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Antihypertensive and anticoagulant properties of glycosaminoglycans extracted from the sturgeon (*Acipenser persicus*) cartilage

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ABSTRACT

Large amounts of valuable waste are produced during sea food processing. This has a great potential for conversion to biologically active proteins and polysaccharides. Among these compounds, sulfated polysaccharides have been considered due to their many biological properties.

The present work was conducted to study anticoagulant activities and angiotensin-I converting enzyme (ACE) inhibitory effects of glycosaminoglycans (GAGs) extracted from the cartilage of sturgeon (*Acipenser persicus*). The enzymatic extraction of sturgeon cartilage was performed in the presence of cetylpyridinium chloride salt. The structure was characterized via electron microscope and Fourier transform infrared spectroscopy (FTIR) analysis. Herein, ACE inhibitory and anticoagulant properties of extracted GAGs were determined.

The amount of GAGs was 6.8±1.3% of cartilage dry weight. GAGs showed good activity in ACE inhibitory – with a highest level of 85.7%. The derived anticoagulant activity indexes, APPT (activated partial thromboplastin time) and TT (Thrombin time) of the extracted polysaccharide showed a prolonging of clotting time, compare to control.

The results of this study revealed that the cartilage extracted GAGs possess promising ACE inhibitory properties and anticoagulant effects. Thus, the product can be substituted for blood reducing drugs and antithrombotic agents at least in laboratory conditions.

INTRODUCTION

A huge amount of fisheries processing are discarded without any attempt to use them [1,2]. Indeed, according to the Food and agricultural Organization (FAO), annual production of aquaculture report this to be more than 106 million tons [3]. Many sea food processing by-products are not disposed directly to aquatic ecosystem due to the environmental problems that they cause. Despite this, the cost of their remediation is too high. Therefore, finding the proper approach as an alternative to disposing of such materials is significant [4,5].One of the suggested suitable approaches for managing such bio-waste is their conversion to an economically valuable products such as biologically-active compounds like polysaccharides [2,6]. Currently, in Iran, by using enzymatic hydrolysis, some discarded biomaterial has been converted to economical and even valuable products with therapeutic properties [1]. Recently, fisheries byproducts particularly cartilage and skin have attracted attention due to the presence of bioactive compounds [7,8]. Glycosaminoglycans (GAGs) are linear, highly negatively charged polysaccharides composed of a variable number of repeating disaccharide units [9]. These compounds play an important physiological role in cell growth, differentiation, morphogenesis, cell migration, as well as host defence and viral infection inflammation, angiogenesis, antihypertensive and blood coagulation [10]. Hypertension or high blood pressure is an important factor for cardiovascular diseases, such as heart failure, atherosclerosis, stroke, as well as coronary heart and kidney diseases. It is known as one of the leading causes of death in developed countries such as Iran [2].

Angiotensin converting enzyme (ACE) plays an important role in regulating and increasing blood pressure. This enzyme acts as catalyzing agent of angiotensin I to Active

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angiotensin II, which is a powerful vasoconstrictor, as well as to inactivate bradykinin, which is a potent vascular dilator [11,12]. Considering that the side effects of most common ACE inhibitors (such as captopril, enalapril, and lisinopril), searching for an alternative for inhibition of ACE that is of marine origins is considered [2].

One of the causes of death in developing countries is the diseases related to improper function of the circulatory system. Herein, the prevention of platelet aggregation and circulating pathway inhibition is achieved by synthetic drugs with anti-thrombotic, anticoagulant, and antiplatelet properties [13,14].

