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Full Length Research Paper

In vitro free radicals scavenging activities of polysaccharide from *Polygonum Multiflorum Thunb*

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The crude polysaccharide (PMTP) was isolated from *Polygonum Multiflorum Thunb* through hot water extraction followed by ethanol precipitation. Through antioxidant assay *in vitro*, the results exhibited PMTP has powerful scavenging abilities, especially on ABTS, DPPH and Hydroxyl radicals. Therefore, the crude polysaccharide from *P. Multiflorum Thunb* should be explored as a novel potential antioxidant.

Key words: *Polygonum Multiflorum Thunb*, polysaccharide, antioxidant activity, *in vitro*, free radicals.

INTRODUCTION

The traditional Chinese herbs, *Polygonum multiflorum Thunb*, have been used in the preparation of herbal medicines in many oriental countries such as China, Japan and Korea for a long time, where dried roots have been used as a tonic and an anti-aging agent in many remedies in traditional Chinese medicine (Chen and Li, 1993; Huang, 1993). Some pharmacological studies have demonstrated that a crude extract of *P. multiflorum Thunb* could produce a hypolipidaemic action in rats (Lv et al., 2007; Zhang et al., 1983) and a vasorelaxant effect on isolated rat aorta (Chang et al., 1990). Many effective constituents of *P. multiflorum Thunb* had been reported, such as gallic acid, catechin, stilbene glycoside, anthocyanin, anthraquinone and so on. Recent research indicates that gallic acid, catechin and 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside in the ethyl acetate fraction of *P. multiflorum Thunb* extracts showed strong antioxidant activities(Chen et al.,1999). The ethanol extract supplemented groups had a lower percentage of lipofuscin and a lower malondialdehyde (MDA) concentration in the brain (Chan et al., 2002). However, little information is available about the polysaccharide from *P. multiflorum Thunb* and its pharmacological activity. Therefore, the aim of present study is to isolate the polysaccharides from *P.multiflorum Thunb* and evaluate the antioxidant activity *in vitro*.

MATERIALS AND METHODS

Plant materials

The plant material of *P. Multiflorum Thunb* were bought from the market of traditional Chinese medicinal materials in Chengdu(China), and were identified according to the identification standard of the Pharmacopoeia of the People's Republic of China (PPRC).

Drugs and reagents

ABTS radical was purchased from Merck. Vitamin C and DPPH radical were purchased from Sigma Co. Dextrans of different molecular weights were purchased from Pharmacia Co. D_2O was purchased from Cambridge Isotope Laboratories, Inc. BTH was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products. Ethanol and all other chemicals and reagents were of grade AR.

Preparation of the crude polysaccharide

Preparation of crude polysaccharide was carried out according to the method of Luo et al. (2009) with some modifications. The powder of *P.Multiflorum Thunb* was extracted successively with petroleum ether and ethanol, at first. After filtered, the residue was further extracted with double-distilled water at 100°C for 2 h three times. Then all extracts were combined, concentrated and filtrated.

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The amylum in the product of condensation was removed using α-amylase. After filteration, the extract was deproteinized 5 times using the Sevag reagent (Navarini et al., 1999), and the polysaccharide was free of proteins as scaned by UV Spectra in 260 nm and 280 nm. After removal of the Sevag reagent, the extract was precipitated by adding ethanol (4 times the volume of aqueous extract), and the mixture was kept overnight at 4°C for the polysaccharides. The precipitate was collected by centrifugation at 4000 rpm for 20 min, washed successively with petroleum ether, acetone and ethanol, the procedure of precipitation was perform iteration, and then dissolved in water and dialyzed against deionized water for 72 h, freeze-drying to yield the crude polysaccharide, which named PMTP.

Molecular weight determination

The molecular weights of PMTP were determined by gel permeation chromatography (GPC) with a Waters HPLC apparatus (Waters 515, Waters Co. Ltd., USA) equipped with an ultra hydrogel column (7.8×300 mm) according to the method of Fan et al. (2009). The operation conditions were described as: mobile phase: 0.2 M phosphate buffer (pH 7.0), flow rate: 0.7 ml/ min, column temperature: room temperature, injection volume: 20 µl, running time: 20 min, and detected by a Waters 2410 refractive index detector (RID). Dextran standards with different molecular weights (2,500, 4,600, 7,100, 10,000, 21,400, 41,100, 84,400, 133,800, 200,000 Da) were used for calibration curve.

