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Running title:

Sodium alginate from *Cystoseira schiffneri*

Abstract

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

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

1. Introduction

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

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

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

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

2. Material and methods

2.1. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH•), butylated hydroxyanisole (BHA), ethylene diamine tetra-acetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-disulfonic acid monosodium salt hydrate (ferrozine), Folin-Ciocalteu's reagent, phloroglucinol, glucose, guluronic acid, sodium carbonate (Na_2CO_3), hydrochloric acid (HCl), ferrous chloride ($FeCl_2$), 3,5 dimethylphenol, trichloroacetic acid (TCA), potassium ferrocyanide ($C_6FeK_4N_6$), sulfuric acid (H_2SO_4), acetone, methanol, chloroform, 95% ethanol and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample collection and preparation

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

2.3. Sodium alginate extraction

Crude sodium alginate extraction was done as previously described by Sellimi et al. (2015). *C. schiffneri* powder (50 g) was defatted and depigmented using maceration twice (24 h, 30°C, 200 rpm) in acetone:methanol (7:3, v:v) (500 ml) followed by chloroform (300 ml). Depigmented and defatted powder was air-dried, then demineralized by incubation (2 h, 60°C, 250 rpm) in 1 l 0.1 M HCl (pH 2). After cooling at room temperature, the residues were recovered using centrifugation (20 min, 4000 ×*g*) in a Rotofix 32 centrifuge (Hettich, Tuttlingen, Germany), washed with distilled water until neutral pH and then treated in 3% Na_2CO_3 solution (pH11) for 2 h at 60°C and 250 rpm to solubilize alginate in the sodium salt form. After centrifugation (20 min, 4000 ×*g*), the supernatant was recovered and two volumes of absolute ethanol were added. Mixtures were left for 12 h at 4°C to precipitate sodium alginate. The precipitated sodium alginate was suspended in distilled water and acidified using 6 M HCl (pH 3) to allow gelation and thereby precipitation of alginate in its acidic form. Precipitated alginic acids were recovered using centrifugation (20 min, 4000 ×*g*), then suspended in distilled water and neutralized to pH 7.5 using 6 M NaOH. The crude sodium alginate was precipitated using two volumes of absolute ethanol, dissolved again in distilled water and lyophilized (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France). The crude alginates extracted from *C. schiffneri* collected at December, April, July and September were referred to AD AA AJ and AS, respectively.

2.4. Chemical analysis

The total sugars were determined using the method of Dubois et al. (1956). Briefly, to 0.3 ml CSSA solution (0.1 g/ml), 1 ml 5% phenol solution and 5 ml 12 NH_2SO_4 were added. The mixture was incubated for 20 min at 30°C then the optical density was measured, using a spectrophotometer (T70 UV-visible spectrometer PG Instruments Ltd., Lutterworth, England), at 490 nm against a standard curve prepared using glucose.

The uronic acids (U) were determined using the method of Scott (1979). To 0.3 ml CSSA solution (0.1 g/ml), 5 ml 12 N H_2SO_4 and 0.3 ml of a solution containing 20 g/l NaCl and 30 g/l H_3BO_3 were added. The mixture was incubated at 70°C for 40 min, then cooled to room temperature for 1 h before adding 0.2 ml 3,5-dimethylphenol. After 10 min at room temperature, the absorbance was measured at 400 and 450 nm against a standard curve of galacturonic acid. U (%) were calculated using equation 1.

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

Where ΔA = the difference in absorbance, V = the total solution volume (ml), D = the sample dilution, Cs = the standard concentration, ΔAs = the difference in the absorbance of the standard (100 μg/ml), m = the mass of the test sample (mg), and 0.91 is the conversion factor of the experimental determination of monosaccharides to polysaccharides.

Total phenolics were determined using slightly modified methods described by Cicco et al. (2009). Phloroglucinol was used as the standard. Briefly, 100 μl of sample solution (2 mg/ml) was mixed with 100 μl 2 N Folin-Ciocalteu's reagentand 800 μl 5% Na_2CO_3solution. The mixture was incubated at 40°C for 20 min, then absorbancies were measured at 760 nm against a standard curve of phloroglucinol.

