

Ophiopogon Japonicus Polysaccharide Plays an Anti-esophageal Cancer Role Through Antioxidant Activity

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Abstract: As a natural product with a variety of biological activities, plant polysaccharide has become a research highlight in the field of antioxidant activity and anti-tumor due to its high efficiency and low toxicity. In this study, the antioxidant and anti-tumor activities of *Ophiopogon japonicus* polysaccharides (OJP) in vitro and in vivo were investigated. The results showed that OJP had obvious scavenging ability on hydroxyl radical and superoxide radical in a dose-dependent manner. Moreover, the reducing power of OJP also increased and appeared to be dose-dependent. In vivo, it reduced the accumulation of lipofuscin and induced cell apoptosis of *Caenorhabditis elegans*. In addition, it significantly inhibits the viability of esophageal cancer cell lines Eca109 and TE-1 in vitro and promoted their apoptosis. In conclusion, OJP avoids oxidative damage of organisms and plays an anti-tumor role via its antioxidation. This study aims to promote the in-depth study of plant polysaccharides and provide theoretical reference for the development of antioxidant foods and anti-tumor drugs.

Keywords: Antioxidant; Apoptosis; Esophageal carcinoma; Lipofuscin; *Ophiopogon japonicus* polysaccharides.

1. Introduction

Polysaccharide generally refers to a class of biological macromolecules polymerized from 10 or more monosaccharides, which widely exists in the cell walls of animals, plants, bacteria and fungi. Plant polysaccharides are rich in sources, have antioxidant, anti-inflammatory, anti-tumor and other biological activities, and have attracted much attention due to their low toxicity and side effects [1,2]. When the body is exposed to various harmful stimuli, the oxidation system and antioxidant system in the body are out of balance, and highly active molecules such as reactive oxygen species accumulate excessively which leading to cell aging and tissue damage [3]. There are more than 100 kinds of diseases caused by oxygen free radicals, such as neurodegenerative diseases, cancer, diabetes and cardiovascular diseases [4]. Therefore, when the antioxidant enzymes in the body are difficult to resist the excessive enrichment of free radicals, it is necessary to supplement natural non-toxic antioxidants to alleviate cell damage. Plant polysaccharide is a natural product that can scavenge free radicals and regulate metabolism, which may be due to the complex structure of polysaccharide with different amounts of active hydroxyl and active hydrogen. These active groups specifically recognize and pair with single free radicals, and achieve the function of scavenging free radicals by changing its structure [5]. Feng et al. showed that PNP could improve the activity of antioxidant enzymes and reduce lipid peroxidation [6]. Furthermore, the fucoidan was proved to have a variety of physiological activities such as antiviral, anticoagulant, anti-tumor, antioxidant, lipid-lowering, immune regulation [7,8]. *Ophiopogon japonicus*, as a traditional Chinese medicine, is the dried root of *Ophiopogon japonicus* of a lily plant. Its main chemical components include steroidal saponins, polysaccharides, flavonoids, amino acids, etc. *Ophiopogon japonicus* polysaccharides (OJP) are mainly composed of fructose and glucose, and have a variety of physiological activities such as immune regulation, anti-inflammatory and anti-tumor [9,10].

Caenorhabditis elegans (*C. elegans*) is a fungus eating organism existing in soil. Because of its short life cycle, easy maintenance and thousands of mutant strains available, it is widely used as a model organism to study the effects of anti-oxidation, anti-aging, anti-Alzheimer's disease and anti-diabetes drugs [11]. At present, there are many studies on the anti-diabetes, anti-virus and immune effects of OJP, but there are few reports on its anti-tumor effect through antioxidant activity. In this study, the antioxidant effect of OJP was studied in vitro, and the model animal of *C. elegans* was taken to further understand its antioxidant and anti-tumor effects, and its anti-esophageal cancer effect was also explored in vitro. The findings might provide theoretical reference for natural food development and clinical cancer treatment of OJP.

2. Materials and Methods

2.1. Materials

OJP was purchased from Xi'an Kailai Biological Engineering Co., Ltd. (Xi'an, China) with a purity of 91.88%. Eca109 and TE-1 cells were obtained from ATCC. RPMI1640 was purchased from Gibco (USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Penicillin, Streptomycin, and agarose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Tian hang Biotech Co. Ltd (Hangzhou, China). DAPI staining solution were bought from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China). DNA Ladder Cell Apoptosis Kit was from Beyotime Biotechnology Co., Ltd (Shanghai, China). All other reagents were of the highest grade commercially available.

