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# The brown seaweed *Cystoseira schiffneri* as a source of sodium alginate: Chemical and structural characterization, and antioxidant activities

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1     **The brown seaweed *Cystoseira schiffneri* as a source of sodium alginate: Chemical and**  
2                                   **structural characterization, and antioxidant activities**

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20                                   **Running title:**

21                                   **Sodium alginate from *Cystoseira schiffneri***

25 **Abstract**

26 Sodium alginates were extracted from brown seaweeds (*Cystoseira schiffneri*)  
27 collected from a Tunisian island (Kerkennah, Sfax) in different seasons (December, April,  
28 July and September). The structural features and antioxidant properties of *C. schiffneri*  
29 sodium alginates (CSSA) were characterized. Micro-elementary analysis showed the absence  
30 of nitrogen and sulfur in alginate fractions. All alginate isolates were high in uronic acids  
31 (47.4-66.4%) and ash (24.3-39.4%). ATR-FTIR, NMR and circular dichroism data showed  
32 that all CSSA were of the polyguluronate-type. Average molecular masses determined using  
33 high performance size exclusion chromatography showed variations ranging from 4.49 to  
34 1230 kDa. Thermogravimetric analysis suggested that CSSA were stable until 400°C except  
35 for that collected in September, which was observed to only be stable until 200°C.  
36 Antioxidant activity of CSSA from different seasons was measured using DPPH• radical-  
37 scavenging, reducing power and Fe<sup>2+</sup> chelating ability assays. Antioxidant potential of CSSA  
38 varied significantly with respect to the season and the main factor controlling antioxidant  
39 properties was the molecular mass.

40 **Keywords:** brown seaweed; *Cystoseira schiffneri*; sodium alginate; polyguluronate;  
41 antioxidant activities.

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## 50 **1. Introduction**

51           Seaweeds are known for their bioactive polysaccharides (Sanjeewa et al., 2017; Zhao  
52 et al., 2018; Rengasamy et al., 2020). Algal polysaccharides are biodegradable, non-toxic and  
53 biocompatible (Liu et al., 2015). Alginates are a family of linear copolymers containing  
54 blocks of (1,4)-linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G) residues. Blocks  
55 are composed of consecutive G residues or M residues or alternating M and G residues (Yang  
56 et al., 2011). Structural features of alginates vary depending on species, season, geographic  
57 location, among other parameters (Setyawidati et al., 2018). Alginates possess numerous  
58 biological activities including antioxidant (Kumar et al., 2019; Sellimi et al., 2015),  
59 antimicrobial(Li et al., 2017) and gastroprotective activities (Ammar et al., 2018). Their  
60 biological properties are closely related to the proportion and distribution of M and G residues  
61 as well as the molecular mass (MM).

62           Companies may use synthetic antioxidants, such as butylated hydroxyanisole (BHA)  
63 and butylated hydroxytoluene (BHT) to control oxidation (Lorenzo et al., 2013). However,  
64 the use of these synthetic compounds has been linked to carcinogenic effects  
65 (KahlandKappus, 1993). Moreover, free radicals are the main cause of many diseases, such as  
66 diabetes, cancer and neurodegenerative pathologies (Liu, 2020). A protection could occur  
67 when free radicals were neutralized or trapped by antioxidant compounds (Schramm et al.,  
68 2003). Naturally occurring antioxidants may be able to replace these synthetic compounds.

69           The brown seaweed *Cystoseira schiffneri*, collected from Kerkennah island during 4  
70 seasons, was chosen as a natural matrix to extract and evaluate bioactive polysaccharides. The  
71 annual cycle of the Mediterranean *Cystoseira* passes through a growth period between  
72 February and May, a breeding period from June-July to August-September followed by a rest  
73 period between October and December (Pellegrini et al., 1997). Hence, the current study aims

74 to assess the effect of seasonal variation on the structural features and antioxidant activities of  
75 *C. schiffneri* sodium alginate (CSSA).

## 76 **2. Material and methods**

### 77 *2.1. Reagents*

78 1,1-Diphenyl-2-picrylhydrazyl (DPPH•), butylated hydroxyanisole (BHA), ethylene  
79 diamine tetra-acetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-disulfonic acid  
80 monosodium salt hydrate (ferrozine), Folin-Ciocalteu's reagent, phloroglucinol, glucose,  
81 guluronic acid, sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), hydrochloric acid (HCl), ferrous chloride  
82 (FeCl<sub>2</sub>), 3,5 dimethylphenol, trichloroacetic acid (TCA), potassium ferrocyanide (C<sub>6</sub>FeK<sub>4</sub>N<sub>6</sub>),  
83 sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), acetone, methanol, chloroform, 95% ethanol and other chemicals were  
84 purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 85 *2.2. Sample collection and preparation*

86 The *Cystoseira schiffneri* samples were collected from Kerkennah islands (Tunisia)  
87 around the point (34°39'30.07"N, 11°8'12.27"E) during low tide. Collections were done in  
88 different seasons (December 2015, and April, July and September 2016). The identification of  
89 collected seaweed was made by Pr. Asma Hamza from the National Institute of Marine  
90 Science and Technology (Sfax, Tunisia). Seaweed fronds were washed with sea water to  
91 eliminate impurities, and then transported to the laboratory in dark plastic bag for a period not  
92 exceeding 12 h. Once arrived, seaweed fronds were washed with tap water followed by  
93 distilled water to eliminate salts. After that, they were dried away from sunlight at room  
94 temperature (25°C) for 20 days until reaching stable moisture. Dried fronds were ground  
95 using a coffee grinder (Moulinex, Mayenne, France) and sieved through a 200 µm mesh size.  
96 The powders were conserved at room temperature for a maximum of 8 wk in dried and sealed  
97 dark glass containers.

98           2.3. *Sodium alginate extraction*

99           Crude sodium alginate extraction was done as previously described by Sellimi et al.  
100 (2015). *C. schiffneri* powder (50 g) was defatted and depigmented using maceration twice (24  
101 h, 30°C, 200 rpm) in acetone:methanol (7:3, v:v) (500 ml) followed by chloroform (300 ml).  
102 Depigmented and defatted powder was air-dried, then demineralized by incubation (2 h,  
103 60°C, 250 rpm) in 1 l 0.1 M HCl (pH 2). After cooling at room temperature, the residues were  
104 recovered using centrifugation (20 min, 4000 ×g) in a Rotofix 32 centrifuge (Hettich,  
105 Tuttlingen, Germany), washed with distilled water until neutral pH and then treated in 3%  
106 Na<sub>2</sub>CO<sub>3</sub> solution (pH11) for 2 h at 60°C and 250 rpm to solubilize alginate in the sodium salt  
107 form. After centrifugation (20 min, 4000 ×g), the supernatant was recovered and two volumes  
108 of absolute ethanol were added. Mixtures were left for 12 h at 4°C to precipitate sodium  
109 alginate. The precipitated sodium alginate was suspended in distilled water and acidified  
110 using 6 M HCl (pH 3) to allow gelation and thereby precipitation of alginate in its acidic  
111 form. Precipitated alginic acids were recovered using centrifugation (20 min, 4000 ×g), then  
112 suspended in distilled water and neutralized to pH 7.5 using 6 M NaOH. The crude sodium  
113 alginate was precipitated using two volumes of absolute ethanol, dissolved again in distilled  
114 water and lyophilized (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France). The  
115 crude alginates extracted from *C. schiffneri* collected at December, April, July and September  
116 were referred to AD AA AJ and AS, respectively.

