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Antioxidant and antitumor activities of the polysaccharide from seed cake of *Camellia oleifera* Abel

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ABSTRACT

To explore biomedical potential of the polysaccharide from seed cake of *Camellia oleifera* Abel, we investigated antioxidant and antitumor capacities of the polymer. The results showed that the polysaccharide is capable of scavenging both superoxide anion and hydroxyl radicals *in vitro*. The highest scavenging rate of superoxide anion and hydroxyl radicals is 85% and 76%, respectively. Using the model animal, *Caenorhabditis elegans*, we further show that the polysaccharide can increase antioxidant enzyme activity, decrease lipid peroxidation level, and reduce paraquat-induced oxidative damage at a polysaccharide concentration more than 50 mg/l. We also revealed that the polysaccharide has some ferric chelating ability and strong *in vivo* antitumor activity. The antitumor rate against Sarcoma180 solid tumor grown in BALB/C mice reached 85.6% at the highest dose of 40 × 20 mg/kg days.

1. Introduction

Oxidative stress imposed by reactive oxygen species (ROS) may be the direct or indirect cause of tissue damage and many human diseases [1]. Antioxidants may play an essential role in protecting our bodies from various oxidative damages linked to cancer, diabetes, cardiovascular disease and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases [2]. The synthetic antioxidants, are able to prevent the radical chain reactions of oxidation, present almost unavoidable side effects which might be responsible for liver damage and carcinogenesis [3]. Hence, the search for new sources of natural antioxidants is currently of major interest to scientists.

The seed cake of *Camellia oleifera* Abel is a byproduct of oil refinery of *Camellia oleifera* Abel, its yield is about 2 million ton per annum in China [4], is usually used as fuel. According to the dictionary of Chinese's Materia Medica records the whole plant of *Camellia oleifera* Abel has some biological activity. In the past several years, medicinal plant polysaccharides have been widely studied for their chemical properties and biological activities [5], including antitumor [6], immunostimulation [7], and antioxidation [7]. However, no information is available about polysaccharide extracted from seed cake of *Camellia oleifera* Abel.

In this study, we first investigated the *in vitro* scavenging effect of seed cake polysaccharide of *Camellia oleifera* Abel on superoxide anion and hydroxyl radical, and then the *in vivo* effect of the polysaccharide on antioxidant enzyme activities, lipid peroxidation level and superoxide-mediated paraquat toxicity using the model animal *Caenorhabditis elegans*. We also probed the antitumor activity and ferric chelating ability of the polysaccharide.

2. Materials and methods

2.1. Chemicals and materials

Seed cake of *Camellia oleifera* Abel was provided by Dongfeng oil refinery, Hunan, China. The samples were cut into small pieces and further ground into a fine powder in a high speed disintegrator and dried at 65 °C (Model DFY-500, DaDe Chinese Traditional Medicine Machine Co. Ltd, Zhejiang, China), and passed through a 40 mesh sieve.

Dextrans of different molecular weights, DEAE-Cellulose A52 and Sephadex G-100 were purchased from the Pharmacia Co. (Uppsala, Sweden). Pure monosaccharide standards (D-mannose, L-rhamnose, D-galactose, D-fucose, L-arabinose, and D-glucose) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Fluka. Aqueous solutions were prepared with ultrapure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade.

2.2. Preparation of polysaccharide fractions

The ground materials was first extracted with petroleum ether at 60–90 °C for 12 h, and soaked with 80% ethanol for 4 h twice and removal of the solvent, and then was immersed overnight in

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distilled water at room temperature and then extracted at 90 ± 1 °C for an hour. The extract was centrifuged, and the supernatant was collected and concentrated under reduced pressure at 40 °C. The solution was precipitated with 3 volumes of ethanol, and the precipitate was collected by filtration and redissolved in distilled water. After the removal of proteins by Sevag method, the polysaccharide solution was dialyzed extensively against distilled water and lyophilized. The crude polysaccharides (SCP) was redissolved in deionized water and forced through a filter (0.45 μm), then applied to a column of DEAE-Cellulose A52. After loading with sample, the column was eluted with gradient NaCl aqueous solution (0–1 M), and the procedure was monitored by the phenol–sulfuric acid method mentioned above. The collected fractions were further purified on a Sephadex G-100 gel filtration column and eluted with deionized water, at a flow rate of 9 ml/h. The main polysaccharide fraction was collected and lyophilized to obtain white purified polysaccharide, named as SCP1 and used in further study. Prior to use, the polysaccharide was dissolved in water and diluted into a series of concentrations as indicated.

