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ORIGINAL ARTICLE

Ophiopogon Japonicus Herbal Tea Ameliorates Oxidative Stress and Extends Lifespan in Caenorhabditis Elegans

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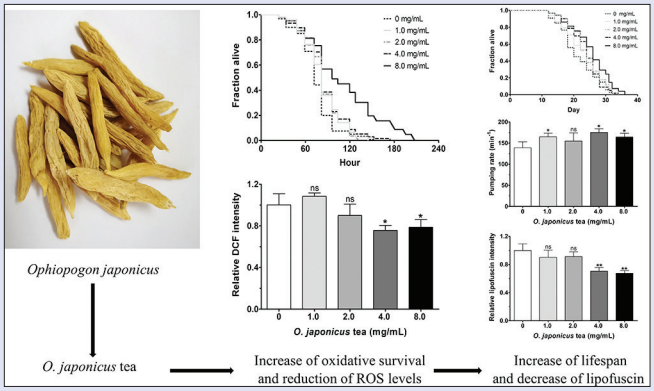
ABSTRACT

Background: *Ophiopogon japonicus* is a medicinal and edible plant widely used in China. Despite a long history of *O. japonicus* tea (OJT) in health promotion, however, the mechanistic studies on its actions are lacking. **Objective:** This study aims to evaluate the antioxidant activity and longevity-promoting potential of OJT using *Caenorhabditis elegans* model. **Materials and Methods:** The antioxidant capacities of OJT were first determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and paraquat survival assay, respectively, and then, further investigated by determining the reactive oxygen species (ROS) level, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activity. The lifespan assay and lipofuscin accumulation assay were performed in a similar fashion to the paraquat survival assay but without paraquat exposure. **Results:** We first show by DPPH scavenging assay that OJT can scavenge free radicals. We then reveal that OJT can not only increase the survival rate of the nematodes but also reduce the endogenous levels of ROS under oxidative stress induced by paraquat. We also show that OJT is capable of increasing the activities of SOD and CAT and reducing the content of MDA in paraquat-exposed *C. elegans*. Further studies indicate that OJT is able not only to extend the lifespan of the nematodes but also to improve the age-related decline of pharyngeal pumping and reduce accumulation of the age pigment lipofuscin in the animals. **Conclusion:** Our data demonstrate the antioxidant activity and age-delaying effect of OJT and thus provide an important insight into the potentials of *O. japonicus* for health promotion.

Key words: Catalase, lipofuscin, malondialdehyde, oxidative survival, reactive oxygen species, superoxide dismutase

SUMMARY

- *Ophiopogon japonicus* tea (OJT) was prepared by infusion of *O. japonicus* tuber powder in hot water
- The antioxidant capacities of OJT were first assessed by 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay *in vitro* and then by paraquat survival assay in *C. elegans*
- The ROS level, MDA content and the activities of SOD and CAT were investigated to evaluate the antioxidant capacities of OJT
- Lifespan assay and lipofuscin accumulation assay were performed to evaluate the anti-aging activity of OJT



Abbreviations used: CAT: Catalase; MDA: Malondialdehyde; OD: Optical density; OJT: *Ophiopogon japonicus* tea; ROS: Reactive oxygen species; SOD: Superoxide dismutase.

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INTRODUCTION

Ophiopogon japonicus (Linn. f.) Ker-Gawl, an evergreen perennial plant of the family Liliaceae, is widely distributed in Southeast Asia. As a traditional Chinese medicine recorded in the earliest known Chinese pharmacopoeia *Shen Nong's Herbal Classics* (Shen Nong Ben Cao Jing, ~110BC), *O. japonicus* has been extensively used to treat a range of disorders such as cardiovascular diseases and inflammation for centuries and is listed in the current Chinese Pharmacopeia. It has also been widely used as traditional food and medicine in other Asian countries, for example as antiphlogistic, expectorant, anticough, and tonic agents in Japan and Vietnam[1] and is considered to have high nutritional and medicinal values.[1-3]

Conventionally, *O. japonicus* is used in herbal prescriptions, for example, Sheng Mai San, a well-known Chinese prescription comprised *Panax ginseng*, *Schisandra chinensis*, and *O. japonicus*, has a long clinical history in the treatment of heart failure and ischemic heart diseases.[4,5] Recently, there is an increasing interest in *O. japonicus* as a source for biologically active compounds. Crude extracts and fractions, as well as isolated constituents such as polysaccharides, saponins and flavonoids,

