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Physicochemical Properties of Pectin from *Retama raetam* Obtained using Sequential Extraction

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Abstract: Water-soluble polysaccharides were extracted sequentially from *Retama raetam*. Dried Alcohol-Insoluble Residues (AIR) of *Retama raetam* were treated sequentially with deionised water, ammonium oxalate and basic solutions and purified by ultrafiltration. The resulting pectin extracts analysed for some physicochemical parameters. The results show that pectin yield (62% dry AIR), uronic acid (260-640 mg g^{-1}), neutral sugars (320-540 mg g^{-1}), degree of methylation (20-31%) and acetylation (2-19%), molar mass (8. 93×10^{4}-3.42×10^{5} g mol^{-1}) and intrinsic viscosity (27- 47 mL g^{-1}) varied with the various extraction methods used. Extraction with ammonium oxalate solution gave the highest pectin yield, with high molar mass and degree of methylation.

Key words: *Retama raetam*, pectins, physicochemical properties, extraction conditions

INTRODUCTION

Polysaccharides have a number of applications in the pharmaceutical, cosmetic and food industries. These numerous applications justify the search for new sources of polysaccharide. Therefore, discovery and evaluation of new polysaccharides from plant becomes a hot research spot. In traditional medicine, extracts of polysaccharide containing plants are widely employed for the treatment of skin and epithelium wounds and of mucous membrane irritation (Bedi and Shenefelt, 2002).

Retama raetam, locally named as R'tm, is a wild plant belonging to the Fabaceae family. It is common to North and East Mediterranean regions. In Tunisia, it is largely abundant. Its abundance in arid area makes it a good candidate for industrial utilisation.

The plant flowers from April to May. The molecular and biochemical mechanisms associated with dormancy and drought tolerance in this desert plant have been elucidated. *Retama raetam* is prescribed for diabetes control and also for treatment of hypertension (Archer and Pyke, 1991; Taylor 1981; Maghrani *et al*., 2003, 2004).

The research carried out concerned on characterization of root-nodulating bacteria on *Retama raetam* (Mahdhi *et al*., 2008). The structural aspects of water-soluble galactomannans isolated from the seeds of *Retama raetam* have been investigated by Ishurd *et al*. (2004) while Kassem *et al*. (2000) focused their search on the extraction of two new flavonoids from *Retama raetam*. Maghrani *et al*. (2005) pointed out the effect of aqueous extract of *Retama raetam* in normal rats.

However, no data exist concerning the macromolecular properties of polysaccharides extracted from *Retama raetam*. The general aim of this research is to evaluate the impact of different extraction conditions on the yield and some physico-chemical characteristics of pectin isolated from *Retama raetam*.

MATERIALS AND METHODS

Materials: *Retama raetam* was harvested in the region of Monastir (Tunisian Sahel) at the flowering period (April). All other chemicals and solvents used were of analytical grade.

Extraction of pectin: The fresh *Retama raetam* (500 g) was washed for 1 h in 4 L of boiling 95% ethanol and the mixture was then allowed to stir mechanically overnight at room temperature. After filtration through a G2 sintered glass funnel, the residue washed with ethanol (5×500 cm^3) and acetone (3×300 cm^3) - named AIS - was dried, to be used as stock material for extraction (Forni *et al*., 1994). They were extracted with hot distilled water for 4 h at 70°C. The residue (R1) will be treated later on during the extraction with oxalate ammonium while the filtrate was precipitated by adding an excess of ethanol (40:60 v:v) 24 h at 4°C. The precipitate was collected by centrifugation (3000 rpm, 20 min) and respectively washed

