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# Extraction and structural characterization of the polysaccharide fraction of *Launaea acanthodes* gum

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ABSTRACT

In this work a physico-chemical characterization of the polysaccharide fraction of the exudates of *Launaea acanthodes*, a common medicinal species in central regions of Iran, was performed.

The extraction of the polysaccharide fraction of the exudates was described. The structure characterization of the polysaccharide was performed with mono and bi-dimensional NMR spectroscopy techniques, which allowed the chemical composition and the relative monomers abundance of the extracted fraction to be estimated. The constituent monosaccharides were predominantly galactose, rhamnose, arabinose and galacturonic acid residues. Interchain association phenomena in the main arabinogalactans component (Mw = 33.550 Da) were evidenced both from high-performance size-exclusion chromatography (HP-SEC–TDA), and by intrinsic viscosity ($[\eta]_0 = 0.323$ dl g$^{-1}$ in water and $[\eta]_0 = 0.115$ dl g$^{-1}$ in $NaNO_3$ 0.1 M at 20 °C) measurement and the Huggings coefficient $\lambda$. Due to the formation of hyperentanglements, a unique function of $C[\eta]_0$ cannot be defined. The technological consequence was that once structured, the polysaccharide network did not express a sufficient degree in polymer orientation and therefore cast films showed weak mechanical properties.



## 1. Introduction

*Launaea acanthodes* (Boiss.) O. Kuntze is an annual herb from the tribe *Lactuceae* and family *Asteraceae* (*Compositae*) which has glabrous bushy and branch stems (Ghahreman, 1986). It is a resistant plant, native in semiarid regions in Iran and grows on Ultramafic soils of Central Iran (Ghaderian & Baker, 2007). The native names of this plant are Charkheh and Charkhak. The *L. acanthodes* is a common medicinal species in central regions of Iran.

This plant exudates a white sap which is obtained by incising the stems close to the ground and, once in contact with air, becomes an opaque yellow matter. It is generally harvested in lumps, consisting of large and irregular masses of a yellowish color and composed of agglutinated tears that have a waxy-like appearance with an acrid taste. Diluted ethanol is its best solvent. Native names of these exudates in the gum-resin form are "Maghal" and "Molke-azragh". The gum-resin exudates has been in traditional pharmaceutical practice in Iran for many centuries, by both oral and topical administration, for "lumbago", "backache" and "footache" (Aynehchi, 1991). Exudates are also widely used for the treatment of gastrointestinal disorders and in wound healing, gastric and gastro duodenal ulcers.

The exudates of this botanic family contain low and relatively high molecular weight compounds. Hot water extract includes alkaloids, terpenoids, saponins and flavonoids, whereas the residue includes proteins, tannins and polysaccharides (Nergard et al., 2004).

Wamegh, Aeinehchi, and Yasa (1986) investigated *L. acanthodes* glycosides. Mahmudi and Yasa (1995) studied the structure of *L. acanthodes* flavonoides by chromatography techniques and separated two flavones. Other studies on this family indicate the presence of pectic polysaccharides: Huang, Gutterman, and Osborne (2004) showed the presence of pectin in *Artemisia sphaerocephala*. Nergard et al. (2005) isolated two polysaccharides, pectin and a pectic arabinogalactan from the dried powdered roots of Vernonia Kotschyana Sch. Bip. ex Walp.

A basic physico-chemical characterization of the polysaccharide fraction of the exudates has not been yet performed. The aim of this study was to isolate and characterize the chemical and rheological properties of *L. acanthodes* polysaccharides by light scattering, NMR spectroscopy and viscosimetry.

