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Anti-Aging Effects of *Lycium ruthenicum* Murr. Granules in *Caenorhabditis elegans*

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Key words
***Lycium ruthenicum* Murr., *Caenorhabditis elegans*, Aging, Oxidative stress, Reactive oxygen species, Antioxidant enzyme.**

ABSTRACT

In order to investigate the anti-aging effects of *Lycium ruthenicum* Murr. granules (HGQ) and potential underlying mechanism of actions, *Caenorhabditis elegans* was exploited as model organisms. The anti-aging capacity of HGQ was determined by lifespan and lipofuscin fluorescence assays in wild-type *C. elegans* N2. Calorie restriction pathway was detected by pharyngeal pumping rate assay and lifespan examination in *sir-2.1* mutant nematodes. The antioxidant activity of HGQ was investigated by determining reactive oxygen species (ROS) level, paraquat-stress survival rate, malondialdehyde (MDA) content as well as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities. We observed that HGQ exerted little effect on the growth of wild-type N2 nematodes at concentration < 0.6 mg/ml, and 0.05 mg/ml of the HGQ significantly prolonged the lifespan and reduced the lipofuscin level in N2 nematodes. However, the HGQ failed to influence the pharyngeal pumping rate in N2 nematodes or to affect the lifespan of *sir-2.1* mutant nematode VC199, indicating the anti-aging effect of HGQ is independent of calorie restriction pathway. Further experiments showed that the HGQ decreased ROS and MDA contents and increased the survival rate and SOD (not CAT or GPx) activities in paraquat-stressed N2 nematodes. Our results suggest that HGQ can effectively delay the senescence of *C. elegans*, and this effect may be related with its significant antioxidant activity.

INTRODUCTION

With improving living standards and health awareness around the world, delaying the senescence has recently gained enormous attentions in China. The agents that claim to prolong lifespan include traditional medicinal, alternative medicine and food items (Breckenridge, 2013). Among them, *Lycium ruthenicum* Murr. has been recorded in the pharmaceutical classics such as Uyghur Medicine, The Four Medical Tantras, and Jing Zhu Materia Medica. This has been reported to enhance immune function, enrich yin and nourish kidney, and delay senescence (Liu, 1999). Recent studies have shown that *Lycium ruthenicum* Murr. contains a variety of bioactive components (Zhao *et al*., 2018), and its mature berries are rich in polyphenols such as anthocyanin and flavone, which have strong pharmacological activities such as anti-oxidation (Wu *et al*., 2016; Chen *et al*., 2018) anti-fatigue (Ni *et al*., 2013), anti-cancer (Zhang *et al*., 2019), and immunity enhancing (Peng *et al*., 2014) properties.

At present, people often extract *Lycium ruthenicum* Murr. in water, and then concentrate and dry to formulate *Lycium ruthenicum* Murr. granules (HGQ). These are then used in tea to enhance human immunity and delay aging. However, there is no systematic research and reports on its anti-aging activity and any underlying molecular mechanisms, and thus warrant studies.

A large number of studies have shown that natural onset of aging is closely related to the generation of excessive free radicals *in vivo*. Therefore, enhancing the body's antioxidant capacity and scavenging excessive free radicals can effectively delay aging (Si *et al*., 2014; Simioni *et al*., 2018). In addition, previous investigations have shown that "calorie restriction" also can delay aging and prolong lifespan through enhancing the activity of longevity genes such as *sir-2* (Testa *et al*., 2014; Al-

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Regaiey, 2016). Polyphenols, including anthocyanin and flavonoid, can exert anti-aging biological activities through their strong anti-oxidation and free radicals scavenging effects (Zilić *et al*., 2012; Loffredo *et al*., 2017; Cirillo *et al*., 2016), and regulations of calorie restriction signaling pathways (Kulkarni and Cantó, 2015; Timmers *et al*., 2011). The study conducted by Shang *et al*. (2017) showed that *Lycium ruthenicum* Murr. is rich in active polyphenol components content (up to 4%), and the average content of anthocyanin can reach to 2.3%. These results provide a foundation to further investigate the anti-aging activity of *Lycium ruthenicum* Murr. granules (HGQ).