117           2.4. *Chemical analysis*

118           The total sugars were determined using the method of Dubois et al. (1956). Briefly, to  
119 0.3 ml CSSA solution (0.1 g/ml), 1 ml 5% phenol solution and 5 ml 12 NH<sub>2</sub>SO<sub>4</sub> were added.  
120 The mixture was incubated for 20 min at 30°C then the optical density was measured, using a  
121 spectrophotometer (T70 UV-visible spectrometer PG Instruments Ltd., Lutterworth,  
122 England), at 490 nm against a standard curve prepared using glucose.

123 The uronic acids (U) were determined using the method of Scott (1979). To 0.3 ml  
124 CSSA solution (0.1 g/ml), 5 ml 12 N H<sub>2</sub>SO<sub>4</sub> and 0.3 ml of a solution containing 20 g/l NaCl  
125 and 30 g/l H<sub>3</sub>BO<sub>3</sub> were added. The mixture was incubated at 70°C for 40 min, then cooled to  
126 room temperature for 1 h before adding 0.2 ml 3,5-dimethylphenol. After 10 min at room  
127 temperature, the absorbance was measured at 400 and 450 nm against a standard curve of  
128 galacturonic acid. U (%) were calculated using equation 1.

$$129 \quad U (\%) = (\Delta A \times V \times D \times C_s \times 0.91 \times 100) / (\Delta A_s \times m)(1)$$

130 Where  $\Delta A$  = the difference in absorbance,  $V$  = the total solution volume (ml),  $D$  = the  
131 sample dilution,  $C_s$  = the standard concentration,  $\Delta A_s$  = the difference in the absorbance of  
132 the standard (100 µg/ml),  $m$  = the mass of the test sample (mg), and 0.91 is the conversion  
133 factor of the experimental determination of monosaccharides to polysaccharides.

134 Total phenolics were determined using slightly modified methods described by Cicco  
135 et al. (2009). Phloroglucinol was used as the standard. Briefly, 100 µl of sample solution (2  
136 mg/ml) was mixed with 100 µl 2 N Folin-Ciocalteu's reagent and 800 µl 5% Na<sub>2</sub>CO<sub>3</sub> solution.  
137 The mixture was incubated at 40°C for 20 min, then absorbancies were measured at 760 nm  
138 against a standard curve of phloroglucinol.

### 139 *2.5. Micro-elementary analysis*

140 Elemental composition of CSSA was done using an EDX analyzer (X-Max<sup>N</sup> SDD  
141 EDX Instrument, Oxford, UK). Results were treated using Oxford AZTEC software 2011  
142 (Oxford Instruments, Abingdon, UK). The samples were metalized previous to analyses using  
143 a Quorum Technologies metalizer (Quorum Technologies, Guelph, ONT, Canada).

### 144 *2.6. Circular dichroism*

145 A Jasco J-815 CD spectrometer (Jasco Corp., Tokyo, Japan) was used to obtain  
146 circular dichroism spectrum in the range of 190-260 nm. The sample solution was at a  
147 concentration of 0.25 mg/ml. A circular dichroism spectrum is often reported in ellipticity

148 degrees ( $\theta$ ) expressed as milli-degrees. The ratio of peak height to trough depth was  
149 calculated using equation 2 (Morris et al., 1980).

150 
$$\text{Peak/trough ratio} = (q_{\text{trough}} - q_{\text{peak}})/q_{\text{trough}}(2)$$

151 *2.7. ATR-FTIR spectroscopy*

152 Attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) spectra of CSSA  
153 were obtained in the range of 4000–600  $\text{cm}^{-1}$  at room temperature using 30 scans and 4  $\text{cm}^{-1}$   
154 resolution. A FTIR Spectrum 100 spectrometer (Perkin-Elmer, Norwalk, CT, USA) with ATR  
155 accessory containing a diamond/ZnSe crystal was used to obtain infrared spectrums.

156 *2.8. NMR spectroscopy analysis*

157  $^1\text{H}$ -NMR spectra were obtained using a Bruker 300 spectrometer (Bruker Biospin AG,  
158 Fallanden, Switzerland) at 60°C. Results were treated using Mestre Nova (MNOVA) software  
159 version 5.3.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). Samples were  
160 deuterium-exchanged using lyophilization with  $\text{D}_2\text{O}$  and then studied as 1% (m/v) solutions in  
161  $\text{D}_2\text{O}$  (99.96%). Signals in the anomeric region (4.4-5.1 ppm) were studied to determine the  
162 CSSA structure. Therefore,  $F_M$ ,  $F_G$ ,  $F_{GG}$ ,  $F_{MM}$ ,  $F_{GM}$  and  $F_{MG}$  blocks of CSSA were calculated  
163 based on the areas of signals (I), (II) and (III) (Fertah et al., 2017) and using equations 3, 4,5,  
164 6 and 7. In addition, M/G ratio can be calculated based on the equation 8.

165 
$$F_G = A_I / (A_{II} + A_{III}) \quad (3)$$

166 
$$F_M = 1 - F_G(4)$$

167 
$$F_{GG} = A_{III} / (A_{II} + A_{III}) \quad (5)$$

168 
$$F_{MG} = F_{GM} = F_G - F_{GG}(6)$$

169 
$$F_{MM} = F_M - F_{MG}(7)$$

170 
$$M/G = F_M / F_G(8)$$



171        *2.9. MM distribution*

172            The number-average molecular mass ( $M_n$ ), the average molecular mass ( $M$ ) and  
173 polydispersity index (PDI) of CSSA were determined using a Waters Alliance model  
174 GPCV2000 (Waters, Milford, MA, USA). High performance size exclusion chromatography  
175 (HPSEC) equipped with a multi-angle laser light scattering (MALLS) detector from Wyatt  
176 (Wyatt Technology, Santa Barbara, CA, USA). Before injection, the apparatus was calibrated  
177 with toluene and normalized with a solution of polyethylene oxide (72 kDa) in 0.1 M NaCl  
178 and the samples were filtered through a 0.45  $\mu\text{m}$  pore size membrane (Merck, Darmstadt,  
179 Germany). The sample (100  $\mu\text{l}$ ) of 3 mg/ml was injected in a column TSK-G2000 SWXL (7.8  
180  $\times$  300 mm). Elution was carried out at 25°C using 0.1 M NaCl as eluent at a flow rate of 0.5  
181 ml/min. The refractive index increment ( $dn/dc$ ) was 0.155. Data collected from the refractive  
182 index detector (RID) and MALLS were treated using ASTRA software version 4.72.03  
183 (Wyatt Technology, Goleta, CA, USA).

184        *2.10. Thermogravimetric analyses*

185            Thermogravimetric analyses (TGA) of the extracted sodium alginates were done on a  
186 TGA Q500 instrument (TA Instruments, Newcastle, DE, USA). Nitrogen flow rate was 60  
187 ml/min and the temperature increase from 20 to 1000°C by 20°C/min.

188        *2.11. Rheological analysis*

189            Rheological analyses of sodium alginates solutions (3%,m/v) from different seasons  
190 were done on a Physica MCR 301 rheometer (Anton-Paar GmbH, Graz, Austria) using the  
191 dispositive plan-plan 50 with a measurement gap of 0.1 mm and temperature of 20°C.  
192 Viscosity was measured as a function of shear rate (0.1-100  $\text{s}^{-1}$ ). Storage modulus ( $G'$ ) and  
193 loss modulus ( $G''$ ) were measured as a function of frequency (2-100 Hz).

