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Structural and enhanced memory activity studies of extracts from *Panax ginseng* root

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ABSTRACT

The aim of this paper is to study low molecular mass carbohydrate polymer from *Panax ginseng* root. The aqueous extracts of *P. ginseng* root were loaded onto a macroporous resin, then forced through a Cellulose Super Filtration System, and loaded onto a Carbon–Celite column and eluted with water, 5% ethanol, 30% ethanol and 95% ethanol. To determine if 30% ethanol elution from a Carbon–Celite column could enhance memory in scopolamine-induced memory deficit rats, Mirros water maze task was used. The purification of the 30% ethanol elution was performed on Sephadex G-15 and Bio-gel P2 columns. GC–MS analysis of methylated derivatives as well as ESI-MS measurement were used for structural confirmation. Results showed that 30% ethanol elution could enhance memory in scopolamine-induced memory deficit rats with intraperitoneal administration. Oligosaccharides and peptides were isolated from 30% ethanol elution. The oligosaccharides were identified as maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose.

1. Introduction

The *Panax ginseng* root is a well-known component herb of Chinese traditional medicine, it is valuable because it is widely used clinically for the treatment of gastrointestinal disorders and as an erythropoietic and a tonic, and in addition it takes 4–6 years for its harvest. Infusions or extracts made from the roots of a number of *P. ginseng* were reported to be effective in alleviating a large number of medical complaints. Ginseng is composed of two major fractions, saponin and nonsaponin fraction. Many ginseng saponins (ginsenosides) have been purified and evaluated, it has been reported that ginsenosides Rb_1 or Rg_1 or both improve learning and memory in γ maze and stepdown avoidance tasks. In addition, these purified individual saponins have been reported to stimulate synaptosomal choline uptake, stimulate acetylcholine release, alter 5-HT concentrations in the brain, potentiate neurite outgrowth stimulated by nerve growth factor, increase expression of choline acetyltransferase and nerve growth factor messenger RNA, and stimulate synapse formation. Long-term oral administration of ginsenoside from American ginseng exert a protective effect against scopolamine-induced learning deficits (Duff Sloley et al., 1999). The nonsaponin fraction of red ginseng could improve learning and memory functions in aged rats with oral administration, and these amelioration effects of the nonsaponin fraction might be attributed partly to augmentation of long-term potentiation (LTP) in the mossy fibre-CA3 synapses (Kurimoto et al., 2004).

To our knowledge, there is no report on the structural analysis and enhanced memory activity studies of oligosaccharides and peptide from Chinese *P. ginseng* root until now. In this paper, we describe the isolation and structural characterisation of oligosaccharides and peptide from the *P. ginseng* root, and its enhanced memory effect against scopolamine-induced memory deficit rats.

2. Experimental

2.1. General methods

Total carbohydrates, uronic acid, protein and saponin contents were determined by the phenol–sulphuric acid (Dubois, Gilles, Hamilton, Reberse, & Smith, 1956), carbazole (Chaplin, 1986), Bradford methods (Bradford, 1976) and vanillina-perchloric acid (Gui & Zhou, 2003), respectively, using D-glucose (⩾99%, China), D-glucuronic acid (⩾99%, Sigma Chemical Co., USA), bovine serum albumin (⩾99%, Sigma Chemical Co., USA) and ginsenoside (from Jilin University) as respective standards. HPLC (Shimadzu, Japan) equipped with refractive index detector (Shimadzu, Japan) on an OH-pak column (SB-802, Shodex, Japan) and with GPC software (China) were used to obtain the purity and molecular mass of each sample. Standard dextrans (Sigma Chemical Co., USA) and each sample were eluted with 0.7% sodium sulphate at 0.5 ml/min. The calibration curve was obtained by standard dextrans, molecu-

