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Full Length Research Paper

Influence of preparation methods on the yield, components and antioxidant activities of polysaccharides from *Palmaria palmata*

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The aim of this study was to clarify the influences of extraction methods and ethanol concentrations of precipitation on the extraction ratios, components and antioxidant activities of polysaccharides from *Palmaria palmate*. Based on the DPPH/hydroxyl radicals scavenging tests, the order of antioxidant activities of extracts was A75 (microwave-assisted extraction (MAE) with 75% ethanol precipitation) > A0 (MAE without precipitation) > B75 (hot reflux extraction (HRE) with 75% ethanol precipitation) > B0 (HRE without precipitation). A75 was purified by anion-exchange chromatography and three fractions (F1 to F3) were obtained, and their antioxidant activities decreased in the order: F3 > F2 > F1. But F4 (the lyophilized supernatant of 75% ethanol precipitation of A0) showed the strongest activities among the crude polysaccharides and their fractions. The weaker activities of B0 than A0 (purity was 50.97 ± 0.86% and the content of F3 was 23.21 ± 0.88%) was due to the coactions of lower purity (39.55 ± 0.70%), total lack of F3, and the stronger activity of A75 was owed to the higher purity (60.31 ± 1.42%). The results indicated that MAE with ethanol precipitation possessed higher selectivity for polysaccharides from *P. palmate* than HRE and it was necessary to attach importance to study the bioactivities of minor components.

Key words: *Palmaria palmata*, polysaccharides, microwave-assisted extraction, heat-reflux extraction, ethanol precipitation, antioxidant activity.

INTRODUCTION

Oxidative stress *in vivo* or *in vitro* may cause many degenerative or pathological processes and deterioration of various food products, which are mainly due to the excessive free radicals and unbalanced mechanisms of antioxidant protection (Babizhayev and Costa, 1994; Das et al., 1997; Moskovitz et al., 2002; Smith et al., 1996). So, antioxidants and their chemical properties are the subjects of avid research and development. In order to develop a variety of secure natural antioxidants, many natural products with antioxidant activity were isolated from fruits and vegetables (Duan and Sun, 2012; Chowdhury et al., 2012; Prommuak et al., 2008; Sun et al., 2008; Wang et al., 2009; Wei et al., 2012; Zhang et al., 2003; Zhu et al., 2008), in which, polysaccharides from seaweeds had attracted considerable attentions (Cho et al., 2011; Hu et al., 2001; Rocha de Souza et al., 2007; Ruperez et al., 2002; Xue et al., 2001).

There are various ways to extract polysaccharides from natural materials, such as microwave-assisted extraction (MAE), hot reflux extraction (HRE) (Anju et al., 2011; Robic et al., 2009), enzymic and ultrasonic methods. At present, many researches have reported the application of MAE for polysaccharides extraction from plants due to the advantages of high purity, considerable reduction in time and solvent (Hayat et al., 2009; Wakte et al., 2011; Xiao et al., 2008). Precipitation is a universal technique for isolation of polysaccharides from crude extract (Wang et al., 2005; Yang et al., 2008). Majority of reports on precipitation were using ethanol to final concentration of 75% (v/v) (Chung et al., 1982; Zhao et al., 2008), and only a few reports performed precipitation using different

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concentrations of ethanol, such as 50, 70 and 80% (McCleary, 2007; Xia et al., 2010). However, there is no comprehensive answer about whether MAE and 75% ethanol is the optimal preparation method when the antioxidant activities of polysaccharides are considered.

Seaweeds are ubiquitous components of diets in Asia, particularly in China, Japan, Korea and Indonesia. In the past decades, excessive studies had indicated that seaweed polysaccharides showed remarkable physiological and pharmacological activities, such as antivirus, anticancer, anti-inflammation and antioxidant activities (Rocha de Souza et al., 2007; Wang et al., 2010).

Palmaria palmate is a kind of red seaweed that grows attached to rocks by a “holdfast” in the North Atlantic and Northwest Pacific. It was commonly used in Ireland and Atlantic Canada both as food and drug, and is shipped around the globe at present. In the experiment, the crude polysaccharides of *P. palmate* were extracted by MAE and HRE, and fractionated by ion-exchange chromatography. The worth noting was that the influence of different preparation techniques on the yield and antioxidant activities of polysaccharides was analyzed.