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of *O. japonicus* are known to have antioxidant, antithrombotic, anti-inflammatory, and other activities.[3,6,7] For example, the polysaccharide fractions from *O. japonicus* tuber are shown to have strong *in vitro* free radical-scavenging and macrophage-activating capacities, indicating the antioxidant and immunomodulatory potentials of *O. japonicus*.[8] Interestingly, a steroidal saponin isolated from *O. japonicus* can extend the replicative lifespan and increase the oxidative survival of yeast, suggesting an anti-aging activity of *O. japonicus*.[9]

In addition to medical applications, *O. japonicus* is also widely used in general diet. For example, *O. japonicus* has been used to make herbal tea by simply infusing in hot water, which is purportedly invented by Sun Simiao (581-682), a well-known Chinese doctor and herbalist in the Sui and Tang dynasty. According to the folklore, *O. japonicus* tea (OJT) is useful in increasing energy, improving overall health and promoting longevity.[1] Despite a long history of OJT in health promotion, however, there are very few mechanistic studies on its actions. As a herbal tea, OJT is in essence a non-concentrated decoction, i.e., an aqueous extract containing a mixture of saccharides, amino acids, polyphenols, and other high-polar ingredients. Given that many orally taken decoctions can modulate oxidative stress,[10,11] the primary aim of this study is, therefore, to evaluate the antioxidant activity and longevity-promoting potential of OJT. We first determined the scavenging effect of OJT on free radicals using *in vitro* chemical assays, and then investigated its antioxidant and anti-aging activities in *Caenorhabditis elegans*, a simple yet powerful nematode model widely used in aging studies.

MATERIALS AND METHODS

Strains and maintenance

Both *C. elegans* (wild-type N2) and *Escherichia coli* (OP50 and NA22) strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, USA). All experiments were performed at 20°C unless otherwise stated.

Herbal tea preparation

The fresh tuberous roots of *O. japonicus* were collected from Quanzhou, Fujian, China, and identified by Professor Yan Wang (School of Traditional Chinese Medicine, Guangdong Pharmaceutical University). A voucher specimen (No. 01-MD-2015YS) has been deposited at the Center for Bioresources and Drug Discovery, Guangdong Pharmaceutical University. To make the herbal tea, the tuberous roots of *O. japonicus* were extracted twice with deionized water at 100°C. First, 100 g of the tuber powder was infused with 1 L of deionized water for 30 min in a shaking bath equipped with temperature control, and the extract was filtered through cotton wool. Then, a second infusion was prepared by adding 0.5 L of boiling deionized water for 30 min followed by filtering through cotton wool. The two infusions were combined and freeze-dried as OJT powder, which was stored at-20°C until use. The powder was dissolved in deionized water to the indicated concentrations before use.

Free radical scavenging assay

The scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined as previously described.[12] Briefly, 100 μL of OJT was mixed with 100 μL of 0.1 mmol/mL DPPH• in ethanol. The mixture was vortexed vigorously and allowed to stand for 30 min in the dark, and the absorbance was measured at 517 nm against a blank. The DPPH radical scavenging activity was expressed as a percentage of inhibition, which was calculated as follows:

DPPH radical scavenging activity (%) = $([A_0 - A_1]/A_0) \times 100$

Where A_0 and A_1 are the absorbances of the control and the sample, respectively.

Food clearance assay

Food clearance assay was conducted essentially as described.[13] *E. coli* OP50 was grown overnight and resuspended at an optical density (OD) of 0.85 in the nematode S medium. OJT was diluted with the *E. coli* suspension to the indicated concentrations, and 100 μL of the final mixture was added per well into 96-well plates. Approximately 50–80 synchronized L1 nematodes were subsequently added and incubated at 20°C. The absorbance of the culture was determined at 570 nm every day for 5 days.

Paraquat survival assay

The oxidative survival assay was performed as described previously using paraquat (Sigma, St. Louis, USA).[14] Synchronized L1 larvae were incubated for 42 h and then 75 μg/mL of 5-fluoro-2′-deoxyuridine (FUdR, Sigma) was added. After further incubation for 24 h, the young adult nematodes were transferred to 96-well plates (20 nematodes per well; >100 nematodes for each treatment) containing *E. coli* NA22 ($OD_{570\,nm}$ = 0.5) and 0–8.0 mg/mL of OJT samples. After incubation for another 24 h, the nematodes were exposed to 50 mM paraquat. The numbers of live and dead nematodes were scored microscopically every 12 h based on their movement and shape.

