roudesli, & Maaroufi (2009) demonstrated that polysaccharide's monosaccharide composition, number and position of sulfate group affecte the anticoagulant activities. Indeed, these results were higher than those reported

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for sturgeon backbone CS (prolonged the aPPT about 1.24 times greater than that of the control at the concentration of 1000 µg/ml) (Gui et al., 2015).

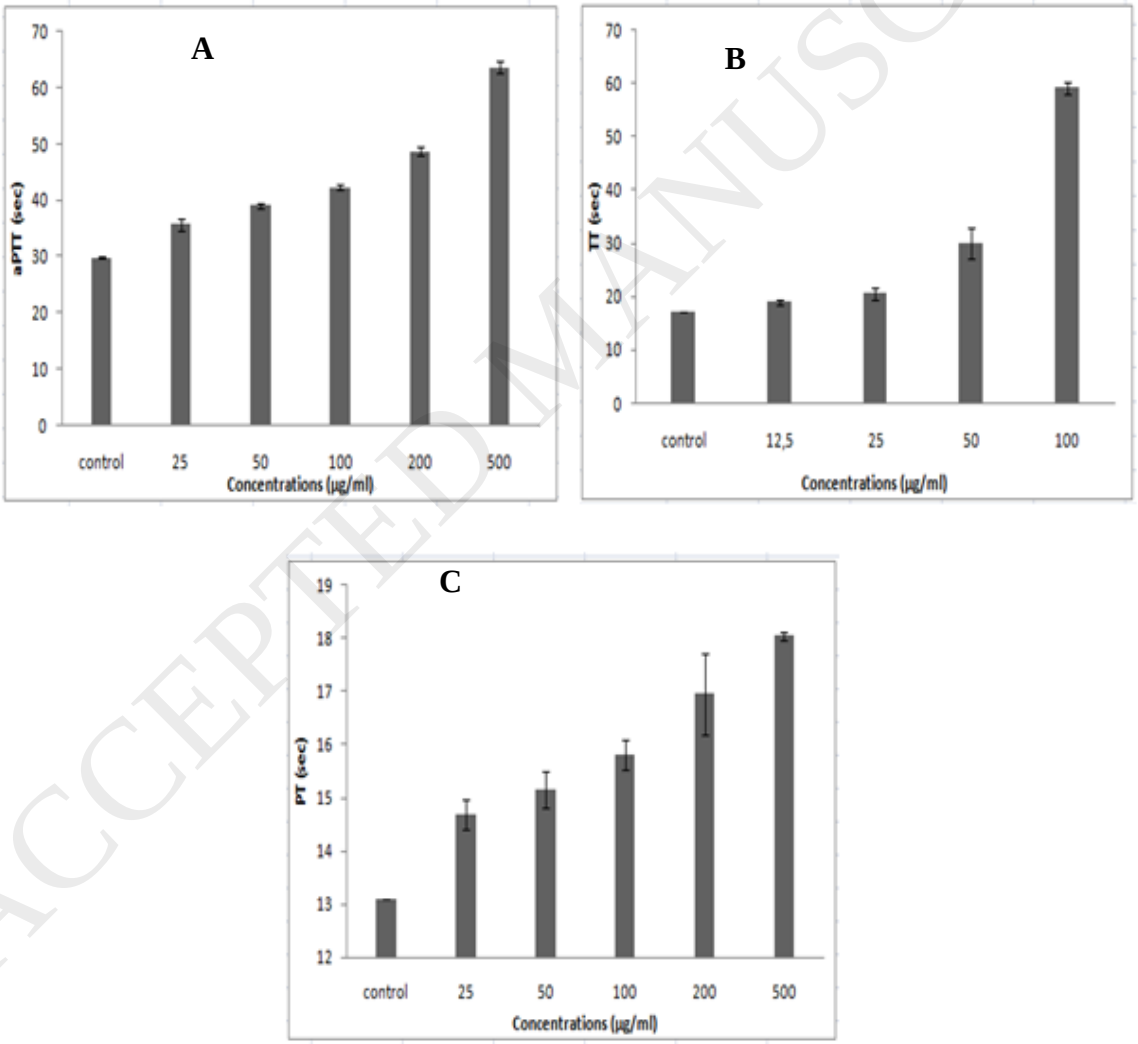
The results of thrombin times of CSSH at various concentrations are illustrated in Fig. 4B which demonstrated that TT was prolonged with increasing concentration of CS. Interestingly, CSSH was prolonged significatively TT about 3.44 (59.25 s ±1.06 s) times greater than that of the control at a concentration of 100 µg/ml. Thus, an increase in TT suggests the thrombin inhibition or fibrin polymerization as thrombin inhibition-dependent clotting time. Therefore CSSH could be a good inhibitor of both the intrinsic and/or common pathways of coagulation. Furthermore, CSSH prolonged TT more than that previously described for the CS from shark cartilage (1.3 fold at the concentration of 1000 µg/ml) (Gui et al., 2015). These results were also higher than those reported for glycosaminoglycans from Norway lobster shell (2.22 times greater than that of the control at the concentration of 100 µg/ml) (Sayari et al., 2016).

