

ORIGINAL ARTICLE

Antioxidant and anti-aging effects of polysaccharide LDP-1 from wild *Lactarius deliciosus* on *Caenorhabditis elegans*

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Popular scientific summary

LDP-1 obtained from *L. deliciosus* had good antioxidant and anti-aging effects. Lipofuscin and ROS level of *C. elegans* was respectively reduced by 22.8 and 42.7%. LDP-1 significantly extended the average lifespan of *C. elegans* by 32.8%. Under heat stress, the life span of *C. elegans* treated with LDP-1 increased by 35%.

Abstract

Background: Edible fungi (mushrooms) have attracted much more concerns due to their abundant nutritions and functional bioactive substances like polysaccharides.

Objective: In this study, the anti-oxidation and anti-aging activities of polysaccharide fraction (LDP-1) from the wild *Lactarius deliciosus* fruiting bodies were systematically evaluated using *Caenorhabditis elegans* (*C. elegans*) as a model.

Methods: Lifetime determination of *C. elegans* (survival status) was observed via microscope. Effects of LDP-1 on *C. elegans* induced by heat and oxidative stress were, respectively, performed in an artificial climate chamber and Juglone solution. Determination of lipofuscin levels in *C. elegans* was observed by laser confocal scanning microscopy. Determination of reactive oxygen species in *C. elegans* *in vivo* was conducted via fluorescence spectrophotometer reader.

Results: The results revealed that LDP-1 could significantly extend the lifespan of *C. elegans*, and the lifetime of *C. elegans* treated with 1,000 µg/mL LDP-1 could be prolonged by 32.8% compared with the control. The survival rates of the experimental *C. elegans* under heat shock and oxidative stress conditions were clearly improved after treated with 1,000 µg/mL LDP-1 (40 and 19.8%, respectively), while under the same circumstances all the *C. elegans* in the blank group died. Fluorescence microscopy analysis confirmed that LDP-1 could effectively reduce the accumulation of lipofuscin and reactive oxygen free radicals in *C. elegans*, where the respective maximum reduction reached 22.8 and 42.7% compared with the control.

Conclusion: These results indicate that LDP-1 had favorable antioxidant and anti-aging effects, which could be explored as potential dietary additives to renovate oxidative damage and slow down aging process.

Keywords: polysaccharide of LDP-1; *Caenorhabditis elegans*; anti-oxidation; anti-aging; *Lactarius deliciosus*

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With the acceleration of the aging population, there is an increasing demand for health products. Delaying senility and preventing various diseases caused by aging are of great significance for prolonging the disease-free health period (1). As an irreversible, long, and gradual complex process (2), aging happens in different organs of the body naturally, which would impair the immune system, deteriorate the normal

functions, and reduce the adaptability (3). More and more researches indicate that aging will greatly increase the incidence of some chronic diseases, such as hypertension, diabetes, atherosclerosis, and Alzheimer's disease (AD) (4, 5). Many theories on aging (e.g. telomere theory and free radical theory) are put forward by far, and among them the free radical-oxidative stress is one of the widely accepted mechanisms (6). The reactive oxygen

species (ROS) like superoxide radicals and hydroxyl radicals are normal metabolites of organisms (7). However, excessive production of ROS will cause a certain degree of damages to the cell molecules like proteins, lipids, and nucleic acids that are manufactured by the body itself (8, 9). The human body has an antioxidant defense system consisting of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) to eliminate harmful ROS (10). The oxidative stress stimulated by the imbalance elimination of ROS has become a major inducement of aging and disease occurrence (11). In order to maintain the homeostasis of the organism, scavenging free radicals is one of the effective ways to prevent diseases and delay aging. Recently, many synthetic chemical antioxidants have been explored for these purposes (8, 12). However, they are limited due to potential liver toxicity and carcinogenesis (13). At present, researchers are focused on developing safe and potent antioxidants, especially original natural ones with low side effects to the human body (14, 15).

Edible mushrooms are not only unique in flavor but also rich in bioactive ingredients containing a variety of natural functional compounds, such as polysaccharides (16), trace elements, and adenosine derivatives (17). It is reported that edible fungal polysaccharides have anti-tumor, anti-aging, immune regulation, and anti-inflammatory bioactivities (18, 19). For instance, the fungal polysaccharides that are extracted from *Agrocybe cylindracea* (20), *Tremella fuciformis* (21), and *Hericium erinaceus* (22) have excellent antioxidant capacity and anti-aging activity. As a rare edible mushroom, the mycelia of *Lactarius deliciosus* are enriched with extracellular enzymes like lignin peroxidase and manganese peroxidase (23). Research has also indicated that polysaccharides from *L. deliciosus* Gray have favorable immune-regulating and antitumor activity in the *in vitro* and *in vivo* tests (24–26). In our previous work, a bioactive polysaccharide component named LDP-1 was obtained from the wild *L. deliciosus* fruiting bodies for the first time, and subsequent studies showed that it had a significant immunomodulatory effect on RAW 264.7 macrophages (27).

In this study, *Caenorhabditis elegans* (*C. elegans*) was chosen as the model to further explore the antioxidant and anti-aging activities of the polysaccharide LDP-1 (28, 29). As known, *C. elegans* model has been widely researched on revealing the genetic and biochemical pathways associated with aging (30). It possesses many advantages like short life cycle, easy to cultivate, transparent body, and simple organs (31, 32). Most importantly, *C. elegans* has many similarities with humans in terms of the aging mechanism (33, 34). In addition, the longevity, reproductive ability, and behavioral response

of *C. elegans* can be used as the vital physiological indicators to evaluate the effect of the bioactive polysaccharide (35). In this study, the antioxidant and anti-aging effects of LDP-1 on the lifespan, lipofuscin accumulation, and ROS levels of *C. elegans* were investigated, which are of great significance to developing potential reagents for preventing and delaying aging.