2.2. Scavenging activity on hydroxyl radical

The Hydroxyl radical (HO[•])-scavenging activity of OJP was measured by an improved Fenton-type reaction. In brief, 1.0 mL of OJP, at different concentrations, was spiked with 1.0 mL of 9 mM FeSO₄, 1.0 mL of 9 mM salicylic acid–

ethanol and 1.0 mL of 9 mM H₂O₂ and incubated at 37°C for 30min. Vc was used as positive control. The absorbance at 510 nm was measured with a visible spectrometer. The percentage of HO·-scavenging activity of OJP was calculated according to the following formula: Scavenging activity against HO· (%) = [1-(Abs. of sample - Abs. of blank)/Abs. of control] × 100.

2.3. Scavenging activity on superoxide radical

The scavenging activity on O₂⁻ of OJP was determined as described in previous study. 200 µL of different concentration sample solution and 3 mL of 0.1 M Tris-HCl were successively added into test tube and incubated at 37°C, 12 µL of 30% mM Pyrogallol solution was added. After 4min, 0.5 mL hydrochloric acid was added immediately to terminate the reaction. The absorbance at 320 nm was measured with a visible spectrometer. O₂⁻-scavenging capability of OJP was calculated according to the following formula: Suppression rate (%) = [1-(Abs. of sample - Abs. Of blank)/Abs. of control] × 100.

2.4. Measurement of reducing power

The reducing power of OJP was measured according to previous procedure [12]. Briefly, 1 mL of different concentration OJP solution was mixed with 1 mL of 0.2 M phosphate buffer saline (pH 6.6) and 1 mL of 1% (w/v) K₃Fe(CN)₆ solution, then incubated at 50°C for 20 min. Afterward, 1 mL of 10% (w/v) TCA solution was added and centrifuged at 3000r/min for 10min. 2.5 mL of the upper layer was combined with 2.5 mL of distilled water and 1 mL of 0.1% (w/v) FeCl₃ solution. Absorbance analysis was followed at 700nm (Vc was used as a positive control). Increased absorbance of the reaction mixture indicates a greater reducing power.

2.5. Fluorescent detection of lipofuscin and apoptosis

2% agarose gel was prepared and poured into a petri dish lid with 1-3 mm thick. After coagulation, a small piece of agar was cut off and placed it on a slide. The nematode was picked out of the medium and exposed to 485nm and 530 nm wavelength blue light by fluorescence microscopy. The apoptosis of nematode cells was stained by 4', 6-diamidino 2-phenylindole fluorescence staining (DAPI), Nematodes were collected using low speed centrifugation with M9 buffer. Each insect sample was stained in photochemical dyes for 10 min, then washed three times with PBS, and observed with a fluorescent microscope that stimulated 360 nm and emitted 400 nm wavelength of ultraviolet light.

2.6. MTT assay

Cytotoxic activities of OJP against Eca109 and TE-1 cell lines were evaluated by the MTT method. Briefly, the tumor cells from logarithmically growing cultures were seeded into 96-well plates at a concentration of 6000 cells per well. After cell adherence, the culture medium was removed and fresh medium containing various concentrations of OJP was added. They were incubated at 37°C, 5% CO₂ for 24 h. Three replicated wells were applied for per concentration. After incubation, the cells were treated with 20 µL of 5 mg/mL MTT and further cultured at 37°C for 4 h. Subsequently, the medium was removed and 100 µL of DMSO was added to each well for 15min. The absorbance of the 96-well plate was measured at 490 nm on a Microplate Reader Bio-Rad 550

after the crystals were fully dissolved.

2.7. DNA ladder experiment

Eca109 and TE-1 cells were treated with different concentrations of OJP for 24 h following to collect culture medium and adherent cells. DNA of each group was collected according to the instructions of DNA ladder kit. The DNA samples were electrophoresed on a 1.2% agarose gel containing 0.04% GoodView nucleic acid dyes. The gel was examined and photographed by an ultraviolet gel documentation system (Bio-Rad, USA).

2.8. Statistical analysis

The data in this paper are processed by Graphpad Prism 5.0, and p<0.05 indicates significant difference. The data is expressed as mean±SD, processed with One-way ANOVA, photographed about active lipofuscin and DAPI staining by fluorescence microscope, and line charts were plotted with GraphPad Prism7.0.

3. Results and Analysis

3.1. In vitro antioxidant activity of OJP

In this study, the in vitro antioxidant capacity of OJP was estimated with ·HO, O₂⁻ and ferric-reducing antioxidant power systems.

