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Short Communication

# Cell-wall polysaccharides from the fruits of *Limonia acidissima*: isolation, purification and chemical investigation

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**Abstract**

Polysaccharides were isolated from the fruits of *Limonia acidissima* by sequential extraction with water, and 1 M and 4 M KOH. The water extract contained pectic polymers substituted with side chains comprising mainly of 1,5-, 1,3,5-linked arabinose together with 1,4-, 1,6-, 1,3,6-linked galactose, and lesser amounts of 1,2,4- and 1,3-linked galactose residues. Galactosyl and arabinofuranosyl groups terminated most of the branched residues. The alkaline extracts contained both pectic and hemicellulosic polymers. The insoluble material consists mainly of cellulose-rich material. 

*Keywords*: *Limonia acidissima*; Rutaceae; Fruit; Cell wall; Pectin; Hemicellulose; Cellulose

## 1. Introduction

*Limonia acidissima* W. and A syn *L.crenulata* Roxb. (Fam. Rutaceae), a tree with green foliage, grows abundantly in different parts of India. Different parts of this plant has been found to contain various types of chemical compounds (Bandara, Gunatilake, Sotheeswaran, Wijeratne & Ranasinghe, 1989; Chatterjee, Sarkar & Shoolery, 1980; Chopra, Nayer & Chopra, 1956; Ghosh, Bandyopadhyay, Thakur, Tamura & Matsumoto, 1989; Jain, Srivastava & Srivastava, 1989; Khan, Siddiqui & Zaman, 1975; Macleod, Peter, Patra, Bandora, Gunatilake & Wijeratne, 1989; Patra, Mishra & Choudhury, 1988; Zarga, 1986). However, the polysaccharides present in the fruits of *Limonia acidissima* have not been investigated before. This communication describes isolation, fractionation and chemical investigation of the polysaccharides present in the fruit.

## 2. Experimental

Air-dried ripe fruits (1.2 kg) of *Limonia acidissima* were extracted sequentially with 2.5 l of petroleum ether (60–80°C) and methanol in a Soxhlet apparatus for 33 and 50 h, respectively. The defatted fruit material (yield 590 g) was separated using tweezers into following two groups: (1) the seeds (S), and (2) the pericarp (P). All subsequential extraction, purification and analysis were carried out using the pericarp (P) or material derived therefrom.

The grounded pericarps (1 g) were sequentially extracted with (i) water, 150 ml for 4 h (twice); (ii) 1 M KOH containing 0.4% $NaBH_4$ (50 ml) at 35°C for 1.5 h (twice); and (iii) 4 M KOH containing 0.4% $NaBH_4$ (50 ml) at 35°C for 1.5 h (twice). All extracts were centrifuged and dialyzed exhaustively; alkaline extracts were acidified to pH 5 with HOAc prior to dialysis. All the dialyzed extracts were concentrated. The water extract was submitted to graded precipitation with ethanol to yield the less soluble fraction PWEL and the more soluble fraction PWEM. The alkaline extracts were diluted with ethanol. In all cases the precipitate was recovered by centrifugation, dehydrated by solvent exchange and finally dried under vacuum over $P_2O_5$.

The resulting KOH unextractable residue was washed thoroughly with water containing acetic acid, and then with deionised water, and finally dried by solvent exchange.

Solutions (150 ml) of PWEL (340 mg) in 10 mM NaOAc buffer pH 6.5 was applied to a column (2.2 × 24 cm) of DEAE-Sepharose CL-6B ($OAc^-$) and then eluted successively with 0.01, 0.1, 0.3, 0.4 and 1 M NaOAc buffer pH 6.5 (160 ml each) in a stepwise manner and finally with 0.2 M NaOH (100 ml). The collected fractions (10 ml) were analysed for neutral sugar and uronic acid content and the

