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Full Length Research Paper

Antioxidant and anti-glycated activities of polysaccharides *in vitro* isolated from *Hedyotis diffusa* Willd.

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The extraction and isolation of four crude polysaccharides from *Hedyotis diffusa* Willd., as well as the evaluation of their antioxidant and anti-glycated activities *in vitro* were investigated. The whole plant of *H. diffusa* was extracted with hot distilled water and three fractions of crude polysaccharides, termed as 50P, 70P and 90P were obtained by graded ethanol precipitation, BP were gained by NaOH solution extracted followed by ethanol precipitation. All of the four polysaccharides were defatted with petroleum ether and protein was removed by the Sevag reagent. Preliminary antioxidant *in vitro* indicated that the four crude polysaccharides showed powerful and dose-dependent scavenging abilities on DPPH· and hydroxyl radicals. For the first time, the anti-glycated activities of the four samples were studied, and they had good potential in inhibiting the non-enzymatic glycation reaction with dose-dependent effects. In particular, 90P showed even better antioxidant and anti-glycated activities than corresponding positive control. Therefore, *H. diffusa* crude polysaccharides have potential value on the prevention and treatment of some diseases caused by oxidant and aging, and worthy of further research and development.

Key words: *Hedyotis diffusa* Willd., polysaccharide, antioxidant activity, anti-glycated activity, *in vitro*.

INTRODUCTION

It is well known that polysaccharides play an important role in the growth and development of living organisms (Yu et al., 2009). In recent years, more and more polysaccharides have been reported to exhibit a variety of biological activities, such as antioxidant (Ye et al., 2008), antiglycation (Yang et al., 2009b), anti-inflammatory (Paiva et al., 2011), antitumour (Yin et al., 2010), antimutagenic (Liu et al., 2008), immuno-modulatory (Cui et al., 2011), etc. Some botanical polysaccharides have been commercially developed into important components of therapeutic drugs and skin care products (Wang and Fang, 2004).

The herb of *Hedyotis diffusa* Willd. (synonym *Oldenlandia diffusa* (Willd.) Roxb., family Rubiaceae), is an annual herbaceous plant distributed in northeastern Asia and southern regions of China (Ko, 1999). *H. diffusa* is well-known as a traditional Chinese medicine for the treatment of hepatitis, tonsillitis, appendicitis, pneumonia, mastitis, urethral infection, and malignant tumors of the liver, lung, and stomach (Liang et al., 2008).

Moreover, it is commonly used in the Orient and tropical Asia (Ahmad et al., 2005), for making teas and botanicals for the relief of “heat”, removal of “toxins” and promotion of diuresis to eliminate “wetness-evil”. In addition, *H. diffusa* is reported to possess various bioactivities in the modern pharmacological investigations, such as anticancer (Chen et al., 2008; Lee et al., 2011; Lin et al., 2011), antifungal (Li et al., 2005), anti-inflammatory (Lin et al., 2002), antioxidant (Lu et al., 2000), immunoloregulation (Meng et al., 2009), hepato protective (Lin et al., 2002), and neuroprotective (Kim et al., 2001) activities.

Previous phytochemical studies revealed the presence of iridoid glycosides (Ding et al., 2010), anthraquinones (Kang et al., 2008), flavones, steroids and phenyl-propanoids (Huang et al., 2008) in the extraction of *H. diffusa*. However, little information is available about the

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polysaccharide from *H. diffusa* and its pharmacological activity. Therefore, the aim of present study is to isolate the polysaccharides from *H. diffusa* and evaluate the antioxidant and anti-glycated activities *in vitro*.

MATERIALS AND METHODS

Plant materials and reagents

The herb of *H. diffusa* was purchased from Lvbaicao drugstore in Guangzhou, Guangdong province of China. DPPH· and Sodium azide were purchased from Sigma-Aldrich (St. Louis, USA). Vitamin C and Bovine Serum Albumin (BSA) were bought from the Puboxin Biotechnology Co., Ltd. (Beijing, China). Glucose, phenol and sulphuric acid were obtained from the Guangzhou Reagent Co. (Guangzhou, China). All reagents used in this study were of analytical grade.