2.3. General methods

The carbohydrate content of the solution was determined by phenol–sulfuric acid method using glucose as standard. Protein content was determined by the Bradford method [8], using bovine serum albumin (BSA) as the standard. IR spectra were recorded using a Fourier transform infrared spectrophotometer (FTIR, Nicolet 5700 Instrument, ThermoCompany, Madison, USA) with KBr pellets in the frequency range 4000–400 cm^{-1}. The monosaccharide component analysis was performed by HPLC method as described by Honda et al. [9]. The molecular weight of SCP1 was determined by HPLC on an Agilent-LC 1100 instrument (Agilent, USA), equipped with a TSK gel 4000 PWXL column and eluted with 0.05 M Na_2SO_4 solution at a flow rate of 0.8 ml/min. Elution was monitored by an Agilent refractive index detector. A series of solutions made from standard Dextrans were run under the same conditions and a standard curve linear over a wide range (10–1000 kDa) was obtained by plotting the elution volume *versus* the logarithm of the corresponding molecular weight.

2.4. Ferric chelating

The ferrous ion chelating ability of the samples (from 0.1 to 2.0 mg/ml) was investigated according to Wang et al. [10].

2.5. In vitro antioxidant assays

The hydroxyl radicals scavenging assay was evaluated using the hydroxyl radical system generated by the Fenton reaction [11]. Briefly, sample was dissolved in distilled water at (control), 1.0–5.0 mg/ml. The reaction mixture contained 1 ml brilliant green (0.435 mM), 0.5 ml $FeSO_4$ (2 mM), 1.5 ml H_2O_2 (3.0%) and 1 ml samples with varying concentrations. After incubation at room temperature for 20 min, the absorbance of the mixture was measured at 624 nm. The superoxide radical scavenging abilities of the sample were assessed by the method of Zhang et al. [12]. Superoxide anion radicals were generated in 4.5 ml Tris–HCl buffer (16 mM, pH 8.0) containing 0.5 ml NBT (300 μM) solution, 0.5 ml NADH (468 μM) solution and one sample (1.0–5.0 mg/ml). The reaction was started by adding 0.5 ml PMS (60 μM) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and measured at 560 nm. Both of scavenging rate (%) of scavenging the superoxide anion radicals and the hydroxyl was calculated as (1 − absorbance of sample/absorbance of control) × 100.

2.6. In vivo antioxidant assays

The *C. elegans* wild-type strain (N2) was obtained from Institute of Development Biology Base of Fudan University, and maintained at 20 °C on NGM plates with *Escherichia coli* OP50 [13]. Synchronization of the worms was performed as described [14]. Food clearance assay was used to determine the suitable range of the polysaccharide concentration [15].

Age-synchronized worms were grown to young adult in S medium and treated with 5-fluoro-2′-deoxyuridine for 2 days at 25 °C to block reproduction. After growing for another 18 days at 25 °C, the worms were treated with SCP1 at indicated concentrations or 50 μg/ml positive control, resveratrol, for 2 days. The worms were harvested, cleaned from bacteria and debris by sucrose floatation, and washed with S medium. The cleaned worms were suspended in 2 ml of 50 mM, pH 7.8 phosphate buffer and homogenized in glass homogenizer. The homogenate was centrifuged at 5000 × *g* and 4 °C for 10 min, and the supernatant was collected for determination of superoxide dismutase (SOD) activity [16], catalase (CAT) activity [17], glutathione peroxidase (GPX) activity [18] and malondialdehyde (MDA) content [19].

To evaluate the *in vivo* antioxidant effect of the polysaccharide against paraquat-induced oxidative stress, the paraquat assay was performed according to Pun et al. [20]. The synchronized young adult worms were treated with the polysaccharide in S medium for 2 days at 25 °C. The worms were then transferred to 96-well plates with about 10 worms per well, and more than 100 worms for each treatment, and exposed to 0.1 mol/l of paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) in S medium containing heat-killed *E. coli*. The numbers of live and dead worms were scored microscopically every 4 h on the basis of their movement, before counting, the plate was gently vibrated to stimulate movement of worms.

2.7. In vivo antitumor test

Animal care and handling were according to the Committee for the Purpose of Control and Supervision of Experiments on Animal's guidelines after research project approval by the Institutional Animal Ethics Committee.

BALB/C mice (male, 6–8 weeks old, weighing 20 ± 2 g) were purchased from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Each of polysaccharide sample as well as control agents were tested on an individual group of animals consisting of 6 BALB/C mice.