In this study, we applied a model organism *Caenorhabditis elegans* to assess the anti-aging impacts of HGQ. The *Caenorhabditis elegans* was adapted mainly due to its relatively short life cycle, simple experimental operation, rich genetic resources, and key-signaling pathway similarities with human. Different approaches were used to assess the lifespan, anti-oxidation and calorie restriction pathways. Collectively, the data proposed the anti-aging activity and provided novel insights into the molecular mechanisms in anti-aging effects of HGQ.

MATERIALS AND METHODS

Strains and materials

Wild-type *C. elegans* N2, mutant strain VC199 [*sir2.1 (ok434)*] and *Escherichia coli* (OP50 and NA22) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota) and maintained under standard conditions.

Lycium ruthenicum Murr. granules were purchased from Hebei Kangping Health Food Co., Ltd. The granules were prepared by concentrating and drying the water extract of *Lycium ruthenicum* Murr.

Reagents and instruments

Agar, tryptone, yeast extract, ampicillin, and 2'-deoxy-5-floxuridine were purchased from Sigma Company, USA. Pierce BCA Protein Quantitative Analysis Kit was purchased from ThermoScientific, USA; Reactive Oxygen (ROS) Assay Kit, Total Superoxide Dismutase (SOD) Activity Assay Kit, Lipid Peroxidation MDA Assay Kit, Catalase (CAT) Assay Kit and Total Glutathione Peroxidase (GPx) Assay Kit were purchased from Beyotime Biotechnology Company, Ltd., China.

Full-wavelength microplate reader (BioTek Co., Vermont, MA, USA); Fluoroskan Ascent FL microplate reader (Thermo Electron Co., Waltham, MA, USA) and grinding machine (Shanghai Jingxin, JX-24) were applied to record diverse assays.

Sample preparation

Appropriate amount of HGQ was taken and dissolved in the liquid S. Medium of *C. elegans* to make 5 mg/ml HGQ sample solution. The solution was filtered through 0.22 μm sterile millipore membrane, and was saved aside as standby until use.

Food clearance assay

The food clearance assay was carried out to determine the range of HGQ concentrations as described (Zhang *et al*., 2016). Synchronized L1 nematodes N2 (10-15/well), *E. coli* NA22 (OD570≈0.70), and sample solution (final concentration 0.01-1.0 mg/ml) were added to a 96 wells plate (final volume 100 μl/well) and incubated at 20°C. The control group was added with S. Medium, and 8 parallel wells were set for each sample concentration. The absorbance of the culture was measured at 570 nm every day from day 1-7 using a full-wavelength microplate reader (BioTek Co., Vermont, MA, USA). The day HGQ added was designed as day 1.

Lifespan assay

The lifespan assay for *C.elegans* was performed in liquid culture at 20°C as described previously (Zhang *et al*., 2012). Briefly, synchronized L1 larvae nematodes were incubated with *E. coli* NA22 in S. Medium for about 42h to L4 larvae and then treated with 100 μg/ml ampicillin, and 75 μg/mL 5-fluoro-2'-deoxyuridine to prevent progeny growth for another 24h until young adult larvae. Then animals were transferred into 96 wells plate (10-15 nematodes/well) and treated with the HGQ (10 wells/concentration). The day of HGQ addition was designated as day 0 during the analysis. The alive animals were counted microscopically every two days based on their movement until all were dead.

Lipofuscin level assay

The lipofuscin assay was performed as described previously (Gerstbrein *et al*., 2005). Nematodes were prepared as described in lifespan assay but black-96 wells plates were used instead, and the fluorescence intensity of lipofuscin was determined by Fluoroskan Ascent FL microplate reader (Thermo Electron Co., Waltham, MA, USA) with 355 nm excitation and 460 nm emission every 3 days from day 1 to day 10.

Pharyngeal pumping rate assay

Pharyngeal pumping rate assay was carried out as previously described (Wang *et al*., 2016). Nematodes were prepared as the method of lifespan assay with a modification of 24-well plate with about 500 nematodes per well. After incubation at 20°C for 4 days, nematodes

were collected and transferred on the NGM plate coated with *E. coli*. OP50. After an adaption of another 30 min at 20°C, the pharyngeal pumping rate was microscopically counted for about 50 nematodes per concentration treatment, and the data was expressed as mean ± SEM of three independent experiments.

