Structural and functional characteristics of pectins from three cultivars of apple (*Malus pumila* Mill.) pomaces

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Abstract

This study aimed to investigate the chemical composition, structural properties, and biological properties of pectin polysaccharides (AP-FS, AP-QG, and AP-HG) isolated from different varieties of apple pomace. Based on the methylation and nuclear magnetic resonance analyses, the structure of AP-FS was determined to be composed of an α-1,4-linked homogalacturonan backbone that exhibited high levels of O-6 methylation. All pectins exhibit potent inhibitory activity against human colon cancer and human liver cancer cells, along with immunostimulatory effects. Among them, AP-FS exhibited the highest activity level. Finally, we further investigated the underlying mechanism behind the effect of AP-FS on RAW 264.7 cells using proteomics analysis. Our findings revealed that AP-FS triggers RAW 264.7 macrophage activation via NOD-like receptor (NLR), NF-κB, and mitogen-activated protein kinase (MAPK) signaling pathways. Therefore, our research contributes to a better understanding of the structure-function relationship among apple pectins, and AP-FS has the potential to be applied to dietary supplements targeting immunomodulation.

Keywords: Apple pectin; Immunostimulatory activity; Structure-function relationship

1. Introduction

Apple fruits are commercially available worldwide, with global production reaching approximately 93.1 million tons in 2021. China stands as the largest producer, accounting for nearly half of the world's output (FAOSTAT). In addition to their consumption in fresh form, derived products such as juice, cider, and sauce enjoy widespread popularity among consumers globally. However, during the production of cider, syrup, or juice, approximately 25% of apple pomace is generated as industrial side-stream by-products [1]. Apple pomace, comprising of peel, flesh, seeds, and stems [1], is a cost-effective source of functional substances such as dietary fiber (such as pectin), polyphenols, organic acids, vitamins, and minerals [2]. However, untreated apple pomace poses environmental hazards and represents a waste of resources. Therefore, the valorization of apple pomace through pectin extraction emerges as an effective method to mitigate environmental pollution risks while contributing to sustainable development within the agro-processing industry. Currently, apple pomace is the second most important commercial source of pectin globally, following citrus fruit peels.

Pectin, an acid heteropolysaccharide abundant in galacturonic acid (GalA) units, is primarily distributed within the primary cell wall and intercellular layer of higher plants [3, 4]. Pectin is composed of three distinct structural domains: homogalacturonan (HG), which accounts for 65% of pectin composition; rhamnogalacturonan I (RGI), comprising 20–35%; and rhamnogalacturonan II (RGII), constituting 10% [5]. Based on its degree of methyl-esterification, pectin can be classified into high methyl-esterified pectin (HMP) with a methyl-esterification degree > 50% and low methyl-esterified pectin (LMP) with a methyl-esterification degree < 50% [6], respectively. The

physiological functions and biological activities of apple pectin have been extensively reported, including modulation of gut microbiota [7], anti-radical activity [8], and antitumor activity [9, 10]. Due to its biocompatibility and biodegradability properties, pectin has wide applications in the biomaterial and food industry [11]. Moreover, it serves as an excellent emulsifier for nanoemulsion development, thereby inhibiting the proliferation of cancer cells [12, 13]. As a functional macromolecule endowed with multiple biological properties, pertinent physicochemical parameters such as the degree of methyl esterification, molecular weight, monosaccharide composition, and conformation/degree of side chain branching directly influence the biological and physicochemical properties of pectins [14].

Stimulating the host's immune system through natural dietary supplements, without causing harm, is a crucial strategy for enhancing immunity in immunocompromised populations, such as cancer patients and the elderly. It has been demonstrated that natural pectin polysaccharides can enhance the host immune system by activating natural killer cells, dendritic cells, and macrophages [15]. Moreover, therapeutic approaches activating macrophages to counteract tumor cell proliferation and spread have gained widespread attention. Accumulating evidence suggests that activated macrophages possess the ability to recognize and eliminate tumor cells, even those resistant to chemotherapeutic agents [16]. Additionally, macrophages can regulate tumor cell proliferation by releasing nitric oxide (NO) and inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β), thereby enhancing immunity. The anticancer and immunomodulatory activities of pectin polysaccharides have been demonstrated [14]. However, despite its potential as a valuable food or pharmaceutical component for anticancer and immunostimulatory purposes, the exact structure–activity relationship of pectin remains limited understanding. In recent years, researchers have focused more

on the potential of apple pectin as a thickening agent due to its viscous properties of pectin, while neglecting its potential biological properties. In particular, there are no detailed reports on the immunostimulatory activity of apple pectin and the evaluation of its structure–activity relationship.

In this study, three types of apple pectin (APs) were isolated and characterized from different apple varieties. The structure of AP-FS was determined via methylation and nuclear magnetic resonance (NMR) analysis. Additionally, the growth inhibitory effects of APs on different cancer cells were investigated through *in vitro* cell experiments, while their immunostimulatory activities were assessed using mouse macrophage RAW 264.7 cells. Finally, we elucidated the potential mechanism of action of AP-FS on RAW 264.7 cells through proteomics analysis. Addressing these concerns would provide a basis for considering the potential utilization of APs as functional ingredients.

2. Materials and methods

2.1. Materials and chemicals

Three apple varieties (‘Fushi’, ‘Qinguan’, and ‘Huaguan’) were harvested in mid-September from Hanyuan County, Ya’an City, China. Human colon cancer cells (HCT116), human liver cancer cells (HepG2), and human stomach cancer cells (SGC7901) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse macrophages (RAW 264.7 cells) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RAW 264.7 cell-specific complete medium containing 10% fetal bovine serum and 1%

streptomycin/penicillin solutions were purchased from Procell Life Science & Technology Co., Ltd. Enzyme-linked immunosorbent assay kits were purchased from NeoBioscience Biotechnology Co., Ltd. Standard monosaccharides, lipopolysaccharide (LPS), Griess reagent, and MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from Sigma-Aldrich. Besides, all the other chemicals and reagents were of analytical grade.

2.2. Extraction and purification of APs

Apples were harvested and utilized for juice extraction. The resulting juice underwent fermentation to produce cider, while the remaining material, known as apple pomace, was subjected to a series of treatments, including freeze-drying and milling, before being sieved through an 80-mesh. Subsequently, crude apple pectins were extracted using the hot water extraction method as described by Guo et al. [17]. Fig. 1A illustrates the flowchart for the extraction and purification of apple pectins (APs) from the apple pomace powders. Briefly, 100 g of apple pomace powders were refluxed with 1000 mL of 80% (*v/v*) ethanol in an ultrasonic bath at 40 ºC for 30 min. Subsequently, the crude apple pectins were extracted twice in a boiling water bath with 2000 mL of ultrapure water for 2 h. Following this, the extracts were precipitated with three volumes of 95% (*v/v*) ethanol overnight at 4°C. The resulting precipitates were washed twice with 70% ethanol, redissolved in pure water at 80°C, and transferred into a dialysis membrane (molecular weight cutoff: 3.5 kDa). The solution was dialyzed at 40°C to expedite the movement of small molecules. Finally, the solution was lyophilized at –40°C for 48 h to obtain crude apple pectins from apple pomace powders.

