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Evaluation of some important physicochemical properties of starch free grewia gum

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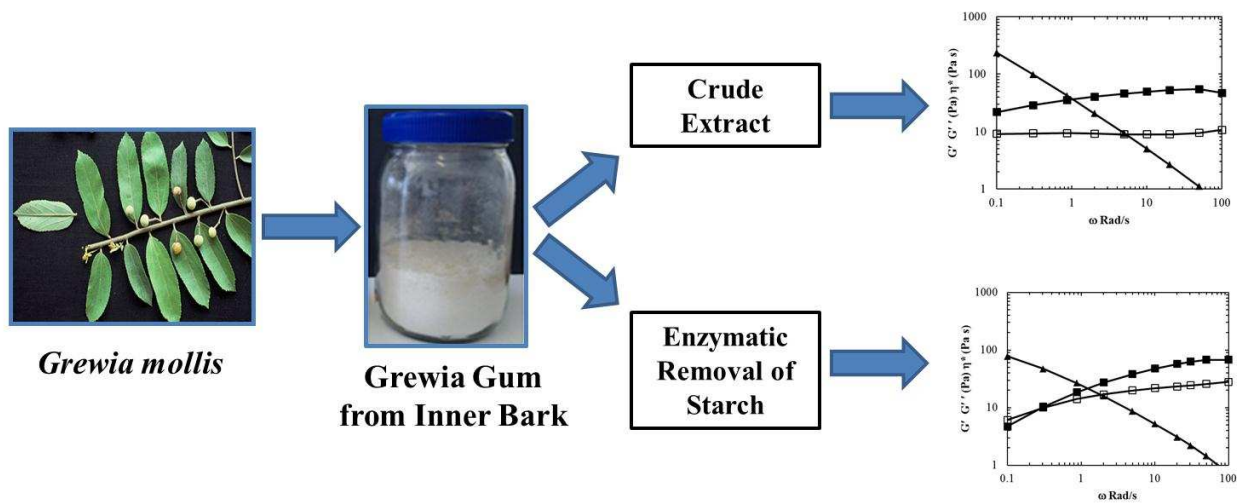
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Graphical Abstract



Highlights:

- High glucose content of grewia gum is the result of high starch content.
- Grewia gum contains highly acetylated uronic acids.
- Removal of starch has an impact on the physicochemical properties of the extracts.
- Rheological behaviour of grewia gum is influenced by changes in pH.

Abstract

Gums obtained by extraction from the inner bark of stems can be found in association with starch, which must be digested in order to obtain a refined polysaccharide isolate. In the present study, grewia gum obtained from the inner bark of the stems of *Grewia mollis* was shown to co-exist with starch and the effect of starch digestion on the physicochemical properties of the resultant polysaccharide was evaluated.

The gum was extracted by maceration of the inner bark in deionized water and isolated by a combination of filtration, centrifugation and finally precipitation with absolute ethanol to produce the crude grewia gum extract (GG). The presence and content of starch in the gum sample was determined followed by enzymatic digestion of the starch using α -amylase (Termamyl 120L) to give a starch-free extract (GGDS). Physicochemical properties of the extracts such as total carbohydrates, total protein, differential sugar composition, NMR, intrinsic viscosity and rheological behaviour of the samples were evaluated.

The GG extract had total carbohydrate content of ~ 60 % out of which 11.8 % was starch, and a protein content of 2.3 %. Samples also contained galacturonic and glucuronic acid which were highly acetylated. Both samples had a higher proportion of galacturonic acid than glucuronic acid and contained rhamnose, arabinose, galactose, glucose and xylose as neutral sugars in varying proportions. Rheological measurements on 2 %w/w dispersions of the extracts show minor differences between both the original extract and the de-starched material but were influenced by changes in pH.

Keywords: *Grewia mollis*; polysaccharides; uronic acids; neutral sugars, starch

1. Introduction

Grewia mollis is a shrub native to sub-Saharan Africa belonging to the Malvaceae family. It is cultivated in the middle belt region of Nigeria where the inner bark from the stems of the shrub is pulverised and often used as a thickener in various food formulations. This crude extract has previously been identified to contain polysaccharides (Okafor, Chukwu & Udeala, 2001; Nep and Conway, 2011a) that consist of five neutral sugars. These have been identified as glucose, rhamnose, arabinose, xylose and galactose (Nep and Conway, 2011a). The mean relative concentration of the sugars in the polysaccharide has been previously reported to be glucose (67.14%), rhamnose (6.2%), xylose (2.72%), galactose (9.61%) and arabinose (12.71%). However, there is little information in literature on the uronic acid composition of the polysaccharide and an explanation of the relatively high glucose content.

