

## Anti-aging activities of black highland barley bran, chrysoeriol, and its glucuronide derivative on *Caenorhabditis elegans*

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### Abstract

The anti-aging activities and underlying mechanisms of black highland barley bran extract (BHBBE) and its major phenolic components, chrysoeriol (CHR) and chrysoeriol-7-O-β-D-glucuronide (CHRG), were investigated in *Caenorhabditis elegans* (*C. elegans*). The effects of BHBBE, CHR, and CHRG at different concentrations were evaluated on lifespan, reproduction, locomotion, stress resistance (heat and ultraviolet [UV] stress), and antioxidant activities (such as superoxide dismutase and catalase activities as well as reactive oxygen species (ROS) and malondialdehyde levels). Furthermore, the expression of longevity-associated genes was examined. Central gene, *daf-16*, in the anti-aging process of BHBBE, CHR, and CHRG was verified by comparing ROS levels in wild-type N2 *C. elegans* with *daf-16(mu86)* mutant strains. Nuclear translocation as a function of DAF-16 protein was assessed. BHBBE, CHR, and CHRG significantly extended *C. elegans*' lifespan, improved locomotion, and enhanced stress resistance, without adversely affecting reproduction. Their anti-aging effects were primarily mediated by DAF-16 nuclear translocation via insulin/insulin-like growth factor 1 signaling (IIS). Furthermore, this IIS signaling pathway was associated with antioxidant capacity and stress resistance. Therefore, BHBBE, a by-product of grain processing, provides a sustainable, safe, and efficient strategy for anti-aging applications.

**Keywords:** black highland barley bran; *Caenorhabditis elegans*; longevity; IIS/DAF-16 pathway; antioxidant

### Introduction

Highland barley (*Hordeum vulgare* L. var. nudum hook. f), a variant of the grass family, is commonly

referred to as naked barley because of its hull-less grains. This important cereal crop is primarily produced in the Qinghai-Tibet Plateau and in other Asian regions, such as China, Japan, Nepal, and India

(Meints *et al.*, 2021). In China, highland barley serves as a staple food for residents in high-altitude areas and is traditionally prepared as Tsampa, a roasted barley flour dish, also known as *Zanba* in Chinese (Obadi *et al.*, 2021). Highland barley, as a nutrient-rich cereal, is widely utilized in brewing, baking, and functional foods (Geng *et al.*, 2022; Lukinac and Jukić, 2022). It has demonstrated significant antioxidant properties, even stronger than common crops, because of its richness in phenolic compounds (Obadi *et al.*, 2021). Among black, white, and blue barley cultivars, black highland barley exhibited the highest polyphenol levels (Yang *et al.*, 2023). This variety was characterized by its black husk (Zhang *et al.*, 2023). Notably, barley bran contains higher polyphenol levels, compared to peeled barley, with black highland barley bran (BHBB) recording a total phenol content of 237.83 mg/g (Blandino *et al.*, 2015; Deng *et al.*, 2024).

Black highland barley bran extract (BHBBE) and its two main components possess robust antioxidant capacity (Demirtas *et al.*, 2013; Deng *et al.*, 2021; Xiang *et al.*, 2021). Polyphenols, as antioxidants, may mitigate aging by eliminating free radicals and modulating key genes in oxidative stress-related pathways. Moreover, these natural polyphenols have shown significant potential for market application (Ding *et al.*, 2017; Okoro *et al.*, 2021). Consequently, the anti-aging effects of BHBBE and its essential polyphenols, chrysoeriol-7-O- $\beta$ -D-glucuronide (CHRG) and chrysoeriol (CHR), are investigated in this study.

*Caenorhabditis elegans* (*C. elegans*) served as a frequently-used anti-aging model because of its short lifespan, and easily observed and conserved longevity pathways (Herndon *et al.*, 2002). Lifespan extension in *C. elegans* led to various physiological changes, including enhanced resistance to heat and ultraviolet (UV) stress and reduced levels of reactive oxygen species (ROS) (Wang *et al.*, 2020).

Genetic mechanisms associated with anti-aging in *C. elegans* are the modulation of insulin/insulin-like growth factor 1 (IGF-1) signaling pathway (IIS) and p38 mitogen-activated protein kinase (MAPK) pathway (Zhang *et al.*, 2020). Extensive research has highlighted the pivotal role of these two pathways in regulating aging and disease processes (How *et al.*, 2021; Park *et al.*, 2021; Xiao *et al.*, 2024; Ziv and Hu, 2011). Key genes, such as *age-1* and *daf-16*, along with major gene regulators, such as *sir-2.1*, *sek-1* and *skn-1*, are involved in these two pathways. Downstream genes, such as *sod-3* and *ctl-1*, influenced the antioxidative capacity of *C. elegans* by regulating activities of enzymes, such as superoxide dismutase (SOD) and catalase (CAT).

Therefore, the anti-aging effects of BHBBE, CHR, and CHRG on *C. elegans* were investigated, including its lifespan, reproductive capacity, stress tolerance (heat and UV resistance), and antioxidant ability. The underlying mechanisms were further explored by examining the nuclear translocation of DAF-16 and analyzing the expression of key aging-related genes, such as *sir-2.1*, *age-1*, *daf-16*, *sod-3*, *ctl-1*, *sek-1*, and *skn-1*. Additionally, the critical role of the DAF-16 pathway was specifically verified by monitoring ROS levels in *daf-16* (*mu86*) mutant strains under hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) stress. This investigation provides important insights into the anti-aging activity and antioxidant mechanism of BHBBE, which may promote its future application in functional foods and nutritional interventions.

## Materials and Methods

### Materials

Black highland barley bran was provided by Tibet Chun Guang Food Co. Ltd. (Tibet, China). The *C. elegans* wild-type strain N2, TJ356 strain zIs356 (*daf-16p::daf-16a/b::GFP* + *rol-6[su1006]*), and *daf-16* (*mu86*) mutant were obtained from the Caenorhabditis Genetics Center (CGC) (University of Minnesota, Minneapolis, MN, USA). The bacterial food source, *Escherichia coli* OP50 (*E. coli* OP50), was also procured from the CGC. Assay kits for SOD (Catalog No. A001-3-2), CAT (Catalog No. A007-1-1), and malondialdehyde (MDA; Catalog No. A003-1-2) were purchased from the Nanjing Institute of Biological Engineering (Nanjing, China). Fluorescent probe 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA; Catalog No. D6883) for ROS detection was obtained from Sigma-Aldrich (St. Louis, MO, US). Additionally, a bicinchoninic acid (BCA) protein assay kit (Catalog No. A045-4-2) for sample normalization was sourced from the Nanjing Jiancheng Bioengineering Institute. All other chemicals and reagents, including AB-8 macroporous resin, were supplied by Chengdu Chron Chemicals Co. Ltd. (Chengdu, China).