The iodine test results of the lyophilized crude pectins were negative, confirming

the absence of starch. Purification of the crude pectin was performed following the protocol reported by Tang et al. [6]. As depicted in Fig. 1, 20 mL of crude pectins (10 mg/mL) was subjected to the DEAE Sepharose Fast Flow column (Ø 4.5 × 60 cm) and the elution was carried out sequentially with deionized water, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl solutions at a flow rate of 3 mL/min, and each elution volume was 1.5 BV. Then, each fraction was concentrated, dialyzed and lyophilized before further purification. As depicted in Fig. 1, the predominant fraction of crude apple pectin designated as AP-01 was primarily eluted with 0.1 M NaCl. Subsequently, 10 mL of AP-01 (10 mg/mL) was further purified utilizing a Sephacryl S-300 HR column (Ø 2.5 × 50 cm) with 0.1 M NaCl as elution at a flow rate of 0.5 mL/min (Distribution coefficient: Kav=0.6). A BSZ-100 automatic collector (Qingpu Huxi Instrument Co., Shanghai, China) was employed for collecting the eluent. Each eluate was promptly transferred into a dialysis membrane (molecular weight cutoff: 3.5 kDa) and dialyzed at 40°C to remove salts. The polysaccharide content of the collected eluate was determined using the phenol–sulfuric acid method [18]. Finally, three apple pectins were obtained and named AP-FS, AP-QG, and AP-HG, respectively.

2.3. Determination of the chemical composition of APs

The quantifications of uronic acids and protein in APs were conducted using the m-hydroxydiphenyl method [19] and Bradford's method [20], respectively. The degree of esterification (DE) was determined through titrimetric analysis following the procedure outlined by Ranganna [21]. Additionally, a PerkinElmer Lambda 1050 UV/Vis spectrophotometer (Perkin Elmer Corp., Massachusetts, USA) was utilized to obtain the UV spectrum of APs solution (0.1%) for evaluating the presence of proteins

or nucleic acids.

2.4. Determination of structural properties of APs

2.4.1. Determination of homogeneity and molecular weight

The molecular weight (M_w) and homogeneity of APs were evaluated using a high-performance gel permeation chromatography (HPGPC) system on a Shimadzu LC-10A system equipped with a BRT105–104–102 column (Ø 8.0 × 300 mm, Borui Saccharide, Biotech. Co. Ltd.) and a refractive index detector. The determination method was conducted following the protocol described in a previous study [22]. Each analysis involved injecting 20 μL of APs (5.0 mg/mL) into the system. Standard curves for calculating M_w were constructed using dextran standards with known M_w. Standard curves for the calculation of M_w were generated using dextran standards (5000, 11600, 23800, 48600, 80900, 148000, 273000, 409800, and 667800 Da).

2.4.2. Rheological properties

The rheological viscosities of the APs were measured utilizing an advanced rotational rheometer (MCR 302, Anton Paar, Austria) equipped with a parallel steel plate (PP50; diameter: 50 mm; gap size: 0.1 mm). This measurement was conducted in accordance with a well-established protocol described by Li et al. [23]. The AP solutions (10.0 mg/mL) were initially homogenized in tubes at 25°C for 10 min, followed by transfer to the rheometer for the purpose of forming a paste. Subsequently, after equilibrating at 25°C for 5 min, the samples underwent shearing with shear rates ranging from 0.1 s^{-1} to 100 s^{-1}. A frequency sweep experiment was conducted using the PP50 probe according to literature references [24]. The test was performed over a

frequency range of 1−1000 rad/s with an amplitude gamma of 2%, then the storage (G′) and loss moduli (G″) were obtained.

2.4.3. Monosaccharide composition

The monosaccharide compositions of the APs were analyzed using an ICS5000 high-pressure ion chromatography system (HPIC, ThermoFisher, USA) equipped with a Dionex CarbopacTM PA20 column (Ø 3 × 150 mm). Following the hydrolysis procedure described by Liu et al. [25], 5.0 mg of APs underwent hydrolysis in 3 M trifluoroacetic acid (TFA, 2.0 mL) at a temperature of 120°C for a duration of 3 h. Subsequently, the resulting hydrolysates were dried using a nitrogen-blowing instrument (UGC-24 M, LiChen, China), re-solubilized in deionized water (5.0 mL), and centrifuged at a speed of 12,000 *rpm* for a duration of 5 min to collect the supernatant for HPIC analysis after dilution by a factor of 100 and injection volume adjustment to be within range from this solution. The HPIC analysis was conducted with the following parameters: column temperature set at 30°C, injection volume and flow rate were 25 μL and 1.0 mL/min, respectively. Additionally, 15 monosaccharide standards were simultaneously executed in accordance with the aforementioned protocol.

2.4.4. Fourier transform infrared spectroscopy (FT-IR) spectroscopy

The FT-IR spectra analysis of three APs was conducted in the range of 400−4000 cm^{-1} using a Nicolet iS10 Fourier transform infrared spectroscopy instrument (ThermoFisher Scientific, Waltham, MA, USA). The DE, as determined by the FT-IR method, was initially calculated utilizing the following equation [26]:

$$DE\ (\%) = \left(\frac{A_{1734}}{A_{1734}+A_{1619}}\right)\times 100$$

where A_{1619} and A_{1734} are the absorption peaks of esterified uronic acids and free uronic acids, respectively.

2.4.5. Methylation and GC-MS analysis

The glycosidic linkages of AP-FS were determined using methylation and GC-MS analysis [27]. Briefly, 3.0 mg of AP-FS was dissolved in 1.0 mL DMSO solution and subjected to ultrasound treatment after the addition of NaOH (40 mg) at 25°C for 30 min. Iodomethane (0.3 mL) was added and stirred for 1 h before terminating the reaction by adding ultrapure water (2.0 mL). The solutions were dialyzed with ultrapure water for 48 hours prior to collection and subsequent evaporation under a stream of N_2 gas until dryness was achieved. The dried methylated sample was hydrolyzed using heated 2 M TFA (1.0 mL) at 100°C for 90 min followed by reduction using 3% $NaBH_4$ (2.0 mL) for 8 h while neutralizing the reduced pectin with acetic acid prior to acetylation using acetic anhydride (1.0 mL). Partially methylated alditol acetates were analyzed using an ion trap MS detector (Agilent6890/5973N GC/MSD System, USA) and an RXI-5 SIL MS capillary column (film thickness: 0.25 μm, 30 m × 0.25 mm i.d.). The column temperature was initially set at 120°C and then ramped up to 250°C at a rate of 3 °C/min per run, while maintaining the detector temperature and injection temperature at 250°C.

2.4.6. NMR spectroscopy

The precise structural properties of AP-FS were determined using a Bruker Avance III™ HD 600 MHz NMR spectrometer (Billerica, Massachusetts, USA) at a temperature of 25°C [28]. Briefly, 50 mg of dry pectin was mixed with 0.55 mL of deuterium oxide (D_2O), subjected to freeze-drying, and subsequently re-solubilized

with 0.55 mL of D_2O before being transferred into an NMR tube. The heteronuclear single quantum correlation spectroscopy (HSQC), the correlated spectroscopy (COSY), the heteronuclear multiple bond correlation spectroscopy (HMBC), and the nuclear Overhauser effect spectroscopy (NOSEY) were conducted to obtain one- and two-dimensional NMR spectra.