In addition to the indigenous use of the extract in foods, previous studies have indicated the potential of *grewia* extract as a pharmaceutical excipient in oral formulations, as a binder or sustained release matrix (Nep & Conway 2011b), as bioadhesive (Nep & Okafor 2006; Nep & Conway 2011c) or as a suspending agent (Nep & Conway 2011d). Extraction conditions are crucial to the functional properties and influence significantly the yield and apparent viscosity of the extracts (Akdowa et al 2014). Other studies have shown that aqueous dispersions of *grewia* extract exhibit pseudoplastic flow with a relatively high viscosity at low concentrations (Okafor, Chukwu & Udeala 2001). Variations in extraction methods have also been explored and have shown to impact on the functional properties of the extract (Ogaji, 2011) providing the potential to diversify the applications from extracts produced using different methods. Although there are several reports of the steady shear behaviour of aqueous dispersions there has been limited analysis of their viscoelastic properties. Understanding the viscoelasticity of aqueous dispersions provides a further insight

to the mechanical behaviour and ultimately could reveal further potential applications of grewia extract. Due to the absence of detailed physical and chemical characterisation from a standardised extraction procedure, translation to industrial application has yet to be achieved despite the extract enjoying the advantage of being of a renewable source, abundant in supply and of relatively low cost of the raw material.

Furthermore, the high glucose content of the gum coupled with the fact that the gum is obtained by extraction from the inner bark of the plant *Grewia mollis*, creates the likelihood of starch being present with the gum. In the present study, the starch content of grewia gum (GG) was evaluated and was subsequently enzymatically digested to give a starch-free extract (GGDS). The physicochemical properties of the two extracts were evaluated, which included a comprehensive evaluation of the uronic and neutral sugar composition, and rheological properties.

2. Materials and Methods

2.1 Materials

All sugar standards D(+) fucose, L-rhamnose, D-glucose, D-galactose, L-arabinose, D(+) xylose, D(+) mannose, D-glucuronic acid and D(+)-galacturonic acid and citrus pectin standards with known degrees of esterification (DE) 34%, 40% and 70% were purchased from Sigma-Aldrich (Poole, Dorset, UK) and standards with degree of esterification of 50% and 60% were prepared by mixing appropriate amounts of two of the procured commercial standards. Termamyl 120L (heat-stable alpha-amylase) was a kind donation from Megazyme, (UK), absolute ethanol, isopropanol, trifluoroacetic acid (TFA), sodium acetate, sodium hydroxide, sodium metabisulphite, perchloric acid, 1% iodine in potassium iodide solution, hydrochloric acid were purchased from Sigma-Aldrich (Poole, Dorset, UK). Dialysis tubing

with molecular weight cut-off of 12500 Da. All other reagents were of analytical grade. Grewia polysaccharide gum samples were extracted in our laboratories as described in the next section.

2.2 Extraction of GG

The fresh inner bark from the stems of *Grewia mollis* was purchased from the local market in Mangu local government area in Plateau state, Nigeria. It was subsequently air-dried (to preserve the material until the time of extraction) and shredded and the shredded material was macerated under ambient conditions in 0.1% w/v sodium metabisulphite for 24 h. The swollen gum was separated from the residue by filtration through a muslin bag. The filtrate was precipitated from solution using 2 volumes of absolute ethanol over a 4 hour period. Further purification was achieved by re-dispersion in water and final precipitation in 2 volumes of absolute ethanol for another 4 hours to give gum fraction named GG which was then oven-dried at 50 °C for 24 h.

2.3 General analyses

Moisture content was determined by oven-drying at 80°C for 24 h. Total protein, determined as nitrogen x 6.25, and ash contents were analysed by an accredited chemical laboratory (Campbell Microanalytical Laboratories, University of Otago, Dunedin, New Zealand). All determinations were performed in duplicate.

2.4 Starch test and quantification

The *grewia* sample GG was tested for starch using 1% v/v iodine in KI solution. Exactly 20 µL were added in 5 mL of 0.5 % polymer dispersions. The standard method of the Association of Official Analytical Chemists (AOAC, 1984) was used for estimation of starch

content. Briefly, 1 mL of 5 mg/mL gum dispersion was added to 3 mL of 70% perchloric acid solution and then heated at 80 °C for 30 min. The mixture was then cooled to room temperature and 20 µL of 1 % iodine in KI solution was added to each of the sample tubes followed by centrifugation at 2000 rpm for 10 min. Absorbance was read at 500 nm. The amount of starch was measured with the help of a calibration curve obtained by using different concentrations of standard soluble starch solutions (0.008-0.125 mg/mL). The blank contained 1 mL deionized water, 3 mL perchloric acid (70 %) and 20 µL of 1 % iodine in KI solution treated in the same manner as the sample and standards.