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## 2. Experimental

### 2.1. Materials

The *L. acanthodes* resin exudates were collected in a central region of Iran. Roots were cut from June to July and kept at 20 °C, in vacuumed plastic bags. All reagents used for the extraction process and characterization procedures were purchased from were Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Isolation and purification of polysaccharides

For the extraction of the polysaccharide fraction of *L. acanthodes* exudates, 10 g of exudate were finely grounded using a blender and then refluxed with ethanol using a Soxhlet apparatus for 24 h in order to remove low molecular weight compounds (impurities). The white powder residue was dried at room temperature and then solubilized and heated in 250 ml of distilled water at 50 °C for 2 h. A vacuum filtration step then followed, through gauze and Whatman grade 54 filter paper (Whatman, UK). The aqueous extraction was repeated on the filtration cake and the total filtrate fraction from the two filtering procedures was concentrated at 40 °C until 30 ml, using a Rotavapor apparatus (Heidolpoh VV2000) and then dialyzed by using a regenerate cellulose dialysis tube (Pierce, IL, USA) with a cut-off of 3500 Da. The dialyzed solution was then placed in a beaker; the volume was adjusted to 250 ml with distilled water and heated at 90 °C for 2 h under magnetic stirring. The procedure (filtration, concentration and dialysis) was then repeated to give a 90 °C crude extract. Eventually, the extract was freeze dried (Lyoflex 04, Edwards, USA) and the white powder obtained was stored in a 20 °C chamber (Sanyo, OMT oven, Analitica De Mori, Milan, Italy).

The yield of water-soluble polysaccharide from *L. acanthodes* was 33% with respect to the raw dry material.

### 2.3. Intrinsic viscosity determination of extracted polysaccharides

To prepare the polysaccharide solution (1.5% w/w), samples were thoroughly dispersed in distilled water or $NaNO_3$ 0.1 M solutions for 1 h at 40 °C to give the "mother solution". Thereafter the "mother solution" was diluted with the appropriate solvent. Only five diluted solutions from 0.31 g/dl up to 0.05 g/dl were considered for the intrinsic viscosity calculations. The diluted solutions were stored in a climatic room (Sanyo, OMT oven, Analitica De Mori, Milan, Italy) at 20 or 40 °C.

Viscosities of the solutions of polysaccharide fraction of the *L. acanthodes* exudate were determined according to the ASTM (D445-97) method (1997), from efflux times with a Cannon–Fenske capillary viscosimeters set (Rheotek, USA) immersed in a water bath (HAAK DC30, Thermo Electron Corporation, USA) at 20 or 40 °C, in order to maintain a constant temperature during the measurement. The kinematic viscosity ($\upsilon$) (cm $s^{-1}$) measurements were repeated four times for each solution tested. Densities ($\rho$) of solutions were measured using A&D densimeter (GF300, A&D Instrument LTD., Japan). From $\upsilon$ and $\rho$ the dynamic viscosity ($\eta$) (Pa s) was easily calculated as $\eta = \upsilon/\rho$. Intrinsic viscosities [$\eta$] (dl $g^{-1}$) and Huggings' coefficients $\lambda$ were obtained from the Huggings equation (Huggins, 1942):

$$\frac{(\eta_{sp})_0}{C} = [\eta]_0 + \lambda[\eta]_0^2 C \quad (1)$$

where $\eta$ = solution viscosity (Pa s), $\eta_{sp} = \frac{(\eta-\eta_s)}{\eta_s}$ is the specific viscosity, with $\eta_s$ = solvent viscosity (Pa s), $C$ = biopolymer concentration in the solution, [$\eta$] = intrinsic viscosity (dl $g^{-1}$). $\lambda$ = Huggins coefficient, index of the degree of polymer–polymer interaction in dilute conditions, and of the extent of coil expansion of the polymer coil.

The subscript "0" means that the specific viscosity is determined in the Newtonian domain.

### 2.4. Rheological properties

The flow behavior of the polysaccharide solutions 1.5% (w/w) in water, stirred at 40 °C for 1 h, was studied by means of a stress controlled rheometer SR5000 (Rheometric Scientific Inc.) equipped with a double wall cuvette. Steady shear tests were performed at 20 °C using a stress range of 0.015–0.1 Pa. Data are expressed as dynamic viscosity ($\eta$) (MPa s) versus shear rate ($\dot{\gamma}$) ($s^{-1}$) or shear stress ($\sigma$) (Pa) versus shear rate ($\dot{\gamma}$) ($s^{-1}$).