MATERIALS AND METHODS

Chemicals

α, α-Diphenyl-β-picrylhydrazyl (DPPH) and 1, 10-Phenanthroline monohydrate were purchased from Aladdin Reagent Database Inc. (Shanghai, China). DEAE-cellulose-52 was purchased from Pharmacia Fine Chemicals (Sweden). All other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Samples of *P. palmate*

P. palmata was purchased from KunshanYihong Seaweeds Co., Ltd. (Jiangsu, China), and authenticated by Professor Sheng-long Zhao (Zhejiang Ocean University, Zhoushan, China). The voucher specimen (No. DC0154) was deposited in School of Food and Pharmacy, Zhejiang Ocean University. The dried *P. palmata* was powdered and degreased by aether at solid-to-liquid ratio of 1:10 for follow-up test.

Preparation of samples by different extraction techniques

MAE was performed on XH-100A microwave extraction apparatus (Bejing Xiang-Hu Technology Development Co., Ltd.). The degreased powder (1.00 g) was extracted at the condition of microwave power of 500 W, material-water ratio of 1:70, extraction time of 10 min and extraction temperature of 70°C. The extract was separated from the residue by centrifugation (4000 rpm for 15 min) and concentrated by rotary evaporation under reduced pressure (50°C, 50 rpm, -0.095 Mpa). The concentrate was repeatedly extracted with Sevag reagent ($CHCl_3$-nBuOH with v/v =4:1) (50 ml × 3) to remove free proteins (Navarini et al., 1999; Nie et al., 2008). After removing the Sevag reagent, the extract was directly freeze-dried and named as A0. The same extracts were precipitated by ethanol of different final concentrations of 70, 75, 80 and 90%, centrifuged (4000 rpm for 15 min) and freeze-dried to obtain the samples of A70, A75, A80 and A90, respectively. The supernatant of ethanol solution was condensed and freeze-dried to obtain the fractions of F4.

HRE was conducted in a boiling water bath. The degreased powder (1.00 g) was placed into a 250 ml glass flask with 70 ml distilled water and extracted for 3 h. The concentrate was repeatedly extracted with Sevag reagent (Navarini et al., 1999; Nie et al., 2008) to remove free proteins. After removing the Sevag reagent, the extract was directly condensed and freeze-dried to yield the sample B0. The extracts were precipitated by 70, 75, 80 and 90% ethanol to gain the samples of B70, B75, B80 and B90, respectively.

Purification of the crude polysaccharide by anion-exchange chromatography

All the crude polysaccharide samples were pre-fractionated by anion-exchange chromatography. 50.0 mg of crude polysaccharides were dissolved in 10 ml distilled water and separated through DEAE-52 cellulose column (1.6 × 60 cm). Fractions were prepared at a flow rate of 1.0 ml/min by stepwise elution with distilled water, 0.1, 0.5 and 1.0 M NaCl solution, and the elution volume of each concentration was 150 ml. Every 5 ml of eluted solution was collected and detected by phenol-sulfuric acid method. The same fractions were pooled, dialyzed, concentrated and lyophilized, and three fractions (F1 to F3) were obtained.

Determination of total polysaccharides content

Total sugar content was determined by phenol-sulfuric acid method using glucose as the standard, at 490 nm (Navarini et al., 1999). The percentage of polysaccharides yield (%) was calculated as the polysaccharides content of extraction divided by dried pretreated sample weight (1.00 g).

DPPH radical-scavenging assay

The DPPH radical-scavenging activity of samples was monitored according to previous report (Luo et al., 2010). Briefly, 2.0 ml aliquot of test sample (in methanol) was added to 2.0 ml of 0.16×10^{-3} M DPPH methanol solution. The mixture was eddied for 1 min and then left to stand at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm. The scavenging effect (%) was calculated by using the follow equation:

$$\text{Scavenging effect (\%)} = [1 - (A_{sample} - A_{sample\ blank})/A_{control}] \times 100$$

Where the $A_{control}$ was the absorbance of the control (DPPH solution without sample), the A_{sample} was the absorbance of the test sample (DPPH solution plus test sample), and the $A_{sample\ blank}$ was the absorbance of the sample only (sample without DPPH solution).