2.5 Starch digestion

The starch content of GG was digested according to the method of Agbenorhevi et al (2011) with slight modifications. Briefly, 3000 mL of 1 % w/v dispersion of GG were treated with Termamyl 120L (1 % v/v) while stirring constantly at 70 °C for 4 h. The termamyl was pretreated by heating at 70 °C for 30 min to deactivate any pectinases and arabinoxylanases. Every 1 h an aliquot of the dispersion was removed and tested for the presence of starch. Starch digestion was complete in 3 h after which the sample did not test positive for starch. Subsequently, protein from the sample was precipitated by adjusting the pH to 4.5 with 2 M HCl and centrifuging at 4400 rpm for 20 min. The supernatant was then dialysed against deionized water for 72 h using cellulose membrane with a molecular weight cut-off of 12500 Da. The dialysed material was precipitated using two volumes of 95% ethanol followed by solvent exchange using one volume of isopropanol. The precipitate was oven dried for 24 hours at 50 °C. This sample was named GGDS.

2.6 FTIR and degree of esterification

FT-IR spectroscopy using a Nicolet 380 FTIR Spectrometer (ThermoElectron Corporation, USA) was carried out on the samples and reference standards with varying degrees of methylation (DM). The spectra were recorded at the transmittance mode from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} with 128 co-added scans. The degree of esterification of grewia polysaccharide samples and pectin standards were determined according to the method of Chatjigakis et al., (1998), Manrique and Lajolo (2002) and Singthong et al., (2004). Briefly, the area of the band at 1730 cm^{-1} (esterified carboxyls) divided by the sum of the areas of the band at 1730 cm^{-1} and 1600 cm^{-1} is proportional to DM. The degree of esterification was extrapolated from the calibration plot of the pectin standards.

2.7 Constituent sugar analysis

The constituent sugar composition of the GG and GGDS preparations was determined high-performance anion-exchange chromatography (HPAEC) after hydrolysis of the polysaccharides present to their component monosaccharides, as described by De Ruiter et al. (1992) with modifications (Wee et al., 2014). Briefly, samples (0.5 mg) were hydrolysed with methanolic acid (3 N, 500 μL , 80 $^{\circ}\text{C}$ 18 h), followed by aqueous TFA (2 M, 500 μL , 120 $^{\circ}\text{C}$, 1 h). Standard sugar mixes were hydrolysed at the same time as the samples. The resulting hydrolysates were dried, re-dissolved in distilled water (0.05 mg mL^{-1}) and aliquots (20 μL) were separated at 30 $^{\circ}\text{C}$ on a CarboPac PA-1 (4 x 250 mm) column equilibrated in 25 mM NaOH and eluted with simultaneous gradients of NaOH (25–10 mM from 0–10 min, then 10–100 mM from 10–30 min and held to 55 min) and sodium acetate (0–500 mM NaOH from 30–55 min) at a flow rate of 1 mL min^{-1} and monitored by pulsed amperometric detection, using the Dionex standard carbohydrate waveform. The sugars were identified

from their elution times relative to standard sugar mixes and quantified from response calibration curves at different concentrations of each sugar.

2.8 Nuclear Magnetic Resonance (NMR) of Samples

GGDS was dissolved in 99.6 atom% D₂O, at a concentration of 100 mg in 3 mL in a 10 mm o.d. tube. The ¹³C NMR spectrum was acquired at 125 MHz on a Bruker Avance DPX-500 spectrometer at 80 °C with data averaged over 6 h. Chemical shifts were measured relative to an internal standard of Me₂SO 39.5 ppm.

2.9 Intrinsic viscosity

Solutions of the polysaccharide samples were prepared in deionized water at concentrations of 0.005 % w/v to 0.2 % w/v. The intrinsic viscosity [η] of grewia solutions was determined at 20 °C with an Ubbelohde capillary viscometer and calculations were made according to the Huggins equation (Huggins, 1942):

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2c \quad (1)$$

where η_{sp} and [η] are the specific and intrinsic viscosities, c = the concentration in g/dL, and k' = the Huggins constant

2.10 Rheological Analysis

2 % w/v dispersions of GG and GGDS (pH 2.5) were prepared by first adjusting the pH of deionised water using 2 M HCl solution and then incorporating the measured amount of the gum while heating to 80 °C in a capped vial with constant stirring for 10 min. The pH was confirmed by final adjustment with 2 M HCl. 2% w/v dispersions of GG and GGDS heated at 80 °C in deionised water had pH values of 5.3 and 5.4, respectively, which were adjusted to pH 5 for comparison. The samples were allowed to cool to room temperature

before rheological tests were performed. Small deformation oscillatory measurements of elastic (G') and viscous modulus (G'') were undertaken to monitor the viscoelastic behaviour of the dispersions using a Bohlin Gemini rheometer (stress controlled) fitted with a 55 mm and 2 ° cone and plate geometry with gap of 70 μm . Amplitude sweeps were performed to ascertain the linear viscoelastic region of the samples. To reveal the mechanical spectrum of the grewia samples measurements of G' , G'' and complex dynamic viscosity (η^*) were taken over a frequency range of 0.1 to 100 rad/s at 1 % strain at 20 °C. To investigate thermal effects on rheology, heating and cooling scans were also performed by measuring G' and G'' (10 rad/s, 1% strain) across a temperature range between 5 - 90 °C using a 2 °C/min temperature ramp. Moisture evaporation was minimized during the measurements by coating the periphery of the sample with light silicone oil and using a solvent trap on the geometry.