Materials and methods

Materials

The polysaccharide of LDP-1 (Mw of 9.8×10^5 kDa) from the wild *L. deliciosus* fruiting bodies was obtained according to our previous report (27). LDP-1 with a purity level of 90.15% has well solubility in water and appears in yellow-brown color, which is an acidic polysaccharide fraction. *C. elegans* (N2, wild-type; belongs to Phylumnematoda, Secernentea, Rhabditida, Rhabditidae, and *Caenorhabditis*) and *Escherichia coli* OP50 strains were presented by Professor Ai-Fang Du from the College of Animal Sciences, Zhejiang University. 2, 7 dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tryptone, yeast extract, NaCl, and $MgSO_4$ were bought from Sangon (Shanghai, China). Juglone and agar were purchased from Macklin (Shanghai, China). Other reagents were of analytical grades commercially produced in China.

Cultivation and synchronization of *C. elegans*

Culture and preservation of uracil-deficient *E. coli*

Under aseptic conditions, uracil-deficient *E. coli* OP50 was added to the Luria-Bertani (LB) culture medium at a ratio of 1:100 (v/v) and cultured at 37°C under shaking at 180 rpm until reaching the logarithmic growth phase.

The *E. coli* OP50 was stored as follows: glycerin was added into the OP50 culture that reached the logarithmic growth phase with final concentration of 3% (v/v), put into eppendorf (EP) tubes, stored under -20°C freezing temperature (Siemens, KG29NS221C, Germany) overnight, and then transferred to -80°C condition for long-period preservation.

Cultivation of *C. elegans*

Under aseptic conditions, 100 μ L of OP50 *E. coli* was pipetted into standard nematode growth medium (NGM) plate and spread evenly. The growth rate of uracil-deficient nematodes in NGM was much slower than that of *E. coli*, which can provide nematode food without overgrowth. A piece of agar containing adult eggs was cut and inoculated on the above NGM plate, and then placed into a 20°C biochemical incubator (XUTEMP TEMPTECH Co., Ltd., XT5107-IB150, Hangzhou, China). The obtained nematodes were subcultured every 4–5 days.

Synchronization of *C. elegans*

The nematodes cultured to the oviposition stage were washed with M9 buffer (KH_2PO_4 3.0 g/L⁻¹, Na_2HPO_4 6.0 g/L⁻¹, NaCl 5.0 g/L⁻¹, MgSO_4 1 mol/L⁻¹). The supernatants were discarded, and the nematodes were collected within the buffer. Then the same amount of lysis solution was added and mixed thoroughly, and the eggs were obtained after centrifugation and rinsed twice with M9 buffer at last. The synchronized eggs were diluted with OP50 culture, transferred into NGM medium, and incubated at 20°C for further analysis.

Lifetime determination of *C. elegans*

The LDP-1 solution (5 mg/mL) was diluted with OP50 medium to obtain 125, 250, 500, and 1,000 µg/mL mixed cultures, and pipetted 100 µL onto the NGM plates, respectively. The LDP-1 interfered groups were prepared using the same method for the following experiments.

The synchronized L4 phase nematodes (36) were picked into the respective media of the blank group and the experimental group with thin platinum wire, and 40 nematodes were selected randomly for each group with three parallel contrasts. The experiments were performed in a constant incubator at 20°C, and the sample groups were taken out for record every other day. The survival status of the nematodes was observed via microscope, while the dead nematodes were picked out and recorded. Using a platinum wire the nematodes were gently touched, and if the head and body did not swing and bend, this indicated death. The survival rate (SR) % was calculated by using the following formula:

$$\text{SR}(\%) = \frac{A_1}{A_0} \times 100\% \quad (1)$$

where A_1 is the number of survival *C. elegans*, and A_0 is the total number of experimental *C. elegans*. Each measure was repeated at least eight times.

The following experiments were continued with the same procedures and criteria until all the nematodes in the experimental groups died.

Effects of LDP-1 on *C. elegans* induced by heat stress

The LDP-1 with concentrations of 250, 500, and 1,000 µg/mL was prepared as discussed in the previous section, and an amount of 100 µL was added to NGM media, respectively. Sixty nematodes that synchronized to L4 stage were picked into the blank group and the LDP-1-treated group media with three parallels. The experimental nematodes were transferred to fresh media every other day consecutively. The culture media were placed in a 35°C artificial climate chamber (Jiangnan Instrument Factory, RXZ-128A, Ningbo, China) under the same conditions. At predetermined time intervals, the nematodes were taken out

for record, while the dead ones were picked out. The average growth time calculated by the growth time of each nematode was used as the average lifespan to characterize the influence of polysaccharides on the survival of nematodes.

Effects of LDP-1 on *C. elegans* induced by oxidative stress

The sterilized NGM medium was cooled to approximately 70°C, and the filtered Juglone solution was added to make a final concentration of 250 µg/mL. The synchronized nematodes were cultured to the L4 stage and transferred to different concentrations of LDP-1-treated groups and blank ones. After further cultivation at 20°C for 5 days, 60 nematodes were shifted to the media containing Juglone solution prepared above. Juglone can cause nematodes to die due to oxidative stress. The survived nematodes were recorded every 2 h until all nematodes died.

Determination of lipofuscin in *C. elegans*

C. elegans were cultured synchronously to the L4 stage and transferred to the LDP-1-treated group and blank group media at 20°C for continuing cultivation. The experimental media were replaced with fresh ones every other day. The lipofuscin levels of 15 nematodes that cultured for 5 and 10 days were observed by laser confocal scanning microscopy (LCM) (FV1000, Olympus Corporation, Japan) with respective excitation and emission wavelengths at 365 and 420 nm. All photos were taken at 100× magnification, and the fluorescence intensity of each nematode was quantitatively analyzed using Image J software (Image-Pro Plus5.0, Media Cybernetics Inc., MD, USA).