Antioxidant activities evaluation

ABTS radicals scavenging assay

The radical scavenging activity of PMTP against ABTS was measured using the methods of Re et al. (1999), and Zhao et al. (2005), with some modifications. ABTS was dissolved in 0.01 M PBS (pH 7.4) to a 7 mM concentration. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 16 h before use. The ABTS radical cation solution was diluted to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C for 30 min. Each sample (0.2 ml) with various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 5.0 mg/ml) was mixed with 2.0 ml of diluted ABTS radical cation solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured immediately and recorded. Vitamin C was used as standard. ABTS radicals scavenging effect was calculated as follows:

ABTS scavenging effect (%) = $[A_0-(As-Ab)]/A_0 \times 100$

where Ao: A_{734} of ABTS without sample, As: A_{734} of sample and ABTS and Ab: A_{734} of sample without ABTS.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of PMTP was determined according to the method of Wang et al. (2008) and Luo et al. (2010), with some modifications. Briefly, different concentrations (0.01, 0.03,0.1,0.3,1.0, 3.0 and 5.0 mg/ml) samples were incubated with 2 mM EDTA–Fe (0.5 ml), 3% H_2O_2 (1.0 ml) and 0.360 mg/ml crocus in 4.5 ml sodium phosphate buffer (150 mM, pH 7.4) for 30 min at 37°C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. The hydroxyl radical scavenging effect was calculated as follows:

Hydroxyl radical scavenging effect (%)=[(Ao-As) / Ao]×100

where Ao is the A_{520} of control and As is the A_{520} of sample.

Superoxide anion scavenging assay

Superoxide radical is a very harmful species to cellular components as a precursor of more reactive oxygen species, and is known to be produced *in vivo* and can result in the formation of H_2O_2 via dismutation reaction (Li et al., 2007). In the experiment, measurement of superoxide anion scavenging activity of PMTP was based on the method described by Zhao et al. (2003) and Wang et al. (2008), with some modifications. 4.5 ml Tris-HCl buffer (50 mmol/L, pH 8.2) and 1.0 ml tested samples with various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 5.0 mg/ml) were mixed in tubes with lids. Then the mixture was incubated for 20 min in the water bath at 25°C. Meanwhile, 0.4 ml of 25 mmol/L pyrogallol preheated at 25°C was added immediately. After 4 min, the reaction was terminated by 0.1 ml HCl solution (8 mol/L) and the mixture was centrifuged at 4000 rpm for 15 min. The absorbance of sample and control were determined by UV spectrophotometer at 325 nm. The curve was made based on the absorbance value. Vitamin C was used as the positive control compounds. Scavenging activity was calculated using the following equation:

Superoxide anion scavenging effect(%)=(Ao-As)/Ao ×100

where Ao is the absorbance without sample, and As is absorbance with sample.

DPPH radicals scavenging assay

DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds. The free radical scavenging capacity of the polysaccharide (PMTP) was analyzed by using the DPPH test according to the method of Sun et al. (2010) and Shimada et al. (1992) and Braca et al. (2001), with some modifications. Vitamin C was used as reference material. Briefly, 3 ml of sample (0.01 to 5.0 mg/ml) was added to 1ml of 0.1mM methanol solution of DPPH. The absorbance at 517 nm was measured after the solution was kept at room temperature for 30 min. The DPPH radical scavenging effect was calculated as follows:

DPPH scavenging effect (%) = [Ao-(A-Ab)]/Ao ×100

where Ao:A_{517} of DPPH without sample, A: A_{517} of sample and DPPH, and Ab: A_{517} of sample without DPPH.

Reducing power

The reducing power of PMTP was quantified by the method described earlier by Raza et al. (2007) and Yen et al. (1995) with some modifications. BHT was used as reference material. Briefly, PMTP and BHT were used at differing concentrations (0.01 to 5.0 mg/ml). 1.0 ml of sample was mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then the reaction was terminated by 2.5 ml TCA solution (0.1%) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 6 mmol/l), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