3. Results and Discussion

3.1 Composition of grewia polysaccharide

Table 1 shows the composition of GG and GGDS. GG contained 2.3% protein, 11.8% moisture and 10.2% ash, while GGDS contained 5.2% protein, 11.0% moisture and 8.0% ash. The total sugar content of both GG and GGDS determined by HPAEC was just below 50%, comprising mostly rhamnose, glucuronic acid and galacturonic acid (Table 1). The starch content of GG was determined as 11.8% and was not detected in GGDS. The amount of glucose after starch digestion fell from 6.4% (GG) to 2.1% (GGDS) and indicated that the high proportion of glucose in GG was due to the presence of starch.

3.2 FTIR spectra of GGDS

No major structural differences in the spectra of the two samples can be seen except for the higher intensity of the peaks and bands of GGDS (Figure 1). The broad band between

3400 - 2500 cm^{-1} is attributed to moisture or O-H stretching absorption due to inter- and intra-molecular hydrogen bonds of the glucuronic acid and galacturonic acid backbone (Singthong et al., 2004; Nep and Conway, 2011). The occurrence of the O-H stretch over a broad range of frequencies has been reported to be indicative of several features of a compound including free hydroxyl group stretching bonds that occur in samples in vapour phase and bonded O-H bands of carboxylic acids (Silverstein et al 1991). The band at 2910 cm^{-1} which is superimposed upon the broader O-H band which is typical for pectins is due to C-H absorption (C-H, CH_2 and CH_3 stretching and bending vibrations and may be attributed to methyl groups of the rhamnose or methyl esters of the uronic acids (Filipov, 1992; Kacurakova et al., 2000). The major functional groups are usually in the region between 1000 and 2000 cm^{-1} of the FTIR spectra (Kalapathy & Proctor 2001). The carbonyl bands at 1630-1650 cm^{-1} and 1740-1760 cm^{-1} indicate the free and esterified carboxyl groups, respectively (Gnanasambandam & Proctor 1999). Increase in esterification will also increase the intensities and band area of the esterified carboxyl groups as seen with spectrum of GGDS. The peak at 1730 cm^{-1} has been assigned to esterified carboxylates while the peak at about 1610 cm^{-1} is due to free carboxylates (Singthong et al., 2004). Estimation of the degree of esterification by FTIR spectra indicated that approximately half of the uronic acid residues were esterified (see NMR spectroscopy, section 3.3). The absorption patterns between 1300-800 cm^{-1} are referred to as the fingerprint region within which the region between 1300-1000 cm^{-1} is attributed to C=O stretches (Coimbra et al., 1998).

3.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{13}C NMR spectrum of GGDS showed three major C-1 signals from 99.1 to 104.1 ppm, (Fig. 2). The NMR spectrum of the crude *Grewia* extract (GG) has been reported previously (Nep and Conway, 2010). The prominent signal at 17.3 ppm was assigned to H-6 (CH_3) of

rhamnopyranose. Three signals at about 21 ppm were assigned to O-acetyl groups, while the absence of O-methyl signals (usually visible at ~55 ppm) indicated that the uronic acid residues of GGDS were not methyl esterified. This suggests that the degree esterification, determined by the FTIR was O-acetylation and not methyl esterification. The C=O signals at 173 ppm are very complex probably because of the varying degrees of acetylation of the GalA and GlcA components of the material.

3.4 Effect of starch digestion on some chemical properties of grewia polysaccharide

Table 1 shows the effect of starch digestion on some of the chemical properties of GG and GGDS. Reaction of sample GG with 1 % (w/v) iodine in KI solution resulted in a blue-black pigmentation. In contrast, sample GGDS did not show reactivity with iodine indicating the absence of starch. The content of starch in GG was determined to be about 11.8 %w/w. Total carbohydrates in both samples were comparable. Much higher protein content of GGDS may be attributed to the presence of remaining α -amylase after starch hydrolysis. The FT-IR spectra of GG and GGDS (Fig. 1) show that grewia polysaccharides are pectin-like in nature (Filipov, 1992) and the degree of esterification (DE) was determined to be higher (49.2%) in GGDS than GG (38.4%). Morris, Foster and Harding (2000) have shown from intrinsic viscosity and sedimentation velocity data that conformational differences exist between pectins with different degrees of esterification which may explain the subtle differences in the intrinsic viscosity measurements for GG and GGDS in the both the presence and absence of salt (Table 2).