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lar mass was calculated by GPC software. For gas chromatography (GC), the carbohydrate compositions of the oligosaccharides were analysed as alditol acetates (Jones & Albersheim, 1972; Merkle & Poppe, 1994). GC analysis was conducted on a 9790 series instrument (China), equipped with a flame-ionisation detector and a 1% of OV-225 column (2 mm × 2 m), chromatography was then carried out isothermally at 220 °C. Paper chromatography was performed in *n*-butyl alcohol–ethanol–water (10:1:2, v/v/v), samples were treated with 2 mol/l trifluoroacetic acid at 121 °C for 1.5 h, the dry hydrolysates were dissolved in 70% ethanol, and glucose and fructose were detected with phenylamine-diphenylamine (Zhang, 1999). PGO4 was treated with 6 mol/l hydrochloric acid at 110 °C for 22 h, the dry hydrolysare was dissolved in water to analyse amino acid compositions. Amino acid analysis was conducted on Agilent 1100 (Agilent, USA) of Agilent 9 Eclipse XDB-C18 column (4.6 × 150 mm, Agilent, USA) eluted with 55% acetonitrile (A) and pH 7.2 phosphate buffer (B) at 1.0 ml/min. Amino acid standards were from Sigma Chemical Co.

2.2. Isolation of PGO from the P. ginseng root

The roots of *P. ginseng* were cultivated on Changbai Mountain, Jilin, China, and collected during 9–10 months. The roots (1 kg) were cut into small pieces and decocted with water (5 l) three times, then the aqueous solutions were combined into one portion, and evaporated to a small volume (200 ml). The aqueous extract was loaded onto a macroporous resin (D101) column (9 × 40 cm) and eluted with water (4 l) to remove the saponine. The aqueous elution from macroporous resin was evaporated into a small column (1 l), and then forced through a Cellulose Super filtration System which molecular weight cut-off is 3000 Da to give the high molecular weight fraction (named PGH) and low molecular weight fraction (named PGL). PGL was loaded onto a Carbon–Celite (1:1) column (9 × 40 cm) and eluted with water (4.0 l), 5% ethanol (3.1 l), 30% ethanol (2.5 l) and 95% ethanol (1.8 l) (Naoki et al., 2008). The 30% ethanol elution was evaporated into 30 ml and lyophilised, dry sample named PGO.

2.3. Enhanced memory performance in rats

The young male Kunming rats (weight 20–22 g) were housed in hanging cages (20 × 30 × 20 cm), 2 rats per cage, and maintained on a 12 h light/dark cycle (lights on at 7 am) under conditions of 25 °C and 50% humidity, and allowed free accessing to standard food and water. Rats were randomly divided into 4 experimental groups (10 per group): normal group, memory deficit group, Piracetam group and PGO group. Piracetam which was a commercial drug in china used as positive drug and PGO which was a low molecular mass carbohydrate polymers used as a sample drug. All procedures complied with institutional guidelines for animal care.

The experiment was based on the Mirros water maze task (Duff Sloley et al., 1999; Whishaw, 1985). The apparatus consisted of a circular pool (walls: 1.2 m diameter, 35 cm deep) which contained water to a depth of 23 cm (at a temperature of approximately 30 °C), made opaque with powdered milk. A moveable platform (10 cm × 11.5 cm, 20 cm tall) was submerged in the pool so that it was covered by 3 cm of water, and hence could not be seen.

During the learning period, animals were allowed to swim freely until they found the escape platform. If a rat failed to find the platform within the allotted period (120 s), it was placed on the platform by the experimenter for 30 s. During the learning period, rats were tested sequentially, 4 times per day for 6 days, during which the escape platform was located in a fixed position in the centre of the pool.

During scopolamine trials, the memory deficit group, Piracetam group and PGO group injected with scopolamine (2 mg/kg), the normal group was injected with physiological saline (10 ml/kg). 30 min after the initiation of oral administration of Piracetam or PGO (400 mg/kg per day), the time required for the rats to locate the platform recorded for 3 days.

Subsequently, the experiment with intraperitoneal administration of Piracetam or PGO (40 mg/kg per day) was conducted. The procedure and the rats of the experiment were the same as oral administration. The data was analysed by ANOVA.

2.4. Purification of PGO1, PGO2, PGO3 and PGO4

An amount of 136 mg of PGO was applied into a Sephadex G-15 column (2.5 × 95 cm, from Sigma Chemical Co., USA), and eluted with 750 ml of water (Gao, Rolf, Chen, & Jiang, 1996). Two samples (PGO1 and PGO2) were obtained (Fig. 1).

An amount of 28.4 mg of PGO2 was applied into a Bio-gel P-2 column (1.6 × 85 cm, from Bio-Rad, USA), and eluted with 150 ml of water. Two samples (PGO3 and PGO4) were obtained (Fig. 2).

