



# Ginseng volatile oil prolongs the lifespan and healthspan of *Caenorhabditis elegans*

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**Abstract** Ginseng volatile oil (GVO) is one of the main components of ginseng and has antibacterial and anti-inflammatory properties. In this study, gas chromatography–mass spectrometry (GC–MS) was applied to characterize GVO chemical composition, and 73 volatile components were detected from GVO. *Caenorhabditis elegans* was used as animal model to further elucidate the antioxidant and anti-aging effects of GVO in vivo. The results suggested that GVO significantly prolonged the lifespan of *C. elegans* and promoted its health without damaging

its reproductive capacity. In addition, GVO increased the antioxidant capacity and survival rate of nematodes after heat shock. Transcriptional sequencing showed that autophagy-related genes *atg-4.2*, *atg-7*, *lgg-2*, and *cyd-1* were up-regulated, and superoxide dismutase 1 (*sod-1*) expression was increased after GVO pretreatment. Considering the role of autophagy and antioxidant in aging, the expression of autophagy substrate P62 protein in BC12921 strain was analyzed and found to decrease by more than 50.00% after treatment with GVO. In addition, the lifespan of SOD-1 mutant nematodes was not significantly different from that of the control group. SOD activity and autophagy were activated, which is a clear expression of hormesis. All these results suggest that GVO prolongs the lifespan and healthspan of *C. elegans*, and its biological functions may be related to hormesis.

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## Introduction

Aging is a natural biological process characterized by a decline in the body's physiological and psychological adaptability to the environment. The incidence of cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, and other diseases annually increases with the degree of aging (2016), which is

therefore considered to be the main cause of these chronic diseases (Niccoli and Partridge 2012). Population aging poses a great threat to the international health care system and economic development. Drug therapy can effectively alleviate aging, but long-term anti-aging drugs such as aspirin and metformin can cause adverse reactions such as anti-platelet aggregation, diarrhea, nausea, and abdominal pain (de Vries et al. 2020; Johnston et al. 2019). Therefore, safe and effective anti-aging strategies must be urgently developed.

Traditional Chinese medicine (TCM) has been widely applied in clinical practice, such as COVID-19 and cancer treatment (Xiao et al. 2020; Liu et al. 2019), and delays aging. In particular, *Lonicera japonica*, *Polygonum multiflorum*, *glycyrrhizae radix*, *Lycium barbarum polysaccharides*, and *astragaloside IV* have significant anti-aging activity (Yang et al. 2018; Saier et al. 2018; Ruan et al. 2016; Zhang et al. 2019, 2021). Ginseng (*Panax ginseng* C. A. Meyer) is a kind of TCM with high medicinal value and has the effects of reducing oxidative stress, anti-aging, and preventing senile dementia (Wang et al. 2009, 2011a). As one of the main components of ginseng (0.1–0.5%), the essential oil of ginseng also has a wide range of medicinal value. A small amount of ginseng volatile oil (GVO) has the excitatory effect, and its appropriate use has a sedative function and can paralyze nerves at large doses (Han et al. 2013). GVO also has antibacterial, anti-tumor, and myocardial ischemia effects (Zhang et al. 2013; Jiang et al. 2008; Bae et al. 2001). Whether this substance can delay aging is unclear.

*Caenorhabditis elegans* is an organism with a short natural lifespan, strong reproductive ability, small body size, and easy maintenance in the laboratory environment. Almost all of the gene families involved in mammalian neuron function and the signaling pathways associated with aging are found in nematodes (Guarente and Kenyon 2000; Gershon and Gershon 2002). Therefore, *C. elegans* was used as an in vivo experimental model to investigate the effect and possible mechanism of GVO on life extension.

In this study, GVO significantly prolonged the lifespan of nematodes, delayed their senescence-related physiological functions, and improved their anti-stress ability, and its life-prolonging activity is related to autophagy and antioxidant pathways. The antioxidant capacity and autophagy activity of *C.*

*elegans* increased by GVO may be attributed to the activation of hormesis pathway by it.

## Materials and methods

### CO<sub>2</sub>-based supercritical fluid extraction (SFE)

GVO was extracted from ginseng through SFE with CO<sub>2</sub> as solvent. The extraction conditions were set as follows: (1) extraction pressure, 25 MPa; extraction temperature, 40 °C; (3) extraction time, 1.5 h; and (4) CO<sub>2</sub> flow rate, 10 mL/min. The obtained GVO was stored at low temperature to avoid light for later use.