2.5. Micro-elementary analysis

Elemental composition of CSSA was done using an EDX analyzer (X-Max ^{N} SDD EDX Instrument, Oxford, UK). Results were treated using Oxford AZTEC software 2011 (Oxford Instruments, Abingdon, UK). The samples were metalized previous to analyses using a Quorum Technologies metalizer (Quorum Technologies, Guelph, ONT, Canada).

2.6. Circular dichroism

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

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

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

2.7. ATR-FTIR spectroscopy

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

2.8. NMR spectroscopy analysis

^{1}H-NMR spectra were obtained using a Bruker 300 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) at 60°C. Results were treated using Mestre Nova (MNova) software version 5.3.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). Samples were deuterium-exchanged using lyophilization with D_2O and then studied as 1% (m/v) solutions in D_2O (99.96%). Signals in the anomeric region (4.4-5.1 ppm) were studied to determine the CSSA structure. Therefore, F_M, F_G, F_{GG}, F_{MM}, F_{GM} and F_{MG} blocks of CSSA were calculated based on the areas of signals (I), (II) and (III) (Fertah et al., 2017) and using equations 3, 4,5, 6 and 7. In addition, M/G ratio can be calculated based on the equation 8.

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

$$F_M = 1 - F_G \quad (4)$$

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

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

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

$$M/G = F_M/F_G \quad (8)$$

2.9. MM distribution

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

2.10. Thermogravimetric analyses

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

2.11. Rheological analysis

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

2.12. Antioxidant activity

2.12.1. DPPH• radical-scavenging activity

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

2.12.2. Reducing power

Ferrous reducing activity was measured as previously described by Yildirim et al. (2001). Sample solutions were prepared at different concentrations from 50 to 500 µg/ml. Sample solution (0.5 ml) was added to 1.25 ml 0.2 M sodium phosphate buffer (pH 6.6) and 1.25 ml 1% (m/v) potassium ferrocyanide. Mixtures were incubated for 30 min at 50°C. After incubation, 1.25 ml 10% (m:v) TCA was added and the mixture was centrifuged at 11000 ×*g* for10 min. From each supernatant, 1.25 ml was transferred and diluted with 1.25 ml ultrapure water. Finally, $FeCl_3$ (250 µl) were added and absorbancies were measured at 700 nm after 10 min incubation at room temperature. Blanks without $FeCl_3$ were prepared for each concentration. The reducing power is shown as the extract concentration ($EC_{0.5}$) providing 0.5 absorbance at 700 nm.

2.12.3. Fe^{2+} chelating ability

The CSSA capacity to complex ferric ions were measured using the method reported by Carter (1971). Sample solutions were prepared at different concentrations from 0.1 to 1 mg/ml. Briefly, 100 µl of sample solution, 50 µl 2 mM $FeCl_2$ and 450 µl distilled water were

mixed and incubated for 3 min at room temperature. Volumes of 200 µl 5 mM ferrozine were added and mixtures were incubated again at room temperature for 10 min before measuring absorbancies at 562 nm. Blanks without ferrozine and control without sample were prepared. Results of metal (Fe^{2+}) chelating ability are shown by IC_{50} values (µg/ml), defined as the extract concentration needed to chelate 50% Fe^{2+}.

2.13. Statistical analysis

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

3. Results and discussion

3.1.Extraction yield

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

3.2. Chemical analysis

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

3.3. Elementary analysis

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

3.4. FTIR spectroscopy

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

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

3.5. Circular dichroism

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

3.6. MM distribution

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

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

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

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

3.7. NMR spectroscopy analysis

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

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

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

3.8. Thermogravimetric analysis

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

3.9. Rheological properties

3.9.1 Rotational analysis

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

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

3.9.2. Oscillatory analysis

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

3.10. Antioxidant activity

3.10.1. DPPH• radical-scavenging ability

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

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

3.10.2. Reducing power

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

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

3.10.3. Fe^{2+} chelating ability

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

4. Conclusion

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

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

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Conflicts of interest

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

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Table 1. Extraction yield(g/100 g DM) and chemical composition (g/100 g DM) of the CSSA fractionsfromdifferent seasons.