Determination of ROS in *C. elegans* in vivo

The nematodes were cultured in the blank group and the LDP-1-treated group with the same procedures, and transferred to M9 buffer containing 1 mL of 1 mM H_2DCFDA . After 3 h incubation at 20°C, the experimental nematodes were fixed on a 2% (w/v) agar plates. At the end of the specified treatment time, all of the *C. elegans* were collected and washed three times with M9 buffer. The nematodes were pipetted into 96-well microtiter plates containing H_2DCFDA . The samples were recorded every 20 min using fluorescence spectrophotometer reader (Synergy Mx, BioTek, VT, USA) at excitation/emission wavelengths of 485 and 525 nm, respectively (11). The following formula was used to calculate the fluorescence ratio (FR, %) indicating the relative ROS level.

$$\text{FR}(\%) = \frac{F_1}{F_0} \times 100\% \quad (2)$$

where F_0 is the fluorescence intensity of the blank group, and F_1 is the fluorescence intensity of the positive

control group or the experimental group, with eight parallels in each group.

Statistical analysis

All measurements were performed at least in triplicate. The statistical significance ($P < 0.05$) between two samples was evaluated by one-way analysis of variance method, followed by the Turkey's test using OriginLab software (OriginPro 9.0.0, OriginLab Co., MA, USA). All data were expressed as mean \pm standard deviation (SD).

Results and discussion

Cultivation and observation of life cycle of *C. elegans*

The uracil-deficient *E. coli* OP50 grew slowly on NGM medium, which can be provided as food for nematode growth. Considering the reproducibility of the nematodes that are used, we investigated the growth character of OP50 to ensure the performed ones were in the logarithmic growth phase as inclusively as possible. As shown in Fig. 1a, OP50 grew up to the logarithmic growth phase at 37°C and 180 rpm after 3 h in LB medium. In view of the physiological-biochemical characteristics of the microbial growth, OP50 with absorbance value of 0.6 was used for all of the experiments.

The life cycle of the hermaphrodite individual *C. elegans* can be divided into worm eggs, larvae, and adults periods, while from the larvae to adults it showed four distinct phases, namely, L1, L2, L3, and L4 (Fig. 1b1–b4). When the individuals completed the gonad development, it produced the eggs during the L4 phase (Fig. 1b5). In a rich living environment, the average lifespan of nematodes lasted for 1 month, and it took only 3 days for the larvae developing to adults, and 4 days to reproducing next generation (37).

To evaluate whether the polysaccharide of LDP-1 had an effect on nematodes, the larvae were selected as the research model. At this stage, the larvae were in the critical period of development and more sensitive to interfering substance. Meanwhile, the adult nematodes could be used to evaluate the toxicity of the surroundings through indicators of lifespan, swallowing rate, motility, and spawning ability. Furthermore, under peroxidation conditions the nematodes will undergo certain degree of aging with extension of time, such as lipofuscin deposition, slow movement, and decreased feeding rate, which indicates that the nematodes were entering the aging process due to the imbalance of free radical homeostasis. These unique aging characteristics of *C. elegans* can display the potential influence of the target substance. In this study,

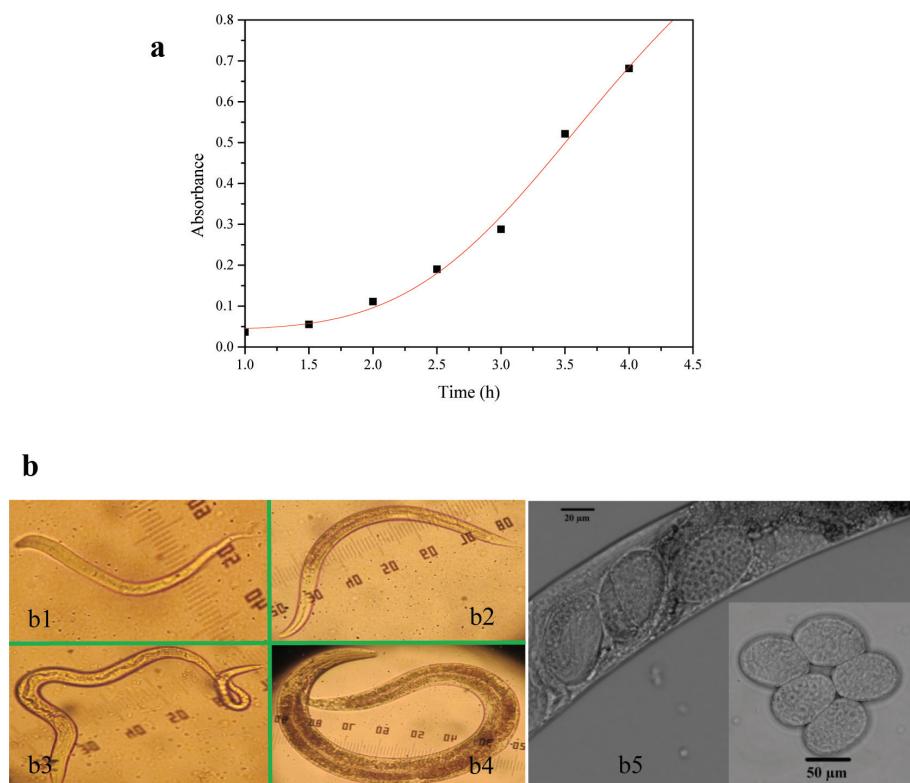


Fig. 1. Cultivation of *E. coli* OP50 and synchronization of *C. elegans*. (a) The logarithmic growth phase of OP50 and (b) the synchronized life process of *C. elegans*. (b1–b4) Morphology of *C. elegans* from larvae to adult phases; (b5) Morphology of *C. elegans* in adult and its eggs.

the nematodes were used as the model to elaborate the antioxidant and anti-aging activity of the polysaccharide LDP-1 with the following experiments.