### Preparation of extracts

Black highland barley bran (2,500 g) was extracted three times with 10 L of 80% ethanol at 70°C. The combined filtrates were concentrated at 50°C to yield BHBBE. The resulting BHBBE was dissolved in an equal volume of distilled water and loaded onto an AB-8 macroporous resin column. The polyphenols CHR and CHRG were separated from BHBBE by step-gradient elution with 0%, 20%, 40%, and 93% (v/v) aqueous ethanol. Fractions were monitored by high-performance liquid chromatography (HPLC), and those enriched in CHR or

CHRG were combined and concentrated. Subsequently, 110 mg of CHR and 610 mg of CHRG were yielded after freeze-drying. The CHR and CHRG was identified using an Agilent 1290 Infinity III HPLC system (Agilent Technologies, USA). The purification was performed by preparative HPLC using a reversed-phase C18 column (XB-C18, 10  $\mu$ m, 10 nm) maintained at room temperature. The separation was achieved under isocratic conditions with a mobile phase comprising acetonitrile and 0.1% formic acid (35:65 v/v) delivered at a flow rate of 1.0 mL/min. Detection was carried out with a UV detector set at 350 nm. For each injection, a volume of 0.4 mL was loaded. The structures of CHR and CHRG were characterized by nuclear magnetic resonance (NMR) spectroscopy. Samples were dissolved in deuterated methanol, and their  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker Avance Neo 400 MHz NMR spectrometer at 25°C (Bruker BioSpin, Billerica, MA, USA).

### Maintenance and synchronization of *C. elegans*' strains

*C. elegans* was cultured at 20°C on Nematode Growth Medium (NGM) fed with *E. coli* OP50 that was heat-inactivated (65°C, 30 min). The gravid nematodes were collected after treatment with M9 buffer ( $\text{KH}_2\text{PO}_4$  +  $\text{Na}_2\text{HPO}_4$  + NaCl). Eggs were released by vigorously shaking gravid nematodes in a solution containing bleach (1 mL 5% NaClO + 0.5-mL 0.5-M NaOH) for 10 min. After centrifugation to remove bleach, eggs were washed for three times with M9, and incubated overnight on NGM plates seeded with *E. coli* OP50. Synchronized L4 nematodes were collected after 48 h of incubation.

According to our previous findings (Wang *et al.*, 2023), BHBBE, CHR, and CHRG exhibited optimal *in vitro* anti-oxidant activity at concentrations ranging from 200 to 1,000  $\mu\text{g}/\text{mL}$ ; therefore, concentrations of 200, 500, and 1,000  $\mu\text{g}/\text{mL}$  were selected for subsequent experiments.

### Lifespan assay

*C. elegans* was treated with varying concentrations of BHBBE, CHR, and CHRG to evaluate their impact on lifespan. Synchronized L4 nematodes were transferred to NGM plates supplemented with *E. coli* OP50, 50- $\mu\text{M}$  5-Fluorouracil 2'-deoxyribose (FUdR), and corresponding concentrations (200, 500, and 1,000  $\mu\text{g}/\text{mL}$ ) of BHBBE, CHR, and CHRG at 20°C, with 30 nematode specimens per plate. FUdR could prevent the reproduction of nematodes without interfering with their development and aging after maturation. Daily observations were made to record the number of deaths until the death of last nematode, and the experiment was repeated thrice. *C. elegans* was considered dead if they did not move or

respond within 10 s on being lightly touched with a platinum wire. Nematodes that escaped from the culture dish or died during the experiment were excluded from data analysis.

### Fecundity assay

In light of the positive relationship between lifespan extension and reproductive capacity in aging research (Son *et al.*, 2011), the effects of BHBBE and its major components were investigated on the fecundity of *C. elegans*. The L4 stage nematodes were treated using the same method as done in the lifespan assay. Nematodes were divided into four groups, with each group containing three gravid adults. These groups were cultured at 20°C on NGM plates seeded with *E. coli* OP50. The treatment groups were supplemented with BHBBE, CHR, and CHRG at concentrations of 200, 500 and 1,000  $\mu\text{g}/\text{mL}$ , while the control group received no supplementation. Subsequently, they were transferred to fresh NGM plates daily until egg-laying was completed. All egg-laying plates were incubated for 48 h to promote hatching, and the number of recorded larvae was considered equivalent to the number of eggs laid.

### Movement assay

*C. elegans*' motility was assessed using the body-swing count test. L4 stage nematodes were treated using the same method as done in the lifespan assay at 20°C. This time point was considered the beginning of the experiment. Over the subsequent 7 days, 10 nematodes were randomly selected daily. Using a platinum wire, nematodes were gently stimulated, and the number of swings performed by each nematode in 1 min was recorded. A swing was counted when *C. elegans* moved from one side to the other, observed either in the head or tail region.

### Stress resistance

*C. elegans* treated with BHBBE, CHR, and CHRG was subjected to acute heat resistance tests. The L4 stage nematodes were treated as described in the lifespan assay and cultured at 20°C for 5 days. Each plate contained 30 nematodes. They were transferred at 37°C on the sixth day until all were dead. Nematode deaths were recorded hourly based on the criteria outlined in the lifespan assay.

Similar to thermotolerance assay, UV stress tests were conducted on 30 randomly selected *C. elegans* from each group. After 5 days of culture at 20°C, the nematodes

were exposed to UV radiation for 2 h on the sixth day. Daily death counts were recorded, and the survival rate was calculated.

### Antioxidant enzyme activity and oxidative stress markers

The effects of BHBBE, CHR, and CHRG on the antioxidant activity of *C. elegans* were evaluated by measuring antioxidant enzyme activities and oxidative damage markers. Synchronized L4-stage nematodes were treated according to the methodology described in the lifespan assay and cultured at 20°C for 5 days. Approximately 1,000 nematodes per group were collected, washed thrice with M9 buffer, and subjected to three rapid freeze-thaw cycles in liquid nitrogen. The samples were then homogenized and centrifuged (12,000 ×g, 4°C, 2 min) to obtain supernatant.

The supernatant was then analyzed using commercial kits to quantify SOD and CAT activities as well as MDA content, and protein concentrations were determined with the BCA method. For ROS detection, 50 µL of supernatant was added to an equal volume of DCFH-DA, and the mixture was incubated for 2 h at 20°C in the dark. Fluorescence was then measured at excitation and emission wavelengths of 485 nm and 538 nm, respectively. ROS levels were expressed as relative fluorescence units (% control).

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

*C. elegans* was cultured on NGM medium supplemented with 200 µg/mL of BHBBE, CHR, and CHRG to investigate their potential lifespan-extending effects and epigenetic regulation. Minimum 2,000 nematodes were cultured per plate. They were then washed thrice with

M9 buffer, and messenger RNA (mRNA) was extracted using the Animal Total RNA Isolation kit (Foregene Co., Ltd., Chengdu, China). The centrifuge protocol was repeated thrice. The concentration and purity of mRNA were assessed using an ultra-micro spectrophotometer. Reverse transcription was conducted using the 5X All-In-One RT MasterMix (with AccuRT Genomic DNA Removal kit) (ABM). RT-qPCR was conducted using EvaGreen Express 2X qPCR MasterMix-No Dye (ABM) following the manufacturer's instructions. The expression of the *daf-16* gene and its downstream target genes was quantified using the *act-1* gene as an internal reference. Relative gene expression levels were calculated using the delta delta crossing thresholds ( $2^{-\Delta\Delta CT}$ ) method. Primer sequences are provided in Table 1.

Intracellular ROS levels were measured in nematodes under oxidative stress conditions in order to investigate the correlation of the *daf-16* gene with the antioxidative effects of BHBBE, CHR, and CHRG. The wild-type N2 and the *daf-16* (*mu86*) mutant strains were used in this experiment. The methods for nematode synchronization and treatment with BHBBE, CHR, and CHRG were consistent with those described in the lifespan assay. After 5 days of treatment, nematodes from each group were collected and exposed to 10-µM H<sub>2</sub>O<sub>2</sub> for 2 h to induce acute oxidative stress. The ROS levels were then measured using the same method as described previously.