Determination of ROS level

The ROS level was determined using the 2'-7'-dichloro-fluorescein diacetate as has previously been described (Wu *et al*., 2006). Briefly, synchronized young adult nematodes were prepared as shown in lifespan assay and then added into a 24-well plate with or without HGQ treatments in three parallel wells for each concentration. After incubation for 2 days at 20°C, 10 µl of paraquat (ROS induction reagent, final concentration of 10 mM) was added, and then the nematodes were cultured for another 2 days at 20°C. Approximately 1500 nematodes per treatment were collected and washed three times with S. Medium and then homogenized in PBST buffer (1*PBS with 0.1%Tween 20) using a grinder at 4°C for 6min (grinding for 45 s, suspending 15 s). The supernatant collected through centrifugation (4°C, 12000 rpm, 10 min) was used as nematode lysate. Pierce BCA Protein Quantitative Analysis Kit (Beyotime, Nanjing, China) and ROS Assay Kit (Beyotime, Nanjing, China) were used to determine the protein concentration and ROS level of the lysate, respectively. The results were normalized by protein content.

Determination of MDA content

The MDA content assay was performed as previously described (Xiao *et al*., 2014). The treatment of nematodes, the preparation of lysate and the quantification of protein content were same as ROS detection. The MDA content was measured using Lipid Peroxidation MDA Assay Kit (Beyotime, Nanjing, China) and results were normalized by protein contents.

Paraquat survival assay

The oxidative survival assay was performed as described previously using paraquat (Wang *et al*., 2014). The preparation of *C. elegans* was performed as shown in lifespan assay. Synchronized young adult nematodes were transferred to 96 wells plates along with HGQ treatment. After incubation for another 24 h, the nematodes were exposed to 60 mM paraquat. The time of HGQ addition was designated as hour 0 and the number of live or dead nematodes were scored microscopically every 12 h based on their movement until all were found dead.

Determination of antioxidant enzyme activity

Antioxidant indices were determined as previously described (Xiang *et al*., 2017; Wang *et al*., 2016). The nematodes and lysate were prepared the same as ROS detection and the protein content of lysate was determined using BCA Assay. The SOD, CAT and GPx activities were measured using respective Assay Kits (Beyotime, Nanjing, China), combined with a full-wavelength microplate reader, and the results were normalized by protein contents.

Statistical analysis

The statistical analysis was performed primarily using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). All experiments were performed more than three times independently. The *C. elegans* lifespan and survival data were analyzed by the Logrank (Mantel-Cox test) method and the representative Kaplan–Meier survival curves were shown. The other experimental data were expressed as mean ± SEM or mean ± SD. One-way analysis of variance (ANOVA) and Dunnett's post-test and un-paired *t*-test were performed to analyse the difference between the experimental and control groups. $p<0.05$ was considered to be significantly different.

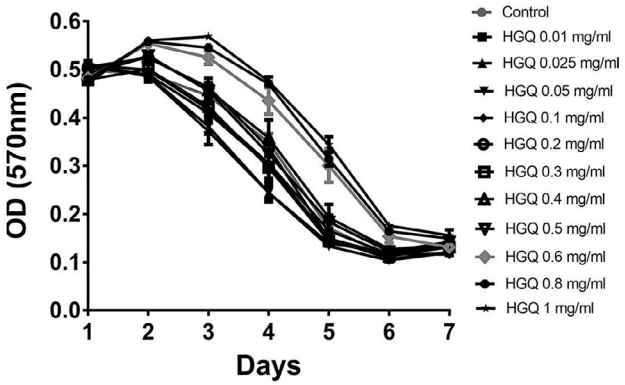

Fig. 1. Influence of HGQ on the food clearance of N2 nematodes. Synchronized L1 nematodes were incubated at 20 °C in 96 wells plates with or without HGQ at the indicated concentrations, and the absorbance at 570 nm was measured daily for 7 days. Results are representative of three independent experiments, and data are shown as mean ± SD of ten parallel wells with 100–150 nematodes per concentration.