Sarcoma 180 tumor cells (5×10^6 cells ml^{-1}) were transplanted subcutaneously into the right groin of the mice. After 24 h of tumor inoculation, SCP1 samples were dissolved in 0.9% aqueous NaCl, then injected intraperitoneally to the mice in the experiment group every other day for 20 days. The equivalent volume of 0.9% aqueous NaCl was injected intraperitoneally in the negative control group. The mice were killed on the next day of the last injection, and the tumors were removed and weighed.

The inhibition ratio ξ and enhancement ratio of body weight f were calculated as follows:

$$\xi = \frac{W_c - W_t}{W_c} \times 100\%$$

$$f = \frac{W_a - W_b}{W_b} \times 100\%$$

where W_c is the average tumor weight of the negative control group, W_t is the average tumor weight of SCP1 group; W_b and W_a are the body weight of mice before and after the assay.

Table 1
Monosaccharide compositions of SCP1.

Monosaccharide	Rhamnose	Fucose	Arabinose	Mannose	Galactose	Glucose
Content (wt%)	5.16	20.77	2.58	5.39	14.32	51.78
Molecular ratio	3.08	13.75	1.87	3.25	8.02	31.24

2.8. Statistical analysis

All data are expressed as means ± standard deviation from a minimum three separate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Isolation and purification of SCP1

The water-soluble crude polysaccharide was obtained as a light-brown powder from the cake by hot-water extraction, ethanol precipitation, deproteinization and lyophilization. The total yield of SCP was 10.8% of the dried material, much higher than the yield of 6.5% from the fruit shell of *Camellia oleifera* Abel [21]. SCP was further purified by DEAE-cellulose A52 column and Sephadex G-100 column chromatography. The main fraction (SCP1) was collected and lyophilized for further study on structure and antitumor bioactivity. SCP1 appeared as a white powder and showed negative iodine–potassium iodide reactions, indicating that it did not contain starch-type polysaccharide. The homogeneity of SCP1 was confirmed by a single symmetrical peak appeared in HPLC.

Based on the equation of the standard curve made by different dextran standards and the retention time of SCP1, the molecular weight of SCP1 was estimated to be 458 kDa.

3.2. Composition and physical property of SCP1

SCP1 was a water-soluble compound. The results showed that the polysaccharide was composed of rhamnose, fucose, arabinose, mannose, galactose and glucose at a molecular ratio of 3.08:13.75:1.87:3.25:8.02:31.24 (Table 1). The monosaccharide's composition is identical to that of WEP2, while the molecular ratio shows much difference [21].

IR spectra of SCP1 showed a typical major broad stretching peak at 3426.5 cm^{-1} for the hydroxyl stretching vibration of the polysaccharide; a weak band at 2921.7 cm^{-1} was attributed to the C—H stretching and bending vibrations. The broad band at 1645 cm^{-1} was due to the bound water. Furthermore, the characteristic absorption bands at 849 cm^{-1} and 889 cm^{-1} indicated that SCP1 contained both α-glycosidic and β-glycosidic linkages [22]. Because of the complexity of polysaccharide structure, only some of the structure information was identified here. The detailed accurate structure of polysaccharide needs to be further studied by two-dimensional NMR and methylation analysis [23].

3.3. Ferric chelating

Iron is the most powerful pro-oxidant among the various metal ions and catalyzes the oxidative changes in lipids, proteins and other cellular components [24]. Metal chelating capacity is significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. Table 2 shows the chelating effects of SCP1 and the standard, EDTA, which acts as a chelating agent and forms complexes readily with diverse metallic ions. The results revealed that SCP1 presented low ferric chelating capacity when compared with EDTA, and the activity doses dependent.

Table 2
Ferric chelating capacity of SCP1 and EDTA.

