Structural and rheological studies of a polysaccharide mucilage from lacebark leaves (*Hoheria populnea* A. Cunn.)

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Abstract

A water-soluble mucilage extracted from the leaves of *Hoheria populnea* was chemically and physically characterised. Monosaccharide composition and linkages were determined by high performance anion exchange chromatography (HPAEC), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Lacebark mucilage was composed of rhamnose, galactose, galacturonic acid and glucuronic acid (2:1:2:1). Proton and ^{13}C NMR spectroscopy, and linkage analysis, revealed a predominantly rhamnogalacturonan I-type (RG I-type) structure comprising of a backbone of →4]-α-D-Gal*p*A-[1→2)]-α-L-Rha*p*-[1→. Data indicated the mucilage likely comprises of a polymer containing several structurally discrete domains or possibly more than one discrete polymer. One domain contains a RG I-type backbone with branching at *O*-3 of Gal*p*A residues to terminal β-D-Glc*p*A residues, another similarly contains a RG I-type backbone but is branched at *O*-4 of the Rha*p* residues to terminal Gal*p*A residues or oligosaccharides containing α-linked 4-Gal*p* and terminal Gal*p*A residues. A possible third domain contains contiguous 2-Rha*p* residues, some branched at *O*-3. Hydrated mucilage exhibited pseudoplastic flow behaviour and viscoelastic properties of an entangled biopolymer network which were consistent from pH 7.5 – pH 1.2 and may prove advantageous in potential end-product applications including oral pharmaceuticals or as a food ingredient.

Keywords: *Hoheria populnea*; lacebark, mucilage, polysaccharide, structure, rheology

1. Introduction

Lacebark (*Hoheria populnea* A. Cunn., Malvaceae) is a small, fast growing tree endemic to New Zealand. Its common name is derived from the lace-like layers of the inner bark which are traditionally woven into kete (woven baskets) and headbands or used as decorative trim on hats and cloaks. In traditional Māori medicine (rongoā) the uses of lacebark are related to the mucilage which is produced when the inner bark layers are soaked in water. The jelly-like mucilage is used externally as an emollient and internally for treating inflammation of the digestive and respiratory tracts [1, 2]

The family Malvaceae includes members such as *Abelmoschus esculentus* (okra), *Althaea officinalis* (marshmallow), *Cola cordifolia* (kola) and *Hibiscus moscheutos* (rose mallow), all of which have a history of traditional medicinal use. Organs and tissues, including the bark, leaves and roots, of many of these plants produce polysaccharide mucilages and have traditionally been used as emollients, demulcents and cough medicines [3-5], Structural studies indicate these polysaccharides have a rhamnogalacturonan I (RG-I) type backbone of →4]-α-D-Gal*p*A-[1→2)]-α-L-Rha*p*-[1→, with two thirds or more of the Gal*p*A residues typically substituted at *O*-3 with β-D-Glc*p*A and branching at *O*-4 of the Rha*p* residues with short galactose side chains also a common feature; they are also often partially *O*-acetylated [4-11]. We have recently partially characterised the physicochemical properties of a mucilage from the inner bark of the stems of *Grewia mollis*, another member of the Malvaceae family [12]. This polysaccharide comprises mostly of rhamnose, galacturonic acid and glucuronic acid and NMR spectroscopy showed that it was partially *O*-acetylated.

There are no reports in the literature of the structure and rheological properties of polysaccharides extracted from members of the genus *Hoheria*. Whilst the traditional uses of

lacebark generally relate to the mucilage obtained from the inner bark, the leaves, which similarly contain mucilage, were also used and represent a more sustainable source of material. Similarly to other Malvaceae species, the mucilage from lacebark may have applications in the pharmaceutical industry as a binder in tablets or as a suspending or emulsifying agent [13]. Thus, in this present study we have investigated the composition and structure of the mucilage extracted from *Hoheria populnea* leaves and evaluated the physicochemical properties of the hydrated polysaccharide.

2. Materials and Methods

2.1. Extraction and isolation of lacebark mucilage

Fresh leaves of *Hoheria populnea* were obtained from Kiwi Herbs Ltd, Lower Hutt, New Zealand and stored frozen until required. Leaves (300 g, frozen) were coarsely ground prior to extraction. Ground leaves were extracted with 80% ethanol (80 °C, 1 h) to remove phenolic and low molar mass materials. The leaves were then extracted with water, twice at 65 °C for 2 h and then at room temperature overnight. Following centrifugation, the combined extracts were mixed with ethanol (1 volume) and left at 4 °C overnight. The precipitated material was recovered by centrifugation and re-dissolved in water, with heating and mixing to aid dissolution. Extracts were then filtered; GD-120 glass fiber filter (Advantec; 0.9 µm) followed by activated carbon (Darco® G-60; using a 1:1 carbon/celite pad). Filtration using a GB-140 glass fiber filter (Advantec; 0.4 µm) removed any carbon fines and the mucilage was then further purified and concentrated using a Vivaflow 200 crossflow device with a polyethersulfone (PES) 100 kDa molecular weight cut-off (MWCO) membrane (Sartorius) prior to freeze-drying.