Anticoagulant activity of CSSH was also proven by the capacity to prolong the prothrombin time (PT). As shown in Fig. 4C the PT determined with the presence of CS at different concentrations, was effectively higher than that of the control clotting time (13.1 s). Furthermore, the values of PT increased with increasing concentrations of CSSH. Thus, PT was significatively prolonged to 18.1 s at the concentration of 500 µg/ml of CSSH. These results indicated that CSSH has a good anticoagulant activity on the extrinsic pathway of coagulation. Therefore, it suggests that CSSH may be employed as natural anticoagulant with no coagulation deficits both in the inhibition of the three classical pathway of coagulation. In this contexte, Gui et al. (2015) reported that CS isolated from sturgeon backbone, shark cartilage and sturgeon skull represent no significant differences in clotting time when using PT test. These results showed a relationship between anticoagulant activity and structure of glycosaminoglycans. Content and position of sulfation, the molecular weight, type of disaccharide composition and the three-

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dimensional structure of the sulfated poolymers influence its interactions with the clotting proteins (Olson, Bjork, & Bock, 2002).

Fig 4. Anticoagulant activity of the chondroitin sulfate isolated from smooth hound cartilage (CSSH) at various concentrations determined by the measurement of (**A**) the activated partial thromboplastin time (aPTT), (**B**) time of thrombin (TT) and (**C**) prothrombin time (TP).



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3.5.2. Anti-proliferative property of CSSH

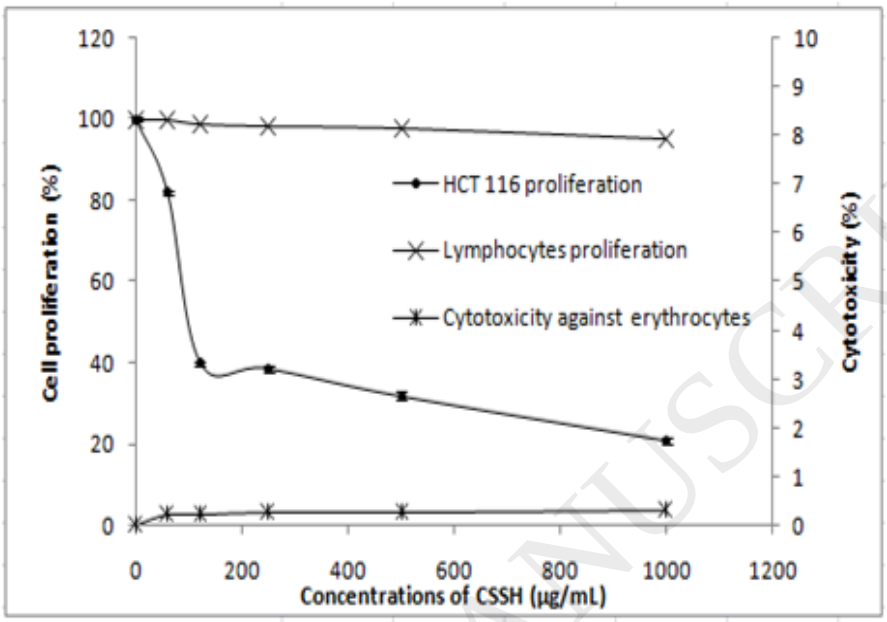
The major factor influencing the antiproliferative activity of chondroitin sulfate is the number and position of sulfate in the polymer. In fact, the action of purified chondroitin sulfate from smooth hound cartilage on cell viability was evaluated using a colorimetric MTT-based test. Fig.5 illustrated the viability of HCT116 cells treated with a various concentrations of CSSH. However, the results indicated that the addition of 1000 µg/mL of CSSH caused significant antiproliferative effect, which was 79% of cell proliferation inhibition. The CI_{50} of CSSH (100 µg/ml) was higher than that of CS/DS from smooth hound skins (65 µg/ml) (Krichen et al., 2017). The evaluation of cytotoxicity against the normal lymphocytes showed that CSSH hasn't toxicity against normal cells in all concentrations. To conclude, this study demonstrated that CSSH, which own a higher sulfate amount (21.48%), has a good antiproliferative effect against HCT116.

3.5.3. Hemolytic property of CSSH

Despite its beneficial effects of heparins for coagulation diseases, their use is limited because its problems on humans healths such as thrombocytopenia and thrombosis syndrome, hemorrhagic complications (Warkentin, 2006) and animal diseases like Mad Cow disease. Thus, it is of great importance to investigate the effect of the CSSH on haemostasis. The action of CSSH was evaluated on bovine erythrocytes. Several concentrations for samples were tested (60 - 1000 µg/mL). As demonstrated in Fig 5, the hemolytic activity of CSSH showed no toxicity against erythrocytes in all concentrations. The results indicated that CSSH has a good antiproliferative effect with no toxicity against lymphocytes cells and erythrocytes. Therefore, it suggests that CSSH may be employed as natural antiproliferative compound with several safety health effects.

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Fig.5. The effect of chondroitin sulfate from smooth hound cartilage (CSSH) on HCT116 human colon cell line, lymphocytes and erythrocytes.



4. Conclusion

In this paper, CS was isolated for the first time from smooth hound cartilage (CSSH) and demonstrated with respect their chemical, structural disaccharide composition and biological activities. Moreover, the purified CSSH has an effective *in vitro* anticoagulant activity, which prolonged blood coagulation time on three classical coagulation assays including aPTT, PT and TT. Furthermore, CSSH exhibited strong antiproliferative activity. The results of the biological activities of CSSH closely related to the disaccharide composition, the molecular weight and especially the number and position of sulfate in the polymer. The comprehensive antithrombotic and antiproliferative effects of CS fom smooth hound cartilage suggested that it may be useful employed as a functional drug with potential anticoagulant and antiproliferative effects.

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