Determination of reactive oxygen species level

To assess reactive oxygen species (ROS) levels under oxidative stress, synchronized young adult nematodes were incubated with or without OJT samples for 24 h and then treated with 2 mM paraquat for 2 days. The determination of ROS levels was performed as described previously.[15] Briefly, bacteria were removed by three repeated washes with M9 buffer and subsequent centrifugation at low speed. The nematodes were collected and dispensed into black 96-well plates (100 animals per well; 800 animals for each treatment) by a COPAS Biosort instrument (Union Biometrica, Inc., Holliston, MA, USA), and then, the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA; Sigma) was added at 50 μM final concentration. The plates were incubated at 20°C for 1 h, and the ROS-related DCF fluorescence was measured by the Fluoroskan Ascent FL plate reader at excitation 485 nm and emission 520 nm.

Determination of antioxidant enzyme activity and malondialdehyde content

Activity of antioxidant enzymes and content of malondialdehyde (MDA) were determined as previously described.[14] Briefly, synchronized young adult nematodes were incubated with or without OJT samples for 24 h and then treated with 2 mM paraquat for 2 days. Approximately 4000 nematodes were then collected and washed three times with M9 buffer, transferred into an Eppendorf tube, and suspended in 600 μL of lysis buffer from the assay kits. The samples were sonicated in an ice bath, and the supernatant was collected by centrifugation (12,000 ×*g*, 5 min, 4°C) for the measurement of superoxide dismutase (SOD) activity, catalase (CAT) activity, and MDA content using commercial chemical assay kits (Beyotime, Haimen, China), respectively.

Lifespan assay

The lifespan assay of nematodes was performed in a similar fashion to the paraquat survival assay but without paraquat exposure. The live and dead nematodes were scored every 2 days until all the nematodes died.

Determination of pharyngeal pumping rate

Age-synchronized young adult nematodes were incubated on NGM agar plates with or without OJT for 4 days and then transferred individually to fresh plates with a lawn of OP50 bacteria. The contractions of pharynx for each nematode were counted manually for 30 s under a dissecting microscope. The pumping rates were measured with 10 nematodes for each group and repeated three times.[16]

Lipofuscin accumulation assay

The accumulation of lipofuscin was assessed essentially as described previously for age pigments.[14] Briefly, day-10 adult nematodes with or without OJT treatment from young adult stage were dispensed to black 96-well plates at 100 nematodes/well by the COPAS Biosort instrument, and the fluorescence of lipofuscin was determined by a Fluoroskan Ascent FL microplate reader (Thermo, Waltham, MA, USA) with 355 nm excitation and 460 nm emission.

Statistical analysis

The statistical analysis was performed primarily by GraphPad Prism 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance. *C. elegans* survival and lifespan data were analyzed by Kaplan-Meier method and Peto's log-rank test using SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant. All experiments were performed at least three times.

RESULTS AND DISCUSSION

In vitro scavenging effect of *Ophiopogon japonicus* tea on 2,2-diphenyl-1-picrylhydrazyl free radicals

Free radicals are known to attack biological macromolecules, including DNA, lipids and proteins, leading to oxidative damage and cellular senescence.[17,18] To examine the antioxidant property of OJT, therefore, we initially tested its ability to scavenge-free radicals using the *in vitro* DPPH radical scavenging methods, which has been extensively used to assess antioxidant activity.[19] As shown in [Figure 1], the DPPH scavenging activity of OJT was increased when its concentration was increased from 1.0 to 16.0 mg/mL, and then, became stable at ~60%. These results demonstrate the *in vitro* antioxidant activity of *O. japonicus* tea.