3.5 Dynamic mechanical measurements

Figure 3 highlights the differences in rheological behaviour on cooling and heating for GG and GGDS at pH 5, 2.5 and 1.2. Gradual adjustment of pH from 5.0 to 1.2 (figure 3)

results in an appreciable difference in the viscoelastic functions. This indicates that conformational changes due to protonation of uronic acids at low pH, affects the mechanical properties of the samples and increased the thermal hysteresis. At higher pH values the polysaccharide adopts an extended conformation that results in extensive inter-molecular interactions between the chains. The mechanical manifestation of such a molecular interplay produces higher storage modulus values compared with the samples at low pH. Conversely, at low pH the compact polysaccharide conformations enhance the intra-molecular interactions that limit polymer hydration, thus diminishing the elastic character of the samples.

This is congruent with the mechanical spectra of both samples that show changes in the moduli with varying frequency as pH is reduced from 5 to 1.2 (figure 4). A notable difference between GG and GGDS is that the differences between G' and G'' are less prominent after starch digestion indicating an increase in the viscous character of the sample.

A direct empirical relationship exists between the rheological response of non-destructive and destructive deformation. According to the Cox–Merz rule, the complex dynamic viscosity η^* (as a function of angular frequency, ω , rad/s) can be superimposed onto the plot of shear viscosity as a function of shear rate (1/s) (Cox & Merz, 1958):

$$|\eta^*(\omega)|_{\omega \rightarrow 0} = |\eta(\dot{\gamma})|_{\dot{\gamma} \rightarrow 0} \quad (2)$$

It follows that the oscillatory and shear flow data should be identical if the polymer solutions are free from high-density entanglements, aggregates or interactions. Biopolymer solutions usually follow the above generalisation at low concentrations but deviations may occur as concentration increases. These aberrations occur as the interacting chain segments are more sensitive to large shear deformations than to small oscillatory perturbations where they have more time to reform in every cycle of oscillation. In the present study, at concentrations of

2% (Figure 5) only the de-starched material follows the Cox-Merz relationship (Figure 5b). This indicates the absence of dominant inter- and intramolecular interactions that have the potential to affect the rheology of the samples. This outcome shows that the rheological response of the GGDS is that of a concentrated entangled polymer solution rather than of a system that has the tendency to form a weak network. Conversely, prior to starch removal Cox-Merz rule was not obeyed which is consistent with the mechanical spectrum in figure 4 indicating an influence of starch to the mechanical behaviour of the GG.

In the present work, we have shown that crude grewia extracts contain large amounts of starch and substantial uronic acid content with the majority consisting of galacturonic acid. The presence of starch may be beneficial in some applications and may explain some of the functional properties previously reported when used as a tablet binder or thickening agent (Nep et al 2010). Enzymatically removing starch opens up the prospect of an additional grade of grewia gum that has different physicochemical properties. This could subsequently encourage the use of grewia gum in new applications.

4. Conclusion

The present study has revealed that the high glucose content of grewia gum is the result of high starch content in the extract. The polysaccharide also contained uronic acids which accounted for approximately 60 % of monosaccharides present. The rheological behaviour showed minor differences between both the original extract and the de-starched material but were influenced by changes in pH. Removal of starch has an impact on the physicochemical properties of the extracts and tailoring extraction procedures may help delivering a polysaccharide with new functional properties.

5. Acknowledgement

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Table 1 Chemical characterisation of GG and GGDS (n=2, mean)

	Weight %	Weight %
	GG	GGDS
Rhamnose	12.3	14.2
Arabinose	0.5	0.2
Xylose	0.3	0.1
Galactose	0.2	0.2
Glucose	6.4	2.1
Glucuronic acid	12.1	13.9
Galacturonic acid	16.3	17.7
Total	48.1	48.4
Starch %	11.8	-
Protein (N x 6.25) %	2.3	5.2
Moisture (loss on drying) %	11.8	11.0
Ash %	10.2	8.0

Table 2 Effect of salt on the intrinsic viscosity of GG and GGDS (n=3, mean \pm s.d).