2.2. General analyses

Moisture content of the extract was estimated by oven-drying (80 °C, 24 h) and measuring the loss of weight. Nitrogen (N) and ash contents were analysed by an accredited chemical laboratory (Campbell Microanalytical Laboratories, University of Otago, Dunedin, New Zealand). Protein content was determined as the N content x 6.25 (assuming the N content of proteins to be 16%; [14]). All determinations were performed in duplicate.

2.3. Chromatographic and spectroscopic analyses

The composition and structure of the lacebark mucilage polysaccharides were analysed essentially as described by Nep et al. [15]. Where data from the analysis of grewia gum is included for comparison, the grewia gum sample is the starch-free material described previously [12].

2.3.1. Constituent sugar analysis

Constituent sugar composition was determined by high-performance anion-exchange chromatography (HPAEC) following hydrolysis of the polysaccharides present to their component monosaccharides. Samples were hydrolysed with methanolic HCl (3 mol L^{-1}, 80 °C, 18 h), followed by aqueous CF_3COOH (2.5 mol L^{-1}, 120 °C, 1 h). The resulting hydrolysates were analysed on a CarboPac PA-1 (4 x 250 mm) column equilibrated in NaOH (25 mmol L^{-1}) and eluted with a simultaneous gradient of NaOH and sodium acetate (1 mL min^{-1}). The sugars were identified from their elution times relative to standard sugar mixes, quantified from response calibration curves of each sugar and expressed as weight percent anhydro-sugar as this is the form of sugar present in a polysaccharide.

2.3.2. Glycosyl linkage analysis

Glycosyl linkage composition was determined by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates (PMAAs), after first reducing uronic acid residues to their dideuterio-labelled neutral sugars [16]. Lacebark mucilage (10 mg) was dissolved in 50 mmol L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (2 mL, pH 4.75) and, following activation with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluenesulfonate (carbodiimide reagent; 400 µL, 500 mg mL^{-1}), free uronic acid residues were reduced overnight with $NaBD_4$. The carboxyl-reduced samples were dialysed (6-8 kDa MWCO) against distilled water, freeze-dried and the sample reduced a second time following carbodiimide activation. Constituent sugar analysis of this material showed that no uronic acid was detected. Carboxyl-reduced mucilage was methylated [17] and during the chloroform extraction step insoluble, particulate material was observed at the water/chloroform interface indicating incomplete methylation [18]. The methylation was repeated, the methylated mucilage hydrolysed with 2.5 mol L^{-1} CF_3COOH and reduced with 1 mol L^{-1} $NaBD_4$ in 2 mol L^{-1} NH_4OH overnight at 25°C. Following removal of excess borate, the resulting alditols were acetylated in acetic anhydride (200 µL, 100 °C, 2 h) and extracted into dichloromethane. The PMAA derivatives produced were analysed by GC-MS (Agilent Technologies, Santa Clara, USA). Identifications were based on peak retention times relative to an internal standard, myo-inositol, and on comparisons of electron impact spectra with the spectra obtained from reference PMAA standards prepared by the method of Doares et al.[19].

2.3.3. Fourier transformed infrared spectroscopy (FTIR)

FTIR spectroscopy was carried out in transmittance mode using a Nicolet 380 FTIR Spectrometer (ThermoElectron Corporation, Waltham, USA) over the range 4000–400 cm^{-1} at 2 cm^{-1} resolution averaging 100 scans. The degree of esterification (DE) of the lacebark mucilage and pectin standards was determined according to the methods of Chatjigakis et al.

[20], Manrique and Lajolo [21] and Singthong et al. [22]. Briefly, the area of the peak at 1730 cm^{-1} (esterified carboxyls) was divided by the sum of the areas of the peaks at 1730 cm^{-1} and 1600 cm^{-1} which is proportional to DE. The DE was extrapolated from the calibration plot of the pectin standards.

