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ORIGINAL ARTICLE

Structural analysis of glycosaminoglycans from *Colla corii asini* by liquid chromatography-electrospray ion trap mass spectrometry

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Abstract

Colla corii asini (*CCA*) made from donkey-hide has been widely used as a traditional animal-based Chinese medicine. Chondroitin sulfate (CS), dermatan sulfate (DS) and hyaluronic acid (HA) are structurally complex classes of glycosaminoglycans (GAGs) that have been implicated in a wide range of biological activities. However, their possible structural characteristics in CCA are not clear. In this study, GAG fractions containing CS/DS and HA were isolated from *CCA* and their disaccharide compositions were analyzed by high sensitivity liquid chromatography-ion trap/time-of-flight mass spectrometry (LC-MS-ITTOF). The result showed that CS/DS/HA disaccharides were detected in the three lower salt fractions from anion-exchange chromatography. The sulfation patterns and densities of CS/DS chains in these fractions differed greatly, while HA chains varied in their chain lengths. The quantitative analysis first revealed that the amount of GAGs in *CCA* varied significantly in total and in each fraction. This novel structural information could help clarify the possible involvement of these polysaccharides in the biological activities of *CCA*.

Keywords *Colla corii asini* · Chondroitin sulfate · Dermatan sulfate · Hyaluronic acid · LC-MS-ITTOF

Abbreviations

AMAC	2-aminoacridone
CCA	*Colla corii asini*
CS	chondroitin sulfate
DS	dermatan sulfate
EIC	extracted ion chromatogram
GAG	glycosaminoglycan
GalNAc	*N*-acetylgalatosamine
GlcA	glucuronate
GlcNAc	*N*-acetylglucosmine
HA	hyaluronic acid
HP	heparin
HS	heparan sulfate
IdoA	iduronate
LC-MS-ITTOF	liquid chromatography-ion trap/time-of-flight mass spectrometry
NaCl	sodium chloride
ΔUA	4,5-unsaturated uronate (generated by chondroitinase excision)
SE-HPLC	size-exclusion HPLC

Haiyue Huang and Su Liu contributed equally to this work.

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Introduction

The major GAGs in animals are heparin sulfate (HS)/heparin (HP), CS/ DS and HA which have important biological activities in embryonic development, morphogenesis, immune response, inflammation mediation, pathogenic infection, tumor progression and invasion, angiogenesis and tissue regeneration [1–4]. These activities of GAGs are related to their structural diversity and ability to interact with a wide variety of

proteins, i.e. enzymes, cytokines, growth factors, and extracellular matrix proteins [5–9].

CS/DS are complex linear polysaccharides which are initially constructed of repeating disaccharide units of glucuronate (GlcA) linked to *N*-acetylgalactosamine (GalNAc). These disaccharides are then subjected to several post-polymeric modification (i.e. epimerization and sulfation) to yield a variety of disaccharide structures. Epimerization occurs at some GlcA and then transforms into iduronate (IdoA), while sulfation occurs at C2 of IdoA and C4 or C6 of GalNAc [10]. HA is the simplest GAG, comprised of repeating disaccharide units of GlcA linked to *N*-acetylglucosmine (GlcNAc) without any sulfo groups in its backbone [11].

CCA, also called E Jiao, is a crude preparation of donkey-hide gelatin that has been widely used as a traditional animal-based Chinese medicine for about 2000 years. *CCA* has been predominantly used for the treatment of gynaecological diseases [12] and some chronic diseases [13]. Also, *CCA* has demonstrated various useful activities in the inhibition of hematological diseases [14, 15], anticoagulation [16], antitumor action [17, 18], immunomodulation [19], anti-inflammation [20], bone repair [21], and anti-aging [22]. However, the relationships between these biological activities and the chemical constituents of *CCA* are still not clear.

Several classes of compounds have been found in *CCA* gelatin, including amino acids, proteins, GAGs, volatile substances, inorganic substances, etc. [13]. Research on the active components within *CCA* have been mainly focused on proteins, peptides and other lower molecular weight ingredients. GAGs are important components in donkey skin. Our recent paper first reported that *CCA* contains a heterogeneous mixture of HS/HP with a variety of like domain structures in their constituent chains [23]. Many medicinal activities have been attributed to *CCA*, including ones in which GAGs are known to be potentially involved. However, the presence and structure of other GAGs, such as DS/CS, HA in *CCA* still remains unclear. Therefore, our current study further explored the structure of CS/DS and HA in *CCA* by high sensitivity LC-MS-ITTOF.