### DAF-16 nuclear localization assay

Visualization of DAF-16 subcellular localization using a DAF-16::GFP reporter strain was employed to examine its potential role in the anti-aging mechanism of BHBBE, CHR, and CHRG. *C. elegans* was treated and cultured as described in the lifespan assay. After 5 days of treatment at 20°C, nematodes were transferred onto a 2% agarose pad on a microscope slide and paralyzed with 10-mM

Table 1. Sequences of gene primers.

Gene	Gene ID	Forward Primer 5'–3'	Reverse Primer 5'–3'
<i>act-1</i> (reference gene)	181097	GTCAGTGGTGACCTGACCT	AGGGGTCTACATGGCAACTG
<i>sek-1</i>	181043	ATGCTCGGTGAGTATTGG	TCATTGATAAACCGAGCC
<i>sod-3</i>	181748	GGCTAAGGATGGTGGAGAAC	ACAGGTGGCGATCTTCAAG
<i>ctl-1</i>	180837	CGGATACCGTACTCGTGATGAT	CCAAACAGCCACCCAAATCA
<i>skn-1</i>	177343	CACGCCGTGCGAAGTA	ATGCTCGGTGAGTATTGG
<i>age-1</i>	174762	CCTGAACCGACTGCCAATC	GTGCTTGACGAGATATGTGTATTG
<i>daf-16</i>	172981	TCAAGCCAATGCCACTACC	TGGAAGAGCCGATGAAGAAG
<i>sir-2.1</i>	177924	ACTGAGATGCTCCATGACAATAAG	GCAAGACGAACCACACGAAC

Note: ROS levels in the *daf-16* mutant of *C. elegans* during oxidative stress.

sodium azide ( $\text{NaN}_3$ ). The localization of DAF-16::GFP was classified as nuclear if  $\geq 50\%$  of intestine cells had positive nuclear GFP punctae, intermediate if more than 10%,  $< 50\%$  of intestine cells had positive nuclear GFP punctae, or cytosol if  $\leq 10\%$  of intestine cells had positive nuclear GFP punctae. For each group, at least 50 nematodes were blindly scored for DAF-16::GFP localization and categorized as nuclear, cytosolic, or intermediate. Data are presented as the percentage of animals exhibiting nuclear translocation (Ke *et al.*, 2020).

### Statistical analysis

Each experiment comprised three biological replicates. The results were reported as mean  $\pm$  standard deviation (SD), with the exception of the lifespan and DAF-16 nuclear translocation assays. Lifespan curves were plotted using the Origin 2021 software (Origin Lab Corporation, Northampton, MA, US). One-way ANOVA and Tukey's test were used to assess the significance of differences in experimental data. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, US), with the significance level set at  $P < 0.05$ .

## Results

### Lifespan extension by BHBBE, CHR, and CHRГ

Chrysoeriol and CHRГ were successfully isolated from BHBBE, and their structures were identified. The isolation was monitored by preparative HPLC (Supplementary Figure S1), and the structural identification was confirmed by NMR spectroscopy (Supplementary Figure S2). Their chemical structures are shown in Figure 1.

The anti-aging effects of BHBBE, CHR, and CHRГ on *C. elegans* were assessed by recording the lifespan (Figures 2(A–C)). Compared to the control group, the survival curves of nematodes treated with varying

concentrations of BHBBE, CHR, and CHRГ shifted to the right, indicating that BHBBE, CHR, and CHRГ remarkably extended the survival rate of wild-type nematodes in a dose-dependent manner ( $P < 0.05$ ). Notably, a significant extension was already achieved at a concentration of 200  $\mu\text{g}/\text{mL}$  for all three compounds ( $P < 0.05$ ).

Mean and median lifespans reflect the overall trend in lifespan, while the median lifespan is not affected by extreme values. Maximum lifespan reveals potential biological limits. Therefore, in order to comprehensively evaluate the effects of BHBBE, CHR, and CHRГ on the lifespan of *C. elegans*, mean, median, and maximum lifespans of nematodes were assessed. In Figures 2(D–F), the mean lifespan of nematodes treated with 500 and 1,000  $\mu\text{g}/\text{mL}$  of BHBBE, CHR, and CHRГ was significantly higher than that of the control group ( $P < 0.01$ ). Remarkably, only CHR at the lowest concentration (200  $\mu\text{g}/\text{mL}$ ) remarkably extended the mean lifespan ( $P < 0.01$ ). Both 500 and 1,000- $\mu\text{g}/\text{mL}$  BHBBE and CHRГ efficiently extended the median lifespan ( $P < 0.05$ ). All concentrations of BHBBE, CHR, and CHRГ significantly extended the maximum lifespan ( $P < 0.01$ ).

### Motility and reproductive capacity

None of the tested compounds (i.e., BHBBE, CHR, and CHRГ) showed statistically significant differences in egg-laying number, compared to the control group ( $P > 0.05$ ), illustrating that BHBBE, CHR, and CHRГ did not affect the reproduction of *C. elegans* (Figure 2G).

The effects of BHBBE, CHR, and CHRГ on the motility of *C. elegans* were assessed by counting body bend frequencies from day 1 to day 7 (Figure 2H). BHBBE and its two major polyphenols improved the motility of nematodes from day 5. BHBBE at 500  $\mu\text{g}/\text{mL}$  and 1,000  $\mu\text{g}/\text{mL}$  efficiently enhanced motility on day 6 ( $P < 0.01$ ). Both CHR and CHRГ at 500 and 1,000  $\mu\text{g}/\text{mL}$  significantly improved motility on day 5 and day 7 ( $P < 0.01$ ).