Hydroxyl radical-scavenging assay

The hydroxyl radical-scavenging activity of samples was measured according to the method of Luo et al. (2010). Briefly, 1, 10-phenanthroline solution (1.0 ml, 1.865×10^{-3} M), phosphate buffer saline (2.0 ml, 0.2 M, pH 7.40), and samples (1.0 ml, 30 μM) were added into a screw-capped tube orderly and mixed homogeneously. The $FeSO_4 \cdot 7H_2O$ solution (1.0 ml, 1.865×10^{-3} M) was then pipetted into the mixture. The reaction was initiated by adding 1.0 ml H_2O_2 (0.03%, v/v). After incubated at 37°C for 60 min in a water bath, the absorbance of reaction mixture was measured at 536 nm against reagent blank. The reaction mixture without any

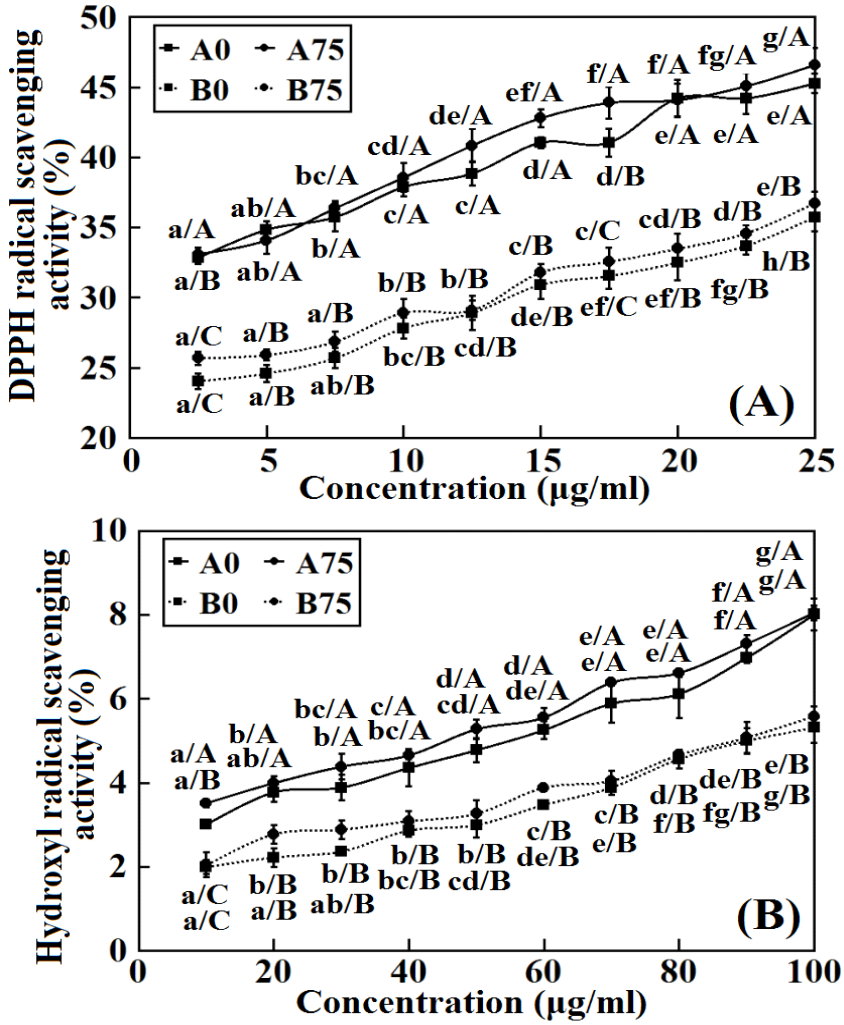

Figure 1. Scavenging activities of different samples on DPPH (A) and hydroxyl (B) radicals. All the values were mean ± SD; SD: Standard deviation; *N* = 3. (A to G) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$) in the same sample. (A to C) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$) in the same concentration.

antioxidant was used as the negative control, and the mixture without H_2O_2 was used as the blank. The hydroxyl radical-scavenging activity (HRSA) was calculated by the following formula:

$$HRSA\ (\%) = [(A_s-A_n)/(A_b-A_n)] \times 100$$

where A_s, A_n, and A_b were the absorbance values determined at 536 nm of the sample, the negative control, and the blank after reaction, respectively.

Statistical analysis

All experiments were performed in triplicate (n = 3), and ANOVA test (using SPSS 13.0 statistical software, SPSS Inc., Chicago, USA) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using LSD tests ($p < 0.05$).