2.5. Structural analysis

2.5.1. Spectroscopic studies

The FT-IR spectrum was acquired using a Bruker (Germany) Vertex 70 FTIR. The sample was pressed into KBr pellets and the spectra were recorded in a transmittance mode over a wavelength range between 4000 and 400 cm^{-1}.

Electro spraying ionisation-mass spectrometry (ESI-MS) spectra was obtained on a FTICRMS 7.OT (IonSpec, USA). For ESI-MS experiments, the spray voltage was set to −3 kV in negative mode, the capillary temperature was at 100 °C.

2.5.2. Methylation analysis

PGO1 and PGO3 were methylated once by the Ciucanu method (Ciucanu & Kerek, 1984; Kalyan & Paul, 1992) respectively. The resulting partially methylated alditol acetates were analysed by GC–MS. GC–MS was performed on Trace-MS (Fingan, USA) using a DB-17HT capillary collum (30 m × 0.25 mm, J&W Scientific, USA). The injection temperature was 200 °C. The column temperature was kept at 50 °C for 2 min after sample injection, increased to 150 °C at 50 °C/min, kept at 150 °C for 1 min, then increased to 250 °C at 4 °C/min. The mass spectra were recorded in the positive ion electron ionisation (EI) mode.

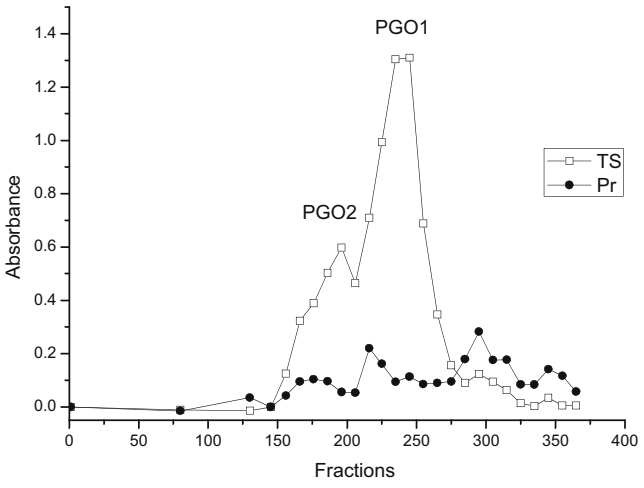

Fig. 1. Elution profile of oligosaccharide fraction (PGO) by gel-filtration chromatography on Sephadex G-15. TS: total sugar, absorbance at 490 nm from the phenol–sulphuric acid assay. Pr: protein, absorbance at 280 nm.

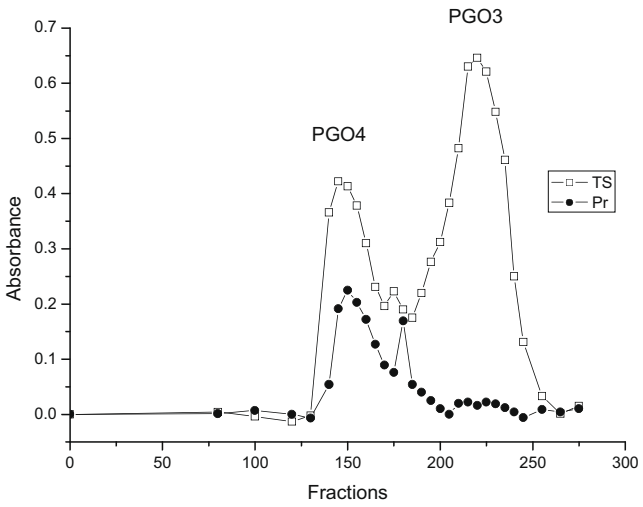

Fig. 2. Elution profile of oligosaccharide fraction (PGO2) by gel-filtration chromatography on bio-gel P-2. TS: total sugar, absorbance at 490 nm from the phenol–sulphuric acid assay. Pr: protein, absorbance at 280 nm.

3. Results and discussion

Compared with properties of the water, 5% ethanol, 30% ethanol, 60% ethanol and 95% ethanol elutions from the Carbon–Celite column, the carbohydrate content of 30% ethanol elution (PGO) was the highest (71.03%), and the molecular mass was from 200 to 1000 (Table 1). So PGO was the low molecular mass carbohydrate polymers, and it was used as a sample in the following studies.

