Partial Structural Characterization of Complex Polysaccharide Isolated from Sugar Maple (Acer saccharum Marsh) Bark and its Anti-Inflammatory Activity

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ABSTRACT
The rationale of the present novel research was to report the properties of a major complex polysaccharide from an aqueous extract of sugar maple bark obtained after catalytic organosolv pulping of the maple bark, which impacted the chemical composition of residual liquor. By using such an aqueous extract of bark, a complex polysaccharide was obtained through ultrafiltration, followed by gel filtration on Sephadex G-50, which was named WSF-3, as the third eluted polysaccharide sub-fraction. Partial polymer and structural characterization revealed that WSF-3 had an average molecular weight of 21.5 kDa and consisted of a backbone of glucose residues (87.2%) linked together by α-(1 → 6) glycosidic bonds. The presence of galactose (9.8%) and rhamnose (3.1%) units may represent polysaccharide impurities as their structures have not been confirmed belonging to the glucan structure. If this was the case, WSF-3 could be similar to dextran, polysaccharide previously reported in sugar maple sap. An anti-inflammatory assay indicated that WSF-3 along with WE and its polysaccharide-rich fraction, all at 100 μg/mL, inhibited TNF-α production in LPS-stimulated RAW 264.7 cells. These results indicate that the concentrated WSF-3 was responsible, at least in part, for the anti-inflammatory effect of WE, suggest that WSF-3 could have therapeutic implications in the treatment of inflammation and inflammatory-related diseases.

KEYWORDS: Sugar maple bark; Complex Polysaccharide; Structural Studies; Anti-inflammatory Activity

Introduction
Every year, Canadian forest industries generate around 17 million m³ of bark as a by-product of wood transformation [1, 2]. Besides its use as a source of energy, bark represents a great potential for the forest industry to get access to new markets through the valuing of bark chemical constituents. In fact, bark is an important source of bioactive compounds that can be used in various areas of human life such as nutraceuticals, cosmetics, and pharmaceuticals [3]. These compounds belong to the extractsives, since they are present in bark in the form of free molecules or bound to glycosides (therefore easily accessible to solvents), but they can also be associated with structural polymers; notably cellulose, hemicelluloses, and lignins [4]. Herein, they are either linked by intermolecular interactions of low energy or they are simply deposited in various cavities of bark cells’ structures[5]. For these reasons, they can easily be solubilised by different solvents (water, ethanol, etc.) depending on their physico-chemical characteristics. The yields of bark aqueous extractions can reach up to 25% on a dry mass basis [2, 3]. These extracts could particularly be attractive for industry since the worldwide
demand for bioactive molecules of natural origin has progressed considerably in recent years.

Recently, our team has undertaken research on the complete valuing of the maple bark residues in the context of integrated biorefinery. Those studies were notably focused on sugar maple bark (Acer saccharum Marsh), infusions of which are used traditionally by Native Peoples in Eastern Canada to treat or prevent various diseases such as diarrhea, back pains, and diuretic and hepatic disorders [6]. The conversion of sugar maple bark residues into new valuable products was performed by a catalytic organosolv process [7]. One of the main steps of this process consists in removing the extractive components from bark using ethanol-water or water only as solvents in order to preserve the efficacy of the catalyst while avoiding its interaction with phenolic extractives and soluble polysaccharides [8, 9]. Therefore, the potential of converting sugar maple bark into valuable products includes the valuing of extractive compounds removed prior to the catalytic organosolv pulping. The extractable polyphenols from sugar maple bark have been intensively studied by our team in recent years [9, 10]. Previous investigations have reported that the sugar maple bark contains unique phenolic glycosides, among which are a few identified molecules such as gallotannins, lignans, coumarins, and coumarin-olignans [10, 11]. These compounds and their extracts have been reported to exhibit a broad spectrum of in vitro and in vivo biological activities, including antioxidant, antitumor, antimicrobial, anti-inflammatory, antidiabetic, and hepatoprotective activities, in addition to promoting osteoblast differentiation [9, 12].

In contrast to phenolic components, oligo- or polysaccharides of sugar maple bark have been less investigated to date. Only few studies reported the presence of inulin, dextran, arabinogalactan, and rhamnogalacturonan type I in maple syrup and in sap of sugar maple [13–15]. Certain studies hypothesized that these polysaccharides could be derived from primary cell wall components of maple trees [15]. Furthermore, we have recently presented an overview of soluble polysaccharides found in sugar maple bark, by applying two different pre-extractions with aqueous versus aqueous ethanol combination [8, 9]. Interestingly, we have observed that the soluble complex sugars and residual monosaccharides released by hydrolysis of hemicelluloses, contribute to the yield of furanic compounds in residual liquor [8].

