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# Structural characterization and DPPH· radical scavenging activity of a polysaccharide from Guara fruits

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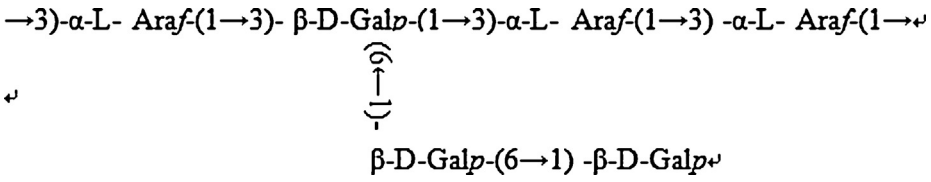
ABSTRACT

The crude polysaccharides were extracted from fruits of *Psidium guajava* Linn. by hot water. After removal of proteins, isolation and purification by DEAE-52 Cellulose chromatography and Sephadex G-75 gel filtration, a polysaccharide (GP70-2) was obtained and structurally characterized. GP70-2 has a relative molecular weight of 74 kDa and was composed of D-galactose and L-arabinose in the ratio of 1:1, with a specific optical rotation of $[a]_D^{25} = +101^\circ$. Structural characterization of this novel polysaccharide was carried out using infrared spectroscopy, methylation analyses, and NMR studies ($^1$H, $^{13}$C, $^1$H-$^1$H-COSY, HMQC, and HMBC). Based on the above data, the following structure was assigned to the repeated core unit of GP70-2:




This polysaccharide showed a concentration dependent DPPH· radical scavenging activity.

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## 1. Introduction

*Psidium guajava* Linn., a fruit-bearing tree, belongs to the Myrtaceae family. *P. guajava* is commonly called guava tree and cultivated in many tropical regions because of its adaptation to a variety of soils, ease of propagation, and rapid fruit bearing. *P. guajava* has a long history of use in traditional medicine and many medicinal properties of this plant have been described (Gutiérrez, Mitchell, & Solis, 2008).

Guavas are green fruits (berries), mostly consumed fresh. In food industry, they are used in the production of jam, ice cream, beverage, syrup, jelly, toffee, cheese, wine, juice, and dehydrated and canned products (Salunkhe & Kadam, 1995). Guava fruits are composed of carbohydrates (13.2%), fats (0.53%), proteins (0.88%) and water (84.9%) (Medina & Pagano, 2003). These fruits are known for their antioxidant and free radical scavenging capacity (Jiménez-Escrig, Rincón, Pulido, & Saura-Calixto, 2001). In modern studies, antimutagenic effects have been found in the water extracts of guava fruits (Grover & Bala, 1993). Guava juice was also reported to notably decrease blood glucose in alloxan-treated and normal diabetic mice after i.p. treatment with 1 g/kg dosage (Cheng & Yang, 1983). Furthermore, a study has determined the significant antihyperglycemic effects of guava which were related to their antioxidative activities (Huang, Yin, & Chiu, 2011). In a clinical study, it was shown that after 12 weeks of guava consumption, the blood pressure of patients decreased by an average 8%, cholesterol levels by 9%, triglycerides by almost 8% while HDL cholesterol increased by 8%. (Singh et al., 1993; Singh, Rastogi, Singh, Ghosh, & Niaz, 1992). These reports demonstrate that carbohydrates of guava fruit play an important role in its bioactivity. Mostly, carbohydrates are deemed important sources of sugar units.

Fruit polysaccharides, very important health factors in foods, are extensively found in edible fruits. In recent years, a deeper understanding of natural and effective polysaccharides has promoted studies dealing with the physical and chemical characterization as well as bioactivity of several fruit polysaccharides (Al-Sheraji et al., 2012; He, Yang, Jiao, Tian, & Zhao, 2012; He et al., 2011; Hu et al., 2011; Simas-Tosin et al., 2012). However, little is known about guava fruit polysaccharides, especially purified carbohydrates. The present work aimed to purify, structurally characterize,

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and evaluate the antioxidant (DPPH· Radical Scavenging) activity of a novel guava water-soluble polysaccharide.

