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Antioxidant stress and anti-aging activity of medicinal plant polysaccharides based on the model of *Caenorhabditis elegans*

Jianchuan Wang[1], Wanyu Li[1], Deli Chen[1], Lin Ma[2]

[1] School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China

[2] Engineering Research Center for Biomass Resource Utilization and Modification of Sichuan Province, China

Abstract

Objective: To study the antioxidant activity *in vitro* and *in vivo* and anti-aging activity of polysaccharide from *Houttuynia cordata*, *Eleutherococcus senticosus*, *Melia azedarach* and *Stellera chamaejasme*.

Method: Water extraction and alcohol precipitation were used to extract polysaccharides from medicinal plants, and the antioxidant activity *in vitro* of polysaccharides was evaluated using ABTS method; Using *Caenorhabditis elegans* as a model, the antioxidant stress activity of polysaccharide was explored, and the oxidative stress level and antioxidant enzyme activity *in vivo* of the nematode were detected with the kit; The anti-aging activity of polysaccharide was studied by life test of nematode and determination of lipofuscin *in vivo*.

Result: The order of antioxidant activity *in vitro* and oxidative stress resistance *in vivo* of 4 medicinal plant polysaccharides at a concentration of 1 mg/mL was *Houttuynia cordata* polysaccharide (HCP)>*Eleuthococcus senticosus* polysaccharide (ESP)>*Melia azedarach* polysaccharide (MAP)>*Stellera chamaejasme polysaccharide* (SCP). This is related to its ability to reduce the levels of ROS and MDA in low nematodes under H_2O_2 stress, as well as to increase the enzyme activity of SOD in nematodes, but it is not closely related to the enzyme activity of CAT. The polysaccharides of the 4 medicinal plants also had no significant effect on the longevity of nematodes and the accumulation level of lipofuscin.

Conclusion: Although HCP, ESP, MAP, and SCP have good antioxidant activity *in vitro* and *in vivo*, they do not have significant anti-aging activity.

Keywords: Medicinal plant polysaccharide, Caenorhabditis elegans, antioxidant stress, anti-aging

Introduction

Caenorhabditis elegans is a harmless, hermaphroditic and independent nematode. Because of its characteristics of easy cultivation, simple individual structure, low cost, rapid reproduction, easy observation, short life cycle, clear genetic background, it is widely used in Molecular genetics, Developmental biology, neuroscience, cell biology and other fields, and is a good Model organism [1]. After genome sequencing, it was found that its genome has high homology with human genome and is related to many human diseases. Therefore, *Caenorhabditis elegans* has unique advantages in aging and longevity, drug activity screening and other studies, and is one of the most commonly used models for anti-aging, antioxidant and other functional evaluation. Up to now, chemical synthetic drugs used for anti-aging, such as aspirin and metformin, will produce adverse reactions in different degrees and directions, with a high risk coefficient [2-3]. The active ingredients of natural medicinal plants, such as *Panax* volatile oil and *Dioscorea alata* polysaccharides [4-5], are safe and effective, but the technical methods for evaluating the active ingredients of traditional Chinese medicine are relatively single [6]. Many studies have shown that there are active substances in Natural product that can delay the aging process. As natural antioxidants, they can eliminate excessive free radicals in the body and slow down the aging and degradation rate of the body.

Materials and Methods

1 Material

1.1 Biomaterials

1.1.1 Plant Materials

Houttuynia cordata, *Eleutherococcus senticosus*, *Melia azedarach and Stellera chamaejasme*.

1.1.2 Test nematodes

Caenorhabditis elegans is provided by Caenorhabditis genetics Center (CGC).

1.1.3 Microbial strains

OP50 *Escherichia coli*

1.2 Main reagents

Total protein quantitative test kit (Nanjing Jiancheng Bioengineering Research Institute, A045-3-1), Malondialdehyde (MDA) test kit (Nanjing Jiancheng Bioengineering Research Institute, A003-1-1), total SOD test kit (Nanjing Jiancheng Bioengineering Research Institute, A001-1-1), CAT test kit (Nanjing Jiancheng Bioengineering Research Institute, A007-1-1), reactive oxygen species (ROS) test kit (Nanjing Jiancheng Bioengineering Research Institute, E004-1-1) H_2O_2 (Chengdu Kelong Chemical Co., Ltd., batch number 2020052501, content ≥30.0%), AB-8 macroporous adsorption resin (Shanghai Maclean's Biochemical Technology Co., Ltd., batch number A875381, content 500g), ABTS and Fluorouracil, etc.