Materials and methods

Materials

CCA was obtained from Shan Dong Dong-E E-Jiao Co., Ltd. (Shandong, China). Eight CS/DS disaccharide standards (generated by the action of chondroitinases) and HA disaccharide standard were obtained from Iduron (Manchester, UK). Pronase (*Streptomyces griseus*) was purchased from Sigma Aldrich (St. Louis, MO., USA). Chondroitinase ABC (*Proteus vulgaris*; EC 4.2.2.4) was purchased from ADHOC (Beijing, China). Superdex™ 75 10/300 GL and DEAE-Sephacel were from GE Healthcare Life Sciences (Uppsala, Sweden).

Methonal (HPLC grade) was provided by Merck KGaA (Darmstadt, Germany). 2-aminoacridone (AMAC, ≥98%), sodium cyanoborohydride (99%), dimethylsulfoxide (≥99.5%) and AR-grade reagents (ammonium hydrogen carbonate, sodium chloride (NaCl), acetic acid) were all purchased from Sigma Aldrich (St. Louis, MO., USA). Sodium hydroxide (≥98%) and AR-grade reagents (sodium acetate, sodium dihydrogen phosphate, disodium hydrogen phosphate) were all obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All HPLC solutions were prepared using MilliQ (Millipore, Watford, Herts., UK) ultra-pure water.

Extraction and partial purification of GAGs

GAGs in *CCA* was prepared by method described in our previous paper [23]. Briefly, *CCA* solution (1.25 g *CCA* in 10 mL of water) was digested by 0.5 mL of pronase (2.5 mg/mL) in 0.24 M sodium acetate and 1.9 M NaCl, pH 6.5 overnight at 37 °C. The GAG-containing fraction was precipitated by saturated sodium acetate/ethanol solution, then was collected by centrifugation and dried at 55 °C. The re-dissolved precipitate was applied to a 5 mL DEAE-Sephacel column pre-equilibrated with 0.1 M NaCl, 15 mM sodium acetate, pH 6.5 containing 0.01% *v*/v Triton X-100. After washing with the same solution, the CS/DS and HA containing GAG fractions were step-wise eluted with 60 mL of different concentrations of NaCl (0.25, 0.5, 1.0, 1.5 and 2.0 M NaCl) in 15 mM sodium acetate, pH 6.5 containing 0.01% (*v*/v) Triton X-100. The collected fractions were then dialyzed against Milli-Q water for 3 days at 4 °C followed by freeze-drying.

Preparation and recovery of GAG disaccharides

As CS/DS and HA chains can both be completely digested by chondroitinase ABC at pH 8.0 [24], GAG fractions from DEAE-Sephacel chromatography were exhaustively digested to CS/DS/HA disaccharides with 10 mIU of chondroitinase ABC in 50 μl of 20 mM, sodium phosphate buffer pH 8.0 containing 0.3% NaCl at 37 °C for 24 h as described by the instruction of enzyme. All digested samples were then separated by size-exclusion HPLC (SE-HPLC) on a Superdex™ 75 10/300 GL column eluted with 0.1 M ammonium hydrogen carbonate at a flow rate of 0.4 mL/min [23]. The disaccharide fractions were collected, incubated at 55 °C for 48 h to remove ammonium hydrogen carbonate, and then concentrated on a rotary vacuum concentrator.

AMAC labelling of CS/DS disaccharides

Dried CS/DS and HA disaccharide standards or purified GAG disaccharides from SE-HPLC (as above) were redissolved in 10 μL of 0.1 M AMAC in 85% dimethylsulfoxide / 15% acetic acid and incubated at room temperature for 20 min. At this point, 10 μL of 1 M sodium cyanoborohydride was added and the mixture was left for 4 h at 45 °C [25]. Finally, the reaction mixtures were diluted with 50% (*v*/v) aqueous dimethylsulfoxide for the following LC-MS analysis.

LC/MS-ITTOF analysis

The structural and compositional analysis of CS/DS and HA disaccharides were carried out on a LC/MS-ITTOF system (Shimadzu Corp., Kyoto, Japan) which was equipped with a binary gradient pump (LC-20 AD), autosampler (SIL-20 AC), degasser (DGU-20A3), photodiode array detector (SPD-M20A), communication base module (CBM-20A) and a column oven (CTO-20 AC).