2.3.4. Nuclear magnetic resonance (NMR) spectroscopy

Lacebark mucilage was exchanged with deuterium by freeze-drying with D_2O (99.9 atom%) three times. Samples were dissolved in D_2O and ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) spectra were recorded on a Bruker Avance DPX-500 spectrometer at 90°C. The ^{1}H and ^{13}C chemical shifts were measured relative to an internal standard of $(CH_3)_2SO$ (^{1}H, 2.70 ppm; ^{13}C, 39.5 ppm; [23]). Assignments were made from heteronuclear single quantum coherence (HSQC) COSY experiments and by comparing the spectra with published data.

2.3.5. Size-exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)

Molar mass was determined using size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). Samples (2 mg/mL in 0.1 mol L^{-1} $NaNO_3$) were allowed to hydrate by standing at room temperature overnight and centrifuged (14,000 x *g*, 10 min) to clarify. The soluble material (100 µL) was injected onto two columns (TSK-Gel $G5000_{PWXL}$ and $G4000_{PWXL}$, 300 x 7.8mm, Tosoh Corp., Tokyo, Japan) connected in series and eluted with 0.1 M $NaNO_3$ (0.7 mL min^{-1}, 60 °C). The eluted material was detected using a UV spectrophotometer (280 nm), a DAWN-EOS MALLS detector (Wyatt Technology Corp., Santa Barbara, USA) and a refractive index (RI) monitor (Waters Corp., Milford, USA). The data for molar mass determination was analysed using ASTRA software (v6.1.84, Wyatt Technology Corp.) using a refractive index increment, *dn/dc*, of 0.146 mL g^{-1} [24].

2.4. Rheological measurements of hydrated lacebark mucilage

Lacebark mucilage was dispersed in deionised water (at concentrations of 0.5, 1 and 2% w/v) with continuous stirring and left overnight at room temperature to fully hydrate prior to analysis. The pH of these solutions was 7.5. A further set of 2% w/v samples at pH 1.2, 2.5 and 4.5 were similarly prepared by firstly adjusting the pH of deionised water to pH 1.2, 2.5 and 4.5, using 1 mol L^{-1} HCl, then dispersing the lacebark mucilage in these solutions with continuous stirring, overnight in a sealed vessel. The pH was measured and re-adjusted if needed prior to analysis.

2.4.1. Viscosity measurements

Steady shear viscosity measurements of the 0.5%, 1% and 2% w/v dispersions of lacebark mucilage at pH 7.5 were performed on a Malvern Kinexus rheometer (Malvern Instruments, UK) fitted with a 55 mm, 2° cone-plate geometry with gap of 70 µm at 25 °C across a shear rate range 0.1–100 s^{-1}.

2.4.2. Modelling of flow behaviour

The flow behaviour of the 0.5%, 1% and 2% w/v dispersions of lacebark mucilage at pH 7.5 was deduced by fitting the shear stress and shear rate to the Ostwald–de Waele power law model (eq. 1).

$$\tau = K\dot{\gamma}^{n} \qquad \text{eq. 1}$$

where τ is shear stress, K is the flow consistency index, $\dot{\gamma}$ is shear rate and n is the flow behaviour index. The goodness of fit was established using the adjusted coefficient of determination where the closer the value is to 1, the better the data fit to the model. The value of n>1 denotes a sample that is shear thickening and n<1 denotes a sample that is shear thinning. In true Newtonian behaviour n=1.

2.4.3. Small deformation oscillatory rheology

Amplitude sweeps were used to determine the linear viscoelastic region and the critical stress required by the samples to yield. The stress was gradually increased using small deformation oscillations from 0.1 Pa to 100 Pa at an angular frequency of 10 rad s^{-1}. All measurements were taken at 25 °C. To characterise the viscoelastic properties of lacebark mucilage prepared at pH 7.5, 4.5, 2.5 and 1.2, small deformation oscillatory measurements of storage modulus (G′) and loss modulus (G″) were taken across a range of angular frequencies from 0.1–100 rad s^{-1} at 25 °C to determine the mechanical spectra of the samples. Measurements were taken at a constant strain of 1% which was previously determined (using amplitude sweeps) to be within the linear viscoelastic region. Moisture loss from samples was minimized during all rheological measurements by applying a thin layer of silicone oil on the periphery of the loaded sample and using a solvent trap on the geometry. All measurements were performed using a Malvern Kinexus rheometer (Malvern Instruments, UK) fitted with a 55 mm, 2° cone-plate geometry with gap of 70 µm.

2.4.4 Statistical methods

All rheological measurements were performed in triplicate and are presented as mean values. Statistical significance ($p < 0.05$) between test groups was determined by one-way analysis of variance (ANOVA) and a Tukey post-hoc test using Primer of Biostatistics version 4.