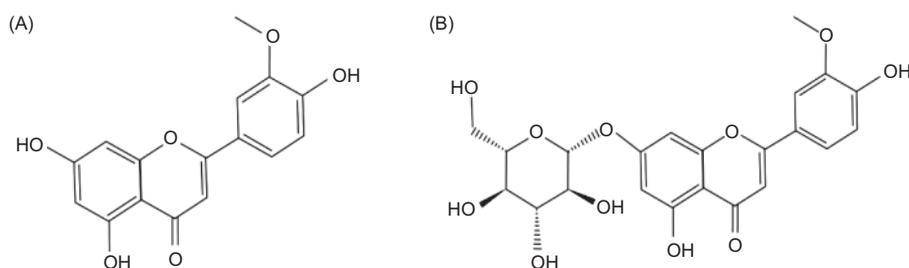
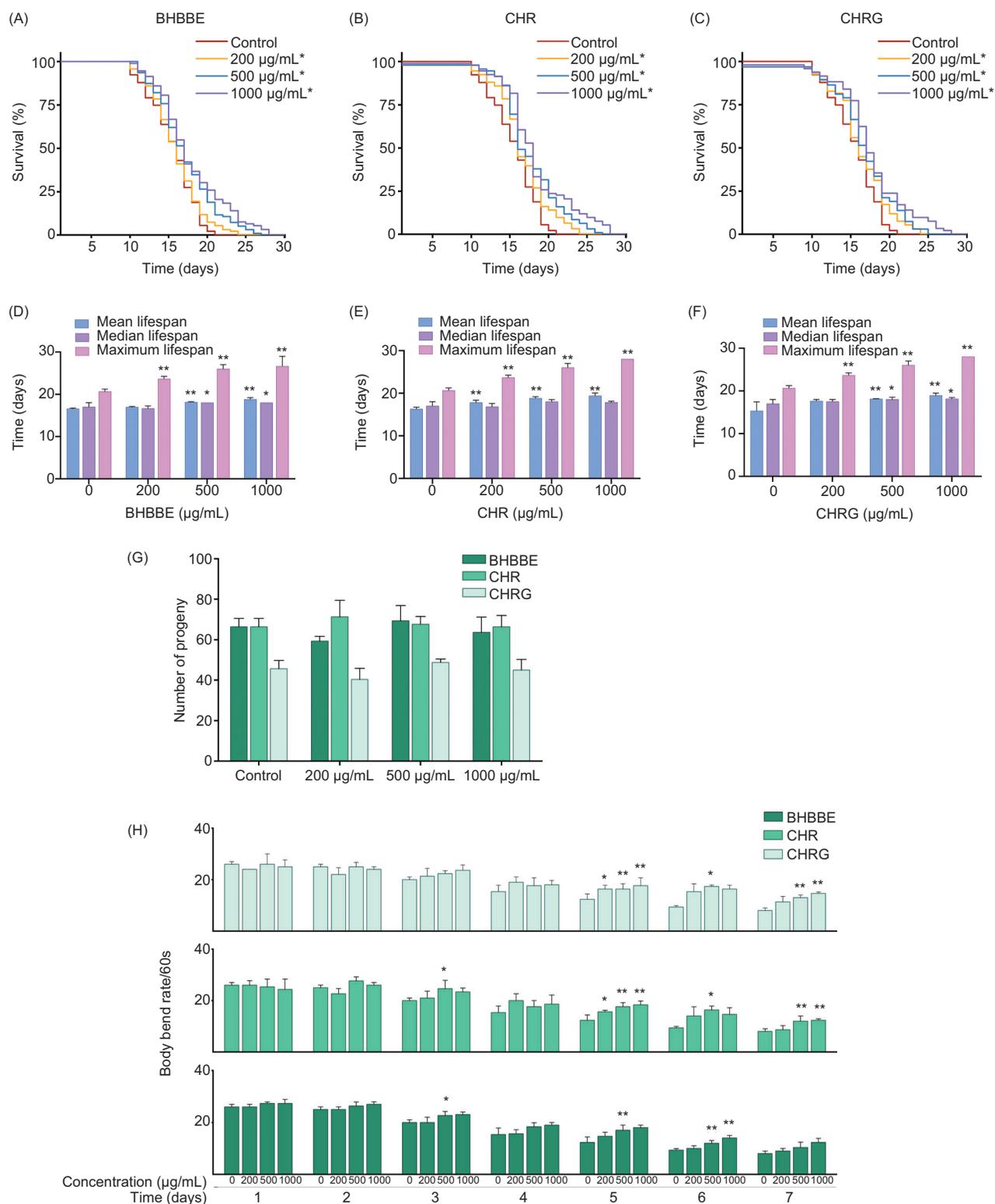


Figure 1. (A) Structure of CHR; (B) structure of CHRГ.



**Figure 2.** Effects of BHBBE, CHR, and CHRГ on lifespan, locomotion, and reproduction of *C. elegans*. (A–C) Survival curves of *C. elegans* treated with 200, 500 and 1,000  $\mu\text{g/mL}$  BHBBE, CHR, and CHRГ, respectively, exhibiting trends in lifespan extension compared to the control. (D–F) Impact of BHBBE, CHR, and CHRГ on the mean, median, and maximum lifespan of *C. elegans*. (G) Number of offspring produced by *C. elegans* treated with BHBBE, CHR, and CHRГ. (H) Frequency of oscillations in *C. elegans* treated with BHBBE, CHR, and CHRГ. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control group.

### Enhancement of heat stress and UV irradiation resistance

The resistance of *C. elegans* to both heat and UV stress was significantly enhanced by treatment with BHBBE, CHR, and CHRG (Figure 3). A progressive increase in lifespan was observed with increasing concentration, and significant prolonging in the lifespan of stressed nematodes by BHBBE, CHR, and CHRG was observed from 200 µg/mL ( $P < 0.05$ ). Given that, 200 µg/mL of BHBBE and its two polyphenols were effective in prolonging of lifespan and improving stress resistance, this concentration was subsequently selected for mechanistic studies.

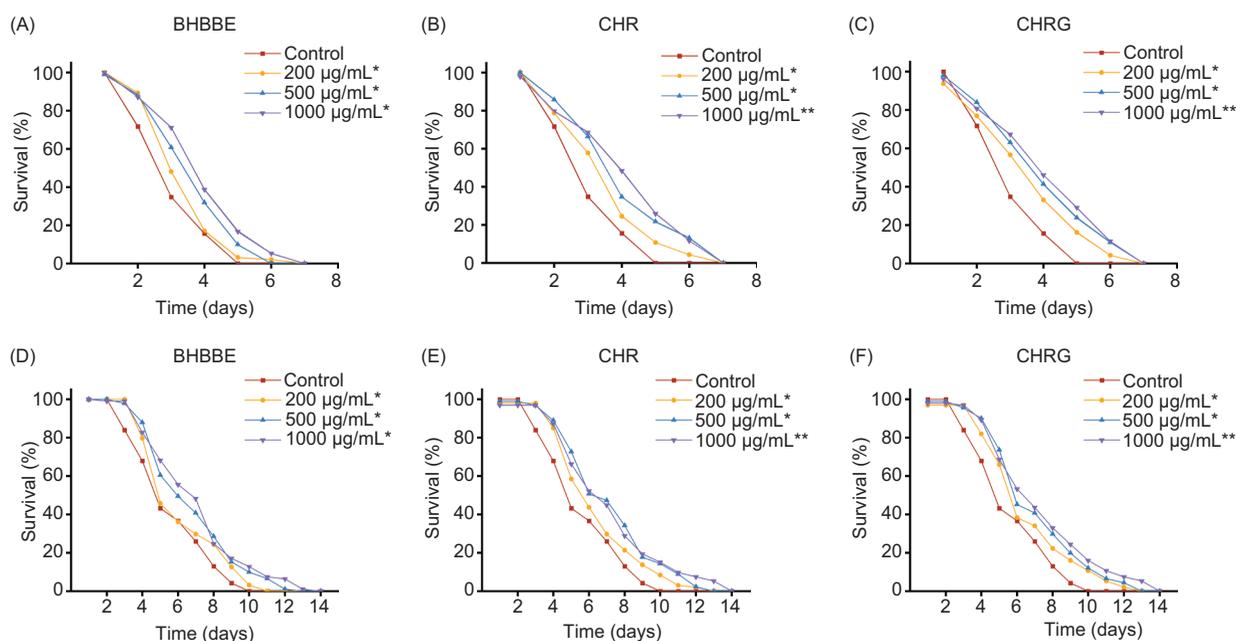
### Enhancement of antioxidant capacity

To explore potential biochemical basis for these phenotypic improvements, the basal activity of key antioxidant enzymes and the level of oxidative damage were measured in cultured nematodes. As shown in Figure 4A, treatment with BHBBE, CHR, and CHRG significantly increased SOD activity in *C. elegans* by 54.8%, 143.6%, and 159.8%, respectively ( $P < 0.05$ ), compared to the untreated control. Similarly, CAT activity was also enhanced (Figure 4B) with increases of 17.9% (ns), 108.5% ( $P < 0.05$ ), and 159.5% ( $P < 0.05$ ). In addition, ROS levels were significantly reduced by BHBBE, CHR, and CHRG, showing the respective decrease of 20.6% ( $P < 0.05$ ), 30.2% ( $P < 0.01$ ), and 34.8% ( $P < 0.01$ ) (Figure 4C).