In the present study, we have hypothesized that the sugar maple bark water-extractable polysaccharides could impact the biological activities of the aqueous extract. In addition to this extract, aqueous ethanol extract was examined in terms of its ability to recover polysaccharides according to the organosolv process developed in our previous studies [8]. Polysaccharides extracted from natural sources have attracted extensive attention to their safety, accessibility, and potent anti-inflammatory activities [16–18]. For this reason, we have investigated the bioactive polysaccharide responsible for the anti-inflammatory potential of aqueous extract via a (sub)-fractionation approach. This approach was guided through a cell-based bioassay measuring TNF-α levels in cell culture of RAW 264.7 cells. Our goal was to establish the procedure of isolation and chemical characterization of the complex polysaccharide present in the aqueous extract. However, the results of chemical characterization of the studied polysaccharides remain preliminary. Nevertheless, we report, for the first time, the partial structure of a major complex polysaccharide from the sugar maple bark, which could be responsible, at least in part, for the anti-inflammatory activity. This polysaccharide could be used in potential therapeutic applications in the treatment of diseases associated with inflammation.

Methods

Materials and Chemical Reagents
Sugar maple barks were provided by Levaco, Inc. (Québec, Canada). Samples were air-dried and ground into a fine powder in a mill (40 and 60 meshes) prior to chemical and biological analyses [8]. Disaccharide and monosaccharide standards (cellobiose, glucose, galactose, mannose, xylose, rhamnose, and arabinose), dextran standards (10, 12.5, 25, 50 and 80 kDa) and lipopolysaccharides (LPS) (from Escherichia coli serotype O55:B5), parthenolide, deuterium oxide (D2O) and gel filtration Sephadex G-50 column were obtained from Sigma (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution, penicillin-streptomycin (P/S) were purchased from WISENT Bioproduct Inc (Montréal, Qc, Canada). Mouse TNF-α DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals and solvents used were of analytical reagent grade.

Extraction and Isolation of Polysaccharide Samples
The sugar maple bark powder was separately extracted with ethanol-water (1/1, v/v; 1 L of final volume for 100 g of bark) mixture or with water alone under reflux for 1 h at 80 °C, according to the protocol reported in our previous study [8, 9]. As schematically summarized in Figure 1, 1 g of each crude extract obtained
was mixed with 80% ethanol in order to separate crude polysaccharides from other compounds (notably polyphenols) and kept at –20 °C overnight. The supernatants were removed after centrifugation at 1000 × g for 15 min. Then, the crude polysaccharide extracts were re-suspended in sequential solvent exchanges with 100% ethanol and centrifuged under the same conditions as described. After being successively washed with acetone, each crude polysaccharide extract was dried overnight in a vacuum oven (Isotemp Vacuum Oven 280A, Fisher Scientific, Ottawa, ON) at 40 °C. Subsequently, they were ultra-filtrated (MW cut-off membrane 5 kDa) against tap water to obtain two fractions. The permeate fractions were eliminated and the retained fractions were lyophilized. The polysaccharide yield was estimated, and the carbohydrate content of each fraction was evaluated by the phenol-sulfuric acid method [19].

**Determination of Monosaccharide Compositions**

Monosaccharide compositions were determined using high-performance liquid chromatography (HPLC 1200 series, Agilent Technologies, Santa Clara CA, USA), equipped with refractive index detector (RID). Briefly, 2 mg of sample (WE, WF, and WSF-1,2,3) was hydrolysed in 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The solution was then evaporated and washed with absolute methanol. After dissolving in H₂O (1 mL), the hydrolysate was filtered through 0.25 µM syringe filter and separated using Rezex RHM-Monosaccharide H+ column (8%) (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) on HPLC-RID system. The column and detector temperatures were set at 75 °C and 55 °C, respectively. The sample was eluted isocratically with 100% nanopure water at a flow rate of 2 mL/min. Monosaccharide standards (arabinose, xylose, rhamnose, galactose, glucose, and fructose) were run through HPLC-RID on the same column. Standard curves were created by plotting areas-under-the-curve (AUCs) against concentrations.