	Concentration (g/l)	Chelating rate (%)
SCP1	0.1	15.2 ± 0.87
	0.5	18.3 ± 0.95
	1.0	22.6 ± 1.38
	1.5	25.1 ± 1.45
EDTA	0.1	25.5 ± 1.87
	0.5	54.7 ± 2.13
	1.0	72.3 ± 2.58
	1.5	95.2 ± 3.21

3.4. In vitro ROS scavenging effect

The delicate balance between reactive oxygen species (ROS) production and clearance is critical to maintain normal physiology of cells. Although most ROS generated under normal conditions can be detoxified by endogenous antioxidant systems, excessive production of ROS often triggers oxidative stress, which is involved in aging and a range of diseases. Since antioxidants are capable of scavenging the deleterious ROS, it is widely accepted that appropriate supplementation of exogenous antioxidants may help reduce ROS-induced oxidative damage. However, due to concerns about the side-effects of some synthetic antioxidants, there is an increasing interest in replacing synthetic antioxidants with natural antioxidants in food, pharmaceutical and cosmetic industries. Among the natural antioxidants are polysaccharides, including those from plant [25,26]. Superoxide anion and hydroxyl radical are the most representative forms of ROS in oxidative reactions. Since the overproduction of ROS, such as superoxide, hydrogen peroxide and hydroxyl radical, is directly associated with oxidative stress, we tested the ability of SCP1 to scavenge ROS. As shown in Fig. 1, the polysaccharides is capable o f scavenging both superoxide anion and hydroxyl radical *in vitro* in a dose-dependent manner; the highest scavenging rates to superoxide anion and hydroxyl radical were 85% and 76%, respectively, demonstrating the *in vitro* antioxidant activity of the polysaccharide. Although hydroxyl radical is the strongest and most harmful ROS, superoxide anion is a relatively weak oxidant species [27]. Nevertheless, superoxide anion can generate other, more toxic forms of free radicals, including hydroxyl radical and singlet oxygen, which may trigger further lipid peroxidation and lead to more damage to cellular components [28]. We

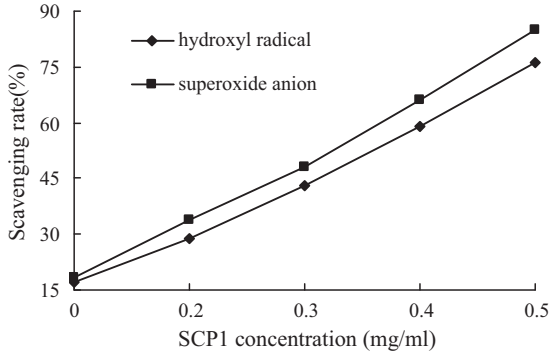

Fig. 1. Scavenging effect of SCP1 on superoxide anion and hydroxyl radical.

Table 3
Effect of SCP1 on the antioxidant enzyme activity and malondialdehyde content of *Caenorhabditis elegans*.

Treatment	Antioxidant enzyme activity			MDA content (mmol/g protein)
	SOD (U/mg protein)	GPX (U/mg protein)	CAT (K/mg protein)	
Control	23.2 ± 1.72	14.2 ± 0.62	32.3 ± 0.87	2.18 ± 0.16
SCP1 (mg/l)				
25	26.1 ± 1.83	16.2 ± 0.64	34.5 ± 0.79	2.15 ± 0.13
50	30.6 ± 1.32	17.3 ± 0.35	36.6 ± 1.02	1.95 ± 0.16
100	35.4 ± 2.01	18.6 ± 0.46	37.9 ± 0.73	1.72 ± 0.07
Resveratrol	35.6 ± 1.23	18.7 ± 0.56	35.1 ± 0.98	1.90 ± 0.08

Table 4
Antitumor activities of SCP1 against Sarcoma180 solid tumor grown in BALB/C mice.

Sample	Dose (mg/kg days)	Enhanced ratio of body weight (%)	Tumor weight (g)	Tumor inhibitory rate (%)
Negative control		35.2	1.15 ± 0.36	–
SCP1	10 × 20	45.8	0.52 ± 0.08	56.8
	20 × 20	48.2	0.40 ± 0.06	73.2
	40 × 20	47.8	0.28 ± 0.05	85.6

have shown that SCP1 is able to scavenge both superoxide anion and hydroxyl radical (Fig. 1). The higher activity of the polysaccharide in scavenging superoxide anion over hydroxyl radical may indicate its preventive effect against ROS formation and oxidative damage. The underlying *in vitro* scavenging mechanisms of SCP1 may involve direct participation in oxidation reaction as well as interaction with hydroxyl radical as discussed for other polysaccharides [10]. Interestingly, SCP1 has some ability to chelate ferric ion (Table 2), which is needed for the generation of hydroxyl radical and decomposition of hydrogen peroxide in Fenton's reaction [29], may also contribute to the scavenging activity of SCP1 on hydroxyl radical.