Regarding MDA content, CHR and CHRG significantly reduced it by 69.4% and 50.8%, respectively ( $P < 0.05$ ), while BHBBE did not cause a significant change in MDA (Figure 4D).

### Gene expression analysis in *C. elegans*

Expressions of the longevity-related genes in *C. elegans*, such as *daf-16*, *age-1*, *sod-3*, *ctl-1*, *sek-1*, *skn-1*, and *sir-2.1*, were adjusted by BHBBE, CHR, and CHRG (Figure 5). Specifically, compared to the control group, expression of the *sod-3* gene was significantly increased by 1.17-fold, 1.15-fold, and 1.29-fold following respective BHBBE, CHR and CHRG treatments ( $P < 0.05$ ). Expression of the *sir-2.1* gene was remarkably increased by 1.11-fold and 1.10-fold by respective BHBBE and CHRG treatments ( $P < 0.05$ ), while it was significantly decreased to 88.86% following CHR treatment ( $P < 0.05$ ). Expression of the *sek-1* gene was efficiently increased by 1.21-fold and 1.23-fold by respective BHBBE and CHRG treatments ( $P < 0.05$ ). Expression of the *daf-16* gene was remarkably increased by 1.30-fold and 1.22-fold by respective CHR and CHRG treatments ( $P < 0.05$ ). Besides, expression of the *age-1* gene was efficiently decreased to 88.00% and 89.00% ( $P < 0.05$ ) by respective BHBBE and CHRG treatments. Expression of the *ctl-1* gene remarkably increased by 1.14-fold by BHBBE treatment ( $P < 0.05$ ). No significant change was observed in the expression of the *skn-1* gene.



**Figure 3.** Survival rate of *C. elegans* treated with BHBBE, CHR, and CHRG under (A–C) heat stress, or (D–F) UV irradiation. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control group.

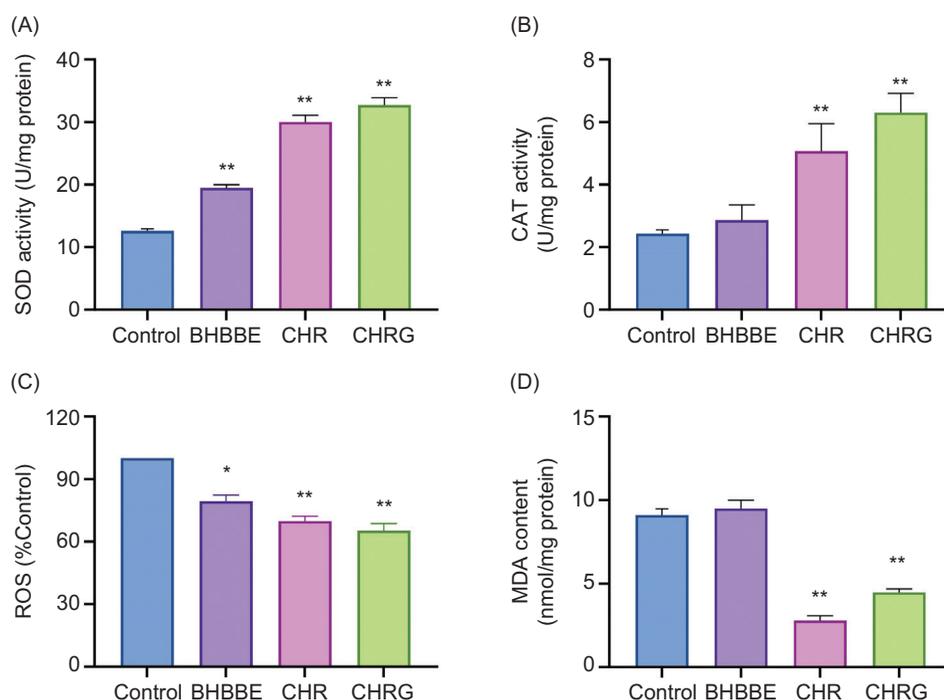


Figure 4. Antioxidant activity of N2 *C. elegans* treated with 200-µg/mL BHBBE, CHR, and CHR.G. (A) SOD activity. (B) CAT activity. (C) ROS level. (D) MDA content. \*P < 0.05; \*\*P < 0.01 vs. control group.

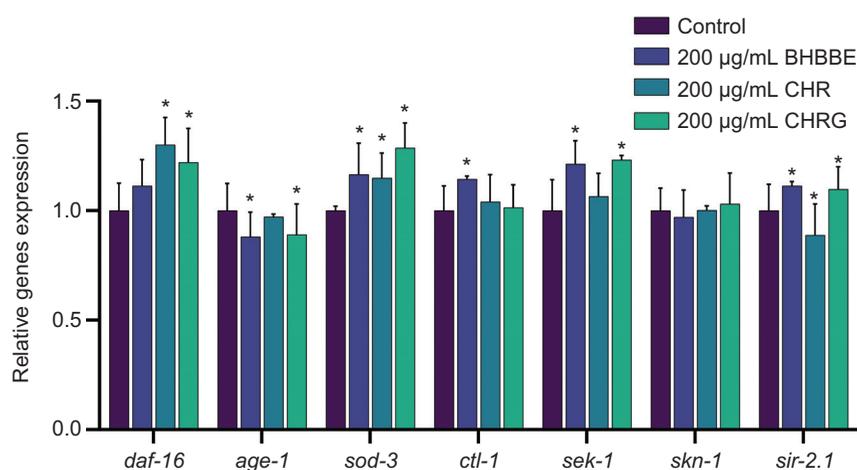


Figure 5. Effects of 200-µg/mL BHBBE, CHR, and CHR.G on the expression of longevity-related genes in *C. elegans*. \*P < 0.05; \*\*P < 0.01 vs. control group.

Overall, treatments with BHBBE, CHR, and CHR.G influenced the lifespan of *C. elegans* by modulating the expression of genes in aging pathways.

**ROS levels in *daf-16* mutant *C. elegans* under oxidative stress**  
 Role of the crucial gene *daf-16* was further verified by comparing N2 wild-type and *daf-16* (*mu86*) mutant *C. elegans* under H<sub>2</sub>O<sub>2</sub> stress (Figure 6). Results demonstrated that the antioxidative capacity of BHBBE,

CHR, and CHR.G was largely dependent on the *daf-16* gene. The efficacy of BHBBE, CHR, and CHR.G in attenuating oxidative stress was evident in N2 *C. elegans*, as shown by the respective reduction of 19.6%, 30.6%, and 33.1% in fluorescence intensity, compared to the control group (Figure 7A). In contrast, *daf-16* mutant nematodes exhibited no significant differences in fluorescence intensity between the treatment and control groups (Figure 7B). These results suggest that

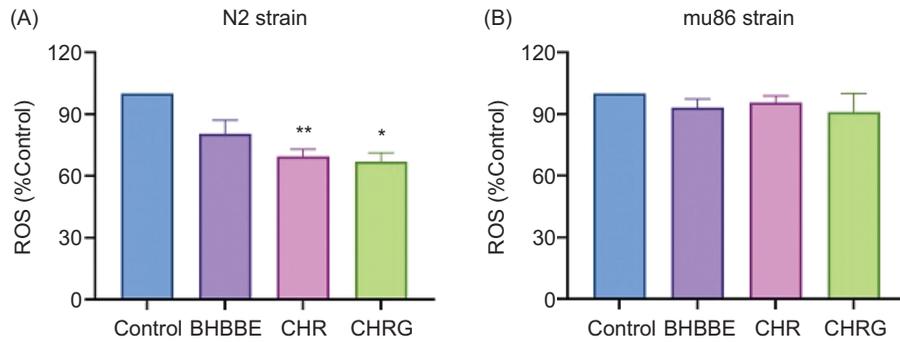


Figure 6. Effects of BHBBE, CHR, and CHR G on ROS levels in (A) wild-type N2, and (B) DAF-16 (mu86) mutant *C. elegans* under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. \**P* < 0.05; \*\**P* < 0.01 vs. control group.