RESULTS

Concentration of HGQ administration

The food clearance rate can indirectly reflect the influence of compounds on the growth and reproduction of *C. elegans* at different concentrations (Voisine *et al*., 2007). In this study, the initial concentration of HGQ was estimated by food clearance assay in wild-type N2 nematodes. The results shown in Figure 1 indicated

that the concentration of < 0.5 mg/ml had no significant influence on the food clearance compared with control, but the concentration of ≥ 0.6 mg/ml prominently decreased the food clearance rate, suggesting that the higher concentration can affect the normal growth of *C. elegans*. Therefore, we considered a concentration range of 0.05 ~ 0.5 mg/ml for subsequent experiments.

Senescence of C. elegans

As lifespan is regarded as an unequivocal anti-aging index (Wang *et al*., 2014), we applied the lifespan assay to assess the anti-aging activity of HGQ in N2 nematodes. The median lifetime of nematodes treated by HGQ at the concentration of 0.05 mg/ml was significantly extended by 7% from 26 days to 28 days ($p < 0.05$) compared with control group (Fig. 2A), while the other concentrations had no obvious influence on the lifespan of N2, suggesting a certain activity of anti-aging effect of HGQ at lower concentrations.

Studies have shown that age-related autofluorescent lipofuscin accumulated in the intestine over time is regarded as a biomarker of aging, and their accumalation is shown to be inversely correlated with longevity (Terman and Brunk, 2004). The model organism of *C. elegans* has transparent body, and the changes in the accumulation of lipofuscin *in vivo* can be quantitatively analyzed based on its optical properties using a fluorescent microplate reader. Therefore, we further studied the anti-aging activity of HGQ using lipofuscin content test. As shown in Figure 2B, compared with the control group, the relative fluorescence intensity of the 0.05 mg/ml HGQ treatment group was significantly decreased on day 4, 7, and 10, indicating that HGQ could significantly reduce lipofuscin content in the body of nematodes at this concentration, and thereby inhibit the aging of nematodes, showing a certain anti-aging activity, while the HGQ sample with a concentration of 0.1-0.5 mg/ml had no significant effect on lipofuscin content.

Calorie restriction pathway of C. elegans

A large number of studies have confirmed that limiting calorie intake has certain anti-aging biological effects and can delay the occurrence of aging-related diseases (Sutphin and Kaeberlein, 2008; Timmers *et al*., 2011). In order to confirm whether the anti-aging activity of HGQ is related to the calorie restriction of *C. elegans*, we detected the pharyngeal pumping rate in N2 nematodes to investigate the influence of HGQ on the food swallowing ability. Compared with the control group, no obvious changes of pharyngeal pumping rate were observed in the nematodes treated by HGQ at the tested concentration, indicating that it had little influence on the feeding and calorie intake (Fig. 3A).

Studies have also shown that the anti-aging effects of calorie restriction pathways is related to the mechanisms such as enhancing the activity of longevity genes such as *sir-2.1* and reversing the expression of longevity genes (Testa *et al*., 2014; Al-Regaiey, 2016). In order to further verify the relationship between the anti-aging activity of HGQ and the calorie restriction pathway, we examined the influence of HGQ on the lifespan of *C. elegans* VC199, which has the mutants defective in the sirtuin-homologue *SIR-2.1*. The results in Figure 3B revealed that the lifespan of VC199 was also prolonged by HGQ at the concentrations of 0.05, 0.1 and 0.3 mg/ml, among which the extensive effect at 0.05 mg/ml was the most significant, similarly as in N2 nematode, suggesting that the anti-aging activity of HGQ was not related to the regulation of gene *sir-2.1*.