3.1.1. Scavenging effect of OJP on hydroxyl radical

HO· is one of the most important active oxygen species which has strong oxidizing capacity and great harm to human. Therefore, the scavenging effect on hydroxyl radical is an important part in the study of OJP antioxidant activity. Fenton reaction (Fe²⁺ + H₂O₂ = HO· + OH⁻ + Fe³⁺) produces HO· which reacts with salicylic acid, then 2,3-dihydroxybenzoic acid is produced which has a special absorption peak at 510 nm. Previous research shows If antioxidants are added to the system, less HO· will be produced and the absorbance decreases [13]. In this experiment, scavenging effect of OJP on hydroxyl radical was determined by Fenton method, all the data were shown in Figure 1. OJP showed the significant scavenging effects (6.94%, 14.97%, 21.35%, 28.63%, and 30.66%) against HO· in a dose-dependent manner (1–8 mg/mL). When the concentration of OJP is 8 mg/mL, the removal rate of hydroxyl radical is 30.66%. It is higher than rubescens polysaccharide, a crude polysaccharide extracted from binding grass by water extraction and alcohol precipitation, which showed 19.56% free radical scavenging activity at 1mg/mL [14]. However, compared with polysaccharides extracted from Chinese water chestnut [15] and osmunda japonica [16], OJP has relatively lower removal rate of hydroxyl radical. These differences may be related to the chemical structure of polysaccharides. In sum, these results show that OJP can help prevent oxidative damage to a certain extent.

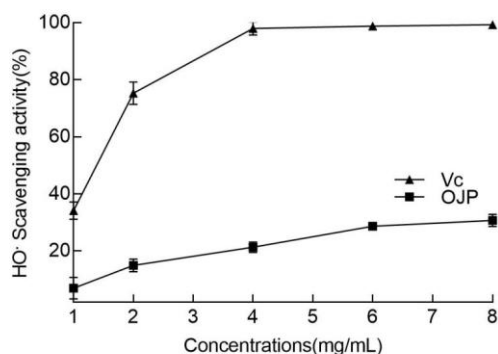


Figure 1. Scavenging ability of OJP to hydroxy free radical. Comparison of HO· radical scavenging by OJP and Vc, and the ladder is the positive control. Data represent the mean $\pm$ S.D. of 5 parallel samples from three independent experiments.

3.1.2. Scavenging effect of OJP on superoxide anion radical

As one of the causes of lipid peroxidation in the body, superoxide anion radical is the first free radical produced in the oxidation process. In Figure 2, OJP showed the ability of removing superoxide anion, but compared with Vc, its clearance rate is lower. In the concentration of 1–8 mg/mL, the scavenging rate of FSP on superoxide anion radicals increased in a dose-dependent manner. Namely, OJP exhibited obvious scavenging activity (9.95%, 13.00%, 24.01%, 32.53%, and 36.96%) against $O_2^{\cdot -}$ in different concentrations respectively. Lou [17] studied the superoxide anion scavenging ability of guava leaf polysaccharide, and the maximum scavenging rate was 22.30%, which was lower than our experimental results. Wang [18] explored the antioxidant effect of *Acer saccharum* polysaccharides ASP-A-c, ASP-A-d and ASP-A-e. When the maximum mass concentration of the three purified polysaccharides was 5 mg/mL, the $O_2^{\cdot -}$ clearance rate of the three plant polysaccharides was higher than 40%, which was higher than the removal rate of OJP with the maximum concentration of 8 mg/mL in this experiment (36.96%).

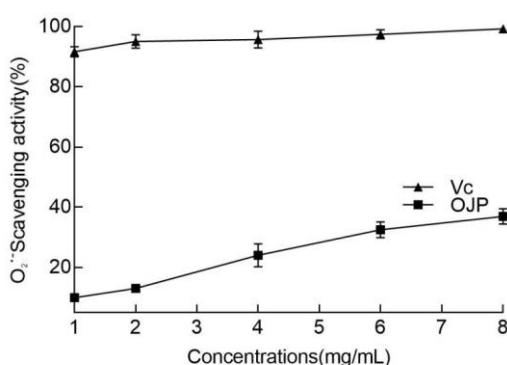


Figure 2. Scavenging ability of OJP to superoxide anion radical. Comparison of $O_2^{\cdot -}$ radical scavenging by OJP and Vc, and the ladder is the positive control. Data represent the mean $\pm$ S.D. of 5 parallel samples from three independent experiments.