2.5. MTT assay

The growth inhibitory effects of APs on different cancer cells were evaluated using the MTT colorimetric method, following the protocol described by Lin et al. [29]. Briefly, HCT116, HepG2, and SGC7901 cells were cultured in DMEM solution supplemented with 5% CO_2 at 37°C. Cancer cell suspensions were seeded at a concentration of 1×10^5 cells/mL in 96-well plates and incubated for 24 h. Subsequently, each well was treated with various concentrations (0.125–1.0 mg/mL) of APs (100 μL). After an additional incubation period of 24 h, MTT reagent was added and the supernatant was aspirated after 4 h of incubation. Formazan dissolving solution (150 μL) was then added and absorbance was measured at 570 nm to determine cell viability.

2.6. Determination of immunostimulatory activities on RAW 264.7 macrophages

2.6.1. Cell culture and stimulation

The immunostimulatory properties of APs, isolated from various cultivars, were evaluated using the RAW 264.7 macrophage model *in vitro* [30]. RAW 264.7 cells were cultured in the complete medium with a CO_2 concentration of 5% at 37°C. To assess the immunostimulatory effects of APs, 100 μL of RAW 264.7 cells (1×10^5 cells/well)

were seeded in a 96-well plate and incubated for 24 h. Subsequently, the cells were treated with APs (0.125−1.0 mg/mL) for an additional period of 24 hours to determine their impact on cell viability using the MTT colorimetric assay as outlined in section 2.5. Blank and positive control groups were established by adding equal volumes of culture medium and lipopolysaccharide (LPS, 1.0 μg/mL), respectively.

2.6.2. Measurement of NO, IL-6, and TNF-α production in RAW 264.7 cells

To assess the effect of APs on the release of nitric oxide (NO) and cytokines (IL-6 and TNF-α) from RAW 264.7 cells, the cells were exposed to varying concentrations (0.125−1.0 mg/mL) of APs for a duration of 24 h. Subsequently, the supernatant was collected and mixed with equal volumes of Griess I and Griess II reagents before measuring absorbance at 540 nm. The NO was determined using $NaNO_2$ as a standard while ELISA kits were employed to measure cytokine release in the supernatant. Additionally, blank control groups received treatment with culture medium only, whereas positive control groups were treated with LPS.

2.6.3. Reverse transcription-polymerase chain reaction (RT-PCR)

To assess the relative gene expression of nuclear factor kappa B (*NF-κB*), NO synthase (*iNOS*), cyclooxygenase (*COX*)-2, and inflammatory cytokines (*IL-6*, *IL-1β*, and *TNF-α*), RNA was extracted using an RT Kit (Accurate Biotechnology Co., Ltd., Changsha, China) after 24 h of cell culture. Real-time PCR analysis was performed using the QuantStudio™ Real-Time PCR System (Thermo Fisher Scientific Inc., USA). The primer sequences utilized for amplification are provided in Table 1, while the $2^{-(\Delta\Delta Ct)}$ method was employed to determine relative quantification of gene expression.

292 *2.6.4. Proteomic analysis*

293 As shown in Fig.6A, the proteomics analysis was conducted using SDT buffer
294 (containing 100 mM Tris-HCl and 4% sodium dodecyl sulfate, pH 7.6) for protein
295 extraction and quantified using the BCA Protein Assay Kit (Bio-Rad, USA). LC-
296 MS/MS analysis was conducted using a Q Exactive mass spectrometer (Thermo Fisher
297 Scientific, USA), followed by hierarchical clustering analysis using Java Treeview and
298 Cluster 3.0 software. Gene ontology (GO) terms were assigned and sequences were
299 annotated using the Blast2GO program. The annotated proteins were compared with
300 the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for KEGG orthology
301 identification and pathway mapping. The enrichment analysis was performed using
302 Fisher's exact test, and the resulting *p*-values were adjusted for multiple testing using
303 Benjamini-Hochberg correction. Pathways with a significance level of $p < 0.05$ were
304 considered statistically significant.

305

306 *2.7. Statistical analysis*

307

308 All experiments were conducted in triplicate, and the data are presented as means
309 ± standard deviation. Data analysis was performed using IBM SPSS Statistics 26
310 software (IBM, New York, USA). Differences among samples were analyzed by one-
311 way analysis of variance (ANOVA) followed by post *hoc* Duncan's *test*. In addition,
312 Origin 2018 software (OriginLab Inc., Northampton, MA, USA) was utilized for charts
313 plotting. Statistical significance was defined at $p < 0.05$.

314

315 **3. Results and discussion**

316

3.1. physicochemical characteristics of APs

3.1.1. Homogeneity and M_w of APs

In this study, three crude APs were extracted from 100 g of apple pomace, resulting in yields of 5.28%, 6.12%, and 5.85% for AP-FS, AP-QG, and AP-HG, respectively. Zhou et al. [31] reported the extraction of apple pectin from thinned-young apples at pH 1.5 with yields ranging from 2.75% to 5.13%. However, Kumar et al. [32] determined that the yield of pectin from apple pomace (pH 2.5) was between 14.55% and 16.75%. These variations in yield could be attributed to differences in apple variety, origin, and harvesting season. The column was subsequently eluted using a DEAE cellulose column with ultrapure water and NaCl solutions at varying concentrations. As shown in Fig. S1A, the eluate from distilled water exhibited minimal polysaccharide content, while the fraction eluted by 0.1 M NaCl (AP-01) exhibited the highest polysaccharide content among all three apples studied. Consequently, further fractionation of the predominant component of apple pectin (AP-01), which was collected using a Sephacryl S-300 HR column as depicted in Fig.S1B, yielded main products that were subsequently utilized for additional analyses.

The HPGPC analysis not only provided molecular weight values but also demonstrated the homogeneity among the analyzed polysaccharides. Molecular weight standard curves and HPGPC chromatograms of APs are presented in Fig. 2A and 2B, respectively, showing symmetrical single sharp peaks with relatively narrow distribution indicating their purity and great homogeneity in molecular weight distribution across all samples tested. The M_w values and number average molecular weights (M_n) of APs were determined using the regression equation: $\lg M_w = -0.196\ R_t + 12.161$ ($R^2 = 0.9903$) and $M_n = -0.1817\ R_t + 11.448$ ($R^2 = 0.9893$), respectively, as