## 2. Materials and methods

### 2.1. Materials

Guava (*P. guajava* Linn.) fruits were purchased from Stone House specialty guava orchard on Haiou Island, Guangzhou city, Guangdong province, China. Voucher specimens were deposited at the College of Pharmacy, Guangdong Pharmaceutical University (Guangzhou, China). Sephadex G-75 gel filtration was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and DEAE-52 cellulose from Whatman Co. (Maidstone, U.K.). Standard monosaccharides, dextran compounds of various molecular weights, trifluoroacetic acid (TFA), sodium hydride ascorbic acid (Vc) and DPPH· (1,1-diphenyl-2-picrylhydrazyl) were obtained from Sigma Chemical Co. (St. Louis, USA).

### 2.2. Extraction and purification of crude polysaccharide extracts

Fresh Guava fruits (50 kg) were sliced into 5 mm thick cubes, then extracted with deionized water 15:1 (v/w) at 80 °C for 3 h and repeated for a total of three times. All the water extracts were combined, concentrated (up to 1/6 initial volume), centrifuged, and filtrated. The supernatant was treated with ethanol at a final concentration of 50% for 24 h at room temperature to obtain crude polysaccharides (P50) after centrifugation at 5000 rpm for 5 min. Then, the concentrated supernatant was precipitated with ethanol at a final concentration of 70% and the process was repeated using 90% ethanol as well. The resulting crude polysaccharide extracts, termed P70 and P90, were obtained and all crude extracts were deproteinized using the Sevag reagent (1-butanol/chloroform, v/v = 1:4). Finally, the supernatant obtained from each polysaccharide extract was dialyzed and lyophilized.

### 2.3. Isolation and purification of the polysaccharide GP70-2

P70 lyophilisate was dissolved in distilled water, centrifuged, and the supernatant was collected and filtrated. The filtrated supernatant was loaded on a DEAE-52 Cellulose chromatography column (2.6 × 40 cm) and eluted with a gradient of 0–1.5 M sodium chloride (400 mL each step). As determined by the phenol-sulfuric acid colorimetric method (Liu, Wong, & Dutka, 1973), fractions eluted with 0 M, 0.1 M and 0.2 M NaCl were collected, dialyzed, lyophilized to obtain three polysaccharides denoted as P70-1, P70-2 and P70-3. P70-2 was purified by gel-filtration (1.6 × 100 cm) on Sephadex G-75 using distilled water as eluent at a flow rate of 0.3 mL/min to yield the purified polysaccharide GP70-2.

### 2.4. Optical rotation identification and relative molecular weight determination

The optical rotation identification and relative molecular weight determination of GP70-2 were carried out according to the method of Li with some modifications (Li, Yan, Hua, & Zhang, 2013).

GP70-2 was dissolved in distilled water completely and ethanol was added to the solution to a final concentration of 65%. A precipitate was obtained after centrifugation at 4000 rpm for 5 min and ethanol was added to the resulting supernatant to a final concentration of 75%. The two precipitates were lyophilized and later resolubilized in distilled water at the same concentration (1 mg/mL). Optical rotation was determined using a P8000 Kruss polarimeter (Germany).

The relative molecular weight of GP70-2 was determined using a high performance gel permeation chromatograph (HPGPC) equipped with a TSK-GEL G-5000$PW_{XL}$ and G-3000 $PW_{XL}$ gel columns in series (Tosoh Biosep, Japan), using a solution of 0.02 M monopotassium phosphate as eluent at a flow rate of 0.6 mL/min and a Waters 2414 refractive index detector (Massachusetts, USA). The columns were calibrated with the Dextran T-series standards of different molecular weights (Dextran T1000, T500, T70, T40, T10, and T5). The column temperature was kept at 35 °C and the molecular weight of GP70-2 was estimated using the calibration curve obtained.

### 2.5. Monosaccharide identification and quantification in GP70-2

Gas chromatography (GC) was used for identification and quantification of the monosaccharide units of GP70-2. The sample (5 mg) was hydrolyzed with 2 M TFA (2 mL) at 120 °C for 6 h in a sealed glass tube and the solution evaporated to dryness. Afterwards, methanol was added and the resulting solution was evaporated to dryness as well and this step was repeated until the pH of the mixture returned to neutral. Acetylization was carried out with 10 mg hydroxylamine hydrochloride and 1 mL pyridine for 30 min at 90 °C. Then, acetic anhydride (1 mL) was added with continuous heating at 90 °C for 30 min. The alditol acetate derivatives were analyzed using an Agilent 6820 GC system (Agilent, USA) with a temperature program set according to previous studies (Li et al., 2013). As references, the following standard monosaccharides were derivatized and analyzed accordingly: L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose.