RESULTS AND DISCUSSION

Comparison of the antioxidant activities of the crude polysaccharides by different extraction techniques

The antioxidant activities of the crude polysaccharides at the concentrations of 2.5 to 25 μg/ml and 10 to 100 μg/ml were detected using the methods of DPPH radical-scavenging and hydroxyl radical-scavenging, respectively. As shown in Figure 1, A75 showed the best DPPH and hydroxyl radical-scavenging activities. For DPPH radical, the scavenging rates varied between 33.08 ± 0.46 and 46.58 ± 1.24%, and for hydroxyl radical, the scavenging rates ranged from 3.52 ± 0.09 to 8.05 ± 0.17%. In other words, the crude polysaccharides, extracted from *P. palmata* by MAE and precipitated with 75% ethanol,

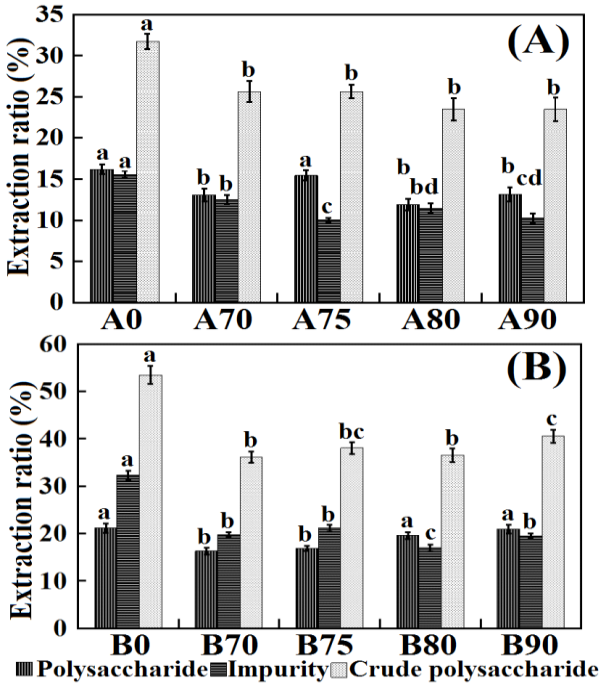

Figure 2. Comparison of the extraction ratios of crude polysaccharides and impurity at different ethanol concentrations by MAE (A) and HRE (B). All the values were mean ± SD; SD, standard deviation; N = 3. (A to C) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$).

had the best antioxidant activity among these four samples, and the following one was A0. However, the gap of the antioxidant activities between A75 and A0 was small. B0 and B75 showed weaker antioxidant activities than their counterparts. But the differences of antioxidant activities between A0 and B0 or A75 and B75 were apparent. The results indicated that it was appropriate to extract the crude polysaccharides by MAE precipitated with 75% ethanol for guaranteeing the best antioxidant activity of the crude polysaccharides from *P. palmata.*

Comparison of the content and purity of the crude polysaccharides by different extraction techniques

The purpose of the experiment was to explore the extraction and purification methods of the antioxidant polysaccharides from *P. palmata*. Figure 2A showed the extraction ratio of the crude polysaccharides declined with the increase of ethanol concentration. The extraction ratio of the crude polysaccharides was 23.50 ± 1.41% by using MAE with 90% (v/v) ethanol precipitation, but the ratio reached 31.80 ± 0.94% when ethanol precipitation was eliminated. Meanwhile, if the extraction ratio of the polysaccharides was considered as the only index, extracting polysaccharides without ethanol precipitation also gained the highest extraction ratio (16.21 ± 0.55%) than those obtained with precipitations (the extraction ratios of polysaccharides were 13.15 ± 0.76, 15.47 ± 0.62, 11.99 ± 0.69 and 13.22 ± 0.85% with precipitations of ethanol at concentrations of 70, 75, 80 and 90%, respectively). The same results also revealed in Figure 2B. More quantity of crude polysaccharides was obtained (53.48 ± 1.91%) using HRE without precipitation than those with precipitations. The extraction ratio of polysaccharides by using HRE without precipitation was 21.15 ± 0.96%, which was higher than the ratio (20.97 ± 0.93%) by using HRE with 90% ethanol precipitation. It was obvious that part components of polysaccharides were lost by using ethanol precipitation. Thus, it was better to choose the extraction method without precipitation for obtaining the whole polysaccharides from raw material, including *P. palmata*.

However, extraction of polysaccharides without precipitation led to more impurity, and this phenomenon was more distinct when using the extraction method of HRE than MAE. The extraction ratio of impurity by using MAE without precipitation was 15.59 ± 0.39%, which was higher than those by using MAE with precipitation. The impurity ratios reduced to 12.55 ± 0.54, 10.08 ± 0.23, 11.51 ± 0.63 and 10.28 ± 0.57% when the ethanol concentrations of precipitations were 70, 75, 80 and 90%, respectively. The extraction ratio of impurity was 32.33 ± 0.95% by using HRE without precipitation. Therefore, the crude polysaccharides, extracted by using MAE or HRE without precipitation, showed the lowest purities.

