Journal Article

Characterization of the polysaccharide from *cola millenii* seeds

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CHARACTERIZATION OF THE POLYSACCHARIDE FROM *COLA MILLENII* SEEDS

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ABSTRACT.

We have isolated and characterized a water soluble polysaccharide from *Cola millenii* seeds. It was found to be composed of a total of 59% neutral sugars (mainly rhamnose, galactose and arabinose ~ 24, 13 and 8% respectively) and 41% uronic acids [mainly galacturonic acid]. The weight and number average molar mass values were found to be 4.7 x 10^6 g/mol and 3.5 x 10^6 g/mol, respectively. The polysaccharide exhibited polyelectrolyte properties with the intrinsic viscosity varying with salt concentration. The polysaccharide formed a highly viscous solution in water with apparent zero shear viscosities of 0.59 - 772 Pa.s at concentrations 0.3 - 2.5wt%. The solutions were shear thinning even at very low concentrations. The mechanical spectra showed gel-like characteristics at concentrations >2 wt%. The rheological behavior indicates the polysaccharide has potential for application as a thickener and suspending agent in food, pharmaceutical and cosmetic formulations.

Key words: *Cola millenii*, polysaccharide, sugar composition, molar mass, rheological properties, polyelectrolyte

1. INTRODUCTION

Cola millenii K Schum is a leguminous plant belonging to the family Sterculiaceae and genus Cola. It is a rain forest plant found in West Tropical Africa including Cote d'Ivoire, Southern Nigeria, Ghana, Togo and Benin. It is a vigorously growing tree or shrub which may attain to a height of 12-20 m and bears edible fruits with characteristic sweetness [1, 2]. It is called monkey kola in English. The pods may contain one to as many as eleven seeds per pod. Studies on different parts of the plant have shown that the plant has antioxidant and antimicrobial activity [2, 3, 4]. The chemical composition highlighting the nutritional, anti-nutritional and mineral elements in *C. millenii* seed has been reported [1, 3, 5]. These authors have found that the seed has a carbohydrate content of 28.73-51.54%. Literature on the composition of *C. millenii* seed is scant. Our previous study showed that *C. millenii* seed is rich in a water soluble polysaccharide [6]. In this study we report its monosaccharide and protein composition, molecular mass distribution, and rheological properties.

2. MATERIALS AND METHODS

2.1 Isolation of Polysaccharide

The *Cola millenii* pods were harvested wild from a forest in Ondo State Nigeria. About ten matured pods were collected from three plants. The pods (Figure 1) were cut and the seeds removed and air dried under ambient conditions. The seed coats were removed and the resulting endosperm pulverized and defatted with n-hexane for 8 h. 50 g defatted seed endosperm powder was dispersed in 400 mL distilled water and the water soluble polysaccharide extracted at room temperature under constant agitation for 2 h. The dispersion obtained was poured into centrifuge tubes and centrifuged at 2500 rpm for 2 h. The supernatant was decanted into a clean container and the residue reconstituted with 200 mL distilled water, agitated for the same period of time and centrifuged again. The combined supernatant was treated with excess isopropanol (1:4, v/v)

to precipitate the polysaccharide. This was reconstituted in a small amount of water and poured into round bottom flasks, frozen and freeze dried.

2.2 Protein Determination

The total nitrogen content of the sample was determined by the micro-Kjeldahl digestion and distillation method. The nitrogen was converted to protein by multiplying by a factor of 6.25.

2.3 Sugar Composition

The sugar composition was determined by HPAEC-PAD using a Dionex ICS-2500 system. Sugar analysis was by methanolysis followed by TFA hydrolysis using myo-inositol as internal standard as reported previously [7].