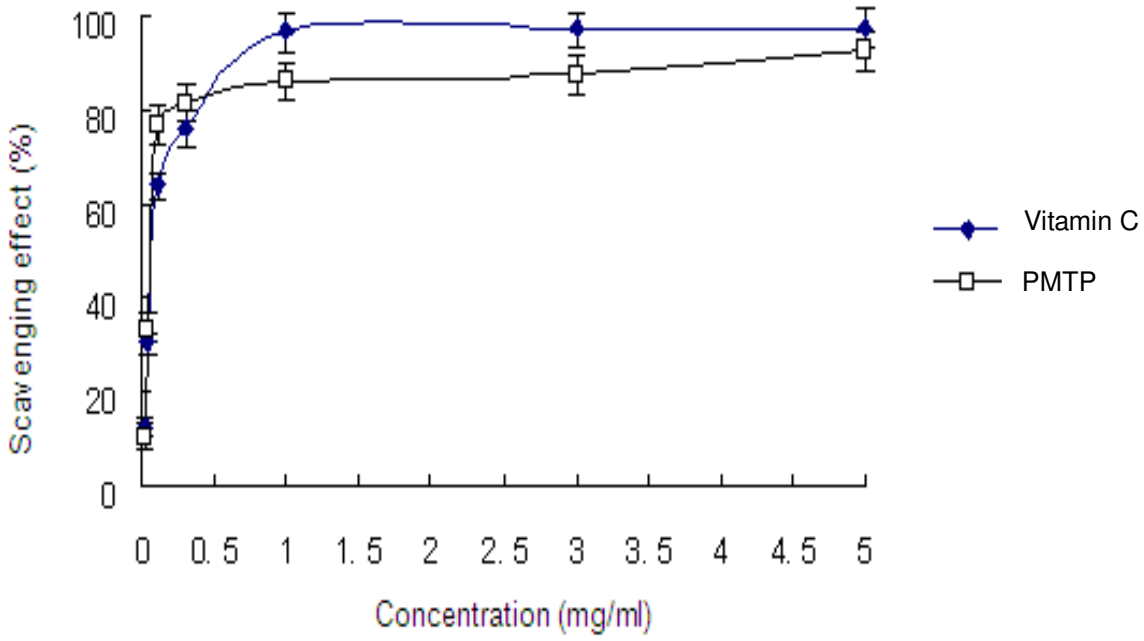

Figure 1. The scavenging effect of PMTP on ABTS radicals. Results are presented as means ± standard deviations (n = 3). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

Statistical analysis

All antioxidant activities processes were replicated three times. Data were statistically analyzed using the SPSS.

RESULTS AND DISCUSSION

Isolation of crude polysaccharide

The crude polysaccharide PMTP was obtained by the method of boiling-water extraction followed by ethanol precipitation. The extracted rate of the polysaccharide (PMTP) was 12.34%. Previous research has indicated that the molecular weight of polysaccharide was an important factor responsible for biological activities. Determination of the molecular weight was therefore the first step for the study of the polysaccharide DDP. The molecular weight (M_w) of PMTP was calculated to be 79.09 kDa based on the calibration curve with standard dextrans.

Effect of scavenging ABTS radicals

ABTS radical assay, can be used in both organic and aqueous solvent systems (Wu et al., 2006), therefore, is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Katalinic et al., 2006; Huang et al., 2008). In this experiment, the isolated polysaccharide from *P. Multiflorum Thunb* was measured and compared to their capacities to scavenge ABTS radical, and vitamin C acted as the comparison standard. The scavenging ability of PMTP on ABTS free radical was shown in Figure 1. The scavenging activities of PMTP and vitamin C correlated well with increasing concentrations. At the low dose of 0.01 mg/ml, the scavenging activities of both samples were poor (PMTP was 10.55% and vitamin C was 12.45%). On the contrary, PMTP exhibited high scavenging power in the higher doses (from 1.0 mg/ml to 5.0 mg/ml), which were closed to that of vitamin C (96.45% at the concentration 1.0 mg/ml, 97.27% at 3 mg/ml and 97.54% at 5 mg/ml). The results indicated that PMTP had strong scavenging power on ABTS radicals, and should be explored as novel potential antioxidants.

Effect of scavenging hydroxyl radicals

The hydroxyl radical in the cells can easily cross cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death. Thus, removing hydroxyl radical is very important for the protection of living systems (Yang et al., 2008). The results of hydroxyl radical-scavenging powers of the PMTP and vitamin C were given in Figure 2. As is illustrated in the figure, both samples exhibited obvious scavenging activity in a concentration-dependent manner. The comparison standard vitamin C showed valuable high radical scavenging activity (97.9%) at the high dose (5.0 mg/ml). PMTP exhibited stronger scavenging effect (81.07%) at the higher dose