1.3 Main instruments

Ultra Clean Workbench (Suzhou Purification Equipment Co., Ltd., SW-CJ-1F), Rotating Evaporator (Shanghai Yarong Biochemical Instrument Factory, RE-52AA), UV Visible Spectrophotometer (Shanghai Yuanxi Instrument Co., Ltd., UV-8000S), Multiskan FC Enzyme Standard Instrument (Thermo Fisher Scientific Oy Ratatie 2, FI-01620 Vantaa, Finland, 1510) Precision thermostatic

incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., BPH-9162), microscope (Shanghai Batuo Instrument Co., Ltd., BTS-300V), high-pressure steam sterilizer (Sanyo Electric Co., Ltd., MLS-3780), vortex mixer (Jintan Tianjing Experimental Instrument Factory, XH-C), low-speed centrifuge (Shanghai Zhixin Experimental Instrument Technology Co., Ltd., SL02), Soxhlet extractor and grinder, etc.

2 Methods

2.1 Extraction of polysaccharides from medicinal plants

2.1.1 Water extraction and alcohol precipitation method for extracting polysaccharides

4 medicinal plants were extracted using water extraction and alcohol precipitation method, with a ratio of 1:15 material to liquid, and subjected to Soxhlet reflux extraction for 6 hours. The extraction solution was concentrated to 20 mL, and 5 times the amount of 95% ethanol was added for alcohol precipitation overnight. On the second day, the precipitates were evaporated in a water bath to obtain crude polysaccharides. The crude polysaccharides were then deproteinized using the Sevage method.

2.1.2 Macroporous resin adsorption and purification

Wet column loading of pre-treated AB-8 macroporous resin (Φ10×80 mm), the polysaccharide sample solution was dynamically loaded onto the column at a ratio of 1:1, stabilized, and allowed to stand for 30 minutes. The adsorbent was collected at a rate of 1 BV/h, and the eluent (I) was collected; Wash with distilled water in a 1:1 ratio and collect the eluent at 1 BV/h to obtain the eluent (II). Combine the eluents (I, II) and dry them under reduced pressure to obtain pure polysaccharides.

2.2 Antioxidant activity of polysaccharides *in vitro*

Prepare medicinal plant polysaccharides into sample solutions with concentrations of 2 mg/mL and 1 mg/mL, and prepare a Vc solution of 0.01 mg/mL as a positive control. The ABTS method was used to determine the free radical clearance rate.

2.3 Simultaneous culture of *Caenorhabditis elegans*

Mix 10 mL 5mol/L KOH solution with 10 mL 10% NaClO solution, and dilute to 100 mL with purified water to prepare a lysis solution. Rinse adult nematodes in their oviposition period with sterilized M9 buffer and collect them in a 1.5 mL sterile centrifuge tube. Centrifuge at 2000 r/min for 3 minutes, and discard the supernatant. Add the lysis solution to a centrifuge tube containing nematode precipitation, and vortex oscillate for 3 minutes to lyse the nematode body; Centrifuge the lysed nematode solution at 2000 r/min for 3 minutes and discard the supernatant. Evenly drop the precipitate onto the surface of NGM solid culture medium containing OP50 *Escherichia coli*, and incubate for 2-3 days in a 22 ℃ constant temperature incubator. When the nematode reaches the L4 stage, it enters the early adult stage and can be used for subsequent experimental research.

2.4 Study on Antioxidant Stress of Medicinal Plant Polysaccharides

2.4.1 Determination of food clearance rate of *Caenorhabditis elegans*

Add synchronized L1 nematodes, food OP50 *Escherichia coli*, and sample solution to a 96-well plate. The control group was replaced with sterile water, and 7 repetitive wells were set for each sample mass concentration. Incubate in a 22 ℃ incubator and measure its OD_{570nm} value for 7 consecutive days.

2.4.2 H_2O_2 oxidative stress of *Caenorhabditis elegans*

The food OP50 *Escherichia coli*, 5-Fluorouracil (final concentration of 30 μg/mL, used to block the development of adult offspring), and polysaccharide samples were added to a 96-well plate together. Pick out the initial nematodes of adults (20 strips per well). The blank group was replaced with sterile water. After incubation at 22 ℃ for 48 hours, add H_2O_2 solution (final concentration of 40 mmol/L). Count the survival status of nematodes every 12 hours with H_2O_2 added as the 0th hour. During the statistical process, nematodes that are stiff and unresponsive to slight vibrations are judged as dead.