342 presented in Table 2. The molecular weight range of pectin extracted from 'Blanca de
343 Asturias' apples using different acidic extraction methods reported by Morales-
344 Contreras et al. [24] was between 59.0 × 10^4 and 86.5 × 10^4 Da. Wikiera et al. [33]
345 extracted four varieties of apple pectins by enzymatic methods with a range of M_w
346 ranging from 31.2 × 10^4 to 45.2 × 10^4 Da. The variation in M_w may potentially be
347 attributed to the diversity, origin, and/or extraction methodology utilized. Consequently,
348 this study successfully obtained apple pectins with a uniform distribution of molecular
349 weights; however, their M_w was comparatively lower than that reported in previous
350 studies.
351
352 *3.1.2. Chemical composition of APs*
353 Crude polysaccharides typically contain significant quantities of protein, phenolic
354 acids, and other impurities, which have a substantial impact on the biological properties
355 of these polysaccharides. Therefore, we analyzed to quantify the protein and phenolic
356 acid content in our samples. As shown in Table 2, no detectable levels of protein or
357 phenolic acids were detected in the APs. Furthermore, the absence of peaks at 260 nm
358 and 280 nm in the UV spectra (Fig. 2C) further corroborated these results by indicating
359 the absence of impurities such as nucleic acids, phenolic acids, and proteins. Moreover,
360 neutral sugar content ranged from 12.83% to 16.95%. The DE values for APs
361 (determined via titrimetric method) ranged between 56.82% and 60.14%. Based on
362 these DE values, the APs were classified as HMP polysaccharides. This is similar to the
363 results of previous studies [24, 33]. Notably, the DE value of pectin is influenced by its
364 origin and the method employed for extraction [11]. No significant differences were
365 observed among AP-FS, AP-QG, and AP-HG with respect to DE. The DE evaluated
366 using the FT-IR method showed similar trends as those observed through titration.

3.1.3. FT-IR spectrum analysis

The main groups of APs were identified through FT-IR analysis (Fig. 2D). The spectra of APs exhibited prominent absorption bands in the range of 3100 cm^{-1} to 3500 cm^{-1}, which can be attributed to O–H bond stretching vibrations. A band associated with C–H stretching was observed between 3000 cm^{-1} and 2840 cm^{-1}. The intense peaks at approximately 1734 cm^{-1} and 1619 cm^{-1} corresponded to the carbonyl C–O stretching of esterified GalA and the carboxylic groups (–COOH) in GalA, respectively. Peaks ranging from 1200 cm^{-1} to 1000 cm^{-1} suggested the presence of C–O–C and C–C linkages within pectin molecules [33]. Furthermore, the region spanning from 1300 cm^{-1} to 800 cm^{-1} served as a characteristic fingerprint for carbohydrates, specifically connecting β-glycosides and/or α-glycosides [34]. Overall, FT-IR analysis confirmed that APs are acidic pectin polysaccharides containing methyl-esterified uronic acids. Notably, significant similarities were observed in absorption peaks within the range of 4000−400 cm^{-1}, indicating comparable structural characteristics among APs extracted from different sources.

3.1.4. Monosaccharide composition analysis

The monosaccharide ratio provides crucial insights into the structural characteristics of pectin [6]. The monosaccharide composition analysis results (Fig. 2E) indicated that APs have a high GalA ratio and lack characteristic monosaccharides (Rha) reflecting the RG-I structural domain, suggesting that HG primarily constitutes the backbone of APs rather than the RG domain. Table 2 summarizes the molar ratios of GalA, Ara, Xyl, Gal, and Glc in APs. These findings are consistent with a previous study on pectin from apple pomace [24]. However, our results contradicted previous

studies reporting the presence of the RG structural domain in apple pectin [33, 35], which may be attributed to differences in extraction and purification processes affecting chemical composition. The monosaccharide composition of polysaccharides extracted under various conditions, including different extraction times, temperatures, and solvents, exhibited significant variations as reported by Chen et al. [36].

3.1.5. Rheological properties of APs

Fig. 2F illustrates the impact of shear rate on the apparent viscosities of three types of APs. The viscosity of APs decreased with increasing shear rate, indicating a shear-thinning fluid behavior. This phenomenon can be attributed to the random coiling of pectin polysaccharides and the conformation rearrangement of pectin molecules [37]. Similar behavior was observed in the studies conducted by Jiang et al. [2] and Wang et al. [38]. The order of apparent viscosities was AP-FS > AP-HG > AP-QG, with lower M_w in AP-HG and AP-QG resulting in relatively lower viscosity. A close relationship between apparent viscosities and M_w was observed. Viscoelastic properties were shown in Fig. 2G, where higher G′ and G″ values were found for AP-FS due to increased polymer chain interactions resulting from its higher M_w. All three types of pectin solutions exhibited more viscous-like characteristics with dominant G″ values over a frequency range of 0.1−1000 rad/s, indicating their potential use as thickeners and stabilizers.

3.2. Precise structural characteristics of AP-FS

3.2.1. Glycosyl linkage analysis

The glycosyl linkages in AP-FS were analyzed using methylation and GC-MS

417 analysis. Fig. S2 presents the GC profile of PMAAs derived from AF-FS, which
418 allowed for the identification of ten major sugar residues based on characteristic iron
419 fragments and retention times. Table 3 provides a summary of the types and molar ratios
420 of these sugar residues, estimated by their peak areas. Gal*p*-(1→ was found to be the
421 most abundant terminal unit with a molar ratio of 0.102, while Ara*f*-(1→ and Glc*p*-(1→
422 were present in minor quantities. The predominant sugar residue was →4)-Gal*p*-(1→,
423 accounting for a molar ratio of 0.568, with unsubstituted residues mainly consisting of
424 →4)-Gal*p*-(1→, →4)-Glc*p*-(1→, and →6)-Gal*p*-(1→.
425
426 *3.2.2. NMR spectra analysis*
427 The precise structural properties of AP-FS were elucidated through NMR
428 spectroscopy (Fig. 3), and the C/H chemical shifts of sugar residues present in AP-FS
429 were determined (Table 4) based on monosaccharide composition, methylation, NMR
430 analysis, and literature reports. The presence of multiple signals in the anomeric carbon
431 region (δ 90 – 110 ppm) observed in the ^{13}C NMR spectra (Fig. 3B), indicated the
432 existence of multiple sugar residues within AP-FS. The DEPT-135 spectrum exhibited
433 a distinct inverted peak signal at δ 60–70 ppm, indicating -CH_2- groups in the sugar
434 residues [39, 40]. Moreover, characteristic signal peaks were observed in both hydrogen
435 and carbon spectra: firstly, the signal assigned to δ 1.22 ppm was identified as Rha
436 residue located at H-6, and cross-peak $\delta_{H/C}$ 1.22/19.71 ppm was detected in HSQC
437 spectrum [41, 42]; secondly, the methyl proton signal of *O*-acetyl group appeared within
438 the range of δ 1.95–2.20 ppm range while no clear methyl signal within acetyl group
439 was observed around δ 20.00 ppm [43, 44]; thirdly, strong signals for C/H atoms of
440 methoxyl (OMe) groups at 3.73/52.82 ppm in the $^{1}H/^{13}C$ HSQC spectrum, and signals
441 for C_6 (Gal*p*A)/H (OMe) at 3.73/170.61 ppm in the $^{1}H/^{13}C$ HMBC spectrum confirmed

the presence of 1,4-*α*-D-Gal*p*A(OMe). The signal at δ 174.85 ppm corresponds to the C-6 absorption peak in the unesterified galacturonic acid residue, with a significantly lower intensity compared with that of the esterified galacturonic acid residue, indicating a high degree of methylation in the galacturonic acid [41, 45].