3. Results and Discussion

3.1. Composition of lacebark leaf mucilage

The yield of mucilage isolated from the lacebark leaves was 1.7%, calculated on a dry weight basis. This mucilage comprised mostly rhamnose, galactose, and both galacturonic and

glucuronic acids, accounting for almost three quarters of the weight of the extract (Table 1). Similarly, we have previously shown that a gum extracted from the inner bark of stems of *Grewia mollis*, a sub-Saharan member of the Malvaceae family, was also rich in rhamnose, galacturonic acid and glucuronic acid [12]; comparable extracts from the inner bark of lacebark showed a similar composition to that of grewia gum (data not shown). In addition, the lacebark leaf extract contained 2.6, 12.1 and 13.5% w/w of protein, ash and moisture, respectively.

3.2. Structural analyses of lacebark leaf mucilage

3.2.1. Linkage analysis

Linkage analysis of the lacebark leaf mucilage showed both linear and branched 2-linked rhamnopyranosyl (Rha*p*) and 4-linked galactopyranosyluronic acid (Gal*p*A) residues (Table 2). More than a third of the Gal*p*A residues were terminally linked, almost all the glucopyranosyluronic acid (Glc*p*A) residues were terminally linked and the galactopyranosyl (Gal*p*) residues 4-linked (Table 2). The presence of 2- and 2,4-Rha*p* is typical of rhamnogalacturonan I (RG I); the presence of 2,3-Rha*p* is unusual but has been reported in pectin-like polysaccharides from the bark and leaves of *C. cordifolia* (Malvaceae) [6, 25], the inner bark of *Ulmus glabra* (Ulmaceae) [26] and in mucilage from the seeds of *Linum usitatissimum* (linseed; Linaceae) [27]. The Gal*p*A residues were both 4- and 3,4-linked; 4-linked Gal*p*A is typical of RG I and 3,4-linked Gal*p*A is typical of RG II, although there was no further evidence for the presence of this pectic polysaccharide [28]. Gum and mucilage polysaccharides structurally related to pectic polysaccharides have been shown to contain 4-linked Gal*p*A branched at *O*-3 with terminal Glc*p*A residues [29, 30]. High levels of 3,4-Gal*p*A have been found in mucilage polysaccharides from other members of the Malvaceae including *H. moscheutos* leaves and roots, *Hibiscus syriacus* flower buds, *Althaea rosea* leaves and roots, *A. officinalis* leaves and roots, *A. esculentus* roots and *Malva sylvestris* leaves [4, 5, 8-10, 31-

34]. Similarly to the lacebark leaf polysaccharide, these mucilage polysaccharides generally have some branching at *O*-4 of 2-Rha*p* residues and 4-Gal*p* disaccharide or short oligosaccharide side chains. The mucilage from lacebark leaves is unusual in having a high content of terminally linked Gal*p*A residues. In contrast, the linkage composition of grewia gum was much simpler, with three major linkages, 2-Rha*p*, 3,4-Gal*p*A and terminal Glc*p*A; it resembled the mucilage extracted from the roots of *Hibiscus moscheutos* [5].

3.2.2. FTIR spectroscopy

The FTIR spectrum of lacebark leaf mucilage was typical of polysaccharides (Fig. 1), containing two major peaks in the region between 3600 and 1800 cm^{-1} corresponding to O–H stretching absorption due to inter- and intramolecular hydrogen bonding (3000–3600 cm^{-1}) and C–H absorption (2930 cm^{-1}), which typically includes CH, CH_2 and CH_3 stretching and bending vibrations [20, 35]. The region of the spectrum below 1800 cm^{-1} indicates the 'fingerprint' region for polysaccharides [36] and was similar in both lacebark mucilage and grewia gum [12]. The peaks between 1200 and 800 cm^{-1} are generally specific to particular polysaccharides, with this region dominated by pyranose ring vibrations overlapped with C-OH stretching vibrations and C-O-C vibrations from glycosidic bonds [36, 37]. The peak at 1415 cm^{-1} showed COO^- symmetric stretching and the peaks at 1600 cm^{-1} and 1724 cm^{-1} indicate free and esterified carboxyl groups, respectively [35]. A peak at ~1730 cm^{-1} can be used to estimate the degree of esterification in uronic acid containing polysaccharides such as pectin and grewia gum [12]. Using this method the degree of esterification of lacebark mucilage was estimated to be ~10%, which was considerably less than that of grewia gum (~50%).