The purities of crude polysaccharide by using MAE were 50.97 ± 0.86, 51.17 ± 0.53, 60.31 ± 1.42, 51.02 ±1.17 and 56.26 ± 1.01% when the ethanol concentrations of precipitations were 0, 70, 75, 80 and 90%, respectively (Figure 4). So the larger quantity and higher purity of polysaccharides was gained by using MAE with 75% ethanol precipitation. In accordance with many other reports (Chung et al., 1982; Zhao et al., 2008), the optimal ethanol concentration for precipitation of crude polysaccharides was very 75%. But the purity of the crude polysaccharides by using HRE with 80% ethanol precipitation reached 53.56 ± 0.76%, which was higher than those (39.55 ± 0.70, 45.14 ± 0.93, 44.45 ± 1.02 and 51.75 ± 1.42%) by using HRE with 0, 70, 75 and 90% ethanol precipitations, respectively. The finding indicated that the optimal ethanol concentration for precipitation was related to the experimental methods and 75% ethanol was not simply the best concentration for precipitation of polysaccharides in any case. Finally, as shown in Figure 3, the method of MAE guaranteed higher purities of the crude polysaccharides than HRE.

Comparison of the components of the crude polysaccharides by different extraction methods using DEAE-52 cellouse column

The crude polysaccharides were separated by using

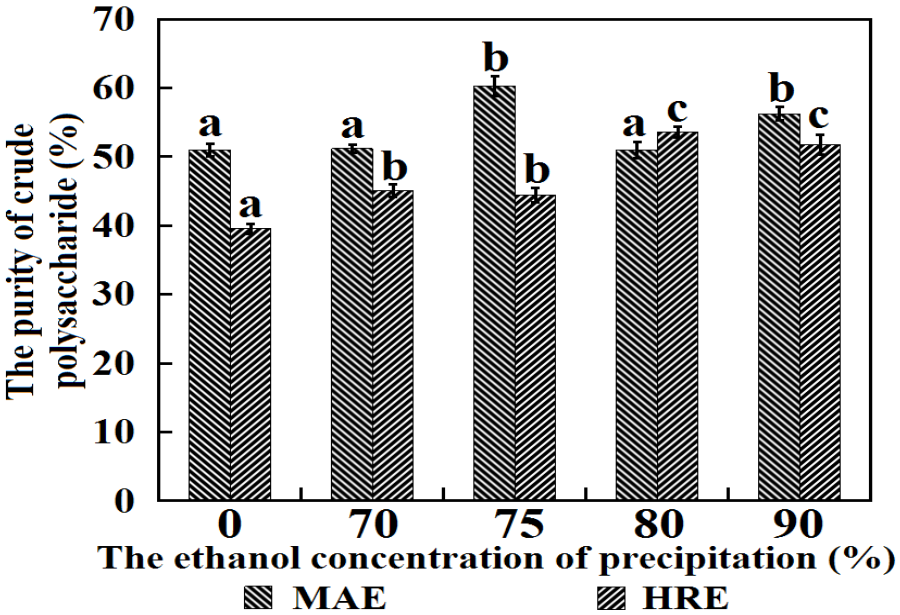

Figure 3. Comparison of the purities of crude polysaccharides on different extraction techniques. All the values were mean ± SD; SD, standard deviation; *N* = 3. (a-c) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$).