Preparation of the crude polysaccharide

Preparation of crude polysaccharide was carried out according to the method of Yin et al. (2010) with some modifications. The whole herb of *H. diffusa* (4000 g) was extracted three times with hot double-distilled water (85 to 90℃) for 3 h each time . The combined aqueous extracts were filtered and concentrated under reduced pressure, and three fractions of crude polysaccharides, termed as 50p, 70p and 90 p, were obtained by graded ethanol precipitation, at final concentration of 50, 70 and 90% of ethanol, respectively.

The residues were further extracted with 0.3 mol/l NaOH three times at room temperature, then, the alkaline extracts were filtered, combined, adjusted to pH 7 with diluted HCl and concentrated under reduced pressure. The aqueous fraction was precipitated with 70% ethanol for 24 h at 4ºC. The precipitate was collected, named as Bp. All of the four fractions of polysaccharides, 50p, 70p, 90p and Bp, were deproteinated by the Sevag reagent (Navarini et al., 1999), defatted with petroleum ether and dialyzed against distilled water for 48 h to remove low molecular weight materials (exclusion limit 3.5 kDa), respectively. Then, the polysaccharides were lyophilized to obtain four crude polysaccharides, termed as 50p, 70p, 90P and BP.

The content of polysaccharides was determined by the phenol-sulphuric acid method (Xie et al., 2010) with D-glucose as standard at 490 nm, wherein glucose was prepared in a series of concentrations to make a standard curve for the polysaccharide calculation. Protein content was estimated by the Lowry method, with bovine serum albumin as standard (Lowry et al., 1951).

Assay for antioxidant activity

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was measured by the method of Sun et al. (2010) with a minor modification. Samples were dissolved in distilled water at 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0 or 10.0 mg/ml. The sample solution (0.5 ml) was mixed with 4.0 ml of reaction buffer [1.0 ml of 50 mM phosphate buffer (pH 7.4) and 0.75 ml of 50 mM phenanthroline, 0.5 ml of 7.5 mM ferrous sulfate and 1.75 ml of ultra-pure water], then, 0.5 ml of 0.1% H_2O_2 was then added to the reaction solution. The reaction solution was incubated for 60 min at 37ºC. The absorbance of the mixture was measured at 536 nm. The inhibition percentage of hydroxyl radical was calculated using the following formula:

$$\text{Scavenging\%} = (A_{sample}-A_{negative\ control})/(A_{blank}-A_{negative\ control}) \times 100\%$$

Here, ultra-pure water (1.0 ml) plus sample solution (0.1 ml) was used as a blank, 0.5 ml of ultra-pure water plus 0.1% H_2O_2 (0.5 ml) was experimented as a negative control, and 0.5 ml of ascorbic acid plus 0.1% H_2O_2 (0.5 ml) was tested as a positive control.

DPPH· radicals scavenging assay

The free radical-scavenging activity of the four polysaccharides were measured by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) test, according to the method of Xie et al. (2010), with some modifications. The 0.1 mM solution of DPPH· in ethanol was prepared daily before UV measurements. 2 ml of various concentrations (25, 50, 75, 100, 150, 200 and 250 µg/ml) of the crude polysaccharides in ultra-pure water were thoroughly mixed with 2 ml of freshly prepared DPPH·. The mixture was shaken vigorously and allowed to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank with an ultraviolet-visible spectrophotometer.

Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Ascorbic acid was used as positive controls. The ability to scavenge the free radical, DPPH· in percent (I%) was calculated using the following equation:

$$I\% = [A_0-(A_2-A_1)]/A_0 \times 100\%$$

Where A_0 is the absorbance of the incubated DPPH· solution without addition of the sample or positive controls, A_1 is the absorbance of the sample without DPPH· solution and A_2 is the absorbance of the incubation mixture containing both the test sample and DPPH· solution.

Assay of anti-glycated activity

The anti-glycated activity was determined by the method of Yang et al. (2009a). A stock solution of 2% (w/w) bovine serum albumin, 1 M glucose and 0.05% (w/w) sodium azide in pH 7.4 phosphate buffer was prepared. Polysaccharide was dissolved in the stock solution to concentration of 0.1, 0.3 and 0.5 mg/ml, respectively. The negative control was free of polysaccharide.