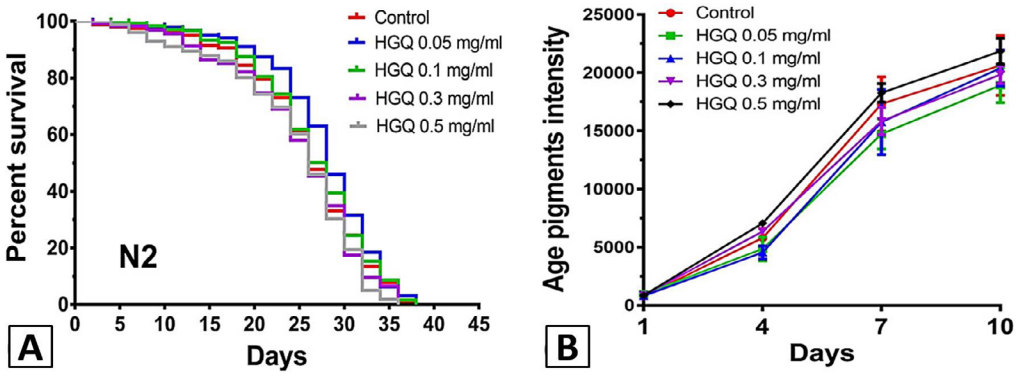

Fig. 2. Influence of HGQ on the senescence of N2 nematodes. Synchronized nematodes were incubated at 20°C in 96 wells plates with or without HGQ at the indicated concentrations, and the representative Kaplan-Meier survial curves are shown from three independent experiments with about 100 animals in each group (A). The fluorescence intensity of lipofuscin in adult nematodes was detected from day 1 to day 10 by a microplate reader at 355 nm excitation and 460 nm emission, and data are shown as mean ± SD of ten parallel wells with 100–150 nematodes per concentration (B).

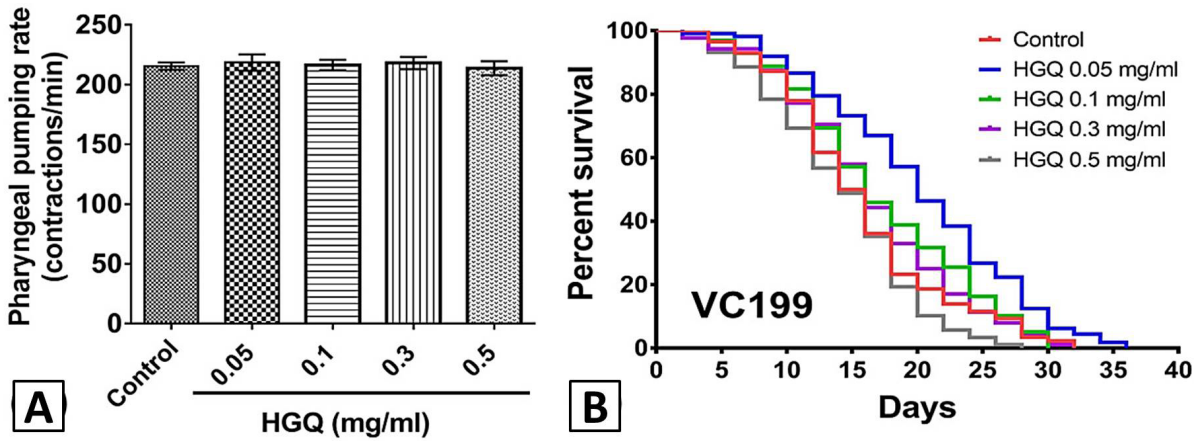

Fig. 3. Influence of HGQ on the calorie restriction pathway in nematodes. Synchronized N2 nematodes were incubated at 20°C in 24-well plates with or without HGQ at the indicated concentrations, and the pharyngeal pump rate was measured on NGM plate at day 4 (A). Data are shown as mean ± SEM of four independent experiments with 40 nematodes assays for each treatment (A). Synchronized *sir-2.1* mutant nematode VC199 were incubated at 20 °C in 96 wells plates with or without HGQ at the indicated concentrations, and the representative Kaplan-Meier lifespan curves are shown from three independent experiments with about 100 animals in each group (B).

