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4 Structural and rheological studies of a polysaccharide 5 mucilage from lacebark leaves (*Hoheria populnea* A. 6 Cunn.)

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31 ## Abstract

32 A water-soluble mucilage extracted from the leaves of *Hoheria populnea* was chemically and
33 physically characterised. Monosaccharide composition and linkages were determined by high
34 performance anion exchange chromatography (HPAEC), gas chromatography-mass
35 spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Lacebark
36 mucilage was composed of rhamnose, galactose, galacturonic acid and glucuronic acid
37 (2:1:2:1). Proton and ^{13}C NMR spectroscopy, and linkage analysis, revealed a predominantly
38 rhamnogalacturonan I-type (RG I-type) structure comprising of a backbone of →4]-α-D-
39 Gal*p*A-[1→2)]-α-L-Rha*p*-[1→. Data indicated the mucilage likely comprises of a polymer
40 containing several structurally discrete domains or possibly more than one discrete polymer.
41 One domain contains a RG I-type backbone with branching at *O*-3 of Gal*p*A residues to
42 terminal β-D-Glc*p*A residues, another similarly contains a RG I-type backbone but is branched
43 at *O*-4 of the Rha*p* residues to terminal Gal*p*A residues or oligosaccharides containing α-linked
44 4-Gal*p* and terminal Gal*p*A residues. A possible third domain contains contiguous 2-Rha*p*
45 residues, some branched at *O*-3. Hydrated mucilage exhibited pseudoplastic flow behaviour
46 and viscoelastic properties of an entangled biopolymer network which were consistent from
47 pH 7.5 – pH 1.2 and may prove advantageous in potential end-product applications including
48 oral pharmaceuticals or as a food ingredient.

49

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

51

52 **1. Introduction**

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

60

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

74

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

77 lacebark generally relate to the mucilage obtained from the inner bark, the leaves, which
78 similarly contain mucilage, were also used and represent a more sustainable source of material.
79 Similarly to other Malvaceae species, the mucilage from lacebark may have applications in the
80 pharmaceutical industry as a binder in tablets or as a suspending or emulsifying agent [13].
81 Thus, in this present study we have investigated the composition and structure of the mucilage
82 extracted from *Hoheria populnea* leaves and evaluated the physicochemical properties of the
83 hydrated polysaccharide.
84

85 **2. Materials and Methods**

86 *2.1. Extraction and isolation of lacebark mucilage*

87 Fresh leaves of *Hoheria populnea* were obtained from Kiwi Herbs Ltd, Lower Hutt, New
88 Zealand and stored frozen until required. Leaves (300 g, frozen) were coarsely ground prior to
89 extraction. Ground leaves were extracted with 80% ethanol (80 °C, 1 h) to remove phenolic
90 and low molar mass materials. The leaves were then extracted with water, twice at 65 °C for
91 2 h and then at room temperature overnight. Following centrifugation, the combined extracts
92 were mixed with ethanol (1 volume) and left at 4 °C overnight. The precipitated material was
93 recovered by centrifugation and re-dissolved in water, with heating and mixing to aid
94 dissolution. Extracts were then filtered; GD-120 glass fiber filter (Advantec; 0.9 µm) followed
95 by activated carbon (Darco® G-60; using a 1:1 carbon/celite pad). Filtration using a GB-140
96 glass fiber filter (Advantec; 0.4 µm) removed any carbon fines and the mucilage was then
97 further purified and concentrated using a Vivaflow 200 crossflow device with a
98 polyethersulfone (PES) 100 kDa molecular weight cut-off (MWCO) membrane (Sartorius)
99 prior to freeze-drying.
100
101

102 *2.2. General analyses*

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

108

109 *2.3. Chromatographic and spectroscopic analyses*

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

114

115 *2.3.1. Constituent sugar analysis*

116 Constituent sugar composition was determined by high-performance anion-exchange
117 chromatography (HPAEC) following hydrolysis of the polysaccharides present to their
118 component monosaccharides. Samples were hydrolysed with methanolic HCl (3 mol L^{-1}, 80
119 °C, 18 h), followed by aqueous CF_3COOH (2.5 mol L^{-1}, 120 °C, 1 h). The resulting
120 hydrolysates were analysed on a CarboPac PA-1 (4 x 250 mm) column equilibrated in NaOH
121 (25 mmol L^{-1}) and eluted with a simultaneous gradient of NaOH and sodium acetate (1 mL
122 min^{-1}). The sugars were identified from their elution times relative to standard sugar mixes,
123 quantified from response calibration curves of each sugar and expressed as weight percent
124 anhydro-sugar as this is the form of sugar present in a polysaccharide.