### 2.6. Methylation analysis of GP70-2

GP70-2 (10 mg) was methylated according to the method of Ciucanu and Kerek (Ciucanu & Kerek, 1984) and the hydroxyl groups of the methylated products examined by IR spectroscopy. The methylated products were hydrolyzed with 2 M TFA, and the excess acid evaporated by co-distillation with distilled water. The hydrolysates were reduced with $NaBH_4$ and finally acetylated with equivalent amounts of acetic anhydride-pyridine. The partially methylated alditol acetates were analyzed by GC–MS (GCMS-QP 2010, Shimadzu, Kyoto, Japan) and the data used to determine the methylated sugar linkages. The acetylated derivatives were loaded into a HP-1 capillary column. The temperature program was set as follows. The initial column temperature of 150 °C was increased to 180 °C at the rate of 10 °C/min, and from 180 °C to 260 °C at 15 °C/min. Subsequently, the temperature was maintained for 5 min at 260 °C with an injection temperature of 220 °C. The ion source of the mass spectrometer was set at 200 °C and the sample (1 μL) was injected with a split ratio of 50:1.

### 2.7. Fourier transform-infrared (FT-IR) analysis

GP70-2 (2 mg) and potassium bromide were mixed, ground, and pressed into a pellet. Spectra were recorded in the absorbance mode from 4000 $cm^{-1}$ to 450 $cm^{-1}$ on a Perkin-Elmer FT-IR spectrometer (Li et al., 2013).

### 2.8. Nuclear magnetic resonance (NMR) spectroscopy

GP70-2 was dissolved in $D_2O$. The $^1H$ and $^{13}C$ NMR spectra of GP70-2 were obtained with a Bruker AV-500 spectrometer (Germany), operating at 500 MHz and 125 MHz for $^1H$ NMR and $^{13}C$ NMR, respectively. Two-dimensional spectra ($^1H$-$^1H$ COSY, HMQC and HMBC) were recorded using standard Bruker procedures.

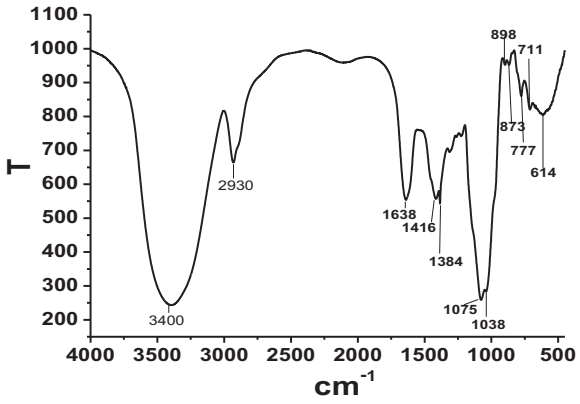


**Fig. 1.** FT-IR spectrum of GP70-2.

### 2.9. Assessment of DPPH· radical scavenging activity

The DPPH· radical scavenging activity was measured according to previous studies (Wang, Gao, Zhou, Cai, & Yao, 2008) with a few modifications. Briefly, 2 mL of 0.2 mM methanolic solution of DPPH· radicals were added to 2 mL of water-solution of GP70-2 at 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, and 10 mg/mL. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at 37 °C in the dark. Ascorbic acid (Vc) was used as the control and distilled water as the blank. The scavenging effect was calculated according to the following equation: Scavenging rate % = $(1 - A_S/A_0) \times 100\%$, where $A_S$ is the absorbance obtained for a sample (GP70-2 or Vc) and $A_0$, the absorbance of the blank.

## 3. Results and discussion

### 3.1. Isolation, purification and general properties of GP70-2

Guava polysaccharides were obtained from guava fruits by hot-water extraction, ethanol precipitation, deproteinization, and lyophilization. After the first purification step by DEAE-52 Cellulose chromatography, fractions P70-1, P70-2, and P70-3 were eluted with 0 M, 0.1 M, and 0.2 M NaCl, respectively. P70-2 was further purified by Sephadex G-75 column to yield GP70-2 with no absorption at 260 nm and 280 nm, indicating the absence of nucleic acids and proteins in the sample. The relative molecular weight of GP70-2 was estimated at 74 kDa and the specific optical rotation in distilled water at room temperature determined to be $[a]_D^{25} = +101°$. Acid hydrolysis of GP70-2 resulted in arabinose and galactose in a molar ratio of 1:1 as determined by GC analyses.