194        2.12. *Antioxidant activity*

195            2.12.1. *DPPH• radical-scavenging activity*

196            Radical-scavenging activity was measured as described by Kirby and Schmidt(1997)  
197 with slight modifications. Sample solutions were prepared at different concentrations from  
198 0.125 to 1.5 mg/ml. In each tube, 500 µl of the sample solution was reacted with 375 µl of  
199 ethanol solution and 125 µl of DPPH• solution (0.2 mM in ethanol) as the free radical source.  
200 The reaction mixtures were incubated for 60 min in the dark at room temperature and the  
201 reduction of DPPH• radical was measured at 517 nm. Control (without sample) and blanks  
202 (without DPPH•) were prepared. Results of DPPH• scavenging activity are shown as IC<sub>50</sub>  
203 values (µg/ml), defined as the extract concentration needed to scavenge 50% DPPH•.

204            2.12.2. *Reducing power*

205            Ferrous reducing activity was measured as previously described by Yildirim et al.  
206 (2001). Sample solutions were prepared at different concentrations from 50 to 500 µg/ml.  
207 Sample solution (0.5 ml) was added to 1.25 ml 0.2 M sodium phosphate buffer (pH 6.6) and  
208 1.25 ml 1% (m/v) potassium ferrocyanide. Mixtures were incubated for 30 min at 50°C. After  
209 incubation, 1.25 ml 10% (m:v) TCA was added and the mixture was centrifuged at 11000 ×g  
210 for 10 min. From each supernatant, 1.25 ml was transferred and diluted with 1.25 ml ultrapure  
211 water. Finally, FeCl<sub>3</sub> (250 µl) were added and absorbancies were measured at 700 nm after 10  
212 min incubation at room temperature. Blanks without FeCl<sub>3</sub> were prepared for each  
213 concentration. The reducing power is shown as the extract concentration (EC<sub>0.5</sub>) providing 0.5  
214 absorbance at 700 nm.

215            2.12.3. *Fe<sup>2+</sup> chelating ability*

216            The CSSA capacity to complex ferric ions were measured using the method reported  
217 by Carter (1971). Sample solutions were prepared at different concentrations from 0.1 to 1  
218 mg/ml. Briefly, 100 µl of sample solution, 50 µl 2 mM FeCl<sub>2</sub> and 450 µl distilled water were

219 mixed and incubated for 3 min at room temperature. Volumes of 200  $\mu$ l 5 mM ferrozine were  
220 added and mixtures were incubated again at room temperature for 10 min before measuring  
221 absorbancies at 562 nm. Blanks without ferrozine and control without sample were prepared.  
222 Results of metal ( $\text{Fe}^{2+}$ ) chelating ability are shown by  $\text{IC}_{50}$  values ( $\mu\text{g/ml}$ ), defined as the  
223 extract concentration needed to chelate 50%  $\text{Fe}^{2+}$ .

### 224 2.13. Statistical analysis

225 All analytical determinations were done in triplicate. One-way analysis of variance  
226 (ANOVA) was done using the statistical package for the social sciences (SPSS) software for  
227 Windows™ (version 17, SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests  
228 ( $p < 0.05$ ) was used to compare the average responses between samples.

## 229 3. Results and discussion

### 230 3.1. Extraction yield

231 The extraction yields of CSSA from different seasons are shown in Table 1. Extraction  
232 yield of AJ was higher than AA and AD. While, AS showed the lowest extraction yield. The  
233 results were comparable to those reported for *Dictyota dichotoma* (20.9%), *Padina*  
234 *boergeseni* (24.3%) and *Sargassum tenerrimum* (32.6%) (Parthiban et al., 2012), but  
235 significantly higher than those of *D. caribaea* (7.4%) and *P. perindusiata* (5.4%) (García-Ríos  
236 et al., 2012). Alginate extraction yield is dependent on the season and thereby the  
237 development stage of *C. schiffneri*. Kumar and Sahoo (2017) reported that the highest alginate  
238 yield from *S. wightii* was obtained in March (32%), however, the lowest one (24%) was  
239 obtained in July. Setyawidati et al. (2018) described that extraction yields of alginate from *S.*  
240 *aquifolium* were higher during the dry season (May, June) (39%) than during the wet season  
241 (November) (24%). On the other hand, *P. boryana* showed extraction yields of 9% during the  
242 wet season against 6% during the dry season. Therefore, the seasonal variation of alginate  
243 yield did not follow one trend line for all species.

244           3.2. *Chemical analysis*

245           Moisture, ash, total phenolics, total sugars and U of CSSA from different seasons are  
246 shown in Table 1. High amounts of U and ash were observed for all seasons, while markedly  
247 low amounts of total sugars were measured. However, traces of phenolic compounds were  
248 found. U and neutral sugars of CSSA were comparable to those of *C. barbata* alginate (58.1  
249 and 9.3%, respectively) (Sellimi et al., 2015). Similarly, Ammar et al. (2018) showed that U  
250 are ~ 60% for three species *C.compressa*, *P. pavonica* and *Dictyopteris*  
251 *membranacea*. Similarly, alginate isolated from *C. compressa* had traces of phenolic  
252 compounds (1.11%) (Hentati et al., 2018). Standard alginates also showed the presence of  
253 impurities. Dusseault et al. (2006) detect proteins (5.9 mg/g) and polyphenols (13.8 AFU) in  
254 food-grade sodium alginate.

255           3.3. *Elementary analysis*

256           The elemental composition of CSSA from all seasons is shown in Table 1. High levels  
257 of carbon, oxygen and sodium while low contents of Mg, Cl, Si and K were measured. Sulfur  
258 and nitrogen were not found, which indicated the absence of proteins and fucans in the  
259 extracted alginates. Elementary analysis therefore suggested that CSSA fractions occurred as  
260 a sequence of U linked predominately to sodium ions.

261           3.4. *FTIR spectroscopy*

262           Fig. 1. shows the spectral data relative to the ATR-FTIR spectroscopy analysis of the  
263 CSSA fractions. The bands at 3354 and 2935  $\text{cm}^{-1}$  were assigned to O–H and C–H stretching  
264 vibrations, respectively (Hentati et al., 2018). Signals at 1605 and 1423  $\text{cm}^{-1}$  were attributed,  
265 respectively to asymmetric and symmetric C=O stretching vibrations. The band at 1025  $\text{cm}^{-1}$   
266 assigned to C-O-C of glycosidic linkage was the polysaccharides characteristic band (Hentati  
267 et al., 2018). The bands, in the anomeric region (750-950  $\text{cm}^{-1}$ ), were assigned to U residues  
268 (Chandía, 2001; Chandía et al., 2004). The peak ratio at 1125 and 1025  $\text{cm}^{-1}$  allows the

269 estimation of M/G ratio (Gómez Ordóñez and RupérezAntón, 2011). Overall, the estimated  
270 M/G ratios of CSSA for the 4 seasons ranged between 0.062 and 0.065 (Table 2), which  
271 suggest that all CSSA were of the polyguluronate-type.