2.4 Molar mass distribution

The molar mass was determined using Gel Permeation Chromatography using multiangle light scattering, refractive index and UV absorbance (wavelength =280 nm) detectors (Optilab DSP, Wyatt Technology Corporation, Santa Barbara Ca93103). The polysaccharide solution (2 mg/mL) prepared as described in Nwokocha & Williams [8] was filtered through a 0.45 μm syringe and injected through a rheodyne into a 200 μL loop connected to a combination of Suprema columns (100Å, 3000Å and 30000Å) packed with 10 μm beads of polyhydroxymethacrylate copolymer. The solvent was 0.1M $NaNO_3$ containing 10^{-6}M NaN_3 which was pumped (Waters: 515 HPLC Pump, Milford, MA 01757, USA) through a degasser (CSI 6150, Cambridge Scientific Instruments, England) at a flow rate of 0.5 mL/min. The total injected mass was 400 μg. The molecular mass distribution was analyzed with Astra software (ASTRA 4.08.09) using the Berry method and a dn/dc value of 0.140 mL/g.

2.5 Rheological Measurements

2.5.1 Capillary viscometry

Cola millenii polysaccharide (0.15 g/100 mL) solution was prepared in different concentrations of NaCl (0.1M, 0.2M, 0.25M). 7 mL of the solution was poured into a capillary viscometer (Type: Cannon 75, J379) and placed in a thermostatic water bath maintained at 25^{o}C to equilibrate. The flow time was measured in triplicate and the average taken.

Serial isoionic dilution was performed in situ for each salt concentration and readings were taken for each dilution. Similar measurements were made for the solvents alone and used to determine the relative viscosity as $\eta_r = t/t_s$. Where t = flow time of polysaccharide solution at each dilution and salt concentration, t_s = flow time of solvent salt solution. The specific viscosity, η_{sp}, was determined as $\eta_{sp} = \eta_r - 1$. The intrinsic viscosity, [η], was determined using the viscometric equations of Fedors ($1/2(\eta_r^{1/2}-1)$ versus 1/c), Huggins (η_{sp}/c versus c) and Kraemer ($\ln(\eta_r)/c$ versus c); where c is polysaccharide concentration.

2.5.2 Shear flow and oscillatory measurements

The polysaccharide solutions (0.3 – 2.5 wt%) were prepared by dissolving the polysaccharide powder in water at room temperature on a roller mixer (Staurt instruments, UK) overnight. The flow and oscillatory measurements were carried out on a Controlled Stress Rheometer (AR 2000, TA Instruments, USA) at 25^{o}C using a 40 mm crosshatched steel plate (994387) for solutions 0.75-2.5wt% and standard-size recessed end concentric cylinders geometry for solutions 0.3 - 0.5wt%. A solvent trap was used in each measurement to minimize moisture loss. In the steady shear measurements the sample was subjected to a stepped flow over a shear rate range of 0.001-1000 s^{-1}. For oscillatory measurements, an oscillation stress sweep was carried out at 1Hz to

locate the linear viscoelastic region. This was followed by a frequency sweep from 0.001 - 628 rad/s using an oscillation stress value in the linear viscoelastic region. The rheological data was analyzed with Rheology Advantage Data Analysis.

3. RESULTS AND DISCUSSION

3.1 Yield and Composition

The water soluble polysaccharide in *Cola millenii* was extracted at a yield of 7.4%. The endosperm residue was still mucilaginous suggesting all the polysaccharide present could not be extracted by the procedure used. The polysaccharide had a protein content of 10.7 %. This is comparable to 11.69% protein reported for *Mucuna flagellipes* polysaccharide [9] and 6.9–10.3% reported for LBG [10].

The monosaccharide composition of the *Cola millenii* seed (Table 1) in order of abundance was galacturonic acid (39.99%), rhamnose (24.24%), galactose (12.98%), arabinose (7.59%), glucose (5.08%), xylose (4.76%), fucose (4.10%) and glucuronic acid (1.26%). Mannnose was not detected in the polysaccharide. The high uronic acid content (41.25%) of the *C. millenii* polysaccharide indicates it would have polyelectrolyte properties. It also could crosslink with calcium ions or other divalent cations to form a polysaccharide hydrogel [11].