125

126 *2.3.2. Glycosyl linkage analysis*

127 Glycosyl linkage composition was determined by gas chromatography-mass spectrometry
128 (GC-MS) of partially methylated alditol acetates (PMAAs), after first reducing uronic acid
129 residues to their dideuterio-labelled neutral sugars [16]. Lacebark mucilage (10 mg) was
130 dissolved in 50 mmol L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (2 mL, pH 4.75)
131 and, following activation with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-
132 toluenesulfonate (carbodiimide reagent; 400 μL, 500 mg mL^{-1}), free uronic acid residues were
133 reduced overnight with $NaBD_4$. The carboxyl-reduced samples were dialysed (6-8 kDa
134 MWCO) against distilled water, freeze-dried and the sample reduced a second time following
135 carbodiimide activation. Constituent sugar analysis of this material showed that no uronic acid
136 was detected. Carboxyl-reduced mucilage was methylated [17] and during the chloroform
137 extraction step insoluble, particulate material was observed at the water/chloroform interface
138 indicating incomplete methylation [18]. The methylation was repeated, the methylated
139 mucilage hydrolysed with 2.5 mol L^{-1} CF_3COOH and reduced with 1 mol L^{-1} $NaBD_4$ in 2 mol
140 L^{-1} NH_4OH overnight at 25°C. Following removal of excess borate, the resulting alditols were
141 acetylated in acetic anhydride (200 μL, 100 °C, 2 h) and extracted into dichloromethane. The
142 PMAA derivatives produced were analysed by GC-MS (Agilent Technologies, Santa Clara,
143 USA). Identifications were based on peak retention times relative to an internal standard, myo-
144 inositol, and on comparisons of electron impact spectra with the spectra obtained from
145 reference PMAA standards prepared by the method of Doares et al.[19].
146
147 *2.3.3. Fourier transformed infrared spectroscopy (FTIR)*
148 FTIR spectroscopy was carried out in transmittance mode using a Nicolet 380 FTIR
149 Spectrometer (ThermoElectron Corporation, Waltham, USA) over the range 4000–400 cm^{-1} at
150 2 cm^{-1} resolution averaging 100 scans. The degree of esterification (DE) of the lacebark
151 mucilage and pectin standards was determined according to the methods of Chatjigakis et al.

152 [20], Manrique and Lajolo [21] and Singthong et al. [22]. Briefly, the area of the peak at 1730
153 cm^{-1} (esterified carboxyls) was divided by the sum of the areas of the peaks at 1730 cm^{-1} and
154 1600 cm^{-1} which is proportional to DE. The DE was extrapolated from the calibration plot of
155 the pectin standards.
156
157 *2.3.4. Nuclear magnetic resonance (NMR) spectroscopy*
158 Lacebark mucilage was exchanged with deuterium by freeze-drying with D_2O (99.9 atom%)
159 three times. Samples were dissolved in D_2O and ^1H and ^{13}C (both ^1H coupled and decoupled)
160 spectra were recorded on a Bruker Avance DPX-500 spectrometer at 90°C. The ^1H and ^{13}C
161 chemical shifts were measured relative to an internal standard of $(CH_3)_2SO$ (^1H, 2.70 ppm; ^{13}C,
162 39.5 ppm; [23]). Assignments were made from heteronuclear single quantum coherence
163 (HSQC) COSY experiments and by comparing the spectra with published data.
164
165 *2.3.5. Size-exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)*
166 Molar mass was determined using size-exclusion chromatography coupled with multi-angle
167 laser light scattering (SEC-MALLS). Samples (2 mg/mL in 0.1 mol L^{-1} $NaNO_3$) were allowed
168 to hydrate by standing at room temperature overnight and centrifuged (14,000 x *g*, 10 min) to
169 clarify. The soluble material (100 μL) was injected onto two columns (TSK-Gel $G5000_{PWXL}$
170 and $G4000_{PWXL}$, 300 x 7.8mm, Tosoh Corp., Tokyo, Japan) connected in series and eluted with
171 0.1 M $NaNO_3$ (0.7 mL min^{-1}, 60 °C). The eluted material was detected using a UV
172 spectrophotometer (280 nm), a DAWN-EOS MALLS detector (Wyatt Technology Corp.,
173 Santa Barbara, USA) and a refractive index (RI) monitor (Waters Corp., Milford, USA). The
174 data for molar mass determination was analysed using ASTRA software (v6.1.84, Wyatt
175 Technology Corp.) using a refractive index increment, *dn/dc*, of 0.146 mL g^{-1} [24].
176