**Average Molecular Weight (Mw) of the Polysaccharide Samples**

The determination of relative average molecular weight of polysaccharide-rich fraction WF and of WSF-1,2,3 was conducted by high-performance size exclusion chromatography (HPSEC) on Agilent 1200 series equipped with two serially linked columns (Varian, Palo Alto, CA, USA), a PLaquagel-OH 30 8 µm column.
(300 mm × 7.5 mm inner diameter, 8 μm) and a PLaquagel-OH 40 8 μm column (400 mm × 7.5 mm inner diameter, 8 μm), using a refractive index detector, eluted with deionized water at a flow rate of 1 mL/min. The column and detector temperatures were controlled at 35 °C and 55 °C during the 40 min of operation time. The injection volume was at 50 μL. The polysaccharide solution was filtered through a 0.22 μm microporous filtering film. The calibration curve of log Mw (molecular weight) of standard dextrans (10, 12.5, 25, 50, 80 kDa) against their elution time (ET) was obtained (log Mw = -0.293ET + 8.5811, R² = 0.99).

Fourier-Transform Infrared (FT-IR) Spectroscopy
Around 2-3 mg of samples were used to obtain FT-IR spectra using a Spectrum-400 spectrometer from Perkin-Elmer (Waltham, MA, USA), which was equipped with an Attenuated Total Reflectance (ATR) crystal. Spectra were recovered at a spectrum resolution of 4 cm⁻¹ and 64 scans, over the range of 4000 to 650 cm⁻¹ in absorption mode. The FT-IR analyses were conducted on powdered samples without any preparation.

Nuclear Magnetic Resonance (NMR) Spectroscopy
About 50 mg of WSF-3 was dissolved with 1 mL of D₂O in a NMR tube. NMR analysis was performed at room temperature on an Agilent 400-MR DD2 system (Agilent Technologies, Santa Clara, CA, USA), by both one-dimensional ¹H and ¹³C NMR and two-dimensional NMR (HSQC, HMBC, COSY and NOESY) spectroscopy. Chemical shifts were referenced to 0.1% tetramethylsilane used as internal standard.

Biological Activity of the Extract and its Fractions
Cell Culture
RAW 264.7 cell lines (murine macrophage) were generously provided by Dr. Jean-Francois Gauchat laboratory (Department of Pharmacology and Physiology, Université de Montréal) and cultured in RPMI 1640, containing 1% of penicillin, 100 μg/mL of streptomycin, and 10% FBS. Cells were incubated in an atmosphere of 5% CO₂ at 37 °C and 10% CO₂ at 37 °C, 5% CO₂. The cytotoxicity assay was carried out to determine the maximal nontoxic concentrations of sugar maple bark extract samples on RAW 264.7 cells, as described in our previous study [20]. Cells were seeded in 96-well plates at a density of 3 × 10⁴ cells/well, incubated, and treated with different concentrations (6.25 to 100 μg/mL) of each sample or 0.1% DMSO (vehicle control, Sigma, St. Louis, MO, USA) and LPS (0.01 to 1 μg/mL) for 18 h at 37 °C. Cell culture media were collected separately for each condition and kept on ice (representing lactate dehydrogenase (LDH) released by damaged cells). Then, cells were lysed by adding culture medium with 1% Triton X-100 (representing cellular LDH) for 10 min at 37 °C, 5% CO₂. The released LDH in the medium and that of obtained in Triton X-100 lysates (cellular LDH) were determined using a commercial detection kit (Roche, Mannheim, Germany) at a wavelength of 590 nm. The ratio of released LDH to total LDH (total LDH = released LDH + cellular LDH) was calculated and the value of each condition was normalized to that obtained from cells treated with 0.1% DMSO (vehicle control) to determine the optimal nontoxic concentration for each sample.

