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Chemical Characterization and Antioxidant activities of Polysaccharides Isolated from the stems of *Parthenocissus tricuspidata*

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ABSTRACT

Four polysaccharides, PTP–1, PTP–2, PTP–3 and PTP–4, were obtained from the water extraction of the stems of *P. tricuspidata* by anion exchange chromatography and gel filtration. The antioxidant activities of four PTPs were investigated, exhibiting different antioxidant activities, in which PTP−4 performed noticeable, with strong superoxide radical activity (comparable to BHT), high DPPH radical activity (78.53% at 1250 μg/mL), moderate hydroxyl radical scavenging activity and reducing power activity. Furthermore, the chemical structure of PTP−4 was measured by FT-IR, GC, ^{1}H and ^{13}C NMR spectra, indicating its mainly composition of the arabinose, xylose, galactose, glucuronic acid, and mannose. Thus, the stems of *P. tricuspidata* could be used as a potential source for natural antioxidant.

Keywords

P. tricuspidata; Polysaccharides; Pro-antioxidant activity

1. Introduction

It is well known that oxidation is an essential biological process to many organisms for the energy production. However, the uncontrolled production of oxygen-derived free radicals is hostile and damaging to cells. When the innate defense in the human body could not been enough for severe oxidative stress, a variety of diseases would happen, including cancer, heart disease, atherosclerosis, inflammation, carcinogenesis, Parkinson's and Alzheimer's diseases, and aging [1−3]. For reducing oxidation damage, many synthetic antioxidants are widely used at present [4], such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [5]. However, synthetic antioxidants are probably toxic and may cause severe food safety problems after high dosage and long-term treatment [6, 7], which lead to their restricted use. Thus, it is essential to find low toxicity or nontoxic natural antioxidants as substitutes for synthetic antioxidants. Polysaccharides are an important source of natural antioxidants [2−4, 8−11]. The search for bioactive polysaccharides with antioxidant activities from plant has become a research hotspot.

The garden plant, *Parthenocissus tricuspidata* (Vitaceae), is a folk medicine in South Asia, for treating arthritis, jaundice, insect bites, and neuralgia [12]. Previous phytochemical studies on this plant had regarded it as a rich source of phenolic compounds [12–14]. However, few reports have been focused on its polysaccharides constituents yet. In 2013, a water-soluble polysaccharide (PT2) was isolated from the adhesive discs of *P. tricuspidata*, showing potential bio-adhesive property [15], which referred that the *P. tricuspidata* would be a potential resource of polysaccharides. In our continuing program to search for bioactive polysaccharides from plants, the crude polysaccharides were extracted from the stems of *P.*

tricuspidata (PTPs), exhibiting strong antioxidant activity against DPPH radicals and moderate antioxidant diversity [16]. However, the chemical characterization of the PTPs were not clear. It is well known that the antioxidant activities of polysaccharides are usually influenced by several factors, such as sugar unit, glycosidic bonds, degree of branching, molecular weight, the conformation, and so on [17, 18]. Thus, in this further study, the isolation of the crude PTPs were conducted, leading to four acidic polysaccharides, PTP–1, PTP–2, PTP−3, and PTP−4, and their in vitro antioxidant activities were evaluated by reducing power and scavenging effects against superoxide, hydroxyl, DPPH radicals. Furthermore, the spectral, and structural properties of the most active polysaccharide, PTP−4, were also observed.

2. Material and method

2.1. Chemicals

DEAE-Sephadex A−25 and Superdex−200 were purchased from Amersham Biosciences Co. 2,2−diphenyl−1−picrylhydrazyl (DPPH) was from Sigma Chemicals Co. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3500Da. Standard monosaccharides (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, glucuronic acid, and galacturonic acid) were purchased from Sigma–Aldrich. All other reagents used were analytical grade. *P. tricuspidata* was collected in Yan-an, Sichuan, China. The stems were washed, air dried and kept in plastic bags at room temperature before using.

2.2. Extraction of the crude polysaccharides from P. tricuspidata

Dried stems of *P. tricuspidata* (60 g) were extracted in 80% EtOH (100 mL, twice) for 2 h, after degreasing by petroleum ether. After filtration, the residue stems were extracted with

boiling deionized water (400 mL, twice) for 1h. The water extracts were combined, concentrated under reduced pressure at 65℃. The proteins in the concentration were removed by the Sevage reagent three times. After the removal of the Sevage reagent, the extract was precipitated with three-fold of the volume of 95% ethanol (v/v) and kept at 4 ℃ overnight. The precipitation was collected by centrifugation, then washed three times with anhydrous ethanol. It was then dissolved in distilled water and subjected to dialysis (cut-off 3500 Da). The dialyzed extract was concentrated using rotated evaporator, and lyophilized to obtain the crude polysaccharide for further studies (see scheme 1).

2.3. Further purification

The crude polysaccharide (100 mg) was dissolved in deionized water and fractionated by a DEAE-Sephadex A−25 column (2.5 cm × 60 cm) eluting with a step gradient of 0.1−2 M NaCl at a flow rate of 2 ml/min. Total sugar content of the elution was determined by the phenol–sulfuric acid method. The elution profile detected by the phenol–sulfuric acid assay showed four big elution peaks namely S1–S4, respectively. To test the homogeneity and molecular weight of S1 to S4, the fractions were determined by size exclusion chromatography on a Hiload™ 16/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta) eluted with 0.9% NaCl at a flow rate of 1 mL/min. Dextran polymers (Pharmacia) of 10, 40, 70, 500 and 2000 kDa were used as calibration standards (see scheme 1) [19].