Nematode survival rate=

$$\frac{\text{Total number of nematodes}-\text{Number of dead nematodes}}{\text{Total number of nematodes}}\times 100\%$$

2.4.3 Determination of oxidative stress level in *Caenorhabditis elegans*

Prepare nematodes according to the oxidative stress experiment, grind the homogenate with a grinder at 4 ℃, and centrifuge to collect the supernatant, i.e. enzyme solution. The total protein quantification test kit, ROS detection kit, and MDA detection kit were used to determine the protein concentration, ROS level, and MDA level of the enzyme solution, respectively. The ROS level was measured using the DCFH-DA probe method, and the results were expressed as DCF fluorescence intensity per mg of enzyme solution protein. The determination result of MDA level is converted into the content of MDA per mg of enzyme solution protein.

2.4.4 Determination of antioxidant enzyme activity in *Caenorhabditis elegans*

The treatment of nematodes, preparation of enzyme solution, and determination of protein concentration are the same as the methods in 2.4.3. Combined with a full wavelength enzyme-linked immunosorbent assay, the total SOD activity detection kit and CAT detection kit are used to determine SOD and CAT activity, respectively. The measurement results are converted into SOD or CAT activity per mg of enzyme solution protein.

2.5 Study on the anti-aging activity of medicinal plant polysaccharides

2.5.1 Life test of *Caenorhabditis elegans*

Add 5-Fluorouracil (final concentration of 30 μg/mL, used to block the development of adult offspring), food OP50 *Escherichia coli*, and polysaccharide sample solution were added together to a 96-well plate. Each sample is equipped with 3 composite pores. Pick out the initial nematodes of adults (20 strips per well). The blank group was replaced with sterile water. Incubate at 22 ℃, starting with the start of administration as the 0th day, and count the survival status of the nematodes every 2 days until all of them die. During the statistical process, nematodes that are stiff and unresponsive to slight vibrations are judged as dead.

Nematode survival rate= ×100%

$$\frac{\text{Total number of nematodes}-\text{Number of dead nematodes}}{\text{Total number of nematodes}}\times 100\%$$

2.5.2 Determination of lipofuscin level in *Caenorhabditis elegans*

Prepare nematodes according to the life test method and incubate at 22 ℃ for 24 hours. The relative fluorescence intensity of lipofuscin was measured with a full wavelength microplate reader. The excitation wavelength was 355 nm, and the emission wavelength was 460 nm, which was recorded as the first day. Measure once every 2 days and continuously 3 times.

Results and Analysis

1 Antioxidant activity *in vitro*

As shown in Table 1, both HCP and ESP exhibit good antioxidant activity *in vitro* at 2 mg/mL and 1 mg/mL, with clearance rates above 99%. At 2 mg/mL, the clearance rate of MAP on ABTS free radicals is relatively high, above 99%. But at 1 mg/mL, the clearance rate decreased to 89.91%. The antioxidant activity *in vitro* of SCP is relatively the worst, with the lowest clearance rates at both concentrations. At 1 mg/mL, its clearance rate is as low as 60.09%.

Table 1: Scavenging rate of ABTS free radicals by 4 medicinal plant polysaccharides

Medical plant polysaccharides	2 mg/mL Clearance (%)	1 mg/mL Clearance (%)
Houttuynia cordata polysaccharide (HCP)	99.34%	99.09%
Eleutherococcus senticosus polysaccharide (ESP)	99.04%	99.06%
Melia azedarach polysaccharide (MAP)	99.12%	89.91%
Stellera chamaejasme polysaccharide (SCP)	82.89%	60.09%

2 Antioxidant stress

2.1 Measurement results of nematode food clearance rate

As shown in Figure 1, the effects of 4 medicinal plant polysaccharides at different concentrations on the clearance rate of nematode food are shown. It can be seen from the figure that with the extension of cultivation time, the content of nematode food generally shows a decreasing trend. Compared with the blank control group curve, the 4 medicinal plant polysaccharides did not significantly affect the food clearance rate of nematodes at a concentration of 1 mg/mL, and the trend of curve changes was almost consistent with that of the control group.