Bacteria were removed by membrane filtration with a pore size of 0.22 µm. Solutions were held in a 15 ml centrifuge tube and incubated in dark at 37ºC for 3 weeks. The content of advanced glycated end products was determined at excitation wavelength of 370 nm and emission wavelength of 450 nm by fluorospectrophotometric method. The percentage of anti-glycated activity (G%) was calculated as

$$G\% = (A_{control}-A_{sample})\ /A_{sample} \times 100\%$$

Where $A_{control}$ represents the fluorescent determination of negative control, and A_{sample} represents the fluorescent determination of sample.

Statistical analysis

All antioxidant and anti-glycated activities processes were carried out in triplicate. Data were statistically analyzed using the SPSS. Each value was expressed as mean ± standard deviation (n = 3).

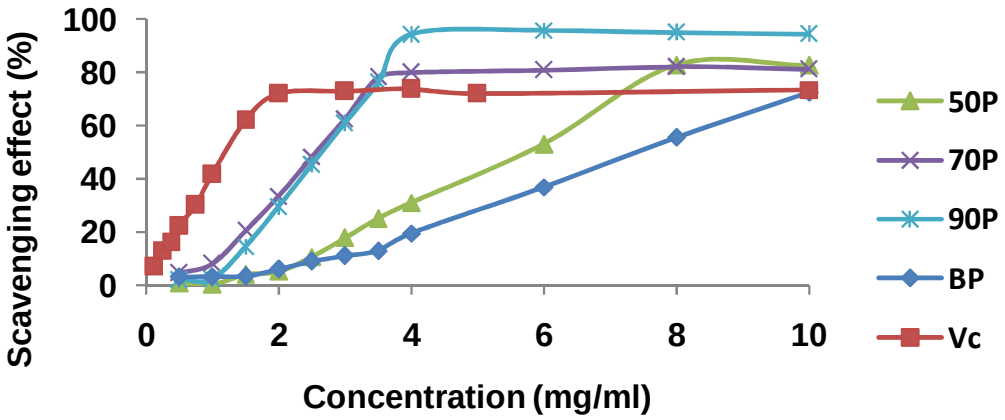

Figure 1. Scavenging effects of 50P, 70P, 90P, BP and vitamin C on hydroxyl radical. Each sample was assayed in triplicate for each concentration. Yan et al. (2009).

RESULTS AND DISCUSSION

Isolation of crude polysaccharide

Four crude polysaccharides, 50, 70, 90P and BP, were obtained from *H. diffusa* by water/alkaline extraction followed by ethanol precipitation, with yields of 3.7, 2.2, 1.5 and 0.5%, respectively. The total extracted rate of the polysaccharide was 7.9%.

Assay for antioxidant activity

Hydroxyl radical scavenging assay

Hydroxyl radicals can easily cross cell membranes, can readily react with most biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death. Thus, removing hydroxyl radicals is important for the protection of living systems (Sun et al., 2010). The results of hydroxyl radical scavenging powers of the four crude polysaccharides, 50P, 70P, 90P and BP, were given in Figure 1.

As is illustrated in the figure, all samples exhibited some scavenging activity in a dose-dependent manner. The comparison standard vitamin C showed valuable high radical scavenging activity at the low dose (<1.5 mg/ml). The four crude polysaccharides exhibited stronger scavenging effect at the higher dose. The scavenging effects of 50P, 70P, 90P and BP at the dose of 0.125 to 10.0 mg/ml were 0.82 to 82.8, 4.6 to 82.1, 2.0 to 95.8 and 3.1 to 72.5%, respectively, and that of vitamin C was about 7.0 to 73.8%.

This result proved that polysaccharides from *H. diffusa* had significant effects on scavenging hydroxyl radicals, and 50P and 90 P were more pronounced than vitamin C at higher dose.

DPPH· radicals scavenging assay

The model of scavenging the stable DPPH· radical is a widely used method for evaluating the free radical-scavenging ability of various antioxidants (Luo, 2011; Sun, 2010; Xie, 2010; Ye, 2008). The method is based on the reduction of DPPH· radical in Ethanol solution at 517 nm in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. It can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations (Xie et al., 2010).