2.4. Chemical analysis

Total carbohydrate content was estimated by the phenol–sulfuric acid method using glucose as standard [20]. Protein content was measured by the method of Bradford [21]. The total

polyphenol was determined colorimetrically using the Follin-Ciocalteau method [22].

2.5. Monosaccharide composition analysis

The hydrolysis of polysaccharide was carried out as previously described [23, 24]. Purified polysaccharide (5 mg) was hydrolyzed using an hydrous methanol containing HCl at 80 ℃ for 24 h. Then the hydrolyzed products were neutralized with methanol-KOH and dried. The above dried product was dissolved with 0.2 mL pyridine adding 3 mg mannitol (as internal references) at 75 ℃ for 30 min. After cooling to room temperature, hexamethyldisilazane (0.2 mL) and trimethylchlorosilane (0.1 mL) were added to the mixture. Then the trimethylsilylated derivatives were analyzed by GC (HP 2010) using a RTX-WAX column and a flame ionization detector. The operation was performed using the following conditions-N_2 : 0.8 mL/min; H_2 : 1.5 mL/min; air: 200 mL/min; injection temperature: 200 ℃; detector temperature: 200 ℃. Sugar identification was done by comparison with reference sugars (rhamnose, arabinose, xylose, mannose, galactose, glucose, ribose, fucose, glucuronic acid, and galacturonic acid).

2.6. IR spectroscopy analysis

For IR spectroscopy, the polysaccharide was mixed with KBr powder, ground and then pressed into a 1 mm pellets for Fourier transform infrared (FI-IR) measurement in the frequency range of 4000–500 cm^{-1}. FI-IR spectra of the polysaccharides were measured on a Perkin Elmer Spectrum Two spectrometer.

2.7. NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra of the main polysaccharides fractions, PTP–4, were recorded on a Bruker spectrometer (600 MHz) after deuterium exchanged three times by freeze-drying in D_2O. Taking tetramethylsilane (TMS) as internal reference, the ^{1}H NMR and

^{13}C NMR spectra of PTP–4 were recorded in D_2O solution on a Bruker AV600 instrument (Bruker, Rheinstetten, Germany) at 25 ℃.

2.8. Antioxidant activity test

2.8.1. Reducing power

The reducing power of purified polysaccharide was carried out by the previous method [25]. In brief, 1.0 mL of polysaccharide solution at various concentrations in 0.2 M phosphate buffer (pH 6.6) was mixed with 1.0 mL of 1% aqueous potassium ferricyanide. Subsequently, the mixtures were incubated at 50 ℃ for 20 min. 1.0 mL of 10% TCA was added to the mixture. After centrifuged (5000 rpm/min for 15 min), the mixture (2.5 mL) was mixed with 0.15 mL of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture demonstrated the increase of reducing ability. Butyl hydroxyl toluene (BHT) was served as positive control.

2.8.2. Superoxide anion radical scavenging activity

The superoxide anion scavenging activity was measured as described in the previous study [25]. With some modification in this test, 0.5 mL sample solution at various concentrations were mixed with Tris-HCl solution (2 mL, 50 mM), distilled water (2 mL) and pyrogallol solution (0.5 mL, 25 mmol) in sequence. The mixtures were incubated at 25 ℃ for 5 min. After the reaction was terminated with hydrochloric acid, the absorbance was measured at 560 nm. Butyl hydroxyl toluene (BHT) was used as positive control. The superoxide anion radical scavenging activity was calculated using the equation as follows: superoxide anion radical scavenging activity (%) = $(A_0 - A_1 + A_2)/A_0 \times 100$, where A_0 was the absorbance of control group (water instead of purified polysaccharide), A_1 was the absorbance of sample in reactive system. A_2 was the final absorbance of sample.

2.8.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of sample was determined according to a previously described method [26]. Accurately 1 mL of sample solution (0.05, 0.1, 0.3, 0.5, 1 and 1.5 mg/mL) was mixed with 20 mM phosphate buffer (pH 7.4, containing 0.1 mM ferric chloride, 0.1 mM EDTA and 2.8 mM deoxyribose), 0.1 mL of 1 mM Vc and 0.5 mL of 20 mM H_2O_2 at 37 ℃ for 90 min. The absorbance of the mixture was measured at 532 nm against a blank. A control containing all the reaction reagents except the sample was prepared and measured. BHT was used for comparison, and the hydroxyl radical scavenging ratio was calculated using the following Eq (1).

$$\text{Scavenging effect (\%)} = (1 - A_s/A_c) \times 100 \quad (1)$$

where A_s is the test sample, and A_c is the absorbance of the control.

2.8.4 DPPH radical scavenging activity

The antioxidant activity of polysaccharides was measured based on the scavenging activity of the DPPH free radical [27] with minor modifications, taking BHT as comparison. Accurately 1 mL of sample solution (0.05, 0.1, 0.3, 0.5, 1 and 1.5 mg/mL) was added to 3 mL of 0.005% MeOH solution DPPH. After 30 min, the absorbance at 517 nm was measured using a spectrometer at 25 ℃, and the percentage scavenging activity was calculated as described in Section 2.8.3.