177 *2.4. Rheological measurements of hydrated lacebark mucilage*

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

185

186 *2.4.1. Viscosity measurements*

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

191

192 *2.4.2. Modelling of flow behaviour*

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

196 $$\tau = K\dot{\gamma}^{n} \qquad \text{eq. 1}$$

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

202

203 *2.4.3. Small deformation oscillatory rheology*

204 Amplitude sweeps were used to determine the linear viscoelastic region and the critical stress
205 required by the samples to yield. The stress was gradually increased using small deformation
206 oscillations from 0.1 Pa to 100 Pa at an angular frequency of 10 rad s^{-1}. All measurements were
207 taken at 25 °C. To characterise the viscoelastic properties of lacebark mucilage prepared at pH
208 7.5, 4.5, 2.5 and 1.2, small deformation oscillatory measurements of storage modulus (G′) and
209 loss modulus (G″) were taken across a range of angular frequencies from 0.1–100 rad s^{-1} at 25
210 °C to determine the mechanical spectra of the samples. Measurements were taken at a constant
211 strain of 1% which was previously determined (using amplitude sweeps) to be within the linear
212 viscoelastic region. Moisture loss from samples was minimized during all rheological
213 measurements by applying a thin layer of silicone oil on the periphery of the loaded sample
214 and using a solvent trap on the geometry. All measurements were performed using a Malvern
215 Kinexus rheometer (Malvern Instruments, UK) fitted with a 55 mm, 2° cone-plate geometry
216 with gap of 70 µm.

217

218 *2.4.4 Statistical methods*

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

222

223 **3. Results and Discussion**

224 *3.1. Composition of lacebark leaf mucilage*

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

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

233

234 *3.2. Structural analyses of lacebark leaf mucilage*

235 *3.2.1. Linkage analysis*

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

252 34]. Similarly to the lacebark leaf polysaccharide, these mucilage polysaccharides generally
253 have some branching at *O*-4 of 2-Rha*p* residues and 4-Gal*p* disaccharide or short
254 oligosaccharide side chains. The mucilage from lacebark leaves is unusual in having a high
255 content of terminally linked Gal*p*A residues. In contrast, the linkage composition of grewia
256 gum was much simpler, with three major linkages, 2-Rha*p*, 3,4-Gal*p*A and terminal Glc*p*A; it
257 resembled the mucilage extracted from the roots of *Hibiscus moscheutos* [5].
258
259 *3.2.2. FTIR spectroscopy*
260 The FTIR spectrum of lacebark leaf mucilage was typical of polysaccharides (Fig. 1),
261 containing two major peaks in the region between 3600 and 1800 cm^{-1} corresponding to O–H
262 stretching absorption due to inter- and intramolecular hydrogen bonding (3000–3600 cm^{-1}) and
263 C–H absorption (2930 cm^{-1}), which typically includes CH, CH_2 and CH_3 stretching and
264 bending vibrations [20, 35]. The region of the spectrum below 1800 cm^{-1} indicates the
265 'fingerprint' region for polysaccharides [36] and was similar in both lacebark mucilage and
266 grewia gum [12]. The peaks between 1200 and 800 cm^{-1} are generally specific to particular
267 polysaccharides, with this region dominated by pyranose ring vibrations overlapped with C-
268 OH stretching vibrations and C-O-C vibrations from glycosidic bonds [36, 37]. The peak at
269 1415 cm^{-1} showed COO^- symmetric stretching and the peaks at 1600 cm^{-1} and 1724 cm^{-1}
270 indicate free and esterified carboxyl groups, respectively [35]. A peak at ~1730 cm^{-1} can be
271 used to estimate the degree of esterification in uronic acid containing polysaccharides such as
272 pectin and grewia gum [12]. Using this method the degree of esterification of lacebark mucilage
273 was estimated to be ~10%, which was considerably less than that of grewia gum (~50%).
274
275 *3.2.3. NMR spectroscopy*