In this area, anticoagulant compounds obtained from marine sources particularly marine seaweeds extensively have been studied [15,16], and research has been devoted to ascertaining how to economically extract and purify biomacromolecules with ACE inhibitory effects from sea food processing by-products [17,18]. The sturgeon species is fished commercially in the Caspian Sea, Iran. Considerable wastes (cartilage and skin) are produced during its processing which are valuable for isolation of bioactive macromolecules such as proteins and sulphated polysaccharides [19]. During the last decade, studies have been undertaken on isolating GAGs from such fish processing by-products. Extracted sulfated polysaccharides from fish cartilage has exhibited a wide variety of biological activities, and multiple clinical benefits have reported from shark and sturgeon cartilage uptake. It is widely used to treat various cancers and has an impressive role in the treatment of osteoarthritis, rheumatoid arthritis, progressive systemic sclerosis (scleroderma) and glaucoma [2,8]. However, only a few pioneering studies have been done regarding the anticoagulation properties of fisheries-waste derived GAGS.

Therefore, the present work was carried out to investigate angiotensin inhibitory effects and anticoagulation properties of sulphated polysaccharides extracted from the cartilage of Persian sturgeon (*Acipenser persicus*).

MATERIALS AND METHODS

Reagents

All solvents and chemical were purchased at highest level of purity from commercial sources. Alcalase®2.4 L serine-protease from Bacilluslicheniformis was obtained from Novozymes® (Bagsvaerd, Denmark). Chemicals required for the isolation and assays including cysteine, cetylpyridinium chloride monohydrate, trichloroacetic acid (TCA), Chondroitin 4-sulphate, angiotensin I-converting enzyme from rabbit lung and the ACE synthetic substrate hippuryl-l-histidyl-L-leucine (HHL) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Platelet poor plasma (PPP) was prepared from whole blood, drawn on 0.13 M sodium citrate (9:1, v/v), obtained by venipuncture of healthy volunteers. Other chemicals and reagents used were of analytical grade.

Preparation of sturgeon cartilage

The cartilages were obtained from the local fish market, and were transported to the biology research lab in the Islamic Azad University branch of Lahijan. They were then cleaned and washed several times with tap water and Phosphate-buffered saline (PBS), respectively. Following this, they were chopped, lyophilized and grounded in the blender to obtain the dried powder [20].

Sample preparation and extraction

Sulfated polysaccharide was extracted from the cartilage according to the method of Krichen *et al*., 2016 [2,20]. A 5 g of sample was dissolved in 250 mL sodium acetate buffer (0.1 M, pH= 6) containing EDTA (5 mM) and cysteine (5 mM). Alacalase (hydrolyzing enzyme) was added, and the mixture was kept for 24 h at 50°C. After cooling at room temperature, the solution was filtered. The filtrate was washed again with distilled water and exposed to cetyl pyridinium chloride (CPC). Subsequently, 10 ml CPC (3% in 0.8 M sodium chloride) was added to the collected filtrate. Finally, the mixture was incubated at 37°C for 24 h at room temperature and centrifuged for 30 min at 5000 g and 4°C to generate a white precipitate.

To remove pyridinium salt from this precipitate, 2 mM sodium chloride (pre-heated to 40°C) was used. After this, ethanol (95%) was added to precipitate the sulfated polysaccharides. The precipitate was washed twice by deionized water and lyophilized in a freeze dryer (BT4KZL-105, USA). The dry matter was referred to as sulfated polysaccharide.

Glycosaminoglycans (GAGs) content assay

The isolated GAGs were subjected to a quantitative dye-binding (1, 9-dimethylmethylene blue) method to measure the in-sample GAGs in the presence of Chondroitin 4-sulfate as standard [21]. A standard curve using different concentration of chondroitin 4- sulfate (1.0, 2.0, 3.0, 4.0 and 5.0 µg) was first constructed. The absorbance was measured at 525 nm by using a microplate reader (ELx800, Biotek instruments, Inc., USA).