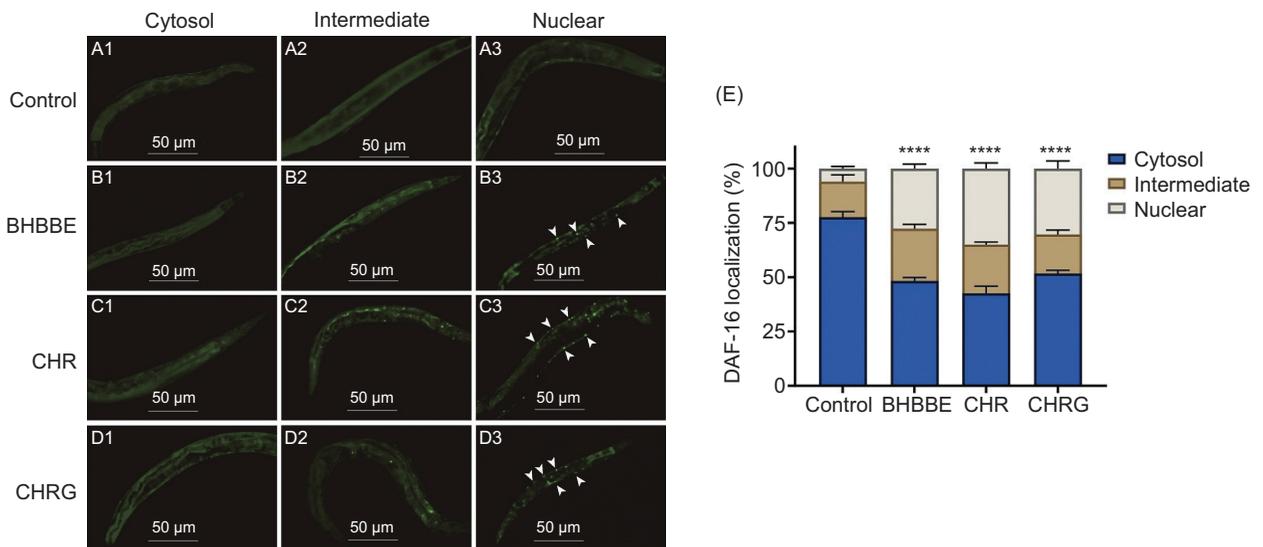


Figure 7. DAF-16 nuclear translocation in *C. elegans*. (A–D) DAF-16::GFP subcellular distribution (cytosolic, intermediate, and nuclear) in *C. elegans* treated with BHBBE, CHR, and CHR G. (E) Quantitative analysis of DAF-16 localization, presented as the percentage of *C. elegans* with DAF-16 predominantly localized to cytosol, intermediate, or nucleus. \*\*\*\**P* < 0.0001 (for nuclear) vs. control group.

the antioxidant effects of BHBBE, CHR, and CHR G appeared to be abolished in the absence of the functional *daf-16* gene.

### DAF-16 nuclear localization

The TJ356 strain contains a DAF-16::GFP fusion protein, which contributes to improving the detectability of DAF-16 protein. The effects of BHBBE, CHR, and CHR G on the localization of DAF-16 protein are shown in Figure 7. DAF-16::GFP was uniformly distributed throughout the cytoplasm in control *C. elegans* (Figure 7A). In contrast, treatment with 200-μg/mL BHBBE, CHR, and CHR G

induced its nuclear translocation, as evidenced by the formation of bright nuclear puncta (white arrows, Figures 7B–D). Quantification revealed that all three treatments significantly increased the proportion of animals exhibiting DAF-16 nuclear translocation, compared to the control (*P* < 0.0001; Figure 7E). Among them, CHR induced the most pronounced nuclear accumulation, followed by CHR G and BHBBE.

### Discussion

Highland barley, a distinct crop native to the plateau regions of China, possesses a range of functionalities,

including the treatment of chronic and metabolic ailments and the enhancement of antioxidant activity (Guo *et al.*, 2020; Li *et al.*, 2022). These nutritional and health advantages can be attributed to the richness of phenolic compounds in highland barley, with approximately 80% of total phenols concentrated in the bran and embryo (Zhu *et al.*, 2015). Thus, phenolic compounds in highland barley bran have a great potential for utilization (Zhang *et al.*, 2021). Dominant phenols, such as CHR and CHR<sub>G</sub>, were found in all 14 varieties of highland barley bran in our earlier research and demonstrated the remarkable antioxidant properties of CHR and CHR<sub>G</sub> (Deng *et al.*, 2021). The antioxidant property closely correlates with delaying aging (da Costa *et al.*, 2016). The anti-aging and antioxidant abilities were strongly related to the treatment of different chronic ailments, such as osteoporosis, neurodegeneration, heart disease, diabetes, and cancer (Higgins-Chen *et al.*, 2021; Rodrigues *et al.*, 2021). Consequently, the anti-aging impact of BHBBE and its dominant phenols, such as CHR and CHR<sub>G</sub>, was assessed on *C. elegans*, and their possible mechanisms were discussed.

The mean lifespan of *C. elegans* was extended by BHBBE, CHR, and CHR<sub>G</sub>, with CHR showing the strongest effect. Compared to CHR, O-glycosylation occurring at C7 position in CHR<sub>G</sub> may substantially alter its biological activity. Hydroxylation at C7 position impeded the interaction with peroxide radicals, resulting in a significant reduction in the antioxidant activity of phenolic compounds (Mishra *et al.*, 2003). Glycosylation was demonstrated to diminish the anticancer activity of CHR (Takasawa *et al.*, 2008). The negative effect potentially stemmed from the substitution of hydroxyl groups with bulky sugar moieties, which led to a reduction of active hydroxyl groups and an increase of steric hindrance. Thus, decline in the anti-aging activity of CHR<sub>G</sub> might be caused by its glycosylation. CHR, as an aglycone of CHR<sub>G</sub>, showed a stronger lifespan extension effect, compared to CHR<sub>G</sub>. The lowest lifespan extension effect was observed in BHBBE. The bran extracts contained 10.85% of CHR, 82.19% of CHR<sub>G</sub>, and other compounds, which may affect BHBBE's lifespan extension, compared to pure CHR and CHR<sub>G</sub>.

A close correlation was observed between reproduction and lifespan (Baxi *et al.*, 2017). Results demonstrated that the lifespan of *C. elegans* was extended by BHBBE, CHR, and CHR<sub>G</sub>, while *C. elegans*' progeny numbers were not affected remarkably. This effect of extending nematodes' lifespan without compromising reproductive health was akin to that observed with epigallocatechin-3-gallate (Song *et al.*, 2024). Additionally, polyphenols from other traditional medicinal plants showed the same effect. For instance, extracts from *Glochidion zeylanicum* leaves remarkably extended the

lifespan of nematodes without impacting their reproductive capacity (Duangjan *et al.*, 2019).