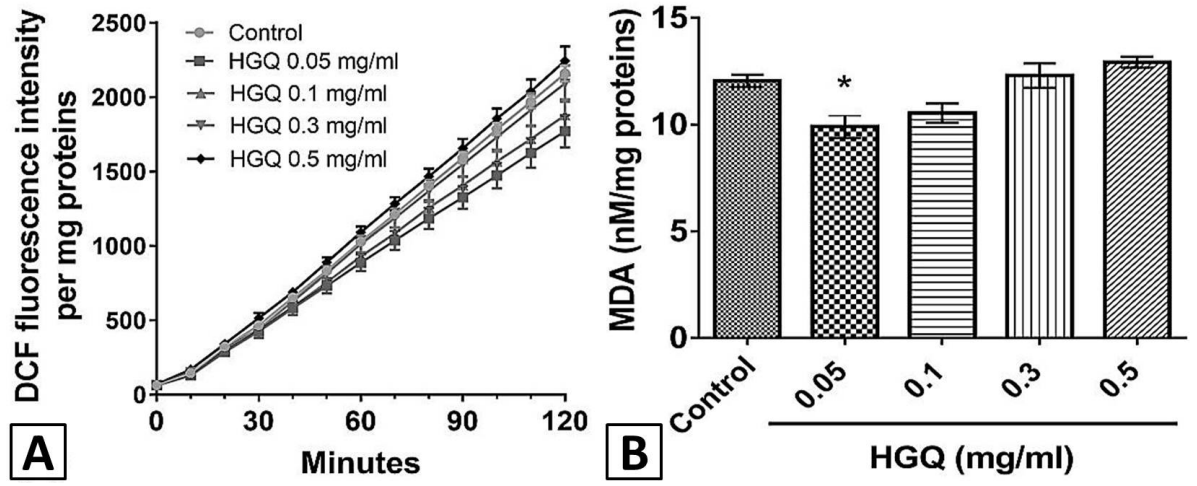

Fig. 4. Influence of HGQ on the oxidative stress level in paraquat-treated N2 nematodes. Synchronized young adult nematodes were pretreated with or without HGQ at indicated concentrations at 20°C for 48 h prior to exposure to 10mM paraquat for 48 h. Approximately 1500 nematodes were collected and homogenized in PBST buffer. The relative level of ROS (A) and MDA content (B) were determined with DCF fluorescence Assay Kit and Lipid Peroxidation MDA Assay Kit. The results werw normalized by protein cotent. Data are shown as mean ± SEM of three independent experiments, and statistical significance is determined by one-way ANOVA followed by Tukey's post hoc test. *p<0.05.

Oxidative stress level in C. elegans

According to the free radical theory of aging, the occurrence of aging is directly related to the formation of excess free radicals *in vivo* and the resulting oxidative stress environment (Finkel and Holbrook, 2000). In order to study whether the anti-aging activity of HGQ is related to the reduction of oxidative stress level, we took the paraquat, a herbicide capable of generating superoxide radicals, to induce oxidative stress in nematodes and then examined the influence of HGQ on the ROS level and malonaldehyde (MDA, ROS-mediated membrane lipid oxidation product) content. Compared with the control group, the 0.05 mg/ml HGQ treatment could significantly reduce the ROS level in paraquat-stressed N2 nematodes, while 0.1 mg/ml treatment exhibited a lowering and non-significant trend (Fig. 4A). The high-concentrations of 0.5

and 0.3mg/ml administration showed a little effect on ROS levels in nematodes.

The MDA content measurement (Fig. 4B) revealed that compared with the control group, the low-concentration of 0.05 mg/ml HGQ treatment significantly reduced the MDA content in paraquat-stressed N2 nematodes. It suggested that, HGQ treatment could effectively reduce the *in vivo* oxidative stress level and exerts certain anti-oxidation capacity in *C. elegans* at low concentrations. Thse results were consistent with the anti-aging activity of HGQ at low concentration treatment.

Antioxidant capacity in C. elegans

In order to further verify the antioxidant capacity of HGQ, we simultaneously determined the nematodes survival rate under 60 mM paraquat oxidative stress. The results shown in Figure 5 indicate that the survival time of N2 nematodes exposed to paraquat was obviously extended after the treatment of HGQ at the concentration of 0.05, 0.1 and 0.3 mg/ml, especially the 0.05 mg/ml treatment exhibiting stable significant difference ($p < 0.05$) compared with control among 3 independent experiments, while no influence observed at the high concentration of 0.5 mg/ml. These results confirmed that the HGQ was capable of reducing oxidative damages in *C. elegans*, especially at the low concentration treatment.