3.2 Molecular Mass Distribution

The refractive index elution profiles of *C. millenii* polysaccharide are presented in Figures 2a and 2b. Figure 2a also shows the M_w of the eluting species and Figure 2b shows the radius of gyration.

The elution peak gave a strong light scattering signal but no UV signal at an absorbance of 280nm [data not shown]. It is evident, therefore, that this peak corresponds to the elution of polysaccharide material and does not contain any proteinaceous moieties. The weight average molar mass, M_w, was found to be 4.7±0.2 x 10^6 g/mol and the number average molar mass, M_n, was 3.5±0.3 x 10^6 g/mol. The average radius of gyration, R_g, was 106.8±0.8 nm (Table 2).

3.3 Intrinsic Viscosity

In Table 3, the intrinsic viscosity values of *C. millenii* polysaccharide were determined by the Fedors, Huggins and Kraemer equations at different NaCl concentrations. The [η] of the polysacchaide was sensitive to salt concentration and decreased with increasing NaCl concentration. This observation is consistent with the high uronic acid composition of the *C. millenii* polysaccharide. In aqueous solution in the absence of electrolyte, the polysaccharide uronic acids ionise leading to intramolecular repulsions resulting in chain expansion and an increase in hydrodynamic volume. In the presence of added NaCl, the intramolecular repulsions are screened resulting in a decrease in the hydrodynamic volume. This is typical polyelectrolyte behaviour [12]. The relationship between intrinsic viscosity and ionic strength for polyelectrolytes in solution is given by Eq. 1

$$[\eta] = [\eta]^{\infty} + SI^{-1/2} \qquad \text{(Eq. 1)}$$

where [η], $[\eta]^{\infty}$ are the intrinsic viscosity and intrinsic viscosity at infinite ionic strength, $I^{-1/2}$ is the inverse square root of ionic strength, S is related to the polymer stiffness. From the plot of [η] against $I^{-1/2}$ (Figure 3), the intrinsic viscosity at infinite ionic strength was extrapolated to be 7.6 dL/g. This represents the hydrodynamic volume of the uncharged molecule.

Smidsrod and Haug [13] defined the stiffness parameter (B) (Eq 2) which compares the chain flexibility of different polyelectrolytes at fixed ionic strength (0.1M NaCl).

$$S = B([\eta]_{0.1M})^{\nu} \qquad \text{(Eq. 2)}$$

Where $[\eta]_{0.1M}$ =10.8 dL/g, the average intrinsic viscosity at 0.1M NaCl, *v* is an exponent experimentally determined to be 1.3±0.1. B was calculated to be 0.015 for *C. millenii* polysaccharide. The flexibility of *C. millenii* polysaccharide is comparable to the value of 0.022 reported for high guluronic acid alginate [14] and higher than the value of 0.005 reported for xanthan gum [13].

3.4 Apparent Viscosity

Figure 4a shows the viscosity shear rate profiles of 0.3 - 2.5wt% of *Cola millenii* polysaccharide in water. The polysaccharide at these concentrations exhibited shear thinning profiles which can best be described by the Cross model (Eq. 3).

$$\frac{\eta - \eta_{\infty}}{\eta_{\circ} - \eta_{\infty}} = \frac{1}{1 + (\tau \times \dot{\gamma})^{n}} \qquad \text{(Eq. 3)}$$

Where η, η_o and η_∞ are viscosity (Pa.s), zero shear viscosity (Pa.s) and infinite shear viscosity (Pa.s) respectively, $\dot{\gamma}$ is shear rate (s^{-1}), τ is Cross relaxation time and n is the rate index (dimensionless).

The parameters of the Cross model are shown in Table 4. The η_o ranged from 0.59 - 772 Pa.s and increased as concentration increased. The onset of shear thinning (critical shear rate, $\dot{\gamma}_{crit}$) shifted to lower shear rates as the concentration increased. This is related to the Cross relaxation time (τ) by ($\tau = 1/\dot{\gamma}_{crit}$); $\dot{\gamma}_{crit}$ marks the onset of shear thinning. The Cross rate index was in the range

0.5603 <n< 0.8633 and increased with polysaccharide concentration. The low values of the standard error of estimates (s.e <20) indicated that the Cross model adequately described the flow properties of *C. millenii* polysaccharide.

