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Antioxidant properties of different molecular weight polysaccharides from *Athyrium multidentatum* (Doll.) Ching

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ABSTRACT

Different molecular weight polysaccharides were prepared by degradation of polysaccharides extracted from *Athyrium multidentatum* (Doll.) Ching rhizome (CPA) with hydrogen peroxide and ascorbic acid. Four low molecular polysaccharides derivatives (CPA-1, CPA-2, CPA-3 and CPA-4) were successfully obtained and had their antioxidant activities investigated employing various established in vitro systems. All CPA derivatives showed pronounced antioxidant activity, and had stronger antioxidant ability than CPA in certain tests. CPA-1 exhibited the strongest scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical among all samples, and the IC_{50} value was 25 μg/mL. CPA-2 possessed the highest scavenging ability against superoxide radical at 200 μg/mL. The scavenging activity of CPA-4 on hydroxyl radical was higher than CPA from 120 to 200 μg/mL. The mechanism on influence the antioxidant activity of CPA and its degraded derivatives was indicated.

1. Introduction

Reactive oxygen species (ROS) in the forms of superoxide anion radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) are highly reactive molecules in living systems, and their uncontrolled generation correlates directly with molecular level of many diseases (Lee & Lee, 2006). Antioxidants play an important role in the biological system against ROS. They can decrease oxidative damage by reacting directly with free radicals, or by enhancing the activity and expression of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Lü, Lin, Yao, & Chen, 2010). However, the synthetic antioxidants such as butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) are not preferred for pharmaceutical and food industries for the toxicological concerns. Therefore, considerable interests have focused on nontoxic and highly active phytochemical antioxidants, such as plant pigments and secondary metabolites.

Pteridphyta, a species of cryptogam with multifarious germplasm and metabolite, is widely distributed in tropical and subtropical area. *Athyrium multidentatum* (Doll.) Ching (AMC) belongs to the family Athyriaceae, which is native to Northeast China. It is 40–100 cm height with short rhizome and ovate-lanceolate leaf blade, and grows near the boundary strip of mountains and forest at an altitude of 300–500 m. Its spear is a unique and nutritious potherb in Changbai Mountain area. Furthermore, the plant is used as an unexplored folk medicine for conditions such as high blood pressure, anxiety and arthritis. Polysaccharide extracted from AMC is a neutral heteropolysaccharide, mainly made of mannose (Man), rhamnose (Rha), glucose (Glc), galactose (Gal) and arabinose (Ara) (Liu et al., 2013). Our preliminary study had shown that the polysaccharide was active as an antioxidant, being strong in scavenging superoxide radical (Liu et al., 2011). Evidence had proved that the molecular weight distributions of polysaccharides had great influence on their biological activities. Conceptually, high molecular weight polysaccharides are less active than low molecular weight ones due to their poor penetration capability on cell-membranes, degradation of high molecular weight polysaccharides might noticeably improve their biological activities (Wu, Zheng, Ning, & Yang, 2007). Zhou, Hu, Wu, Pan, and Sun (2008) reported the low molecular weight fraction from *Sargassum fusiforme* increased the antioxidant activity. Furthermore, our earlier study suggested that polysaccharide fraction with low molecular weight from AMC showed strong hydroxyl radical scavenging activity at high concentrations (Liu et al., 2013). However, the antioxidant activity of degraded polysaccharides from AMC yet remained unclear.

In this study, antioxidant activities of degraded derivatives of polysaccharides extracted from AMC were investigated, including

Abbreviations: AMC, *Athyrium multidentatum* (Doll.) Ching; ROS, reactive oxygen species; CPA, polysaccharides extracted from *Athyrium multidentatum* (Doll.) Ching rhizome; H_2O_2, hydrogen peroxide; TCA, trichloroacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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superoxide, hydroxyl radical and DPPH scavenging activity, and reducing power. The in vitro antioxidant activity and the relationship between chemical property and antioxidant activity were characterized in this paper.

2. Materials and methods

2.1. Materials

A. multidentatum (Doll.) Ching rhizome was collected from Changbai Mountain area of Jilin Province, China (September 2012). The fresh rhizome was air-dried, crushed and kept in plastic bags at room temperature for use. Hydrogen peroxide (H_2O_2, 30%, v/v), 1,1-diphenyl-2-picrylhydrazyl (DPPH), pyrogallol, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and purchased from China National Pharmaceutical Group Co.

