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A polysaccharopeptide complex and a condensed tannin with antioxidant activity from dried rose (*Rosa rugosa*) flowers

T. B. Ng, Z. F. Pi, H. Yue, L. Zhao, M. Fu, L. Li, J. Hou, L. S. Shi, R. R. Chen, Y. Jiang and F. Liu

Abstract

In this study, the fraction (P) from an aqueous extract of dried rose (*Rosa rugosa*) flowers was obtained by ethanol precipitation. P was chromatographed on DEAE-cellulose. The components retained on DEAE-cellulose were eluted with a linear gradient of 0–2 M NaCl solution. Two fractions, eluted at concentrations of 0.5 M NaCl and 1 M NaCl, respectively, were obtained. These two components were designated as P_1 and P_2, respectively. P_1 was further purified using gel filtration on Sephadex G-200. P_1 yielded two peaks, and the two components were designated as $P_{1\text{-}a}$ and $P_{1\text{-}b}$, respectively. $P_{1\text{-}a}$ was a polysaccharide–peptide complex, and $P_{1\text{-}b}$ exhibited chemical properties of a condensed tannin as revealed by FTIR and NMR assay of carbohydrate and protein contents and HPLC-ESI-MS. The molecular masses of $P_{1\text{-}a}$ and $P_{1\text{-}b}$ were 150 kDa and 8 kDa, respectively. Both $P_{1\text{-}a}$ and $P_{1\text{-}b}$ possessed antioxidant activity, with the activity of $P_{1\text{-}b}$ higher than that of $P_{1\text{-}a}$. This study demonstrated that different components from rose flowers exhibited antioxidant activity.

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

T. B. Ng

Department of Microbiology, College of Life Science, Nankai University, Tianjin, China

Z. F. Pi, M. Fu, L. Li, J. Hou, L. S. Shi, R. R. Chen, Y. Jiang, F. Liu

The New Drug Laboratory of Changchun Institute of Applied Chemistry, Chinese Academy of Science, China

H. Yue

Hospital of Tianjin, Tianjin, China

L. Zhao

Correspondence: F. Liu, Department of Microbiology, College of Life Sciences, Nankai University, Tianjin, China. E-mail: liufang312@eyou.com

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Introduction

Oxidative reactions are important to many living organisms for generation of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species, that are continuously produced in-vivo, can bring about cell death and tissue damage (Yang 2002). Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, the superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. The damaging action of the hydroxyl radical is the strongest among free radicals (Liu et al 1997). The enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase constitute the enzymatic defence systems against free radicals (Singh & Pathak 1990).

Rugosa rose, *Rosa rugosa* Thunb., and its horticultural variety *R. rugosa* var. *plena* are well known as fragrant and medicinal plants in east Asia. The traditional use of rose flowers is for controlling pain and diarrhoea, and for treating neurotic gastritis, chronic gastritis, hepatitis, acute mastitis, trauma and blood disorders (Hsu et al 1986). In northern Japan, dried rose petals have been used as antidiarrhoeal and haemostatic agents (Hashidoko 1996). *R. rugosa* is a source of phytochemicals, some of which have interesting biological activity. It was reported that flavonoids extracted from rose expressed antioxidant activity (Kleszczynska et al 1999). The secondary metabolites of *R. rugosa*, defined by structural class, comprise hydrolysable tannins (contained in leaves and petals), flavonoids (leaves), 2-phenoxychromones (leaves), catechin derivatives (roots), monoterpenes (floral parts, leaves), sesquiterpenes (leaves, especially from glandular trichomes) and triterpenes (leaves and roots) (Hashidoko 1996). Secondary metabolites of plants are known to function as chemical messengers or as anti-pest agents (Harborne et al 1993). In the case of *R. rugosa*, the roles of the secondary metabolites would appear to be more versatile than just to confer protection

against pathogenic microorganisms and insect herbivores, whose populations in coastal areas are low.

The majority of published research has shown that antioxidant components in *R. rugosa* are non-polar and of low molecular weight. However, it was reported that many high-molecular-weight components with antioxidant activity (e.g. polysaccharides, tannins) had been found in many plants other than *R. rugosa*. In this study, two new antioxidant components with high molecular weight were obtained. This study may facilitate elucidation of the mechanism of action of *R. rugosa*. Previously we have reported a gallic acid derivative with antioxidant activity from *R. rugosa* (Ng et al 2004). It inhibited generation of superoxide and hydroxyl radicals and lipid peroxidation (Ng et al 2004). It reinstated the activity of the antioxidant enzymes catalase and glutathione peroxidase, and reduced the amount of hepatic, renal and brain malondialdehyde in 9-month-old male senescence-accelerated mice (Li et al 2005).