### 2.5. Nuclear magnetic resonance spectroscopy

$^1H$ and $^{13}C$ NMR spectra were recorded with a Bruker Avance 500 (Brucker, Inc., Germany) at 11.4 T, equipped with a TXI 5 mm probe at 313 K. The samples were solubilized in $D_2O$ with a polymer concentration of 10 mg/ml for the $^1H$ NMR and $^1H/^{13}C$-HSQC experiments and 200 mg/ml for the $^{13}C$ NMR experiments.

### 2.6. High-performance size-exclusion chromatography triple detector array (HP-SEC–TDA) analysis

The HPLC equipment consisted of a Viscotek system equipped with a VE1121 Solvent Delivery pump, a metal free two channel on line degassing device Gastorr 150. The detector system used in this study was a Viscotek mod.302 Triple Detector Array. Right Angle/Low Angle (8°) Laser Light Scattering is the first detector after the columns, with the following technical specifications: cell volume of 10 μl; maximum backpressure on cell of 15PSI; maximum signal of 2.5 V; a 670 nm laser light source. Refractive index (RI) is the second detector with the following technical specifications: cell volume of 12 μl; maximum backpressure on cell of 5PSI; maximum signal of 10 V; light emitting diode (LED) at 660 nm wavelength. Viscometer is the last detector, characterized by four capillaries (0.01″ id × 24″ L) with a differential Wheatstone bridge configuration.

Two TSK gel columns in series (GMPWXL mixed bed column, 7.8 mm ID × 30 cm, Viscotek, $V_0$ = 6 ml, $V_t$ = 11 ml each one) were used. Columns, injector and detectors were maintained at 40 °C. An aqueous solution of 0.1 M $NaNO_3$ pre-filtered on 0.22 μm filter (Millipore) was used as mobile phase at a flow rate of 0.6 ml $min^{-1}$. The system was calibrated with the PEO narrow and broad standards of known Mw, polydispersity and intrinsic viscosity. The narrow standard is particularly useful for determining detector volume offsets and peak broadening parameters.

Analysis of data was performed with Viscotek TriSEC software, version 3.0 using the d$n$/d$c$ value of 0.146 reported in the literature (Sanchez et al., 2008).

For HP-SEC/TDA analyses, samples were dissolved in a 0.1 M $NaNO_3$ aqueous solution to final concentration of 10 mg/ml. The run time was of 60 min.

### 2.7. Preparation of polysaccharide film and mechanical elongation test

Thin films (75 μm thickness) of the isolated polysaccharides were produced by the casting procedure from 1.5% (w/w) water solutions stirred at 40 °C for 1 h. Sixteen grams of the filmogenic solution were poured in standard polypropylene Petri dishes and solvent evaporation was performed in a climatic room (HC0020, Heareus Votsch) at 55% R.H. and 35 °C for 15 h.

Film stripes were cut (50 mm × 15 mm) and their mechanical behavior was tested in tensile mode by means of a Texture Analyzer TAXTplus (Stable Micro System, Godalming, UK). Test speed

was 1 mm $min^{-1}$. Results were expressed as: stress at break ($\sigma_{max}$) (MPa); Young modulus ($E$) (MPa); work at break ($W$) (MJ $m^{-3}$); elongation at break ($\varepsilon$) (%). All parameters were considered for a statistical multivariate analysis that was performed in order to delete the outliers from 15 replicates performed. A PCA protocol was implemented using the software SCAN (Minitab Inc., USA). On the plane composed by the first two principal component PCs, the outliers were deleted qualitatively by visual inspection. Finally, 10 replicates were considered.