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

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

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

Method	Season			
	AD	**AA**	**AJ**	**AS**
FTIR	0.062	0.064	0.065	0.062
Circular dichroism	0	0.022	0.012	0.015
NMR	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.
670

Table 3. Average molecular mass (g/mol) of the CSSAfractions from different seasons.

	Season			
	AD	**AA**	**AJ**	**AS**
$\mathbf{M_n}$	7.62×10^5	8.02×10^4	1.26×10^5	7.85×10^2
M	1.23×10^6	1.16×10^5	2.33×10^5	4.49×10^3
PDI	1.62	1.45	1.85	5.72

AD, AA, AJ and AS represent sodium alginates extracted from *C. schiffneri*collected in December, April, July and September, respectively;Mn, M, and PDI are the number-average molecular mass, the average molecular mass, and the polydispersity index, respectively.

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

Season	F_G	F_M	F_{GG}	F_{MM}	F_{MG}	F_{GM}
AD	0.91	0.09	0.88	0.06	0.03	0.03
AA	0.98	0.02	0.97	0.01	0.01	0.01
AJ	0.97	0.03	0.96	0.02	0.01	0.01
AS	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.
683

Table 5. Antioxidant activities ofthe CSSA fractionsfrom different seasons.

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

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

Figure captions

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

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

Fig.3. ^{1}H-NMR spectra of sodium alginates extracted from *C. schiffneri* collected in December (AD), April (AA), July (AJ) and September (AS).

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

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

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

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

Fig. 1.