The zero shear viscosity for a 0.5% solution was ~1Pa.s which is similar to the zero shear viscosity of xanthan gum at the same concentration reported by Sworn [15]. The high η_o of *C. millenii* indicates its potential as a thickener and suspending agent for particulate dispersions and emulsions.

Figure 4b shows the plot of reduced viscosity (η/η_o) versus Deborah number ($\tau\dot{\gamma}$) for different polysaccharide concentrations. A master curve was observed with deviation for some flow profiles at higher values of Deborah number. As $\tau\dot{\gamma}$ tended to zero, η/η_o approached unity. At higher Deborah number, the flow profiles for those concentrations that approached terminal viscosity deviated from the master curve. Similar deviations have been reported for other polysaccharides at shear rates approaching infinity [16]. Figure 5 shows the plot of log zero shear viscosity versus log concentration in the concentrated solution regime of *C. millenii* polysaccharide. The viscosity dependence of concentration for *C. millenii* polysaccharide was given by $c^{3.3}$ which is in agreement with values reported by Morris, Cutler, Ross-Murphy, Rees and Price [17] for conformational disordered polysaccharides in the concentrated regime.

3.5 Viscoelastic properties

Figure 6 shows the plot of the elastic (G′) and loss (G″) moduli against angular frequency (ω) of solutions of *Cola millenii* polysaccharide at different concentrations (0.5, 0.75, 1, 1.5, 2 and 2.5 wt%). Both G′, G″ showed dependence on ω and the extent of dependence was a function of concentration of the polysaccharide.

For polysaccharide concentrations 0.5, 0.75 and 1wt%, G″ > G′ at lower ω, both moduli increased as ω increased but separation between them decreased until a critical frequency was reached where both moduli crossed over at (G′= G″). Below this point the polysaccharide solution exhibited liquid-like properties (G″ > G′) but above this point the solution exhibited a gel-like response (G′ > G″). For 1.5 and 2 wt%, at low ω, G′ and G″ were independent of ω (G″ ≈ G′), however both moduli increased but G′ increased faster than G″ until G′ > G″ indicating the transition from liquid-like to a gel-like response. For a 2.5 wt% polysaccharide concentration, G′ > G″ all through the range of ω indicating the solution exhibited gel-like behavior. Table 5 shows the variation of the critical angular frequency (ω_{crit}) and G′ G″ crossover point with concentration. As polysaccharide concentration increased, the value of G′ G″ at the crossover point increased while the value of ω_{crit} shifted to lower ω. This observation is consistent with random coil polymers [18].

4. CONCLUSION

The water-soluble polysaccharide extracted from *Cola millenii* seeds has a high molar mass and forms highly viscous and viscoelastic solutions at low concentrations. It is evident that the zero shear viscosity at 0.5% concentration has a similar magnitude to that for xanthan gum at the same concentration and hence it has promise for application as suspending agent and stabilizer in food and other formulations.

Acknowledgements: The authors acknowledge the award of a Leverhulme Trust Visiting Fellowship to Dr Nwokocha. We are grateful to Dr M. Yadhav for carrying out the sugar analysis.

5. REFERENCES

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Table 1. Composition of *Cola millenii* polysaccharide

Monosaccharide	Relative mole %
Fuc	4.10
Rham	24.24
Ara	7.59
Glc	5.08
Gal	12.98
Xyl	4.76
Man	0.00
GalA	39.99
GlcA	1.26

Fuc, Fucose; Rham, Rhamnose; Ara, Arabinose; Glc, Glucose; Xyl, Xylose; Man, Mannose; GalA, galacturonic acid; GlcA, Glucuronic acid.