276 The ^{13}C NMR spectrum of lacebark leaf mucilage (Fig. 2A) resembled that of grewia gum,
277 except that the anomeric region of lacebark mucilage showed C-1 signals at 100.3 and 101.0
278 ppm, and C-6 signals at 60.9 and 61.9 ppm that were not observed in the spectrum of grewia
279 gum [12]. These signals were thus assigned to 4-Gal*p* residues that were not detected in the
280 linkage analysis of grewia gum (Table 2). These two C-1 signals showed H-1 cross-peaks in
281 the ^{1}H NMR spectrum (Fig. 2B) at 5.22 and 5.23 ppm, indicating that the 4-Gal*p* residues are
282 in the α configuration; the presence of such residues has been reported previously only once
283 for the RG I-like polysaccharide from *Cola cordifolia* bark [6], although Sengkhamparn et al.
284 [7] showed the presence of α-linked terminal Gal*p* residues in okra pectin.

285

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

289

290 The C-1 signal at 104.5 ppm with a H-1 cross-peak at 4.73 ppm was assigned to terminal β-
291 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
292 consistent with the presence of α-Gal*p*A residues. There were no signals in the NMR spectra
293 of lacebark mucilage to indicate the presence of methylesterified α-Gal*p*A residues (^{13}C 54
294 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
295 similar polysaccharides from other members of the Malvaceae [6, 36]. However, signals at
296 21.2–21.4 ppm (^{13}C) and 2.14–2.22 ppm (^{1}H) showed the presence of *O*-acetyl groups, similar
297 to that found previously for grewia gum [12], indicating that the esterification identified by
298 FTIR was *O*-acetylation rather than methyl esterification. We have not investigated the
299 location of the *O*-acetyl groups, but Sengkhamparn et al. [7] showed that they were located on
300 both Rha*p* and Gal*p*A residues of pectic polysaccharides extracted from okra pods.

301

302 *3.2.4. Structure of lacebark mucilage*

303 Based on the composition and structural analysis data we deduce that lacebark mucilage
304 polysaccharide predominantly comprises of a repeating disaccharide backbone of →4]-α-D-
305 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
306 *O*-4, respectively, and two thirds of the 4-Gal*p*A residues branched at *O*-3. Side-chain
307 substituents include terminal β-Glc*p*A, terminal α-Gal*p*A and short oligosaccharides
308 containing →4]-α-Gal*p*-[1→. The NMR spectra shows the presence of *O*-acetyl groups, which
309 by comparison with published data are probably located on some of the Rha*p* and Gal*p*A
310 residues [7].

311

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

326 predominance of 2-Rha*p* over 4-Gal*p*A indicates the presence of a possible third domain
327 containing contiguous (1→2)-linked α-L-Rha*p* residues with some of the Rha*p* residues
328 branched at *O*-3. Although uncommon, a similar predominance of Rha over GalA has been
329 reported in RG I from flax seed mucilage and *Arabidopsis* [27, 41] and is evident in the
330 mucilages from many of the Malvaceae species studied [9, 10, 31, 33]. The presence of
331 branching at *O*-3 of 2-Rha*p* residues is unusual for mucilages from Malvaceae species, having
332 only been previously reported in the leaves and bark of *Cola cordifolia* [6, 25]. While branching
333 at *O*-3 of Rha*p* residues is rare in RG I polysaccharides there is also evidence of this branching
334 pattern in RG I from flax seed mucilage [27] and pectic-type polysaccharides from the inner
335 bark of *Ulmus glabra* (Wych elm) [26]. As far as we are aware, within the Malvaceae the
336 presence of 4-linked α-Gal*p* is unique to the mucilage from lacebark leaves and the bark of *C.*
337 *cordifolia* [6].
338
339 *3.3. Size-exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)*
340 Size-exclusion chromatography of lacebark leaf mucilage and grewia gum showed that ~90%
341 of the material eluted between 10.5–13.5 mL; both samples also contained a small late-eluting
342 peak (Fig. 3). The mass-average molar mass (M_w) of the major peak of lacebark mucilage was
343 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
344 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
345 mol^{-1}) was slightly smaller than that of lacebark mucilage, but its molar mass range was much
346 greater with a Đ of 1.67; the z-average mean square radius was 81.0 nm.
347
348 *3.4. Rheological measurements*
349 To understand the flow properties of hydrated lacebark mucilage both steady shear and
350 dynamic measurements of viscoelasticity were performed. This provided an insight into the