Survival and lifespan of C. elegans treated with LDP-1

The lifespan is one of the valuable indicators in evaluating the physiological characteristics of nematodes. It displays the growth status of nematodes and potential toxicity of the target substance. Herein, the polysaccharide of LDP-1 with concentrations of 125, 250, 500, and 1,000 µg/mL (w/v) were set as the parallel groups for treating the nematodes. The toxicity of the prepared LDP-1 solutions was investigated. As shown in Fig. 2, LDP-1 within concentrations of 125–1,000 µg/mL had no significant influence on the SRs of the treated nematodes ($P > 0.05$), indicating the polysaccharide of LDP-1 was nontoxic and safe to the wild-type nematodes. In subsequent experiments, three level concentrations of 250, 500, and 1,000 µg/mL were selected to further explore the effects of polysaccharide LDP-1 on the lifespan of wild nematodes.

As shown in Fig. 3a, all the nematodes in the blank group (Control) died after 20 days cultivation. In contrast, the lifespan of the experimental nematodes that were treated with LDP-1 was significantly longer than that of the blank group, and showed a concentration dependence. After 20 days, the SRs of the experimental nematodes treated with 250, 500, and 1,000 µg/mL

LDP-1 solutions were 16, 17.6 and 21.2%, respectively. The average lifespan of the blank group and LDP-1-treated groups (250, 500, and 1,000 µg/mL) was, respectively, 11.7, 13.55, 15.157, and 15.538 days, with a maximum life extension of 32.8% compared with the blank one (Fig. 3b). Liu et al.'s research showed that the polysaccharide of CP2-c2-s2 from *Cordyceps militaris* could prolong the lifespan of nematodes by 16.58% with a concentration of 1,500 µg/mL treatment (32). The polysaccharide of LDP-1 showed favorable life-extending activity in nematodes.

Survival and lifetime of C. elegans under heat stress

The nematodes were transferred from the optimal surrounding (20°C) to a 'higher temperature' (above 30°C) environment. Under heat stress conditions, the protective effect of the active polysaccharide on the nematodes can be assessed through the SR. As can be seen from Fig. 4a, the LDP-1-treated nematodes had better resistance to heat stress than the normal cultured nematodes (Control). The nematodes in the blank group died after 6.5 days under heat stress treatment. In comparison, the SR of the 1,000 µg/mL LDP-1-treated group was still reached 40% in the same period. The average lifetime of the experimental nematodes that were treated with the active polysaccharide LDP-1 was significantly longer than that of the blank group (Fig. 4b). The average lifetime of the nematodes treated with 250, 500, and 1,000 µg/mL LDP-1 was, respectively, increased by

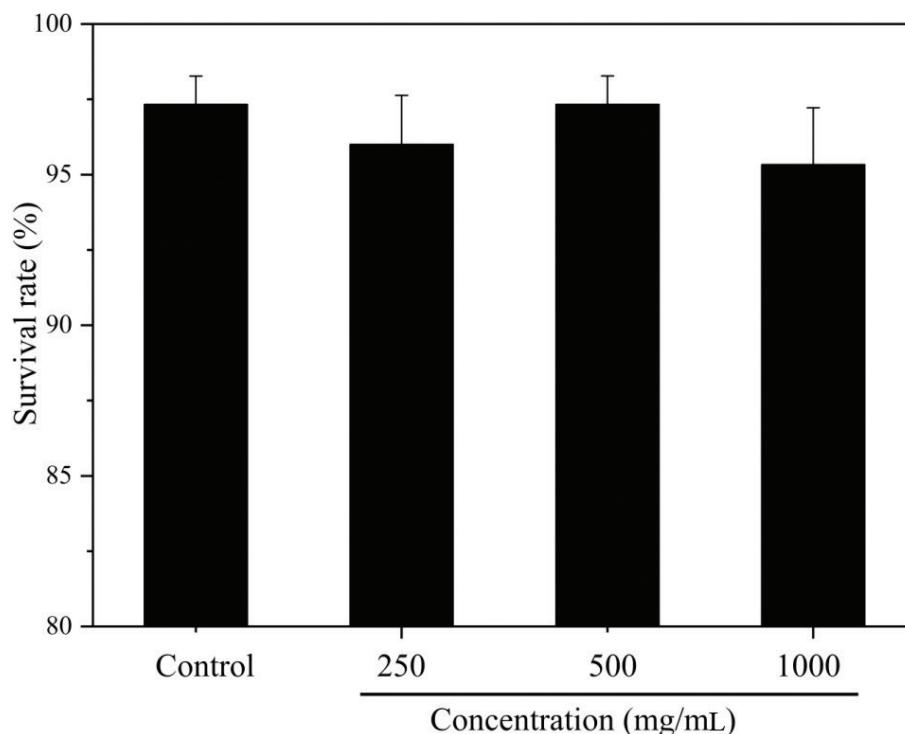


Fig. 2. Toxicity test of LDP-1 against *C. elegans*. Significant differences were compared from the Control at * $P < 0.05$.