3.1.3. Total reducing force of OJP

The sample reduces $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$ which reacts with Fe^{3+} and produces Prussian blue. Then, prussian blue is detected at the wavelength of 700 nm. The higher the absorbance value, the stronger the reduction ability of the sample. As shown in Figure 3, in the broad range of 1–8 mg/mL, the phenomenon of concentration-dependence was

notable, and ferric-reducing antioxidant power values (increased absorbance at 700 nm) of OJP ranged from 0.12 to 0.43. The data presented here indicates that OJP has potential to be explored as an antioxidant although it is inferior to the reference Vc in antioxidant capacity. The reduction ability may be affected by the molecular weight of polysaccharides, that is, the lowest molecular weight polysaccharides have the highest reduction ability. Similarly, some polysaccharides from chestnut, water shield and *Polygonatum odoratum* showed very low reduction capacity [19–21].

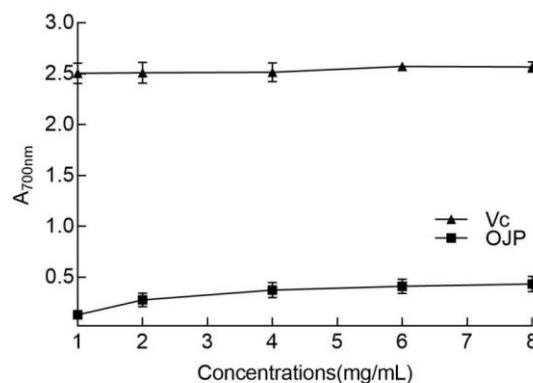


Figure 3. Total reducing power of OJP. Comparison of $O_2^{\cdot -}$ radical scavenging by OJP and Vc, and the ladder is the positive control. Data represent the mean $\pm$ S.D. of 5 parallel samples from three independent experiments.

The data presented here indicates that OJP has potential to be explored as an antioxidant although it is inferior to the reference Vc in antioxidant capacity.

3.2. Effect of OJP on lipofuscin and cell apoptosis in nematode

Lipofuscin [22] is an indicator of oxidative stress and aging of nematodes, which is a kind of spontaneous fluorescent pigment. The content of lipofuscin gradually increases with the age of nematode. Furthermore, excessive lipofuscin precipitation will cause the damage to nematodes and the aging of nematode will be accelerated in the end. As shown in Figure 4, the spontaneous fluorescence intensity of nematode in OJP treatment group appeared to be lower than that in control group. Therefore, OJP can slow down the accumulation of lipofuscin which delays the aging process of nematode. DAPI staining is often used for the detection of cell apoptosis [23], and the change of cell morphology such as envelope fold or blistering, highly condensed nuclear chromatin, marginalisation, etc. are the characters of cell apoptosis. DAPI staining results in Figure 4 showed that the nuclei in the control group chromatin is highly aggregated and morphologically changed, while OJP is relieved to some extent. The degree of apoptosis was dose-dependent. In previous research, *Cyclocarya paliurus* polysaccharide can significantly reduce the accumulation of brown pigment in *C. elegans* [24]. Zhang Xiaohan et al. [25] showed that nematode senescence leads to accumulation of lipofuscin in the body, and phloretin can significantly reduce the content of lipofuscin with a dose dependent effect. Furthermore, Suetomi et al. [26] reported that during the aging process of nematodes, accompanied by cell apoptosis, apoptotic cells appear bright blue after DAPI staining and natural active substances alleviate the degree of cell apoptosis to a certain extent. The results of this study are consistent with these

previous studies.

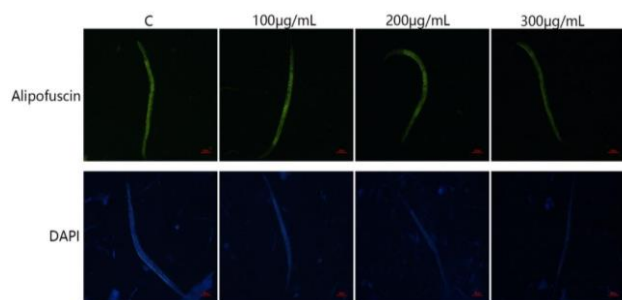


Figure 4. Effect of OJP on lipofuscin accumulation and DAPI staining in *C. elegans*. *C. elegans* was treated with different concentrations of OJP (0 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL) for 5d. Then, the nematodes were picked out of the medium and observed by fluorescence microscopy. The apoptosis of nematode cells was stained by DAPI for 10 minutes, then observed with a fluorescent microscope that stimulated 360 nm and emitted 400 nm wavelength of ultraviolet light. The data shown are representative of three independent experiments.