The aging of *C. elegans* was accompanied by a gradual decline in its motility, which reflected the health status of nematodes (Hsu *et al.*, 2009; Li *et al.*, 2019). The locomotion of nematodes was observed to slow down with aging. BHBBE, CHR, and CHR<sub>G</sub> enhanced *C. elegans*' locomotion, evidently after 5 days of treatment. Epigallocatechin-3-gallate increased the body bend frequency, head thrash ability, and pharyngeal pumping rate of nematodes (Brown *et al.*, 2006). Olive polyphenols also had a strong beneficial effect on the locomotion of *C. elegans* (Di Rosa *et al.*, 2020). Thus, polyphenols such as CHR, CHR<sub>G</sub>, and BHBBE that improved motility and anti-aging effect had a promising application in functional foods.

Besides, BHBBE and its two main polyphenols resisted the aging of *C. elegans* triggering from environmental stress. BHBBE, CHR, and CHR<sub>G</sub> could still extend the lifespan of nematodes after exposure to 37°C heat stress or UV irradiation for 2 h. Blueberry polyphenols also enhanced stress resistance and extended the lifespan of *C. elegans* (Wang *et al.*, 2018).

Oxidative activity played a crucial role in *C. elegans* aging and age-related diseases, such as neurodegenerative diseases, cardiovascular diseases, diabetes, and cancers (Maglioni and Ventura, 2016; Salmon *et al.*, 2010). The accumulation of ROS and its damage is the primary cause of aging according to free radical theory (Brintz, 2020). SOD and CAT, as key antioxidant enzymes, protected cells by eliminating ROS or other oxidative products (Jomova *et al.*, 2023). Excessive free radical induced lipid peroxidation, and thus produced oxidative products such as MDA. High SOD and CAT activity indicated strong free radical scavenging ability, while low MDA showed reduced free radicals and promoted antioxidant effect.

The *daf-16* gene may be the pivotal gene for the anti-aging and anti-oxidative effects of BHBBE and its two polyphenols. Because ROS levels were significantly reduced by two phenols in wild-type N2 nematodes under oxidative stress, no notable change was observed in the *daf-16* mutants. The *daf-16* gene is a crucial one in the IIS pathway. The IIS pathway was a highly conserved aging progress that controlled stress resistance and lifespan of *C. elegans* (Morley *et al.*, 2002). The *age-1* gene negatively regulated the *daf-16* gene expression, while *sir-2.1* as a member of Sirtuin family, positively regulated DAF-16 protein translocation (Kobayashi *et al.*, 2005; Zečić and Braeckman, 2020). Expressions of the *age-1* gene could be inhibited by up-regulating the *sir-2.1* gene by BHBBE, CHR, and CHR<sub>G</sub>, which show the possible anti-aging mechanism of IIS pathway. Improvement in

BHBBE, CHR, and CHRG in DAF-16 nuclear translocation was also observed. The DAF-16 transcription factor, an ortholog of human FOXO proteins in IIS pathway, was capable to activate the expression of the *sod-3* and *ctl-1* genes (Motta *et al.*, 2020; Sun *et al.*, 2017; Wu *et al.*, 2012). These activated genes up-modulated the activity of anti-oxidative enzymes SOD and CAT that decreased oxidative products such as ROS and MDA (Braeckman *et al.*, 2003; Yang *et al.*, 2021). BHBBE, CHR, and CHRG enhanced antioxidant enzyme activity (SOD and CAT), reduced ROS levels, and decreased MDA content. BHBBE, CHR, and CHRG also increased the expression of the *sod-3* gene, and BHBBE increased the expression of the *ctl-1* gene. Therefore, BHBBE, CHR, and CHRG up-regulated the activities of SOD and CAT by activating the *sod-3* and *ctl-1* genes, thereby bolstering the antioxidant defense system in *C. elegans*. Additionally, the expression of the *sod-3* gene enhanced SOD activity and improved muscle function (Damiano *et al.*, 2019). Therefore, BHBBE, CHR, and CHRG may enhance SOD enzyme activity and improve nematodes' locomotion by up-regulating the *sod-3* gene.

Results showed that BHBBE and CHRG increased the expression of the *sek-1* gene, which is an upstream gene of the p38 MAPK pathway in *C. elegans* and is closely associated with oxidative stress response (Inoue *et al.*, 2005). The transcription factor SKN-1 was related to enhance antioxidant capacity and promote longevity, and the *sek-1* gene may interact with IIS pathway through the *daf-16* gene (Tanaka-Hino *et al.*, 2002; van der Hoeven *et al.*, 2011).

Potential mechanism of BHBBE, CHR, and CHRG to prolong longevity is summarized in Figure 8. Thus, the *daf-16* gene was a crucial target through the anti-aging,

stress-resistant, and anti-oxidant pathways in *C. elegans*. Environmental stress, such as UV radiation, high-temperature, and oxidation, could reduce lifespan by phosphorylating DAF-16 protein (Davis, 2000; Oh *et al.*, 2005). BHBBE, CHR, and CHRG extended the lifespan of nematodes subjected to heat stress and UV irradiation, presumably through the phosphorylation of DAF-16 protein in IIS pathway. Among them, CHR was the most efficient to enhance the *daf-16* gene expression and to extend lifespan. BHBBE and CHRG reduced the *age-1* gene expression while increasing the *sir-2.1* gene expression. BHBBE, CHR, and CHRG also increased the expression of the *sod-3* and *ctl-1* genes, thereby enhancing SOD and CAT activity as well as anti-aging effect of *C. elegans*. Thus, the longevity and antioxidant effects of BHBBE and CHRG are mediated by the *age-1*, *sir-2.1*, and *sek-1* genes, which regulate the translocation of DAF-16 protein.

## Conclusions

Black highland barley bran extract and its primary phenolic compounds, CHR and CHRG, significantly extended the lifespan, enhanced locomotor capacity, and improved stress resistance in *C. elegans* without affecting fecundity. Furthermore, the anti-aging effects of BHBBE, CHR, and CHRG were associated with the promotion of nuclear translocation of DAF-16 in IIS pathway. The activation of DAF-16 was correlated with improved antioxidant and stress phenotypes, ultimately contributing to the extension of lifespan. CHRG was most effective in up-regulating the *daf-16* gene expression and extending lifespan of *C. elegans*. This research provides a mechanistic foundation for developing high-polyphenol cereals, such as rye, anthocyanin-rich wheat, and oats, into anti-aging functional foods.

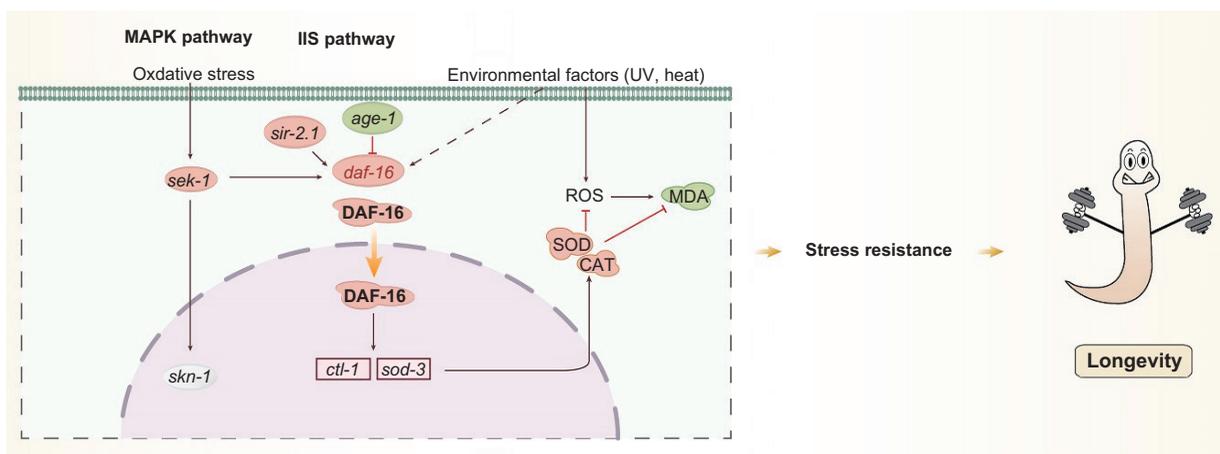


Figure 8. The possible mechanisms of BHBBE, CHR, and CHRG against aging of *C. elegans*.