Intrinsic viscosity (dL/g):	GG	GGDS
Deionised water	3.78 \pm 0.05	4.40 \pm 0.44
0.1N NaCl	2.78 \pm 0.04	2.53 \pm 0.06

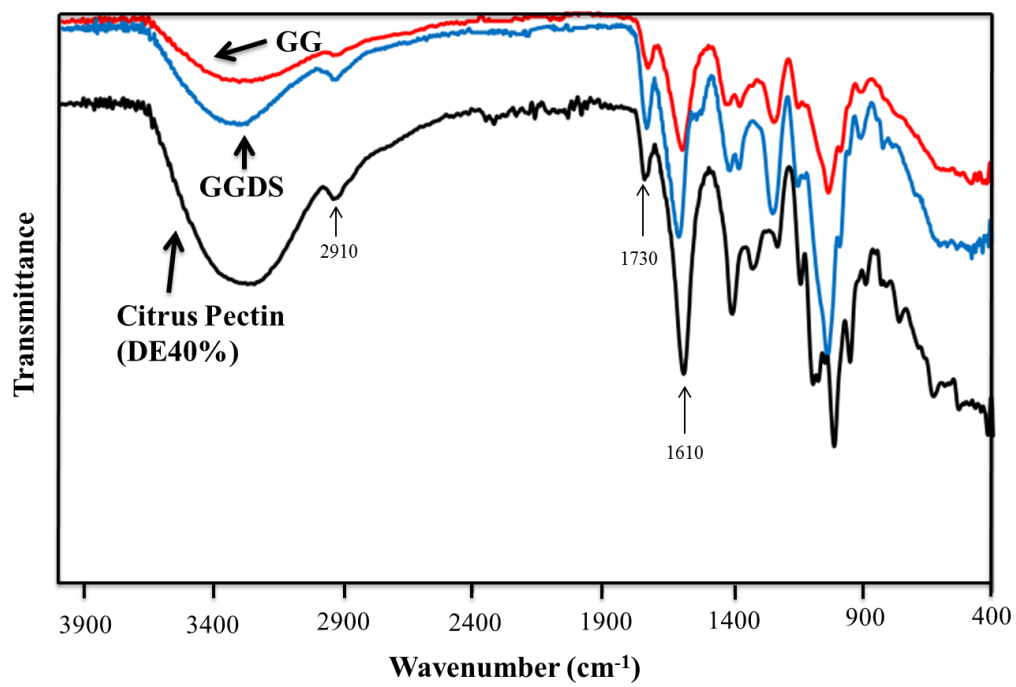


Figure 1: Fourier transform infrared spectra of GG, GGDS and citrus pectin

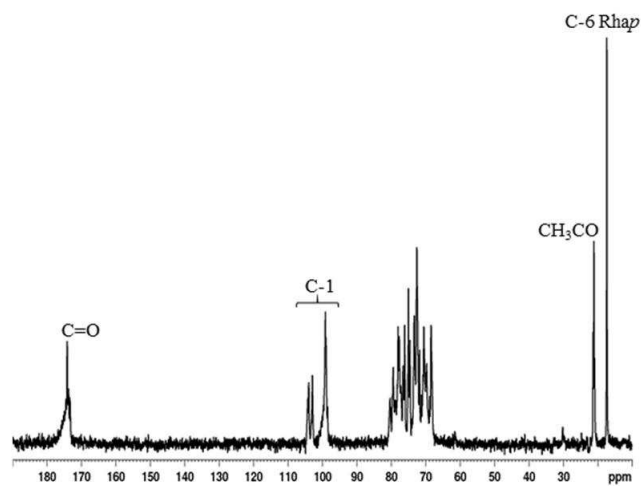


Figure 2: ^{13}C NMR of starch free grewia gum (GGDS).