Herein the previous-mentioned model was used to determine inhibitory activities of the water-soluble polysaccharide on DPPH· radicals. Figure 2 depicts the DPPH·-scavenging power of four crude polysaccharides and vitamin C. Obviously, the scavenging effects of polysaccharides were increased with increasing concentrations. At concentrations of 0.1 to 1.0 mg/ml, the scavenging abilities of the 50, 70, 90P and BP on DPPH· radicals were in the range of 12.0 to 72.2, 19.5 to 91.8, 30.8 to 92.0 and 4.6 to 29.0%, respectively.

At the concentration of 1.0 mg/ml, 70 and 90P were observed to possess strong DPPH· radical scavenging effects, with value of around 91.8 and 92.0%, and scavenging effects of ascorbic acid and 50P were 97.9 and 72.2%, respectively.

These results indicate that 70 and 90 P have noticeable effect on scavenging DPPH· radicals, especially at high concentration. However, the radical scavenging activity of 50 P was lower than that of vitamin C used in this study.

Assay of anti-glycated activity

Protein glycation plays an important role in the development of diabetic vascular complications *in vivo*. Schiff alkali and Amadori products are formed in the initial stage

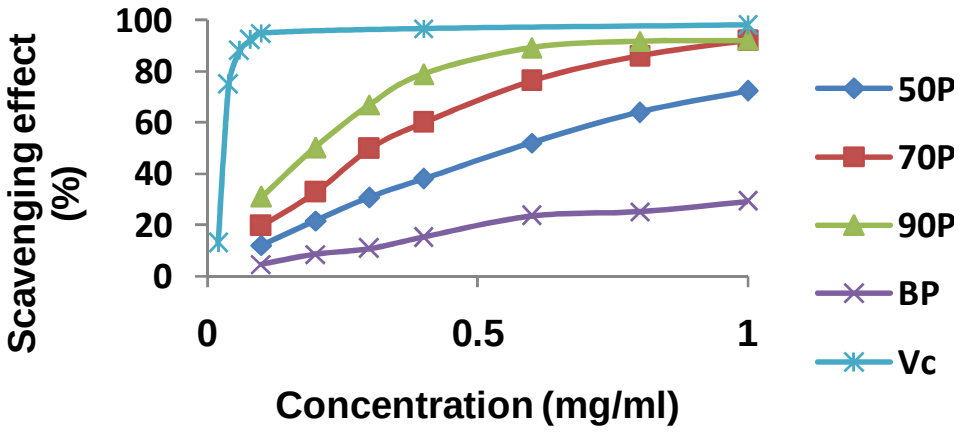

Figure 2. Scavenging effects of 50P, 70P, 90P, BP and vitamin C on DPPH· radical. Each sample was assayed in triplicate for each concentration.