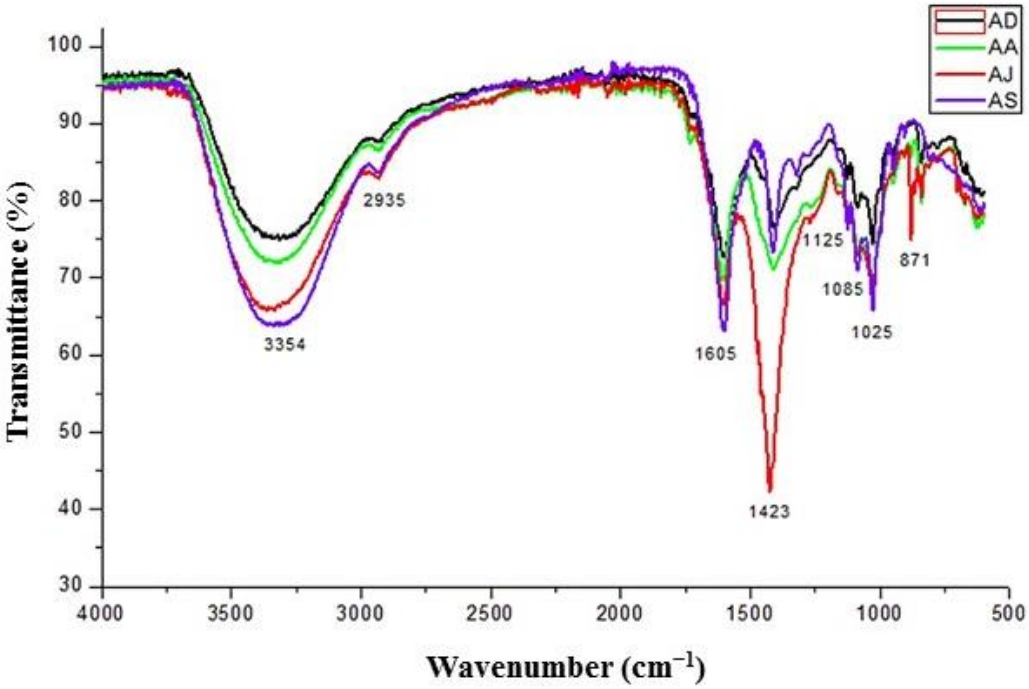
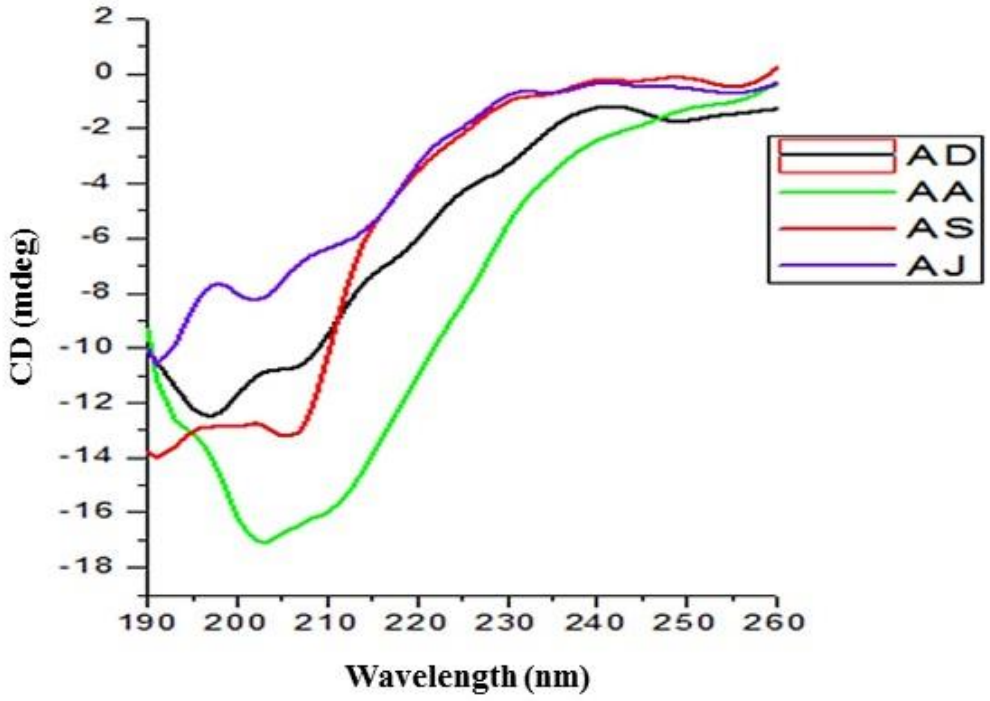

AD
AA
AJ
AS
Transmittance (%)
Wavenumber (cm^{-1})
3354
2935
1605
1423
1125
1085
1025
871

Fig. 2.