Anti-Inflammatory Assay
RAW 264.7 cells were cultured in RPMI 1640 media supplemented with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep, and 10% FBS, at 37 °C and 5% CO₂. Briefly, 1 mL of cells was transferred (3 × 10⁴ cells/well) into a 96-well plate and stimulated with culture medium, or LPS (0.01 to 1 μg/mL) or LPS in the presence of studied samples (WE, WF, WSF-1,2,3) dissolved in DMSO for 24 h. LPS at 1 μg/mL released the optimal level of TNF-α in RAW 264.7 cells without causing cytotoxicity. Therefore, this concentration of LPS was used as a standard in all experiments. All samples were used at optimal non-toxic concentration of at 100 μg/mL, except for the WE and WSF-5, which were assayed at 6.25, 12.5, 25, 50 and 100 μg/mL. Parthenolide (10 μg/mL, Sigma-Aldrich, St-Louis, MO, USA) and 0.1% DMSO were used as positive and vehicle controls, respectively. After incubation, cells were centrifuged at 450 × g for 10 min at room temperature. Cell culture supernatants were separated and stored at −80 °C for subsequent analysis. The anti-inflammatory activity of each sample (WE, WF, WSF-1,2,3) was evaluated by measuring TNF-α levels in cell culture supernatants with DuoSet® ELISA development kits according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis
Data are expressed as means of three repeated determinations ± standard deviation (SD). A one-way ANOVA were used to determine the statistical significance of the differences between the various experimental and control groups. P values of 0.05 or less were considered to be statistically significant.
Results

Extraction and Fractionation

As summarized in Table 1, the yield of the crude aqueous extract, expressed per g / 100 g of dry bark, (WE, 7.9 ± 0.5%) was higher than that of ethanol-aqueous mixture (EtOH, 5.9 ± 0.6%). The analysis of total carbohydrate content revealed that carbohydrates were more concentrated in WE (569.7 ± 2.3 mg/g) that in EtOH (461.1 ± 1.3 mg/g), confirming that water remains the best solvent for extracting soluble polysaccharides. For this reason, WE was chosen and fractioned using an ultra-filtration technique with a 5 kDa cut-off membrane. The fraction enriched in polysaccharides (WF) was sub-fractioned using gel filtration on a Sephadex G-50 column, leading to three major sub-fractions. The main sub-fraction, WSF-3, obtained at a yield of 0.42 ± 0.1%, was more concentrated in sugar with 545.4 ± 2.2 mg/g of total sugar, compared to WSF-2 and WSF-1 with 478.9 ± 1.4 mg/g and 453.2 ± 2.3 mg/g of total sugar, respectively.

Chemical Compositions

HPLC analysis of aqueous extract (WE), polysaccharide-rich fraction (WF) and their sub-fractions WSF-1,2,3 was conducted to determine their monosaccharide compositions. As presented in Table 2, the obtained results indicate that the glucose residues were the most important in WE, WF and WSF-1,2,3. Apart from glucose, other monosaccharides, namely galactose (9.8–27.5%), rhamnose (3.1–27.5%) and arabinose (5.3–24.9%) were also identified in all examined samples. However, fructose was detected only in WF and WSF-1. Interestingly, the sub-fraction WSF-3 was composed mainly of glucose (87.2%), with lower quantities of galactose (9.8%) and rhamnose (3.1%).

Molecular Weight of Aqueous Fractions and Sub-Fractions

The average molecular weight of WSF-1,2,3 was measured by HPSEC-RID as illustrated in Figure 2. The studies of these HPSEC-RID profiles revealed that the WSF-2 fraction could contain more than two polysaccharides, while the WSF-1 fraction contains only two. As a result, the two fractions must be subjected to isolation and structural identification of their polysaccharides, which will be investigated in further studies. In contrast, WSF-3 was eluted as a single and symmetrical peak, indicating that WSF-3 was a homogeneous polysaccharide. Based on the calibration curve determined with standard dextrans, the relative average molecular weight of WSF-3 was calculated to be 21.5 kDa.

FT-IR Spectrum of WSF-3

The assignments of the absorption bands in FT-IR spectrum of WSF-1,2,3 are made according to the data available from the literature [21, 22]. As presented in Figure 3, the absorption at 3251 cm\(^{-1}\) corresponds to the O-H stretching vibration in sugar residues, in which glucose residues are the most important. The band at 2917–2881 cm\(^{-1}\) corresponds to C-H vibration and that at 1595 cm\(^{-1}\) could be indicative of the presence of water bound to the polysaccharide. Further analysis, notably \(^{13}\text{C}\)-NMR will confirm the origin of this group. The absorption peaks at 1413 and at 1377 cm\(^{-1}\) may be due to the angular deformation of C-H, while the strong peak at 1015 cm\(^{-1}\) was ascribed to the stretching vibrations of the C-OH side groups and the C-O-C glycosidic bond vibrations. In the anomeric region (900–700 cm\(^{-1}\)), the characteristic absorption peak at 891 cm\(^{-1}\) was assigned to the \(\alpha\)-pyranose configuration of glucose units [23, 24], revealing the presence of the \(\alpha\)-glycosidic bonds [25].