2.9 Statistical analysis

All the experiments were performed in triplicate and the date were represented as mean ± standard deviation (SD). All statistical analyses were performed by using commercially available statistical software.

3. Results and Discussion

3.1. Isolation and purification of the polysaccharide

The crude polysaccharide was extracted from *P. tricuspidata* and then purified by DEAE-Sephadex A−25 column, and four fractions were obtained: S1 to S4 from 0.1, 0.3, 0.4 and 0.5 M NaCl elution, respectively (Fig. 1a). Almost no polysaccharide was detected in 1.0–2.0M NaCl elution. The four polysaccharide fractions were, respectively, pooled, dialyzed and lyophilized. The yields of the four fractions were 9.00%, 16.00%, 15.40%, 27.40%, S1 to S4, respectively, appeared as only a single and symmetrically sharp peak on Superdex−200 (1.6 cm × 60 cm) with the AKTA purifier system, indicating the four polysaccharide fractions (S1 to S4) were homogenous polysaccharides (Fig. 1b to 1e).

3.2. Chemical analysis

The total carbohydrate content, protein, polyphenol content, average molecule weight of PTP−1, PTP–2, PTP–3, and PTP–4 were shown in Table 1. The polysaccharide contents of PTP−1, PTP–2, PTP–3, and PTP–4 was 83.6%, 87.9%, 81.8% and 89.1%, respectively. No protein was detected in purified polysaccharides. These indicated that the protein existing in *P. tricuspidata* polysaccharide was free. According to the calibration curve, log Mw =8.51–0.069V (V was the elution volume), the average molecule weight of PTP−1, PTP–2, PTP–3, and PTP–4 were calculated to be 7.94×10^5, 1.02×10^6, 1.34×10^6 and 1.70×10^6 Da, respectively.

3.3. Structural characterization of PTP−4

The structural characterization of PTP−4, which showed the best antioxidant activities was analyzed by GC, IR, ^{1}H and ^{13}C NMR spectrum.

Fig. 2b showed the GC of trimethylsilylated derivatives of the hydrolysis products of PTP−4. Based on the retention time of the derivatization of monosaccharide standards, the monosaccharide types of PTP−4 were identified, which was composed of arabinose, xylose, galactose, glucuronic acid, mannose, galacturonic acid, rhamnose, glucose, with a molar ratio of 9.7:6.6:5.2:4.6:4.5:1.5:1.2:1 (see in table 2). It indicated that the arabinose, xylose, galactose, glucuronic acid, and mannose were the main monosaccharide compositions of PTP−4.

The infrared spectrum of PTP−4 ranged from 400 cm^{-1} to 4000 cm^{-1} was shown in Fig. 3. A broad, strongly represented intense peak at 3412 cm^{-1} was due to the stretching vibration of O–H bonds [28, 29]. The signal at around 2926 cm^{-1} could be associated with the stretching vibration of the C–H bond in the sugar ring [30]. The relative strong absorption peak at 1611 cm^{-1}represented the characteristic stretching vibration of the C–O bond [31]. The polysaccharide did not have the absorption peaks at 1541 cm^{-1}, indicating that they did not contain protein [31], which was consistent with the Bradford method result. The band around 1412 cm^{-1} was assigned to the bending vibration of the C–H bond. The polysaccharide peaks between 1000–1200 cm^{-1} were assigned to their C–O–C and C–O–H. The strong absorption band around 1050 cm^{-1} was assigned to the skeletal modes of pyranose rings in the monosaccharide of PTP–4. The results showed that PTP–4 had strong absorption peaks of 1074 cm^{-1}, indicating that PTP–4 were rich in Ara, Gal, Gal A and Man. Those results were consistent with the monosaccharide composition analyses.

NMR spectroscopy has simplified the procedure for the structural analysis of carbohydrates and become the most powerful technique for the structural analysis of complex polysaccharides, providing detailed structural information of carbohydrates, such as the monosaccharide composition, α- or β-anomeric configurations, the linkage patterns and sequences of the sugar units in polysaccharides [32]. Most of the α-anomeric protons usually appear in the 5–6 ppm region while most of the β-anomeric protons in the δ_H 4–5 ppm range. ^{1}H and ^{13}C NMR spectrum of the PTP–4 in D_2O was shown in Fig. 4. In ^{1}H NMR spectrum of PTP−4, the signals appeared at 5.1–5.5 ppm indicated that PTP−4 were composed of an amount of α-glycoside bonds, and the peaks around 4.5 ppm showed it also contained β-glycoside bonds. The signals at δ_H 1.9 ppm was assigned to acetyl groups. The signals around δ_H 1.3 were assigned to the methyl groups in rhamnosyl residues. In the ^{13}C spectrum, the signals from the principal sugars, arabinose, xylose, galactose, glucuronic acid, and mannose units were dominating. The signals in the region for the resonances of carbonyl groups (carboxyl and ester carbonyls) at δ_C 173.3 −188.2 ppm corresponded to the C–6 of unesterified or esterified galacturonic acid and glucuronic acid units. The anomeric peaks were at δ_C 108.7, 108.5, 107.0, 106.8, 103.0, 101.9, 100.8, 100.0, 98.2, and 97.4 ppm, indicating several kinds of anomeric configuration for monosaccharide residue. Usually, in a ^{13}C spectrum, the signals derived from α-anomeric carbons usually appear in the δ_C 95−101 ppm region while most of the β-anomeric carbons appear in the range δ_C 101−105 ppm [32]. Also, predominant signals between δ_C 58.0 to δ_C 82.0 ppm were assigned to the oxygen substituted carbon of monosaccharide residues, in which the signals at δ_C 80.0−83.0, and 68.8−70.0 confirmed the existences of (1 → 3/4) glycosidic linkages and (1 → 6)