### 3.2. FT-IR spectrum analysis of GP70-2

The FT-IR spectrum recorded in the region 4000–450 $cm^{-1}$ for GP70-2 is shown in Fig. 1. A characteristic peak was found around 3400 $cm^{-1}$, due to the hydroxyl stretching vibrations in GP70-2. The band at 2930 $cm^{-1}$ resulted from C–H stretching vibrations while the band found at 1638 $cm^{-1}$ indicated the absorbed water. The bands at 898 $cm^{-1}$ and 873 $cm^{-1}$ are the characteristic "anomeric region" absorption of β-linkage of pyranose and α-linkage of furanose (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). Together with the bands at 1073 $cm^{-1}$ and 91039 $cm^{-1}$, we suggested that GP70-2 consists of β-linkages of galactopyranoses and α-linkages of arabinofuranoses, in agreement with the following NMR data.

**Table 1**
Glycosidic linkage composition of methylated GP70-2.

| Sugars | PMAA | Linkage | Molar ratios |
|---|---|---|---|
| D-Gal*p* | 2,4-$Me_2$-Gal | 3,6)-Glc-(1→ | 1.13 |
| D-Gal*p* | 2,3,4-$Me_3$-Gal | →6)-Gal-(1→ | 1.02 |
| D-Gal*p* | 2,3,4,6-$Me_4$-Gal | Gal-(1→ | 1.00 |
| L-Ara*f* | 2,5-$Me_2$-Ara | →3)-Ara-(1→ | 3.08 |

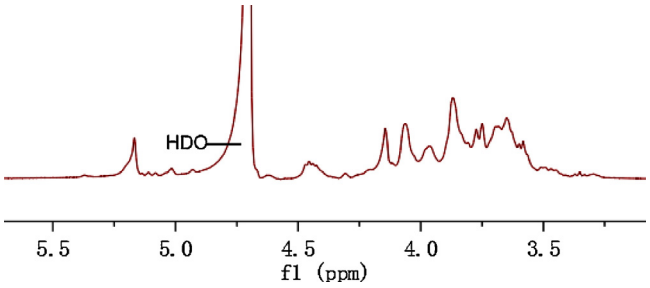


**Fig. 2.** $^1$H NMR spectrum of GP70-2.

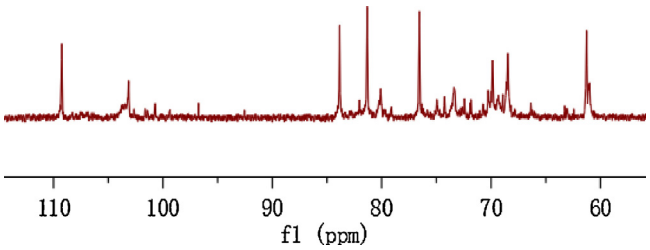


**Fig. 3.** $^{13}$C NMR spectrum of GP70-2.

### 3.3. Methylation results of GP70-2

The fully methylated product of GP70-2 was hydrolyzed with acid, converted into partially methylated alditol acetate (PMAA) and analyzed by GC–MS. The related linkage patterns and corresponding percentage in GP70-2 are shown in Table 1. The data revealed the presence of 1,3,5,6-qua-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol, 1,5-di-*O*-acetyl-2,3,4,6-qua-*O*-methyl-D-galactitol, and 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl-L-arabinitol as products in a relative molar ratio of 1.01: 1.02: 1.00: 3.08. These findings demonstrate that GP70-2 is composed of (1 → 3, 6), (1 → 6) and (1 → ) linked galactose, (1 → 3) linked arabinose.

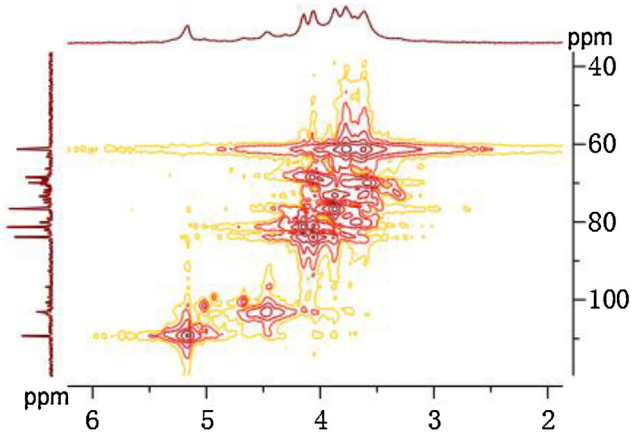


**Fig. 4.** HMQC spectrum of GP70-2.

**Table 2**
Chemical shifts for the resonances of glycosyl residues in GP70-2 obtained from $^{1}H$ and $^{13}C$ NMR spectra.