Increase of oxidative survival rate and decrease of ROS Level by *Ophiopogon japonicus* tea in *Caenorhabditis elegans*

Although DPPH radical scavenging assay is widely used to study *in vitro* antioxidant properties, it does not necessarily predict *in vivo* antioxidant activity.[20] Therefore, we attempted to evaluate the *in vivo* antioxidant capacity of OJT using *C. elegans* models. To do so, we first determined the appropriate concentrations to be used in the nematodes by food clearance assay. The nematodes were grown in liquid culture and treated with 0–16.0 mg/mL of OJT, and the consumption rate of bacteria was monitored by absorbance on a daily basis. As shown in [Figure 2], the absorbance of the control, as well as that of the nematodes treated with ≤8.0 mg/mL of OJT, was significantly reduced within 5 days, indicating that treatment with up to 8.0 mg/mL of OJT did not affect the nematodes. However, the animals treated with 16.0 mg/mL of OJT displayed an apparent delay in food consumption, suggesting that the herbal tea may have side effect on the nematodes at >16.0 mg/mL. Therefore, in the following experiments, 1.0–8.0 mg/mL of OJT was used as indicated.

Survival of animals under increased oxidative stress is undoubtedly a direct index of their antioxidant competence. Therefore, we determined the effect of OJT on the survival rate of *C. elegans* exposed to paraquat, a strong redox agent that is known to increase intracellular superoxide anion levels and may result in generation of additional toxic hydrogen peroxide and hydroxyl radicals.[21] The nematodes were pretreated with 0–8.0 mg/mL of OJT for 24 h and then exposed to 50 mM paraquat, and the live and dead nematodes were scored every 12 h. As shown in [Figure 3a and Table 1], the survival rates of the nematodes pretreated with 1.0–8.0 mg/mL of OJT were all increased as compared to that of the control nematodes exposed to paraquat only; in particular, the mean survival time of the nematodes treated with 8.0 mg/mL of OJT was increased >40%. These results demonstrate the *in vivo* antioxidant activity of the herbal tea.

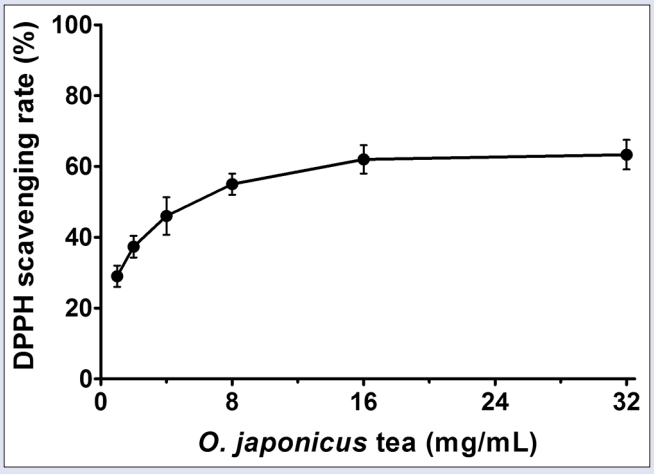

Figure 1: DPPH radical scavenging effect of *Ophiopogon japonicus* tea. The mixture of 100 μL of OJT and 100 μL of DPPH• was allowed to stand for 30 min and the absorbance was read at 517 nm. The DPPH radical scavenging activity was expressed as a percentage of inhibition relative to the control. DPPH: 2,2-diphenyl-1-picrylhydrazyl

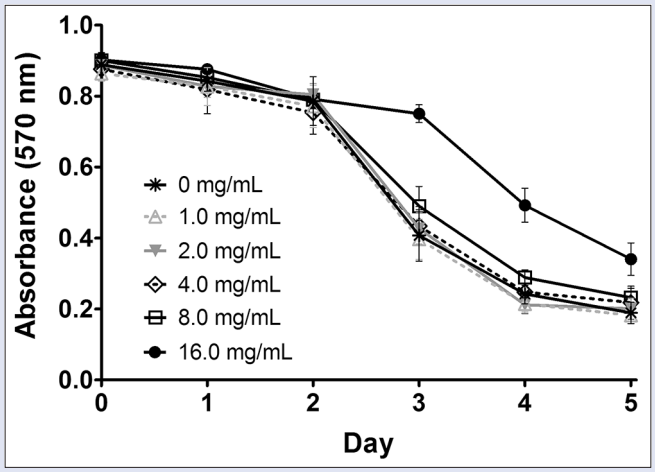

Figure 2: Concentration range of *Ophiopogon japonicus* tea for *Caenorhabditis elegans*. Synchronized L1 nematodes were incubated with *Escherichia coli* and OJT at the indicated concentrations in 96-well plates at 20°C, and the absorbance of the culture was daily measured. Data are representative of three independent experiments and presented as mean ± SD. SD: Standard deviation; OJT: *Ophiopogon japonicus* tea