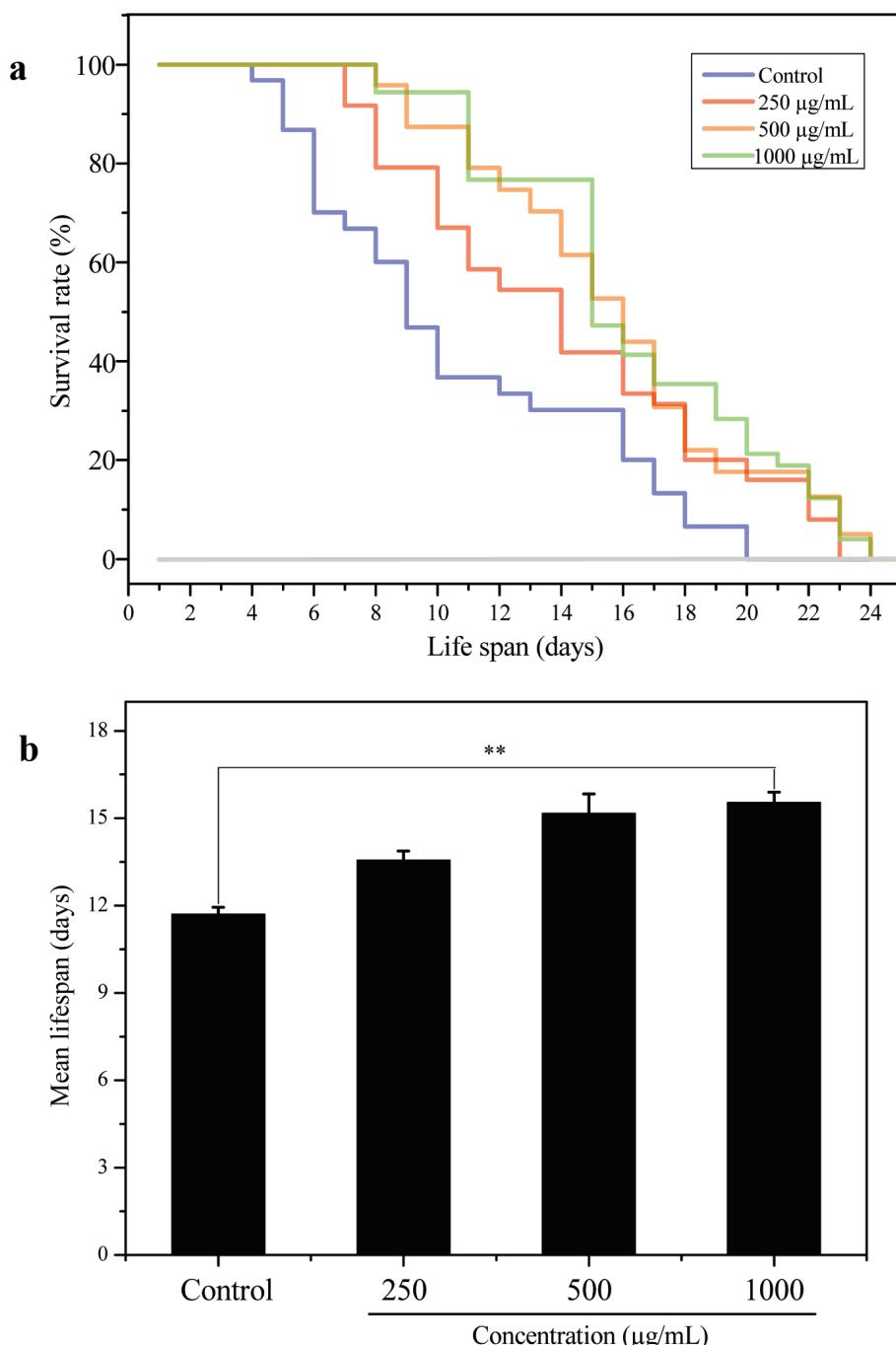


Fig. 3. Determination of *C. elegans* lifespan treated with polysaccharide LDP-1. (A) Survival rate of *C. elegans*; (B) Average life expectancy of *C. elegans*. Significant differences were compared from the Control at **P < 0.01.

14.5, 35, and 16.6% compared with the blank group. These results confirmed that the LDP-1 had good effects in anti-heating stress.

Regulation effect on *C. elegans* under oxidative stress

Compared with the heat stress response, the oxidative stress to the nematodes induced by environment has a similar principle. That is to say, the outside oxidative stimulation combined with the nematode's autoimmune

response system will initiate the resistance to the external environment, and thereby improve the tolerance so as to prolong their lifespan.

As shown in Fig. 5a, the nematodes in the blank group died after 14 h cultivation; comparatively, the experimental nematodes that were treated with different concentrations of LDP-1 showed good antioxidant capacity. After 16 h treatment, the SR of the nematodes (1,000 μg/mL LDP-1) was still up to 19.8%. Meanwhile, the average