3.2.3. NMR spectroscopy

The ^{13}C NMR spectrum of lacebark leaf mucilage (Fig. 2A) resembled that of grewia gum, except that the anomeric region of lacebark mucilage showed C-1 signals at 100.3 and 101.0 ppm, and C-6 signals at 60.9 and 61.9 ppm that were not observed in the spectrum of grewia gum [12]. These signals were thus assigned to 4-Gal*p* residues that were not detected in the linkage analysis of grewia gum (Table 2). These two C-1 signals showed H-1 cross-peaks in the ^{1}H NMR spectrum (Fig. 2B) at 5.22 and 5.23 ppm, indicating that the 4-Gal*p* residues are in the α configuration; the presence of such residues has been reported previously only once for the RG I-like polysaccharide from *Cola cordifolia* bark [6], although Sengkhamparn et al. [7] showed the presence of α-linked terminal Gal*p* residues in okra pectin.

A C-1 signal at 99.0 ppm, which showed H-1 cross-peaks at 5.40 and 5.30 ppm, was assigned to α-Rha*p* residues. A C-methyl signal at 17.4 ppm showed H-6 cross peaks at 1.26 and 1.32 ppm, corresponding to unbranched and branched α-Rha*p* residues, respectively [7, 38].

The C-1 signal at 104.5 ppm with a H-1 cross-peak at 4.73 ppm was assigned to terminal β-Glc*p*A [29, 39], and a C-1 signal at 98.5 ppm with H-1cross-peaks at 5.09 and 5.06 ppm was consistent with the presence of α-Gal*p*A residues. There were no signals in the NMR spectra of lacebark mucilage to indicate the presence of methylesterified α-Gal*p*A residues (^{13}C 54 ppm, ^{1}H 3.8–3.9 ppm) or 4-*O*-methyl Glc*p*A residues (^{13}C 60 ppm, ^{1}H 3.5 ppm), as shown for similar polysaccharides from other members of the Malvaceae [6, 36]. However, signals at 21.2–21.4 ppm (^{13}C) and 2.14–2.22 ppm (^{1}H) showed the presence of *O*-acetyl groups, similar to that found previously for grewia gum [12], indicating that the esterification identified by FTIR was *O*-acetylation rather than methyl esterification. We have not investigated the location of the *O*-acetyl groups, but Sengkhamparn et al. [7] showed that they were located on both Rha*p* and Gal*p*A residues of pectic polysaccharides extracted from okra pods.

3.2.4. Structure of lacebark mucilage

Based on the composition and structural analysis data we deduce that lacebark mucilage polysaccharide predominantly comprises of a repeating disaccharide backbone of →4]-α-D-Gal*p*A-[1→2)]-α-L-Rha*p*-[1→, with 10% and 24% of the 2-Rha*p* residues branched at *O*-3 and *O*-4, respectively, and two thirds of the 4-Gal*p*A residues branched at *O*-3. Side-chain substituents include terminal β-Glc*p*A, terminal α-Gal*p*A and short oligosaccharides containing →4]-α-Gal*p*-[1→. The NMR spectra shows the presence of *O*-acetyl groups, which by comparison with published data are probably located on some of the Rha*p* and Gal*p*A residues [7].

Tomoda and co-workers isolated a mucilage polysaccharide from the roots of *H. moscheutos* (Malvaceae) with a →4]-α-D-Gal*p*A-[1→2)]-α-L-Rha*p*-[1→ backbone repeat unit and terminal β-Glc*p*A attached to *O*-3 of the Gal*p*A, while a similar polysaccharide from the leaves had a more complex structure and included branched Rha residues and short galacto-oligosaccharide side-chains [5, 9]. Similar complex, more highly branched mucilage polysaccharides have also been isolated from various organs of other members of the Malvaceae, including *Hibiscus syriacus* [32], *Althaea rosea* [8, 33], *Althaea officinalis* [4, 10], *Abelmoschus esculentus* [34, 40] and *Malva sylvestris* [31]. We speculate that the polysaccharides isolated from lacebark leaves along with those from the leaves of *H. moscheutos* and various organs of other Malvaceae members are, in fact, two or three separate molecules or possibly one polymer containing several structurally discrete domains. Two domains have an RG I-type backbone; one branched at *O*-3 of 4-Gal*p*A residues to terminal Glc*p*A, similar to the polysaccharides from the roots of *H. moscheutos* and grewia gum, the other is branched at *O*-4 of the 2-Rha*p* residues to 4-Gal*p* and terminal Gal*p*A residues. The