According to the NMR analysis, the polysaccharide samples exhibited several anomeric regions signal peaks that were significant for structural analysis. The chemical shifts of these anomeric region signals were $\delta_{H/C}$ 4.88/100.25 ppm, $\delta_{H/C}$ 5.02/99.41 ppm, $\delta_{H/C}$ 5.05/99.41 ppm, $\delta_{H/C}$ 4.55/103.92 ppm, $\delta_{H/C}$ 4.46/102.93 ppm, $\delta_{H/C}$ 4.42/103.08 ppm, $\delta_{H/C}$ 5.16/109.25 ppm, $\delta_{H/C}$ 5.08/106.99 ppm, $\delta_{H/C}$ 5.01/107.29 ppm. The above corresponding residues were designated as $_{Me}GA_4$, GA_4, GA_t, G_t, $G_{1,3,6}$, $G_{1,6}$, A_t, $A_{1,3,5}$, and $A_{1,5}$. In addition, the reducing terminal signals assigned to $\delta_{H/C}$ 5.22/92.13 ppm and $\delta_{H/C}$ 4.52/95.92 ppm were designated as residues R_α and R_β, respectively. The chemical shifts of anomeric carbon and proton signals are assigned and summarized in Table 4.

The anomeric proton and carbon signals δ 4.88 ppm (H-1) and δ 100.25 ppm (C-1), respectively, were observed for residue $_{Me}GA_4$ based on COSY (Fig. 3D) and HSQC (Fig. 3E) spectra, indicating *α*-linkage of residue $_{Me}GA_4$. Based on these findings, the $_{Me}GA_4$ residue was assigned as *α*-Gal*p*A. Furthermore, the H-1 chemical shift of $_{Me}GA_4$ residue was determined to be at δ 4.88 ppm, while the chemical shifts of H-2 to H-4 were identified from the COSY spectrum as δ 3.63 ppm, δ 3.92 ppm, and δ 4.38 ppm, respectively. Similarly, the HSQC spectrum provided the chemical shifts of C-1 to C-4 as δ 100.25 ppm, δ 67.72 ppm, δ 67.84 ppm, and δ 78.94 ppm, respectively. Additionally, the cross peaks of H-5 and C-5 were observed at $\delta_{H/C}$ 5.00 – 5.08/70.51 ppm in the HSQC spectrum. The HSQC and HMBC spectra exhibited cross peaks at $\delta_{H/C}$ 3.73/52.82 ppm and $\delta_{H/C}$ 3.73/170.61 ppm, respectively, indicating that the signal at δ 170.61 ppm originated from the C-6 position presence of the esterified galacturonic

acid residue. Therefore, the chemical shift at C-6 was determined to be δ 170.61 ppm, indicating the presence of methyl esterification. The down-field chemical shifts of C-1 and C-4 indicated that the $_{Me}GA_4$ residue was substituted at the C-1 and C-4 positions. The down-field shift in H-5 was attributed to the introduction of methoxy, resulting in the proton's chemical shift.

Based on these results, in combination with the methylation results with literature reports [15, 45, 46], the sugar residue $_{Me}GA_4$ was designated as →4)-*α*-Gal*p*A-6-OMe-(1→. Using a similar method as described above, the hydrogen and carbon signals of the other sugar residues were deduced. Consequently, the sugar residue $G_{1,3,6}$, GA_4, R_α, R_β, GA_t, G_t, $G_{1,6}$, A_t, $A_{1,3,5}$, and $A_{1,5}$ were designated as →4)-*α*-Gal*p*A-6-OMe-(1→, →4)-*α*-D-Gal*p*A-(1→, →4)-*α*-D-Gal*p*A, →4)-*β*-D-Gal*p*A, *α*-D-Gal*p*A-(1→, *β*-D-Gal*p*-(1→, →6)-*β*-D-Gal*p*-(1→, *α*-L-Ara*f*-(1→, →3,5)-*α*-L-Ara*f*-(1→, and →5)-*α*-L-Ara*f*-(1→, respectively.

The sequential arrangement of individual sugar residues can be further deduced by utilizing the HMBC and NOESY spectrum (Fig. 3F). In the HMBC spectrum, the cross peak observed between H-4 (δ 4.38 ppm) and C-1 (δ 100.25 ppm) of the $_{Me}GA_4$ residue ($_{Me}GA_4$ H-4/$_{Me}GA_4$ C-1), as well as the cross peak detected between H-1 (δ 4.88 ppm) and H-4 (δ 4.38 ppm) of the $_{Me}GA_4$ residue in the NOESY spectrum ($_{Me}GA_4$ H-1/$_{Me}GA_4$ H-4), provided evidence for an α-1,4-linkage between residues. Furthermore, in the NOESY spectrum, a cross peak was observed between H-1 (δ 5.02 ppm) of GA_4 residue and H-4 of $_{Me}GA_4$ residue (δ 4.38 ppm), provided evidence for a *α*-1,4-linkage between GA_4 residue and $_{Me}GA_4$ residue, resulting in a structure of a structure (→4)-*α*-D-Gal*p*A-(1→4)-*α*-D-Gal*p*A-6-OMe-(1→). Based on these findings, the structure of AP-FS was determined to be composed of an α-1,4-linked homogalacturonan backbone that exhibited high levels of O-6 methylation. The possible structure of AP-FS is illustrated

in Fig. 3G. Due to the high molecular weight and viscosity properties of pectin, obtaining more precise branched linkage patterns based on NMR spectra proved challenging. In future studies, we intend to obtain polysaccharide fragments suitable for study by NMR spectroscopy by enzymatic digestion in order to elaborate upon AP-FS exact structural features.

3.3. MTT assay

Cancer is primarily caused by the uncontrolled proliferation and unrestricted metastasis of cancer cells, leading to high mortality rates. Numerous studies have confirmed the anticancer activity of pectin and its close relationship with its complex structure [14]. To assess the inhibitory potential of APs on cancer cells, we investigated *in vitro* growth inhibitory effects against HCT116, HepG2, and SGC7901 cells. Our results demonstrated a dose-dependent inhibition of HCT116 and SGC7901 cells by APs (Figs. 4A and 4B). At a concentration of 1.00 mg/mL, AP-FS exhibited the lowest viability for cell growth on HCT116 cells at 68.55 ± 1.79%, followed by higher values for AP-HG (76.70 ± 1.06%) and AP-QG (80.07 ± 4.66%). Similarly, at this same concentration level (1.00 mg/mL), we observed that AP-FS had the lowest viability for cell growth on SGC7901 cells at 67.26 ± 3.63%, followed by higher values for AP-HG (70.68 ± 4 .31%) and AP-QG (73.98 ± 4 .31%). However, they were not significantly different. For HepG2 cells (Fig. 4C), the cell viabilities after treatment with APs from apple pomace exceeded 80%, indicating that the inhibitory effects of APs on HepG2 cells were not significant. In our investigation into monosaccharide composition and M_w, we found that compared with other types of pectins extracted from APs, AP-FS had a higher M_w as well as GalA content which may contribute to its inhibition of cancer

cells.