ROS is a major source of oxidative stress and one of the key factors causing senescence, and compounds with ROS-eliminating ability can protect organisms from oxidative damage. For example, a polyphenol-enriched extract from cocoa seeds is shown to reduce the amount of cellular ROS and related toxicity caused by ochratoxin A, a mycotoxin.[22] Therefore, we further examined the effect of OJT on ROS levels in the nematodes exposed to paraquat. The nematodes were first treated with OJT at the indicated concentrations for 24 h and then exposed to 2 mM paraquat for 2 days, and the ROS level was assessed using the DCFH-DA fluorescence assay. As shown in [Figure 3b], the relative DCF fluorescence intensity of the nematodes treated with 4.0 and 8.0 mg/mL of OJT was significantly lower than that of the paraquat-exposed control nematodes ($P < 0.01$), indicating reduced ROS levels after OJT treatment. Taken together, our results demonstrate that the free radical-scavenging and ROS level-reducing activities of OJT may significantly contribute to its antioxidant capability against paraquat toxicity.

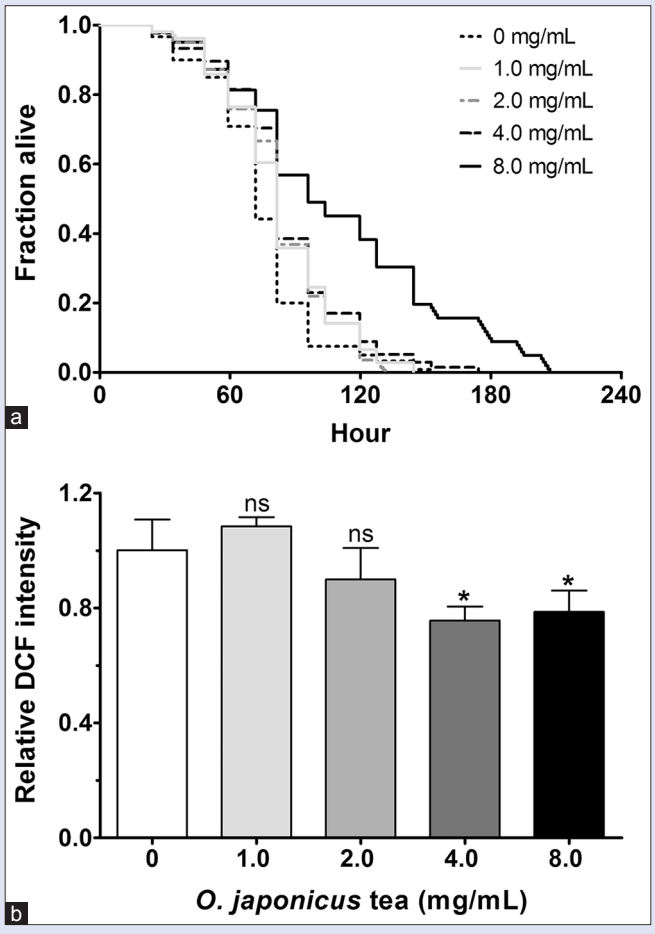

Figure 3: Effect of *Ophiopogon japonicus* tea on oxidative survival and ROS levels in *Caenorhabditis elegans*. (a) Increase of survival rate by OJT in paraquat-exposed nematodes. After 24 h of OJT pretreatment, the nematodes were exposed to 50 mM paraquat for survival analysis at 12-h intervals. (b) Reduction of ROS levels by OJT in paraquat-exposed nematodes. After 24 h of OJT pretreatment, the nematodes were exposed to 2 mM paraquat for 2 days and then subjected to DCFH-DA fluorescence assay. The ROS level was indicated by relative DCF fluorescence intensity. *$P < 0.05$; ns, $P > 0.05$. DCFH-DA: 2′,7′-dichlorofluorescin diacetate; ROS: Reactive oxygen species; OJT: *Ophiopogon japonicus* tea

Increase of antioxidant enzyme activities and decrease of malondialdehydecontent by *Ophiopogon japonicus* tea in *Caenorhabditis elegans*