The results (Table 2) of the water maze task showed that PGO group with oral administration reduced the platform search time compared with memory deficit group, but it used more time than Piracetam group and normal group. So PGO displayed a little enhanced memory activity but not significant.

To confirm this activity, the drugs were taken by peritoneal cavity injection. The results (Table 3) showed that, compared with the memory deficit rats, both injection of PGO and Piracetam significantly reduced the platform search time, but the PGO group reduced the platform search time more than the Piracetam group. Compared with the normal group, the PGO group used more platform search time on day 7 and 8, but less time on day 9. The result showed that PGO could not immediately recover the memory of memory deficit rats, it needs 2 days. This finding suggests that PGO has the enhanced memory effect against scopolamine-induced memory deficit rats with intraperitoneal administration, and better than Piracetam.

HPLC analysis of PGO showed seven peaks, therefore, it should be separated. Purification of PGO1 and PGO2 were carried out by Sephadex G-15 column from PGO (Fig. 1). Fractions 220–260 and 175–219 were collected as PGO1 and PGO2, respectively.

In Fig. 1, the total sugar of PGO2 was low, in order to improve the sugar content, PGO2 should be separated. To isolate the components of PGO2, Bio-gel P2 column was used, for its resolution was higher than the Sephadex G-15 column. The result of the purification of PGO3 and PGO4 was shown in Fig. 2. Fractions 190–240 and 135–165 were collected as PGO3 and PGO4, respectively.

The result of paper chromatography on PGO1, PGO2, PGO3 and PGO4 implied that there were only glucose and no fructose content. And all of them also contained no saponin which was tested by the vanillina-perchloric acid method. The yields and chemical compositions of PGO1, PGO3 and PGO4 are given in Table 4. PGO1 and PGO3 only contained carbohydrate. Sugar composition analysis of PGO1 and PGO3 by GC showed that glucose alone accounted for 100% indicating they are glucan. The sugar compositions of PGO4 analysed by GC were arabinose, galacturonic acid and glucuronic acid. The protein content of PGO4 was 20.74%, but its molecular mass was less than 2000, So PGO4 contained peptide. Amino acid analysis of PGO4 showed that Cys and Tyr contents (total 9.16%) were higher; others were Asp, Glu, Arg, Gly, Thr, Pro, Ala, Val, Ile, Leu, Lys and Hyp. HPLC analysis of PGO1, PGO3 and PGO4 implicated that they did not show a single peak, so all of them were a mixture.

The FT-IR spectra of PGO1 and PGO3 showed the presence of bands typical for those of saccharides (data not shown) and the assignment of 847 indicated the presence of α-linked sugar residues (Zhang, 1999).

The ESI-MS spectrometric analysis of PGO1 and PGO3 are shown in Fig. 3 and Fig. 4. The full-scan mass spectrum was in negative mode. In Fig. 3, the peaks at m/z 377.08559, 539.13950 and 701.19592 were assigned to the $[M+Cl]^-$ ion of disaccharide, trisaccharide and tetrasaccharide. And the peaks at *m/z* 341.10926, 503.16272, 665.21614 were assigned to the deprotoned ion $[M-H]^-$ of trisaccharide and tetrasaccharide (Liu, Cui, Liu, & Song, 2004). The mass spectrum of PGO1 illustrated that it was consisted

Table 1
Physicochemical properties and molecular mass of water, 5% ethanol, 30% ethanol, 60% ethanol and 95% ethanol elutions.

	Water elution	5% ethanol elution	30% ethanol elution	60% ethanol elution	95% ethanol elution
Carbohydrate (%)[a]	2.02	25.07	71.03	36.97	11.84
Uronic acid (%)[a]	tr[b]	5.65	12.88	6.76	nd[c]
Protein (%)[a]	7.21	18.71	6.75	25.87	29.23
Molecular mass (Da)	100–400	100–1000	200–1000	300–2000	100–3000

[a] Calculated as weight percent of applied material.
[b] Trace.
[c] Not detected.

Table 2
Results of water maze experiment in rats with oral administration of drug ($\bar{X}$±SD, $n = 10$).