Separations were performed on an ODS-2 HYPERSIL column (4.6 × 250 mm, 5 μm; Waters, Milford, MA, USA) at a flow rate of 0.3 ml/min at 45 °C. Eluent A was 40 mM ammonium acetate (pH 5.6) in water, whereas eluent B was methanol. A linear elution gradient was applied for isocratic 15% B in 5 min, then from 15% to 41% B in 45 min. The photodiode array detection was performed from 190 to 800 nm.

The mass spectrometer was equipped with an electrospray ionization source and was operated in the negative mode. Mass spectroscopic analyses were carried out on a full-scan mass spectrometer with a mass range of 200–1500 m/z. Liquid nitrogen was used as the nebulizing gas at a flow rate of 1.5 L/min. The curved desolvation line and heat block temperatures were both 200 °C. The interface voltages were set at −3.5 kV, and the detector voltage was 1.6 kV. The IT and TOF area vacuums were maintained at 1.8e-002 Pa and 1.6e-004 Pa, respectively.

Calculation

Quantitative analyses of CS/DS and HA disaccharides by fluorescence were performed using calibration curves as described by Volpi [26]. Briefly, increasing amounts of AMAC labelled CS/DS disaccharide standards (20, 30, 40, 70 and 120 ng) and HA disaccharide standard (240, 260, 280, 300 and 320 ng) were all analyzed by LC-MS, then the linearity of each disaccharide was assessed based on the amount of disaccharide and its peak intensity measured in the extract ion chromatogram (EIC).

Results and discussion

Partial purification of GAGs

Our most recent paper has developed an effective method for the isolation of the GAG fraction from the complex *CCA* mixture [23]. In order to investigate the structure and heterogeneity of the CS/DS and HA chains in *CCA*, the crude GAG fraction containing free GAG chains or/and GAG-peptide complexes was isolated by protease digestion and ethanol precipitation, then was further separated by stepwise salt-elution, using 0.2, 0.5, 1.0, 1.5 and 2.0 M NaCl steps, giving D1 to D5 fractions respectively. Large amounts of material were eluted in the low salt fractions (D1 and D2), while relatively small amounts of material were eluted in the 1.0 (D3), 1.5 (D4) and 2.0 M (D5) NaCl steps (See Fig. S1). These suggested that D1 and D2 might probably correspond to non-sulfated or low-sulfated GAGs, while D3 to D5 might be the relatively highly sulfated GAGs.

As CS/DS species can be sulfated to varying degrees, all the above five elution fractions (D1 to D5) were dialyzed and then freeze dried for subsequent enzymatic digestion, followed by disaccharide purification and analysis.

Purification of GAG disaccharides

SE-HPLC is a useful tool for purifying the GAG (likes HS/HP) disaccharides of *CCA* from other anionic contaminants for subsequent LC-MS-ITTOF structural analysis [23]. Therefore, the obtained D1 to D5 fractions were completely digested by chondroitinase ABC to disaccharides, which were then further purified by SE-HPLC on Superdex™ 75 column in 0.2 M ammonium hydrogen carbonate. As shown in Fig. 1D, the eight standard CS/DS disaccharides (all originally derived from chondroitinase-digested CS/DS and therefore contained the same characteristic uronate C4–5 unsaturation as our disaccharide samples) were eluted from the Superdex™ 75 column between 38 and 44 min. All the profiles for the five fractions (D1-D5, corresponding to Fig. 1A-1E, respectively) from DEAE-Sephacel chromatography contain a wide range of anionic contaminants, from low molecular weight ones to high molecular weight ones (mainly other GAGs, i.e. HS/HP [23], which are not degradable by chondroitinase ABC). The isolated disaccharide samples derived from D1 to D5 fractions were heated to remove ammonium hydrogen carbonate and then were AMAC-labelled by reductive amination for the following disaccharide analysis.