2
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CD (mdeg)
190
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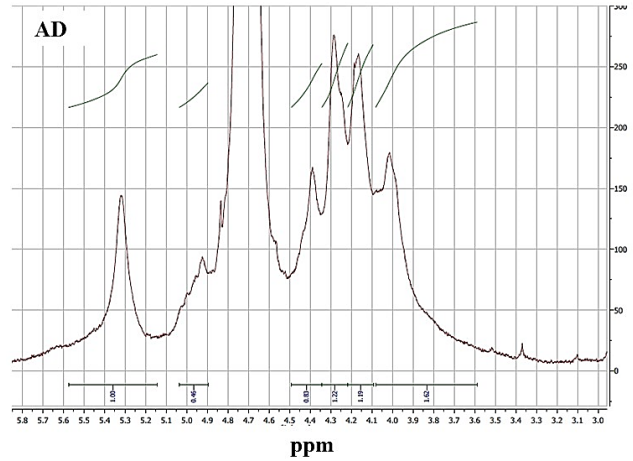
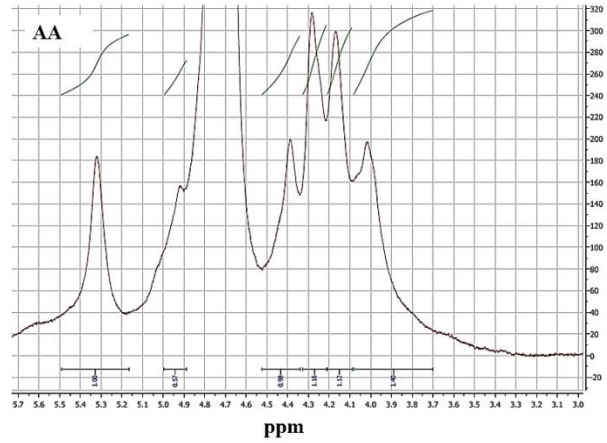
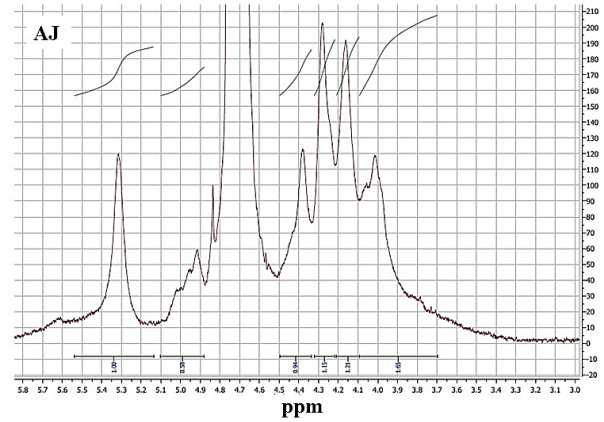
Wavelength (nm)
AD
AA
AS
AJ

Fig. 3.







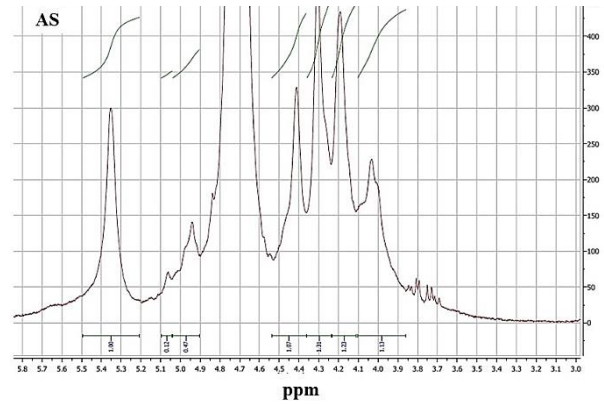


Fig. 4.