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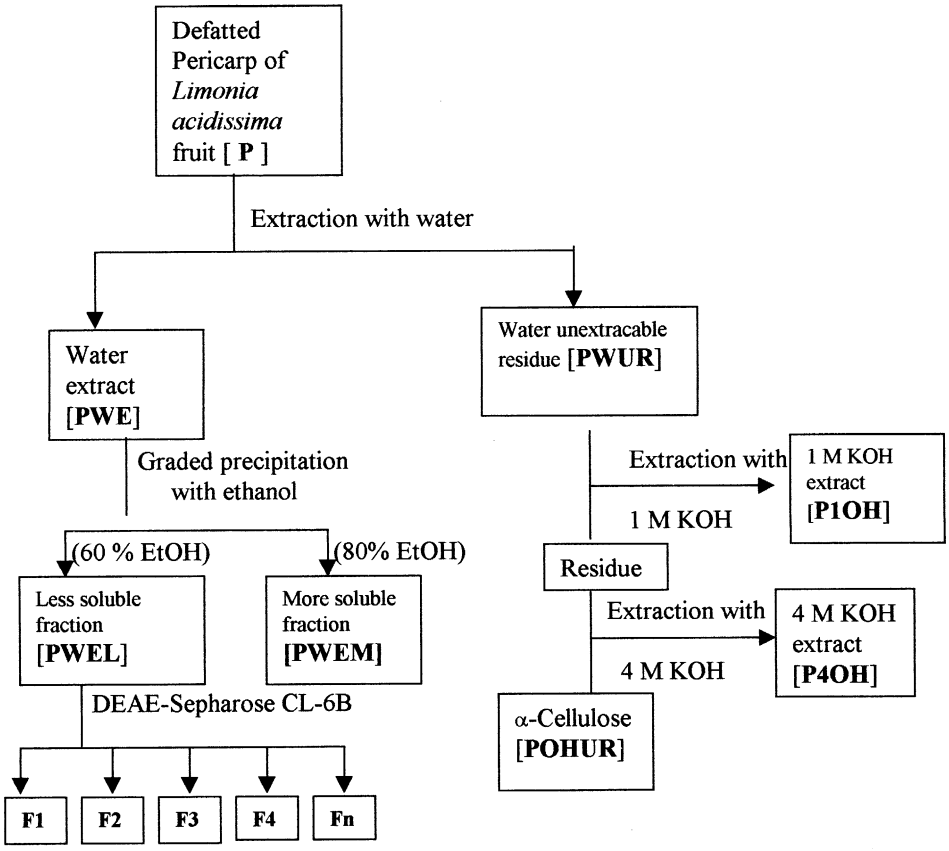


Fig. 1. Scheme for extraction and fractionation of polysaccharides obtained from the pericarp of *Limonia acidissima* fruit.

Table 1
Yields and sugar composition of the fractions isolated from the defatted pericarp of *Limonia acidissima* fruit by sequential extraction with inorganic solvents (see experimental for identification of fractions). Note: nd: not determined, tr: trace

| | P | PWEL | PWUR | P1OH | P4OH | POHUR |
|---|---|---|---|---|---|---|
| Yield[a] | 100 | 2.1 | 60.5 | 17.8 | 7.5 | 24.6 |
| NS[b] | 34 | 32.5 | 28 | 47.4 | 48.7 | 59.4 |
| UA[b] | 14.4 | 38.2 | 13.9 | 4.9 | 7.7 | 12.5 |
| Protein[b] | nd | 15 | nd | 25 | 22.4 | nd |
| Rha[c] | 1.5 | 1.8 | 1 | 1.3 | 1.54 | 1.1 |
| Fuc[c] | 0.6 | 0.3 | 0.7 | 0.6 | 2.4 | tr |
| Ara[c] | 19.9 | 18.5 | 17.4 | 23.7 | 14.8 | 6.4 |
| Xyl[c] | 7.2 | 4.3 | 8.9 | 14.4 | 19.7 | 1.8 |
| Man[c] | 3.9 | 7.2 | 2.8 | 5.7 | 4.5 | 1.7 |
| Gal[c] | 15.9 | 14.0 | 9.5 | 19.4 | 14.9 | 4.9 |
| Glc[c] | 24.1 | 3.6 | 30.5 | 25.8 | 30.2 | 68.2 |
| UA[c] | 26.9 | 50.3 | 29.0 | 9.1 | 12.0 | 16.1 |
| DM | nd | 11.1 | nd | nd | nd | nd |
| DA | nd | 3.4 | nd | nd | nd | nd |
| Ara/Gal | 1.25 | 1.32 | 1.77 | 1.22 | 1.15 | 1.3 |
| UA/Rha | 17.9 | 27.9 | 29 | 7 | 7.8 | 14.6 |

[a] Percentage weights of the P dry weight.
[b] Percentage weights of fraction dry weight.
[c] Percentage mol.

appropriate ones were combined, concentrated, dialyzed and diluted with ethanol to yield five fractions.