Activity of antioxidant enzymes in C. elegans

Antioxidant enzymes are key substances in the cellular antioxidant system. Enhancing the activity of antioxidant enzymes helps to clear excess ROS and alleviate oxidative stress (Finkel and Holbrook, 2000). In order to explore the mechanism of HGQ's antioxidant activity, we detected the influence of HGQ on the activity of SOD, CAT and GPx in *C. elegans*. Compared with the control group, HGQ treatment at concentrations of 0.05 and 0.3 mg/ml could significantly enhance the SOD activity (Fig. 6A), however the effects on the enzyme activities of CAT and GPX were non-significant (Fig. 6B, C). These results indicate that the free radical scavenging activity of HGQ should be mainly related to the increase of SOD activity in nematodes.

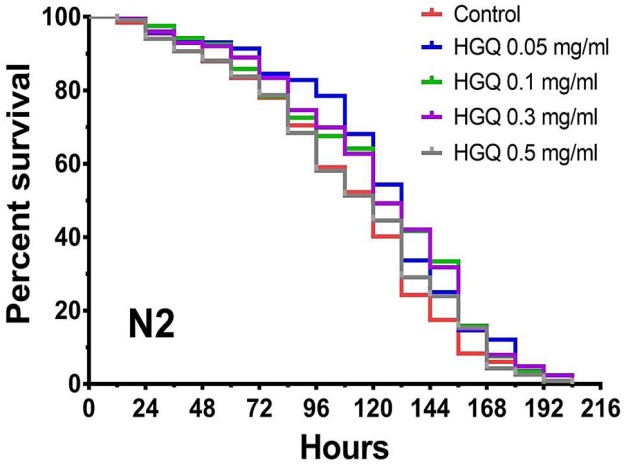

Fig. 5. Influence of HGQ on the survival rate of paraquat-stressed N2 nematodes. Synchronized nematodes were treated with or without HGQ at indicated concentrations at 20°C for 24 h prior to exposure to 60mM paraquat, and the live/dead nematodes (~100 for each treatment) were scored every 12 h until all dead. The representative Kaplan-Meier survival curves are shown from three independent experiments, and data are compared for significance by the log-rank test.

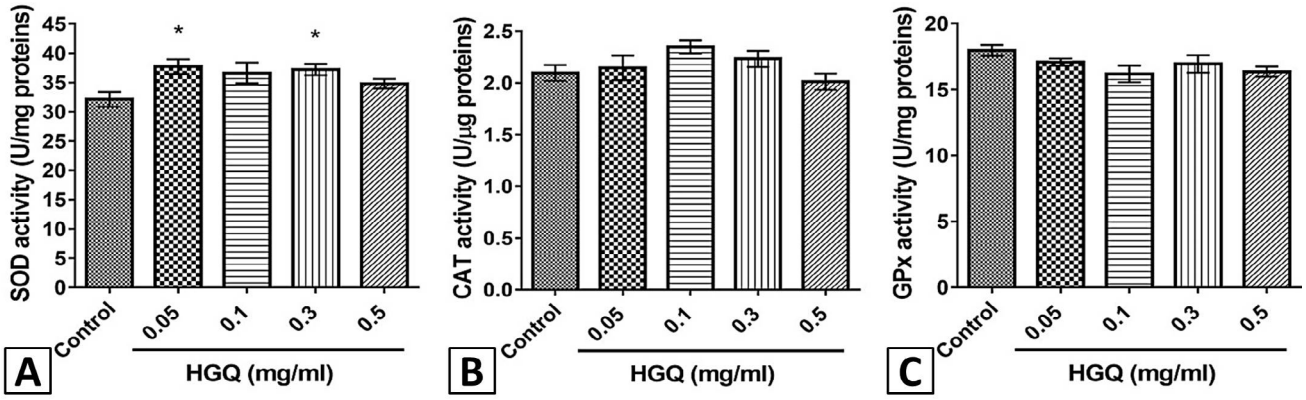

Fig. 6. Influence of HGQ on the antioxidant enzyme activity in N2 nematodes. Synchronized nematodes were prepared as in Figure 4 until lysis. The SOD (A), CAT (B) and GPx (C) activity were determined, respectively with Total Superoxide Dismutase Assay Kit, Catalase Assay Kit and Total Glutathione Peroxidase Assay Kit. The results were normalized by protein content. Data are shown as mean ± SEM of three independent experiments, and statistical significance is determined by one-way ANOVA followed by Tukey's post hoc test. *p<0.05.