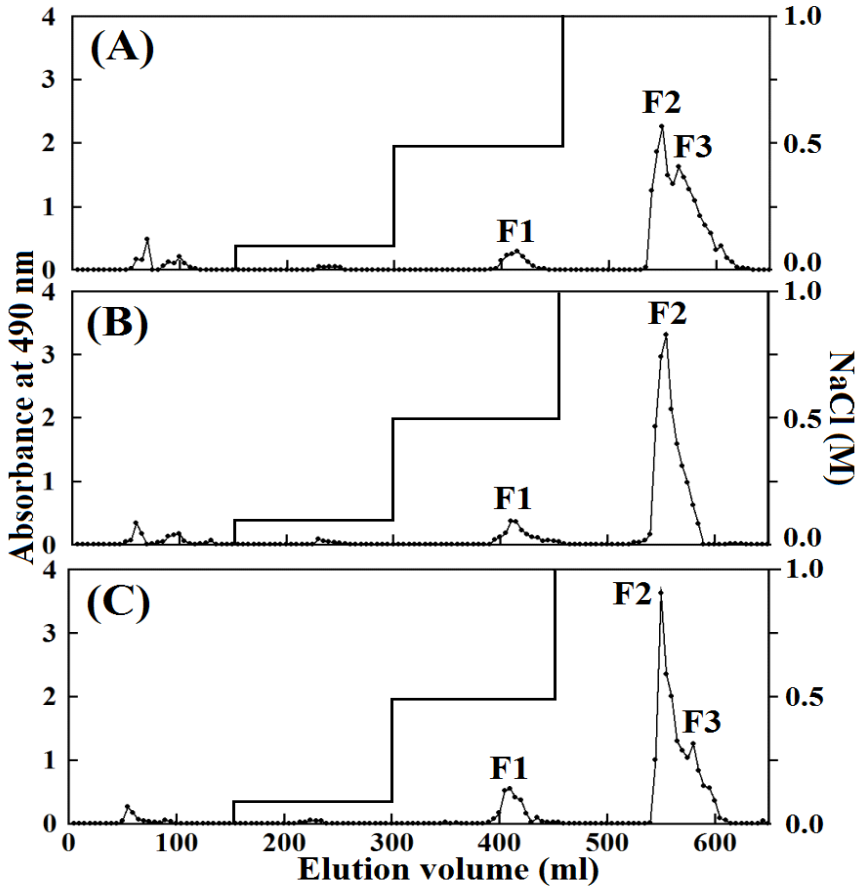

Figure 4. Elution profiles of A0 (A), B0 (B) and A75 (C) from *P. palmata* by DEAE-52 cellouse ion-exchange chromatography.

Table 1. Comparison of the proportions of different fractions by different extraction methods.

Extraction method	Percentage content of crude polysaccharide (%)				
	F1	F2	F3	Others	Total
MAE without precipitation	3.32 ± 0.15$^{a/A}$	18.79 ± 0.23$^{b/A}$	23.21 ± 0.88$^{c/A}$	5.65 ± 0.08$^{d/A}$	50.97 ± 1.35$^{e/A}$
HRE without precipitation	3.98 ± 0.18$^{a/A}$	31.73 ± 0.74$^{b/B}$	0.00 ± 0.00$^{c/B}$	3.84 ± 0.30$^{a/B}$	39.55 ± 1.22$^{d/B}$
MAE with precipitation	7.25 ± 0.47$^{a/B}$	34.94 ± 1.01$^{b/C}$	12.44 ± 0.62$^{c/C}$	5.68 ± 0.38$^{a/A}$	60.31 ± 2.48$^{d/C}$

All the values were mean ± SD; SD, standard deviation; *N* = 3. (A to E) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$) in the same row. (A to C) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$) in the same column.

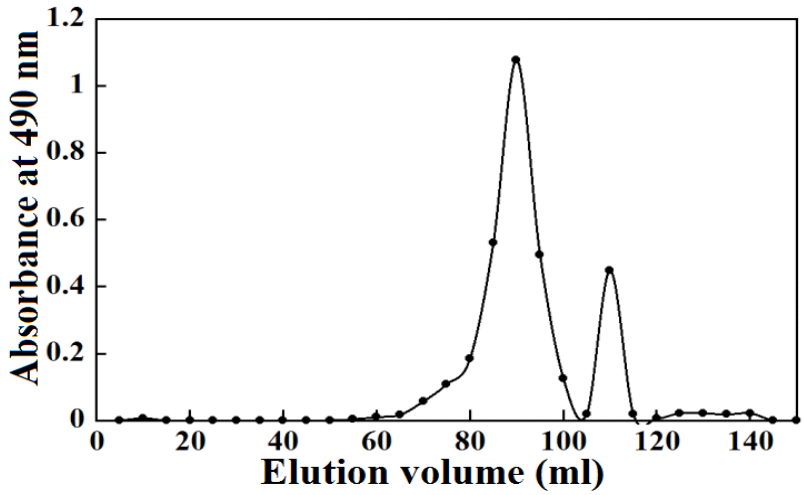

Figure 5. Elution profile of the lyophilized supernatant of ethanol precipitation from *p. palmata* by DE-52 ion-exchange chromatography.