glycosidic linkages, respectively [33]. The signal at δ_C 19.0 to 22.5 ppm represented acetyl groups of the monosaccharide units and the signals around 15.8 ppm were confirmed to the CH_3 of rhamnosyl residues. All the NMR chemical shifts were compared with previously reported values.

3.4. Antioxidant activities analysis

Reducing power could be used as an indicator to measure the potential antioxidant capacity because of their close relationship [25]. As shown in Fig. 5a, PTP–1 to PTP–3 showed very low reducing capacities, whereas PTP–4 presented stronger reducing power, reaching to 0.693 at 267 μg/mL, lower than BHT.

Superoxide anions radical is considered as the initial free radicals formed from the mitochondrial electron transport system. Fig. 5b illustrated the different superoxide radical scavenging abilities of the four polysaccharides in dose-dependent manners at all tested concentrations, in which, PTP–4 showed the highest level radical scavenging effect, with IC_{50} value at 450 μg/mL, comparable to BHT. When the concentration at 455 μg/mL, the percentage inhibition reached 51.14% (BHT 55.85%).

Removing hydroxyl radicals are extremely important to protect living systems, because it can easily cross cell membranes, and cause tissue damage or cell death [26]. Four *P. tricuspidata* polysaccharides presented different level of the hydroxyl radical scavenging effects (Fig. 5c), in which PTP–4 expressed outstanding ability, increasing from 3.65% to 38.77% as the concentration increased from 0.0625 mg/mL to 1.0 mg/mL. These results indicated that PTP–4 had a moderate scavenging ability for hydroxyl radicals, weaker than BHT.

DPPH is a free-radical compound that has been widely used to determine the free radical scavenging abilities of various natural compounds for the convenient and reproducibility [34]. Results in Fig. 5d demonstrated the DPPH scavenging activities of the four *P. tricuspidata* polysaccharides, in which only PTP−4 exhibited outstanding scavenging ability, in a dose-dependent manner. The scavenging ratio at the concentration of 1250 μg/mL was 78.53%, with IC_{50} value at 630 μg/mL, which was still lower than BHT.

According to the antioxidant activity analysis, the antioxidant activities of *P. tricuspidata* polysaccharides in various reactive systems were different, among which PTP−4 performed the best. In a comprehensive consideration of the above results, it suggested that the PTP−4 had a certain antioxidant activity. Different activity in different tests may be due to its specific structures.

3.5. The analysis of structure -antioxidant activities relationships for PTP–4

It is widely believed that the bioactivity of polysaccharides is affected by their structure characteristics, such as chemical composition, molecular mass, types of glycosidic linkage, and conformation. Differences in origin materials, extraction procedures, and even drying technologies that influence the physiochemical properties, structure, or conformation of polysaccharides will lead to differences in antioxidant activity [36–38].

Among all the influence factors mentioned above, a number of reports suggested that the antioxidant potency is mainly associated with molecular weight of polysaccharides. It was supposed that polysaccharides with lower molecular weights would have more reductive hydroxyl group terminals (on per unit mass basis) to accept and eliminate the free radicals [39–45]. However, inconsistent findings were also described. For example, A. Kardošová and

E. Machová [46] revealed the effect of molecular weight was not significant based on similar antioxidant levels of polysaccharides and oligosaccharides. Cheng et al. [47] obtained polysaccharides from *Epimedium acuminatum* with higher molecular weight displayed better antioxidant actions, which was consistent with the results of PTP-4.

Secondly, uronic acid is considered to be another important indicator reflecting the antioxidant activity of the polysaccharides. Many acidic polysaccharides which contained a certain amount of uronic acid, were reported as potent antioxidants [48–51]. It is supposed that the presence of electrophilic groups like keto or aldehyde in acidic polysaccharide facilitates the liberation of hydrogen from O-H bond [52]. PTP-4 was obtained from the 0.5 M NaCl elution, indicating highest content of uronic acid. According to the results of monosaccharide analysis, there was 17.6% uronic acid in PTP−4 (glucuronic acid 13.4%, galacturonic acid 4.2%).

Third, it was revealed that compositions and ratios of monosaccharide would be of concern in modulating the antioxidant properties. Meng et al. [53] referred that the antioxidant activity was significantly correlated with the content of mannose (?? < 0.01) and glucose (?? < 0.05), whereas galactose was not correlated (?? > 0.05). PTP-4 was just at a high content of mannose (13.2%).