2.2. Preparation of crude polysaccharides

AMC rhizome (800 g) was soaked in methanol at room temperature for 10 d. The methanol was filtered off and the residues were refluxed in 6400 mL distilled water for 3 h. The hot solution was collected and concentrated to about 100 mL under reduced pressure, then dialyzed against tap water for 48 h and against distilled water for 24 h using 3500 Da Mw cutoff dialysis membranes. Finally, the solution was concentrated and precipitated with anhydrous ethanol. The resultant precipitate was washed three times with anhydrous ethanol and dried at 60 °C to give AMC rhizome polysaccharides (CPA, mean yield 3.2%).

2.3. Degradation of CPA

CPA was degraded by hydrogen peroxide and ascorbic acid. In brief, CPA (0.5 g) was dispersed in 100 mL distilled water, then hydrogen peroxide (1 mM) and ascorbic acid (1 mM) were introduced directly into the solution. The reactive mixture was stirred at room temperature for 4 h, and dialyzed against tap water for 48 h and against distilled water for 48 h using 300 Da Mw cutoff dialysis membranes. Finally, the resultant was concentrated and lyophilized to give degraded polysaccharides. Other degraded polysaccharide derivatives were prepared by adding increased proportions of hydrogen peroxide (2, 3, 5, 10 and 20 mM) and ascorbic acid (2, 3, 5, 10 and 10 mM), separately.

2.4. Analytical methods

Total sugar content was determined by the phenol–sulfuric acid colorimetric method, using glucose as standard. Sulfate content was analyzed by the barium chloride–gelatin method of Kawai, Seno, and Anno (1969). Infrared spectra were recorded from polysaccharide powders in KBr pellet on a Fourier-transform infrared spectrophotometer (AVATAR360, USA) in the frequency range 4000–500 cm^{-1}. Molecular weights were determined on a high performance liquid chromatograph (SHIMADZU LC-20A, Japan) with a gel-filtration chromatographic column of TSK-GEL G3000PW^{XL} (TOSOH, Japan). The column temperature was maintained at 40 °C, and the mobile phase was 2.84% Na_2SO_4 buffer with a flow rate of 0.5 mL/min. All the samples were filtered through 0.45 μm filter membrane before analyzed. The detection was carried out at 35 °C with a refractive index detector (RID).

2.5. Antioxidant activity assays

2.5.1. Reducing power assay

The reducing power was determined using a modified method of Yamaguchi, Takamura, Matoba, and Terao (1998). 0.5 mL of different concentration of samples (40–200 μg/mL) was mixed with 1 mL phosphate buffer (0.2 M, pH 6.8) and 2 mL potassium ferricyanide (1%, w/v). The mixture was incubated at 50 °C for 20 min, then added 1 mL of TCA (10%, w/v) and 0.5 mL of ferric chloride (0.1%, w/v). The reacting solution was shaked vigorously and left stillness for 10 min. Increased absorbance at 700 nm indicated increased reducing power.

2.5.2. Superoxide radical scavenging assay

The scavenging activity of the superoxide radical was assayed according to a slightly modified method of Zhang, Gong, Meng, Sun, and Gao (2010). Superoxide radicals were generated in the pyrogallol autoxidation system. Briefly, 4 mL of Tris–HCl buffer (50 mM, pH 8.2) and 1 mL of varying concentrations of samples (40–200 μg/mL) were incubated at 37 °C for 10 min, then added 0.5 mL of pyrogallol (50 mM) pre-incubated at 37 °C. After reacted at room temperature for 6 min, the reaction was terminated by 0.5 mL HCl (8 M). The absorbance was measured at 320 nm against the blank. In the control, Tris–HCl buffer was substituted for samples. The superoxide radical scavenging activity was calculated by the following equation:

$$\text{Scavenging effect}\,(\%) = \left(1 - \frac{A_{\text{sample}\,320}}{A_{\text{control}\,320}}\right) \times 100 \quad (1)$$

2.5.3. Hydroxyl radical scavenging assay

Measurement of hydroxyl radical scavenging activity was determined by an improved method of Cao, Li, and Wang (2008). Concretely, 1 mM H_2O_2 (1 mL), 2 mM ferrous sulphate (1 mL), and 6 mM salicylate (1 mL) were mixed and incubated at 37 °C for 15 min. Then 1 mL of different concentration of samples (40–200 μg/mL) was added and allowed to stand at room temperature for 5 min. Hydroxyl radical was detected at 520 nm. In the control, distilled water was substituted for the samples. Lower absorbance of the mixture indicated higher scavenging activity. The hydroxyl radical inhibition percentage was calculated according to the following formula:

$$\text{Scavenging effect}\,(\%) = \left(1 - \frac{A_{\text{sample}\,520}}{A_{\text{control}\,520}}\right) \times 100 \quad (2)$$