3.5. Effect of SCP1 on antioxidant enzyme activity and lipid peroxidation level in C. elegans

Oxidative stress develops when an imbalance between ROS production and elimination occurs. Therefore, we examined the effect of SCP1 on the antioxidant defense system of *C. elegans*, a favorable animal model in oxidative stress and aging studies with a short life span [30]. As shown in Table 3, the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), was significantly increased when the worms were treated with SCP1 for 2 days at concentration >50 mg/l. When the polysaccharide was at 100 mg/l, the increase level of the antioxidant enzyme activity was at a comparable scale with the treatment of 25 mg/l resveratrol, a known polyphenolic antioxidant [31]. On the other hand, the level of lipid oxidation product MDA was decreased about 21.1% when the worms were treated with 100 mg/l of SCP1 (Table 3). All these data show that SCP1 has *in vivo* antioxidant effect.

3.6. Effect of SCP1 on superoxide-mediated paraquat toxicity in C. elegans

To test the effect of the polysaccharide SCP1 on oxidative stress-mediated toxicity in general, we examined the survival rate of paraquat-challenged *C. elegans*. Paraquat, a highly toxic herbicide to animals and humans, is known to increase an intracellular superoxide anion level, which may result in generation of more toxic hydrogen peroxide and hydroxyl radicals [32]. As shown in Fig. 2, SCP1 increased the survival rate of the worms exposed to paraquat, suggesting the reduction of paraquat-induced oxidative stress and toxicity.

3.7. In vivo antitumor activity

A variety of polysaccharides from different biological sources has been shown to enhance the immune system. The immunostimulating potential of these biopolymers is described as mainly promoting the activity of phagocytosis of the respective macrophages and granulocytes, inducing the production of TNFα and interleukins, and, finally, acting on the complement system. According to reports, more than 100 kinds of plant polysaccharides were extracted. Due to the wide source and no cytotoxicity being applied to organisms the research on the plant polysaccharides has become a hot domain in the medical field [33].

The inhibition ratio of SCP1 *in vivo* assay was summarized in Table 4. SCP1 exhibited remarkable antitumor activity against Sarcoma180 cells compared to the negative control group. As shown in Table 4, the tumor inhibition ratio of SCP1 increased as the dose increased. At the highest dose 40 mg/kg days, the tumor inhibition rate reached 85.6%. Many investigators reported that polysaccharides with antitumor action differ greatly in their chemical composition, configuration and physical properties. In some reported cases, minor changes in structure, molecular weight or chain conformation may have dramatic effects on potency.

Antitumor activity assays implied that SCP1 seemed to have a powerful tumor-fighting properties *in vivo*. Furthermore, the enhancement ratios of body weight indicated that the polysaccharide might not have cytotoxicity, which kills normal cells as well as cancer cells, which would greatly promote the development of antitumor polysaccharides from seed cake of *Camellia oleifera* Abel.

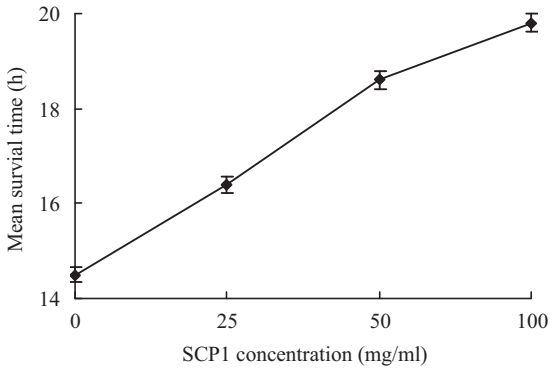

Fig. 2. Effect of SCP1 on the mean survival time of paraquat-treated *Caenorhabditis elegans*.

4. Conclusion

The results showed that SCP1 is capable of scavenging both superoxide anion and hydroxyl radicals in a dose-dependent manner both *in vivo* and *in vitro*. At the same time, SCP1 can increase the activities of antioxidant enzymes (SOD, CAT and GPX) and decrease the content of lipid peroxidation product MDA in *C. elegans* at a comparable scale with resveratrol treatment. In addition, SCP1 displayed promising activity in the antitumor assay and can increase the survival rate of paraquat-challenged *C. elegans*. Furthermore, the enhancement ratios of body weight indicated that the polysaccharide might not have cytotoxicity, it can be assumed to be potentially useful as a safe antitumor agent for industries. Finally, the biological activity observed in SCP1 provides a scientific basis for the use of the "waste" in traditional medicines.

Doubtless more detailed studies on the physicochemical properties and bioactivities of SCP1 would be helpful for its potential medical application. The next challenge is to determine other properties of SCP1 and identify correlations between pharmacological effects and structure.

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