4. Conclusions

In summary, four polysaccharides (PTPs) were prepared via a series of steps from the stem of *P. tricuspidata*. The antioxidant activities of four PTPs were investigated, exhibiting different antioxidant activities, in which PTP−4 performed noticeable, with strong superoxide

radical activity (comparable to BHT), high DPPH radical activity (78.53% at 1250 μg/mL), moderate hydroxyl radical scavenging activity and reducing power activity. Furthermore, the chemical structure of PTP−4 was measured by FT-IR, GC, ^{1}H and ^{13}C NMR. It was mainly composed of the arabinose, xylose, galactose, glucuronic acid, and mannose. Therefore, it was indicated that PTP−4 could be used as a potential natural antioxidant. Its antioxidant activities may be related to the high molecular weight, as well as high contents of uronic acid and mannose. Accordingly, the stems of *P. tricuspidata* could be used as a potential source for natural antioxidant.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1 Major chemical composition of MDPs

Fragments	PTP−1	PTP−2	PTP−3	PTP−4
Total carbohydrate (%)	83.6±0.9	87.9±1.8	81.8±1.3	81.8±1.2
Protein (%)	–	–	–	–
Polyphenol (%)	0.8±0.1	0.6±0.3	0.9±0.2	0.7±0.2

Table 2 The monosaccharides composition of PTP-4

	Ara	Rha	Xyl	GlcA	Man	Gal	GalA	Glc
monosaccharides composition (mol%)	28.6	3.5	19.1	13.4	13.2	15.0	4.2	2.9

Captions to illustrations

Scheme 1 Procedure of extraction and purification of polysaccharides from *Parthenocissus tricuspidata*

Fig.1. (a) Purification of the polysaccharides obtained from *P. tricuspidata*. The crude polysaccharide was applied to a column of DEAE-Sephadex A−25 and eluted as described in Section 2. The fractions containing the polysaccharides were pooled and named as S1 to S4, respectively. (b) S1 obtained on DEAE-Sephadex A−25 was applied to a Superdex−200 and eluted as described in Section 2. (c) S2 obtained on DEAE-Sephadex A−25 was applied to a Superdex−200 and eluted as described in Section 2. (d) S3 obtained on DEAE-Sephadex A−25 was applied to a Superdex−200 and eluted as described in Section 2. (e) S4 obtained on DEAE-Sephadex A−25 was applied to a Superdex−200 and eluted as described in Section 2.

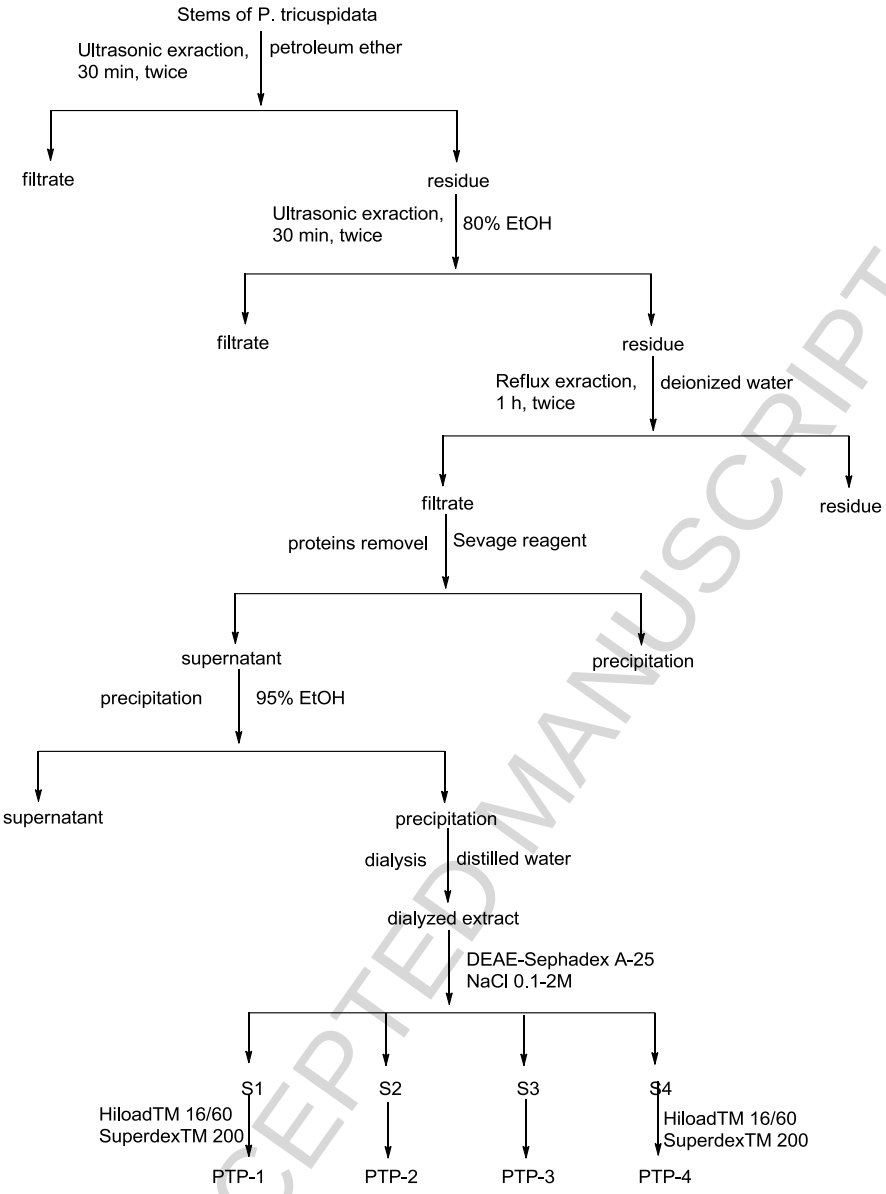
Fig. 2. GC profile of trimethylsilylated derivatives of the standards monosaccharides (a) and hydrolysis products of PTP−4 (b).