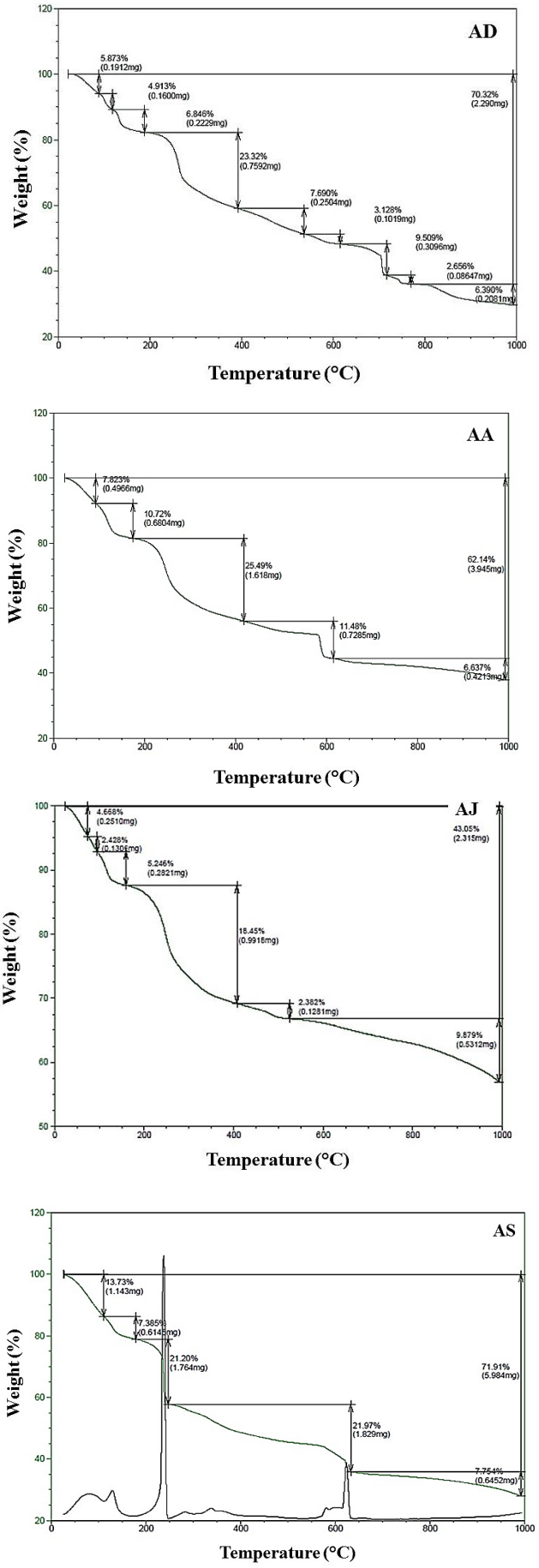

AD
5.873% (0.1912mg)
4.913% (0.1600mg)
6.846% (0.2229mg)
23.32% (0.7592mg)
7.690% (0.2504mg)
3.128% (0.1019mg)
9.509% (0.3096mg)
2.656% (0.08647mg)
6.390% (0.2081mg)
70.32% (2.290mg)
Weight (%)
Temperature (°C)
AA
7.823% (0.4966mg)
10.72% (0.6804mg)
25.49% (1.618mg)
11.48% (0.7285mg)
6.637% (0.4213mg)
62.14% (3.945mg)
Weight (%)
Temperature (°C)
AJ
4.668% (0.2510mg)
2.428% (0.1305mg)
5.246% (0.2821mg)
18.45% (0.9918mg)
2.382% (0.1281mg)
9.879% (0.5312mg)
43.05% (2.315mg)
Weight (%)
Temperature (°C)
AS
13.73% (1.143mg)
7.385% (0.6148mg)
21.20% (1.764mg)
21.97% (1.829mg)
7.754% (0.6452mg)
71.91% (5.984mg)
Weight (%)
Temperature (°C)

Fig. 5.

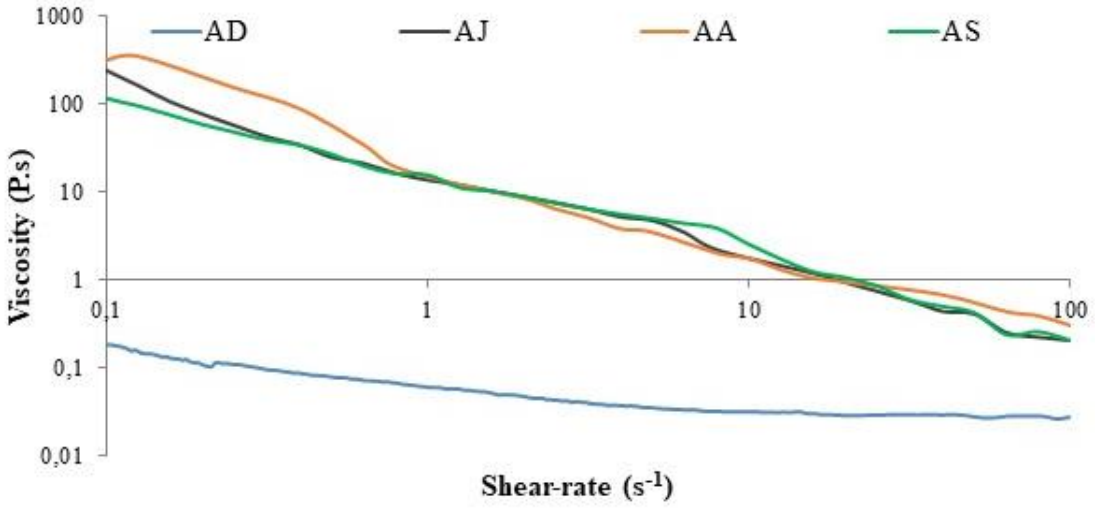

AD
AJ
AA
AS
1000
100
10
1
0,1
0,01
0,1
1
10
100
Viscosity (P.s)
Shear-rate (s-1)

Fig. 6.