Materials and Methods

Reagents

DEAE-cellulose anion exchanger, thiobarbituric acid (TBA), cytochrome C, butylated hydroxyanisole (BHA) and nitroblue tetrazolium (NBT) were obtained from Sigma. Sephadex G-200 (5–600 kDa) was obtained from Amersham Biosciences. 2,2′-Azo-bis-(2-amidinopropane)-dihydrochloride (AAPH) was purchased from Wako (Osaka, Japan). All other chemicals were of the highest quality available.

Animals

Male Kunming mice, 18–22 g, were used in this study. They were housed under normal laboratory conditions (21 ± 2°C, 12-h light–dark cycle) with free access to standard rodent chow and water. The experiments using animal tissues were conducted in accordance with the guidelines and with the approval of Animal Research Ethics Committee, the Chinese University of Hong Kong.

Anion exchange chromatography on DEAE-cellulose

Dried rose (*Rosa rugosa* Thunb.) flowers from Mainland China were cut up into small pieces, soaked overnight in distilled water at room temperature, and then boiled under reflux for 2 h. The extract was evaporated under reduced pressure to remove the solvent. Water and then 4 volumes of ethanol were added, and the mixture was allowed to stand at 4°C overnight. The precipitate (P) was collected by filtration, lyophilized, dissolved in 10 mM Tris-HCl buffer (pH 6.5), and centrifuged to remove insoluble materials and subjected to the next isolation step. Simultaneously, a solution of the precipitate was scanned from 200 nm to 800 nm with Smartspect 3000. The maximum absorption wavelength was found to be 280 nm. Therefore, the wavelength 280 nm was used to monitor the purification of P.

The lyophilized powder (P) (50 mg) was dissolved in 10 mM Tris-HCl buffer (pH 6.5), and centrifuged to remove insoluble materials. The solution was chromatographed on a column of DEAE-cellulose (2.5×35 cm) that had previously been equilibrated with, and was then eluted with, 10 mM Tris-HCl buffer (pH 6.5). After eluting with 250 mL of the buffer, the column was eluted with a linear gradient of 0–2 M NaCl solution. The flow rate was 50 mL h^{-1} and the fraction size was 2.5 mL. The absorbance at 280 nm was measured. Fractions were collected and dialysed against de-ionized water. Simultaneously, the carbohydrate content of the fractions was estimated with the anthrone-H_2SO_4 method (Kawagishi et al 1990). All fractions were lyophilized and monitored for antioxidant activity using the method described below.

The fraction (P_1) eluted at the concentration of 0.5 M NaCl was subjected to the next isolation step.

Gel filtration on Sephadex G-200 and molecular weight determination

The molecular weight of the fraction was determined by gel filtration on Sephadex G-200 (5–600 kDa). Bovine albumin (BSA), egg albumin and cytochrome C were used as standards and distilled water was used as eluant. The elution volume of dextran blue 2000 was used as void volume.

The P_1 powder was applied to a Sephadex G-200 column (2.5×100 cm) in distilled water and was eluted with the same buffer at a flow rate of 18 mL h^{-1}. Fractions of 3 mL were collected. The eluate was monitored with a UV detector at 280 nm. The eluted fractions were lyophilized.

Chemical analysis

Carbohydrate and protein contents of the fractions were quantitatively determined with the colorimetric anthrone-H_2SO_4 method (Kawagishi et al 1990) and Lowry-Folin method (Lowry et al 1951), respectively. Tannin was determined as described by Dalzell & Shelton (1997).

Assay of lipid peroxidation

The brains and kidneys of normal mice were dissected and homogenized in ice-cold Tris-HCl buffer (20 mM, pH7.4) to produce a 10% homogenate. The homogenate was centrifuged at 14 000 rev min^{-1} for 15 min. A 200-μL volume of the supernatant was incubated with the test sample in the presence of 10 μM $FeSO_4$ and 0.1 mM ascorbic acid at 37°C for 1 h. The reaction was stopped by addition of 0.4 mL trichloroacetic acid (TCA, 28% w/v) and 0.6 mL thiobarbituric acid (TBA, 1% w/v) in succession, and the solution was then heated at 100°C for 15 min. After centrifugation to remove precipitated protein, the absorbance of the malondialdehyde (MDA)–TBA was measured at 532 nm (Liu et al 1997). Butylated hydroxyanisole (BHA) was used as positive control. The inhibition ratio (%) was calculated by using the following formula:

$$\text{Inhibition ratio } (\%) = (A - A_1)/A \times 100\% \quad (1)$$

Where A was the absorbance of control, and A_1 was the absorbance of the tested sample.