Fig. 3. IR spectrum of PTP−4

Fig. 4. The NMR spectra of PTP−4

Fig. 5. The antioxidant activities of PTPs

Illustrations



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Scheme 1.

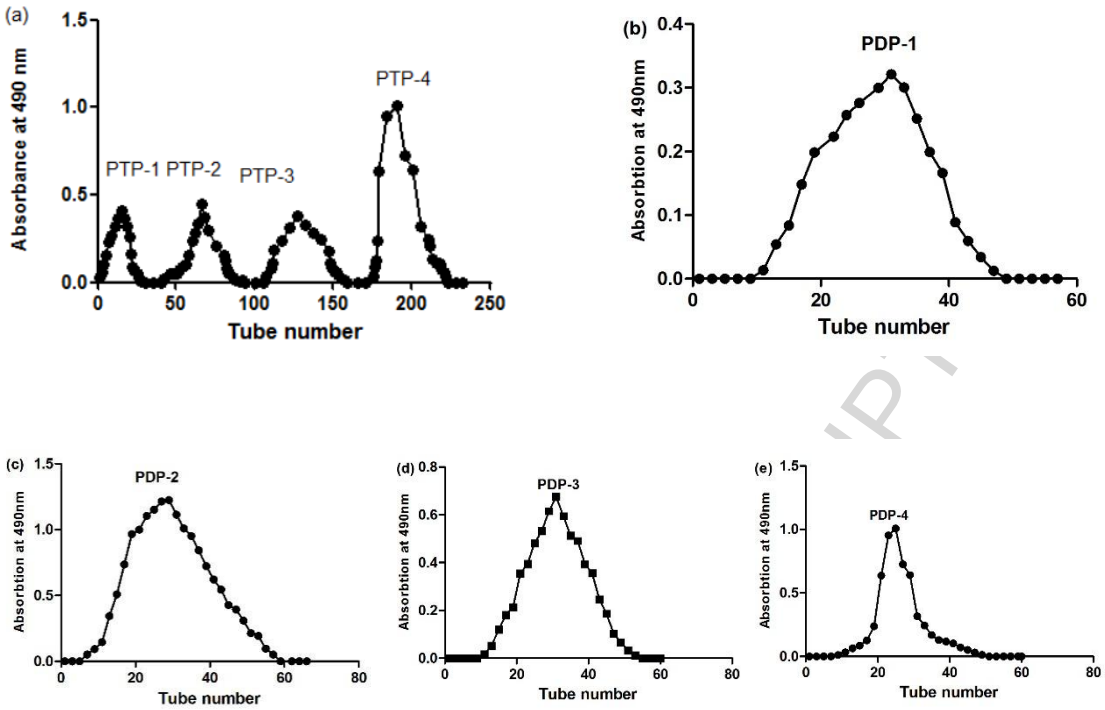

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Fig.1.