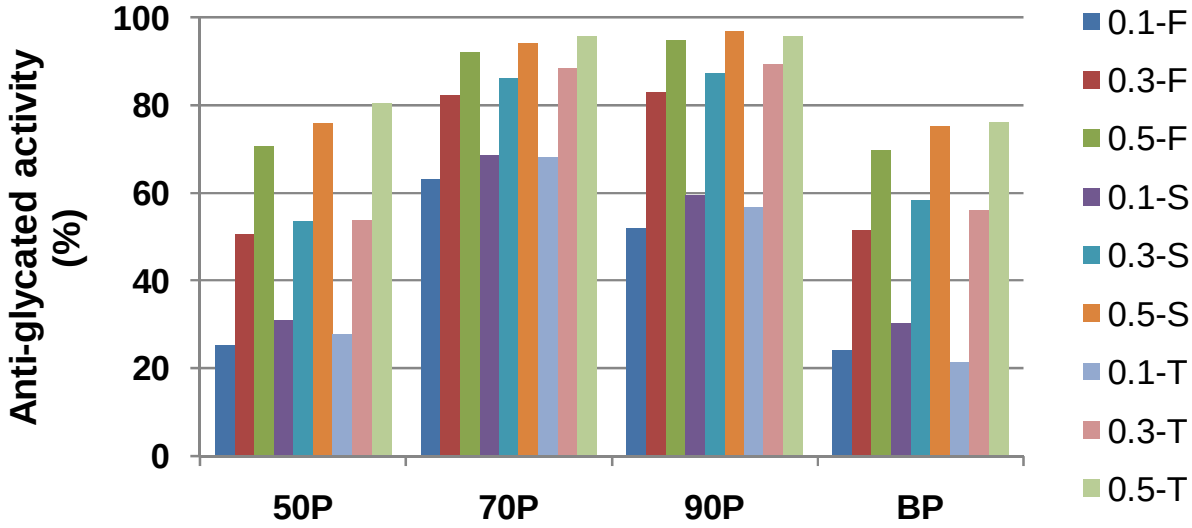

Figure 3. Anti-glycated activities of four polysaccharides from *H. diffusa* during different incubation periods. Each sample was assayed in triplicate for each concentration. 0.1-F: the anti-glycated activity at 0.1mg/ml at the first week. 0.3- F: the anti-glycated activity at 0.3 mg/ml at the first week. 0.5- F: the anti-glycated activity at 0.5 mg/ml at the first week. 0.1-S: the anti-glycated activity at 0.1 mg/ml at the second week. 0.3- S: the anti-glycated activity at 0.3mg/ml at the second week. 0.5- S: the anti-glycated activity at 0.5 mg/ml at the second week. 0.1-T: the anti-glycated activity at 0.1 mg/ml at the third week. 0.3- T: the anti-glycated activity at 0.3mg/ml at the third week. 0.5- T: the anti-glycated activity at 0.5 mg/ml at the third week.

of non-enzymatical reaction between protein and glucose, which are transformed into irreversible advanced glycation end products after oxidation, rearrangement and crosslinking (Metz et al., 2003). Therefore, inhibiting the formation of advanced glycation end products will be beneficial to human health.

In this work, three concentrations (0.1, 0.3 and 0.5 mg/ml) were used to evaluate the inhibition effect of four polysaccharides on the formation of advanced glycated end products. The results are present in Figure 3. There were positive correlation between the anti-glycated activities of 50P, 70P, 90P and BP and its acting concentrations. At the first week, significant anti-glycated activities were observed for four samples. The G % of 70P and 90P were above 50% at the concentrations of 0.1 mg/ml, significantly higher than those of 50 P, BP and aminoguanidine (Yang et al., 2009a).

The anti-glycated activities of samples at the second week were higher than those at the first week. When a concentration of 0.5 mg/ml was used, the G % of 70 and

9P were 94.2 and 96.8% at the second week, much higher than those of 5P, BP and aminoguanidine.

However, there were little changes of G % between the second week and the third week. Aminoguanidine has been used clinically as a glycation inhibitor, and as positive control *in vitro* in anti-glycated assay, which exhibited activity of less than 5% at the first week, and 60% at 0.1 mg/ml, 80.6% at 0.5 mg/ml on the second week (Yang et al., 2009a). The previous data showed that 70P and 90 P had higher anti-glycated activity than aminoguanidine under the experimental conditions.

Conclusion

In the present study, four polysaccharides (50P, 70P, 90 P and BP) were isolated from *H. diffusa* by water/alkaline extraction and ethanol precipitation. Hydroxyl radical scavenging activities *in vitro* indicated that the four crude polysaccharides exhibited strong scavenging effect at the high dose, in which 50P and 90P were more significant than vitamin C at higher dose. DPPH· radicals scavenging assay showed that the scavenging effects of four polysaccharides were increased with increasing concentrations, and 70P and 90 P have noticeable effect at high concentration, which closed to the positive control (vitamin C). However, 50P was lower than that of vitamin C.

In this work, we also firstly studied the anti-glycated activity *in vitro* of the polysaccharides from *H. diffusa.* The four polysaccharides had good and stable anti-glycated activity, and there were positive correlation between the activities and their acting concentrations. Moreover, the anti-glycated activities of 70P and 90P were much higher than those of 50 P, BP and aminoguanidine within three weeks. All the experimental results of activities showed that 90P had the most powerful antioxidant and anti-glycated activities *in vitro* among the four polysaccharides.

However, the antioxidant and anti-glycation mechanism of 90P is still not clear. At present, the exact structure of 90P and the relationship between structure and activities are in progress.

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