	Dosage (mg/kg)	Platform search time (s) on day 7	Platform search time (s) on day 8	Platform search time (s) on day 9
Normal group	–	79.968 ± 11.234	79.116 ± 11.449	68.178 ± 8.588
Memory deficit group	–	86.985 ± 12.353	90.899 ± 8.284	87.098 ± 8.030
Piracetam group	400	67.274 ± 9.491[a]	61.193 ± 9.190[a]	73.503 ± 6.424[a]
PGO group	400	84.125 ± 9.445[b]	85.817 ± 7.665[b]	86.426 ± 9.369[b]

[a] Piracetam $P < 0.05$.
[b] PGO $p < 0.05$, compared with memory deficit group.

Table 3
Results of water maze experiment in rats with intraperitoneal administration ($\bar{X}$±SD, n = 10).

	Dosage (mg/kg)	Platform search time (s) on day 7	Platform search time (s) on day 8	Platform search time (s) on day 9
Normal group	–	70.768 ± 10.1078	74.338 ± 11.1142	77.428 ± 8.1177
Memory deficit group	–	95.960 ± 8.1564	96.648 ± 10.027	102.370 ± 5.1633
Piracetam group	40	86.347 ± 9.7191[a]	92.839 ± 7.5810[a]	87.265 ± 6.7747[a]
PGO group	40	76.666 ± 8.6787[b]	90.002 ± 9.0535[b]	76.758 ± 9.4407[b]

[a] Piracetam $P < 0.05$.
[b] PGO $p < 0.01$, compared with memory deficit group.

Table 4
State, physicochemical properties, molecular mass, yield and sugar composition of PGO1, PGO3 and PGO4.

State	PGO1 White powder	PGO3 White powder	PGO4 Pale yellow powder
Carbohydrate (%)[a]	94.01	98.17	54.17
Uronic acid (%)[a]	tr[c]	tr[c]	23.54
Protein (%)[a]	tr[c]	nd[d]	20.74
Molecular mass (Da)	300–700	800–1200	600–1400
Yield (%)[a]	25.50	10.56	24.56
Sugar composition (mol%)[b]			
Glucose	100	100	nd[d]
Arabinose	nd[d]	nd[d]	31.42
Galacturonic acid	nd[d]	nd[d]	32.70
Glucuronic acid	nd[d]	nd[d]	35.88

[a] Calculated as weight percent of applied material.
[b] Mole percent of total carbohydrate content.
[c] Trace.
[d] Not detected.

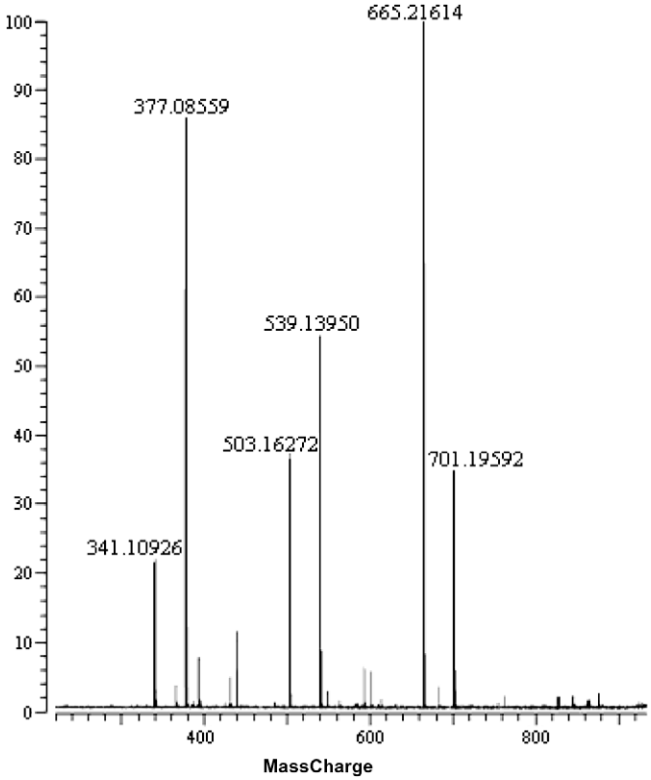

Fig. 3. The full-scan mass spectrum of PGO1 by ESI-MS spectrometric in negative mode.

of disaccharide, trisaccharide and tetrasaccharide, which have molecular masses of 342, 504 and 666 Da. In Fig. 4, the peaks at *m/z* 863.25274, 1025.30787 and 1187.37617 were assigned to the $[M+Cl]^-$ ion of pentasaccharide, hexasaccharide and heptasaccharide. And the peaks at *m/z* 827.26706, 989.32050 and 1151.38884 were assigned to the deprotoned ion $[M-H]^-$ of pentasaccharide, hexasaccharide and heptasaccharide. The mass spectrum of PGO3 illustrated that it was consisted of pentasaccharide, hexasaccharide and heptasaccharide, which have molecular mass 828, 990 and 1152 Da.