351 mechanical behaviour and potential industrial applications as a thickener or suspending agent.
352 Measurements of apparent viscosity increased significantly ($p<0.05$) with increasing
353 concentration (0.5%, 1% and 2% w/v) and also showed significant pseudoplastic flow
354 behaviour in all samples with viscosity decreasing with increasing shear rate (Fig. 4). The
355 calculated exponents of flow behaviour, when modelled according to the Ostwald–de Waele
356 power law, showed that increasing concentration caused the flow behaviour index (*n*) to
357 decrease, indicating greater pseudoplastic behaviour at higher concentrations (Table 3). To
358 determine the onset of shear thinning behaviour the data was fitted to the Cross equation (eq.
359 2).
360

361 $$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{1+(C\,\dot{\gamma})^m} \quad \text{(eq. 2)}$$

362 where η_0 is zero shear viscosity, η_{∞} is the infinite shear viscosity, *m* is the rate constant (a
363 measure of the degree of dependence of viscosity on shear rate in the shear thinning region)
364 and *C* is the consistency constant given in dimensions of time. The reciprocal, $1/C$, is defined
365 as the critical shear rate and is an indicator of the shear rate at the onset of shear thinning.
366 Parameters elucidated from the Cross model fitting of the measured viscosity vs shear rate data
367 (supplementary data, S1) are given in table 4. These parameters revealed that the onset of shear
368 thinning began at significantly lower shear rates ($p<0.05$) with increasing polymer
369 concentration ($1/C$ = 0.34, 0.1 and 0.08 s^{-1} for 0.5%, 1.0%, and 2.0% respectively). Moreover,
370 the dependence of viscosity on shear rate in the shear thinning region (*m*) increased with an
371 increase in concentration, indicating an increase in the extent of pseudoplastic behaviour. This
372 kind of flow behaviour is particularly useful in pharmaceutical liquid formulations and in foods
373 as a suspending agent and viscosifier by providing increased viscosity on storage preventing
374 sedimentation and then easy dispensing following the application of shear.
375

376 To gain an insight into the viscoelastic properties of lacebark leaf mucilage, small deformation
377 oscillatory measurements were performed on 2% w/v solutions prepared at a range of pH
378 values. Stress sweep measurements were used to evaluate the critical stress required by the
379 samples to yield. The value of critical stress was lowest at pH 1.2 (~5 Pa) and highest at pH
380 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
381 has previously been observed in other similar anionic polysaccharides [12, 35] and has been
382 attributed to reduced intermolecular associations between polymer chains [12]. In this case
383 however, it should be noted that the differences in critical stress with reducing pH are relatively
384 small which points to the material maintaining some level of intermolecular entanglement even
385 at low pH. To investigate this further frequency sweeps were performed on the same samples.
386 The mechanical spectra obtained were characteristic of entangled polymer solutions with G″
387 greater than G′ at low frequencies of oscillation (where the period of oscillation is sufficient to
388 allow disentanglement to occur). At higher frequencies, however, elastic deformation
389 dominated with G′ greater than G″, indicating that polymer entanglement was the dominating
390 intermolecular interaction between the chains (Fig. 6). On further examination of the
391 mechanical spectra only subtle differences were observed at different pH values indicating the
392 mucilage was fairly resistant to changes in pH. Characteristic relaxation time (τ) calculated
393 from the inverse of the angular frequency at which G′ and G″ cross ($\tau = \omega_c^{-1}$), as described by
394 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
395 7.5. Although this points to a more extended conformation at pH 7.5 with increased polymer
396 entanglement between the chains, the relatively small differences in relaxation times indicate
397 a reduction in pH has only minimal effect on the kind of transient networks observed.
398 Furthermore, the relaxation time was unaffected when reducing the pH from 4.5 to 1.2
399 indicating that the lacebark mucilage was fairly resistant to acidic pH variations. Indeed, the
400 loss tangent (ratio of loss modulus to storage modulus) when plotted against angular frequency