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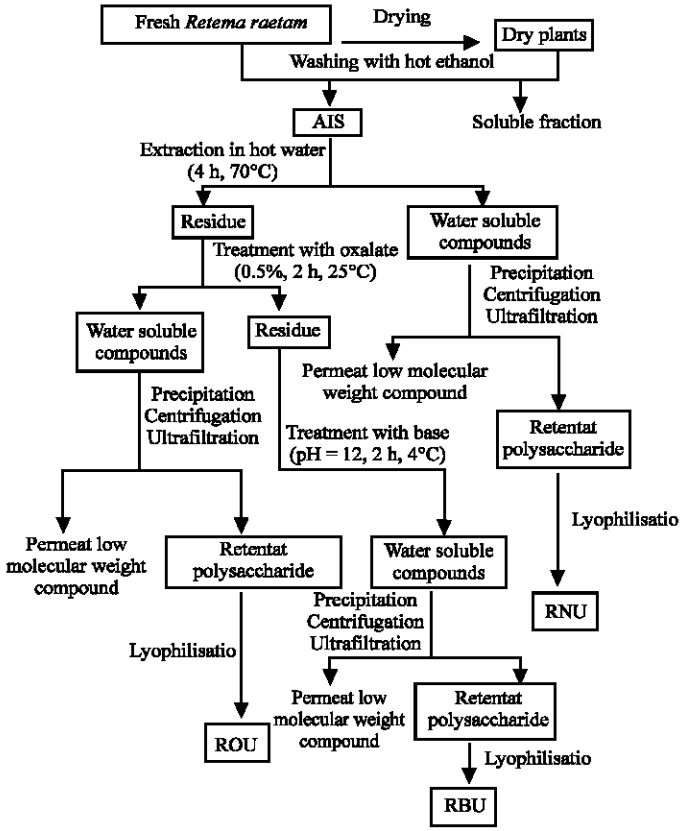

Fig. 1: Procedure diagram used for extraction and purification of water soluble polysaccharides from *Retama raetam*

several times with 70% ethanol and finally with acetone and then vacuum-dried at, giving a powder RNU. The residue (R1) is treated with the oxalate $K_2C_2O_4.H_2O$ (0.5%) at 25°C, after filtration, precipitation and freeze-dried, we obtained ROU. The residue (R^2) is treated in basic medium with (NaOH; pH = 12, t = 2 h, 4°C) to give product RBU (Fig. 1).

Uronic acid and neutral sugars contents: The uronic acid contents of the peels, AIR and extracted pectins were determined colorimetrically by the automated m-phenyl phenol method (Thibault, 1979). Neutral sugars were analysed as their alditol acetate derivatives by Gas Liquid Chromatography (GLC) after acid hydrolysis. The pectin was hydrolysed with 1 M H_2SO_4 (3 h, 100°C). The peels and AIR were prehydrolysed with 13 M H_2SO_4 (30 min, 25°C), diluted to 1 M and heated (2 h, 100°C). The individual neutral sugars obtained were reduced with $NaBH_4$, acetylated and analysed by GLC according to (Harris *et al*., 1983).

Degree of methylation (DM) and acetylation (DAc): The degree of methylation (DM) is defined as the number of moles of methanol per 100 moles of galacturonic acid. The degree of acetylation (DAc) is defined as the percentage of galacturonosyl residues esterified with one acetyl group. DAc is generally low in native pectins, ranging between 3 and 15% (Voragen *et al*., 1995). The botanic origin and the extraction procedure determine the content of galacturonic acid, DM and DAc in pectins.

SEC-MALLS: Solutions were prepared by dissolving the polysaccharide samples overnight in 0.1 M $LiNO_3$. The pH was adjusted to the desired value by adding phosphate buffer (I = 0.025 mol L^{-1}; pH = 6.3). The concentration of solutions (0.1 wt.%) was chosen such that the overlap concentration of polymer C* was not exceeded, but large enough to give a measurable light scattering response.

The samples injected through a 100 μL loop, were eluted on a TSK-GEL GMPWXL column (Tosohaas) with a flow rate of 0.5 mL min^{-1}. The eluent and samples were filtered prior to use, through a 0.1 and 5 μm hydrophilic membrane (Millex® SV, Millipore) respectively. A MALLS detector (Dawn™ DSP, Wyatt Technology Co) coupled with a refractive index detector (Optilab, Wyatt Technology Co.) as concentration detector, was used to

obtain on-line determination of the absolute molar mass (M) and of the root mean square radius of gyration ($<r_g^2>^{0.5}$) for each elution fraction of ca 0.01 mL which allow the molar mass and size distributions to be calculated for the SEC profile using ASTRA software (Wyatt Technology Co.) (Podzimek, 1994). Well-adapted representations of experimental data such as $<r_g^2>^{0.5}$ versus M for example, $<r_g^2>^{0.5} = K. M^x$ (Eq. 1) give information about polymer conformation in solution. Zimm plot (first-order fitting) of the data collected for each slice (Zimm, 1948), observed to be linear over a large range of molar mass, was used to determine M and $<r_g^2>^{0.5}$ from extrapolation of the intercept and the initial slope.

