# Isolation and characterization of polysaccharides from *Fraxinus angustifolia* infusions

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## ABSTRACT

In Trás-os-Montes region the infusions prepared from the dried leaves of "freixo" (*Fraxinus angustifolia*) is used for the prevention of high levels of cholesterol, blood pressure and uric acid. In this work, infusions from the dried leaves of "freixo" were prepared and the high molecular weight material (HMWM) was obtained and fractionated. The results evidenced the presence of pectic polysaccharides, frequently referred as biologically active, with distinct proportions of homogalacturonans (HG) and rhamnogalacturonan (RG) domains. Additional work is in progress in order to evaluate the biological activity of the various isolated fractions and to establish a structure-activity relationship.

## 1. INTRODUCTION

A great diversity of plant infusions is being used for medicinal purposes. In Trás-os-Montes region, one of the most used is the one prepared from the dried leaves of the narrow-leafed ash "freixo" (*Fraxinus angustifolia*). This infusion is used for the prevention of high levels of cholesterol, blood pressure and uric acid [1]. Although, the bioactivity and potential health benefits of the most popular plant infusions, such as *Camellia sinensis* and *Matricaria recutita*, are well documented [2, 3], the use of most of them is based on an empirical knowledge transmitted across several generations. The reported health benefits are associated with the extraction of biologically active substances, such as phenolic compounds and polysaccharides, among others. Recent studies have shown that pectic polysaccharides isolated from plant infusions exhibit a number of beneficial therapeutic properties, such as anti-ulcer, anti-tumoral and immunobiological activities, being thought that the mechanisms involved in these effects are due to the modulation of innate immunity and, more specifically, macrophage function [4]. Pectic polysaccharides are a very complex group of heteropolysaccharides composed of homogalacturonan (HG) and rhamnogalacturonan (RG) domains [5]. The HG domain is composed of partially methyl-esterified α-1,4-galacturonic acid residues, which can be interrupted by ramified domains, called rhamnogalacturonan I (RG-I) or rhamnogalacturonan II (RG-II) domains. RG-I consists of a backbone alternating 1,4-linked GalA and 1,2-linked Rhap units. The rhamnose residues are frequently branching points, primarily on position 4. Frequently, Type II arabinogalactans

(AG-II) may be found as side chains linked to the rhamnose residues, although AG-I occasionally also may be present. AG-II is a branched polysaccharide containing ramified chains of 1,3-linked and 1,6-linked β-D-Galp units. The arabinosyl units might be attached through position 3 of the 1,6-linked galactosyl side chains. RG-II domain is characterized by a homogalacturonan backbone of 9-10 residues of 1,4-linked GalA, substituted with four different oligosaccharide side chains containing some uncommon sugars, such as 2-O-Me-Fuc, 2-O-Me-Xyl, Api, AceA, Kdo and Dha.

## 2. MATERIALS AND METHODS

### 2.1 Infusions and preparation of polymeric fractions

The infusions of *Fraxinus angustifolia* were prepared by boiling the dried leaves in hot water during 4 h, divided in two periods of 2 h. The infusions were filtered, dialyzed (12-14 kDa) and freeze-dried to obtain the HMWM, which was fractionated by ethanol precipitation to give the material that precipitated in 50% ethanol ($Et_{50}$), 75% ethanol ($Et_{75}$), and the supernatant solution (SN).

### 2.2 Anion exchange chromatography

The $Et_{50}$ and $Et_{75}$ fractions were further fractionated by anion exchange chromatography on DEAE-Sepharose FF. The samples were suspended in potassium phosphate buffer pH 6.5 and sequentially eluted in the phosphate buffer (fraction A), phosphate buffer with 0.25 M NaCl (fraction B) and 1.00 M NaCl (fraction C). Fractions were collected, dialyzed and lyophilized.

### 2.3 Sugar and linkage analysis

Neutral sugars were released by acid hydrolysis and were analyzed as their alditol acetates by GC-FID. Uronic acids were determined colorimetrically by 3-phenylphenol. Polysaccharides were methylated with methyl iodide, hydrolyzed, derivatized partially methylated alditol acetates and analyzed by GC-MS.

### 2.4 FT-IR spectroscopy

FT-IR spectra of $Et_{50}$, $Et_{75}$ and SN extracts were acquired with a Specac ATR in a Perkin-Elmer Spectrum BX instrument at a resolution of 8 $cm^{-1}$ and 128 co-added scans. Spectra for each sample were recorded, at least, in triplicate, in the absorbance mode from 4000 to 550 $cm^{-1}$.

## 3. RESULTS AND DISCUSSION

The HMWM obtained in the 1$^{st}$ 2h contained 57.0% of glycosidic material, while the obtained in the 2$^{nd}$ 2h was richer, with 76.1%, as illustrated in **Table 1**. This suggested a preferential extraction of polysaccharides during the 2$^{nd}$ 2h, whereas in the 1$^{st}$ 2h other types

of compounds, such as phenolics, may also be co-extracted, which was supported by the darker colour, characteristic of the presence of phenolic compounds, of the HMWM from the 1st 2h.