FT-IR analysis

Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy of GAGs from fish scale was performed using FT-IR spectrophotometer (FTIR, 8400S spectrophotometer, Japan). Herein, 10 mg of the samples (extracted GAGs and heparin) were mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum. The spectra were taken between wave numbers in 4000 and 450 cm^{-1}, and automatic signals were collected in 32 scans at a resolution of 4 cm^{-1}. The transmission spectrum of the sample and standard were recorded. All assays were performed at room temperature in a dry atmosphere.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) using a Hitachi S-2500C microscope was applied to study the surface morphology of the sulfated polysaccharide extracted from sturgeon cartilage. The sample was first coated with gold layer and observed while applying a tension of 15 kV. The dimensions of the extracted polysaccharide was measured by way of digital image analysis software.

ACE inhibitory activity

The inhibitory activity of ACE was determined in triplicate according to the method of Nakamura *et al*., 1996 [22]. A sample solution (50µl) containing different concentrations (0.2-1 mg/mL) of extract was mixed with 50 µl of ACE solution (25 units/mL) and incubated for 5 minutes at 37°C. After this, 150 µl of HHL (hippuryl-l-histidyl-L-leucine) 3.8 mM in 50 mM sodium borate buffer was added to solution and incubated for 60 minutes at the same temperature. The reaction was stopped by adding 250 µL of chloride acid (1 M). The liberated hippuric acid (HA) was extracted with ethyl acetate (0.5 mL). After centrifugation at 3000 rpm for 15 minutes, 0.2 ml of the upper layer was transferred to the test tube and dried at 80°C for 1 hour. The residue was then dissolved in 1 mL of distilled water, and the absorbance of the extract was measured at 228 nm by ultraviolet spectrophotometer (UV/Visible spectrophotometer, Jenway, UK). The average amount of three assays at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE inhibition (\%)} = \frac{\text{B-A}}{\text{B-C}} \times 100$$

where:
A - refers to the absorbance of hippuric acid (HA) produced in the presence of ACE inhibitor
B - refers to the absorbance of HA produced in the without ACE inhibitor, in this case borate buffer (100 mM, pH = 8.3) was used instead of extracted polysaccharide
C - refers to the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay)

The concentration of sulfated polysaccharide (mg/mL) capable of inhibiting 50% of ACE was defined as the IC50 (Inhibition Concentration) value. This value was calculated for each sample using non-linear regression from a plot of percentage ACE inhibition versus sample concentrations.

Clotting assays. Anticoagulation assay performed in a laboratory with citrated human platelet poor plasma (ppp). Human plasmas was collected from healthy individual donors (11 different donors) and mixed with 3.2% sodium citrate solution (9:1 v/v blood to sodium citrate). The plasma was obtained by centrifugation at 5400 g for 30 min at 4°C. Afterwards, the plasma was maintained at -60°C until use [23].

The anticoagulant activity of the extracted sulfated polysaccharide was determined by classical coagulation assays: activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). The sample was dissolved in physiological serum. All analyses were performed in triplicate, and mean values were taken.

Activated partial thromboplastin time (APTT) assay

The 10 µl extracted GAGs of different concentrations (10, 50, and 100 µg/ml) was mixed with 90 µl of human pooled plasma, and incubated at 37°C for 3 min according to the Tingbø method. Thereafter, 100 µl of APTT reagent was added to the samples and incubated at 37° C for 10 min. The clotting time was then measured after adding 100 µl $CaCl_2$ (0.05), using a coagulometer (SY-B032, SUNNYMED) [17]. Heparin (1 IU/mL) in different concentrations and NaCl (50 µg/ml) were used as the positive and negative control, respectively.

Prothrombin time (PT) assay

To determine the PT indicator, the human pooled poor plasma (90 µl) was mixed with 10 µl of the Heparin (different concentrations), NaCl (50 µg/ml) and different amounts of extracted polysaccharide (50, 100, and 200 µg/ml). It was then incubated at 37°C for 10 min. After this, 200 µl of pre-incubated (37°C) PT reagent (thromboplastin reagent) was added and the the clotting time was again recorded using a coagulometer.