## Data Availability

The authors confirmed that the data supporting the findings of this study are available within the article and its supplementary material.

## Mandatory Disclosure on Use of Artificial Intelligence

The authors declare that no AI-assisted tools were used in the preparation of this manuscript. All references have been manually verified for accuracy and relevance.

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## Author Contributions

Gang Liu: conceptualization, fund acquisition, and methodology; Shuyuan Fan: investigation and writing—original draft; Peng Zeng: visualization and methodology; Yunshuang He and Yuehan Shen: investigation; Changbin Lin: data curation and supervision; Chen Xia: fund acquisition and project administration; Peijun Li: formal analysis; and Xueli Li: fund acquisition and writing—review and editing.

## Conflict of Interest

The authors declared that they had no conflict of interest.

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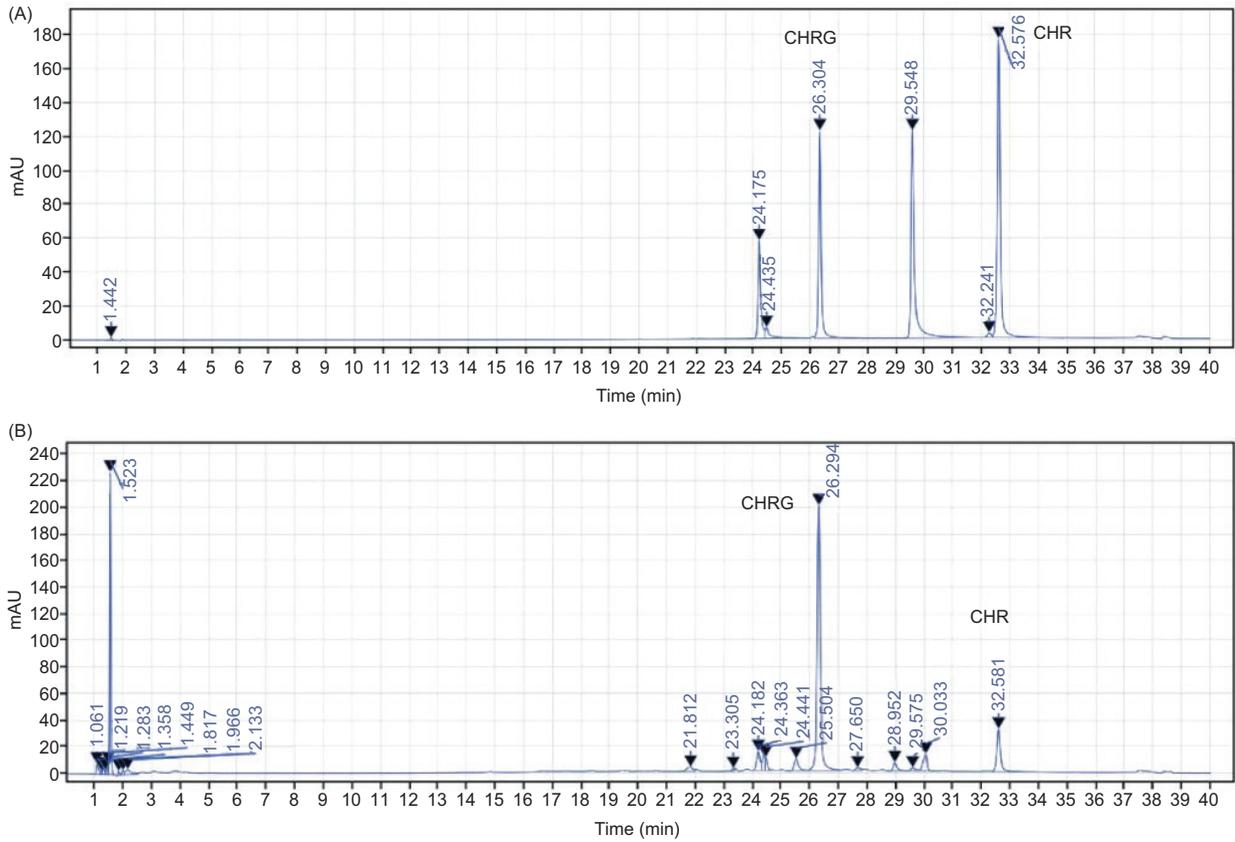
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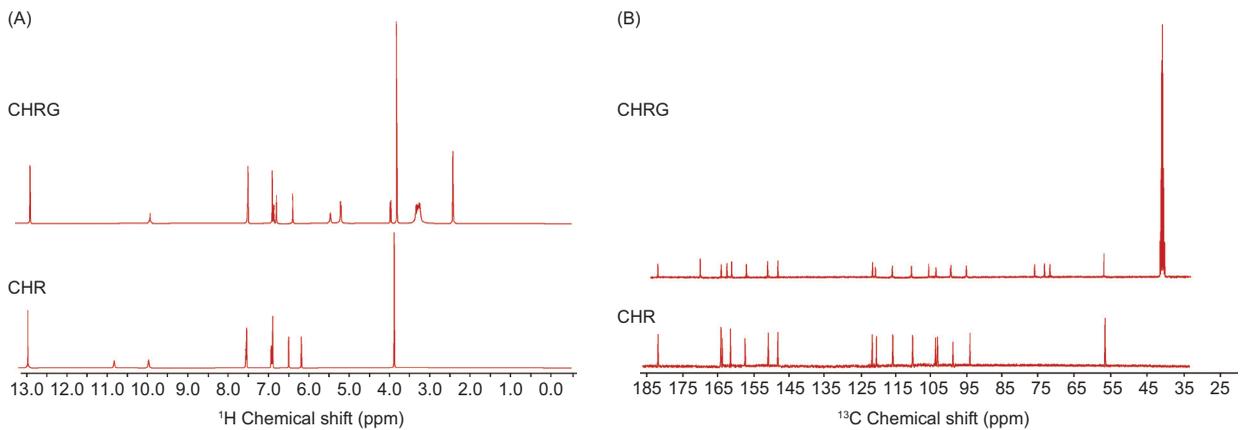
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Supplementary



**Figure S1.** Purification chromatogram of CHR and CHRg from BHBBE by preparative HPLC. (A) HPLC profile of standard references: nordihydroguaiaretic acid, lignin, CHR, and CHRg. (B) HPLC chromatogram showing the isolated CHR and CHRg fractions from the BHBBE sample.



**Figure S2.** (A) <sup>1</sup>H-NMR and (B) <sup>13</sup>C-NMR spectra for the structural identification of CHR and CHRg.