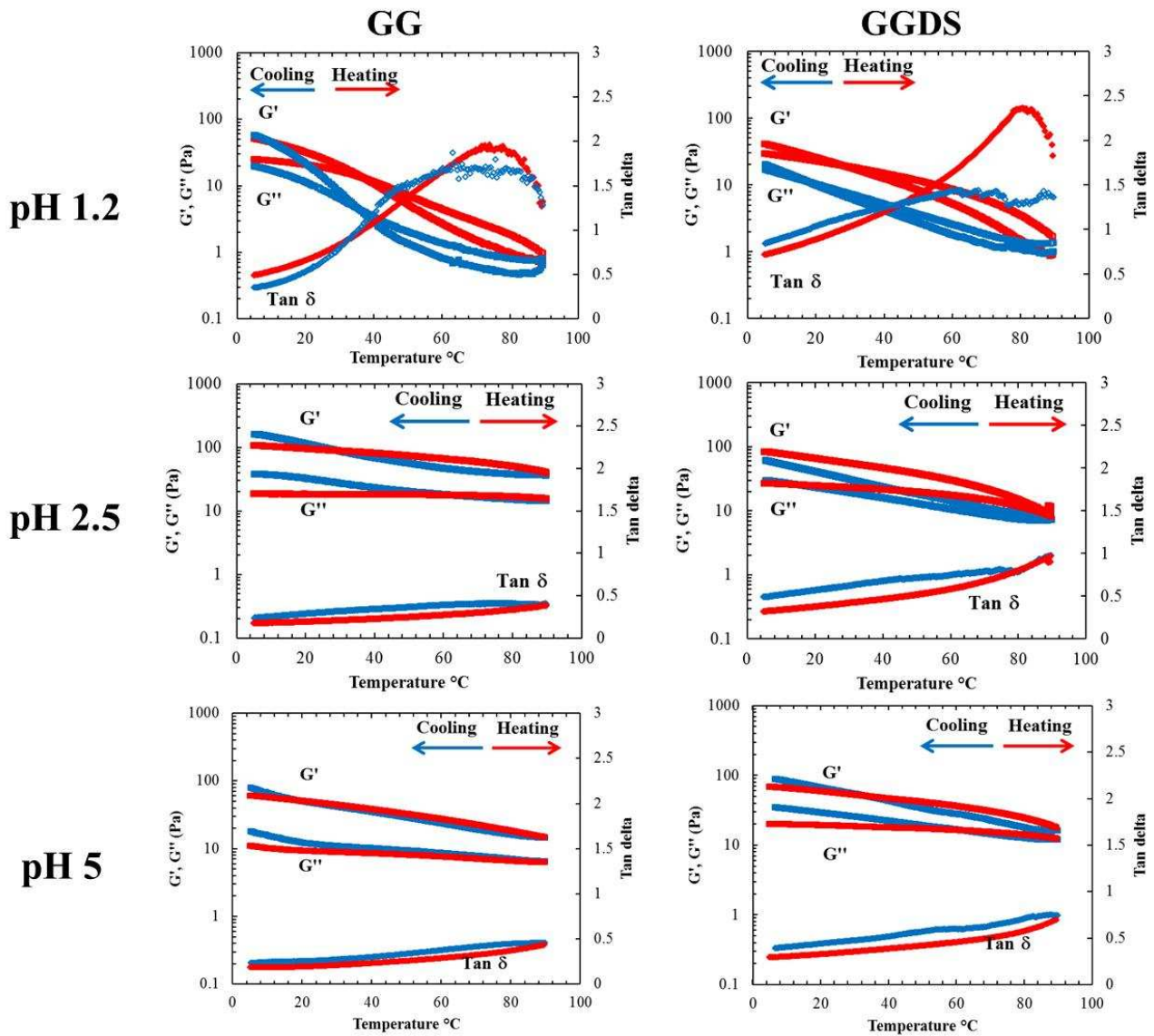


Figure 3: Oscillatory rheological measurements on cooling and heating for 2 % w/v GG and GGDS at pH 5, 2.5 and 1.2 (scanning rate of $2\text{ }^{\circ}\text{C min}^{-1}$, 0.5 % strain, 10 rad s^{-1}). Red squares correspond to heating and blue squares correspond to cooling.