### 272 3.5. Circular dichroism

273 The M/G ratios of sodium alginate fractions could also be determined from circular  
274 dichroism spectra based on the empirical correlation between the peak (~195 nm) to depth  
275 (~205 nm) ratio (Morris et al., 1980). The CSSA spectra showed that peaks ~195 nm were  
276 almost absent for all samples (Fig. 2). Therefore, the calculated M/G ratios did not exceed  
277 0.022 (Table 2). The CSSA fractions were, therefore, composed essentially by G residues,  
278 which confirmed the FTIR data. Ramos et al. (2018) reported that low M/G ratio of alginates  
279 resulted in strong and big gels with interactions with calcium ions. The CSSA fractions seem  
280 to be interesting for strong gels applications and this during all algae development stages.

### 281 3.6. MM distribution

282 MM distributions of CSSA were determined and results are shown in Table 3. For all  
283 seasons, the CSSA fractions were polydisperse (PDI>1). The polydispersity may be due to the  
284 use of the whole thalli. Handå et al. (2013) reported that alginate MM varied as a function of  
285 the thalli part. Hence, the MM of alginate was an average over the whole distribution.

286 The highest MM was obtained for AD, followed by AJ, AA and AS. The AA and AJ  
287 fractions had MM comparable to commercial sodium alginates CR8133 (M = 90-180 kDa)  
288 and CR8223 (M= 250-350 kDa), respectively (Ramos et al., 2018). MM of CSSA for all the  
289 seasons, except for December, were observed to be lower than those of sodium alginates  
290 extracted from 8 species of *Saccharina* (511-616 kDa) (Suzuki et al., 2016).

291 The important variation noted between seasons for MM may be related to the life  
292 cycle of *C. schiffneri*. In December at the rest period thalli are formed entirely of old stems  
293 characterized by high MM. In April, during the growth period new stems with low MM

294 appear on old stems and continue growing gaining higher MM. In September, after  
295 reproduction it appears new thalli formed entirely by the new stems that are characterized by  
296 low MM (Handå et al., 2013)

### 297 *3.7. NMR spectroscopy analysis*

298 <sup>1</sup>H-NMR spectra of CSSA from the different seasons are shown in Fig. 3. Characteristic  
299 bands were observed to be around 1.5, 2.0, 4.5, 5.0 and 5.5 ppm for all the seasons. The  
300 distribution of M and G could be studied based on signals from the anomeric region (Fertah et  
301 al., 2017). Signal (I) that correspond to G-1 anomeric proton of G was obtained at 5.35 ppm.  
302 Signal (II), that was due to the overlap between the M anomeric proton (M-1) and the C-5 of  
303 the alternating blocks (GM-5), was obtained at 4.93 ppm. Similarly, signal (III) characterizing  
304 H-5 G proton (GG-5G) was obtained at 4.84 ppm. The calculated  $F_G$ ,  $F_M$ ,  $F_{GG}$ ,  $F_{MM}$ ,  $F_{MG}$  and  
305  $F_{GM}$  are shown in Table 4. The results of the calculated M/G ratios (Table 2) were consistent  
306 with the circular dichroism and FTIR spectroscopy data, in which the CSSA fractions were  
307 defined as polyguluronates. Higher M/G ratios were described by Mohammed et al. (2020)  
308 (M/G=0.45), Okolie et al.(2020) (M/G=0.87), Davis et al. (2003) (M/G=0.19) and Larsen et  
309 al. (2003) (M/G=0.45). There were no previous studies describing comparable or lower M/G  
310 ratios. The CSSA fractions might be the first reported as polyguluronates. The results for  
311 structural composition showed through the year high  $F_G$  and  $F_{GG}$  values and low  $F_M$ ,  $F_{MM}$  and  
312  $F_{MG}$  values. These results suggested that CSSA fractions were composed essentially by G  
313 blocks punctuated with low amounts of M and MG blocks. Gelling properties of alginate are  
314 related to G blocks responsible for the “Egg Box” structure (Xiao et al., 2015). High  $F_{GG}$   
315 values for CSSA fractions with high M/G ratio suggested the possibility to form a strong and  
316 rigid gel.

317 Results for the M/G ratio determined using three methods suggested that through the  
318 year the CSSA fractions were of the polyguluronate-type with low amounts of M. However,

319 the values obtained from one method to another. Hence, it should be pointed out that the M/G  
320 ratio calculated from FTIR spectra were only an approximation. The circular dichroism,  
321 which determines the ratio of M to G based on empirical correlation, gives more accurate  
322 results. Overall, NMR spectra gave the best calculations not only for the M/G ratio but also  
323 for GGG, MGM, MG, MM, GGM and MGG. Hence, Hentati et al.(2018) reported that NMR  
324 is the only reliable and accurate method to study the alginate structure.

### 325 *3.8. Thermogravimetric analysis*

326 The mass losses of CSSA from the 4 seasons as function of temperature are shown in  
327 Fig. 4. Idris et al. (2012) reported that the combustion had three phases namely drying (50-  
328 220°C), pyrolysis (220-500°C) and char combustion (>500°C). The first mass loss was  
329 attributed to the evaporation of non-bonded water and it occurred between 50 and 150°C  
330 (Mallick et al., 2018). The second mass loss, between 150 and 220°C, was assigned to the loss  
331 of bonded water. Fig. 4 shows that the most important mass loss was observed around 400°C  
332 for all CSSA fractions, except for AS (around 200°C), which was due to the degradation of  
333 the osidic monomers. The decrease of temperature degradation of AS compared to others  
334 alginates could be related to their lower MM. Mallick et al. (2018) reported that low MM  
335 molecules had high volatility and could therefore accelerate degradation. Differences among  
336 seasons could be explained by the MM differences and the presence of minerals. López-  
337 González et al. (2014) reported that alkali metals catalyzed combustion that was characterized  
338 by multiple and small mass losses.

### 339 *3.9. Rheological properties*

#### 340 *3.9.1 Rotational analysis*

341 The viscosities of AA, AJ and AS decreased with shear rates indicating a shear-  
342 thinning behaviors (Fig. 5). However, Newtonian behavior was observed for AD at shear rates  
343  $>10 \text{ s}^{-1}$  indicating that the CSSA flow has encountered more resistance at high shear rate

344 (Benchabane and Bekkour, 2008). Comparable viscosities were observed for all the seasons  
345 except for AD that showed low viscosity. The difference of AD behavior from other CSSA  
346 fractions might be related to its higher MM giving more intermolecular entanglements.  
347 Comparable results were shown by Rezende et al. (2009) for alginate showing shear-thinning  
348 behaviors. Hentati et al. (2020) reported that at low concentrations (0.25-2%) of *C. compressa*  
349 alginate, shear-thinning behavior was observed at low shear-rates that turn to Newtonian  
350 behavior at high shear-rates. However, at high concentrations (3-5%) only shear-thinning  
351 behaviors were shown. The shear-thinning behavior observed for the CSSA fractions  
352 facilitates the flow compared with a Newtonian fluid allowing high flow rates for equal  
353 pressures (Rezende et al., 2009).

#### 354 *3.9.2. Oscillatory analysis*

355 The increase in  $G'$  for AD with increasing frequencies suggested that elastic behavior  
356 becomes dominant (Fig. 6). However, for AA, AJ and AS,  $G'$  remained almost constant with  
357 the increase of frequency indicating no elastic behavior for alginate solutions (Böhning et al.,  
358 2019). All samples showed viscous liquid-like behavior as the  $G''$  values were greater than  
359 the  $G'$  for all the samples (Hentati et al., 2020). The elastic behavior of AD seemed to be due  
360 to the high MM. However, the CSSA from other seasons should be considered as non-viscous  
361 liquids, which could be important for some manufacturing uses (Rezende et al., 2009).