Table 1. Yields and total carbohydrate of extracts, fractions and sub-fractions of sugar maple bark.

<table>
<thead>
<tr>
<th></th>
<th>Yield (%)(^1)</th>
<th>Total carbohydrate(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>5.9 ± 0.6</td>
<td>461.1 ± 1.3</td>
</tr>
<tr>
<td>WE</td>
<td>7.9 ± 0.5</td>
<td>569.7 ± 2.2</td>
</tr>
<tr>
<td>After ultrafiltration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtOHF</td>
<td>1.3</td>
<td>567.4 ± 2.2</td>
</tr>
<tr>
<td>WF</td>
<td>2.8</td>
<td>576.6 ± 2.2</td>
</tr>
<tr>
<td>After Sephadex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSF-1</td>
<td>0.7 ± 0.1</td>
<td>453.2 ± 2.3</td>
</tr>
<tr>
<td>WSF-2</td>
<td>0.6</td>
<td>478.9 ± 1.4</td>
</tr>
<tr>
<td>WSF-3</td>
<td>0.42 ± 0.1</td>
<td>545.4 ± 2.2</td>
</tr>
</tbody>
</table>

\(^1\) Yield is expressed per g/100 g of dry bark.  
\(^2\) Results are expressed as mg/g of dry sample.

Table 2. Monosaccharide compositions of ethanol-aqueous and aqueous extracts, aqueous extract polysaccharide-enriched fraction and sub-fractions WSF-1,2,3.

<table>
<thead>
<tr>
<th>Monosaccharide compositions</th>
<th>WE</th>
<th>WF</th>
<th>WSF-1</th>
<th>WSF-2</th>
<th>WSF-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>61.6</td>
<td>50.7</td>
<td>58</td>
<td>45.9</td>
<td>87.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
</tr>
<tr>
<td>Galactose</td>
<td>26</td>
<td>29.6</td>
<td>29.5</td>
<td>21.7</td>
<td>9.8</td>
</tr>
<tr>
<td>Xylose</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.3</td>
<td>2.5</td>
<td>N/d</td>
<td>N/d</td>
<td>N/d</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4.3</td>
<td>7.7</td>
<td>4.7</td>
<td>7.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Arabinose</td>
<td>6.8</td>
<td>12</td>
<td>5.3</td>
<td>24.9</td>
<td>N/d</td>
</tr>
</tbody>
</table>

N/d: not detected.
**Figure 2.** HPSEC-RID profiles of polysaccharide-rich fraction (WF) and WSF-1,2,3.