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Table 2. Molecular mass parameters of *Cola millenii* polysaccharide

Parameter	Peak
M_w (g/mol)	$4.7\pm0.2 \times 10^6$
M_n (g/mol)	$3.5\pm0.3 \times 10^6$
M_w/M_n	1.40±0.06
R_g (nm)	106.8±0.8

M_w = mass average molecular mass; M_n = number average molecular mass, R_g = radius of gyration; *Results are mean ± std of two determinations

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Table 3. Intrinsic viscosity, [η], of *Cola millenii* polysaccharide at different NaCl concentrations using different equations

NaCl (aq)	[η]		
	Huggins	Kraemer	Fedors
0.1M	9.7	11.1	11.67
0.2M	9.35	9.98	10.10
0.25M	8.07	8.70	8.70

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Table 4. Parameters of shear viscosity versus shear rate at different concentrations Cola millenii polysaccharide according to Cross model

Conc (wt%)	η_o (Pa s)	η_∞ (Pa s)	τ (s)	n	s.e
0.3	0.5864	7.047E-3	4.51	0.5603	6.594
0.5	2.023	0.01925	4.213	0.6988	19.45
0.75	9.75	0.02823	9.506	0.7292	12.25
1	21.25	0.02656	14.93	0.7335	8.555
1.5	90.11	0.03711	38.31	0.7506	11.62
2	177.1	0.0568	25.98	0.8178	9.978
2.5	772.2	0.1049	33.88	0.8633	15.40

η_o, η_∞ = zero and infinite shear viscosity, respectively; τ = Cross relaxation time; n = rate index; s.e.= standard error

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Table 5. Parameters of oscillation at different concentrations *Cola millenii* polysaccharide

Conc. (wt%)	ω_{crit} (rad/s)	G (Pa)
0.50	0.3564	0.184
0.75	0.0986	0.283
1	0.06999	0.4066
1.50	0.00681	0.5778
2	0.003047	0.7083

ω_{crit} = angular frequency at crossover point; G = modulus at crossover

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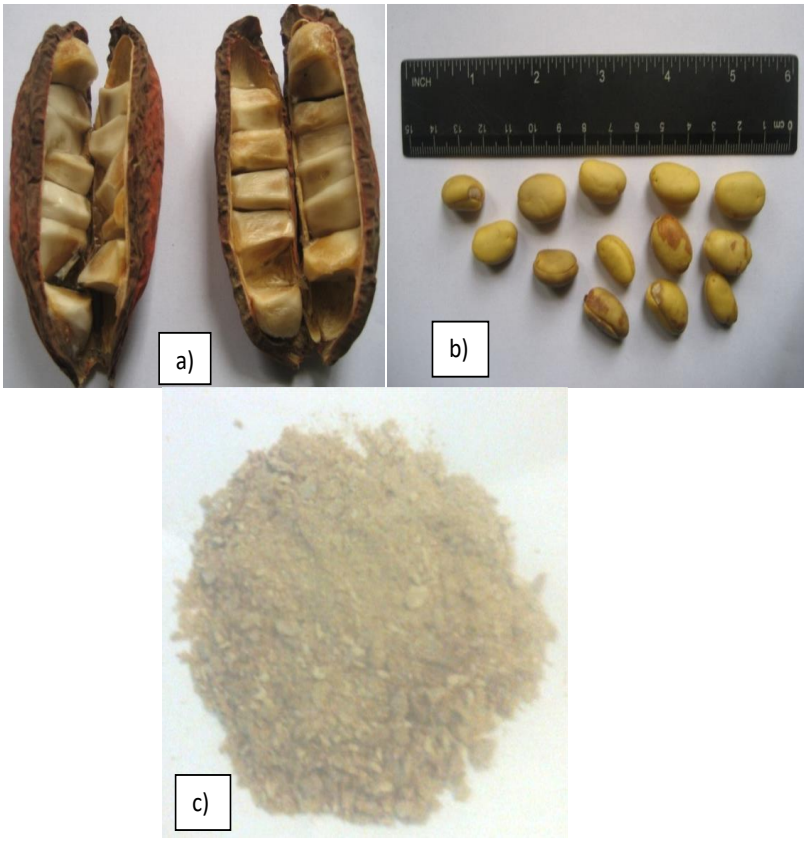