Assay of erythrocyte haemolysis

Blood was obtained from male adult mice by extirpating the eyeball and collected in tubes containing 0.15 M NaCl. Erythrocytes were washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged at 2500 rev min^{-1} for 10 min (Miki et al 1987).

Erythrocyte haemolysis was mediated by peroxyl radicals in this assay system (Sugiyama et al 1993). A 12.5% suspension of erythrocytes in pH 7.4 phosphate-buffered saline (PBS) was added to the same volume of 200 mM AAPH solution (in PBS) containing samples to be tested at different concentrations. The reaction mixture was shaken gently during incubation at 37 °C for 1 h. The reaction mixture was then removed, diluted with 19 volumes of PBS and centrifuged at 2500 rev min^{-1} for 10 min. The absorbance A of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 19 volumes of distilled water to achieve haemolysis, and absorbance B of the supernatant, obtained after centrifugation, was measured at 540 nm. The inhibition ratio (%) of erythrocyte haemolysis was calculated by using the following formula:

$$\text{Inhibition ratio } (\%) = (1 - A/B) \times 100\% \quad (2)$$

L-Ascorbic acid was used as a positive control.

Assay of superoxide dismutase (SOD) of erythrocyte activity

Erythrocytes were obtained by using the same method as described in the assay of erythrocyte haemolysis. In this experiment, the suspension of erythrocytes was prepared in 10 mM PBS (pH 7.4) containing samples to be tested at different concentrations. The reaction mixture was shaken gently during incubation at 37 °C for 3 h. It was then removed and the SOD extraction was prepared as follows.

The erythrocytes were obtained by centrifugation at 2500 rev min^{-1} for 10 min. Three volumes of ice-cold distilled water were added to the erythrocyte suspension to produce lysis of the erythrocytes. Ice-cold ethanol (95% v/v) (0.1 mL) was mixed with 0.2 mL erythrocyte lysate. After shaking lightly for 30 s, 0.1 mL ice-cold chloroform was added to the mixture and extraction was performed on a shaker for 1 min. The reaction mixture was centrifuged at 3500 rev min^{-1} for 8 min and the supernatant was collected.

The supernatant was assayed for SOD activity photochemically using an assay system consisting of *p*-nitroblue tetrazolium (NBT), methionine and riboflavin (Giannopolitis & Reis 1997; Rodriguez et al 1998). The reaction mixture was composed of 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 10 mM sodium carbonate and the SOD extraction sample in an appropriate volume. The initial reaction rate was determined as increase of absorbance at 560 nm under a fluorescent lamp. The reaction mixture was then illuminated under a fluorescent lamp for 5 min. PBS was used as a negative control. The absorbance, A, of sample and the absorbance, B, of control were measured at intervals at 560 nm. One unit of SOD activity is the amount of enzyme that inhibits NBT photoreduction by 50%. The SOD activity was calculated using the following formula:

$$\text{SOD activity (unit)} = (B - A)/(B \times 50\%) \quad (3)$$

HPLC

After the two chromatographic steps, the active fractions were subjected to reverse-phase HPLC, FTIR spectroscopy and NMR spectroscopy, as described by Ng et al (2004).

Statistical analysis of data

The data from the assays were analysed using one-way analysis of variance followed by Duncan's multiple range test.

Results

P was chromatographed on DEAE-cellulose. The adsorbed components on DEAE-cellulose were eluted with a linear gradient of 0–2 M NaCl. Two components, designated as P_1 and P_2, were eluted at a concentration of 0.5 M NaCl and 1 M NaCl, respectively. The carbohydrate content of P_1 was higher than that of P_2, as indicated by OD620 using the anthrone-H_2SO_4 method (Figure 1).