3.3. OJP decreased the viability of Eca109 and TE-1 cells

As shown in Figure 5, the antitumor activity of Eca109 cells was gradually enhanced with the increase of drug concentration, and the difference between 300 µg/mL, 400 µg/mL, 500 µg/mL and 600 µg/mL groups and the control group was extremely significant. For TE1 cells, in the concentration of 25-200 µg/mL group, there was no statistical differences compared with the control group. However, OJP with the concentration of 300 µg/mL, 400 µg/mL, 500 µg/mL and 600 µg/mL significantly decreased the cell viability. In previous research, Mariem et al. reported that watermelon peel polysaccharide had a significant cytotoxic effect on human laryngeal cancer Hep-2 cells in a dose and time dependent manner [27]. OJP also inhibit the growth of s180 sarcoma and ascites tumor, and had a certain inhibitory effect on solid tumors of hepatocellular carcinoma [28].

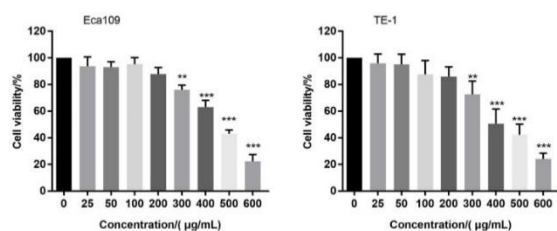


Figure 5. OJP decreased the viability of Eca109 and TE-1 cells. Eca109 and TE-1 cells were cultured with various concentrations of OJP (C, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL and 600 µg/mL) for 48h. Then, MTT assay was used to detect the viability of each group. Data represent the mean $\pm$ S.D. of 5 parallel wells from three independent experiments

3.4. OJP induced the apoptosis of Eca109 and TE-1 cells

We explored whether the decreasing of the viability of Eca109 and TE-1 cells was related to the apoptosis which appeared to cleave their DNA into very precise fragments of 180–200 bp. DNA fragments in this way form a ladder which can generally be detected by agarose gel electrophoresis. Our study revealed that middle and high dose of OJP-treated

Eca109 cells showed obvious typical ladders while faint ladder bands appeared in TE-1 cells (Figure 6). These data suggested that OJP induced the apoptosis of Eca109 and TE-1 cells in a dose-dependent manner. Tian et al. found that *Pinellia ternata* polysaccharide induced dose dependent apoptosis in Hep-G2 cells [29].

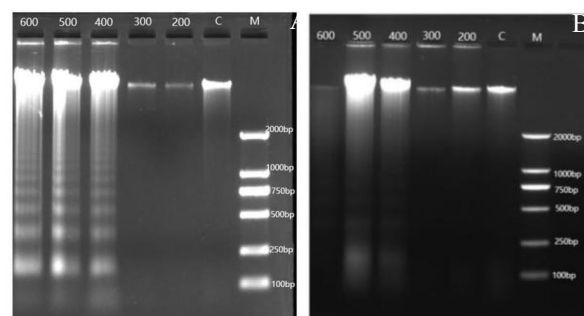


Figure 6. OJP induced the appearance of typical ladders. Eca109 and TE-1 cells were treated with different concentrations of OJP. Fragmented DNA was extracted and assessed by agarose gel electrophoresis and ethidium bromide. Data shown are representative of three separate experiments. Lane M: DNA marker. A: Eca109 cells were treated without OJP. B: TE-1 cells were treated without OJP. The concentrations of OJP were 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL and 600 µg/mL respectively. The data shown are representative of three independent experiments.

4. Conclusion

OJP has been proven to have multiple physiological activities and potential medicinal value. In this experiment, we first studied the antioxidant activity of OJP in vitro, then investigated its antioxidant activity in vivo, and even explored its regulation on the viability and apoptosis of esophageal cancer cell lines. This study found that OJP has significant antioxidant effects in vitro, effectively delays the aging of nematodes, significantly inhibit the proliferation of esophageal cancer cell lines, and promote their apoptosis. In summary, these findings provide experimental basis for the antioxidant research of OJP, as well as theoretical basis for the development of natural antioxidants and anti-tumor drugs. However, the molecular mechanism of OJP antitumor is relatively complex, and further molecular level research needs to be carried out in the future.

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