Disaccharide analysis of GAG fractions

By combination with fluorescent labelling, LC-MS has recently become widely used to analyze CS/DS/HA disaccharides derived from biological samples [26, 27]. In this study,

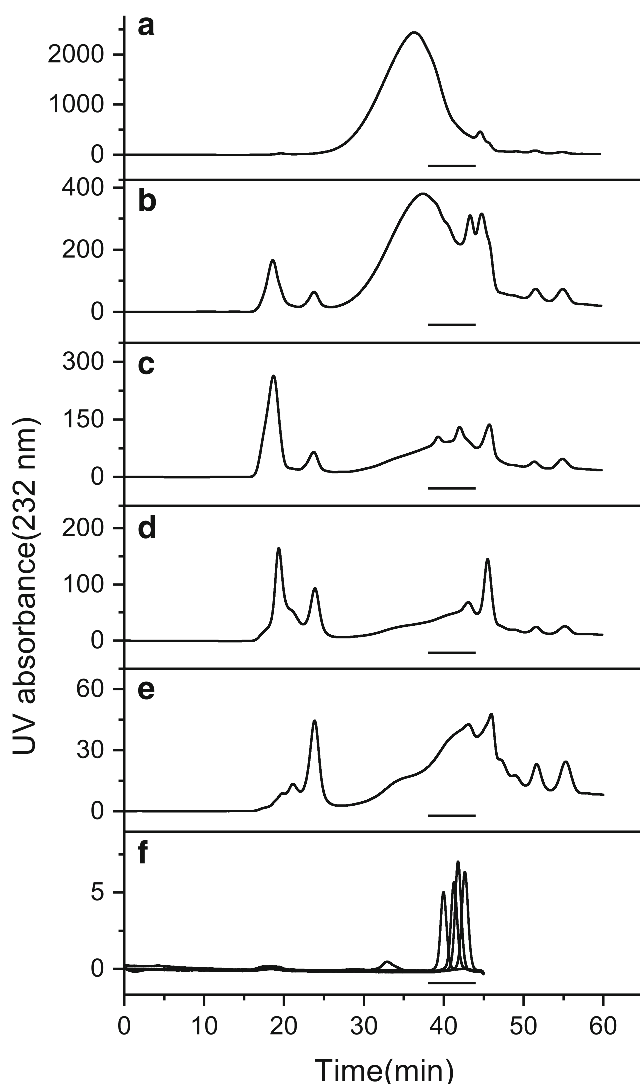

Fig. 1 Purification of CS/DS and HA disaccharides by SE-HPLC on a Superdex™ 75 column. Five fractions, D1 (A), D2 (B), D3 (C), D4 (D) and D5 (E) from DEAE-Sephacel chromatography (Fig. S1) were digested with chondrotinase ABC and the released disaccharides were then purified by SE-HPLC. Elution was monitored by UV absorbance at 232 nm. The eight standard CS/DS disaccharides were eluted between 38 and 44 min on this column (F), so this fraction range (indicated by horizontal bars), was collected from each of the D1-D5 digested samples (profiles A-E, respectively)