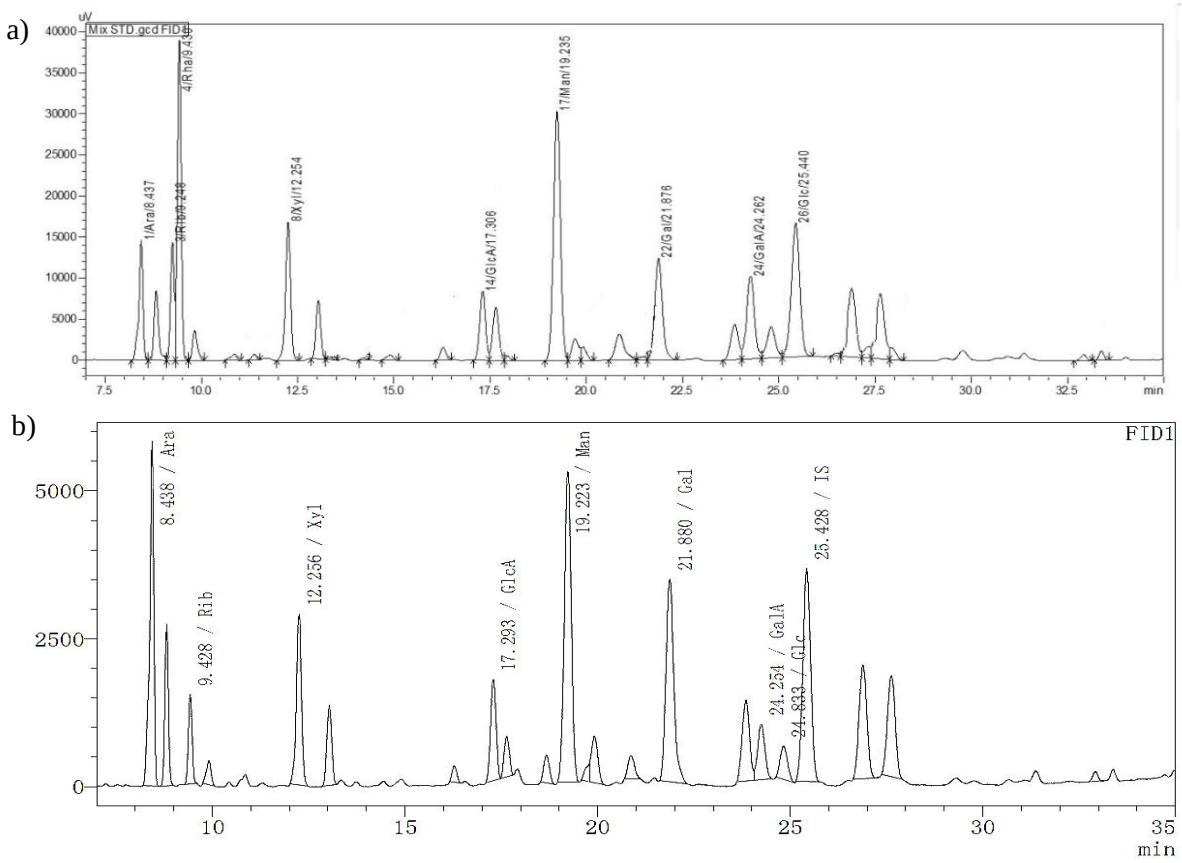

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Fig.2.

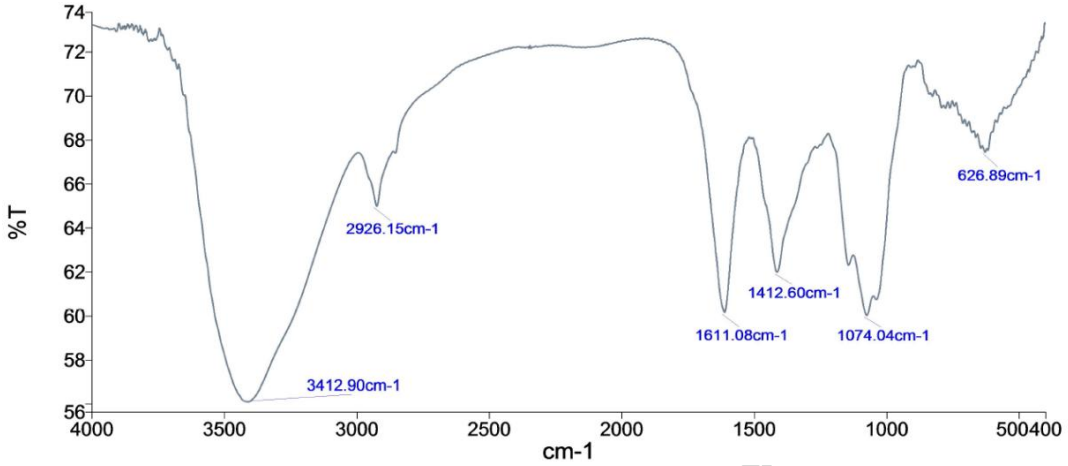
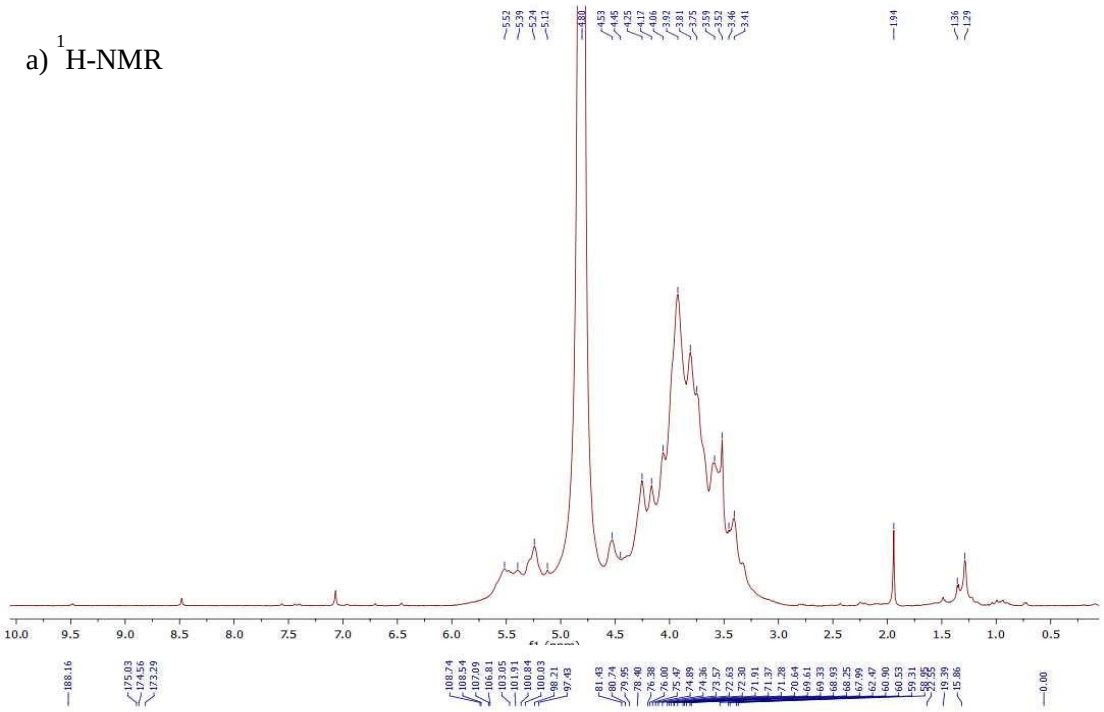