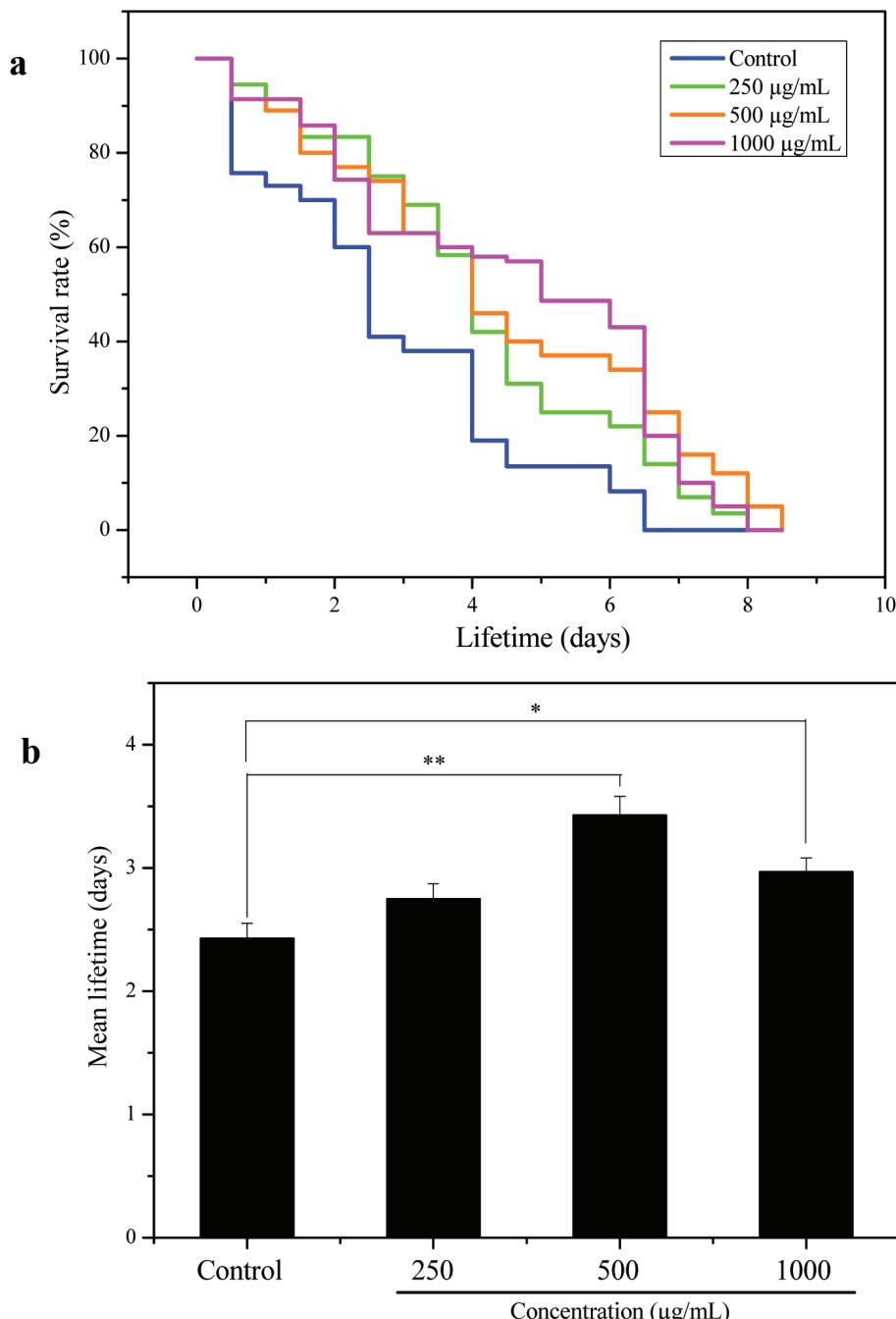


Fig. 4. Determination of *C. elegans* lifespan under a heat shock stress environment. (a). Survival rate of *C. elegans* and (b) average lifespan of *C. elegans* under heat stress conditions. Significant differences were compared from the Control at * $P < 0.05$ and ** $P < 0.01$.

lifespan of the nematodes presented a rising trend from 6.8 to 10.8 h compared with the control (Fig. 5b). Comparatively, similar research was conducted on the Puer tea water extract by Fei et al. (38), which manifested that the concentration of 2,500 µg/mL had a significant antioxidant effect on nematodes. Thus, LDP-I could improve the anti-oxidative stress ability of the nematodes effectively.

Determination of lipofuscin in *C. elegans* treated with LDP-I

Lipofuscin contained in *C. elegans* has spontaneous fluorescence characteristics, which can be observed under a fluorescence microscope. During the normal aging process, lipofuscin gradually accumulates and becomes increasingly strong in terms of fluorescence intensity, which is an important indicator for evaluating the aging process of the nematodes (39). As shown in Fig. 6a, compared with