#### 362 *3.10. Antioxidant activity*

##### 363 *3.10.1. DPPH• radical-scavenging ability*

364 The DPPH• radical-scavenging test reflects the ability of a sample to stabilize free  
365 radicals by donating a proton. Fig. 7 shows the concentration dependent radical-scavenging  
366 activity of CSSA and the  $IC_{50}$  values are shown in Table 5. The AS with the lowest MM  
367 showed the lowest  $IC_{50}$  value and therefore the best activity. However, radical-scavenging  
368 activity of CSSA remained lower than that of BHA. Overall, the CSSA fractions were



369 observed to show higher antiradical activity than alginate hydrolysates ( $IC_{50}=10.4$  mg/ml)  
370 (Zhu et al., 2016). However, Hentati et al. (2018) reported that *C. compressa* sodium alginate  
371 with MM of 1.105 g/mol had higher activity ( $IC_{50} = 560$   $\mu$ g/ml) than those of AD, AA and  
372 AJ, but remained lower than that of AS. A positive correlation between radical-scavenging  
373 activity at 1500  $\mu$ g/ml and MM ( $R^2=0.31$ ) was observed. Therefore, the main factor  
374 controlling the antioxidant properties of sodium alginates seems to be the MM. Accordingly,  
375 Kelishomi et al. (2016) showed that radical-scavenging activity of alginates was inversely  
376 proportional to the MM. Higher MM polysaccharides had a more compact structure, resulting  
377 in stronger intramolecular hydrogen bonds and thus restricting the hydrogen groups.  
378 Therefore, the access of active groups to oxidant molecules is more difficult.

### 379 3.10.2. Reducing power

380 The ability of CSSA to stabilize oxidants by donating an electron was measured and  
381 results are shown in Fig. 7. The CSSA fractions showed a reducing power in a concentration-  
382 dependent manner and the  $EC_{0.5}$  values are shown in Table 5. The highest activities were  
383 obtained for AS and AD followed by AJ. However, the  $EC_{0.5}$  values of CSSA from all seasons  
384 remained lower than that of BHA. All CSSA fractions, except AA, showed higher reducing  
385 power than that of *C. barbata* sodium alginate (absorbance of 0.7 at 0.5 mg/ml) (Sellimi et al.,  
386 2015). Comparable results to AJ were reported for *C. compressa* sodium alginate that showed  
387 absorbance  $\sim 1$  at 0.5 mg/ml (Hentati et al., 2018). The reducing power of polysaccharides  
388 was directly related to their MM and to the hydroxyl and carboxyl groups of U (Raza et al.,  
389 2011; Shang et al., 2018). However, the present study showed contradictory results, where the  
390 reducing abilities of CSSA fractions were not correlated with either their MM or their U.  
391 However, inconsistent results were also described by others. Cheng et al. (2013) indicated that  
392 polysaccharide fractions from *Epimedium acuminatum* with high MM had high antioxidant  
393 activity. Kardošová and Machová (2006) reported that for some polysaccharides and

394 oligosaccharides the MM was not correlated with the antioxidant levels. Hence, the MM-  
395 antioxidant activity relation of alginate is still uncertain.

### 396 3.10.3. $Fe^{2+}$ chelating ability

397 The chelating abilities of CSSA was dependent on the concentration and the results are  
398 shown in Fig. 7, and the  $IC_{50}$  values are shown in Table 5. The highest activity was obtained  
399 for AS followed by AD and AJ, while the lowest  $Fe^{2+}$  chelating ability was measured for AA.  
400 Nevertheless, the CSSA from all the samples showed lower chelating abilities than EDTA.  
401 The AS, which had the lowest MM, had the highest  $Fe^{2+}$  chelating ability. These results were  
402 consistent with the results of Wang et al. (2016) who reported that the  $Fe^{2+}$  chelating ability of  
403 polysaccharides decreased with increased MM. However, AD with higher MM showed higher  
404 chelating ability. Similarly, Cheng et al. (2013) reported that polysaccharide fractions from  
405 *Epimedium acuminatum* with high MM had more antioxidant activity. Kardosová and  
406 Machová (2006) showed that MM of some polysaccharides and oligosaccharides was not  
407 significantly related to the antioxidant properties. The chelating ability was mostly due to OH,  
408 -COOH, -C=O and -O-groups (Wang et al., 2016). The relationships between antioxidant  
409 activity and physico-chemical properties or structural features have not been comprehensively  
410 elucidated and confirmed. Conflicting results have been observed in the literature. Extraction  
411 source and methods, and even drying procedures can influence the results. Furthermore, other  
412 antioxidant substances, such as polyphenol may be retained in polysaccharides fractions and  
413 should be taken into account. Overall, the antioxidant potential of polysaccharides is not  
414 determined by a single factor but a combination of several related ones (Wang et al., 2016).

## 415 4. Conclusion

416 The seasonal variation effect on structural features and antioxidant activities of sodium  
417 alginate extracted from *C. schiffneri* (CSSA) was studied. The isolated alginates fractions  
418 were of the polyguluronate-type and most of them were stable until 400°C. The CSSA

419 showed season-dependent structural characteristics and antioxidant activities. The techno-  
420 functional properties of the CSSA should be studied and compared to commercial alginates.  
421 The CSSA might be used in a variety of applications including food and biomedical  
422 formulations.

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### 428 **Conflicts of interest**

429 The authors confirm that they have no conflicts of interest with respect to the study  
430 described in this manuscript.

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633 **Table 1.** Extraction yield(g/100 g DM) and chemical composition (g/100 g DM) of the CSSA  
 634 fractions from different seasons.

Parameters	Season			
	AD	AA	AJ	AS
639 <b>Yield</b>	25.1±0.4 <sup>c</sup>	28±1 <sup>b</sup>	38.4±0.3 <sup>a</sup>	21±1 <sup>d</sup>
640 <b>Moisture*</b>	11.3±0.1 <sup>b</sup>	10.0±0.4 <sup>c</sup>	10±0.5 <sup>d</sup>	12.6±0.4 <sup>a</sup>
641 <b>Neutral sugars</b>	9.2±0.4 <sup>a</sup>	6.3±0.4 <sup>b</sup>	5.5±0.4 <sup>c</sup>	6.1±0.3 <sup>b</sup>
642 <b>Uronic acids</b>	47±1 <sup>d</sup>	53±2 <sup>c</sup>	66±2 <sup>a</sup>	62±1 <sup>b</sup>
643 <b>Total phenolics‡</b>	1.47±0.01 <sup>a</sup>	1.03±0.02 <sup>b</sup>	0.92±0.01 <sup>c</sup>	0.66±0.02 <sup>d</sup>
644 <b>Ash</b>	39.4±0.1 <sup>a</sup>	37±0.5 <sup>b</sup>	24.3±0.4 <sup>d</sup>	29±1 <sup>c</sup>
645 <b>C</b>	35±2 <sup>b</sup>	40±1 <sup>a</sup>	28±1 <sup>c</sup>	39.6±0.3 <sup>a</sup>
646 <b>O</b>	45.4±0.1 <sup>a</sup>	43±2 <sup>b</sup>	41±1 <sup>c</sup>	46±1 <sup>a</sup>
647 <b>Na</b>	16±1 <sup>b</sup>	14.9±0.4 <sup>b</sup>	26±1 <sup>a</sup>	10.5±0.3 <sup>c</sup>
648 <b>S</b>	0	0	0	0
649 <b>N</b>	0	0	0	0
650 <b>Mg</b>	0	0.17±0.01 <sup>c</sup>	0.25±0.01 <sup>b</sup>	0.44±0.03 <sup>a</sup>
651 <b>K</b>	0.18±0.01 <sup>c</sup>	0.53±0.01 <sup>a</sup>	0.44±0.04 <sup>b</sup>	0.17±0.01 <sup>c</sup>
652 <b>Cl</b>	0.39±0.01 <sup>b</sup>	1.24±0.1 <sup>a</sup>	0.35±0.04 <sup>b</sup>	1.38±0.1 <sup>a</sup>
653 <b>Si</b>	0.36±0.08 <sup>b</sup>	0.27±0.01 <sup>c</sup>	0.57±0.05 <sup>a</sup>	0.26±0.02 <sup>c</sup>