3.4. Immunostimulatory activities

3.4.1. The production of NO, IL-6, and TNF-α

Prior to investigating their potential immunostimulatory activity, the cytotoxicity of APs on macrophages was assessed. As shown in Fig. 4D, treatment with APs (0.125–1.0 mg/mL) resulted in survival rates exceeding 95% compared with the blank control group. Morphological alterations were observed in RAW 264.7 cells treated with varying concentrations of APs and LPS (Fig. S3), indicating macrophage activation characterized by irregular polygonal shape and increased pseudopod formation that correlated positively with APs concentration [47]. An increase in pseudopods significantly enhances the adhesion capacity and immunologic effects of macrophages [47]. Moreover, we investigated the effects of APs on the release of NO, IL-6, and TNF-α release in RAW 264.7 cells; our results demonstrated that all tested APs activated RAW 264.7 cells and stimulated the secretion/release of NO, TNF-α, and IL-6. Among all tested APs at a dosage level up to 1.0 mg/mL (Fig. 4E-G), AP-FS exhibited the most pronounced effect. Specifically, we observed production levels for NO to be 24.06 ± 1.10 μM, for TNF-α to be 16.19 ± 0.74 pg/mL, and for IL-6 to be 738.69 ± 19.70 pg/mL, respectively. These results are similar to previous findings demonstrating the immunostimulatory effects of natural polysaccharides derived from wild *Lactarius deliciosus* [48], *Poria cocos* [49], and *Rubus chingii Hu* [50].

3.4.2. Effects of AP-FS on the mRNA expression of TNF-α, IL-1β, IL-6, COX-2, iNOS, and NF-κB genes

To further evaluate the immunostimulatory effects of AP-FS, we investigated the

mRNA expression levels of transcription factor *NF-κB*, inflammatory mediators *COX-2* and *iNOS*, as well as pro-inflammatory cytokines *IL-6*, *IL-1β*, and *TNF-α* genes. It is worth noting that *iNOS* primarily contributes to NO synthesis in macrophages. Excessive expression of *iNOS* enhances NO production, and induces the expression of *COX-2* [51]. As shown in Fig. 5A, AP-FS upregulated the mRNA expression levels of *COX-2* and *iNOS* genes in a dose-dependent manner. Similarly, AP-FS promoted the expression levels of *TNF-α*, *NF-κB*, *IL-6*, and *IL-1β* genes. *TNF-α*, predominantly produced by activated macrophages, plays a crucial role in inducing the production and secretion of diverse immunomodulatory mediators and inflammatory cytokines [52]. Furthermore, *IL-1β* and *IL-6* are considered significant inflammatory mediators that trigger acute immune responses leading to fever [52]. These findings further supported the activation potential of RAW 264.7 cells.

3.4.3. Proteomic analysis

To explore the potential mechanisms underlying the immunostimulatory activity of apple pectin on macrophages, we conducted label-free proteomics analysis to compare protein expression levels between control and AP-FS treated groups. Three biological replicates were performed for each group. In total, 4936 proteins were quantified. Differentially expressed proteins were proteins with a fold change less than 0.5 or greater than 2.0 and a p-value < 0.05. Among the AP-FS-treated cells, we identified 83 down-regulated proteins and 88 up-regulated proteins using volcano plot analysis (Fig. 5B). Furthermore, hierarchical clustering analysis (Fig. S4) revealed distinct differences in protein expression between the control and AP-FS groups. The clustering patterns demonstrated that differentially expressed proteins from replicate groups with the same treatment clustered together, indicating a high level of reproducibility in our experimental treatments.

Furthermore, the proteome's molecular mechanisms were comprehensively investigated through GO analysis (Fig. 5C) and KEGG enrichment analysis (Fig. 5D and Table S1). The differential regulation of proteins was predominantly associated with biological processes such as stimulus-response, stress response, and immune system functions. Regarding cellular component classification (CC), these proteins accounted for 16 GO terms, where most differentially regulated proteins were mainly related to cells, cell parts, and organelles. The most enriched categories under molecular functions (MF) were binding and catalytic activity, indicating that differential proteins primarily contributed to metabolic processes. KEGG pathway analysis revealed upregulation in pathways related to the immune system such as cytosolic DNA-sensing pathway, NOD-like receptor (NLR), Toll-like receptor (TLR), RIG-I-like receptor (RLR), IL-17 signaling pathway. Additionally, significant enrichment was observed in the signaling pathways associated with signal transduction, including cytokine-cytokine receptor interaction, TNF signaling, and NF-κB signaling. These findings indicated a substantial upregulation of immune system-related pathways. NLR signaling represents a crucial family of cytoplasmic recognition receptors that can activate immune responses through various inflammasomes and related signaling pathways [53]. Proteomic analysis revealed the involvement of 11 altered proteins 11 altered proteins (e.g., NACHT, LRR, and PYD domains-containing protein 3; interferon-activable protein 204; nuclear factor NF-kappa-B p105 subunit; immunity-related GTPase family M protein1; and 2-5-oligoadenylate synthase 1A) in the activation of NOD-like receptor signaling

3.4.4. Analysis of potential signaling pathways

The NLR, MAPK and NF-κB signaling pathways were emphasized as topical

592 immunostimulatory pathways. Based on our findings, we proposed a potential signaling
593 pathway (Fig. 6B) for AP-FS-induced macrophage activation. As shown in Fig. 6B,
594 polysaccharides exerted their immunostimulatory activity by activating TLRs (e.g.,
595 TLR2 and TLR4), which subsequently triggered intracellular transcriptional pathways
596 [54]. CD14 may also assist the TLR4 receptor in driving the AP-FS-induced
597 immunostimulatory phenotype. Activation of downstream signaling pathways and
598 cytokine expression can be induced by the activation of TLR2/TLR4, which mediates
599 signal transduction through MyD88 to activate the IκB kinase-dependent pathway
600 primarily via MyD88 activation, leading to phosphorylation and subsequent
601 degradation of the IκB subunit in the NF-κB complex. This process results in the release
602 of p65 and p50 into the nucleus, where they regulate the production of inflammatory
603 mediators/cytokines [55]. In this study, AP-FS treatment upregulated NF-κB and P50
604 protein expression levels, thereby enhancing the release of inflammatory cytokines,
605 indicating activation of the NF-κB signaling pathway. Moreover, activation of the
606 MAPK signaling pathway increased RAW 264.7 cells viability and was implicated in
607 cytokine expression. The MAPK pathway encompasses extracellular regulated protease
608 kinase (ERK) as well as stress protein kinases (JNK and P38) [56], which have been
609 reported to participate in various physiological processes ranging from cell proliferation
610 to apoptosis across multiple cell types. Notably, P38 plays a pivotal role in immune
611 system regulation by activating *TNF-α* gene expression. Our findings revealed that AP-
612 FS treatment significantly augmented P38 expression levels, suggesting that AP-FS
613 induced immune responses in RAW 264.7 cells through simultaneous activation of both
614 the NF-κB and MAPK signaling pathways.
615
616 *3.5. Structure-activity analysis*