| Glycosyl residues | Chemical shifts, $\delta$ (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | C-1<br>H-1 | C-2<br>H-2 | C-3<br>H-3 | C-4<br>H-4 | C-5<br>H-5 | C-6<br>H-6 |
| →3)-α-L-Ara-(1→ | 109.17 | 81.32 | 83.89 | 83.87 | 61.18 | – |
| A | 5.21 | 4.14 | 3.96 | 4.05 | 3.78 | – |
| →3,6)-β-D-Gal-(1→ | 103.13 | 73.21 | 76.96 | 69.87 | 76.63 | 68.92 |
| B | 4.46 | 3.79 | 3.86 | 4.04 | 3.87 | 3.69 |
| →6)-β-D-Gal-(1→ | 103.12 | 73.17 | 70.33 | 69.87 | 79.25 | 69.12 |
| C | 4.47 | 3.86 | 3.76 | 3.97 | 3.68 | 3.58 |
| β-D-Gal-(1→ | 103.12 | 73.39 | 72.44 | 70.03 | 73.39 | 60.99 |
| D | 4.47 | 3.60 | 3.59 | 3.95 | 3.68 | 3.77 |

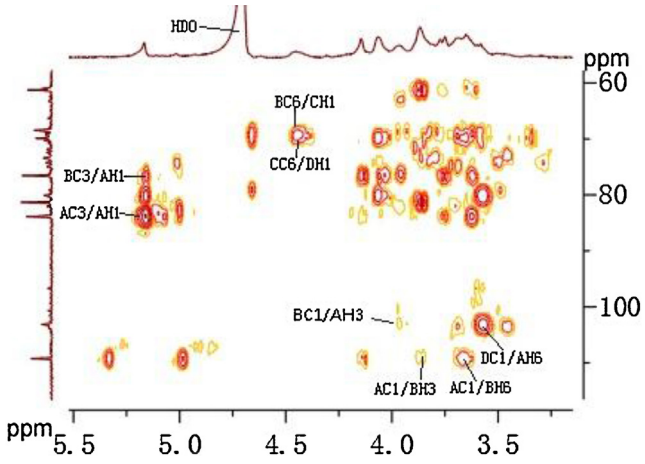


**Fig. 5.** HMBC spectrum of GP70-2.

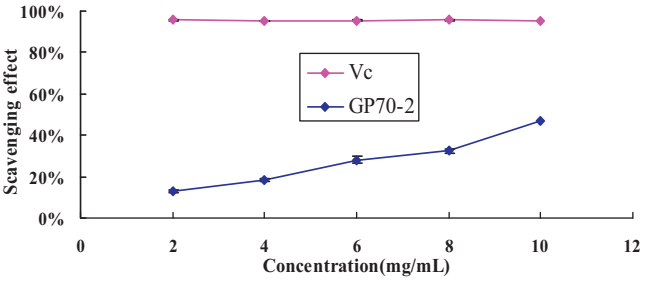


**Fig. 6.** DPPH· radical scavenging effect of GP70-2 and Vc. Data are means ±*SD* of values obtained in three separate experiments.

### 3.4. NMR data of GP70-2

The structural features of GP70-2 were further identified by 1 and 2 dimensional NMR spectroscopy. According to the characteristic signals, the $^{1}H$ and $^{13}C$ spectra of GP70-2 was completely analyzed by two-dimensional $^{1}H$-$^{1}H$COSY and HMQC NMR experiments, and the linkage sites as well as sequence among the linked residues identified by HMBC.

The $^{1}H$ NMR spectrum (Fig. 2) showed two overlapping peaks in the anomeric region ($\delta$ 4.3–5.5 ppm), one signal ($\delta$ 5.2 ppm) recognized for α-anomeric protons and the other ($\delta$ 4.4 ppm) identified as β-anomeric protons (Li, Fan, & Ding, 2011). And the $^{13}C$ NMR spectrum of GP70-2 was shown in Fig. 3.