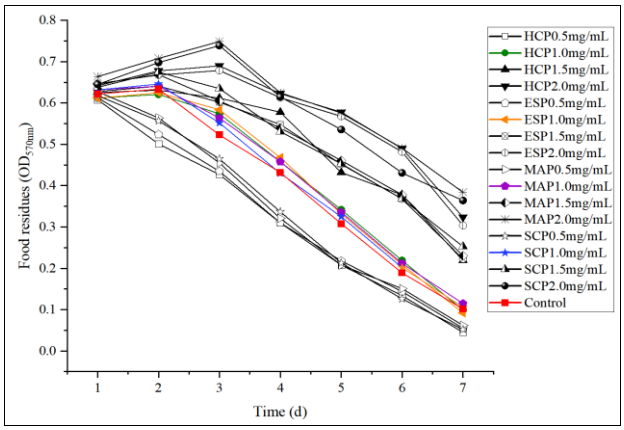

Fig 1: Effect of 4 medicinal plant polysaccharides on the food clearance rate of nematodes

2.2 Effects of polysaccharides on the survival rate of nematodes under antioxidant stress

The effects of 4 medicinal plant polysaccharides on the antioxidant stress of nematodes at a concentration of 1 mg/mL are shown in Figure 2. It can be seen that all 4 medicinal plant polysaccharides have certain antioxidant stress effects on nematodes. The order of resistance to oxidative stress is HCP>ESP>MAP>SCP. This is also consistent with the order of antioxidant activity *in vitro*. Among them, HCP had the most significant effect on prolonging the time to live of nematode after oxidative stress. It not only prolonged the median time to live of nematode under H_2O_2 stress, but also extended the average time to live and maximum time to live of nematode. The resistance of SCP to oxidative stress of nematodes is relatively lowest. As the duration of oxidative stress prolongs, its resistance to oxidative stress also decreases. This is also related to the lowest clearance rate of ABTS free radicals at a concentration of 1 mg/mL *in vitro*.

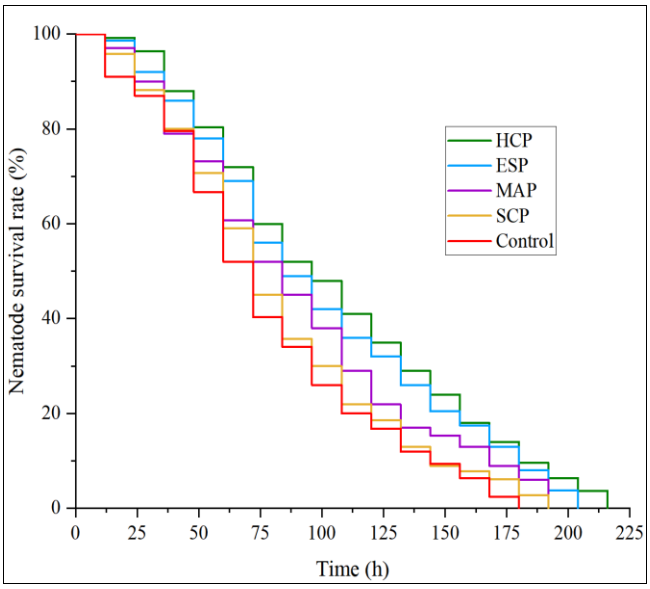

Fig 2: Effects of 4 medicinal plant polysaccharides at a concentration of 1 mg/mL on antioxidant stress in nematodes

2.3 Effects of polysaccharides on oxidative stress levels in nematodes

The effects of 4 medicinal plant polysaccharides at a concentration of 1 mg/mL on the levels of ROS and MDA *in vivo* under oxidative stress of nematodes are shown in Figure 3-1 and Figure 3-2, respectively. As the detection time prolongs, the ROS level increases. The excessive accumulation of MDA can also affect the normal life activities of nematodes. Compared with the control group, all 4 medicinal plant polysaccharides can reduce the ROS levels in nematodes. Among them, HCP has the most significant effect on reducing ROS levels in nematodes; 4 medicinal plant polysaccharides can significantly reduce the MDA content in nematodes. The effects of HCP, ESP, and MAP on reducing MDA content in nematodes reached extremely significant levels. The above results indicate that the antioxidant stress activity *in vivo* of 4 medicinal plant polysaccharides against nematodes is related to their ability to reduce the levels of ROS and MDA in nematodes under H_2O_2 stress conditions.