The uronic acids present in the major fraction F2 (40 mg) obtained from anion exchanger, were reduced twice by the method of Taylor and Conrad (1972), as described by Ray and Lahaye (1995a) to yield the carboxyl-reduced polysaccharide (F2R, yield 25 mg).

Methylation of F2 fraction and its carboxyl-reduced derivative (F2R) were carried out by the methods of Blakeney and Stone (1985). Conversion to the partially methylated alditol acetate (PMAA) and GC and GC–MS on columns of SGE BP 1 and DB-225 (JW) were done as described (Ray & Lahaye, 1995a).

Protein was estimated by the methods of Lowry, Rosebrough, Lewsfarr and Randall (1951) using bovine serum albumin as standard. Acetic acid and methanol contents were estimated by HPLC according to Voragen, Schols and Pilink (1986). The degree of acetylation (DA) and degree of methylation (DM) were calculated as the molar ratios of acetic acid and methanol to uronic acid. The uronic acid content was determined by the m-hydroxydiphenyl assay as described by Ahmed and Labavitch (1977) using galacturonic acid as standard. Neutral sugars were released by Saeman or 1 M $H_2SO_4$ and analysed as their alditol acetates (Blakeney, Harris, Henry & Bruce, 1983) by GLC on columns of SGE BP 225 and DB-225 (JW) as

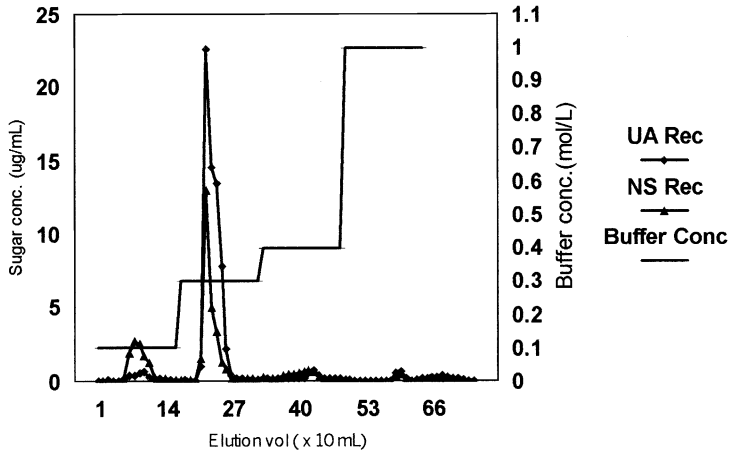


Fig. 2. Anion exchange chromatography of PWEL fraction, isolated from the pericarp of *Limonia acidissima*fruit by extraction with water, on DEAE-Sepharose CL-6B column. F1–4: Pooled fractions were eluted with NaOAc buffer pH 6.5, the digit corresponds with the order of elution. Fn: Fraction eluted with 0.2 M NaOH.

described (Ray & Lahaye, 1995b). Phenolic acids were determined by HPLC after saponification and extraction as described (Thibault, 1988). The sugars in the acid hydrolysates were also analysed by TLC.

## 3. Results and discussion

The flow-sheet for the isolation and fractionation are given in Fig. 1. The yield and chemical composition of the *Limonia acidissima* fruit (pericarp) polysaccharides extracted sequentially with various inorganic solvents are given in Table 1. Water alone extracted 7.3% of the material present in the defatted pericarp (P). This extract, which represents 12% of the uronic acid present in P, was then subjected to a graded precipitation with ethanol. The less soluble fraction (PWEL) obtained at an ethanol concentration of 60% (v/v) represents 27.8% of the material present in the water extract. The sugar composition of this fraction indicates the probable presence of pectic polymers. The ratio of acidic to neutral sugars was around 50 galacturonic acid molecules for 50 neutral sugar residues. The degree of acetylation and the degree of methylation of the PWEL fraction were 3.4 and 11.1%, respectively. No phenolic acids were detected. The more soluble fraction (PWEM), obtained at ethanol concentration of 80%, accounted for 5.2% of the P and contained 64.1% neutral sugar and 17.3% uronic acid. The water un-extractable residue (PWUR) represented 60.5% of the defatted fruit powder and contained 41.9% carbohydrate material.