Thrombin time (TT) assay. In the TT assay, 90 µl human pooled poor plasma (PPP) were mixed with 10 µl of different concentrations of extracted polysaccharide (10, 50 and 100 µg/ml) and incubated at 37°C for 2 min. Following this, 100 µl of thrombin was subsequently added and the TT was recorded.

Statistical analysis

The Kolmogorov-Smirnov test was applied for data normalization. Because of the normal distribution of data, one way analysis of variance (ANOVA) was applied to compare the mean values of anticoagulant properties. All data were expressed as mean ± standard deviation (SD) with n = 3, and statistical analyses were performed with SPSS 19 software package, and the Duncan's Multiple-Range Test. The significance of differences was defined at $p < 0.05$.

RESULTS

The GAGs content in isolated samples from sturgeon cartilage was measured by dye binding assay. A calibration curve was constructed between the different concentration values of Chondroitin 4-sulfate and the respective absorbance values (0.017, 0.020, 0.031, 0.042, 0.051 and 0.057) at 556 nm. This revealed the linear relationship (Fig. 1). The yield of GAGs calculated was 6.8±1.3%.

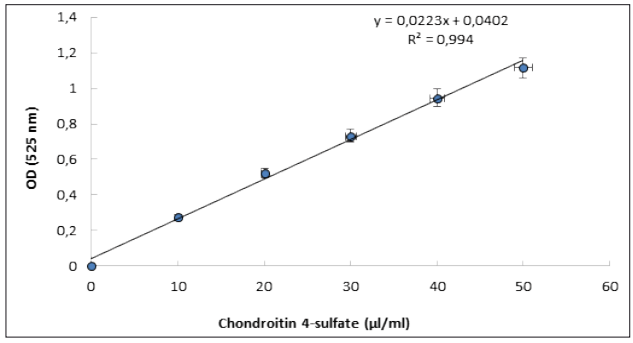


Figure 1. Calibration curve of different concentrations of Chondroitin 4-sulfate. Values expressed as means ± SD (*n* = 3)

FTIR spectroscopy was applied to confirm the composition and the structural integrity of the extracted GAGs (Fig. 2a). The FTIR analysis of the GAGs from fish scales revealed a pattern similar to the heparin (Fig. 2b), and proved a strong OH band at 3200-3500 cm^{-1} and a strong OH band at 1620-1660 cm^{-1}, indicating the groups available in glycosaminoglycan well. More absorbance at 1239 cm^{-1} and at 821 cm^{-1} was assigned to the stretching of S=O (sulfate group).

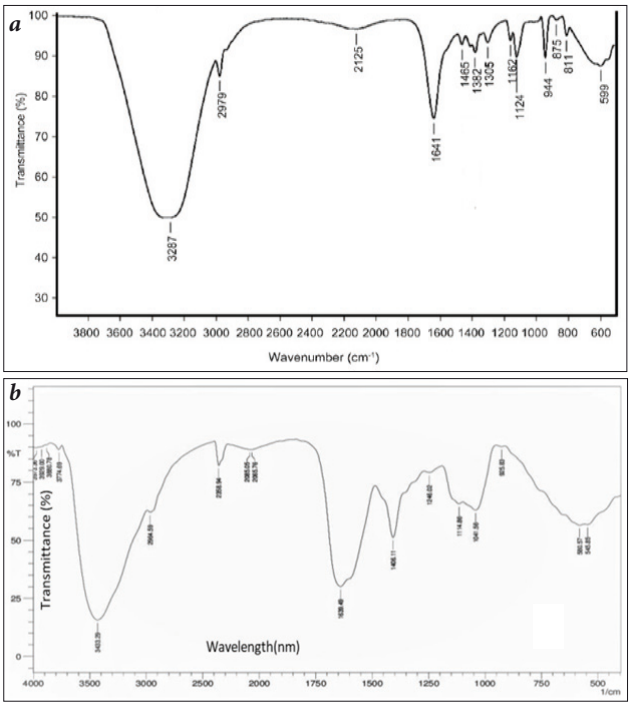


Figure 2. a) FT-IR spectrum in a KBr pellet of the sulfated glycosaminoglycan from fish (*R. frisi kutum*) scale in the wave number range from 4000 to 450 cm^{-1}. b) FTIR spectrum of Standard heparin (sigma CO. USA)

Scanning electron microscopy (SEM) is physical method which is used to characterized surface morphology of sulfated polysaccharide from sturgeon cartilage. Scanning electron microscopy images of the extracted sulfated polysaccharide are seen in Figure 3. The SEC images show the spherical shape and complex structure.