### 2.8. Scanning electron microscopy

Microscopy investigation was performed by using dried strips fragments of films which were positioned on specimen stubs with cross-section oriented up and coated with a thin layer (nm) of gold by DC sputtering (AGAR B 7340, Agar Scientific Ltd., Stansted, UK). Digital images of film cross-section were collected by using a LEO EVO 40 scanning electron microscope (Zeiss, Oberkochen, Germany) at a tilt angle of 0° to the electron beam, with a 20 kV acceleration voltage.

## 3. Result and discussion

### 3.1. Isolation and structural characterization of polysaccharide fraction

The structure characterization of the polysaccharide was performed with mono and bi-dimensional NMR spectroscopy techniques, which allowed to estimate the chemical composition and the relative monomers abundance of the extracted fraction.

The assignments of anomeric signals in $^{13}C$ spectrum are reported in Figs. 1 and 2, and were made as described earlier (Duan, Wang, Dong, Fang, & Li, 2003; Polle, Ovodova, Shashkov, & Ovodov, 2002).

These analyses allowed to evaluate the efficiency of the extraction and purification home-made process that was developed. The procedure provides satisfactory results since the extracted fraction is not contaminated by proteins and chemical compounds coming from the raw resin. Nevertheless, in Fig. 1 is it possible to point out the presence of ethanol due to the Soxhlet purification step.

### 3.2. HP-SEC–TDA analysis

High-performance size-exclusion chromatography (HP-SEC) with different columns and eluents, represent the most commonly used method for the measurement of molecular weight and size distribution of polysaccharides. One of the advantages of a triple detector array (TDA) assembly is that chromatographic calibrations are not necessary. The elaboration of the light scattering signals (Right Angle Light Scattering – RALS and Low Angle Light Scattering – LALS) and concentration detector (RI) responses gives the weight average mean molecular weight (Mw) and the number-average mean molecular weight (Mn), while the viscosity detector provides the intrinsic viscosity ($[\eta]$) values. Moreover, it is possible to obtain structural information about the hydrodynamic (Rh) and gyrational (Rg) radius as described in the literature (Bertini, Bisio, Torri, Bensi, & Terbojevich, 2005).

The RALS and LALS chromatographic profiles (Fig. 3) show the presence of aggregated structures ($V$ eluted 12 ml) that are excluded from the calculation of the molecular weight distribution. The molecular weight values Mn = 18,300, Mw = 33,500, the intrinsic viscosity $[\eta]$ = 0.096 (dl $g^{-1}$), the hydrodynamic radius Rh = 3.35 (nm) and the radius of gyration Rg = 4.50 (nm) were obtained for the polysaccharide with OMNISEC software data analysis.

The chromatograms show a bimodal distribution: the rise in the light scattering peaks with only a moderate viscosity rise, compared to the refractive index peak, indicates a higher ratio of molecular weight to intrinsic viscosity of the early eluting species. It could be concluded that the sample is branched.

The low values of the radius account for a structural compactness of the macromolecules under the analysis conditions that were adopted. This result is confirmed from the Mark–Houwink (Fig. 3) plot obtained through the double logarithmic plot of intrinsic viscosity versus molecular weight shown in Fig. 3 obtained using HP-SEC–TDA. Regression analyses provided scaling exponent of 0.5 and log $k$ value of −3.00, in good agreement with the value reported by Sanchez et al. (2008) and that shows a random-coil conformation and a high structural density.

### 3.3. Viscosimetric characterization

Rheological measurements were performed to further evaluate the structure of the macromolecular system under study according to fundamental rheological interpretations that are lacking in the case of the polysaccharides extracted from *Launaea alcantodes* exudates. In specific, the viscous properties in the Newtonian domain of the arabinogalactan from *L. alcantodes* were determined and results were discussed within a theoretical framework.

Flow behavior of the extracted arabinogalactans was evaluated for 1.5% w/w water solution at 20 and 40 °C. The polymer solution is Newtonian in flow, with a viscosity equal to 1.60 and 1.25 MPa s, respectively, at the two temperatures investigated.