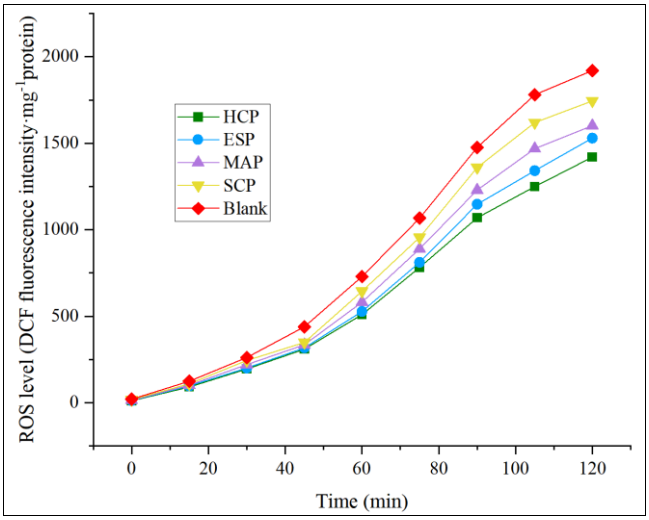

Fig 3-1: ROS Level Impact

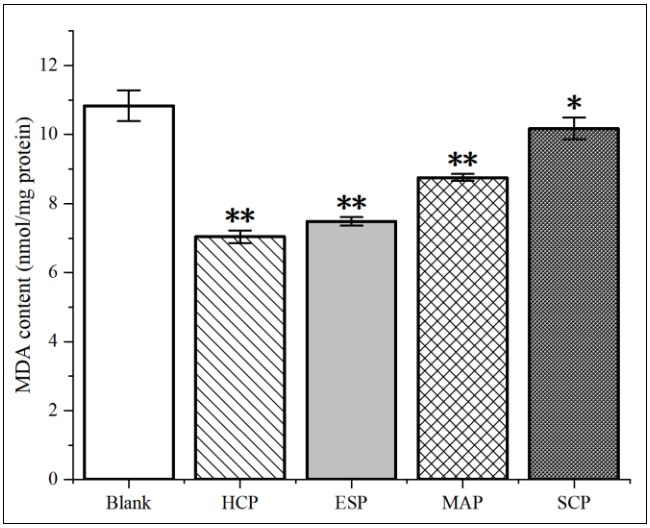

Fig 3-2: MDA Level Impact

2.4 Effects of polysaccharides on antioxidant enzyme activity *in vivo*

Enhancing the activity of antioxidant enzymes helps to reduce oxidative stress damage and maintain normal life activities in the body. As shown in Figures 4-1 and 4-2, the effects of 4 medicinal plant polysaccharides at a concentration of 1 mg/mL on the activity of antioxidant enzymes in nematodes under oxidative stress are shown. Compared with the blank group, all 4 medicinal plant polysaccharides can enhance the activity of SOD in nematodes under oxidative stress, with the effects of HCP and ESP reaching significant levels; HCP and SCP did not significantly enhance the activity of CAT in nematodes under oxidative stress, while ESP and MAP had a certain reduction in CAT activity in nematodes under oxidative stress, which was not significant. The above results indicate that the antioxidant stress activity of 4 medicinal plant polysaccharides against nematodes *in vivo* is related to their ability to enhance SOD activity in nematodes under H_2O_2 stress conditions, but not related to CAT enzyme activity.