predominance of 2-Rha*p* over 4-Gal*p*A indicates the presence of a possible third domain containing contiguous (1→2)-linked α-L-Rha*p* residues with some of the Rha*p* residues branched at *O*-3. Although uncommon, a similar predominance of Rha over GalA has been reported in RG I from flax seed mucilage and *Arabidopsis* [27, 41] and is evident in the mucilages from many of the Malvaceae species studied [9, 10, 31, 33]. The presence of branching at *O*-3 of 2-Rha*p* residues is unusual for mucilages from Malvaceae species, having only been previously reported in the leaves and bark of *Cola cordifolia* [6, 25]. While branching at *O*-3 of Rha*p* residues is rare in RG I polysaccharides there is also evidence of this branching pattern in RG I from flax seed mucilage [27] and pectic-type polysaccharides from the inner bark of *Ulmus glabra* (Wych elm) [26]. As far as we are aware, within the Malvaceae the presence of 4-linked α-Gal*p* is unique to the mucilage from lacebark leaves and the bark of *C. cordifolia* [6].

3.3. Size-exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)

Size-exclusion chromatography of lacebark leaf mucilage and grewia gum showed that ~90% of the material eluted between 10.5–13.5 mL; both samples also contained a small late-eluting peak (Fig. 3). The mass-average molar mass (M_w) of the major peak of lacebark mucilage was determined to be 2.31 x 10^6 g mol^{-1}, ranging from 1.8–3.0 x 10^6 g mol^{-1}; the dispersity (Đ) was 1.02 and the z-average mean square radius was 90.4 nm. The M_w of grewia gum (1.80 x 10^6 g mol^{-1}) was slightly smaller than that of lacebark mucilage, but its molar mass range was much greater with a Đ of 1.67; the z-average mean square radius was 81.0 nm.

3.4. Rheological measurements

To understand the flow properties of hydrated lacebark mucilage both steady shear and dynamic measurements of viscoelasticity were performed. This provided an insight into the

mechanical behaviour and potential industrial applications as a thickener or suspending agent. Measurements of apparent viscosity increased significantly ($p<0.05$) with increasing concentration (0.5%, 1% and 2% w/v) and also showed significant pseudoplastic flow behaviour in all samples with viscosity decreasing with increasing shear rate (Fig. 4). The calculated exponents of flow behaviour, when modelled according to the Ostwald–de Waele power law, showed that increasing concentration caused the flow behaviour index (n) to decrease, indicating greater pseudoplastic behaviour at higher concentrations (Table 3). To determine the onset of shear thinning behaviour the data was fitted to the Cross equation (eq. 2).

$$\eta = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1+(C\,\dot{\gamma}\,)^m} \qquad \text{(eq. 2)}$$

where η_0 is zero shear viscosity, η_∞ is the infinite shear viscosity, m is the rate constant (a measure of the degree of dependence of viscosity on shear rate in the shear thinning region) and C is the consistency constant given in dimensions of time. The reciprocal, $1/C$, is defined as the critical shear rate and is an indicator of the shear rate at the onset of shear thinning. Parameters elucidated from the Cross model fitting of the measured viscosity vs shear rate data (supplementary data, S1) are given in table 4. These parameters revealed that the onset of shear thinning began at significantly lower shear rates ($p<0.05$) with increasing polymer concentration ($1/C$ = 0.34, 0.1 and 0.08 s^{-1} for 0.5%, 1.0%, and 2.0% respectively). Moreover, the dependence of viscosity on shear rate in the shear thinning region (m) increased with an increase in concentration, indicating an increase in the extent of pseudoplastic behaviour. This kind of flow behaviour is particularly useful in pharmaceutical liquid formulations and in foods as a suspending agent and viscosifier by providing increased viscosity on storage preventing sedimentation and then easy dispensing following the application of shear.

