Antioxidant activities *in vitro* and *in vivo* of water-soluble polysaccharide isolated from *Sparganium stoloniferum* Buch.-Ham

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Abstract: In this study, the crude polysaccharides from *Sparganium stoloniferum* Buch.-Ham were prepared using hot-water extraction and further deproteinzated by Sevage method. The purified fraction of crude polysaccharide was obtained using a DEAE-52 cellulose chromatography and named with WSSP. Then, the antioxidant capacities of WSSP were assessed in vitro and *in vivo*. The results in vitro indicated that the WSSP possessed notably free radical scavenging capacity. And the antioxidant abilities were dose-dependent and increased with increasing dose of sample. The findings *in vivo* showed the gavage administration of WSSP can increase SOD and TAOC activities, and decrease MDA levels in tissue and serum of mice. Therefore, the WSSP may serve as a potential antioxidant.

Keywords: Polysaccharide; Antioxidant activity; *Sparganium stoloniferum* Buch.-Ham; In vitro and *in vivo*

INTRODUCTION

Free radicals, including superoxide anion, hydrogen peroxide, single oxygen, and hydroxyl radical, may cause the detriment of cellular components of DNA, proteins and lipids, and result in various conditions such as tumor, aging, heart disease and cardiovascular disease (Halliwell *et al*., 1995; De Lima *et al*., 2004; Lu *et al*., 2010). To lower damage to the human body, the most effective way is with the help of antioxidants. Furthermore, antioxidants are usually used in food products for preservation. However, at present, synthetic antioxidants are widely utilized. It is controversial whether such antioxidants are safe, and thus their use would be scrutinized for their potential toxicity. Increasing attention, therefore, has been paid to the natural antioxidant in recent years. Many studies have demonstrated that polysaccharides isolated from medicinal plant have evident antioxidant effect on free radicals and can be exploited as novel potential antioxidants (Chen *et al*., 2008; Ye *et al*., 2011; Chen *et al*., 2012).

Sparganium stoloniferum Buch.-Ham, which belongs a aquatic species distributed in China, Japan and Korea, has been shown to possess antioxidant activity (Xu *et al*., 2009; Lee *et al*., 2010; Wang *et al*., 2012), anti-platelet and anti-thrombotic actions (Lu *et al*., 1999), analgesic and anti-inflammatory effect (Ma *et al*., 2009), and anti-cancer activity (Sun *et al*., 2010; Sun *et al*., 2011). Several major functional compounds including polyphenol (Xu *et al*., 2009; Lee *et al*., 2010; Wang *et al*., 2012; Wu *et al*., 2012), steroidal and saponins (Zhang *et al*., 1996; Zhang *et al*., 1996) were believed to be important contributors to the activities. However, no information is available on polysaccharide from *Sparganium stoloniferum* Buch.-Ham. In this study, in vitro and *in vivo* antioxidant properties of polysaccharide from *Sparganium stoloniferum* Buch.-Ham were evaluated. The findings obtained from this study may offer a scientific reference to explore polysaccharide from this medicinal plant as natural antioxidant or food additive.

MATERIALS AND METHODS

Chemicals and materials

Sparganium stoloniferum Buch.-Ham was gathered from the Nanjing City of Jiangsu Province, China and identified by Professor Qinan Wu. The materials were cut into slices and dried at 65^{o}C in an electric dry oven, and then powered to pass through a 40-mesh sieve. The power was kept in sealed polyethylene bags at 4^{o}C until use.

Ascorbic acid and 2,2′ -azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared by an EPED super purification system (EPED, Nanjing, China). All other chemicals were analytical grade from Nanjing Chemical Reagent Co., Ltd.

Preparation of water-soluble polysaccharide

The power defatted was prepared as previously described (Liu *et al*., 2009). Briefly, the power was performed in a Soxhlet apparatus using petroleum ether (boiling point: 60-90^{o}C) and 80% ethanol pretreatment three times to remove some coloured substances, oligosaccharides, monosaccharides, and small molecule compounds. The solvent was volatilized to dryness and the defatted powder was gained. The defatted power was extracted using water at 95^{o}C for three times. In order to collect the supernatant,

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centrifugation of the sample at 4,000 rpm for 15min was performed. The solution was condensed approximately to one-fifth of sample volume and anhydrous ethanol was slowly added to the concentrated solution to a final concentration of 80% (v/v) and maintained overnight at 4°C. After centrifugation at 4,000rpm for 20min, the precipitate was obtained, and dissolved using deionized water and employed to Sevag method to eliminate free protein (Staub, 1965). The deproteinization sample was re-precipitated in 80% ethanol. The precipitate was obtained and washed successively with acetone and ethanol, and then solubilized in deionized water and dialyzed against deionized water for 72h. The crude polysaccharide was obtained by freeze-drying. The crude polysaccharide was dissolved using water and then used to a column (30cm×2.0cm) of DEAE-cellulose. The water-soluble fraction of polysaccharide was eluted using 200mL of denioized water and then freeze-dried, which was named WSSP.