DEAE-52 cellouse column. Chromatographic elution profiles of A0 and B0 were shown in Figures 4A and B. Three main fractions were obtained from the crude polysaccharides obtained by using MAE without precipitation: F1 from 0.5 M NaCl elution, F2 and F3 from 1.0 M NaCl. Based on the weight, F1, F2 and F3 accounted for about 3.32 ± 0.15, 18.79 ± 0.23 and 23.21 ± 0.88% of the crude polysaccharides, respectively, and the latter two fractions were deemed as the key components of A0 (Table 1). But only two main fractions were obtained from the crude polysaccharides by using HRE without precipitation: F1 from 0.5 M NaCl elution and F2 from 1.0 M NaCl. Based on the weight, F1 and F2 accounted for about 3.98 ± 0.18 and 31.73 ± 0.74% of crude polysaccharides, and F2 was considered as the key component of B0 (Table 1).

Comparison of the components of the crude polysaccharides precipitated at different ethanol concentrations by using DEAE-52 cellouse column

Using DEAE-52 cellouse column, the chromatographic elution profile of A75 was shown in Figure 4C. Comparing Figures 4A with 4C, there were three same main fractions in the two figures. However, their contents in the crude polysaccharides changed significantly (Table 1). F2 became the major component and the content by weight increased from 18.79 ± 0.23 to 34.94 ± 1.01% when the crude polysaccharides were gained with 75% ethanol precipitation. By comparison, the content of F3 declined in the crude polysaccharides. So the results indicated that the compositions of the crude polysaccharides from *P. palmata* were affected by the extract method with or without precipitation. The composed fractions of the crude polysaccharides did not change, but there was a dramatic change in the contents of each fraction. This might be caused by the partial loss of F3 fraction when the precipitation method was used in the preparation process of crude polysaccharides.

As aforementioned, ethanol precipitation caused a loss of polysaccharides. To expatiate this point clearly, the fractions of the crude polysaccharides, abandoned by precipitation, was prepared. The supernatant of ethanol solution was separated from precipitates by centrifugation condensed and freeze-dried. As showed in Figure 5, the lyophilized supernatant (F4) was composed of two main components.