The ACE inhibitory effect of sulfated polysaccharide in different concentrations displayed a dose dependent trend (Fig. 4). Herein, the lowest value was measured at 0.2 mg/mL concentration (61.3%) and the highest ACE inhibitory activity was 85.7% at highest concentration (1 mg/mL) of the extracted polysaccharide. Inhibition of this extract or IC50 value was 0.17±0.05 mg/ml, which showed a significant difference to captopril (IC50 = 0.003±0.002 µg/ml) as standard ($p<0.05$).

The anticoagulant activities of extracted sulphated polysaccharide was measured by three coagulation indicators, APPT, PT and TT, using heparin sulphate and normal saline as standard and negative control, respectively. Sturgeon sulfated polysaccharide induced significant anticoagulant activities, as indicated by the APTT and TT indexes, at concentrations between 10-100 µg/mL. In addition, the anticoagulant effects were dose-dependent. The values for both indexes were, however, lower than heparin sulphate (Table 1). The clotting times were also significantly prolonged – about 2.07 and 1.3 folds greater than control in terms of APTT and TT, respectively ($p<0.05$). Of note, the prothrombin time (PT assay) is normally applied to detect bleeding disorders. This index was not significantly affected by the extracted sulphated polysaccharide ($p>0.05$).

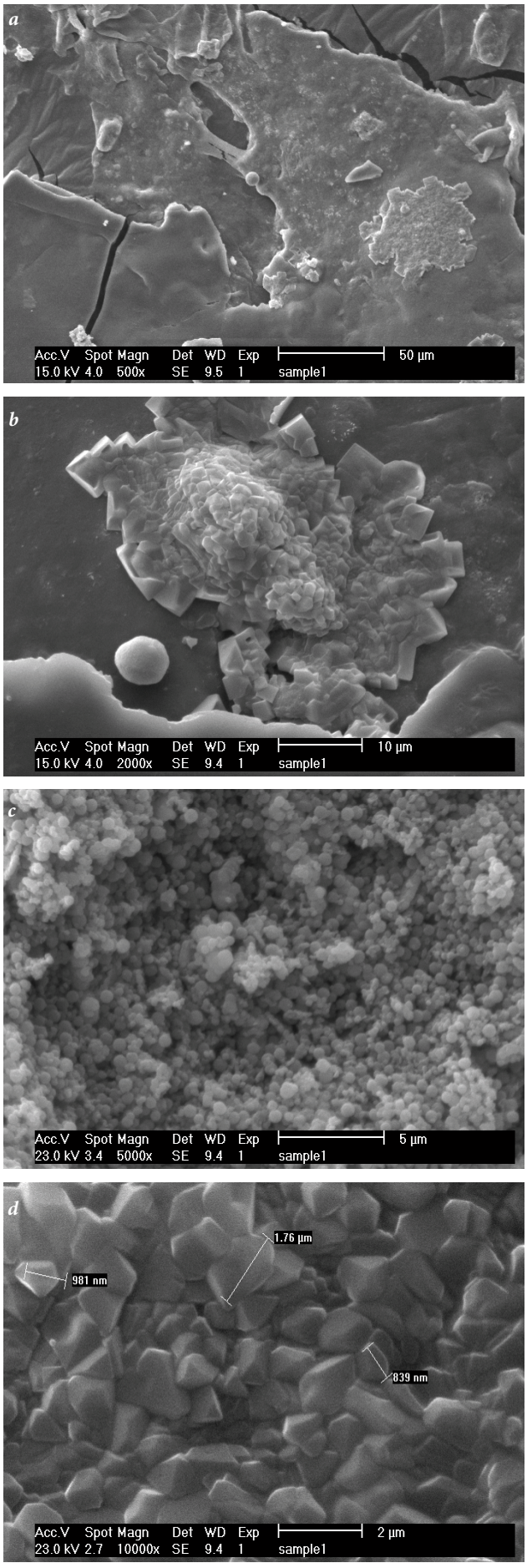
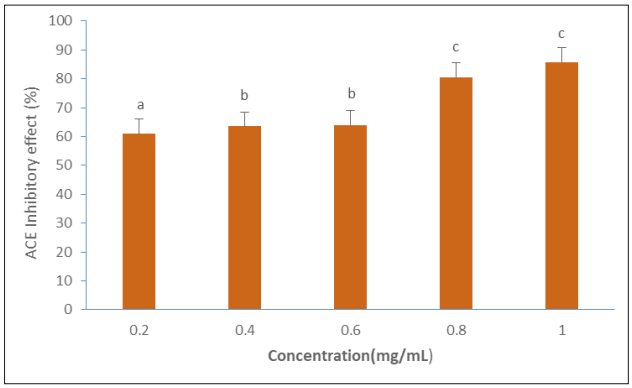