The above results indicated that AP-FS could promote the activation of RAW 264.7 cells through the NLR, MAPK, and NF-κB signaling pathways. It has been reported that the structural properties of polysaccharides (e.g., the linkages of glycosidic linkages, M_w, and monosaccharide composition) are closely associated with their biological properties. Previous studies have demonstrated that high-methoxyl homogalacturonan pectin containing (1→4)-*α*-D-GalA residues might be implicated in immunostimulatory effects. According to the report by Wang et al. [57], high-methoxyl homogalacturonan pectin derived from *Hippophae rhamnoides* L. Berries has been found to induce downstream signal transduction and pro-inflammatory cytokine production, thereby stimulating the activation of RAW 264.7 macrophages. Furthermore, analysis of monosaccharide composition revealed that AP-FS mainly consisted of Ara, Gal, Xyl, and Glc, which might potentially influence its immunostimulatory activity. In accordance with the findings reported by Du et al. [56], polysaccharides containing Glc and Gal are more readily recognized by TLR2, suggesting a correlation between immunostimulatory activity and the presence of these two monosaccharides; however, it should be noted that AP-FS only contained a minimal amount of these monosaccharides. Moreover, AP-FS with a highest M_w of 16.82 kDa exhibited significant immunostimulatory activity, which is consistent with previous studies suggesting that polysaccharides with an M_w over 100 kDa possessed better immunostimulatory properties [56]. However, these findings are not absolute as some studies have proposed that the arabinogalactan structural motifs of RG-I may be more crucial for immunostimulation than HG [58]. Additionally, various reports have indicated that certain natural pectin polysaccharides, such as purple passion fruit (*Passiflora edulia* Sims) peel [59], raspberry [60], and okra (*Abelmoschus esculentus*

L.) [61], exhibited remarkable anti-inflammatory effects. According to Ma et al. [62], ultrasonic pretreatment reduced the M_w of pectin and contributed to inhibitory activity against HT-29 colon cancer cells. These discrepancies in results may arise from complex structural differences among polysaccharides, particularly in terms of M_w, monosaccharide composition, glycosidic linkages, and tertiary structures. overall, our study provided evidence that apple pectin can activate macrophages; AP-FS may possess immunomodulatory properties capable of enhancing immune response. Our work contributes to understanding the structure-activity relationship of pectin polysaccharides.

4. Conclusion

Three pectins (AP-FS, AP-QG, and AP-HG) were isolated from three apple varieties ('Fushi', 'Qinguan', and 'Huaguan'). The chemical composition and structural characteristics of the APs were determined using HPGPC, HPIC, and FT-IR spectroscopy. Furthermore, we investigated and compared the inhibitory potential of the three pectins against cancer cells. Our results demonstrated that all APs significantly inhibited the growth of HCT116 and SGC7901 cells *in vitro* in a dose-dependent manner, while showing no significant inhibitory activity on HepG2 cells. Additionally, we examined the immunostimulatory activities of APs using mouse macrophage RAW 264.7 cells. Our findings revealed that all APs could activate RAW 264.7 cells by inducing NO, TNF-α, and IL-6 production as well as upregulating *TNF-α*, *IL-1β*, *IL-6*, *COX-2*, *iNOS*, and *NF-κB* genes via NLR-, MAPK-, and NF-κB-signaling pathways. Among them, AP-FS exhibited the most significant effect. We found that compared with other types of pectins extracted from APs, AP-FS had a higher M_w as well as GalA

content (→4)-*α*-D-Gal*p*A-(1→ residues) which may contribute to its inhibition of cancer cells and immunostimulatory activities. This study supports the potential of apple pectin, especially AP-FS, in inhibiting cancer cell growth and immune regulation. Currently, the structural analysis of polysaccharides faces numerous challenges, including their high molecular weight, lack of ultraviolet absorption, and poor reproducibility in structure identification. In this study, we analyzed the main chain structure and possible branched chain compositions of AP-FS using literary data, monosaccharide composition, methylation analysis, and NMR results. However, due to the high molecular weight and viscosity of pectin, obtaining precise branching patterns via NMR spectroscopy proved challenging. In future studies, we intend to obtain polysaccharide fragments suitable for study by NMR spectroscopy by enzymatic digestion in order to elaborate upon AP-FS exact structural features.

CRediT authorship contribution statement

Huan Guo: Methodology, Investigation, Writing–original draft, Writing–review & editing. **Hang Li**: Investigation, Methodology. **Wenyi Ran**: Data curation. **Wenyue Yu**: Software. **Yue Xiao**: Writing–review & editing. **Renyou Gan**: Writing–review & editing. **Hong Gao**: Conceptualization, Supervision, and Writing–review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Figure Captions

Fig. 1 Extraction and purification procedure of APs. (AP-FS, AP-QG, and AP-HG, purified pectin derived from 'Fushi', 'Qinguan', and 'Huaguan' apple pomace, respectively)

Fig. 2. (A) Regression curves for molecular weight standards, (B) HPGPC spectrum of APs, (C) UV spectrum of APs, (D) FT-IR spectrum of APs, (E) HPIC spectrum of APs, (F) Apparent viscosities of APs, (G) Frequency sweeps of APs (MIX, the mixed standard of monosaccharides; 1-15: Fucose, Fuc; Glucosamine hydrochloride, GalN; Rhamnose, Rha; Arabinose, Ara; D-glucosamine hydrochloride, GlcN; Galactose, Gal; Glucose, Glc; *N*-Acetyl-D-Glucosamine, *N*-Acetyl-D-Glc; Xylose, Xly; Mannose, Man; Fructose, Fru; Galacturonic acid, GalA; Guluronic acid, GulA; Glucuronic acid, GlcA; and Mannuronic acid, ManA).

Fig. 3. Nuclear magnetic resonance (NMR) spectra of AP-FS. A: ^{1}H-NMR spectrum; B: ^{13}C-NMR spectrum; C: DEPT-135 spectrum; D: ^{1}H-^{1}H COSY spectrum; E: HSQC spectrum; F: HMBC spectrum; G: NOESY spectrum.

Fig. 4. (A-C) Growth inhibitory effects of APs on different cancer cells. A: Human stomach cancer cells (SGC7901); B: Human colon cancer cells (HCT116); C: Human liver cancer cells (HepG2); (D) Cytotoxicity of AP-FS on RAW 264.7 macrophages; (E-G) Effects of APs on NO, IL-6, and TNF-α secretion in RAW 264.7 macrophages;

928 **Fig. 5.** (A) Effects of APs on the mRNA expression of *TNF-α*, *IL-1β*, *IL-6*, *COX-2*,
929 *iNOS*, and *NF-κB* (Nitric oxide, NO; Interleukin, *IL*; Tumor necrosis factor, *TNF*;
930 Inducible nitric oxide synthase, *iNOS*; Nuclear factor kappa B, *NF-κB*; and
931 Cyclooxygenase, *COX*); (B) Volcano map, (C) GO enrichment analysis of differentially
932 expressed proteins identified in AP-FS stimulated RAW 264.7 macrophages, (D)
933 KEGG enrichment analysis of differentially expressed proteins (up-regulated)
934 identified in AP-FS stimulated RAW 264.7 macrophages.