**Table 1.** Glycosidic content, expressed in mass percentage, and monosaccharide composition of the HMWM and various fractions obtained by ethanol precipitation, expressed in molar percentage.

| | **Glycosidic content (mass%)** | **Monosaccharide Composition (mol %)** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Rha** | **Ara** | **Xyl** | **Man** | **Gal** | **Glc** | **UA** |
| **1st 2 h** | 57.0 | 1.8 | 5.3 | 0.9 | 3.0 | 8.6 | 9.4 | 71.1 |
| $Et_{50}$ | 81.0 | 2.1 | 3.5 | 1.6 | 0.5 | 3.2 | 3.0 | 86.3 |
| $Et_{75}$ | 55.8 | 3.4 | 11.2 | 2.6 | 2.6 | 12.8 | 9.8 | 57.8 |
| SN | 33.0 | 8.4 | 17.0 | 1.2 | 11.0 | 5.3 | 33.9 | 23.3 |
| **2nd 2 h** | 76.1 | 1.6 | 6.8 | 0.8 | 1.7 | 8.1 | 4.8 | 76.4 |
| $Et_{50}$ | 90.1 | 1.6 | 4.3 | 1.5 | 0.2 | 2.9 | 1.1 | 88.5 |
| $Et_{75}$ | 84.7 | 2.5 | 10.6 | 2.6 | 1.1 | 9.5 | 4.1 | 69.8 |
| SN | 38.8 | 5.3 | 35.7 | 1.2 | 8.0 | 4.5 | 22.3 | 23.1 |

The HMWM from both the 1st and 2nd 2 h displayed high proportions of uronic acids (UA), besides exhibiting significant proportions of Ara, Gal and Glc, indicating the presence of pectic polysaccharides, as showed in **Table 1**. However, as Glc is not usually referred as a component of pectic polysaccharides, it can be deduced that other types of compounds are also being extracted in combination with these polysaccharides. With the purpose of separating the polysaccharides present in the HMWM from both 1st and 2nd 2h, a fractionation method based on ethanol precipitation was performed. As showed in **Table 1,** the $Et_{50}$ and $Et_{75}$ fractions, from both the 1st and 2nd 2h, were richer in glycosidic material (55.8-90.1%), while the SN fractions showed a relatively low content of glycosidic material (33.0-38.8%). **Table 1** also shows that the $Et_{50}$ fractions contained higher proportions of UA, with more than 85.0%. Although in small proportions, neutral sugars such as Ara (mostly terminally- and 5-linked, with small proportions of 3- and 3,5-linked residues) and Gal (terminally-, 3-, 6- and 3,6-linked residues) were detected. Rha (terminally-, 2-, and 2,4-linked) residues were also detected. This and the very high proportions of UA, may indicate the presence of pectic polysaccharides enriched in HG domains, but also comprising RG-I domains rich in neutral sugars, particularly Ara and Gal organized as AG-II chains, frequently referred as potentially biologically active [4]. Although exhibiting high proportions of UA, the $Et_{75}$ fractions evidenced a less acidic nature, reflected by the presence of lower amounts of UA (57.8-69.8%), suggesting the presence of a lower proportion of HG domains and higher proportions of RG-I domains. In fact, the $Et_{75}$ fractions exhibited higher proportions of Ara, Gal, and Glc, which, although in different proportions, featured the same type of linkages detected in the $Et_{50}$ fractions, indicating the presence of the same type of polysaccharides but with distinct proportions of HG and RG regions. The $Et_{50}$ and $Et_{75}$, from both the 1st and 2nd 2h, which were the richest in glycosidic material, were further fractionated by anion exchange chromatography, each yielding a non-retained fraction A and two retained fractions B and C. Fractions A and B exhibited higher amounts of glycosidic material, with the ones originated from the $Et_{50}$ material displaying

the highest proportions of UA (81.7 and 86.4%). Contrary to what was observed for the fractions originated from the $Et_{50}$ material, the A fraction from $Et_{75}$ material exhibited higher proportions of UA than the B fraction.

**Table 2.** Yield, glycosidic content, expressed in mass percentage, and monosaccharide composition of the various fractions obtained by anion exchange chromatography, expressed in molar percentage.

| | Yield (mass %) | Glycosidic Content (mass%) | Monosaccharide Composition (mol %) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Rha | Ara | Xyl | Man | Gal | Glc | UA |
| ***Et*$_{50}$ 1$^{st}$ 2 h** | | | | | | | | | |
| A | 22.3 | 83.8 | 0.5 | 3.9 | 3.5 | 1.3 | 4.3 | 4.8 | 81.7 |
| B | 59.4 | 86.3 | 1.9 | 5.1 | 1.9 | 0.0 | 4.2 | 0.4 | 86.4 |
| C | 18.3 | 22.1 | 7.8 | 8.6 | 1.9 | 0.8 | 10.4 | 8.2 | 62.4 |
| ***Et*$_{75}$ 1$^{st}$ 2 h** | | | | | | | | | |
| A | 54.3 | 85.9 | 0.9 | 7.9 | 3.9 | 3.7 | 10.8 | 5.7 | 67.1 |
| B | 31.2 | 55.8 | 5.7 | 17.6 | 1.7 | 0.3 | 13.5 | 2.8 | 58.2 |
| C | 14.5 | 13.9 | 17.9 | 5.4 | 0.6 | 1.4 | 5.9 | 43.8 | 25.0 |

FT-IR spectroscopy, showed peaks at 1630 and 1750 $cm^{-1}$ characteristic of carboxylate and ester groups [6], suggesting that some UA units might be methylesterified, contributing for the non retention of the material. Fractions C showed the lowest amounts of glycosidic material and proportions of UA, indicating that the interaction with the stationary phase was promoted by other negatively charged species, probably phenolic compounds, which contributed for the darker colour of these fractions.

## 4. CONCLUSIONS

The HMWM from the infusions of *Fraxinus angustifolia* showed a monomeric composition and a pattern of glycosidic linkages diagnostic of the presence of pectic polysaccharides. Through fractionation procedures it was possible to isolate fractions comprising pectic polysaccharides with distinct proportions of HG and RG-I domains. FT-IR spectroscopy analysis suggested that the UA units might be methylesterified to some extent. The RG-I domains contained AG-II, frequently reported as potentially biologically active. Additional work is in progress in order to evaluate the biological activity of these polymers.

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