Figure 3. Scanning electron micrographs of the sulfated glycosaminoglycan; a) ×500; b) ×2000; c) ×5000 and d) ×10000

Table 1. Anticoagulant activities of extracted sulfated glycosaminoglycan from Sturgeon cartilage

Sample	APTT(s) at different concentrations (µ/mL)			PT(s) at different concentrations (µ/mL)			TT(s) at different concentrations (µ/mL)	
	10	50	100	10	50	100	10	50
Negative Control	27.23±0.6[a]	-	-	9.51±0.4[a]	-	-	15.51±0.2	-
Extracted sulphated polysaccharide	29.41±0.2[a]	43.25±0.4[b]	56.41±0.2[c]	9.48±0.1[a]	9.65±0.2[a]	9.84±0.2[a]	16.5±0.8[a]	17.9±0.6[b]
Heparin sulphate	34.5±1.6[a]	57.5±3.4[b]	86.3±0.3[c]	11.2±0.3[a]	11.7±0.9[a]	10.7±1.0[a]	18.1±0.3[a]	20.17±1.2[b]

Each value is the mean ± SD of triplicate measurements
(-) Not detected
Different letters indicate significant differences ($p<0.05$)



Values are means of three replications ± SD

Figure 4. Angiotensin I-converting enzyme inhibitory activities of extracted sulfated glycosaminoglycan at different concentrations. Values are means of three replications ±SD

DISCUSSION

Due to the global demand for new bioactive compounds, sea food processing by-products have attracted attention in the search for natural substances with therapeutic benefits [1,24]. Herein, production and characterization of their bioactive products (antimicrobial, antioxidants, anti-tumor, anticoagulation and antihypertensive properties) has been the subject of world-wide research.

The amount of GAGs derived from Sturgeon fishery by-products in this present work is estimated as 6.84±1.13%. In a previous studies, the amount of sulfated glycosaminoglycan from *Salmo salar*, *Somniosus microcephalus*, *Galeus melastomus*, *Deania calcea*, *Amblyraja hyper borean* and *Acipenser sinensis* were about 10.31, 13.96, 7.53, 6.66, and 15.51 g of dry weight, respectively [19,25-27]. In other similar work, the sulfated polysaccharides extraction yields from grey triggerfish and smooth hound skins were reported to be 8.62 and 9.86%, respectively [2,10].