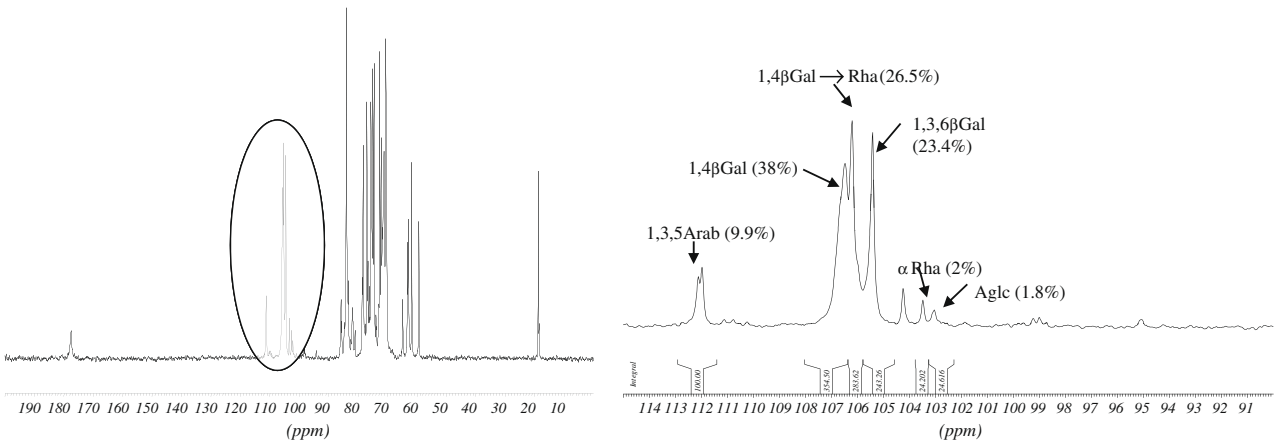


**Fig. 1.** $^{13}C$ NMR spectra of the extracted polysaccharide fraction.

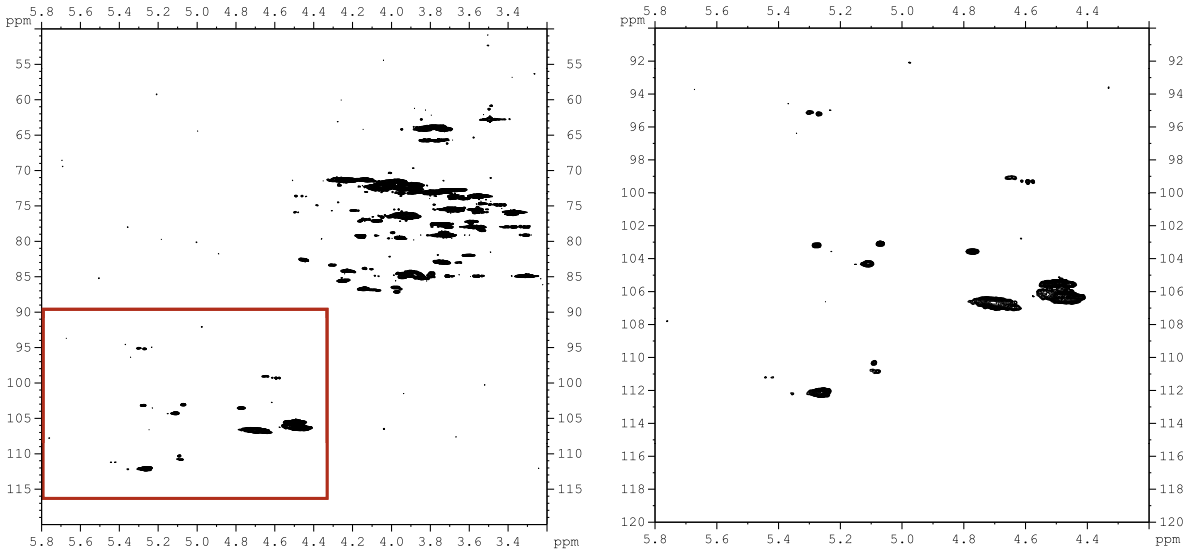


**Fig. 2.** NMR bi-dimensional analysis (HSQC) of the extracted polysaccharide fraction.

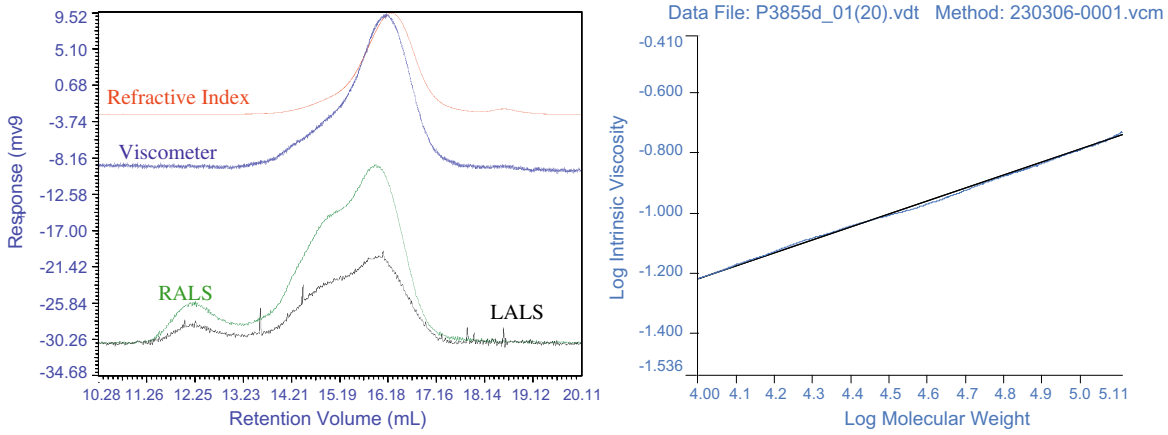


**Fig. 3.** HP-SEC–TDA chromatogram in 0.1 M $NaNO_3$, $T$ 40 °C and Mark–Houwink plot.

The intrinsic viscosity $[\eta]_0$ of the extracted arabinogalactan in water solution domain was then studied in the shear rate Newtonian at 20 °C by applying the Hugging equation (1).

The calculated value of the hydrodynamic volume is $[\eta]_0 = 0.323$ dl $g^{-1}$ and the value of the Huggings coefficient $\lambda$ is higher than 1 ($\lambda = 1.42$), confirming the interchain association phenomena that were previously evidenced.

A new solvent for the polysaccharide, $NaNO_3$ 0.1 M, the same used for the previous structural characterization, was then tested and the calculation of the intrinsic viscosity were repeated both at 20 and 40 °C. The intrinsic viscosity $[\eta]_0$ reduces in presence of salt concentration ($[\eta]_0 = 0.115$ and 0.102 dl $g^{-1}$ at 20 and 40 °C, respectively), as expected for a polyelectrolyte.

The decrease of $[\eta]_0$ is considered to be due to changes in solvent quality that also adjust the Huggings' coefficients ($\lambda$): this index varies according to solvent composition but remains higher than in water solutions of arabinogalactans. A general trend for polysaccharides is a decrease in $\lambda$ when the expansion factor $\alpha$ increases (Launay, Cuvelier, & Martinez-Reyes, 1997). Moreover, it is generally accepted that a modification in $[\eta]_0$ caused by excluded volume effects is directly related to the expansion factor $\alpha$:

$$\alpha = \left(\frac{[\eta]_0}{([\eta]_0)_\Theta}\right)^{\frac{1}{3}} \quad (2)$$

where $[\eta]_0$ is the intrinsic viscosity and $([\eta]_0)_\Theta$ is the intrinsic viscosity in theta conditions.