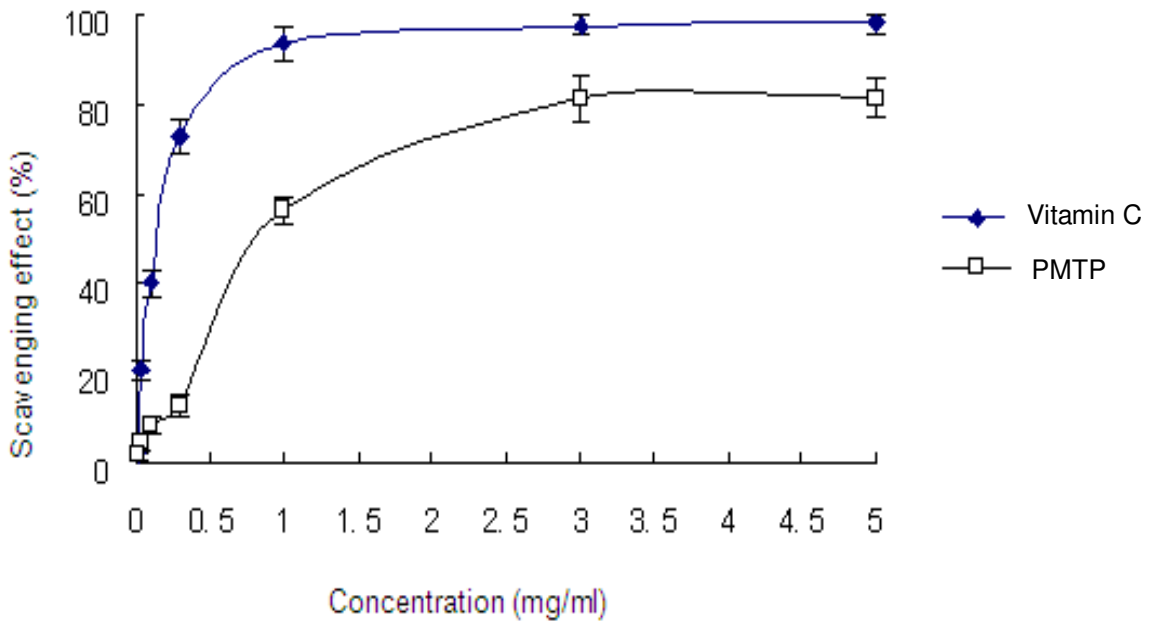

Figure 2. The scavenging effect of PMTP on hydroxyl radicals. Results are presented as means ± standard deviations (n = 3). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

(3.0 mg/ml). Beyond the concentration of 3.0 mg/ml, the increase of scavenging effect on hydroxyl radical was unconspicuous. Therefore, PMTP has an appreciable scavenging power on hydroxyl radicals.

Effect of scavenging superoxide radicals

Superoxide anion radical is known as an initial radical and plays an important role in the formation of other reactive oxygen-species, such as hydrogen peroxide, or singlet oxygen in living systems (Stief et al., 2003). In the experiment, the scavenging ability of PMTP on superoxide free radical was shown in Figure 3. As seen from the figure, the activities of all the samples increased in a concentration dependent manner. The comparison standard vitamin C showed valuable high radical scavenging activity (87.12 to 89.46%) in the higher doses (from 3.0 mg/ml to 5.0 mg/ml). But PMTP exhibited very low radical scavenging activity at every concentration point. Therefore, the polysaccharide PMTP has no significant effects on superoxide free radical scavenging.

Effect of scavenging DPPH radicals

DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol (Xu et al., 2009). The effect of antioxidants on DPPH radical scavenging was conceived to be due to their proton-donating ability. In DPPH test, the antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine (Blois et al., 1958). Based on this principle, the antioxidative activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In this experiment, the DPPH free radical scavenging effect of PMTP was measured, and the results are shown in Figure 4. The results indicated that comparison standard vitamin C and PMTP showed in all concentrations dose-dependent DPPH radical scavenging activities. Furthermore, the scavenging activities of both increased very significantly with increasing concentrations. When the concentration got to 1.0 mg/ml, the scavenging effects increased indistinctively. At the high dose of 5.0 mg/ml, vitamin C exhibited very high scavenging effect (99.77%), and PMTP also have strong scavenging activity on DDPH radical (87.15%). Therefore, it was obvious that the polysaccharide of PMTP has strong antioxidant activity in the higher doses on DPPH radical scavenging.