It is well established that intracellular ROS level is balanced by a set of antioxidant enzymes such as SOD and CAT, which are an important part of the antioxidant system. These enzymes not only scavenge free radicals but also maintain the cellular homeostasis in a living system.[23] However, the endogenous antioxidant system is not always effective to scavenge excessive ROS, leading to interruption of cellular homeostasis and acceleration of aging process.[24] Accordingly, supplementation of exogenous antioxidants is commonly regarded as a promising strategy to promote health and retard aging.[25,26] To assess the effect of OJT on the endogenous antioxidant defense system, we examined the activities of the antioxidant enzymes SOD and CAT and the content of the lipid peroxidation product MDA in *C. elegans*. After pretreatment with OJT at the indicated concentrations for 24 h and then exposure to 2 mM paraquat for 2 days, the SOD and CAT activities were increased while the MDA content was decreased in the nematodes treated with 1.0–8.0 mg/mL of OJT as compared to those of the control nematodes exposed to paraquat alone, respectively [Figure 4]. Together, our results suggest that the upregulation of antioxidant enzyme activities by the herbal tea may, at least in part, be responsible for the reduction of ROS levels.

Increase of lifespan and pumping rate and decrease of lipofuscin by *Ophiopogon japonicus* tea in *Caenorhabditis elegans*

A considerable correlation is known to exist between longevity and enhanced tolerance to stress including oxidative, heat shock, and ultraviolet stresses.[27] As shown above, *O. japonicus* tea is capable of enhancing tolerance of *C. elegans* to oxidative stress and thus may have the potential to delay aging process. Therefore, we determined the effect

Table 1: Effect of *Ophiopogon japonicus* tea on the survival time in paraquat-intoxicated *Caenorhabditis elegans*

OJT (mg/mL)	Survival time (h)	Total nematodes
0	73.6±3.2	313
1.0	81.9±2.2	308
2.0	84.6±5.4*	410
4.0	88.9±5.1**	381
8.0	105.8±16.3***	304

*P<0.05; **P<0.01; ***P<0.001. After pretreatment with OJT at the indicated concentrations for 24 h, the nematodes were exposed to 50 mM paraquat and the survival status was recorded every 12 h until all dead. The data for survival time are presented as mean±SEM and the total nematodes are the number of animals from three independent experiments. OJT: *Ophiopogon japonicus* tea; SEM: Standard error of mean

Table 2: Effect of *Ophiopogon japonicus* tea on the adult lifespan of *Caenorhabditis elegans*

OJT (mg/mL)	Mean lifespan (day)	Total nematodes
0	20.6±1.8	371
1.0	21.2±2.1	385
2.0	21.6±1.6	292
4.0	21.9±1.5*	347
8.0	23.6±1.8**	345

*P<0.05; **P<0.01. The nematodes were treated with OJT at the indicated concentrations from young adult and the number of live nematodes was scored every 2 days until all dead. The data for mean lifespan are presented as mean±SEM and the total nematodes are the number of animals from three independent experiments. OJT: *Ophiopogon japonicus* tea; SEM: Standard error of mean