Lower values of $\alpha$ should then expected for the AG in $NaNO_3$ 0.1 M solution with respect to solutions in water, as it was concluded from the analysis of the Huggings coefficient $\lambda$.

Premises so far given let therefore to conclude that, due to the high Huggings' coefficient values, the viscosity data do not obey the master curve based on the "corresponding state principle" (Simha & Zakin, 1960) that relates the Newtonian specific viscosity $(\eta_{sp})_0$ to the reduced concentration $C[\eta]_0$ (Figs. 4 and 5).

It is evident that the master curves that are here presented are limited to the dilute regime of concentration (maximum of reduced concentration = −1.5), that is when $C < C^*$, where polymer coils have "infinite dilution" dimensions. Results, therefore, show that $(\eta_{sp})_0$ depends on the arabinogalactan concentration jet in the dilute regime: it can then be assumed that chain dimensions have not attained their unperturbated values, but a solvent-dependence exists that can be justified in terms of the expansion coefficient.

The master curve slopes are equal to 1.42, 1.28, 1.5 for AG solutions in water at 20°, in $NaNO_3$ 0.1 M at 20 °C and in $NaNO_3$ 0.1 M at 40 °C, respectively. Typically, in the dilute regime the slope of the master curve is in the range 1.2–1.3 (Launay et al., 1997). The fairly high values of the master curve slopes that are here

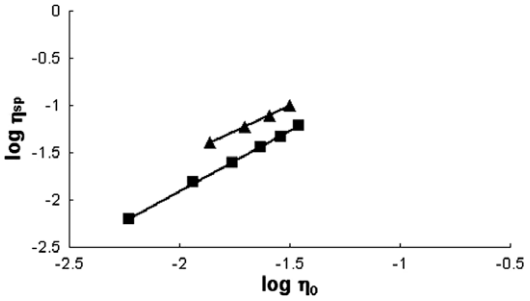


**Fig. 4.** Zero shear rate specific viscosity versus reduced concentration for AG in water (triangle) and $NaNO_3$ (square) at 20 °C.

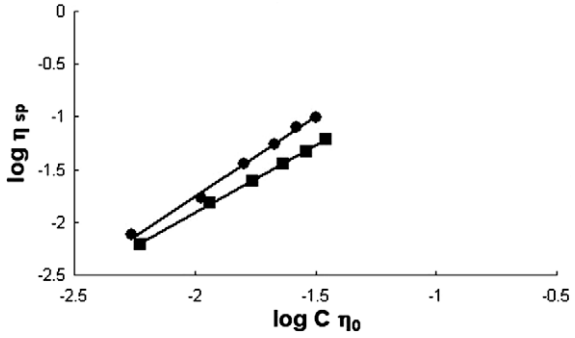


**Fig. 5.** Zero shear rate specific viscosity versus reduced concentration for AG in $NaNO_3$ at 40 °C (round) and $NaNO_3$ at 20 °C (square).

obtained for the extracted arabinogalactan account for the non-superimposed-results on the $\log(\eta_{sp})_0 - \log C[\eta]_0$ master curves: it has been claimed (Launay et al., 1997) that slopes higher than 1.5 could be explained by the creation of hyperentanglements.

In addition, due to purely topological entanglements, further intermolecular association could be formed between unsubstituted regions (Morris, Culter, Ross-Murphy, Ress, & Price, 1981). Therefore, for the different arabinogalactans solvents that have been used in this study, $(\eta_{sp})_0$ is no longer a unique function of $C[\eta]_0$ if the interaction parameter $\lambda$ varies.

A further observation is that $(\eta_{sp})_0$ is temperature dependent. Order–disorder transition of AG chains may have occurred upon heating. Specific viscosity reflects changes not only in conformation and size, but also in intermolecular interactions. It provides simple means to probe changes at supermolecular scales in diluted polymer solutions (Lefebvre & Doublier, 2005) as a function, in this case, of temperature.