Gel filtration of P_1 on Sephadex G-200 (5–600 kDa) yielded two fractions (absorbance peaks), $P_{1\text{-a}}$ and $P_{1\text{-b}}$ (Figure 2). $P_{1\text{-a}}$ was a white powder, whereas $P_{1\text{-b}}$ was a filemot powder. The molecular weight of each fraction was determined by gel filtration on Sephadex G-200, using BSA,

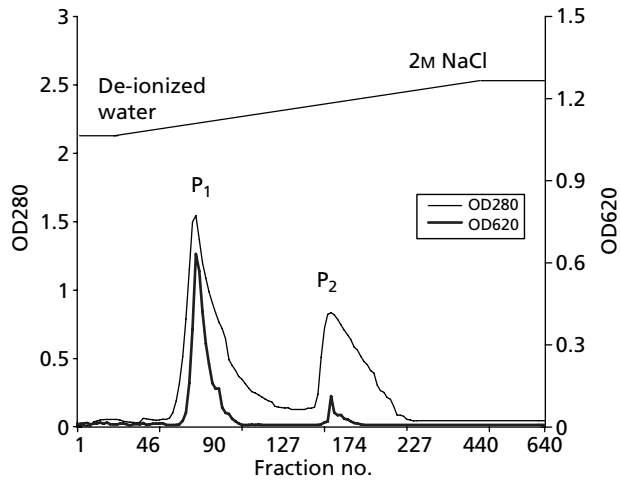

Figure 1 Elution profile of fraction P from DEAE-cellulose column. OD280 measures protein while OD620 measures carbohydrate.

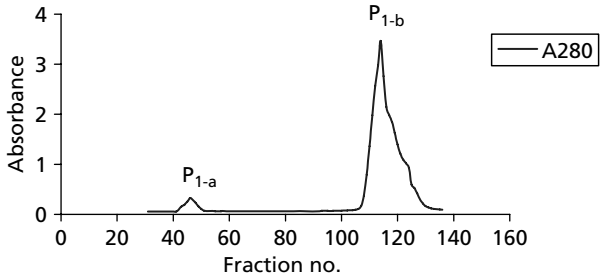

Figure 2 Elution pattern of fraction P_1 from Sephadex G-200 column.

Table 1 Carbohydrate and protein content of the chromatographic fractions

Fraction	Yield (g/100 g rose)	Carbohydrate (%)	Protein (%)
P	3.49	22.37 ± 0.061^{b}	28.46 ± 0.041^{e}
P_1	0.72	30.74 ± 0.181^{c}	5.68 ± 0.021^{b}
P_2	0.35	23.27 ± 0.098^{b}	2.98 ± 0.038^{a}
P_{1-a}	0.04	66.83 ± 0.035^{d}	7.19 ± 0.017^{c}
P_{1-b}	0.57	13.30 ± 0.028^{a}	13.55 ± 0.043^{d}

Groups with different superscripts are statistically different when data are subjected to statistical analysis by one-way analysis of variance followed by Duncan's multiple range test. Date represent means ± s.d., n = 5.

Table 2 Effects of chromatographic fractions from rose flower extract on inhibition of erythrocyte haemolysis

Sample	Concentration ($\mu g\,mL^{-1}$)	Inhibition (%)
P	250	95.55 ± 1.68
	125	94.64 ± 0.37
	62.5	93.46 ± 0.34
P_1	250	98.76 ± 2.39^{a}
	125	70.82 ± 5.44^{b}
	62.5	24.64 ± 6.25^{c}
P_2	250	76.68 ± 11.45^{a}
	125	36.46 ± 20.44^{b}
	62.5	13.76 ± 11.23^{c}
P_{1-a}	250	38.43 ± 3.86^{a}
	125	13.25 ± 1.66^{b}
	62.5	12.22 ± 7.82^{b}
P_{1-b}	250	98.79 ± 0.36^{a}
	125	70.22 ± 1.84^{b}
	62.5	42.63 ± 7.26^{c}
Gallic acid derivative	50	96.51 ± 2.639
L-Ascorbic acid	250	98.68 ± 0.36^{a}
Control (PBS)	—	16.50 ± 0.48^{b}

Responses to different concentrations of the same sample with different superscripts are statistically different from one another when data are subjected to statistical analysis by one-way analysis of variance followed by Duncan's multiple range test. Data represent means ± s.d., n = 5.

egg albumin and cytochrome C as standards. P_{1-a} gave a very broad, small single peak on Sephadex G-200, with a molecular weight of about 150 kDa. P_{1-b} gave a single, sharp peak on Sephadex G-200 with a molecular weight of about 8 kDa. The total contents of carbohydrate and protein are shown in Table 1. It is possible that nucleic acids, lipids, etc., were present in the fractions since the percentages of protein and carbohydrate did not add up to 100%.