2.5.4. DPPH free-radical scavenging assay

DPPH free-radical scavenging activity was measured according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). Briefly, a 0.14 mmol/L solution of DPPH in anhydrous ethanol (2 mL) was added 2 mL of sample solution at different concentrations (10–50 μg/mL). The mixture was shaken vigorously and reacted at room temperature for 5 min. The absorbance was measured at 517 nm. In the control, sample was substituted with 95% alcohol. The scavenging activity of the DPPH radical was calculated by the following equation:

$$\text{Scavenging effect}\,(\%) = \left(1 - \frac{A_{\text{sample}\,517}}{A_{\text{control}\,517}}\right)) \times 100 \quad (3)$$

2.6. Statistical analysis

All the data are shown in means ± S.D. ($n = 3$) within significance $p < 0.05$ after passing Duncan's multiple-range test, and processed with Excel and Statistica (2003).

Table 1
Chemical composition (%, dry weight) of CPA and its degraded derivatives (CPA-1, CPA-2, CPA-3 and CPA-4).

Sample	Total sugar	Molecular weight (Da)	Sulfate
CPA	80.01	16,894	1.79
CPA-1	84.14	14,528	0.54
CPA-2	70.86	12,370	0.62
CPA-3	62.61	11,548	1.34
CPA-4	52.43	6403	1.15

3. Results and discussion

3.1. Chemical analysis

Four degraded polysaccharides (CPA-1, CPA-2, CPA-3 and CPA-4) were obtained by degradation of CPA with hydrogen peroxide (2, 5, 10 and 20 mM) and ascorbic acid (2, 5, 10 and 10 mM). Two degraded derivatives were rejected for their abnormal molecular weights of 17,758 and 19,399 Da, which were prepared with hydrogen peroxide and ascorbic acid in the molar ratios of 1:1 and 3:3 (mM/mM). The yields of CPA-1, CPA-2, CPA-3 and CPA-4 were 46.48, 42.76, 50.88, and 56.22%, respectively. The chemical properties of all samples are summarized in Table 1. The molecular weights of CPA, CPA-1, CPA-2, CPA-3 and CPA-4 were 16,894, 14,528, 12,370, 11,548 and 6403 Da, and the total sugar contents were 80.01, 84.14, 70.86, 62.61 and 52.43, respectively, which indicated successfully the degradation of CPA. Except CPA-1, the sulfate and total sugar contents of degraded derivatives were lower than CPA. We suppose some vice reactions led to the loss of some sugars and sulfate group in the samples. The antioxidant effect of polysaccharide depends on several structural parameters such as the molecular weight, type of sugar, and glycosidic branching (Melo, Feitosa, Freitas, & de Paula, 2002). As shown in Fig. 1, IR spectra of all samples were similar; it was to say the degradation reaction did little destruction on the main structure of CPA. Therefore, the antioxidant properties of the degraded polysaccharides might primarily be attributed to the total sugar and sulfate contents, and molecular weight, which have been discussed in this research.

3.2. Reducing power

In the reducing power assay, Fe^{3+}/ferricyanide complex was reduced to the ferrous form (Fe^{2+}) by the tested samples. The green and blue test solution was spectrophotometrically determined at 700 nm. The reducing power of CPA and its degraded derivatives is depicted in Fig. 2. Higher absorbance value means stronger reducing power. Except CPA-3, the reducing power of all samples was concentration dependent. The absorbance of CPA, CPA-1, CPA-2, CPA-3 and CPA-4 was 0.734, 0.687, 0.605, 0.429 and 0.420 at 0.2 mg/mL, which was much higher than BHT and those extracts from *Hypsizigus marmoreus* and *Armillaria mellea* (Hazra, Biswas, & Mandal, 2008; Lee, Jian, Lian, & Mau, 2008; Lung & Chang, 2011). CPA exhibited the strongest reducing power among the samples, the reducing power was found to be decreased in the order of CPA > CPA-1 > CPA-2 > CPA-3 > CPA-4 from 40 to 200 μg/mL, which was positively correlated with their molecular weights. It meant that higher molecular weight exhibited stronger reducing power. Schepetkin et al. (2008) found polysaccharides extracted from *Opuntia polyacantha* with high molecular weight possessed the most active macrophage immunomodulatory activity. Leung et al. (2004) reported that biological activity of mannans from *Aloe vera* was correlated with increased size. The reducing power of natural antioxidants is related to their capability of donating electrons. We suppose that the high molecular weight polysaccharide had more repetitive structures and electron donors such as hydroxide groups, which could reduce Fe^{3+} to Fe^{2+}. Our data indicated that the reducing power of all tested samples was a significant indicator of their potential antioxidant activity.