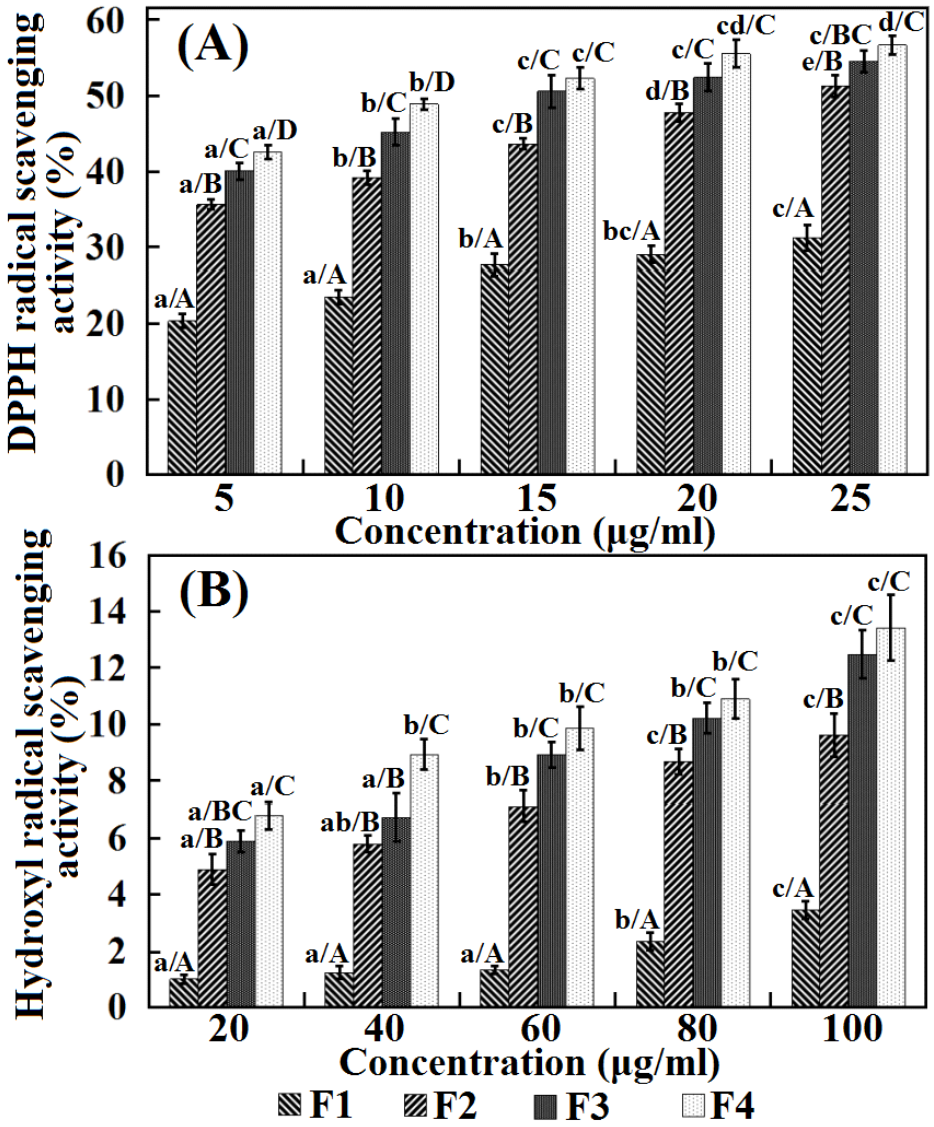

Figure 6. Scavenging activities of different samples on DPPH (A) and hydroxyl (B) radicals. All the values were mean ± SD; SD, standard deviation; *N* = 3. (A to E) Column wise values with same superscripts of this type indicated no significant difference ($p > 0.05$) in the same sample. (A to C) Column wise values with same superscripts of this type indicated no significant difference ($P > 0.05$) in the same concentration.

Comparing the antioxidant activities of the polysaccharides components

The antioxidant activities of each component (F1, F2, F3 and F4) of the polysaccharides from *P. palmata* were tested. As shown in Figure 6, the radical-scavenging activities of four components increased with increased concentration, and their activities were listed in a decreasing order as followed: F4 > F3 > F2 > F1. The result indicated that F4 had the strongest antioxidant activity. At the concentrations of 5, 10, 15, 20 and 25 μg/ml, DPPH radical-scavenging activities of F4 were 42.62 ± 0.93, 48.93 ± 0.70, 52.37 ± 1.46, 55.63 ± 1.80 and 56.78 ± 1.24%, respectively; and at the concentrations of 20, 40, 60, 80 and 100 μg/ml, hydroxyl radical-scavenging activities of F4 were 6.78 ± 0.46, 8.92 ± 0.54, 9.86 ± 0.77, 10.89 ± 0.70 and 13.42 ± 1.17%, respectively. But, due to the limited quantity, its contribution to the crude polysaccharide could be ignored. However, it was true that some components of the polysaccharides, which had wonderful antioxidant activity, might be abandoned when ethanol precipitation was used during extraction process. The main components of F3 in the crude polysaccharides of A75 and A0 had a better antioxidant activity than F2, and the result revealed that the high antioxidant activities of the crude polysaccharides extracted by MAE were due

to the high ratio of F3. The finding indicated that MAE possessed higher selectivity for antioxidant polysaccharides from *P. palmate* than HRE.

Conclusion

It was obvious that different extraction techniques resulted in different impacts, which was confirmed by acquisition of different components with different antioxidant activities from the crude polysaccharides of *P. palmate*. The extraction yields of the polysaccharides by MAE and HRE were similar. However, the purity and antioxidant activity of the crude polysaccharides obtained by MAE were higher than those of HRE. The main reason was that the polysaccharides obtained by HRE lost the highly active fraction of F3. On the other hand, it was clear that the polysaccharides, which were extracted with the additional process of ethanol precipitation, always had higher purities on the expense of some polysaccharides components. The A75 chromatograms of DEAE-52 ion-exchange clarified that F4 (the lyophilized supernatant from the 75% ethanol precipitation of A0) was lost entirely and F3 fraction was abandoned partially. It was worth to mention that F4 had the strongest antioxidant activity among the crude polysaccharides and their fractions, but it was usually discarded during the prepared process due to the low content. The finding suggested that it was necessary to attach importance to study the biological activity of minor components.

In present experiment, different extraction methods brought different polysaccharides with different antioxidant activities, which might be due to the structure-extraction relationships of them. Therefore, clarifying the influence of extraction methods on the structures of polysaccharides from *P. palmata* and the structure-activity relationships of them will be our new direction in the future.

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Abbreviations: MAE, Microwave-assisted extraction; **HRE,** hot reflux extraction; **A75,** MAE with 75% ethanol precipitation; **A0**, MAE without precipitation; **B75,** HRE with 75% ethanol precipitation; **B0,** HRE without precipitation; **DPPH,** α, α-diphenyl-β-picrylhydrazyl.

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