The effects of chromatographic fractions from the rose flower extract on erythrocyte haemolysis are shown in Table 2. Fractions P, P_1 and P_{1-b} showed strong inhibiting activity. However, P_2 and P_{1-a} were relatively ineffective and they had little activity at low concentrations ($62.5\,\mu g\,mL^{-1}$). The activity of P_1 and P_{1-b} was concentration dependent.

Compared with P_1, P_2 was relatively ineffective in inhibiting erythrocyte haemolysis. Therefore, only P_1 was studied in the following experiments. P_2 will be investigated in the future.

The effect of each fraction on lipid peroxidation in mouse brain and kidney homogenates is shown in Table 3. P and P_1 exhibited potent activity in inhibiting lipid peroxidation in mouse brain and kidney, while the effects of P_{1-a} and P_{1-b} were meager. P_1 gave rise to P_{1-a} and P_{1-b}, and the inhibiting effect of P_1 may be attributed to a combined action of P_{1-a} and P_{1-b}. P_{1-b} gave a blue coloration after reaction with $K_3Fe(CN_6)/FeCl_3$ and was precipitated with lead acetate.

Table 4 shows the effect of each fraction on superoxide dismutase (SOD) activity of erythrocytes. All fractions enhanced erythrocyte SOD activity with the effect of P_{1-b} being the strongest.

P_{1-a} yielded a peak with a small hillock when subjected to HPLC. The results showed that P_{1-a} is a complex of

Table 3 Effect of chromatographic fractions from rose flower extract on lipid peroxidation in mouse brain and kidney homogenates

Sample	Concentration ($\mu g\,mL^{-1}$)	Brain MDA inhibition (%)	Kidney MDA inhibition (%)
P	250	73.59 ± 1.48^{d}	73.56 ± 1.53^{c}
P_1	250	36.88 ± 1.52^{c}	72.86 ± 1.33^{c}
P_{1-a}	250	16.47 ± 1.78^{a}	18.86 ± 1.68^{b}
P_{1-b}	250	26.57 ± 2.26^{b}	12.44 ± 1.93^{a}
Gallic acid derivative	250	93.27 ± 1.83^{e}	89.03 ± 1.34^{d}
BHA	250	76.88 ± 1.26^{d}	75.68 ± 0.96^{c}

Groups with different superscripts are statistically different when the brain or kidney MDA data are subjected to statistical analysis by one-way analysis of variance followed by Duncan's multiple range test. Data represent means ± s.d., n = 5.

Table 4 Effect of chromatographic fractions from rose flower extract on erythrocyte superoxide dismutase (SOD) activity

Sample	Concentration ($\mu g\,mL^{-1}$)	SOD activity (U)
P	500	1.622 ± 0.039^{c}
P_1	500	1.424 ± 0.009^{a}
$P_{1\text{-a}}$	500	1.467 ± 0.011^{b}
$P_{1\text{-b}}$	500	1.518 ± 0.035^{c}
Control (PBS)	—	1.244 ± 0.166^{a}

Groups with different superscripts are statistically different when data are subjected to statistical analysis by one-way analysis of variance followed by Duncan's multiple range test. Data represent means ± s.d., n = 5.

polysaccharide–peptide. FTIR spectrum of $P_{1\text{-a}}$ showed that 3422 cm^{-1} ($\nu_{O\text{-}H}$ and $\nu_{N\text{-}H}$) and 2932 cm^{-1} ($\nu_{C\text{-}H}$) are the regions of characteristic absorbance of polysaccharide. In addition, 1654–1624 cm^{-1} ($\nu_{C=O}$), 1404 cm^{-1} ($\gamma_{C\text{-}O}$) and 1019 cm^{-1} ($\nu_{C\text{-}O}$) are also the regions of absorbance of carbohydrates. 833.9 cm^{-1} ($\gamma_{C\text{-}H}$) indicated that the bond is α-type (data not shown).

$P_{1\text{-b}}$ yielded a single peak when subjected to HPLC. The results showed that $P_{1\text{-b}}$ is a single fraction. FTIR spectrum of $P_{1\text{-b}}$ showed that 3423 cm^{-1} ($\nu_{O\text{-}H}$) and 2932 cm^{-1} ($\nu_{C\text{-}H}$) are the regions of characteristic absorbance of carbohydrates. The results were consistent with the assay of total contents of carbohydrate and protein. In addition, the absorbance of 1743 cm^{-1}, 164 cm^{-1} and 1300–1000 cm^{-1} indicated the presence of C = O, C–C and C–O in $P_{1\text{-b}}$, respectively (data not shown).