Summarizing, the assessment of the rheological behavior of arabinogalactan in diluted solutions validates the structural evaluations performed by means of HP-SEC–TDA analysis. It can be concluded that the proved resistance to solubilization of arabinogalactan from *L. acanthodes* exudates could influence the polymer networking capability.

### 3.4. Mechanical properties and electronic microscopy analysis

In view of planning possible technological application, the processability of the isolated polysaccharides was therefore tested in terms of behavior under mild conditions of thermal stress. Following the casting film-making procedure, the solvent was evaporated from the aqueous solutions in a convective air climatic chamber at 55% R.H. and 35 °C. The film formation by casting procedure is a useful way to investigate the structuring ability of the arabinogalactan extracted from the *L. acanthodes* exudates. In the context of this work, the film making trials have no other practical application reasons. Upon dehydration, the arabinogalactans tailor a continuous network and result in an opaque film whose morphology was observed by means of electronic microscopy and whose mechanical properties were evaluated in terms of tensile breaking stress. The three-dimensional structure is fragile, with a feeble breaking resistance: average breaking stress ($\sigma_{max}$) is equal to 0.96 (±0.24) MPa, Young modulus ($E$) 0.39 (±0.16) MPa, work at break ($W$) 2.22 (±0.68) (MJ $m^{-3}$) and elongation to break ($\varepsilon$) 3.96 (±0.68) %. The SEM images justify these macro-scale evidences (Fig. 6): polymers are not well oriented and the polysaccharide network does not express sufficient degree of polymer orientation.

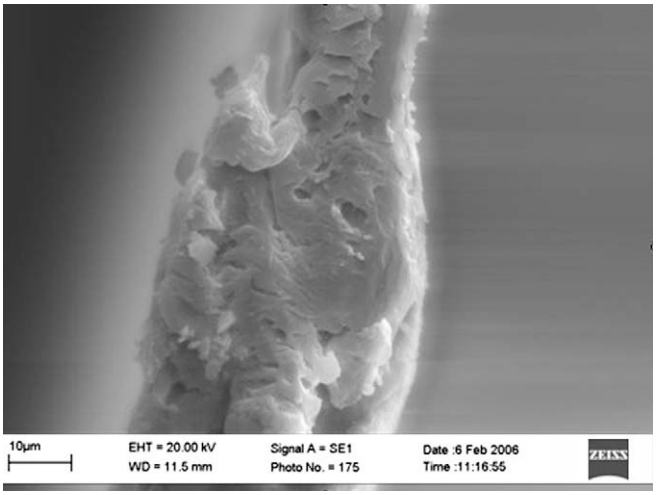


**Fig. 6.** SEM transversal section of the film obtained by casting.

## 4. Conclusions

The extract of *L. acanthodes* exudates contained several types of extractable polysaccharides, predominantly constituted by galactose, rhamnose, arabinose and galacturonic acid residues. Interchain association phenomena in the main arabinogalactans component were evidenced. Advanced viscosimetric investigations on this polysaccharide indicate that, a unique function of $C[\eta]_0$ cannot be defined because of formation hyperentanglements. The technological consequence is that once structured, the polysaccharide network does not express a sufficient degree of polymer orientation and therefore shows weak mechanical properties.

Analysis of the collected data reveals relevance regarding the comparison between a classical viscosimetric method plus data fitting with the Huggings equation and the recent HP-SEC–TDA approach in the determination of the intrinsic viscosity $[\eta]$ of the arabinogalactans under study: the $[\eta]$ values obtained in $NaNO_3$ 0.1 M, at 40 °C are 0.102 and 0.096 dl $g^{-1}$, respectively. Besides a structural characterization of a polysaccharide fraction from the exudates of a herbal material of great interest in Iran, the scientific impact of this work lies in the contribution in validation of the size-exclusion chromatography triple detector array, a high-performance biophysical technique for polysaccharide characterization.

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