Table 2
Partially methylated alditol acetates (mol%) from the F2 fraction, and its carboxyl reduced derivative (F2R) [see experimental for the identification of fractions]

| Partially methylated alditol acetate[a] | F2 | F2R |
|---|---|---|
| 2,3,5-$Me_3$-Ara | 16.5 | 7.2 |
| 2,3-$Me_2$-Ara | 19.0 | 11.2 |
| 2-Me-Ara | 10.5 | 6.4 |
| 2,3-$Me_2$-Xyl | 3.4 | 2.6 |
| 3,4-$Me_2$-Rha | 1.1 | 1.2 |
| 3-Me-Rha | 2.1 | 2.4 |
| 2,3,4,6-$Me_4$-Gal | 6.6 | 3.0 |
| 2,4,6-$Me_3$-Gal | 1.8 | 2.2 |
| 2,3,6-$Me_3$-Gal | 12.9 | 50.1 |
| 2,3,4-$Me_3$-Gal | 6.1 | 6.4 |
| 3,6-$Me_2$-Gal | 2.5 | 1.4 |
| 2,4-$Me_2$-Gal | 9.1 | 2.2 |
| 2,3,6-$Me_3$-Glc | 1.0 | 1.4 |
| 2,3,6-$Me_3$-Man | 6.4 | 2.4 |

[a] 2,3,5-$Me_3$-Ara = 2,3,5-tri-*O*-methyl-1,4-di-*O*-acetyl arabinitol etc.

Most of the polysaccharides containing xylose were extracted with alkali. The 1 M KOH soluble fraction (P1OH), which constituted 17.8% of the P, contained substantial amounts of arabinose, xylose, galactose and glucose, with some deoxyhexose and mannose and 4.9% galacturonic acid (Table 1). The sugar composition of this fraction suggested that it contained a mixture of pectic and hemicellulosic polymers. The galacturonic acid to rhamnose ratios of pectic polymers extracted with alkali is small compared with that of water extracted one. The 4 M KOH soluble fraction (P4OH) accounted for 7.5% of the P and was rich in arabinose, xylose, galactose, glucose and uronic acid indicating the presence of pectic and hemicellulosic polymers. The dilute alkali treatments (1 M KOH) dissolve ~6.1% of uronic acid originally present in P, whereas ~4% of uronic acids were solubilised by 4 M KOH. The final residue (POHUR) after extraction with 4 M KOH gave a yield of 24.6% of P and contained 68.2 mol% glucose, of which 59.1 mol% is of cellulosic origin. It represented 21.4% of the uronic acid originally present in the P. In total, 32.6% of the P could be extracted using sequential extraction with inorganic solvents. The total carbohydrate content of the polymers soluble in various inorganic

solvents suggested that they contained significant amount of noncarbohydrate material.

The polysaccharides present in the PWEL fraction was further resolved by anion exchange chromatography into five (F1, F2, F3, F4 and $F_n$) charged fractions (Fig. 2). The major fraction, F2 accounted for 56.7 and 83.4% of the neutral sugars and uronic acid recovered from the anion exchanger, respectively, and contained galacturonic acid (57.5 mol%), arabinose (20.6 mol%) and galactose (12.8 mol%) as the major sugar together with smaller quantities of rhamnose (2.4 mol%), mannose (3.2 mol%), glucose (1.1 mol%), xylose (1.9 mol%) and trace amount of fucose residues.

The results of methylation analysis indicated that galacturonic acid (Gal*p*A) residues are 1,4-linked (Table 2). Rhamnose is 1,2-linked with branching via C-4. Arabinoses are in furanose form and are 1,5-, 1,3,5- and terminally linked. The presence of 1,6- and 1,3,6-linked galactosyl, and 1,3- and terminal arabinosyl residues was indicated.