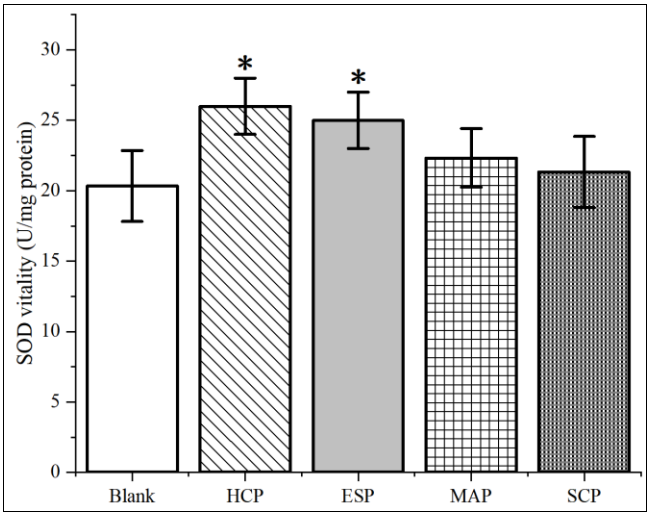

Fig 4-1: Impact of SOD Activity

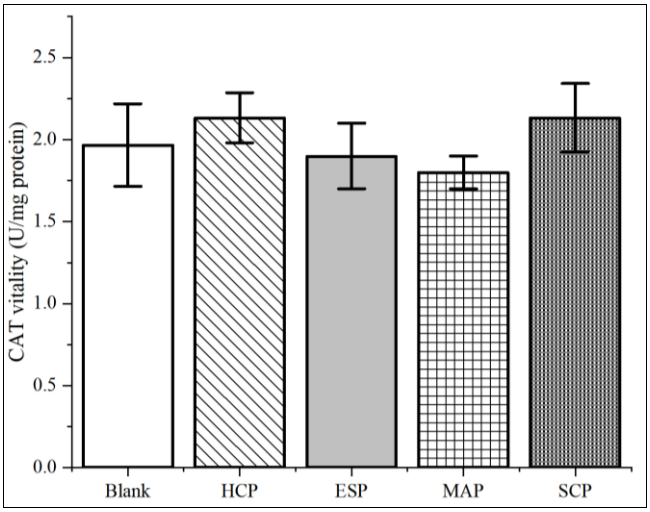

Fig 4-2: Impact of CAT Activity

3 Anti-aging

According to the free radical theory of aging, the production of aging is directly related to the generation of excessive free radicals in the body and the resulting oxidative stress environment. On the basis of determining that 4 kinds of medicinal plant polysaccharides have certain biological activities of free radical scavenging and oxidative stress resistance to *Caenorhabditis elegans*, their anti-aging activities were studied. Exploring the effects of 4 medicinal plant polysaccharides at a concentration of 1 mg/mL on the survival curve of nematodes, the results are shown in Figure 5-1. Compared with the control group, there was no significant difference in the lifespan curve of nematodes in the 1 mg/mL treatment group of 4 medicinal plants. Even the HCP treatment group with the best antioxidant effect did not extend the lifespan of nematodes; At the same time, the accumulation level of lipofuscin, the aging marker of *Caenorhabditis elegans elegans*, was also detected, as shown in Figure 5-2. Compared with the control group, the accumulation of lipofuscin in nematode was not significantly affected by the 4 kinds of medicinal plant polysaccharides. The above results indicate that although these 4 medicinal plant polysaccharides have certain antioxidant effects, they do not have significant anti-aging activity to prolong the lifespan of nematodes.