Figure 1. *Cola millenii* a) seed pods; b) seed endosperms; c). seed polysaccharide

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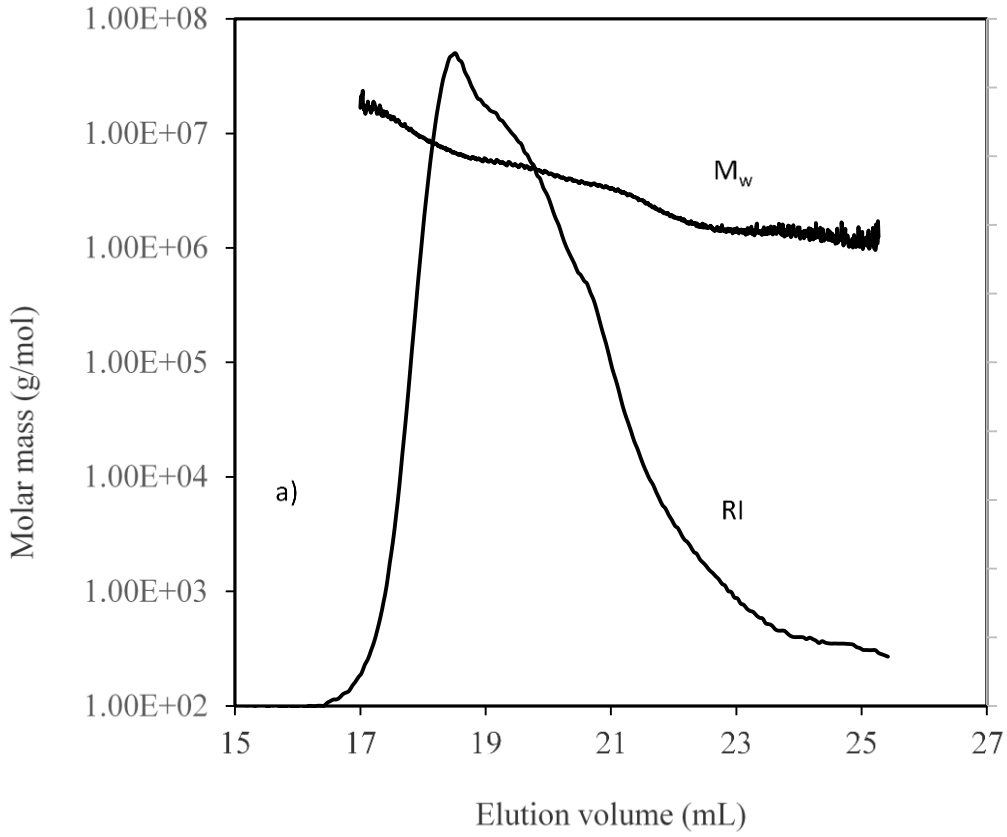