661 AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri* collected in December, April, July  
 662 and September, respectively; \*The moisture is expressed as g/100 g lyophilized sodium alginate;‡Total  
 663 phenolics were expressed as g eq. phloroglucinol/100 g DM; Each value represents the mean±SD ( $n = 3$ );  
 664 <sup>a,b,c,d</sup>Different letters within different seasons of harvest (same column) indicate significant differences ( $p < 0.05$ ).

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667 **Table 2.** M/G ratios of the CSSA fractions from different seasons.

Method	Season			
	AD	AA	AJ	AS
<b>FTIR</b>	0.062	0.064	0.065	0.062
<b>Circular dichroism</b>	0	0.022	0.012	0.015
<b>NMR</b>	0.093	0.024	0.037	0.080

668 AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri* collected in December, April, July  
 669 and September, respectively.

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671 **Table 3.** Average molecular mass (g/mol) of the CSSA fractions from different seasons.

	Season			
	AD	AA	AJ	AS
<b>M<sub>n</sub></b>	7.62×10 <sup>5</sup>	8.02×10 <sup>4</sup>	1.26×10 <sup>5</sup>	7.85×10 <sup>2</sup>
<b>M</b>	1.23×10 <sup>6</sup>	1.16×10 <sup>5</sup>	2.33×10 <sup>5</sup>	4.49×10 <sup>3</sup>
<b>PDI</b>	1.62	1.45	1.85	5.72

672 AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri* collected in December, April, July  
673 and September, respectively; M<sub>n</sub>, M, and PDI are the number-average molecular mass, the average molecular  
674 mass, and the polydispersity index, respectively.

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680 **Table4.**Structural composition of the CSSA fractions from different seasons.

<b>Season</b>	<b>F<sub>G</sub></b>	<b>F<sub>M</sub></b>	<b>F<sub>GG</sub></b>	<b>F<sub>MM</sub></b>	<b>F<sub>MG</sub></b>	<b>F<sub>GM</sub></b>
<b>AD</b>	0.91	0.09	0.88	0.06	0.03	0.03
<b>AA</b>	0.98	0.02	0.97	0.01	0.01	0.01
<b>AJ</b>	0.97	0.03	0.96	0.02	0.01	0.01
<b>AS</b>	0.92	0.08	0.9	0.06	0.02	0.02

681 AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri* collected in December, April, July  
682 and September, respectively.

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684 **Table 5.** Antioxidant activities of the CSSA fractions from different seasons.

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	Season				Standards	
	AD	AA	AJ	AS	BHA	EDTA
<b>DPPH•</b>						
<b>scavenging activity</b>	>1500	>1500	>1500	190±10 <sup>a</sup>	14.1±0.2 <sup>b</sup>	
<b>Reducing power</b>	51±1 <sup>b</sup>	>500	208±6 <sup>a</sup>	51±2 <sup>b</sup>	25.2±0.1 <sup>c</sup>	
<b>Fe<sup>2+</sup>chelating ability</b>	145±1 <sup>c</sup>	511±17 <sup>a</sup>	211±6 <sup>b</sup>	43±1 <sup>d</sup>		10.0±0.2 <sup>e</sup>

686 AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri* collected in December, April, July  
 687 and September, respectively; Results of DPPH• scavenging and metal (Fe<sup>2+</sup>) chelating assays are shown as  
 688 IC<sub>50</sub> values (µg/ml), defined as the extract concentration needed to scavenge 50% of DPPH• and to chelate 50%  
 689 of Fe<sup>2+</sup>, respectively. The reducing power (OD 700) is shown as the extract concentration (EC<sub>0.5</sub>) providing 0.5  
 690 absorbance at 700 nm; Each value represents the mean±SD (*n* = 3); <sup>a,b,c,d,e</sup> Different letters within different  
 691 seasons of harvest (same row) indicate significant differences (*p*<0.05).  
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693 **Figure captions**

694 **Fig. 1.** ATR-FTIR spectroscopy of sodium alginates extracted from *C. schiffneri* collected in  
695 December (AD), April (AA), July (AJ) and September (AS).

696 **Fig. 2.** Circular dichroism spectra of sodium alginates extracted from *C. schiffneri* collected in  
697 December (AD), April (AA), July (AJ) and September (AS).

698 **Fig. 3.** <sup>1</sup>H-NMR spectra of sodium alginates extracted from *C. schiffneri* collected in  
699 December (AD), April (AA), July (AJ) and September (AS).

700 **Fig. 4.** Thermogravimetric analysis of sodium alginates extracted from *C. schiffneri* collected  
701 in December (AD), April (AA), July (AJ) and September (AS).

702 **Fig. 5.** Viscosity of sodium alginates extracted from *C. schiffneri* collected in December  
703 (AD), April (AA), July (AJ) and September (AS).

704 **Fig. 6.** Viscoelasticity of sodium alginates extracted from *C. schiffneri* collected in December  
705 (AD), April (AA), July (AJ) and September (AS).

706 **Fig. 7.** Antioxidant activities of sodium alginates extracted from *C. schiffneri* collected in  
707 December (AD), April (AA), July (AJ) and September (AS).