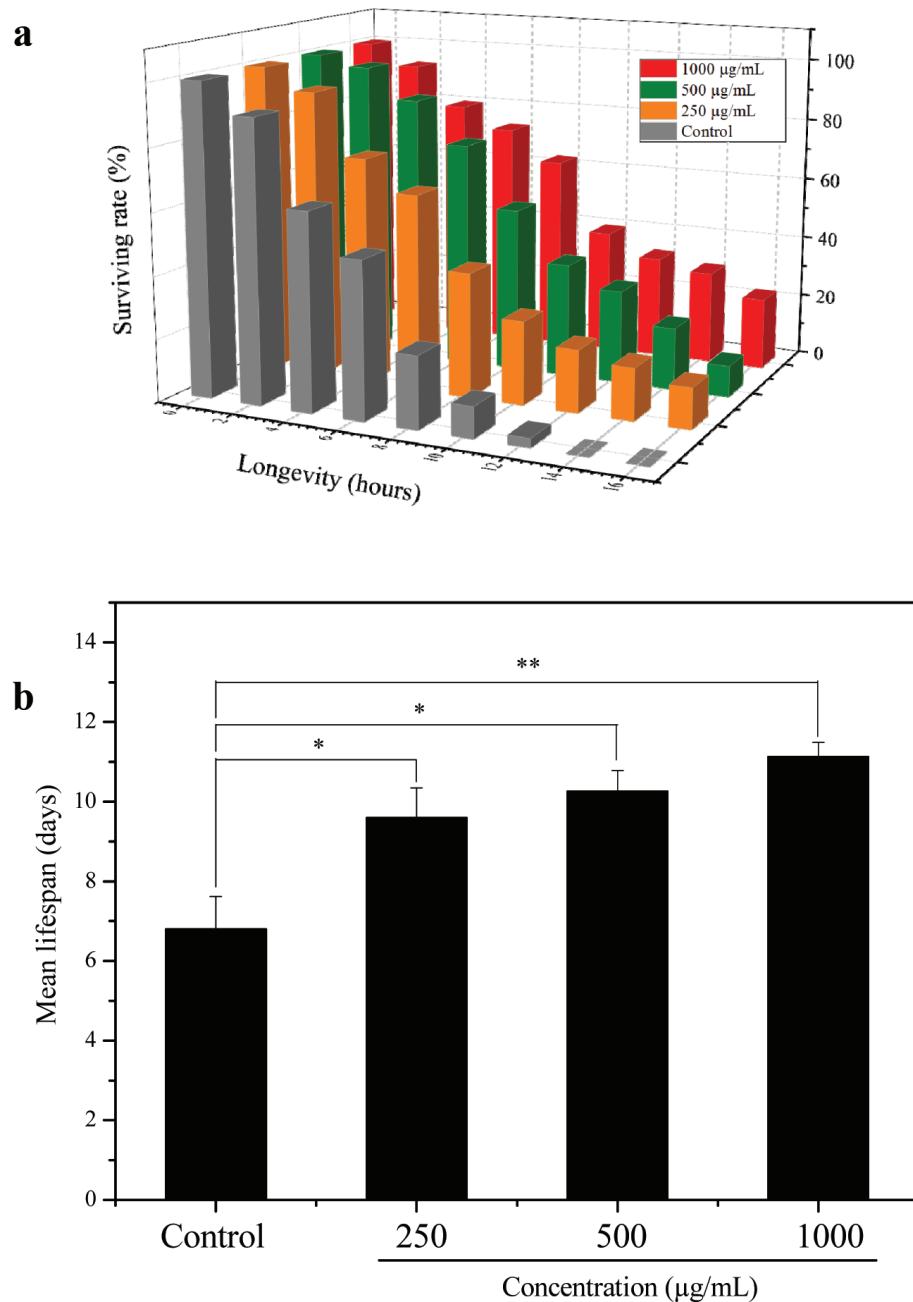


Fig. 5. Determination of *C. elegans*' lifespan under an oxidative stress environment using Jugularia as the oxidant. (a) Survival rate of *C. elegans* and (b) average life expectancy of *C. elegans* under oxidative stress conditions. Significant differences were compared from the Control at * $P < 0.05$ and ** $P < 0.01$.

the blank groups (Control), the fluorescence intensity of lipofuscin in the experimental nematodes became weaker clearly with the increasing concentrations in both 5 and 10 days later. Quantitative analysis shows that the lipofuscin accumulated in the experimental nematodes (1,000 µg/mL) on the 5th day was reduced by 20% compared to the blank groups. With the extension of the treatment time, the lipofuscin accumulation in the nematodes increased on the whole; however, it still presented a weaker image in the experimental groups. After 10 days treatment, the

lipofuscin accumulated in the worms was reduced by 22.8% in the LDP-1 interfered groups compared with the blank group (Fig. 6b). These results indicate that LDP-1 could significantly delay the aging process of nematodes.

Analysis of ROS levels in *C. elegans* under oxidative stress

The free radical aging theory indicates that aging is caused by molecular damage due to the imbalance accumulation of oxygen free radicals (40). What is more is that the faster the individual's metabolic rate is, the more ROS