To gain an insight into the viscoelastic properties of lacebark leaf mucilage, small deformation oscillatory measurements were performed on 2% w/v solutions prepared at a range of pH values. Stress sweep measurements were used to evaluate the critical stress required by the samples to yield. The value of critical stress was lowest at pH 1.2 (~5 Pa) and highest at pH 7.5 (~7.5 Pa) with samples at pH 4.5 and pH 2.5 at ~6.5 Pa (Fig. 5). This trend in behaviour has previously been observed in other similar anionic polysaccharides [12, 35] and has been attributed to reduced intermolecular associations between polymer chains [12]. In this case however, it should be noted that the differences in critical stress with reducing pH are relatively small which points to the material maintaining some level of intermolecular entanglement even at low pH. To investigate this further frequency sweeps were performed on the same samples. The mechanical spectra obtained were characteristic of entangled polymer solutions with G″ greater than G′ at low frequencies of oscillation (where the period of oscillation is sufficient to allow disentanglement to occur). At higher frequencies, however, elastic deformation dominated with G′ greater than G″, indicating that polymer entanglement was the dominating intermolecular interaction between the chains (Fig. 6). On further examination of the mechanical spectra only subtle differences were observed at different pH values indicating the mucilage was fairly resistant to changes in pH. Characteristic relaxation time (τ) calculated from the inverse of the angular frequency at which G′ and G″ cross ($\tau = \omega_c^{-1}$), as described by de Freitas et al. [42], was the same (0.63 s) at pH 1.2, 2.5 and 4.5, compared with 1.59 s at pH 7.5. Although this points to a more extended conformation at pH 7.5 with increased polymer entanglement between the chains, the relatively small differences in relaxation times indicate a reduction in pH has only minimal effect on the kind of transient networks observed. Furthermore, the relaxation time was unaffected when reducing the pH from 4.5 to 1.2 indicating that the lacebark mucilage was fairly resistant to acidic pH variations. Indeed, the loss tangent (ratio of loss modulus to storage modulus) when plotted against angular frequency

showed that there was no significant difference ($P<0.05$) between the samples at different pH across the frequency range measured (Fig. 6B).

The nature and complexity of the relaxation processes can also be visualized using Cole-Cole plots of G′ vs. G″ (Fig. 6C). A semicircle in the Cole–Cole plot signifies a system with a single relaxation time, non-semicircle plots however, indicates the existence of more than one relaxation time, as occurred in the lacebark mucilage. This further supports the findings of the structural analysis that the lacebark mucilage consists of either more than one separate molecule or a single polymer containing structurally discrete domains.

The nature of the physical interactions of biopolymers can be assessed by the Cox–Merz rule which is an empirical relationship that exists between the rheological response of destructive and non-destructive deformation. If biopolymer solutions are free from high density physical aggregation or interactions (other than simple entanglement) complex viscosity, η^* (as a function of angular frequency, ω), obtained from oscillatory measurements should be almost identical to that of shear viscosity (as a function of shear rate, $\dot{\gamma}$) [43-45]. When this relationship was examined for the lacebark mucilage at 2% w/v (Fig. 7) the data was in agreement with the Cox-Merz rule indicating the absence of aggregation or specific physical interactions and further supporting the interpretation that the rheological behaviour is that of an entangled concentrated biopolymer solution.

The uronic acid components of the polysaccharides found in mucilages from the Malvaceae have a negative charge at neutral pH, which can cause intra-molecular repulsion resulting in an extended conformation [46, 47]. This favours inter-molecular interactions between the chains rather than self-association. If the pH is lowered to below the pK_a of the uronic acid however, the charge is lost which can either lead to over association between polymer chains (gelation

or precipitation) or to intra-molecular association resulting in a more compact conformation which reduces entanglement between chains (reducing viscosity). In both cases this is manifested in a distinctive change in the mechanical behaviour. Only minimal changes however, were observed for lacebark mucilage. A reasonable explanation for this behaviour is the structural peculiarity of the polysaccharides present in the lacebark mucilage, whereby steric hindrance from the oligosaccharide side-chains prevents strong intra-molecular association when in the deprotonated state at low pH, retaining a relatively extended conformation and thus causing the intermolecular association to remain more favourable. Furthermore, there is also the possibility of hydrophobic forces from the acetyl groups which could influence molecular conformation and therefore the mechanical behaviour. From the chemical and structural analysis of the lacebark mucilage, it is proposed that a more extended conformation and branching of neutral sugar residues provide multiple short-range attachment points for intermolecular entanglement that dominate over the electrostatic repulsion expected from the deprotonated glucuronic acid residues at pH's > pK_a of the uronic acid. At pH's < pK_a of the uronic acid, it is thought that steric and hydrophobic forces dominate maintaining an extended conformation allowing the branched oligosaccharide side-chains to remain available for intermolecular entanglement, manifesting in similar rheological behaviour. The relatively small variation in the rheological behaviour between pH 7.5 and pH 1.2 may indicate potential uses of lacebark mucilage in gastro retentive pharmaceutical formulations.