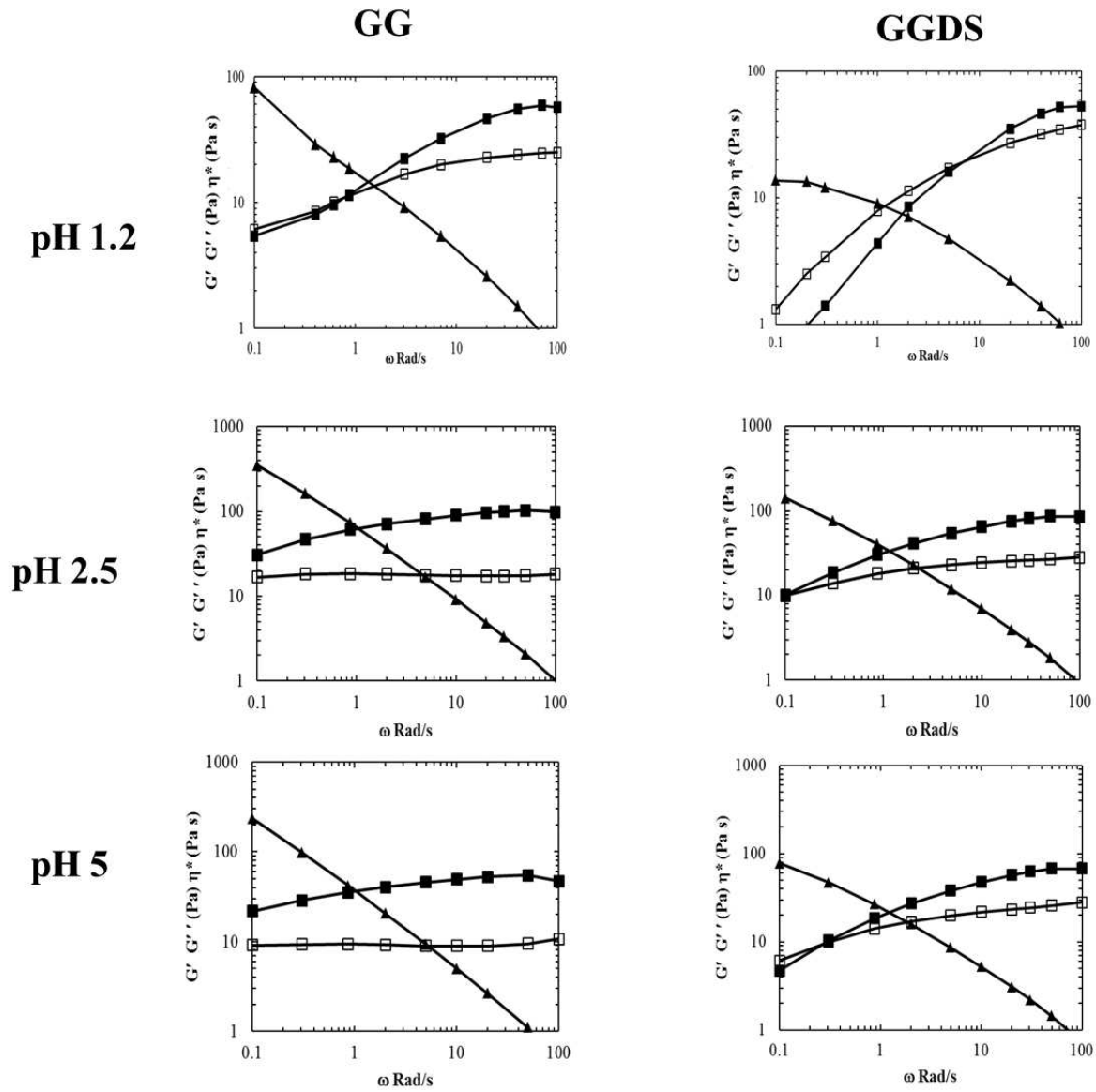


Figure 4: Mechanical spectra of 2% w/v GG and GGDS at pH 5, 2.5 and 1.2, (0.5% strain, 20 °C).

Filled or open squares correspond to G' or G'' , respectively, whereas filled triangles to η^* .

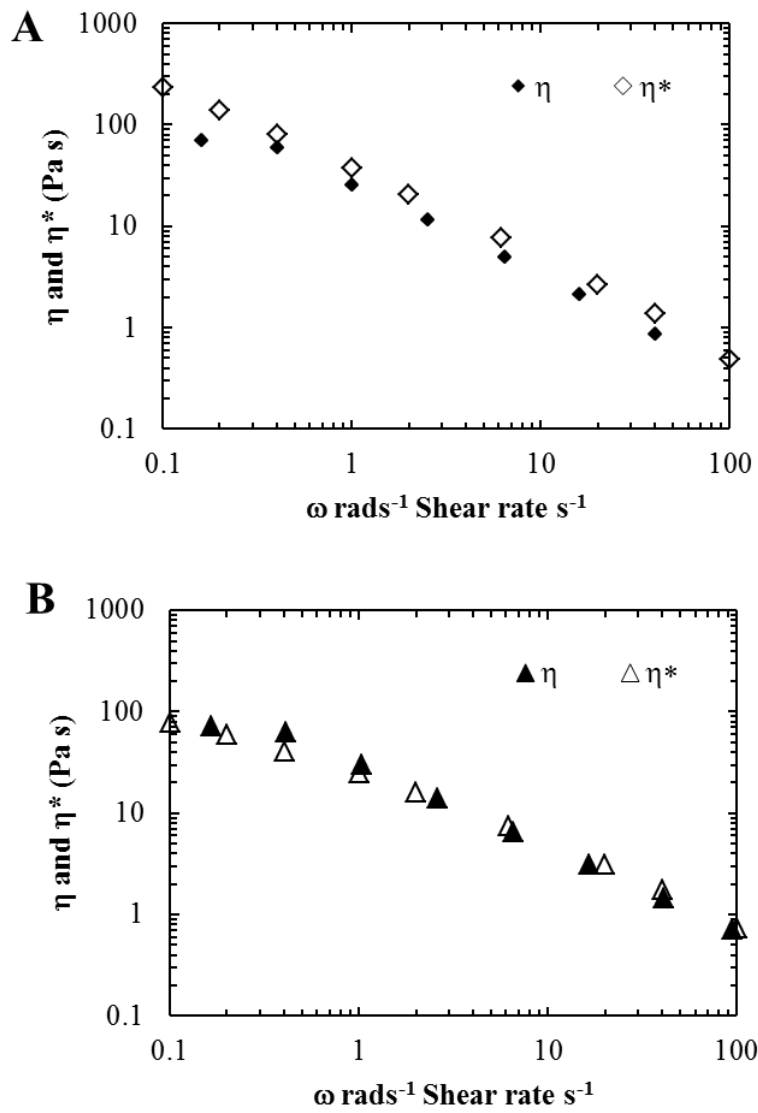


Figure 5: Cox–Merz rule applicability for 2% w/v grewia gum dispersions a) GG and b) GGDS. Measurements were taken at pH 5 and 20 °C.