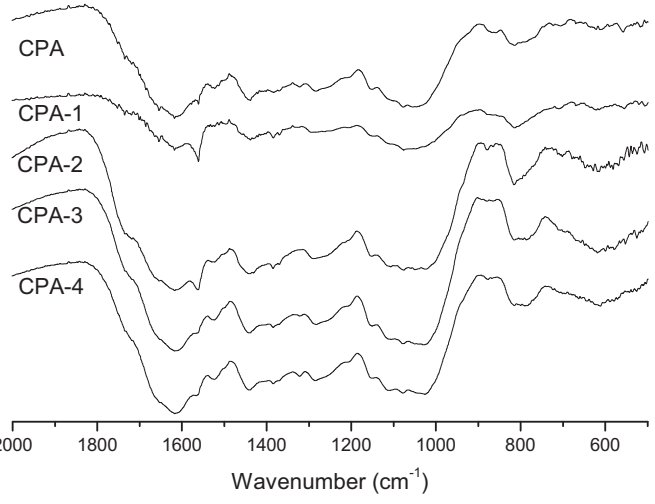

Fig. 1. FT-IR spectra of CPA and its degraded derivatives (CPA-1, CPA-2, CPA-3 and CPA-4).

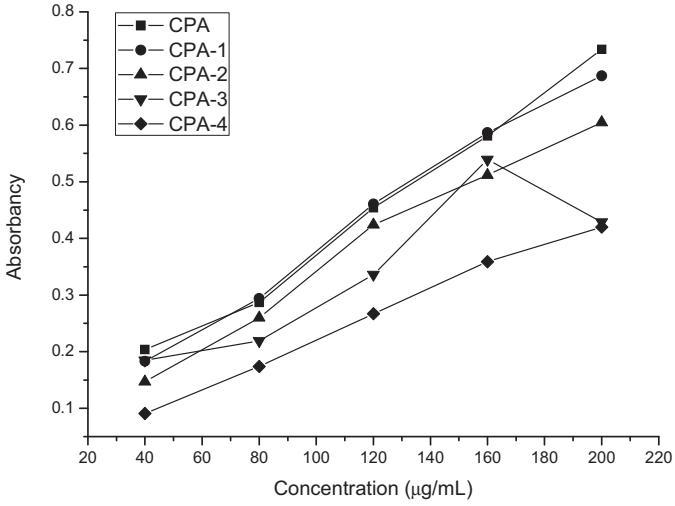

Fig. 2. Reducing power of CPA and its degraded derivatives. Values are means ± S.D. ($n = 3$).

3.3. Superoxide radical scavenging activity

Superoxide anion radical is less reactive and much longer lifetime than other radicals. However, it can take part in further reactions leading to the formation of more reactive oxygen species such as hydroxyl radical and singlet oxygen, and cause tissue damage and various diseases (Wade, Jackson, Highton, & van Rij, 1987). Therefore, the scavenging effect on superoxide radical is an important way to illustrate the mechanism of antioxidant activity. As shown in Fig. 3, the scavenging effects of all samples on superoxide radicals were complicated. CPA-2 possessed the most active scavenging effect on superoxide radicals among the degraded derivatives. The scavenging capability of CPA-2 was 2.97, 3.07, 6.11, 8.21 and 10.71% from 40 to 200 μg/mL, which was in a dose–response pattern. The scavenging effects of CPA-1 and CPA increased at the concentrations greater than 80 and 160 μg/mL, respectively. At the concentration of 200 μg/mL, the inhibitory effects of CPA, CPA-1, CPA-2, CPA-3 and CPA-4 were 9.68, 7.81, 10.71, 8.97 and 8.57%. CPA-1 and CPA-2 presented ascending