In the ^{1}H NMR spectrum of $P_{1\text{-b}}$, many peaks appeared at δ 5.0 ppm and δ 3.5–4.5 ppm, indicating the presence of the proton of a glucose ring. The peak at δ 175.58 ppm in ^{13}C NMR of P1-b indicated the presence of C = O. The peaks at δ 55–80 ppm showed the presence of C–O, C–N or C–H (data not shown).

The chemical analysis, NMR spectrum and FTIR spectrum showed that $P_{1\text{-b}}$ was a complex fraction with a high molecular weight.

Conclusion

In this study a polysaccharopeptide complex and a condensed tannin were isolated from dried rose flowers. Both exhibited antioxidant activity in a number of assay systems.

Discussion

In an earlier study (Ng et al 2004) polysaccharides were prepared from a boiled water extract of rose flowers. In this study a crude polysaccharide preparation was obtained with an additional step of precipitation with ethanol. Ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex 200 were employed in this study instead of ion-exchange chromatography on CM-cellulose and gel filtration on Sephadex 75 (Ng et al 2004).

Anion-exchange chromatography of P on DEAE-cellulose yielded two fractions, P_1 and P_2 (Figure 1). Table 1 shows that the yield of P_1 was higher than that of P_2, while the effect of P_1 on inhibition of erythrocyte haemolysis was stronger than that of P_2 (Table 2). P_1 was selected for further study. P_2 will be studied in a future experiment.

Various antioxidant compounds in rose flowers, such as flavonoids, hydrolysable tannins and catechin derivatives have been reported (Kleszczynska et al 1999). All of these compounds possess a small molecular weight. Both fractions $P_{1\text{-a}}$ and $P_{1\text{-b}}$ demonstrate antioxidant activity and their molecular weights are relatively high. The carbohydrate content of $P_{1\text{-a}}$ is 66.83% and its protein content is 7.19%. Hence $P_{1\text{-a}}$ is a polysaccharide–peptide complex. Very few reports on the antioxidant activity of polysaccharide–peptide complexes exist in the literature (Shin et al 2001; Ruperez et al 2002). $P_{1\text{-a}}$ exhibits some antioxidant activity.

The contents of carbohydrate and protein of $P_{1\text{-b}}$ are 12.2% and 11.7%, respectively. $P_{1\text{-b}}$ gives a blue colour after reaction with $K_3Fe(CN)_6/FeCl_3$ and is precipitated with lead acetate (data not shown). The molecular weight of $P_{1\text{-b}}$ is 8 kDa and the NMR spectrum and FTIR spectrum shows that $P_{1\text{-b}}$ is a complex component. It is difficult to infer the structure with NMR and FTIR spectroscopy. So, HPLC-ESI-MS was used to analyse the snippets of $P_{1\text{-b}}$. The $K_3Fe(CN)_6/FeCl_3$-spray test and $Pb(CH_3COO)_2$ precipitation test reveal that $P_{1\text{-b}}$ possesses some chemical properties of condensed tannin.

Compared with $P_{1\text{-a}}$, $P_{1\text{-b}}$ has stronger antioxidant activity in inhibiting erythrocyte haemolysis and enhancing SOD activity in erythrocytes. But their effects on inhibiting lipid peroxidation in mouse brain and kidney are both weak, while P_1 has strong inhibiting ability. It has been shown that crude polysaccharide extracts exhibit stronger antioxidant activity than a purified polysaccharide fraction because crude extracts are identified as being rich in antioxidants (e.g. carotenoids, riboflavin, ascorbic acid, thiamine, nicotinic acid) (Luo 2004). In this study, P is a crude polysaccharide preparation prepared by ethanol precipitation. As it undergoes purification, components with antioxidative activity, such as anti-haemolytic activity, are removed by processes such as dialysis. This probably accounts for the reduction in antioxidant activity upon further purification. P_1 is composed of $P_{1\text{-a}}$ and $P_{1\text{-b}}$ and its stronger inhibiting effect may be due to the combined action of $P_{1\text{-a}}$ and $P_{1\text{-b}}$ (e.g. synergism between $P_{1\text{-a}}$ and $P_{1\text{-b}}$). Though the cruder fractions exhibit stronger antioxidant activity, the study of purified fractions may facilitate elucidation of the mechanism of action.

The importance of these findings is that they demonstrates the existence of multiple antioxidant components in dried rose flowers, Chinese medicinal material that allegedly can beautify the skin after consumption. The present observation about the antioxidant activity of the polysaccharide–peptide complex has only been infrequently reported in the literature (Shin et al 2001; Ruperez et al 2002).

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