The spectra of the extracted GAGs exhibit peaks at the range of 1500 cm^{-1} to 1750 cm^{-1}. This bears relation to that of C = O stretching groups. The presence of peaks in the range of 979.77 cm^{-1} and 1242.07 cm^{-1} bears a relationship to that of the stretching groups of C-O and bending groups of C-OH that are standard in the heparin sample. In contrast, the spectrum of isolated GAGs show a major peak at 1650.9 cm^{-1}. This is that of the glycosaminoglycan group. In the FTIR spectrum, the strong absorbance band at 1646 cm^{-1} (C=O stretching) and two medium size bands at 1378 and 1412 cm^{-1} (O-C=O bending) distinctly evidenced uronic acids [25,28].These FTIR results show a good agreement with previous studies on extracted GAGs from fish by-products [19,29,30].

The scanning electron microscopy of sulfated glycosaminoglycan derived from sturgeon cartilage show a spherical shape with irregular particles. Similar structures were reported in sulfated polysaccharides from Tunisian fish skins, grey triggerfish (*Balistes capriscus*), smooth hound (*Mustelus mustelus*) and also Brazilian mushrooms (*Agaricus blazei*) [2,31,32]. In contrast, an amorphous solid was observed for the structure of a sulfated derivative of polysaccharide from *Agaricus brasiliensis* (*Brazilian mushroom*) [32]. This difference can be attributed to the species studied, and to variations in the isolation and analysis method [2,31]. Various studies have also shown that the protein hydrolysate from fish and shellfish can affect the level of plasma cholesterol and can reduce its level in animal models [14,33].

The enzymatic extraction of polysaccharide from sturgeon cartilage produced sulfated polysaccharides with a strong ACE inhibitory effect [34]. The IC50 value for the derived sulfated polysaccharide in the present study revealed good agreement with IC50 values recorded for polysaccharides from squid.

In similar studies, the value of IC50 for the sulfated polysaccharide isolated from the skin of two Tunisian fishes: grey triggerfish (*Balistes capriscus*) and smooth hound (*Mustelus mustelus*) (SHSP), were reported as 0.16 and 0.18 mg/mL, respectively [2].

In the widespread use of synthetic ACE inhibitory drugs in the treatment of hypertension, some side effects have been recorded. These include hypotension, reduced renal function, angioedema, skin rash, increased potassium level and cough. Therefore, ACE inhibitory sulfated polysaccharides can be good alternatives to standard synthetic agents [33,34].

In order to determine the amount of inhibition of the coagulation cascade, anticoagulant tests (APTT, PT, TT) were used. The observed prolonged times of APTT and TT with the increasing levels of GAGs indicate within the coagulation pathway, the existence of an effective intrinsic inhibitory mechanism, as well as the presence of thrombin activity with fibrin polymerization [25,35]. However, the different concentrations of extracted GAGs did not have any significant effect in the PT indicator trials. Thus, GAGs isolated from cartilage cannot modulate the extrinsic inhibitory mechanism.

The same results have been obtained by Krylov *et al*., 2011 for extracted GAG compounds such as chondroitin sulphate from sturgeons and shark cartilages [25]. They also observed a dose-dependent concentration in APTT and TT values in the presence of the extracted GAGs. The noted prolongation time of clotting reveals that GAG compounds can modulate intrinsic coagulation factors and thrombin activity [36,37]. However, the anticoagulant property of GAG compounds were impacted by several factors. Among these are species, structural composition and the glucose amine sulphation pattern (6,38). It must be underlined, therefore, that, while increased levels of mono-sulphated disaccharide lead to stronger anticoagulant activity [37], blood

clotting is a complex mechanism. Thus, further studies need be undertaken to investigate the anticoagulant mechanism of isolated GAGs.

CONCLUSION

The GAGs extracted from sturgeon cartilage showed potent ACE inhibitory activity and anticoagulant properties. The results are comparable with commercial anticoagulants like heparin. Thus, sturgeon fishery by-products can be a beneficial source of items of pharmaceutical and nutraceutical interest.

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