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709 **Fig. 1.**

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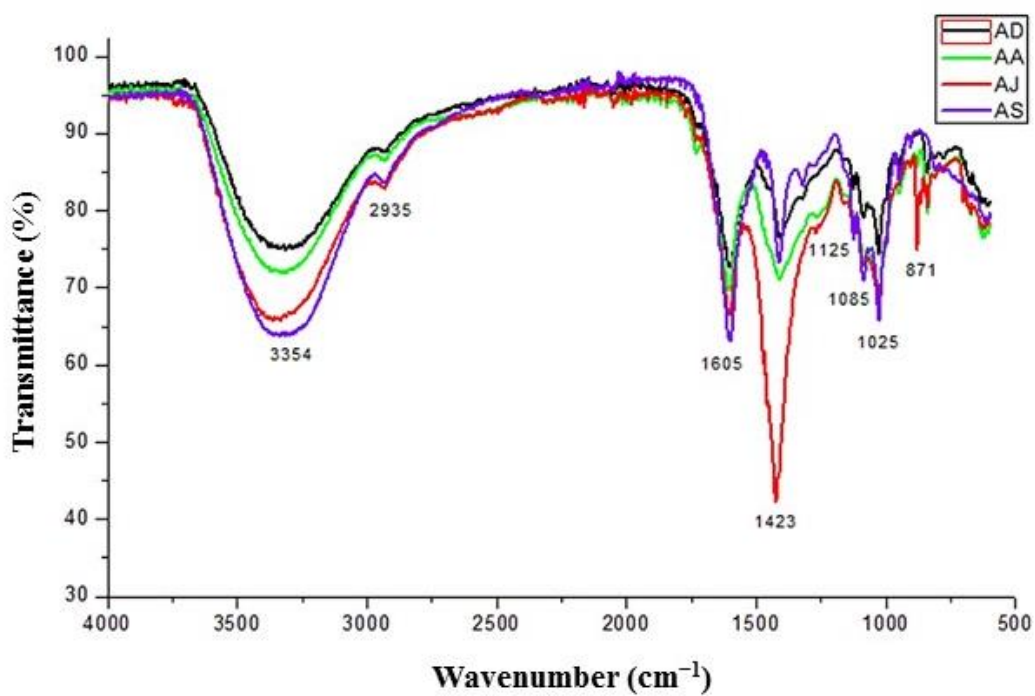
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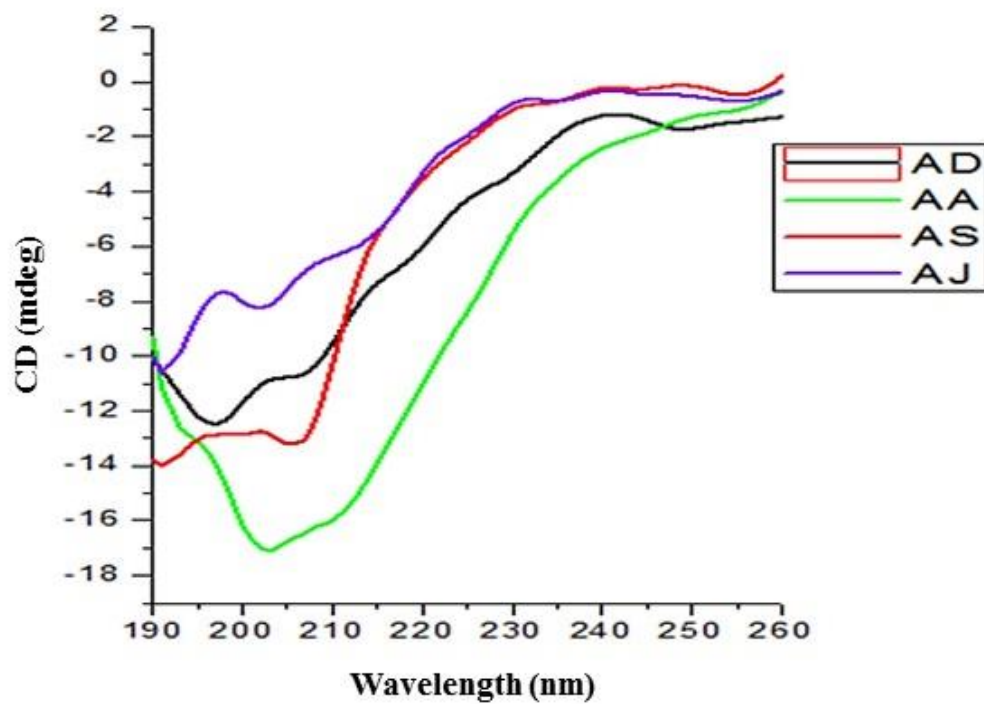
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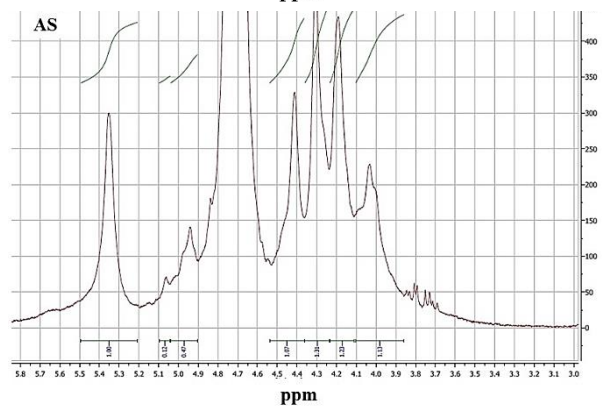
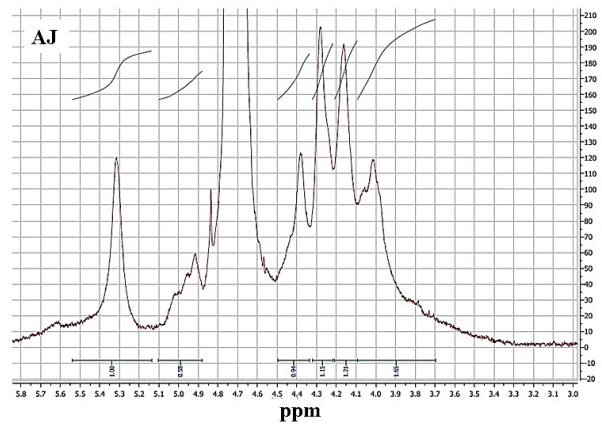
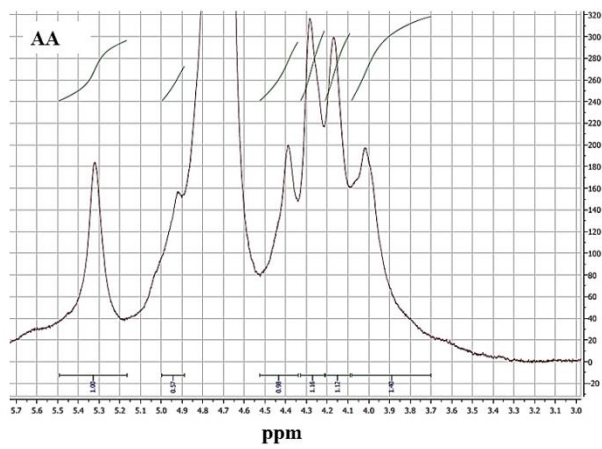
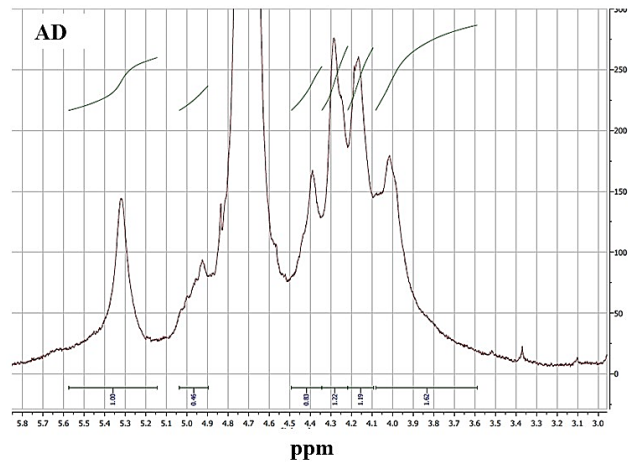
728 **Fig. 2.**