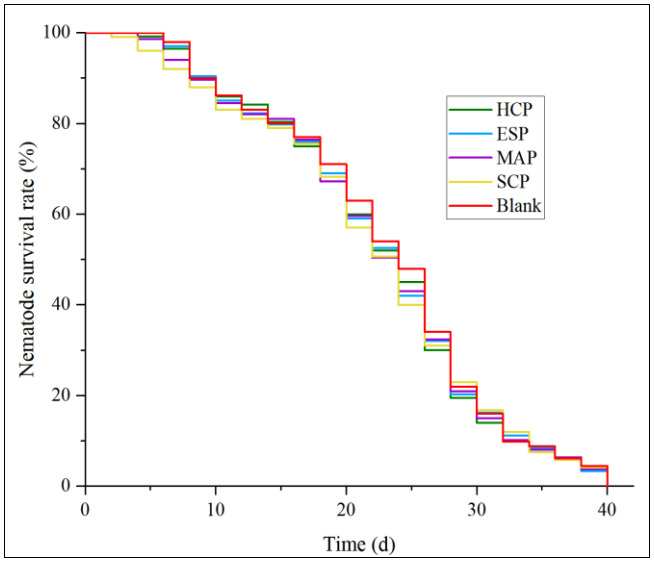

Fig 5-1: Nematode lifespan curve

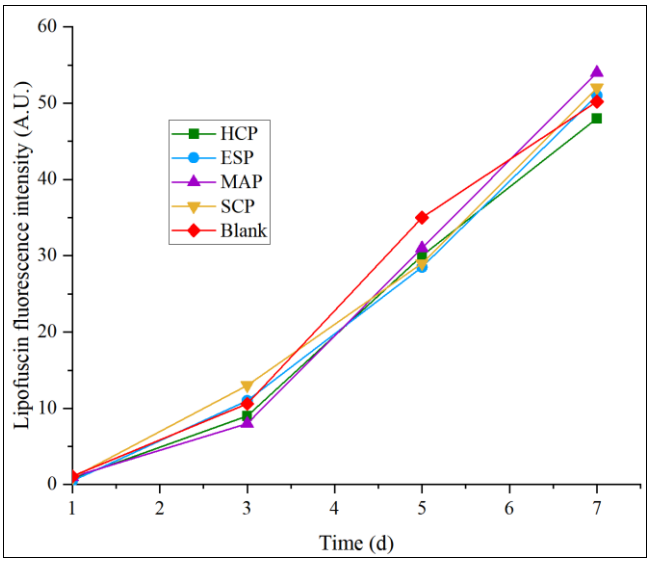

Fig 5-2: Effect of lipofuscin level

Discuss

Medicinal plant polysaccharides generally have good antioxidant and anti-aging activities [7]. At present, *Caenorhabditis elegans is* widely used as a Model organism for anti-oxidation and anti-aging *in vivo*, which is widely used for the evaluation of anti-oxidation stress and life extension of active components of medicinal plants. In this experiment, 4 medicinal plant polysaccharides showed strong antioxidant activity *in vitro* and *in vivo* antioxidant stress activity at 1 mg/mL. The strength of its activity has the same trend, which further indicates that it has good antioxidant activity. When the active ingredients of medicinal plants act on organisms, the mechanisms are complex and difficult to explore. Through preliminary exploration, it was found that the antioxidant stress activity of polysaccharides from 4 medicinal plants is related to their ability to reduce the oxidative stress level in nematodes under H_2O_2 stress conditions and increase some antioxidant enzyme activities. The results can provide a clear and effective antioxidant index and explore the direction of its mechanism of action. In addition, the antioxidant activity *in vivo* and *in vitro* of HCP was optimal at 1 mg/mL. This may be related to its multiple mechanisms that affect oxidative stress both *in vivo* and *in vitro* [8], as well as other antioxidant active components such as polyphenols and volatile oils in *Houttuynia cordata* [9]. On the whole, the antioxidant activity of polysaccharide from *Houttuynia cordata* and *Eleutherococcus senticosus*, two medicinal and food homologous plants, is significantly better than that of *Melia azedarach* and *Stellera chamaejasme*. This may be related to the higher safety of plant polysaccharides derived from medicinal and food sources, while medicinal plants themselves have a certain degree of toxicity and have an impact on the oxidative stress of nematodes.

Polysaccharides, as a natural antioxidant active ingredient, also have certain anti-aging effects. One of the anti-aging mechanisms of polysaccharides is to enhance antioxidant enzyme activity and eliminate free radicals [10], such as *Dendrobium nobile* [11], *Cornus officinalis Sieb* [12], and *spirulina platensis*[13] polysaccharides, which all have certain oxidative stress resistance and delayed nematode aging. In this study, although the 4 medicinal plant polysaccharides exhibited good antioxidant stress activity *in vivo*, none of them showed significant activity in prolonging the lifespan of nematodes. The reason for the unsatisfactory results of its anti-aging experiment may be due to the single concentration of polysaccharides in the experiment, which limits the research on anti-aging; The aging characteristics of *Caenorhabditis elegans are* not obvious, difficult to observe, and highly subjective; Biological experiments are unstable and susceptible to external factors. There is currently limited research on the antioxidant stress and anti-aging effects of HCP, ESP, MAP, and SCP. Therefore, further in-depth research is needed on its antioxidant and anti-aging mechanisms and detailed action targets.

Conclusion

All 4 medicinal plant polysaccharides exhibit good antioxidant *in vitro* and antioxidant stress activity *in vivo* at a concentration of 1 mg/mL. The activity comparison order is HCP>ESP>MAP>SCP, but these 4 medicinal plant polysaccharides did not show significant anti-aging activity against nematodes. The research results provide a theoretical basis for the in-depth development and mechanism of action of medicinal plant polysaccharides in antioxidant stress and anti-aging.

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