935

936 **Fig. 6.** (A) Schematic diagram of proteomics analysis; (B) Possible molecular
937 mechanism of AP-FS activating RAW 264.7 cells **(**Nitric oxide, NO; Interleukin, *IL*;
938 Tumor necrosis factor, *TNF*; Inducible nitric oxide synthase, *iNOS*; Nuclear factor
939 kappa B, *NF-κB*; Cyclooxygenase, *COX*; and Activator protein-1, AP-1).
940

Table 1. The sequences of primers used in this study.

Gene	Upstream primer sequence5′−3′	Downstream primer sequence5′−3′
β-actin	CAGCTGAGAGGGAAATCGTG	CTCCAGGGAGGAAGAGGATG
IL-1β	TTCATCTTTGAAGAAGAGCCCAT	TCGGAGCCTGTAGTGCAGTT
IL-6	CATCCAGTTGCCTTCTTG	TCTGTTGGGAGTGGTATC
iNOS	CTGCAGCACTTGGATCAGGAAGCTG	GGGAGTAGCCTGTGTGCACCTCGAA
NF-κB	GAGGCGTGTATTAGGGGCTA	ACGCTCAGGTCCATCTCCTT
COX-2	CCATGTCAAAACCGTGGTGAATG	ATGGGAGTTGGGCAGTCATCAG
TNF-α	TTCTGTCTACTGAACTTCGGGGTGATCGGTCC	GTATGAGATAGCAAATCGGCTGACGGTGTGGG

Table 2 Chemical analysis, M_w, and monosaccharide composition of APs

	AP-FS	AP-QG	AP-HG
Yields (%[1])	~0.60	~1.07	~0.87
Neutral sugar (%[1])	12.83 ± 0.57 [b]	16.95 ± 0.48 [a]	13.11 ± 0.53 [b]
Uronic acid (%[1])	86.39 ± 1.04 [a]	81.24 ± 1.16 [c]	84.76 ± 0.89 [b]
Protein contents (%[1])	N.D.	N.D.	N.D.
Phenolic acids content (%[1])	N.D.	N.D.	N.D.
DE using FT-IR method (%[2])	54.87 ± 1.26 [a]	52.15 ± 1.01 [b]	53.65 ± 1.50 [ab]
DE using titrimetric method (%[2])	60.14 ± 2.46 [a]	56.82 ± 2.08 [a]	58.88 ± 2.22 [a]
M_w (×10[4] Da)	16.82	13.15	13.33
M_n (×10[4] Da)	10.47	8.32	8.42
M_w/M_n	1.61	1.58	1.58
Monosaccharides and molar ratios			
Arabinose	1	1	1
Galactose	0.72	0.75	0.62
Glucose	0.14	0.11	0.42
Galacturonic acid	3.88	2.95	4.15
Xylose	0.10	0.08	0.10

Values represent mean ± standard deviation and statistical analysis was carried out by ANOVA plus *post hoc* Ducan's test, and statistical significance ($p < 0.05$) was indicated with different letters (a-c). Abbreviations: %[1] Weight percentage; %[2] Molar percentage. DE, degree of esterification; N.D. Not determined.

Table 3 Methylation analysis of AP-FS.

Peak	Retention time (min)	Methylated sugar	Mass fragments (m/z)	Molar ratio	Type of linkage
1	10.341	2,3,5-Me_3-Ara*f*	45,71,87,101,117,129,145,161	0.019	Ara*f*-(1→
2	16.836	2,3,4,6-Me_4-Glc*p*	45,71,87,101,117,129,145,161,205	0.018	Glc*p*-(1→
3	17.695	2,3,4,6-Me_4-Gal*p*	45,71,87,101,117,129,145,161,205	0.102	Gal*p*-(1→
4	18.754	2-Me_1-Ara*f*	45,58,85,99,117,127,159,201	0.031	→3,5)-Ara*f*-(1→
5	21.089	2,3,6-Me_3-Gal*p*	45,87,99,101,113,117,129,131,161,173,233	0.568	→4)-Gal*p*-(1→
6	21.31	2,3,6-Me_3-Glc*p*	45,87,99,101,113,117,129,131,161,173,233	0.113	→4)-Glc*p*-(1→
7	23.548	2,3,4-Me_3-Gal*p*	45,87,99,101,117,129,161,189,233	0.024	→6)-Gal*p*-(1→
8	24.14	2,6-Me_2-Glc*p*	45,87,97,117,159,185	0.054	→3,4)-Glc*p*-(1→
9	26.846	2,3-Me_2-Gal*p*	45,71,85,87,99,101,117,127,159,161,201,261	0.048	→4,6)-Gal*p*-(1→
10	28.268	2,4-Me_2-Gal*p*	45,87,117,129,159,189,233	0.022	→3,6)-Gal*p*-(1→

Table 4 ^{1}H NMR and ^{13}C NMR spectral assignments for AP-FS

Glycosyl residues			Chemical shift δ（ppm）						
			1	**2**	**3**	**4**	**5a/5b**	**6a/6b**	**-OMe**
$_{Me}$**GA$_4$**	→4)-*α*-D-Gal*p*A-6-OMe-(1→	**H**	4.88	3.63	3.92	4.38	5.00/5.08		3.73
		C	100.25	67.72	67.84	78.94	70.51	170.61	52.82
GA$_4$	→4)-*α*-D-Gal*p*A-(1→	**H**	5.02	3.64	3.92	4.32	4.64		
		C	99.41	67.72	67.84	78.65	71.20	174.85	
R$_α$	→4)-*α*-D-Gal*p*A	**H**	5.22	3.74	3.94	4.51	--		
		C	92.13	67.83	67.72	78.64	--	--	
R$_β$	→4)-*β*-D-Gal*p*A	**H**	4.52	3.39	3.68	4.21	--		
		C	95.92	71.09	71.78	78.94	--	--	
GA$_t$	*α*-D-Gal*p*A-(1→	**H**	5.05	3.65	4.04	4.34	3.68		
		C	99.41	67.72	67.98	70.25	70.78	174.85	
G$_t$	*β*-D-Gal*p*-(1→	**H**	4.55	3.60	3.67	3.82	3.64	3.66/3.74	
		C	103.92	71.51	72.05	68.14	74.31	60.25	
G$_{1,3,6}$	→3,6)-*β*-D-Gal*p*-(1→	**H**	4.46	3.59	3.67	4.05	3.85	3.85/3.96	
		C	102.93	69.67	79.91	68.14	73.47	68.47	
G$_{1,6}$	→6)-*β*-D-Gal*p*-(1→	**H**	4.42	3.61	3.69	3.83	3.97	3.62/3.74	
		C	103.08	71.36	69.67	68.84	73.89	65.46	
A$_t$	*α*-L-Ara*f*-(1→	**H**	5.16	4.14	3.86	4.04	3.62		
		C	109.25	81.47	76.26	83.58	60.98		
A$_{1,3,5}$	→3,5)-*α*-L-Ara*f*-(1→	**H**	5.08	4.06	3.97	4.22	3.75/3.87		
		C	106.99	80.90	83.69	81.17	65.46		
A$_{1,5}$	→5)-*α*-L-Ara*f*-(1→	**H**	5.01	4.06	3.93	4.01	3.71/3.82		
		C	107.29	80.90	76.42	83.67	66.31		