401 showed that there was no significant difference ($P<0.05$) between the samples at different pH
402 across the frequency range measured (Fig. 6B).
403 The nature and complexity of the relaxation processes can also be visualized using Cole-Cole
404 plots of G′ vs. G″ (Fig. 6C). A semicircle in the Cole–Cole plot signifies a system with a single
405 relaxation time, non-semicircle plots however, indicates the existence of more than one
406 relaxation time, as occurred in the lacebark mucilage. This further supports the findings of the
407 structural analysis that the lacebark mucilage consists of either more than one separate
408 molecule or a single polymer containing structurally discrete domains.
409
410 The nature of the physical interactions of biopolymers can be assessed by the Cox–Merz rule
411 which is an empirical relationship that exists between the rheological response of destructive
412 and non-destructive deformation. If biopolymer solutions are free from high density physical
413 aggregation or interactions (other than simple entanglement) complex viscosity, η^* (as a
414 function of angular frequency, ω), obtained from oscillatory measurements should be almost
415 identical to that of shear viscosity (as a function of shear rate, $\dot{\gamma}$) [43-45]. When this relationship
416 was examined for the lacebark mucilage at 2% w/v (Fig. 7) the data was in agreement with the
417 Cox-Merz rule indicating the absence of aggregation or specific physical interactions and
418 further supporting the interpretation that the rheological behaviour is that of an entangled
419 concentrated biopolymer solution.
420
421 The uronic acid components of the polysaccharides found in mucilages from the Malvaceae
422 have a negative charge at neutral pH, which can cause intra-molecular repulsion resulting in an
423 extended conformation [46, 47]. This favours inter-molecular interactions between the chains
424 rather than self-association. If the pH is lowered to below the pK_a of the uronic acid however,
425 the charge is lost which can either lead to over association between polymer chains (gelation

426 or precipitation) or to intra-molecular association resulting in a more compact conformation
427 which reduces entanglement between chains (reducing viscosity). In both cases this is
428 manifested in a distinctive change in the mechanical behaviour. Only minimal changes
429 however, were observed for lacebark mucilage. A reasonable explanation for this behaviour is
430 the structural peculiarity of the polysaccharides present in the lacebark mucilage, whereby
431 steric hindrance from the oligosaccharide side-chains prevents strong intra-molecular
432 association when in the deprotonated state at low pH, retaining a relatively extended
433 conformation and thus causing the intermolecular association to remain more favourable.
434 Furthermore, there is also the possibility of hydrophobic forces from the acetyl groups which
435 could influence molecular conformation and therefore the mechanical behaviour. From the
436 chemical and structural analysis of the lacebark mucilage, it is proposed that a more extended
437 conformation and branching of neutral sugar residues provide multiple short-range attachment
438 points for intermolecular entanglement that dominate over the electrostatic repulsion expected
439 from the deprotonated glucuronic acid residues at pH's > pK_a of the uronic acid. At pH's < pK_a
440 of the uronic acid, it is thought that steric and hydrophobic forces dominate maintaining an
441 extended conformation allowing the branched oligosaccharide side-chains to remain available
442 for intermolecular entanglement, manifesting in similar rheological behaviour. The relatively
443 small variation in the rheological behaviour between pH 7.5 and pH 1.2 may indicate potential
444 uses of lacebark mucilage in gastro retentive pharmaceutical formulations.
445

446 **4. Conclusions**

447 The structure and some physicochemical properties of mucilage isolated from the leaves of the
448 New Zealand endemic tree *H. populnea* have been investigated. The results show that the
449 mucilage is mostly high molar mass polysaccharide, comprising a rhamnogalacturonan I-type
450 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

617 **Table 2. Glycosyl linkage composition (mol%) of carboxyl-reduced lacebark mucilage**
618 **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-	-	-

619 [a] 2-Rha*p* deduced from 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, etc.
620 [b] Values are the averages of duplicate analyses.
621
622
623 **Table 3. Power law exponents for increasing concentrations of lacebark mucilage**

624

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

625
626
627
628 **Table 4. Parameters derived from regression fitting according to the Cross equation for**
629 **increasing concentrations of lacebark mucilage**

630

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

631

632 **Figure captions**

633 Figure 1. Fourier transform infrared spectrum of lacebark mucilage.

634

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

636

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

639

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

643

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

647

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

652

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

655

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