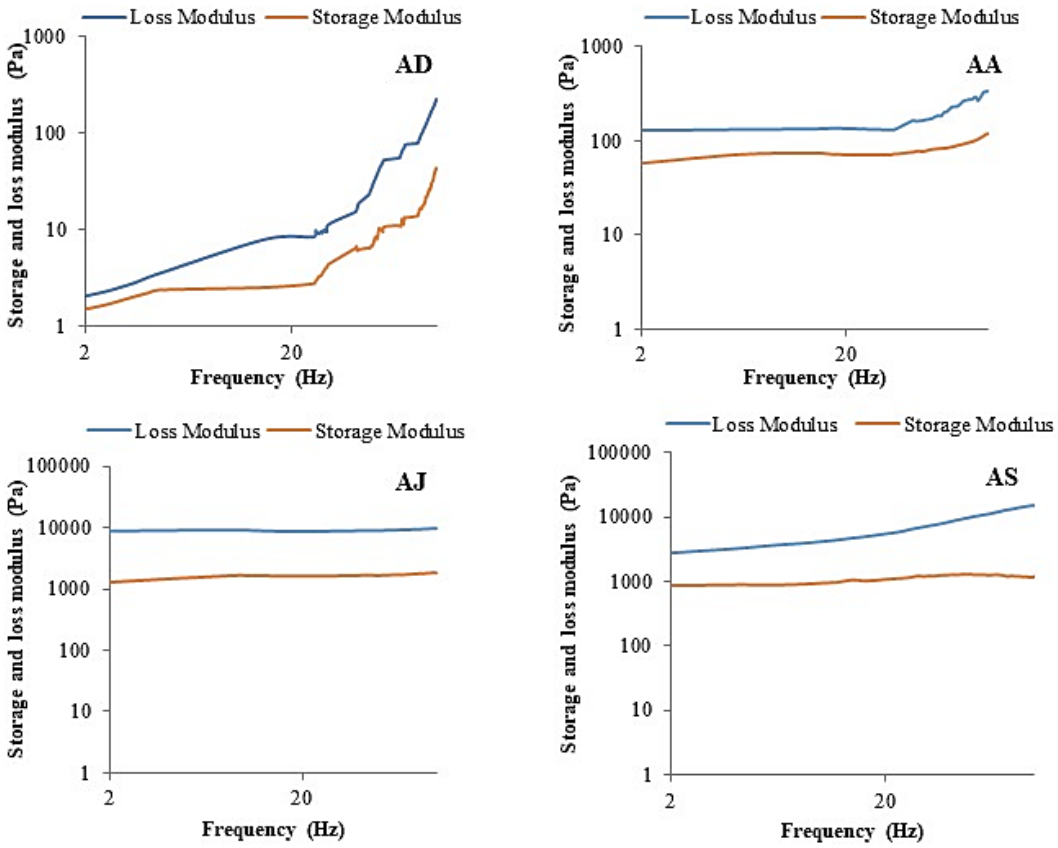
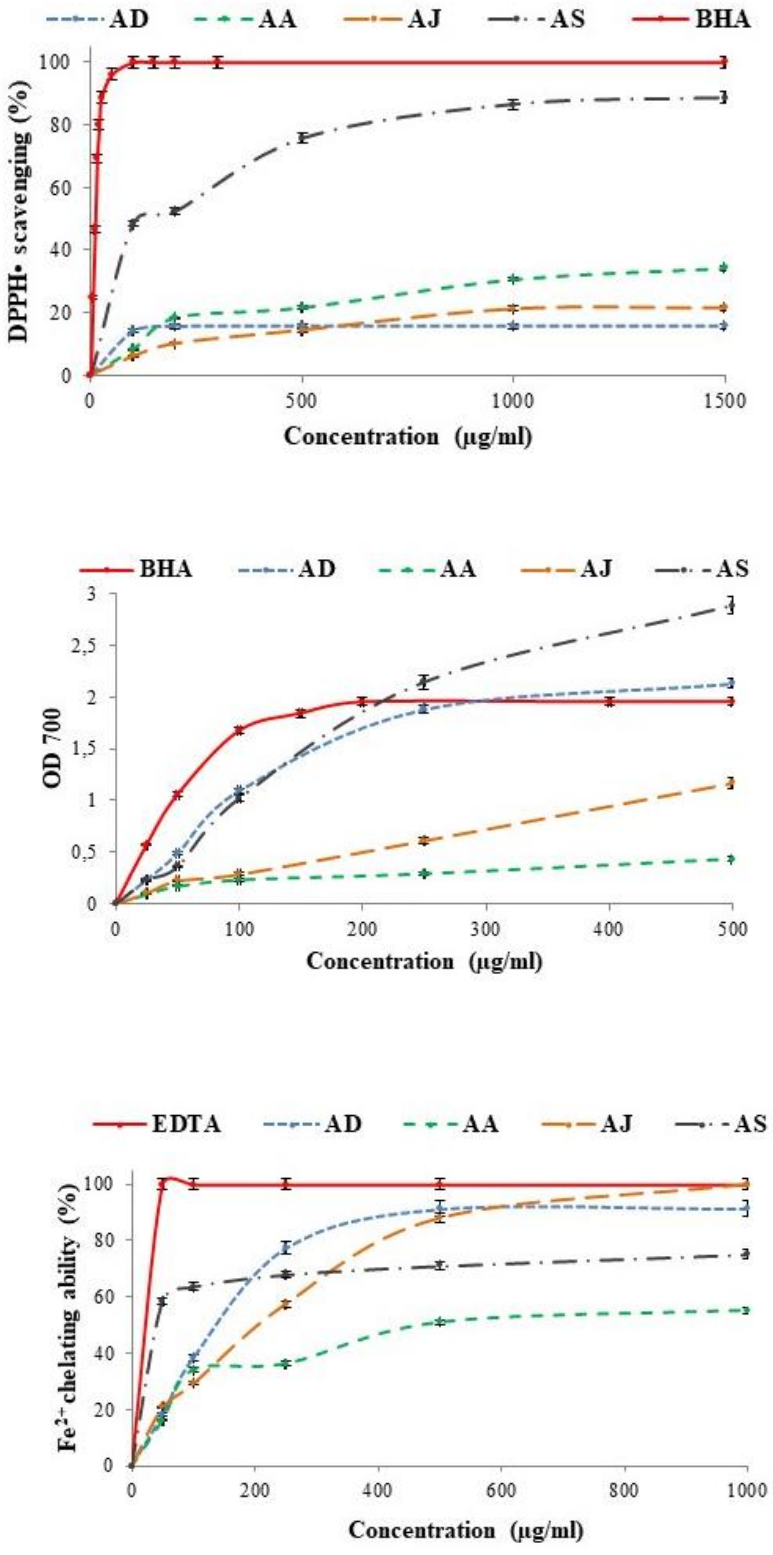

Loss Modulus
Storage Modulus
AD
AA
AJ
AS
Storage and loss modulus (Pa)
Frequency (Hz)
1
10
100
1000
10000
100000
2
20

Fig. 7.


AD
AA
AJ
AS
BHA
DPPH• scavenging (%)
Concentration (µg/ml)
BHA
AD
AA
AJ
AS
OD 700
Concentration (µg/ml)
EDTA
AD
AA
AJ
AS
Fe2+ chelating ability (%)
Concentration (µg/ml)