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746 **Fig. 3.**

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789 **Fig. 4.**

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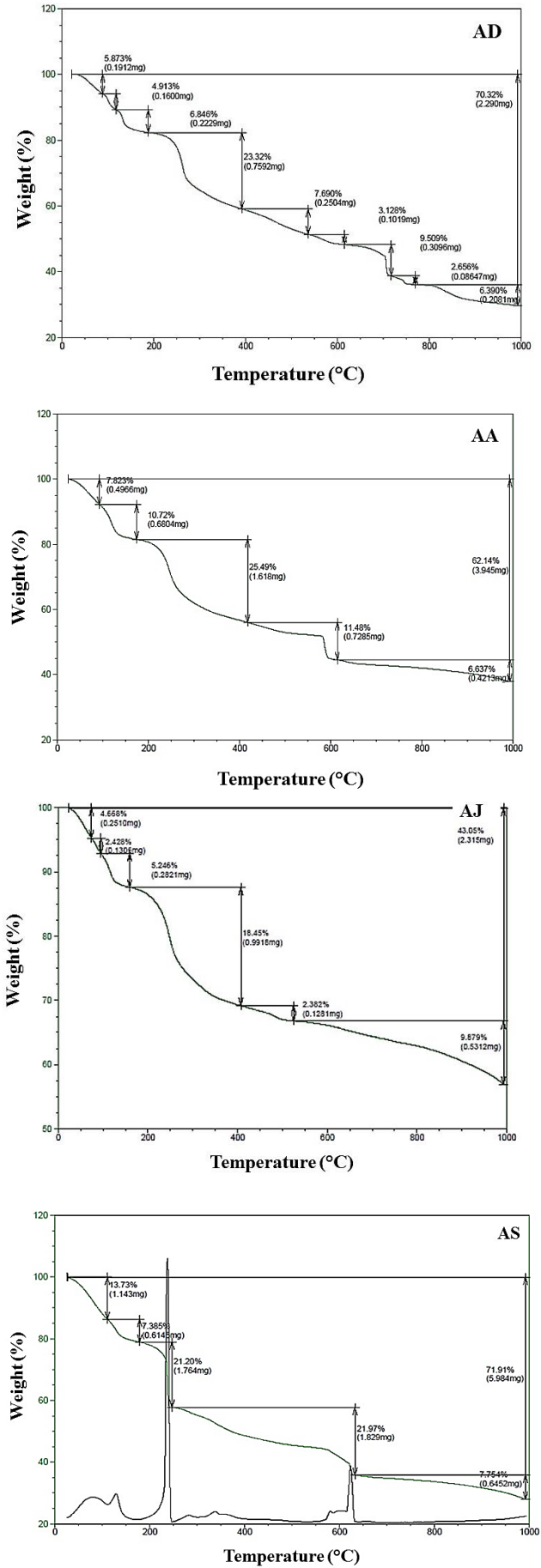
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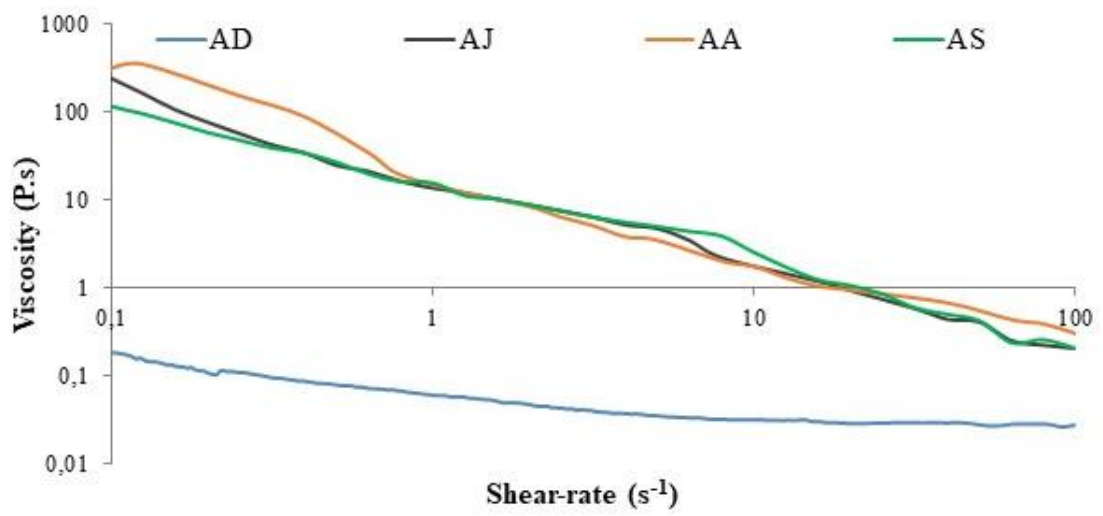
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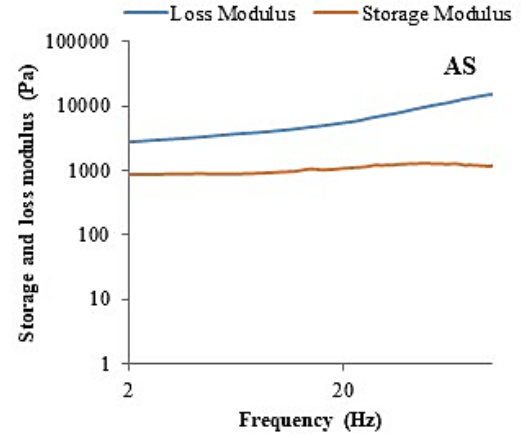
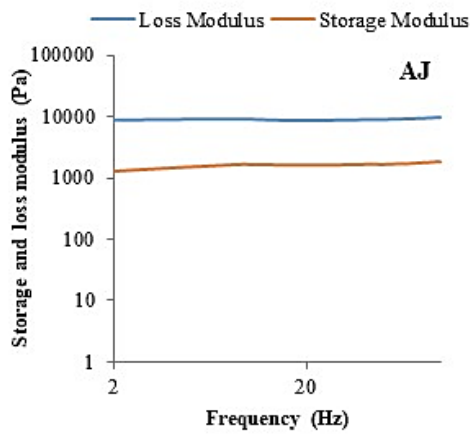
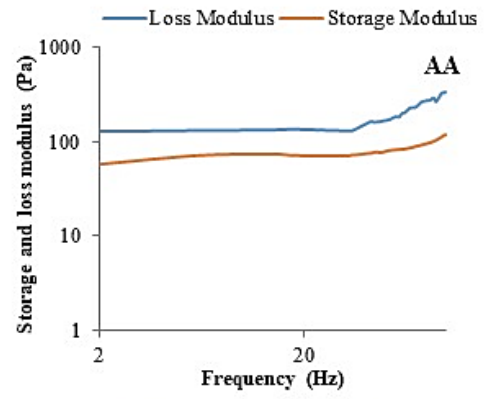
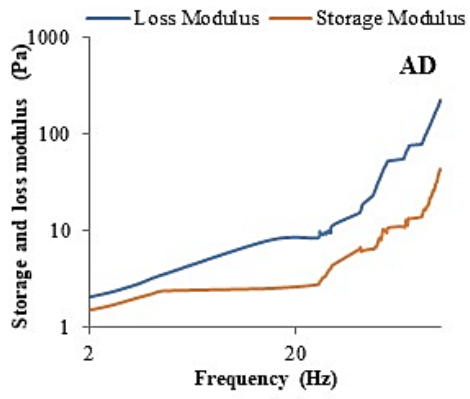
817 **Fig. 5.**

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838 **Fig. 6.**

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863 Fig. 7.

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