Treatment of the light scattering data and determination of the polysaccharide concentration in each fraction eluted requires an accurate knowledge of the refractive index increment of pectin samples in a given eluent, which were determined at 25°C using the interferometric refractive index detector (Optilab-DSP, Wyatt Technology Co.) equipped with a P2L cell operating at the same wavelength as the light scattering laser (632.8 nm).

Polyelectrolyte behaviour: Titration of the different samples was carried out after conversion of the carboxylic residues under their acidic form to determine the Acid Equivalent Weight (AEW) related to the content of free acid moieties. In fact, a fraction of the carboxylic acid functions present in pectins is naturally esterified with O-methyl groups. So, the discrepancy between the content of galacturonic residues inferred from the sugar composition and that from the potentiometric titrations is indicative of the methylation degree of galacturonic acid functions.

Viscosity measurements: Intrinsic viscosity is another important feature of the polymer behaviour because it is related to the dimension in solution and the molar mass of macromolecules. As the polysaccharides extracted are polyelectrolytes, repulsion between charged segments affects the dimension in solution that is to say that the viscosity will depend strongly on the ionisation degree of the chain. In order to minimise the effect of dilution upon ionisation, measurements were performed at high ionic strength (NaCl) to shield the anionic charges of macromolecules.

RESULTS AND DISCUSSION

Extraction yields: The yields were calculated in comparison with the corresponding mass of dry plants. Lipids and pigments, were removed by the ethanolic treatment (Selvendran and Du Pont, 1980).

From the extraction data in Table 1, it appears that the yield of *Retama* pectin extraction varied from 4-12% dry weight of AIR, depending on the extraction condition used. The highest yields were obtained with oxalic (ROU) and the lowest with water (RNU). In comparison, yields for lime (9-30%) (Koubala *et al*., 2008) were lower under water extraction conditions.

Uronic acid and neutral sugars contents: *Retama raetam* pectins had galacturonic acid (260-640 mg g^{-1}) and total neutral sugars (320-540 mg g^{-1}) contents that varied widely, depending on the extraction method used (Fig. 2). Water-extracted pectins were particularly rich in uronic acids while ROU and RBU extracted pectins were rich in total neutral sugars and poorest in uronic acid (Table 2).

Degree of methylation (DM) and acetylation (DAc): Results in Table 3 show that water-extracted and oxalic ammonium extracted pectins were more methylated (DM 29-31%) than were basic extracted pectins (DM 20%). Compared to those of *Ambarella* pectins and the DM of lime pectins were significantly higher (58-82%). Retama pectins exhibited low degrees of acetylation (2-19%) for all three methods of extraction. The basic extraction process yields a pectin of low degree of esterification (2%) as a result of saponification of the ester groups whereas the oxalic extraction process yields a high degree of esterification (31%).

Table 1: Yields and extractabilities of pectins obtained under different extraction conditions from *Retama raetam*

Sample	RNU	ROU	RBU
Rdt %	0.42	1.18	0.56

Table 2: Sugar compositions of polysaccharides extracted from *Retama raetam*

Sample	RNU	ROU	RBU
Neutral sugars (%)	320	540	430
Galacturonic acid (%)	640	320	260

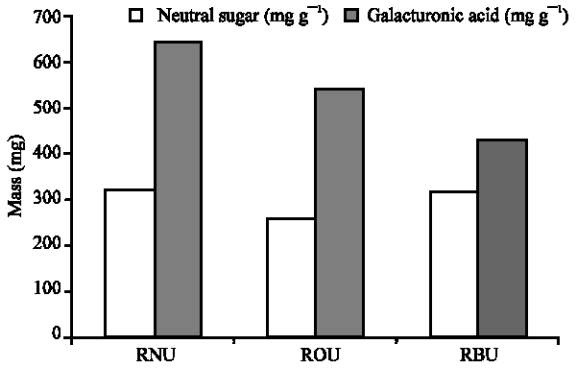

Fig. 2: Sugar composition of different polysaccharide from *Retama raetam*

Table 3: Macromolecular data for polysaccharides extracted obtained by SEC-MALLS (0.1 M $LiNO_3$, T = 25°C)