4. Conclusions

The structure and some physicochemical properties of mucilage isolated from the leaves of the New Zealand endemic tree *H. populnea* have been investigated. The results show that the mucilage is mostly high molar mass polysaccharide, comprising a rhamnogalacturonan I-type backbone with, unusually for pectic-type polysaccharides, side-chains of β-D-Glc*p*A attached

at *O*-3 of the 4-Gal*p*A backbone residues, of α-Gal*p*A and oligosaccharides containing 4-linked α-galactose residues attached at *O*-4 of the 2-Rha*p* backbone residues, as well as some branching at *O*-3 of a small proportion of the 2-linked Rha*p* backbone residues. At this stage, it is not possible to determine whether these structures are all present within one polysaccharide chain or whether the mucilage preparation comprises several different, structurally-related, high molar mass polysaccharides. In the hydrated state, the mucilage exhibits viscoelastic behaviour consistent with intermolecular entanglement within the linear viscoelastic region and this behaviour appears to have relatively small pH dependence when compared with similar mucilages. The rheological behaviour at both acidic and neutral pH may be advantageous if this material is applied as a food ingredient or in oral pharmaceutical dosage forms.

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Author contributions

IS, AS and SC conceived and designed the research, carried out the analyses, interpreted the data and wrote the manuscript. GM and MG contributed to the molar mass, FTIR and rheological analyses and editing of the manuscript.

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610 **Table 1. Chemical composition (weight %) of the mucilage from lacebark leaves; the**
611 **chemical composition of gum from the inner bark of Grewia stems is included for**
612 **comparison.**

Sugar	Lacebark Leaf[a]	Grewia Inner bark[b]
Fucose	0.3	-
Rhamnose	22.3	14.2
Arabinose	0.2	0.2
Xylose	-	0.1
Galactose	11.4	0.2
Glucose	-	2.1
Mannose	-	-
Galacturonic acid	26.0	17.7
Glucuronic acid	14.5	13.9
Total	74.7	48.4
Protein (N x 6.25)	2.6	5.2
Moisture (loss on drying)	13.5	11.0
Ash	12.1	8.0

613 [a]Values are the averages of duplicate analyses.
614 [b]data from [12].
615

616

Table 2. Glycosyl linkage composition (mol%) of carboxyl-reduced lacebark mucilage and grewia gum.

Sugar	Deduced linkage[a]	Lacebark Leaf	Grewia Inner bark
Rha*p*	2-	22.4[b]	34.6[b]
	2,3-	3.2	0.1
	2,4-	8.0	0.2
Gal*p*	4-	15.1	0.9
Glc*p*	4-	1.5	5.0
Gal*p*A	Terminal	12.1	0.3
	4-	7.2	2.0
	3-	0.1	0.2
	3,4-	14.4	26.3
Glc*p*A	Terminal	15.7	30.3
	4-	0.3	-
	6-	-	0.1
	3,4-	-	-

[a] 2-Rha*p* deduced from 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, etc.

[b] Values are the averages of duplicate analyses.

Table 3. Power law exponents for increasing concentrations of lacebark mucilage

Concentration wt%	K (Pa s)	n	R^2
0.5	0.16	0.77	0.99
1.0	1.04	0.60	0.98
2.0	9.65	0.37	0.98

Table 4. Parameters derived from regression fitting according to the Cross equation for increasing concentrations of lacebark mucilage

Concentration wt%	η_0	η_∞	m	C	1/C
0.5	0.7	0.0007	0.373	2.95	0.34
1.0	12.2	0.012	0.506	10.08	0.10
2.0	125	0.125	0.692	11.05	0.08

Figure captions

Figure 1. Fourier transform infrared spectrum of lacebark mucilage.

Figure 2. Selected regions of the ^{13}C (A) and ^{1}H (B) NMR spectra of lacebark leaf mucilage.

Figure 3. Molar mass analysis by size-exclusion coupled with multi-angle laser light scattering (SEC–MALLS) of lacebark leaf mucilage (black) and grewia gum (red).

Figure 4. Measurement of viscosity vs. shear rate at 25 °C for solutions of lacebark leaf mucilage prepared at concentrations of 0.5%, 1.0% and 2.0% w/v at pH 7.5. Data shown are the means ± SD.

Figure 5. Stress sweep for a 2% w/v solution of lacebark leaf mucilage at various pH values. Dotted lines indicate the point where values of critical stress were taken. Full colour version available online.

Figure 6. A) Mechanical spectra (1% strain, 25°C) of lacebark leaf mucilage showing the frequency dependence of G′, G″, B) Loss tangent vs angular frequency (Means ± SD) and C) Cole-Cole plots of G′ vs G″, for 2% w/v solution of lacebark leaf mucilage at various pH values.

Figure 7. Cox–Merz rule applicability for a 2% w/v solution of lacebark leaf mucilage. Measurements were taken at pH 7.5 and 25 °C.

Figure S1. Cross fitting of viscosity vs shear rate data for lacebark leaf mucilage prepared at concentrations of 0.5%, 1.0% and 2.0% w/v at pH 7.5