In conclusion, this study has shown that the major polysaccharides in the cell walls of *Limonia acidissima* fruit are cellulosic, hemicellulosic and pectic polymers. The water-soluble material comprises mainly of pectic polysaccharides. These polymers gave viscous solution, were lightly esterified, and contained acetyl groups. Alkaline extracts contained both pectic and hemicellulosic polymers. Glucose and xylose were the predominant sugars in the 1 and 4 M KOH extract, which may indicate the probable presence of xyloglucan in this extract. The isolation of oligosaccharides for further structural analysis of these polysaccharides is in progress.

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## References

Ahmed, A., & Labavitch, J. M. (1977). A simplified method for accurate determination of cell wall uronide content. *Journal of Food Biochemistry*, *1*, 361–365.

Bandara, B. M. R., Gunatilake, A. A. L., Sotheeswaran, S., Wijeratne, E. M. K., & Ranasinghe, M. A. S. K. (1989). Insecticidal properties of an active constituent of *Limonia acidissima*. *Journal of Natural Science Council Srilanka*, *17*, 237–239.

Blakeney, A. B., & Stone, B. A. (1985). Methylation of carbohydrates with lithium methylsulphinyl carbanion. *Carbohydrate Research*, *140*, 319–324.

Blakeney, A. B., Harris, P., Henry, R. J., & Bruce, A. B. (1983). A simple rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research*, *113*, 291–299.

Chatterjee, A., Sarkar, S., & Shoolery, J. N. (1980). 7-Phenylacetoxy coumarin from *Limonia crenulata*. *Phytochemistry*, *19*, 2219–2220.

Chopra, R. N., Nayer, S. L., & Chopra, I. C. (1956). In *Glossary of Indian medicinal plant* (pp.154) CSIR, New Delhi.

Ghosh, P., Bandyopadhyay, A. K., Thakur, S., Tamura, T., & Matsumoto, T. (1989). Acidissim, a new tyramine derivative from *Limonia acidissima*. *Journal of Natural Products*, *52*, 1323–1326.

Jain, A. K., Srivastava, S. K., & Srivastava, S. D. (1989). Some new constituents from *Limonia crenulata* and their anti-inflammatory activity. *Indian Journal of Pharmacological Science*, *51*, 32–35.

Khan, H., Siddiqui, S. A., & Zaman, A. (1975). Chemical extraction of *Heserathusa crenulata*. *Journal of Industrial Chemical Society*, *52*, 177–178.

Lowry, O. H., Rosebrough, N. J., Lewsfarr, A., & Randall, R. J. (1951). Protein measurement with the folin reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Macleod, J. K. M., Peter, D. R., Bandora, B. M. R., Gunatilake, A. A. L., & Wijeratne, E. M. K. (1989). Acidissimin, a new limonoid from *Limonia acidissima*. *Journal of Natural Products*, *52*, 882–885.

Patra, A., Mishra, S. K., & Choudhury, S. K. (1988). Constituents of *Limonia acidissima*. Applications of two-dimensional NMR spectroscopy in structure elucidation. *Journal of Industrial Chemical Society*, *65*, 205–208.

Ray, B., & Lahaye, M. (1995a). Cell-wall polysaccharides from the marine green algae *Ulva rigida* (Ulvales, Chlorophyta) 2:Chemical structure of ulvan. *Carbohydrate Research*, *274*, 313–318.

Ray, B., & Lahaye, M. (1995b). Cell-wall polysaccharides from the marine green algae Ulva rigida (Ulvales, Chlorophyta)- 1:Extaction and chemical composition. *Carbohydrate Research*, *274*, 251–261.

Taylor, R. L., & Conrad, H. E. (1972). Stoichiometric depolymerisation of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. *Biochemistry*, *11* (8), 1383–1388.

Thibault, J. F. (1988). Characterization and oxidative crosslinking of sugar-beet pectins extracted from cossets and pulps under different conditions. *Carbohydrate Polymers*, *8*, 209–223.

Voragen, A. G. J., Schols, H. A., & Pilnik, W. (1986). Determination of the degree of methylation and acetylation of pectins by HPLC. *Food Hydrocolloids*, *1*, 65–70.

Zarga, M. H. A. (1986). Three new simple indole alkaloids from Limonia acidissima. *Journal of Natural Products*, *49*, 901–904.