Extraction process	Raw material	DM (%)	DA (%)	[η] (mL g^{-1})	⟨Mw⟩ (g mol^{-1})	dn/dc (mL g^{-1})
Water	RNU	29	6.6	35	3.07×10^5	0.14
Oxalic	ROU	31	19	27	3.42×10^5	0.14
Basic	RBU	20	2	47	8.93×10^4	0.14

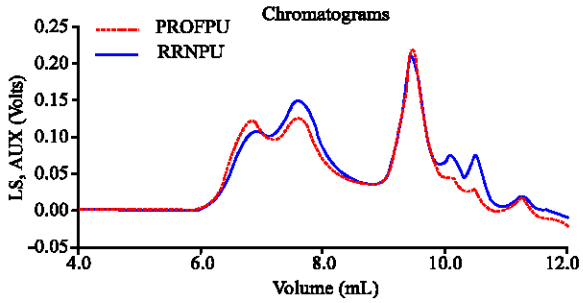

Fig. 3: Light scattering response at 90° (dotted line) from SEC /MALLS analysis of ROU and of RNU in $LiNO_3$ 0.1 M at 25°C, D = 0.5 mL min^{-1}

The DAc values of water extraction RNU (6.6%), were similar to *Ambarella* extracted pectins and to that of lime pectin extracted with water (6.0%) (Koubala *et al*., 2008).

Macromolecular features: SEC-MALLS experiments were carried out in 0.1 M $LiNO_3$ at a near neutral pH in order to determine molar mass and size information of the biopolymers studied.

The chromatograms of RNU and ROU (Fig. 3) introduce practically two elute population at the neighbourhoods respectively of 7 and 9.5 mL. These population is of strong mass this is explained by the great intensity of signal of diffusion of the light. Figure 3 shows the SEC elution profile observed for polysaccharides RNU and ROU. As can be seen, the function log (molar mass) = f (Ve) calculated from the experimental values is linear over the major part of the peak with a deviation for higher elution volumes. This phenomenon is indicative of non-SEC effects under the present experimental conditions due to partial polymer adsorption on the chromatographic support likely caused by hydrophobic interaction.

The weight average molar mass (⟨M_w⟩) of *Retama* pectins varied from 8.93×10^4 to 3.42×10^5 g mol^{-1} and their corresponding intrinsic viscosities ([η]) from 23.55-68.57 mL g^{-1} (Table 3). These results show the important variability of molar mass and intrinsic viscosity of pectins according to the extraction conditions used. Macromolecular parameter values depend, not only on the extraction process used, but also on the plant material used. These differences of molar masses could also be due to the type of method used for the determination of molecular parameter. According to Ralet *et al*. (2002), the water (in)-solubility and the aggregation of pectin could influence molar mass measurement.

Table 4: Average macromolecular characteristics of polysaccharides extracted from *Retama raetam* determined by SEC/MALLS (0.1M $LiNO_3$, 40°C, pH 6.3)

Samples	M_n	M_w	I_p	R_g	dR_g/dM_w
RNU	9.830×10^4	3.07×10^5	3.12	21.5	0.25
ROU	1.022×10^5	3.42×10^5	3.34	26.8	0.10
RBU	5.710×10^3	8.93×10^4	5.00	41.9	0.13

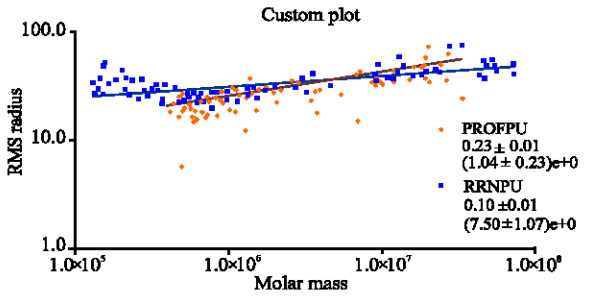

Fig. 4: Relation between the RMS and molar mass for the SEC high molar mass fraction of polysaccharides extracted from *Retama* (RNU and ROU)

The value of the specific refractive index increment was called 0.14 mL g^{-1} for RNU, ROU and RBU.