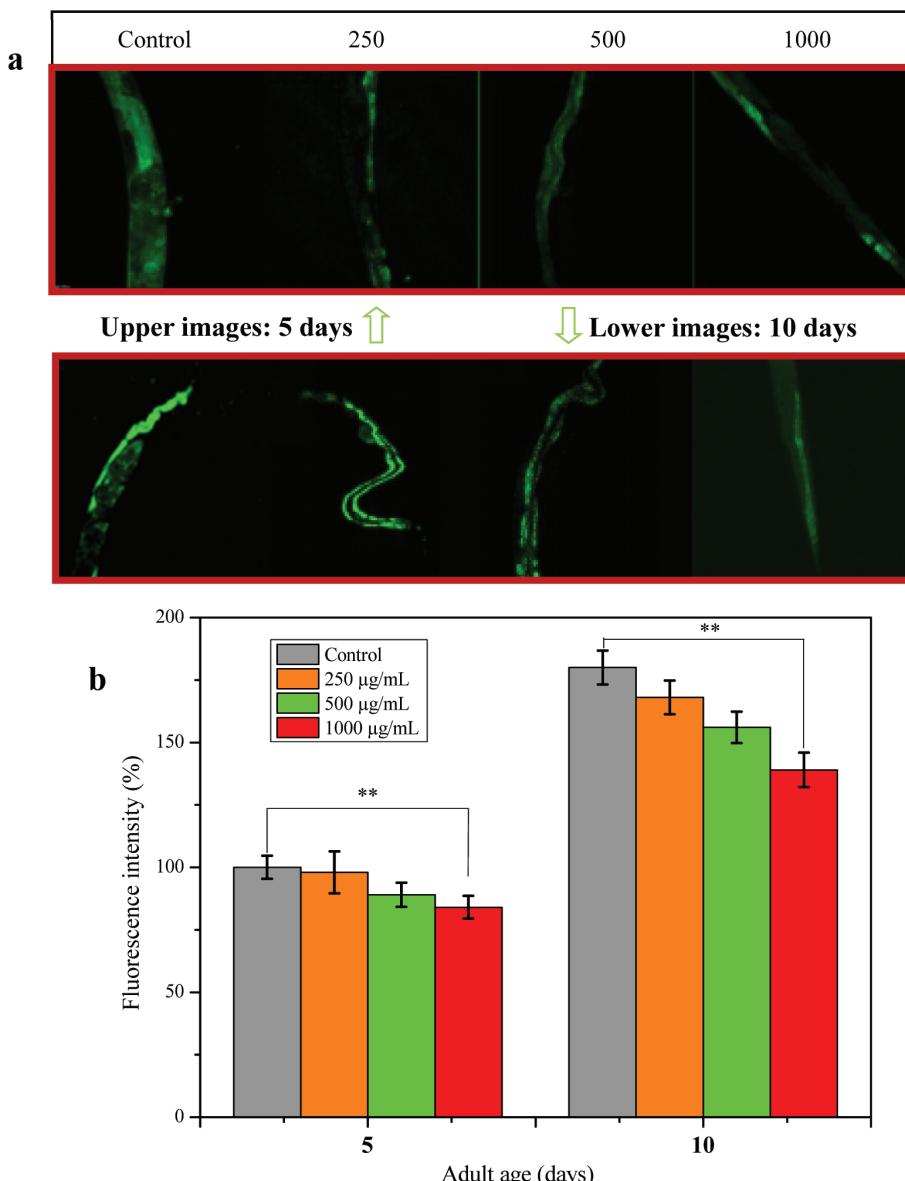


Fig. 6. Effects of the polysaccharide LDP-1 on the lipofuscin accumulation in *C. elegans* growth process. (a) Observation of the lipofuscin accumulation in *C. elegans* under fluorescent microscope; (b) Fluorescence intensity analysis of the produced lipofuscin. Significant differences were compared from the Control at ** $P < 0.01$.

it produces; under this circumstance, with the accumulation of oxidative damage it will speed up the aging process undoubtedly. Therefore, it is of great significance to assess the ROS levels in experimental nematodes.

As can be seen from Fig. 7, under the normal conditions, the ROS levels were tested in the blank group and LDP-1-treated groups after 5 days. It manifested that there were no significant differences in the ROS levels compared with the blank group (Control). However, when the experimental nematodes treated with 100 µg/mL Juglone solution for 3 h, the ROS levels of the LDP-1 interfered nematodes were significantly

reduced than that of the blank group, with a maximum decrease by 42.7%. Wang et al. (41) showed that the maximum clearance rate of ROS via the bioactive peptides was greater than 20%. Comparatively, the clearance rate of LDP-1 was twice than that of it. It further confirmed that LDP-1 had favorable antioxidant activity.

Conclusions

In this article, the antioxidant and anti-aging activity of polysaccharide LDP-1 was mainly investigated with *C. elegans* as the model. The results showed that LDP-1

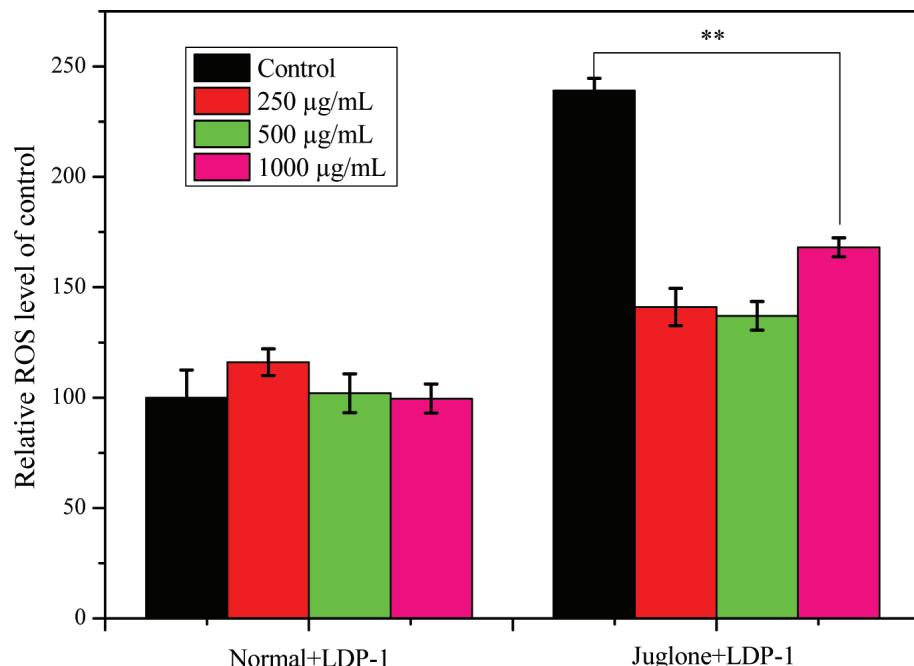


Fig. 7. Determination of ROS levels in *C. elegans* under normal environment and oxidative stress conditions. Comparisons were obtained from the first point of the Control at ** $P < 0.01$.

had favorable life-extending characteristics. It proved that LDP-1 could increase the lifespan of nematodes under heat shock and oxidative stress environments. The fluorescence intensity observed from the lipofuscin accumulation of the LDP-1-treated groups became weaker, which confirmed that LDP-1 could clearly delay the aging process of nematodes and decrease the ROS levels. On the basis of our previous report, LDP-1 showed good antioxidant, anti-aging, and immunomodulatory effects. Thus, it could be further explored as potential additive agents applied in functional foods and health care products.

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Conflict of interest and funding

There are no conflicts of interest to declare. The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

Authors' contribution

Xiao-Hui Wang: Methodology and Writing-original draft.
Xiao-Du Cheng: Experimentation and Data curation.

Dong Wang: Data processing. Zhi Wu: Graphic design. Yan Chen: Conceptualization. Qing-Xi Wu: Conceptualization, Investigation, Writing-review & editing, Project administration & Funding acquisition and Supervision.

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