**Figure 3.** FT-IR profiles of the WSF-1,2,3.
NMR Analyses of WSF-3

The structural features of WSF-3 were further studied by NMR spectral analysis. As displayed in the Figure 4, the $^1$H and $^{13}$C NMR spectra for WSF-3 revealed the three peaks corresponding to the protons at and to the anomeric carbons in the chemical shift ranges ($\delta$) of 4.2–5.8 ppm and 97.6 ppm, respectively. Literature data reported $\alpha$-anomeric peaks at $\delta_{H} / \delta_{C}$ 4.8–5.8 / 98–103 ppm and that of $\beta$-anomer at $\delta_{H} / \delta_{C}$ 4.2–4.8 / 103–106 ppm [26]. Thus, the anomeric proton and carbon of WSF-3 found at $\delta_{H}$-1 / $\delta_{C}$-1 4.81 / 97.6 ppm is a signal characteristic of glucosyl residues linked through $\alpha$-$(1 \rightarrow 6)$ (87.2%) linkages [26–28]. The presence of $\alpha$-$(1 \rightarrow 6)$ glucosyl residue can also be confirmed by the scalar coupling constant $^{3}J_{H_{1}-H_{2}}$ which is around 3.4 Hz, corresponding to that reported in literature data, normally around 3–4 Hz for the $\alpha$-anomer [26, 29]. On the other hand, the two remaining minor peaks appearing at $\delta$5.0 and 5.13 could be attributed to anomic protons of D-galactose (9.8%) and D-rhamnose, respectively, which were detected after hydrolysis of WSF-3. However, it was difficult to confirm the full assignments of protons in D-galactose and D-rhamnose residues, due to their low amount in the WSF-3 structure. Furthermore, due to the fact their integration values are not consistent with the backbone of glucose residues, it cannot be excluded that these sugars represent impurities that contaminated WSF-3 during the extraction process. Nonetheless, the ring protons of glucose residues could be unequivocally detected as they appeared from 63.2 to 4.1 ppm: $\delta = 3.33$ (dd, 1H, $J = 3.2$ Hz, $H-2$), 3.35 (t, 1H, $J = 9.2$ Hz, $H-3$), 3.55 (t, 1H, $J = 9.6$ Hz, $H-4$), 3.75 (d, $J = 8.4$, 1H, $H-5$), 3.58 (d, $J = 9.76$, 1H, $H-6a$) and 3.83 (d, $J = 7.48$, H-6b). HSQC spectrum showed that the carbon signal appearing at $6C$-6 65.41 had two correlation peaks, which included H-6a ($63.75$) and H-6b ($83.83$) (Figure S1). The four remaining signals observed at 673.28, 70.06, 69.39, and 71.29 correspond to C-4, C-5, C-3, and C-2 hydrogens, respectively. However, there is no signal in the 170–180 ppm region, which could be attributed to the existence of C=O group from non-esterified uronic acid or from acetyl esters in the studied polysaccharide. Therefore, the absorbance band at 1595 cm$^{-1}$ in FT-IR spectrum should be attributed to the presence of water bound to the polysaccharide, as its signal is found in the same area [30]. Full assignments of the proton and carbon resonances were also secured from the COSY, NOESY and HMBC data (Figures S2–S5). The sequence of the glucosyl residues was further confirmed by 2D-NOESY and HMBC spectra. As displayed in the Figure 5, the anomic proton (H-1) of glucose residue

Figure 4. $^1$H NMR (A) and 2D-NOESY (B) spectra of WSF-3.
A had a strong inter-residue NOE connected to H-6a,b of residue B in addition to intra-residue connection to H-2 (Figures S4–S5). However, the strongest inter-residue connection was established between protons H-1 and H-6a, confirming, hence again, the presence of α-(1→6) glycosidic linkage between glucose residues in this polysaccharide. To confirm the configurations at the glycosidic linkages, the direct coupling constants (1\(^J\)\(C–H\)) of C-1 of glucose units were also obtained from HMBC spectrum. The high values of ~170 Hz for these glucose residues indicate that the protons at C-1 are equatorial, therefore the configuration at C-1 of glucose units is correctly determined as α-D-glucose (placing the -OH group in axial position). Based on the above analysis, WSF-3 can therefore be assigned as an α-glucan closely related to dextran constituted of glucose residues linked together with α-(1→6) glycosidic bonds [15].

**Cytotoxicity Assay**
The LDH test was used to determine the maximal non-toxic concentration of the aqueous extract, polysaccharide-rich fraction and sub-fractions on RAW 264.7 cells. The results (Figures S7 and Table S1) demonstrated that none of the maple bark samples affected the viability of 264.7 RAW cells at the concentrations tested (10 and 100 µg/mL) in comparison to DMSO (0.1%). Therefore, a concentration of 100 µg/mL was used for all extract, fractions and sub-fractions.

**In vitro Anti-Inflammatory Effects**
The LPS-stimulated RAW 264.7 cell-based bioassay was used to study the anti-inflammation effects of maple bark aqueous extract (WE), polysaccharide-rich fraction (WF), and sub-fractions WSF-1,2,3. Bacterial lipopolysaccharide (LPS) was used to induce TNF-α production in macrophage cells. Of note, TNF-α is one of a number of potent pro-inflammatory cytokines that possess multiple effects, including the activation of pro-inflammatory cells, the induction of several pro-inflammatory activation of immune proteins, as well as edema formation, and neutrophil migration. The levels of TNF-α in culture supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) method. LPS at concentrations ranging from 0.01 to 1 µg/mL have been tested to induce RAW 274 macrophages. Treatment with LPS at 0.01 µg/mL did not release TNF-α while 1 µg/mL released the optimal level with 1778.9 ± 97.9 pg/mL of TNF-α from RAW 264.7 cells as compared to the baseline level of TNF-α in vehicle-treated cells (34.6 ± 6.2 pg/mL); the former was set at 100% and the latter at 0% (Figure 6A). When LPS-stimulated RAW264.7 cells were treated with maple bark extract samples at 100 µg/mL, only WE, WF and WSF-3 significantly inhibited TNF-α production by 20.8 ± 4.9% (p < 0.0001), 30.7 ± 4.1% (p < 0.0001) and 33.1 ± 1.8% (p < 0.0001), respectively, as compared to LPS alone. Additionally, there was no significant difference between the inhibitory effect of WF and that of WSF-3. The possible LPS contamination in maple bark samples has not been evaluated in this study, but we assume that this factor would not have had a substantial impact on these results. To further delineate the effects of maple bark extracts on TNF-α production, we correlated the latter with the sugar compositions of all samples at 100 µg/mL using linear regression analysis. As summarized in Figure 6B, a significant and negative correlation between the anti-inflammatory effect and the molar proportion of glucose in the polysaccharides.
of all extract was observed \((r = -0.81; p = 0.045)\). Finally, we evaluated the apparent anti-inflammatory activity of WE and WSF-3 at various concentrations and found that significant inhibitory effects on TNF-\(\alpha\) production were present only at concentration of 50 \(\mu\)g/mL and greater (Figure 7).