In vitro antioxidant activities

Scavenging effect on hydroxyl radical

Scavenging effect on hydroxyl radical was assessed using the method described by the report (Smirnoff *et al*., 1989) with a few modifications. Briefly, 1mL of WSSP solution was mixed using 1mL of 9 mmol/L $FeSO_4$ and 1 ml of 9 mmol/L ethanol salicylic acid solution. The reaction was generated by the mixture of 1mL of 8.8mmol/L H_2O_2. After the 30 min incubation at 37°C, and then the absorbance of the mixed solution was detected at 510 nm against blank. Ascorbic acid was used for comparison. The hydroxyl free radical scavenging capability was calculated as follows:

Hydroxyl radical scavenging activity (%)=[1 − (A_i − A_0)/A_c]×100 (1)

where A_i, A_0 and A_c represent the absorbance of mixture, mixture without H_2O_2 and mixture without sample, respectively.

Scavenging effect on ABTS radical

The ABTS radical cation ($ABTS^+$) was used to measure the antioxidant effect of WSSP by our previously published literature (Wang *et al*. 2012). The ABTS, a colorless dianion salt of sodium, can become a colorful $ABTS^+$ under oxidation by potassium persulphate. Briefly, 5mL of ABTS solution (7mM) and 5mL of potassium persulphate (2.45mM) were mixed, and the reaction mixture was allowed to lay at 25°C in the dark for 14-16h. This mixture was then diluted using ethanol to achieve an absorbance of 0.700±0.020 at 734nm. The $ABTS^+$ solution (3.6mL) was added with 0.4mL of the WSSP solution. The mixtures were incubated for 30 min and the absorbance was measured at 734 nm against a blank. Ascorbic acid was used as positive reference. The scavenging effect (%) of $ABTS^+$ was evaluated according to the following equation:

$ABTS^+$ radical scavenging effect (%)=(1−A_i/A_s)×100 (2)

where A_s is the absorbance of pure $ABTS^+$, A_i is the absorbance of $ABTS^+$ in the presence of sample.

Reducing power evaluation

The Fe^{3+} reducing power of WSSP was detected according to the report of Wang *et al* with slight modifications (Wang *et al*., 2012). At various concentrations, 1mL sample was mixed with 2.5mL phosphate buffer (0.2M, pH 6.6) and 2.5mL potassium ferricyanide (1%, w/v). The mixed solution was incubated for 20 min at 50°C. And then, 1mL TCA (10%, w/v) was added, followed by centrifugation at 3000 rpm for 10 min. Next, 2.5mL of the supernatant was mixed with 2.5mL deionized water and 0.5mL ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10min. The absorbance at 700 nm was recorded as the reducing power. Increased absorbance of the reaction mixed solution showed the increased reducing power of the WSSP. Ascorbic acid was used as the control.

In vivo antioxidant activities

Male KM mice (20 ± 2g) were purchased from the Laboratory Animal Center of Jiangsu University (Zhenjiang, China) and housed on a 12h light-dark cycle at a temperature 25 ± 2°C and a 40-60% of relative humidity. They allowed free access to food and water during the experiment. The study received clearance from the Institutional Animal Ethical Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Nanjing University of Chinese Medicine (Nanjing, China). Twenty-four mice were randomly divided into two experimental groups of 12 mice each, including a control group and WSSP group (30mg/kg body weight). The control group was given a 0.2-mL physiological saline solution (0.9% w/v) once daily for 30 consecutive days and the WSSP group was given a 0.2-mL WSSP solution once daily for 30 consecutive days. Twenty-four hours after the last drug administration, blood samples were collected from the eyepit of the mice and centrifuged for serum. Liver, kidney and heart were dissected out and washed immediately with saline solution to eliminate blood. The activities of superoxide dismutase (SOD) and total antioxidant activity (TAOC), as well as the level of malondialdehyde (MDA) were evaluated using commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) according to the instruction manuals.