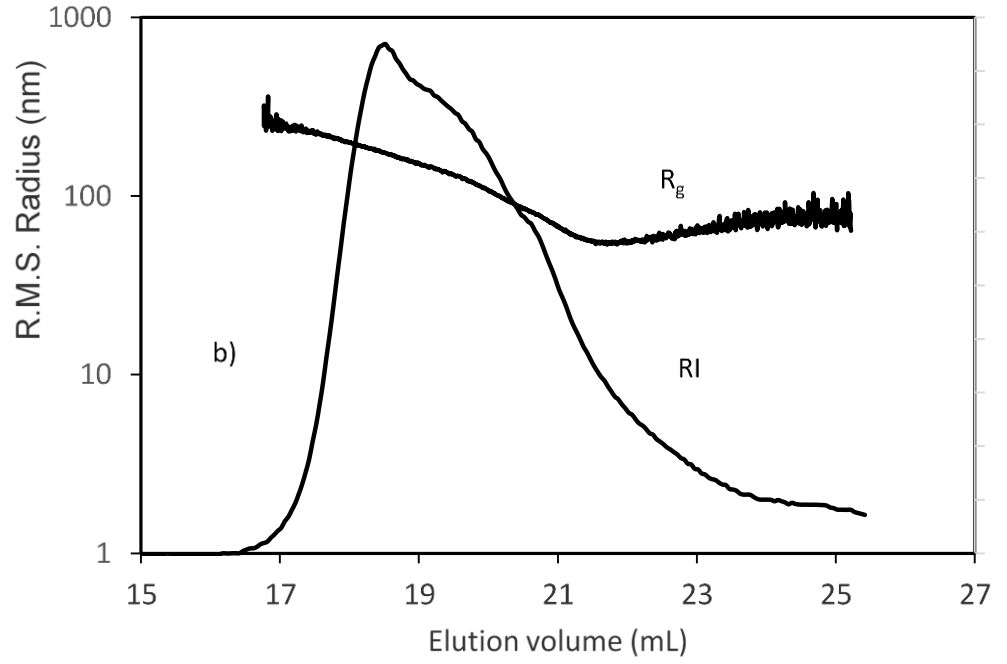

Figure 2. (a). Molar mass (M_w) and Refractive index (RI) versus Elution volume; (b). R.M.S. radius (R_g) and Refractive index (RI) versus Elution volume

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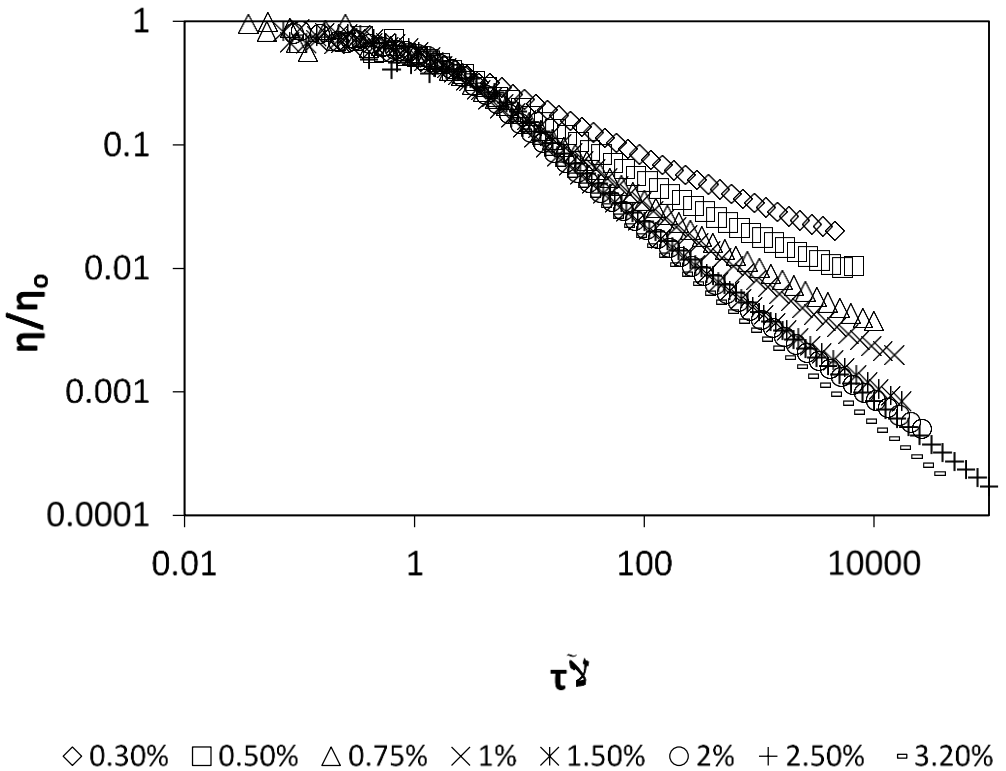

Figure 4b. Master curve: Reduced viscosity (η/η_o) versus Deborah number ($\tau\tilde{\lambda}$)