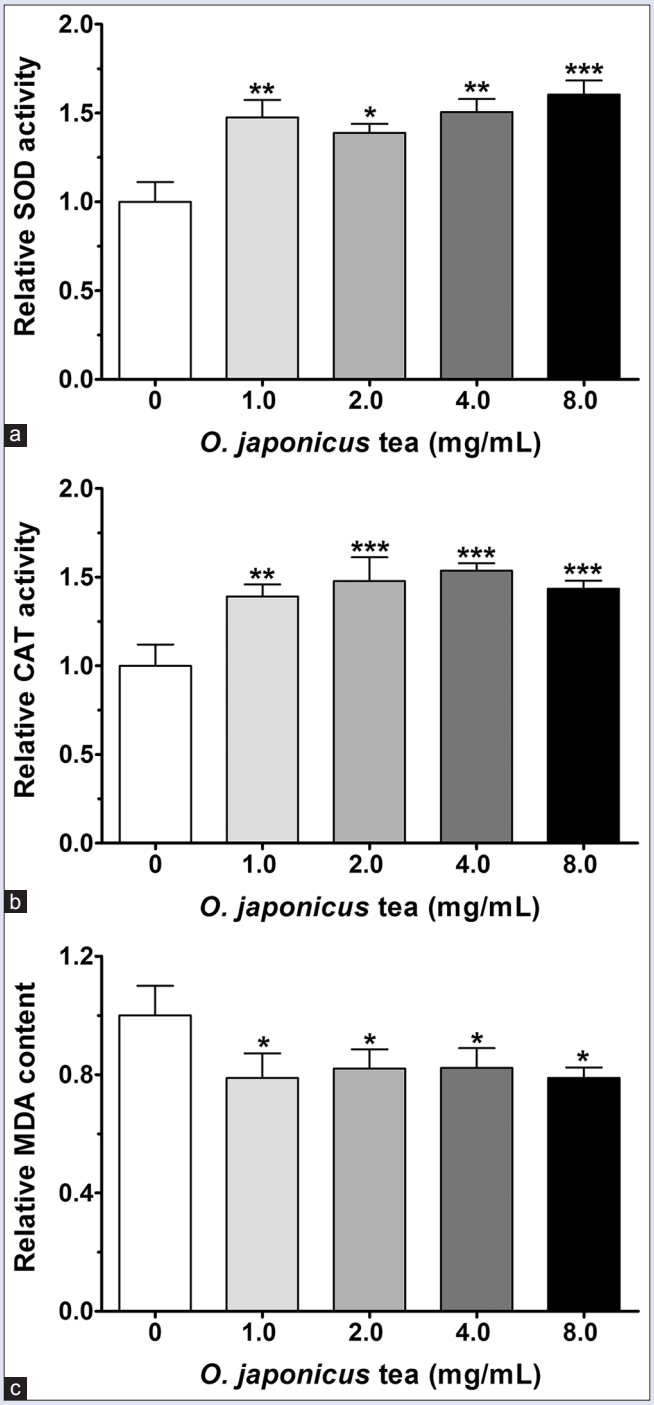

Figure 4: Effect of *Ophiopogon japonicus* tea on the antioxidant enzyme activities and MDA content in *Caenorhabditis elegans*. After pretreatment with 0–8.0 mg/mL of OJT for 24 h and then exposure to 2-mM paraquat for 2 days, the nematodes were lysed for determination of SOD (a) and CAT (b) activities and MDA content (c). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. OJT: *Ophiopogon japonicus* tea; MDA: Malondialdehyde; SOD: Superoxide dismutase