Effect of reducing power

Research has revealed that there is a direct correlation between antioxidant activities and reducing power (Yildirim et al., 2001). To measure reductive power of PMTP, the Fe^{3+}-Fe^{2+} transformation in the presence of different concentrations sample were investigated, BHT was used as reference material. The reductive capabilities of PMTP and reference material were exhibited as

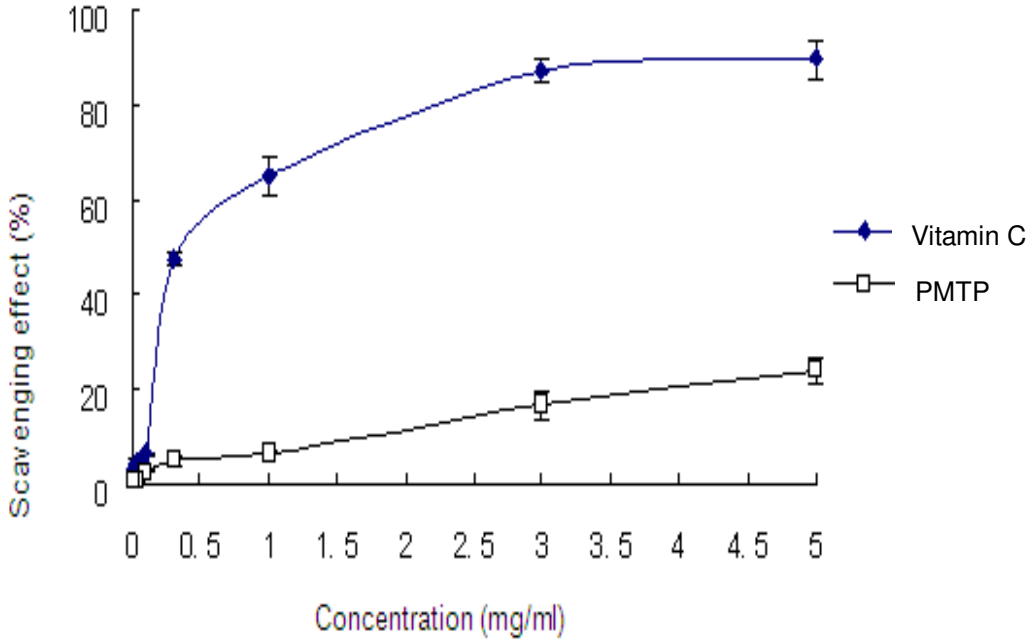

Figure 3. The scavenging effect of PMTP on superoxide radicals. Results are presented as means ± standard deviations (n = 3). Differences are considered to be statistically significant if P < 0.05 when compared to control.