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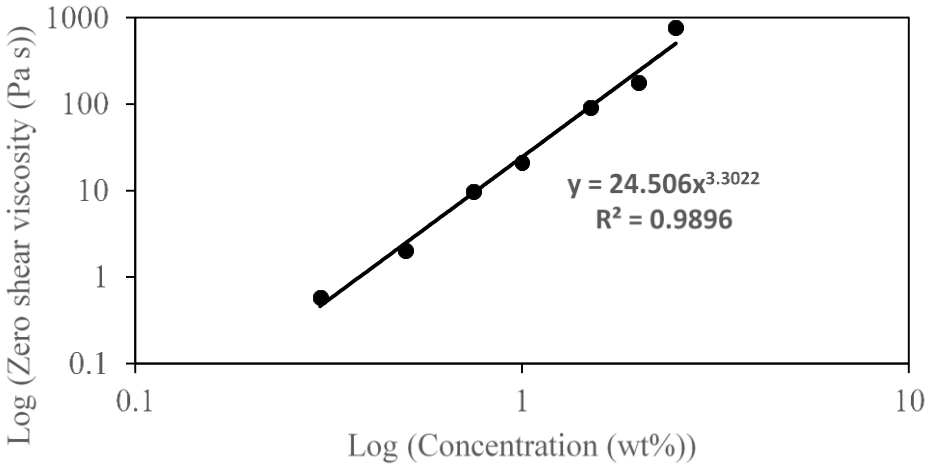

Figure 5. Log Zero shear viscosity versus Log Concentration

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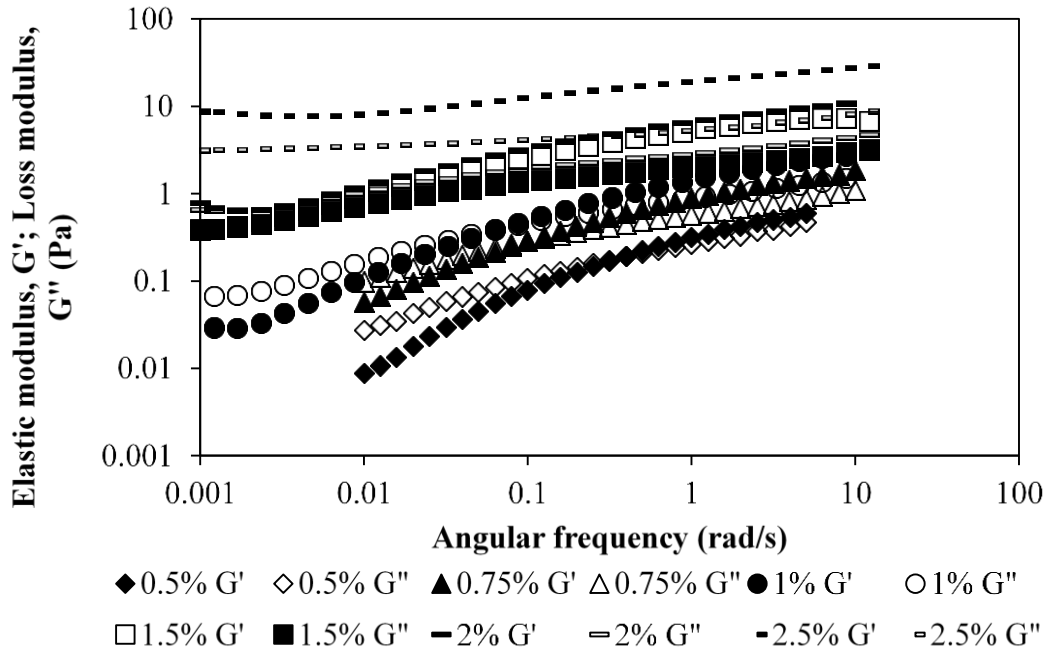

Figure 6. Elastic, G′ and loss, G″ moduli versus angular frequency, ω

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