Fig.3.

a) ^{1}H-NMR



b) ^{13}C-NMR

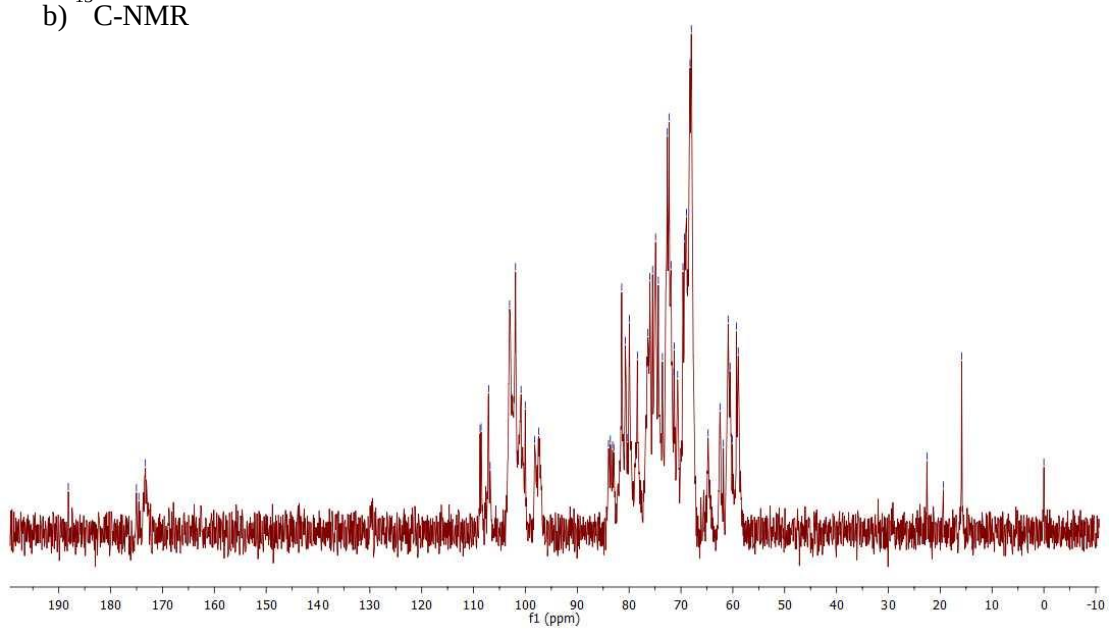


Fig.4.

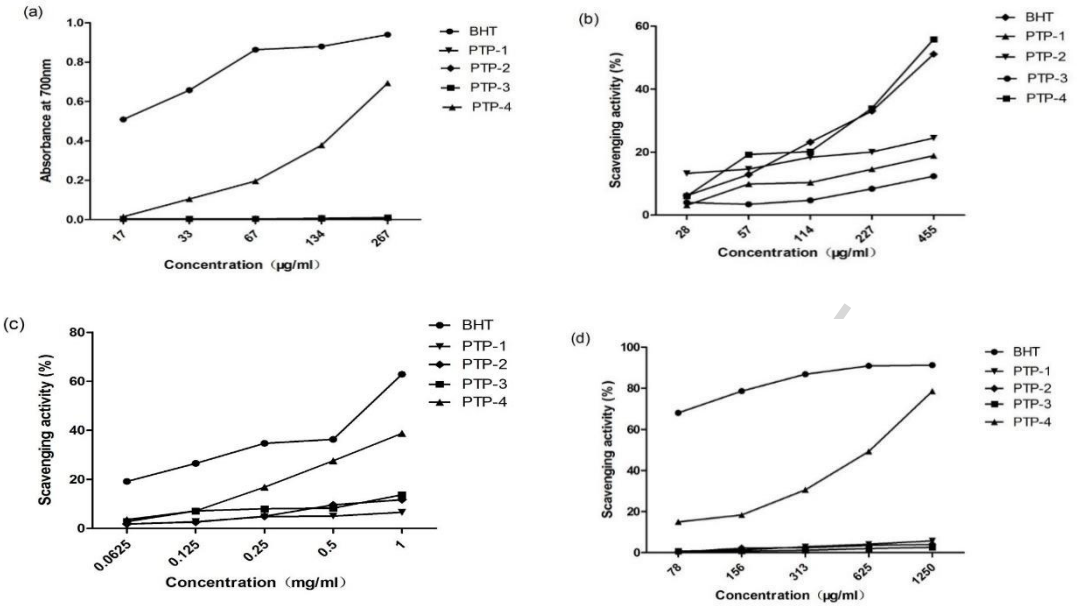


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Fig.5.