Complete dissolution of different samples was difficult and the presence of aggregates could not be avoided even after sonication (hazy solutions) so that the macromolecular features above determined may be only the characteristics of the soluble part. Further investigations on this compound were not undertaken.

The weight-average values of molar mass and root mean square radius of gyration determined for each fraction are reported in Table 4. It must be noted that the $\langle r_g^2\rangle^{0.5}$ determination for the slices of higher elution volume was affected by a retention on the stationary phase involving a tailing of molecules having high molar masses due to a non-size exclusion mechanism, resulting in a poor accuracy of the values obtained (Fig. 4).

SEC-MALLS experiments, owing to the simultaneous determination of the mass and the size for each elution volume, allow one to reach information about the polymer conformation. The value of the slope x resulting from the log-log plot of the root mean square radius of gyration as a function of the molar mass (Eq. 1), reveals whether the macromolecule is a sphere (x = 0.33), a random coil

(x = 0.5-0.6) or a rod (x = 1.0) (De Gennes, 1979). As can be seen from the slope values given in Table 4, both these samples have spherical structure. In spite of the data scattering, slopes can be estimated to be ca 0.3 for RNU, ROU and RBU, so spherical conformation.

Polyelectrolyte behaviour: Titration of different carbohydrates was carried out after conversion of the carboxylic residues under their acidic form to determine the AEW related to the content of free acid moieties.

AEW_1 was determined for each sample from the potentiometric titration according to Eq. 1:

$$AEW_1 = \frac{m}{C_e \times V_e} \quad (1)$$

pK_a was determined according to Eq. 2 :

$$pK_a = pH - \log\frac{\alpha}{1-\alpha} \quad (2)$$

$$\alpha = \frac{V}{V_e} + \frac{V_0 + V}{C_e \times V_e}([H_3O^+] - [OH^-])$$

where, m was the mass of the polymer sample, V_0, V_e respectively the polymer and NaOH volume of the endpoint, C_e the concentration of NaOH (Fig. 5).

In fact, a fraction of the carboxylic acid functions present in pectins is naturally esterified with O-methyl groups. The results presented in Table 3 show that all of samples are lowly methoxylated (DM<50%).

From the data of Table 5, it was concluded that the polysaccharides extracted in water (RNU) are low charged anionic polymers whereas the pectin extracted on NaOH can be viewed as a moderately charged anionic polymer (Fig. 6).

Table 5: Potentiometric titrations of polysaccharides extracted from *Retama raetam*

Samples	$(pKa)_0$
RNU	1.57
ROU	2.65
RBU	2.72

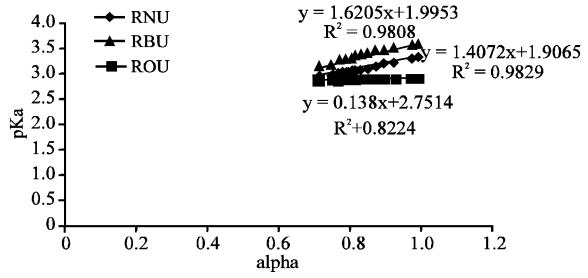

Fig. 5: Relation between pKa and alpha of different sample

Viscosity measurements: The influence of the NaCl concentration on the relative viscosity of different sample is shown in Fig. 7. The repulsion between charged sites prevails in pure water causing the chain to adopt an extended conformation evidenced by the maximum viscosity. A salt concentration as low as 0.1 M results in a dramatic decrease of viscosity indicating decreasing dimensions of the polysaccharide macromolecules related to a weakening of the electrostatic interactions by shielding of the polyion charges. The viscosity decrease slowed down in the range of ionic strength 0.1-0.3 M and reached a limiting value at high salt concentration meaning that the polyelectrolyte character is minimised in this domain.