The HMQC spectrum (Fig. 4) showed two cross peaks in the anomeric region, indicating that two monosaccharides were present in GP70-2. Together with the results of GC–MS analyses, these data suggest that the two signals at 109 ppm and 103 ppm represented the peaks of residue A and the overlapping peaks of residue B, residue C and residue D, respectively.

According to the data generated in methylation assays, 1,3-Ara*f* was expected to be the most abundant residue according to the glycosyl composition and previously reported data (Capek, Matulová, Navarini, & Suggi-Liverani, 2010; Capek, Matulová, Navarini, & Suggi-Liverani, 2013; Dong et al., 2010; Gutiérrez de, Martı′nez, Sanabria, León de Pinto, & Igartuburu, 2005). The downfield shifts of C-3 ($\delta$ 83.89 ppm) and the reported values of standard methyl glycosides indicated that residue A was linked at C-3 (Joseleau, Chambat, Vignon, & Barnoud, 1977). Accordingly, the proton and carbon signals of residues B, C and D were assigned in the same way as described for A, using $^{1}H$-$^{1}H$ COSY and HMQC spectra along with previous literature data (Ivanova, Trofimova, Es'kova, & Babkin, 2012; Braz de Oliveira et al., 2013; Xu, Dong, Qiu, Cong, & Ding, 2010; Yan, Yin, Zhang, Yang, & Yu, 2013). Thus, combining the information derived from the $^{1}H$, $^{13}C$, HMQC and $^{1}H$-$^{1}H$ COSY spectra with the methylation analysis data, a complete description of all the linkage patterns was proposed (Table 2).

In order to determine the linkage sites and sequence among residues, the HMBC (Fig. 5) spectrum was analyzed. A cross peak was observed between H-1 ($\delta$ 5.21 ppm) of residue A and C-3 ($\delta$ 76.96 ppm) of residue B (BC3/AH1) suggesting that C-1 of residue A was linked to O-3 of residue B. Similarly, the cross peaks at 68.92/4.47 ppm (BC6/CH1), 103.12/3.58 ppm (DC1/CH6) and 103.13/3.96 ppm (BC1/AH3) suggested that residue C was linked to O-6 of residue B, residue D to O-6 of residue C, residue B to O-3 of residue A. Besides, the strong cross peak 83.89/5.21 ppm (AC3/AH1) associated with the proportion of residue A in all residues demonstrated that residue A was linked to O-3 of another one.

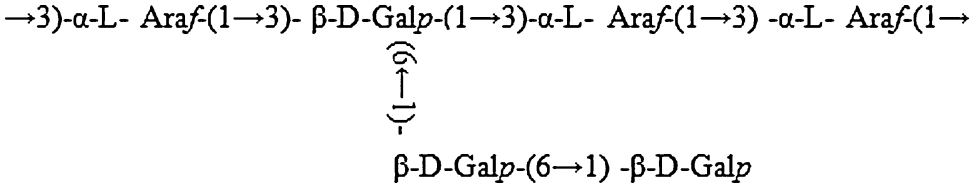


**Fig. 7.** Predicted structure of the repeating unit of GP70-2.

### 3.5. DPPH· radical scavenging activity of GP70-2

Scavenging of DPPH· radicals is the basis of a common antioxidant assay. Antioxidants can protect against the damage caused by free radicals that have been implicated in the etiology of large number of major diseases (Devasagayam et al., 2004). As shown in Fig. 6, GP70-2 displays concentration dependent radical scavenging effects although weaker than that of Vc in the same concentrations. $IC_{50}$ was estimated at 10.8 mg/mL.

## 4. Conclusions

The crude polysaccharide extracts of guava fruits were obtained by hot water extraction and ethanol precipitation. The purified GP70-2 was obtained by DEAE-52 Cellulose chromatography and Sephadex G-75 gel filtration. The relative molecular weight of GP70-2 was 74 kDa. GP70-2 is composed of D-galactose and L-arabinose in the molar ratio 1:1 and its specific optical rotation was found to be $[a]_D^{25} = +101^\circ$. The structure of GP70-2 comprises a backbone of (1 → 3) linked α-L-Ara and (1 → 3,6) linked β-D-Gal with branches consisting of (1 → 6) linked β-D-Gal and terminal β-D-Gal. The proposed structure of GP70-2 is shown in Fig. 7. GP70-2 has DPPH· radical scavenging activities. Further studies are essential in order to find other biological effects of GP70-2.

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