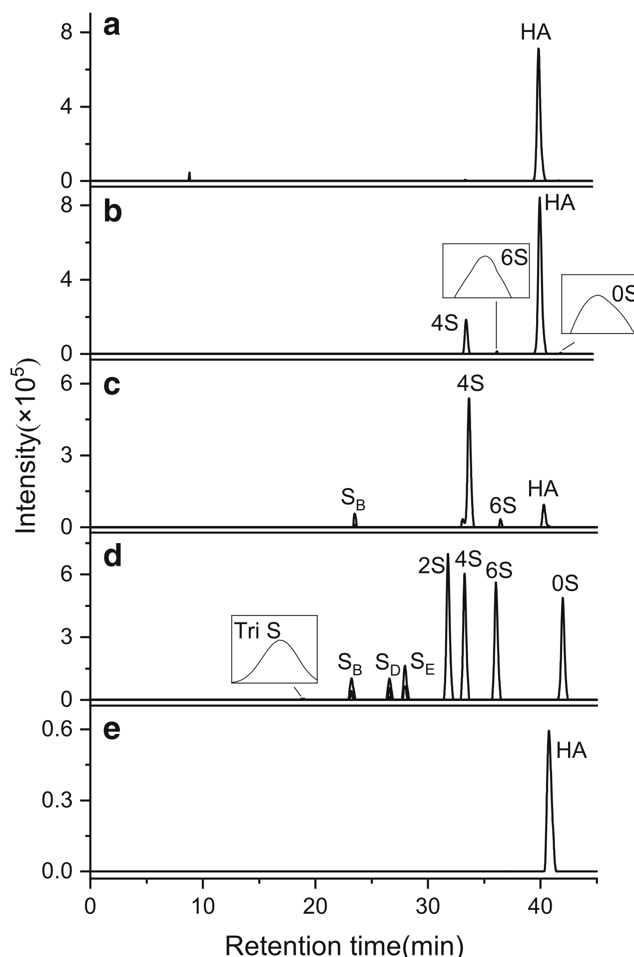

Fig. 2 Extracted ion chromatograms of AMAC labeled CS/DS and HA disaccharides analyzed by LC/MS-ITTOF. Profiles A-C correspond to the EIC profiles of disaccharides in the fractions D1-D3 (Fig. S1). Profile D is the EIC of the eight AMAC labeled standard CS/DS disaccharides, which are (TriS) ΔUA(2S)-GalNAc(4S,6S), (SB) ΔUA(2S)-GalNAc(4S), (SD) ΔUA(2S)-GalNAc(6S), (SE) ΔUA-GalNAc(4S,6S), (2S) ΔUA(2S)-GalNAc, (4S) ΔUA-GalNAc(4S), (6S) ΔUA-GalNAc(6S) and (0S) ΔUA-GalNAc. Profile E is the EIC of standard HA disaccharide

disaccharide compositional analysis of the AMAC-labelled disaccharide samples were performed by reverse-phase LC-MS using EIC detection (shown in Fig. 2A-2E**)**. Because chondroitinase ABC has activity to both CS/DS and HA chains [24], the eight standard CS/DS and HA disaccharides were AMAC labeled and then quantitatively analyzed by LC-MS (Fig.2D&E; chemical structures shown in Table 1). The calibration curves for all disaccharide standards showed a linear response with high correlation coefficients (provided in Table 2). The preparation of GAG and its disaccharides were performed in duplicate. The resulting average disaccharide compositions are shown in Table 3.

By comparison with the EIC profiles of CS/DS and HA standard disaccharides, we detected CS/DS and HA disaccharides in D1-D3 fractions (Fig.2A-2C) which were eluted from DEAE column at 0.25, 0.5 and 1.0 M NaCl, suggesting that CS/DS and HA were present in *CCA*. However, no CS/DS/HA disaccharides were found in D4 and D5 fractions which had been eluted from DEAE column at higher salt concentrations (i.e. 1.0 and 1.5 M NaCl), suggesting that the constituents in D4 and D5 fractions are mainly highly sulfated HS/HP [23].

CS/DS disaccharides were detected in the EIC profiles of D2 and D3 fractions. D2 fraction contained three CS/DS disaccharides, ΔUA-GalNAc(4S), ΔUA-GalNAc(6S) and ΔUA-GalNAc at the level of 94.3%, 4.1% and 1.6% (See Table 3). In D3 fraction, three disaccharides, ΔUA-GalNAc(4S), ΔUA-GalNAc(6S) and ΔUA-GalNAc(4S,6S)

Table 1 Structures and *m/z* values of chondroitinase-generated CS/DS and HA disaccharides

(CS/DS) (HA)

CS/DS/HA disaccharides	Structure	R_2	R_4	R_6	Theoretical mol mass	Theoretical AMAC-m/z	Observed ions AMAC-m/z
Tri S	ΔUA2S-GalNAc4S6S	SO_3H	SO_3H	SO_3H	618.9819	812.0585	812.0590
S_B	ΔUA2S-GalNAc4S	SO_3H	SO3H	H	539.0251	732.1017	732.1022
S_D	ΔUA2S-GalNAc6S	SO_3H	H	SO_3H	539.0251	732.1017	732.1022
S_E	ΔUA-GalNAc4S6S	H	SO_3H	SO_3H	539.0251	732.1017	732.1022
2S	ΔUA2S-GalNAc	SO_3H	H	H	459.0683	652.1448	652.1454
4S	ΔUA-GalNAc4S	H	SO_3H	H	459.0683	652.1448	652.1454
6S	ΔUA-GalNAc6S	H	H	SO_3H	459.0683	652.1448	652.1454
0S	ΔUA-GalNAc	H	H	H	379.1115	572.1880	572.1886
HA	ΔUA-GlcNAc	-	-	-	379.1115	572.1880	572.1886

were detected at the level of 65.9%, 2.6% and 31.5%. Table 3 also shows comparisons of the overall levels of 2-*O*-, 4-*O*-, 6-*O*-sulfation and total sulfation, as calculated from the disaccharide compositions. The level of 4-*O*-sulfation of CS/DS chains in both fractions were significantly higher than 6-*O*-sulfation. 2-*O*-sulfation of CS/DS does not exist in D2 fraction, however it occurred in D3 fraction at level of 31.5%. Consequently, the overall sulfation density of CS/DS chains in D2 fraction is 33.1% lower than the ones in D3 fraction. These results showed that the sulfation pattern and density of CS/DS chains in both fractions are clearly significantly different, suggesting that CS/DS in *CCA* is structurally heterogeneous.