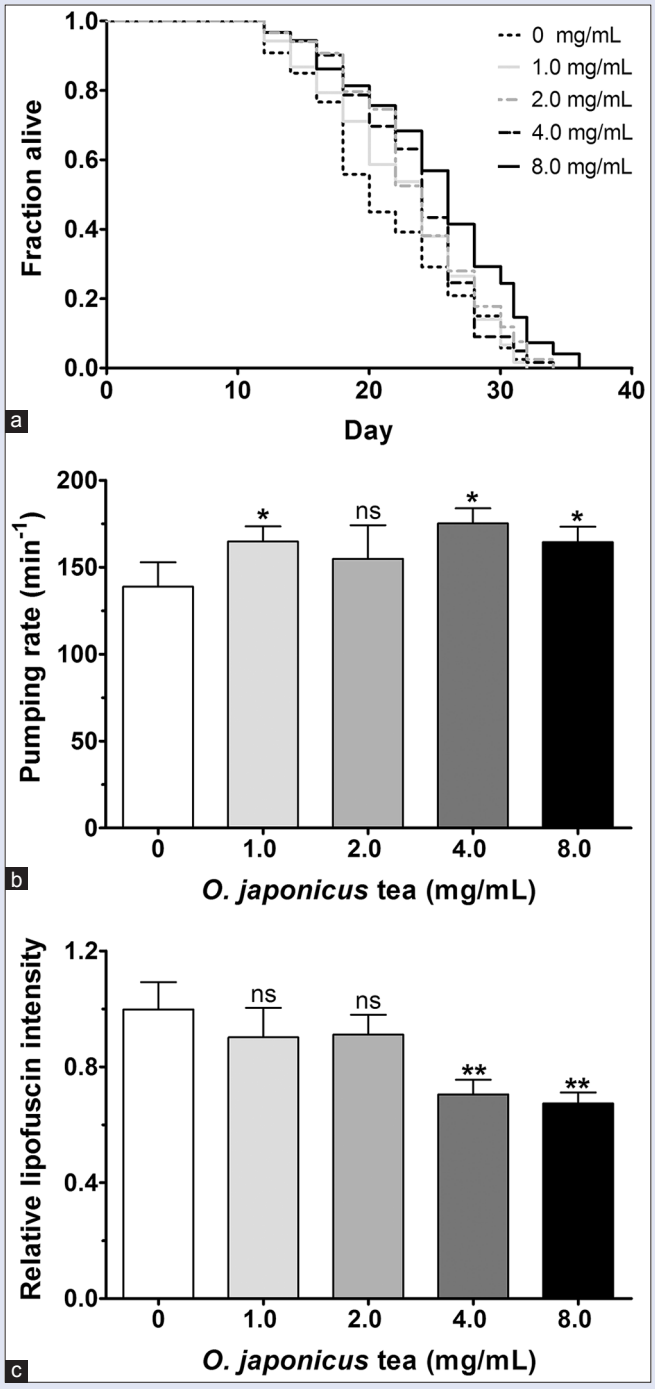

Figure 5: Effect of *Ophiopogon japonicus* tea on lifespan, food-intake behavior and lipofuscin content in *Caenorhabditis elegans*. (a) The adult lifespan of nematodes treated with 0–8.0 mg/mL of OJT. (b) Pharyngeal pumping rate of nematodes treated with 0–8.0 mg/mL of OJT. Pharyngeal pumping was counted individually for 30 s, and the average pumping rate was calculated for each treatment. (c) Relative lipofuscin fluorescence level of nematodes treated with 0–8.0 mg/mL of OJT. The fluorescence intensity of intestinal lipofuscin was determined at day 10 after treatment. *$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$. OJT: *Ophiopogon japonicus* tea; ns: Not significant

of OJT on lifespan, which is unequivocally the most direct antiaging index,[28] using nematode models. As shown in [Figure 5a and Table 2], the lifespan of the nematodes treated with 4.0 and 8.0 mg/mL of OJT was indeed extended as compared to that of the control nematodes, demonstrating the age-delaying effect of the herbal tea.

In addition to lifespan itself, other age-associated changes in *C. elegans* include pharyngeal pumping rate and intestinal lipofuscin level, both of which are important physiological parameters to measure the health

condition of nematodes.[29,30] As aging progresses, pharyngeal pumping rate decreases and intestinal lipofuscin content increases.[30,31] Therefore, we examined the effect of OJT on these physiological functions. The nematodes were treated with 1.0–8.0 mg/mL of OJT and the pumping rate was determined on day 4. As shown in [Figure 5b], the pharyngeal pumping rate of nematodes treated with OJT was slightly increased, indicating that the herbal tea was able to prevent the age-related decline of pharyngeal pumping and that its lifespan-extension effect was not due to reduced food intake, which is a potential cause of lifespan extension through dietary restriction.

Intestinal lipofuscin, a marker for cellular damage during aging, is composed primarily of lipid peroxidation products and oxidized proteins that resist proteolytic degradation.[32] Recent studies suggest that antioxidant treatment can retard age-associated accumulation of lipofuscin.[33,34] Therefore, we tested whether *O. japonicus* tea had an effect on the lipofuscin content in *C. elegans*. The nematodes were treated with 1.0–8.0 mg/mL of OJT and the relative content of lipofuscin was determined based on its fluorescence intensity. As shown in [Figure 5c], the relative fluorescence of Day-10 nematodes after treatment from the young adult stage with 4.0 and 8.0 mg/mL of OJT was significantly decreased, indicating a reduction of lipofuscin accumulation by the herbal tea. Taken together, our results suggest that *O. japonicus* tea can extend lifespan and counteract aging process independent of dietary restriction.

CONCLUSION

In this study, we demonstrate that OJT, the herbal tea prepared from *O. japonicus* tuber, is capable of not only scavenging DPPH-free radicals *in vitro* but also increasing the survival rate and reducing the ROS levels of *C. elegans* under oxidative stress induced by paraquat. We also reveal that the herbal tea can increase the activities of the antioxidant enzymes SOD and CAT and reduce the content of the lipid peroxidation product MDA in paraquat-exposed *C. elegans*. Further studies indicate that OJT can extend the lifespan and slightly increase the pharyngeal pumping rate of *C. elegans*. Interestingly, OJT is also shown to reduce the accumulation of lipofuscin, a typical age pigment. Taken together, our findings demonstrate the antioxidant and anti-aging capacities of *O. japonicus* tea.

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Conflicts of interest

There are no conflicts of interest.

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