The values of intrinsic viscosity reported in Table 6, demonstrate a similar trend for polymer conformation as that inferred from the SEC-MALLS data. The large decrease of intrinsic viscosity on going from RBU to ROU (Fig. 8) indicates not only the lowering of molecular weight but also the chain folding due to a higher content of galacturonic acid units. It is noteworthy that the values of overlap polymer concentrations C^* estimated using

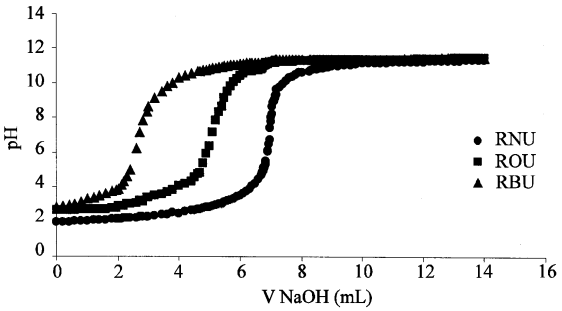

Fig. 6: Titration of different polysaccharide from *Retama raetam*

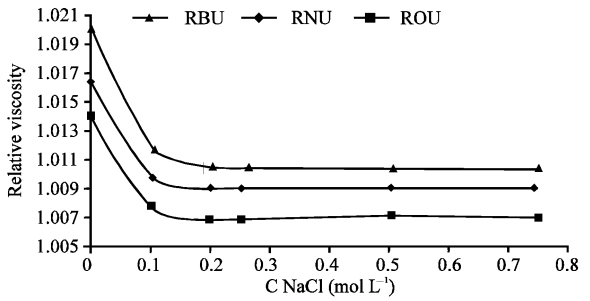

Fig. 7: Variation of relative viscosity of RNU, ROU and RBU

Table 6: Viscometric characteristics of polysaccharides extracted from *Retama raetam*

Sample	[η] mL g^{-1}	K' Huggins	C^*
RNU	35	0.35	0.04
ROU	27	0.15	0.05
RBU	47	0.29	0.03

C^*: Overlap polymer concentrations

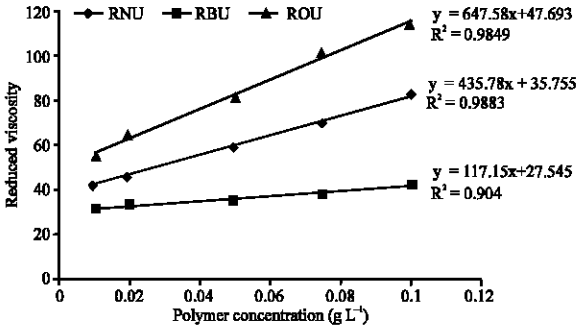

Fig. 8: Variation of reduced viscosity of RNU, ROU and RBU

equation using the equation: $C^* = 1.5/[\eta]$ (Morris *et al*., 1980), confirm the fact that the determination of the molar mass, root mean square radius and intrinsic viscosity were performed in the dilute regime.

CONCLUSION

Different polymers were extracted from *Retama raetam* by sequential extraction. The results showed that *Retama raetam* are a source of pectins with high molecular weight. The extraction conditions had a significant impact on the characteristics of the extracted pectins. Ammonium oxalate allowed high extraction yields, the recovered pectins being of high degree of methylation, high molar mass and high intrinsic viscosity. Basic extraction gave low yields of pectins with low uronic acids and DM. Water extraction gave the lowest yields of pectins with high degrees of methylation and high intrinsic viscosity. On the whole, oxalate extraction of pectins from *Retama raetam* provides good yields, which are biochemically interesting due to their high molar mass.

These investigations revealed that the polysaccharides from *Retama raetam* have a sugar composition typical of pectin with a high and medium degree of esterification of galacturonic residues respectively. SEC-MALLS experiments gave information of the distribution of the molar mass and root mean square radius. Information on the conformation of these polysaccharides was obtained by applying the relation between the root mean square radius and the molar mass by viscometric measurements. Spherical conformation was found for the former samples.

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