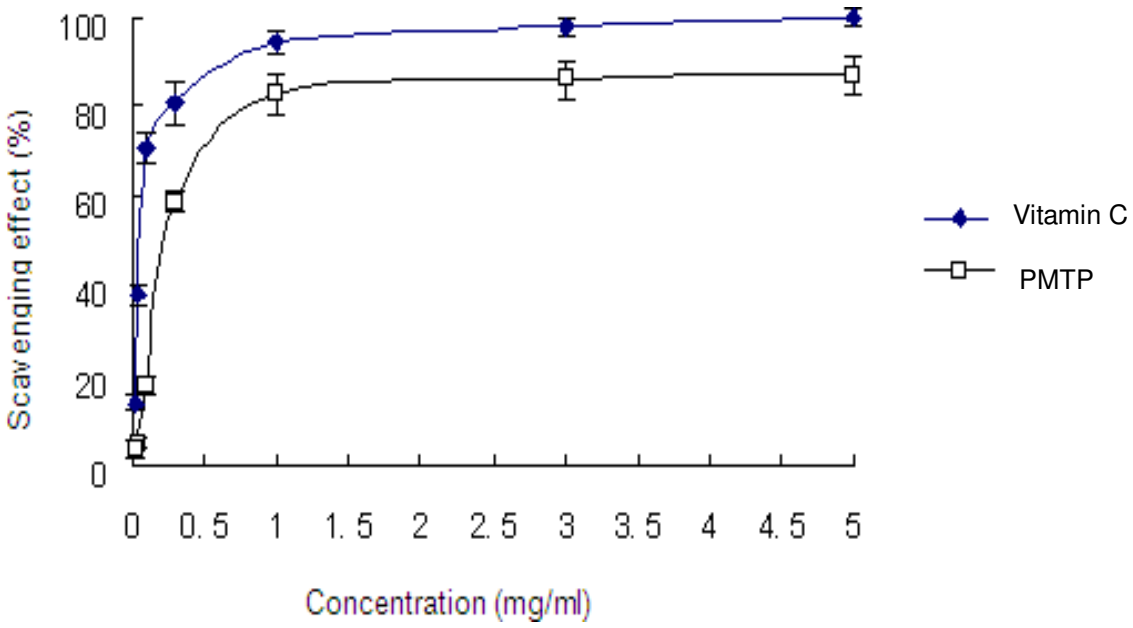

Figure 4. The scavenging effect of PMTP on DPPH radicals. Results are presented as means ± standard deviations (n = 3). Differences are considered to be statistically significant if P < 0.05 when compared to control.

shown in Figure 5. From the result, reducing powers of all samples were in a concentration-dependent manner. In the high doses (from 1.0 to 5.0 mg/ml), BHT exhibited higher absorbance (2.103), which indicated that BHT has greater reducing power. PMTP has lower absorbance than BHT at every concentration. At the high dose (5.0 mg/ml), the absorbance of PMTP was 0.78, which indicated that PMTP has a certain extent reducing capacity, but compared to the reference, it was poor. The results suggested that PMTP has no significant reducing

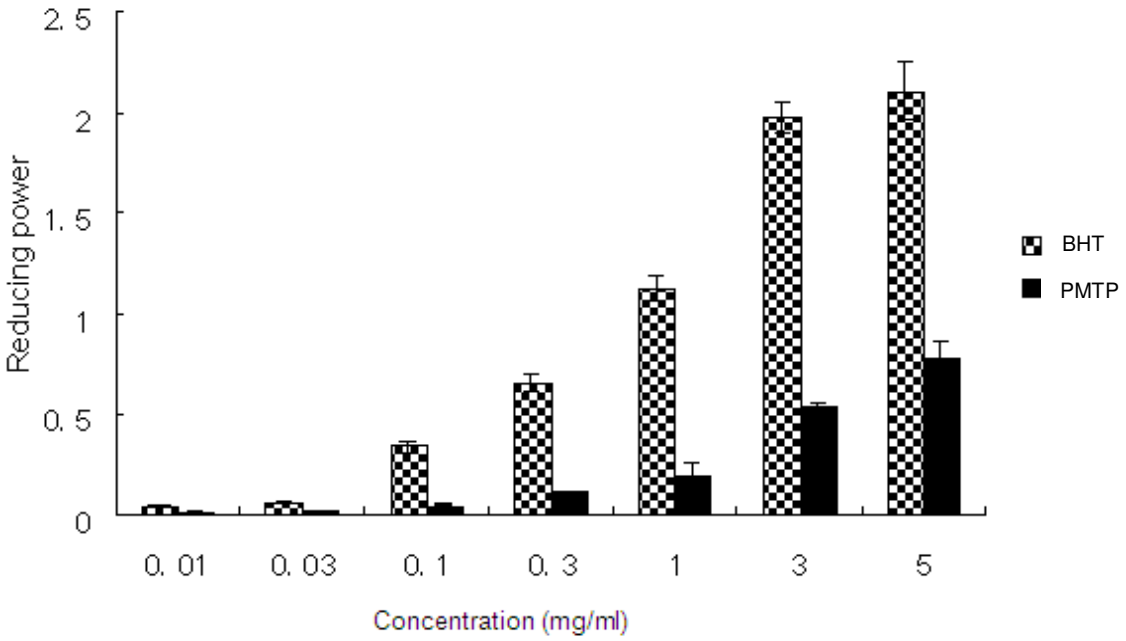

Figure 5. The effect of reducing power. Results are presented as means ± standard deviations (n = 3). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

capacity.

Conclusion

In the present study, the polysaccharide (PMTP) was isolated from *P.multiflorum Thunb* by water extraction and ethanol precipitation. Free radicals scavenging activities *in vitro* indicated that PMTP has significant radicals scavenging abilities on ABTS, DPPH and Hydroxyl radicals. The scavenging effects were powerful, which closed to the positive control (Vc). Therefore, the polysaccharide PMTP should be explored as novel potential antioxidants. On the other hand, PMTP exhibited a weak reducing capacity and scavenging effect on superoxide anion radical compared to the reference. Therefore, further investigation of its antioxidant activities *in vivo* elucidate and the mechanism of action relevant to its anti-oxidative activity will be carried out in our later work.

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