**Discussion**

The present study was conducted in the context of the comprehensive valorization of sugar maple bark extract potential uses due to its contents in numerous bioactive compounds, among which the polysaccharides. Thus, the results of this study are reporting for the first time the isolation and the chemical characterization of the main polysaccharide found in sugar maple bark aqueous extract. The polysaccharide named WSF-3 consists of glucose residues linked together by \(\beta-(1 \rightarrow 6)\) glucosidic bonds. However, the lesser number of galactose and rhamnose residues detected in the backbone of glucose residues may represent impurities that made their way into the polysaccharide during the extraction process. Therefore, WSF-3 seems to be an \(\alpha\)-glucan closer to dextran, which contains principally repeating units of \(\alpha-1,6\)-linked glucose. Dextran along with inulin are the common oligosaccharides that have previously been

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**Figure 6.** Anti-inflammatory activity of extract, fraction and sub-fractions obtained from sugar maple bark at 100 \(\mu\)g/mL in LPS-stimulated RAW 264.7 cells (Panel A). DMSO was used as a vehicle in both unstimulated and LPS-stimulated controls and parthenolide (Parth, 5 \(\mu\)g/mL in DMSO) was used as a positive control. Pearson’s correlation coefficients obtained \((r = -0.81; p = 0.045)\) between TNF-\(\alpha\) production and the glucose compositions in all sample (Panel B). The sugar maple extract and fractions originated from the same batch of extraction. Data are mean \(\pm\) standard deviation (SD), \(n = 3\).

**Figure 7.** Anti-inflammatory activity of polysaccharide fraction (Panel A) and sub-fraction (Panel B) extracted from sugar maple bark in LPS-stimulated RAW 264,7 cells. DMSO was used as a vehicle in both unstimulated and LPS-stimulated controls. Data are mean \(\pm\) standard deviation (SD), \(n = 3\).
Plant-derived polysaccharides are interesting bioactive molecules, particularly in the context of their demonstrated role to modulate human immune responses [16–18]. Indeed, gut epithelial and immune cells express Toll-like receptors (TLRs) that recognize molecular patterns from commensal and pathogenic microbes in order to maintain a healthy and dynamic equilibrium between fending off potential harmful intruders and tolerating the beneficial ones [33–35]. Among such molecular patterns recognized by TLRs are lipopolysaccharides such as the LPS used in our RAW 264.7 macrophage bioassay as a positive control [36, 37]. Indeed, macrophages respond to LPS by secreting a myriad of pro-inflammatory modulators such as nitric oxide and cytokines TNF-α, IL-1β, and IL-6. It is well established that the overproduction of pro-inflammatory cytokines, including TNF-α and IL-1β, has an important role in the pathogenesis of various inflammatory diseases [38]. In this context, plant-derived polysaccharides that stimulate macrophages can help enhance immune responses, while other polysaccharides that mitigate the production of TNF-α and IL-1β can be seen as promising anti-inflammatory agents. We therefore evaluated the effects of maple bark extracts, notably those enriched in polysaccharides, on TNF-α production in RAW 264.7 macrophages. All samples derived from the hot aqueous extract of maple bark were quite innocuous as seen from their lack of cytotoxic effect on the cells in concentrations up to 100 µg/mL (Figures S7). When LPS was subsequently employed to induce an inflammatory response in these cells, WE, WF and the sub-fraction WSF-3 significantly decreased TNF-α production, thereby suggesting a good anti-inflammatory potential. Given that the WSF-3 sub-fraction yielded an effect as important as its parent WE fraction, it is plausible that the pure α-glycan is responsible, in good part, for the anti-inflammatory activity of the plant extract. This polysaccharide can thus be added to the list of numerous polysaccharides originating from natural sources, which are known to exhibit immunomodulatory activity.