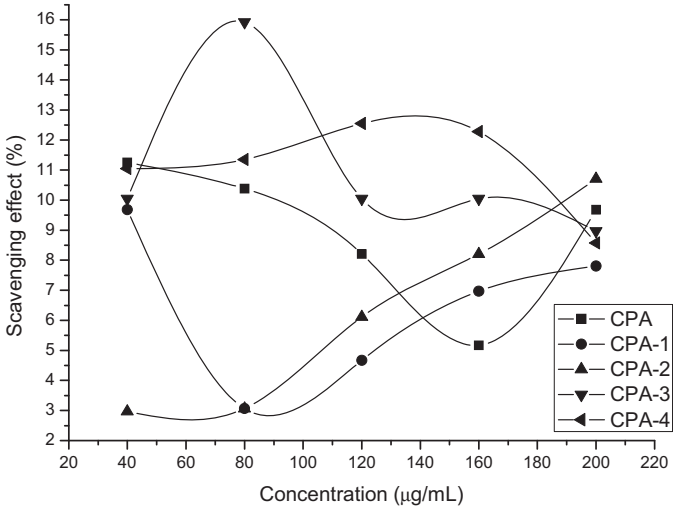

Fig. 3. Scavenging effect on superoxide radicals by CPA and its degraded derivatives. Values are means ± S.D. ($n = 3$).

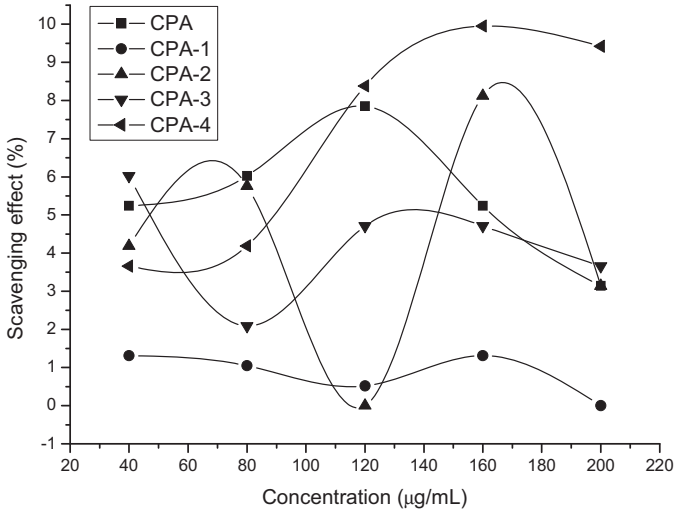

Fig. 4. Scavenging effect on hydroxyl radicals by CPA and its degraded derivatives. Values are means ± S.D. ($n = 3$).

curves at 80–200 μg/mL, oppositely, CPA-3 and CPA-4 exhibited decreased dose–response curves. That was to say, lower molecular weight polysaccharides did not show stronger scavenging activity on superoxide radicals. Total sugar and sulfate content could affect the scavenging capability of the samples against superoxide radicals. Wang, Zhang, Zhang, Zhang, and Li (2009) found oversulfated fucoidan had much greater antioxidant than fucoidan. Sulfation derivatives of neutral *Auricularia auricular* polysaccharides had much stronger scavenging activity on the superoxide radical than Vitamin C and the native non-sulfated polysaccharide (Zhang et al., 2011). However, the antioxidant mechanism has not yet been fully understood, and a further study of the relationship between polysaccharide structure/configuration and antioxidant activity was necessary.