The ESI-MS spectrums of PGO2 and PGO4 showed too many peaks, and it can not identify any structure from them.

The methanolysate of permethylatesd PGO1 exhibited two peaks, which corresponded to 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (*t*, retention time of methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside; 8.54 min) and 2,3,6-tri-*O*-methyl-D-glucopyranoside (*t*, 9.12 min). The 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside represented the non-reducing terminal. And the 2,3,6-tri-*O*-methyl-D-glucopyranoside indicated that the glycosidic linkage is (1→4)-linked (Ciucanu & Costello, 2003). From this finding, the structures of the disaccharide, trisaccharide and tetrasaccharide are α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranose, α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranose and α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranose, which are maltose, maltotriose and maltotetraose, respectively. And the methanolysate of permethylatesd PGO3 exhibited two peaks the same as PGO1. So PGO3 was also shown to contain only a (1→4)-linkage. The pentasaccharide, hexasaccharide and heptasaccharide are maltopentaose, maltohexaose and maltoheptaose, respectively.

4. Conclusions

To summarise, this study presents, for the first time, that PGO from *P. ginseng* root can significantly enhance memory performance in scopolamine-induced memory deficit rats. PGO contains oligosaccharides and peptides. Structural analysis showed that the oligosaccharides are maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. The structural analysis of PGO4 will be done soon. To finding the active parts of PGO, the oligosaccharides and peptides will be used in enhanced memory experiment respectively.

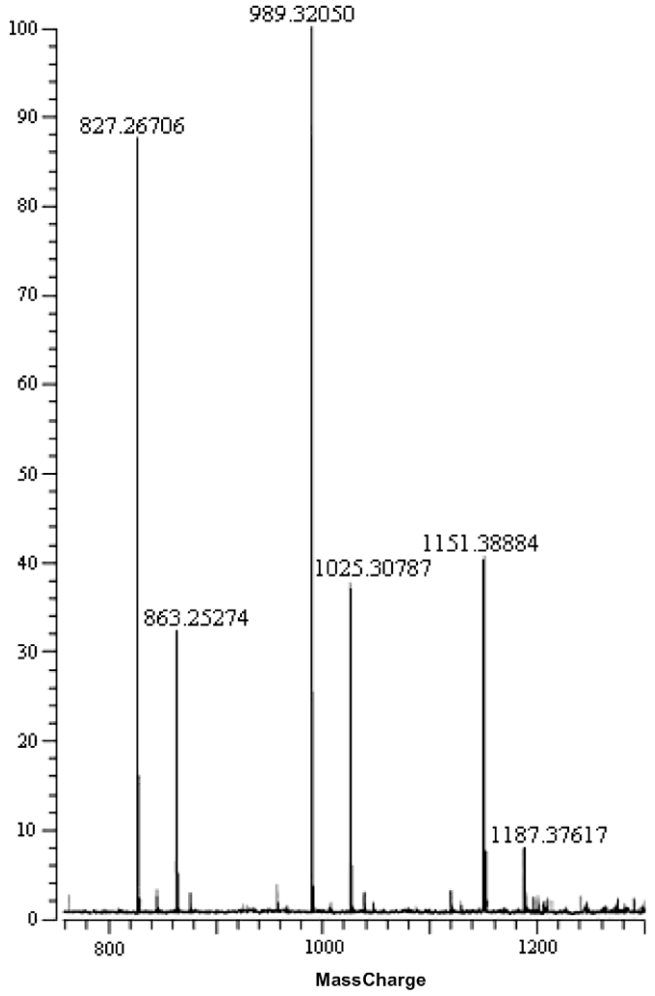

Fig. 4. The full-scan mass spectrum of PGO3 by ESI-MS spectrometric in negative mode.

Acknowledgements

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