In contrast, HA disaccharide was found in D1 to D3 fractions, indicating that the length of HA in three fractions may be different, probably having higher molecular weight of HA in D3 fraction, which could be due to the natural polydispersity of HA or/and the degradation of HA during the production of *CCA*. *CCA* was prepared from *Equues asinus* L. donkey-hide by decoction under pressure and concentrating [28]. These production procedures might cause the degradation of GAGs, such as CS/DS [29] and HA.

Calculation by quantitative disaccharide analysis (Table 4) showed that the amounts of HA from 1 g of *CCA* were 1508, 1745 and 405 ng in D1 to D3 fractions respectively. In

Table 2 The equations of peak area as a function of amount for each disaccharide

CS/DS/HA disaccharides	Equations	R^2
Tri S	y = 0.0048x-0.2956	0.9957
S_B	y = 0.1061x-2.5235	0.9824
S_D	y = 0.0984x-1.8687	0.9850
S_E	y = 0.1597x-2.3168	0.9962
2S	y = 0.7054x + 0.1879	0.9987
4S	y = 0.6113x-0.2639	0.9981
6S	y = 0.6151x-0.2498	0.9993
0S	y = 0.5474x + 0.2329	0.9979
HA	y = 0.1716x-34.568	0.9932

Table 3 Disaccharide composition of CS/DS in D2 and D3 fractions

CS/DS disaccharides	Composition (%)	
	D2	D3
Tri S	n.d.[a]	n.d.
S_B	n.d.	31.5%
S_D	n.d.	n.d.
S_E	n.d.	n.d.
2S	n.d.	n.d.
4S	94.3%	65.9%
6S	4.1%	2.6%
0S	1.6%	n.d.
2-*O*-Sulfation	n.d.[a]	31.5%
4-*O*-Sulfation	94.3%	97.4%
6-*O*-Sulfation	4.1%	2.6%
Total sulfation	98.4%	131.5%

[a] not detected

Table 4 Amount of GAGs in D1 to D5 fractions

Purification steps	Amount [a] (ng/g of *CCA*)		
	CS/DS	HA	HS/Heparin[d]
D1	n.d.[b]	1508	120
D2	55	1745	1970
D3	283	405	2970
D4	n.d.	n.d.	70
D5	n.d.	n.d.	20
Total[c]	338	3658	5150

[a] Calculated by quantitative disaccharide analysis

[b] Not detected

[c] Total amount of GAGs from D1 to D5 fractions

[d] Published data in Du et al. (2019)

contrast, the amounts of CS/DS disaccharides were 55 and 283 ng in D2 and D3 fractions. The total amount of CS/DS (338 ng) was 10 times lower than that of HA (3658 ng), and 15 times lower than HS/HP (5150 ng) [23].

Our current study demonstrated the distribution and the possible structure of HA and CS/DS in *CCA*. *CCA* has shown clinical functions of anti-aging and bone repair, but their possible mechanisms are still unknown. The presence and polydispersity of HA might be one of the important factors contributing to these activities, as HA has a role in tissue regeneration and cosmetic, nutricosmetic effects [7, 30, 31]. Also, CS/DS has been related to blood coagulant and tumor invasion and metastasis [6, 32, 33], therefore the structural characterization of CS/DS from *CCA* may provide possibilities for further elucidating the medicinal properties of *CCA*.

In conclusion, this paper shows that *CCA* contains a heterogeneous mixture of CS/DS and HA, in which CS/DS possesses a heterogeneous structure with different sulfation patterns and densities in its constituent chain, while HA structure might vary in its chain length. The presence of these complex GAGs raises the question as to whether they may contribute in any way to the biological/medicinal properties attributed to *CCA*.

Heading

Structural analysis of GAGs from *CCA*

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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