3.4. Hydroxyl radical scavenging activity

Hydroxyl radicals are the most reactive and dangerous species among ROS. They can cause oxidative damage to biomolecules including DNA, lipids and proteins, and induce severe tissue damage or cell death (Spencer et al., 1994). Therefore, it is important to reveal the radical reactions between hydroxyl radicals and antioxidants since there are no enzymatic systems known to neutralize them in any living beings. In this experiment, Fenton reactions were used to investigate the hydroxyl radical scavenging capacities of CPA and its degraded derivatives. The scavenging ability of all samples was decreased in the order of CPA-4 > CPA-3 > CPA-2 > CPA > CPA-1 at 200 μg/mL (Fig. 4). CPA-4 showed the strongest scavenging activity at the concentrations of 120–200 μg/mL. At 160 μg/mL, the scavenging effect of CPA-4 was 9.95%, whereas the scavenging effect of BHA was only 12.2% at the concentration 1.0 ± 0.1 mg/mL (Mau, Chang, Huang, & Chen, 2004), which was lower than CPA-4. These results suggested that the scavenging activity was improved with the diminishing of the molecular weight of CPA, which was in accordance with Wu et al. (2007). There are three types of mechanisms about scavenging of hydroxyl radicals: electron transfer ($\cdot OH + R \rightarrow OH^- + R^+$), hydrogen abstraction ($RH + \cdot OH \rightarrow R\cdot + H_2O$), and addition to an aromatic ring or to a double bond yielding an addition product ($\cdot OH + R = R \rightarrow HO\text{-}R\text{-}R\cdot$) (Peshev, Vergauwen, Moglia, Hideg, & van den Ende, 2013). Hernandez-Marin and Martínez (2012) predicted that carbohydrates scavenged hydroxyl radicals through hydrogen atom transfer (hydrogen abstraction) from C—H. The scavenging mechanism was different from that of reducing power, which might provide a good explanation for the reducing power and free-radical scavenging capacity of CPA and its degraded derivatives.

3.5. DPPH free-radical scavenging activity

DPPH radical is an unwavering nitrogen-centered radical that has a characteristic absorption at 517 nm. Excellent DPPH scavenging activity confers superior antioxidant activity, and the mauve color of DPPH solution fades upon the scavenging capacity of the tested samples. As shown in Fig. 5, the scavenging ability of all samples was concentration related. The scavenging effect of all samples was found in the following order CPA-1 > CPA > CPA-2 > CPA-3 > CPA-4, which was similar to their reducing power. The inhibition rates of CAP, CPA-1 and CPA-2 were 49.76, 63.35 and 50.23% at 30 μg/mL. IC_{50} is the half maximal (50%) inhibitory concentration of a substance, and commonly used as a measure of antagonist drug potency in pharmacological research. The IC_{50} values obtained for ascorbic acid and BHT were 0.660 ± 0.014 mg/mL and 0.350 ± 0.029 mg/mL, respectively (Roy, Amdekar, Kumar, & Singh, 2011), the scavenging effects of CAP and its degraded derivatives on DPPH were much greater than ascorbic acid and BHT. DPPH

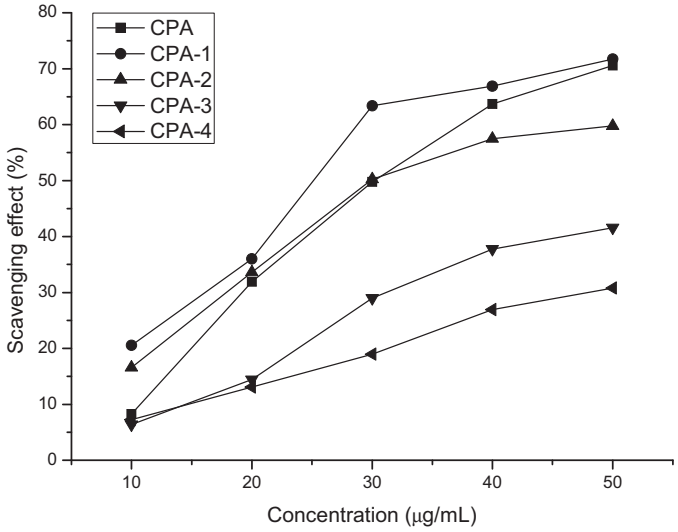

Fig. 5. Scavenging effect of CPA and its degraded derivatives on DPPH radicals. Values are means ± S.D. ($n = 3$).

radical was scavenged by antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Matthäus, 2002). It was evident that CPA and its degraded derivatives showed strong proton donating ability and could serve as free radical inhibitors or scavengers, acting possibly as antioxidants.

4. Conclusion

To sum up, CPA was successfully degraded into four low molecular weight derivatives (CPA-1, CPA-2, CPA-3 and CPA-4) by hydrogen peroxide and ascorbic acid. The relationship between antioxidant ability and molecular weight was discussed. Our results showed that high molecular weight polysaccharides showed prominent reducing power and DPPH radicals scavenging capability, nevertheless, low molecular weight polysaccharides exhibited relatively stronger free-radical scavenging activity, especially hydroxyl radical. The effect of molecular weight on antioxidant ability was significant and complicated. The identification of toxic and safety is required for the development of CPA and its degraded derivatives as natural antioxidant in pharmaceutical and food industries.

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