STATISTICAL ANALYSIS

All experiments were carried out three replicates, and the results were presented as mean ± standard deviation (SD). Statistical analysis was conducted using SPSS for Windows, Version 18.0 (SPSS Institute, Inc., Cray, NC, USA). Statistical significance was determined at P<0.05.

RESULTS

In vitro antioxidant activities

Scavenging effect of WSSP on hydroxyl radical

The hydroxyl radicals are very strongly reactive oxygen species generated in biological systems and can reduce disulfide bonds in proteins, specifically fibrinogen, resulting in various diseases including atherosclerosis, cancer and neurological disorders (Lipinski H. 2011). In the study, the hydroxyl radical formed Fenton reagent was applied to assess the scavenging effect of the WSSP. In fig. 1, the results indicated antioxidant ability possessed a dose-dependent manner, which implied that the WSSP can prevent oxidative damage in the human body. However, the scavenging capacity on hydroxyl radical is weaker than that of positive control.

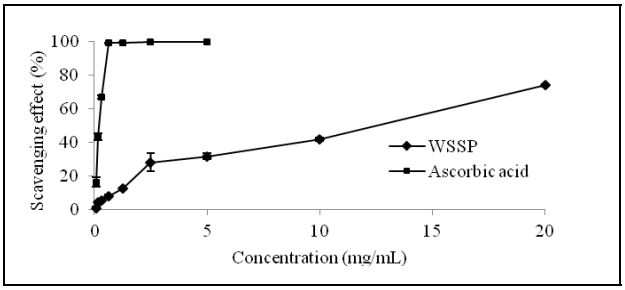

Fig. 1: Scavenging effect of WSSP and ascorbic acid on hydroxyl radical

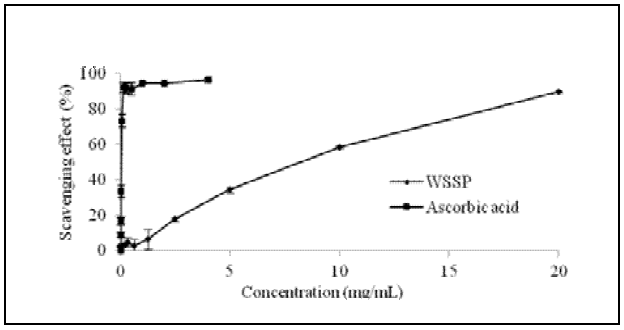

Fig. 2: Scavenging effect of WSSP and ascorbic acid on ABTS

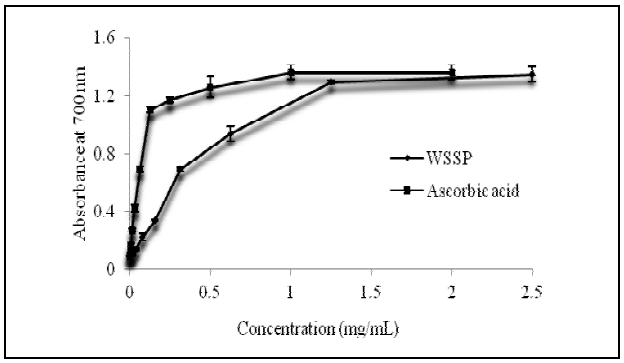

Fig. 3: Reducing power of WSSP and ascorbic acid.

Scavenging effect of WSSP on ABTS

The ABTS radical cation scavenging evaluation, which employs a specific absorbance (734nm) at a wavelength well separated from the visible region and requires a short reaction time, has been widely used to assess the total antioxidant effect in various samples (Chen *et al*., 2011). The scavenging activity of WSSP on ABTS free radical is showed in fig. 2. It may be found to be very useful in scavenging $ABTS^+$ radical and the increase showed concentration-dependent. Moreover, the WSSP indicated strongly $ABTS^+$ radical scavenging activity at a high concentration of 20mg/mL. When compared to positive reference, the $ABTS^+$ scavenging effect of the WSSP was comparable at high concentration. Therefore, the results showed that the WSSP has obvious effect on ABTS free radical scavenging at a high concentration.

Reducing power

Reducing power is a significant mechanism of antioxidant action and extensively used as an important indicator of potential antioxidant ability (Jayaprakasha *et al*., 2011). Many researches have confirmed that the antioxidant activity is direct related to reducing power (Yildirim *et al*., 2001; Sun *et al*., 2011; Wang *et al*., 2012). In the reducing power evaluation, the presence of the anti-oxidant can cause the reduction of the Fe^{3+}/ferricyanide complex to the $Fe^{2+/}$ferrous form. The mount of Fe^{2+} can be then monitored by detecting the formation of Perl's Prussian blue at 700nm (Sun *et al*., 2009). As shown in fig. 3, a higher absorbance at 700 nm shows a stronger reducing power of the WSSP. A concentration-dependent reducing power of the WSSP was found. It was observed that reducing power of WSSP was weaker than that of ascorbic acid. However, the WSSP notably possessed anti-oxidant activity at high concentration.