DISCUSSION

Due to the rich polyphenolic active substances, *Lycium ruthenicum* Murr. has been extracted and dried to make *Lycium ruthenicum* Murr. granules (HGQ) in tea to be exploited as an anti-aging agent. However, studies were still lacking to provide systematic scientific verification on its biological effects. The model organism *C. elegans* has the advantages at overall animal level such as short life cycle, intact organ system, and simple and economical operation. Thus it has been widely used in the fields of biomedicine and health food in recent years. For example, Wang *et al*. (2019) and Zhang *et al*. (2019) have used the model organism *C. elegans* to study the antioxidant and anti-aging activities of abalone protein hydrolysate and rose tea polyphenol. Therefore, we studied the activity effect and the mechanism of HGQ using the model *C. elegans*.

Our results revealed that HGQ could significantly extend the median lifespan and reduce the accumulation of lipofuscin in the wild-type *C. elegans* N2 at a low concentration of 0.05 mg/ml treatment, showing a good anti-aging activity. The pharyngeal pumping rate assay and the lifespan examination of *sir-2.1* mutant nematode showed that HGQ not only could not affect the food and energy intake of nematodes, but also exhibited an irrelevance between the life-extension and *sir-2.1* gene, indicating the anti-aging effect of HGQ is independent of calorie restriction pathway. This result is consistent with Zhang *et al*. (2018) finding, which have revealed that polysaccharides from *Dendrobium* could effectively delay the aging and increase the anti-stress ability of *C. elegans* N2, but it also did not affect the nematode's pharyngeal pumping rate and energy intake.

Further studies have found that HGQ can effectively reduce the ROS level and MDA content *in vivo*, increase the survival rate and enhance the activity of SOD in *C. elegans* N2 under paraquat oxidative stress, showing a good antioxidant capacity. Interestingly, this activity also showed a drug concentration-dependence with better effect at 0.05 mg/ml treatment, similarly with the HGQ's anti-aging activity, which suggesting a considerable contribution of the antioxidant to the anti-aging effect of HGQ. The researches of Chen *et al*. (2016) and Wang *et al*. (2014) have also shown the similar results that both Hongshan flower Chinese cabbage extract and *Angelica* protolysate can delay the aging of *C. elegans* N2 through its good antioxidant activity *in vivo* and *in vitro*. So, our results suggest that HGQ can effectively delay the senescence of *C. elegans*, and this effect may be related with its significant antioxidant activity.

Previous findings (Song *et al*., 2014; Chen *et al*.,2018; Liu *et al*., 2013) have shown that the crude methanol extract, polyphenols and polysaccharide components from the *Lycium ruthenicum* Murr. exhibit free radical scavenging and antioxidant activities. Therefore, we reasoned that the anti-aging activity of HGQ should be related to its rich antioxidant active components. However, a study conducted by Shang *et al*. (2017) have revealed that there were great differences in the chemical composition of *Lycium ruthenicum* Murr. from different producing areas. For example, the free radical scavenging ability of *Lycium ruthenicum* Murr. from Alatanaobao, Alxa Right Banner, Inner Mongolia is the strongest, while the reducing ability of *Lycium ruthenicum* Murr. from Soumber, Mongolia is the strongest. Therefore, further studies will combine the raw materials of different producing areas to analyze the active function, thus providing a more optimized and sufficient theoretical basis for the deep development of *Lycium ruthenicum* Murr. granules.

CONCLUSIONS

In summary, data presented in this study indicate that *Lycium ruthenicum* Murr. granules can effectively delay the aging of *C. elegans*, and the anti-aging activity may be related to its significant antioxidant effect, but not related to the calorie restriction pathway. These finding provide foundational benefits of the HGQ in human healthcare and highlight the need of future studies in this domain.

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Statement of conflict of interest

We declare no conflicts of interest in this study.

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