According to literature, many polysaccharides might be used as immunomodulators to help enhance or normalize the immune system. Several factors underlie the immunomodulatory activity of polysaccharides, some of the most important being monosaccharide compositions, glycosidic bond types and molecular weight [39, 40]. Monosaccharides arabinose, mannose, xylose or galactose appear to play the largest role in the immune-enhancing activities of polysaccharides in macrophages, whereas glucose residues do not appear to have a clear contribution [17, 41, 42]. However, studies reported that many β-glucans induced immunomodulatory effect mediated by some receptors such as Dectin-1 [43–45]. In this context, we evaluated the relationship between the monosaccharide compositions of our sugar maple bark extract and their biological activity. We found a substantial and statistically significant inverse correlation between TNF-α production and the content in glucose moieties of our maple bark fractions and sub-fractions.

The mechanisms by which such anti-inflammatory activity is exerted remain to be determined. Indeed, recent literature shows that polysaccharides containing mannose or glucose are most likely recognized by Toll-like receptor 2 (TLR2), CR3, or MR [17, 34]. Furthermore, other studies have demonstrated conclusively that TLR4 and TLR2 are able to directly recognize polysaccharides like mannans and α-glucans [34]. However, as mentioned, the toll-like receptors represent an important family of innate immune receptors, which act as the first line of host immunity defense against various pathogens, notably through pro-inflammatory signaling pathways [46]. Indeed, lipopolysaccharide (LPS), recognized by TLR-4, which is, along with TLR-2, dominant in immune cells, is one of the most potent initiators of inflammation [35]. The modulation of TLR-2 and TLR-4, as well as the downstream expression of pro-inflammatory cytokines may inhibit inflammation. Future studies with maple bark extracts should thus explore the modulation of such pathways and notably examine if the high level of α-1,6 linked glucose present in WSF-3, as well as other bioactive maple bark samples, can contribute to their anti-inflammatory activities.

Conclusions

In continuation of our previous studies regarding the integrated valorization of sugar maple bark, we investigated the water-extractable polysaccharides of this bark. Among the sub-fractions obtained from the aqueous extract, we successfully isolated a major complex polysaccharide (WSF-3). The chemical structure has been partially elucidated and assigned as a α-(1 → 6) glucan composed of glucose. However, it is acknowledged that galactose, and arabinose units...
detected in WSF-3 may represent impurities resulting from the extraction process. The average molecular weight was determined to be 21.5 kDa. The WSF-3, along with its parent polysaccharide-rich fraction (WF) and original aqueous extract (WE), all elicited significant inhibitions of TNF-α production in LPS-stimulated RAW 264.7 cells. Such inhibitions were found to be proportional to the molar glucose content of each extract or fraction. These results suggest that major complex polysaccharide (WSF-3) present in polysaccharide-rich extracts of sugar maple bark, displays an interesting anti-inflammatory potential.

**List of Abbreviations**

- COSY: correlated spectroscopy
- DMSO: dimethyl sulfoxide
- FT-IR: Fourier-transform infrared spectroscopy
- FBS: fetal bovine serum
- HPSEC-RID: high-performance size exclusion chromatography- refractive index detector
- HPLC: high-performance liquid chromatography
- HMBC: heteronuclear multiple bond correlation
- HSQC: heteronuclear single quantum coherence
- LDH: lactate dehydrogenase
- LPS: lipopolysaccharides
- IL-6/1β: interleukin 6/1β
- NMR: nuclear magnetic resonance
- NOESY: nuclear Overhauser Enhancement Spectroscopy
- TLR: toll-like receptors
- TNF-α: tumor necrosis factor-α

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Ethics Approval and/or Participant Consent**

No animals or human subjects were involved.

**Authors’ Contributions**

PBK: made contributions of the design of this study, collected, analysed, and interpreted of the data, drafted the manuscript and gave final approval of the version to be published.

PSH and TS: lead the design and planning, revised the manuscript critically, and gave final approval of the version to be published.

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