In vivo antioxidant activities and MDA levels

As shown in Table 1A-B, compared with normal group, there was no difference in SOD activities in liver ($P>0.05$). However, significant difference could be observed in kidney, heart and serum ($P<0.05$). Seen from Table 1A-B, TAOC of serum and tissue significantly increased in WSSP group compared with normal group ($P<0.05$). It was found in Table 1A-B, the level of MDA in kidney was no significant difference between WSSP group and normal group ($P > 0.05$). However, gavage administration with WSSP decreased the MDA level in liver, heart and serum compared with normal group, confirming the role of WSSP as a potential antioxidant.

DISCUSSION

It is well-known that free radicals can result in various diseases. In recent years, natural polysaccharides have received great attention due to their antioxidant activities. In present study, a novel WSSP was exhibited to possess the evident antioxidant capacities to scavenge hydroxyl and ABTS radical, and reducing power (Fig. 1-3), which is similar to antioxidant activity of water-soluble polysaccharide from *Dendrobium denneanum* (Luo *et al*., 2011).

Table 1A: Effect of WSSP on liver and kidney SOD, T-AOC and MDA in mice

Group	Liver			Kidney		
	SOD (U/mg prot)	T-AOC (U/mg prot)	MDA (nmol/mgprot)	SOD (U/mgprot)	T-AOC (U/mg prot)	MDA (nmol/mgprot)
Normal group	63.42±3.02	4.28±0.07a	6.57±0.89a	103.80±2.79a	4.78±1.20a	36.88±2.95
WSSP group	69.20±5.77	5.27±0.15b	4.08±0.47b	201.98±23.31b	15.88±1.38b	30.30 ±3.46

Table 1B: Effect of WSSP on heart and serum SOD, T-AOC and MDA in mice

Group	Heart			Serum		
	SOD (U/mg prot)	T-AOC (U/mg prot)	MDA (nmol/mgprot)	SOD (U/mgprot)	T-AOC (U/mg prot)	MDA (nmol/mgprot)
Normal group	137.77±7.84a	1.47±0.13a	16.01±2.20a	16.48±0.92a	30.83±1.23a	9.21±1.41a
WSSP group	224.41±4.14b	1.69±0.06b	10.31±0.30b	18.06±0.38b	52.62±3.77b	6.21±0.30b

The data are the means ± standard deviations (n=12) and evaluated by one-way ANOVA followed by t-test to estimate inter-group differences, values followed by different letters in same column are significantly different ($P<0.05$).

Furthermore, our studies demonstrated that WSSP can increase SOD and T-AOC activities, and decrease the MDA level in *vivo*. SOD, which belongs to a major antioxidant enzyme in organism, converts superoxide radicals into hydrogen peroxide, which then is decomposed to oxygen and H_2O, thereby reducing the damage done by superoxide radicals (Li *et al*., 2007). The results from our study are agreement with previous findings that polysaccharides from sweet potato vines (PSPV) can increase the SOD activity (Luo *et al*., 2006). The possible cause may be that this synthesis of the enzyme is increased by gavage administration of WSSP. Oxidative stress takes place while TAOC is overwhelmed by over production of reactive oxygen species (ROS) (Li *et al*., 2007). The oxidative stress caused by ROS may lead to pathologic state (Smaga *et al*., 2012). TAOC can reflect the ability of nonenzymatic antioxidant defense system. Assay of TAOC from serum and tissue, therefore, can obtain a more precise implication of the correlation between antioxidants and free radicals. The data obtained from this present study demonstrated that the intake of WSSP can markedly enhance the TAOC in all tested mice. MDA, a naturally occurring product of lipid peroxidation, has been documented a primary biomarker of free radical mediated damage and oxidative stress (Del Rio *et al*., 2005). Therefore, MDA level in tissue and serum, as an important index of lipid peroxidation, was measured. The results obtained from this study showed the MDA level decreased compared with normal group.

CONCLUSION

The findings of this present study demonstrated that WSSP possessed markedly antioxidant activities in vitro and *in vivo*. WSSP may have an utilization as a potential additive in the food and pharmaceutical industries. Further investigation on WSSP structures and other biological activities are in progress.

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