### Gas chromatography–mass spectrometry (GC–MS)

The structure and purity of the compounds were characterized by GC–MS using Agilent ChemStation data system. The chromatographic conditions were as follows: db-1 capillary column; helium as carrier gas, constant flow 1.0 mL/min; and injector temperature, 280 °C. For the heating procedure, the initial temperature of 60 °C was kept for 3 min and then increased to 280 °C at 10 °C/min. Afterward, 1 µL was injected for 5 min. The relative contents of GVO can be obtained through area normalization.

### *Caenorhabditis elegans* strains and maintenance

*Caenorhabditis elegans* strains including N2, Bristol (wild type), BC12921 (rCesT12G3.1::GFP+pCeh361) and sod-1(FX776) and *Escherichia coli* OP50 (*E. coli* OP50) were reserved in the laboratory. All *C. elegans* specimens were cultured on standard nematode culture medium (NGM) and maintained in accordance with the standard rules (Brenner 1974). Unless otherwise stated, all nematodes were cultured at 20 °C and fed on OP50. Eggs were collected within 4 h to obtain age-synchronized nematodes through oviposition synchronization.

### GVO administration

First, dimethyl sulfoxide (DMSO) was used to dilute GVO, and the mixed solution was then added to *E. coli* OP50 at specified concentrations (0, 12.5, 25, and 50 µg/mL). The final DMSO content of 0.1%

was used in each dose. NGM with GVO solution was dried and used to culture *C. elegans* for biological assays.

#### Lifespan assay

Age-synchronized L4-stage hermaphrodites were selected and treated with different concentrations of GVO (0, 12.5, 25, and 50 µg/mL). The first day of treatment with GVO was recorded as Day 0, and the nematodes were transferred to the newly prepared plate on each subsequent day. Survival, death, and loss were recorded. Deaths due to eggs hatching inside the body were not included in the data. Living status was determined by the reaction of the worm picker.

#### Adversity resistance assay

*Caenorhabditis elegans* specimens at L4 stage were randomly placed in NGM plates containing different concentrations of GVO. After three consecutive days of administration, the animals were heat-stimulated at 35 °C for 4 h and then placed back at 20 °C for recovery for 12 h. The survival rate was recorded.

#### DPPH free radical scavenging

The in vitro antioxidant capacity of GVO was determined by examining its absorption of stable free radical DPPH (Sigma-Aldrich GmbH, Steinheim, Germany) (Blois 1958). In brief, 100 µL of different concentrations of GVO were mixed with 100 µL of 200 µM DPPH (diluted with methanol) and reacted in the dark for 30 min. The absorption value of the mixture was measured at 517 nm. The free radical scavenging ability of methyl xanthine was calculated by the following formula:

$$DPPH\text{scavengingeffect}(\%) = \frac{A_0 - A_1}{A_0} \times 100\%,$$

where A0 represents the light absorption value in the presence of only DPPH, and A1 represents the light absorption value in the presence of GVO.

#### Measurement of reactive oxygen species (ROS)

The synchronized L4-stage N2 worms exposed to different concentrations of GVO for 8 days were

collected to measure endogenous ROS levels by using the ROS kit (Beyotime, Shanghai China) with H<sub>2</sub>DCF-DA as the molecular probe (Wolfe and Liu 2007). M9 buffer (NaCl 5 g, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 15.12 g, MgSO<sub>4</sub> 0.25 g, and KH<sub>2</sub>PO<sub>4</sub> 3 g in 1 L of H<sub>2</sub>O) was used to collect and wash the nematodes at least three times. Each group was then added with 10 µL of 10 mM H<sub>2</sub>DCF-DA, and the mixture was shaken and incubated at 37 °C for 1 h. The nematode was repeatedly cleaned with M9 buffer to remove excess probes, and then neatly placed on 2% agarose pads. Fluorescence was quantified at excitation/emission wavelengths of 488 nm/525 nm with FV3000 confocal laser microscope (Olympus, Japan).

#### Fluorescence quantification of age pigment

As the worm ages, its gut produces an autofluorescence pigment called lipofuscin (Zhou et al. 2018; Lin et al. 2019). Adult nematodes treated with or without GVO for 8 days were collected and anaesthetized with levamisole (Aladdin, Shanghai, China) and immobilized on 2% agarose pads for determination its lipofuscin levels. Fluorescence intensity was observed using BX53 fluorescence microscope (Olympus, Japan).

#### Motility and body size assay

The nematodes were treated with different concentrations of GVO according to the above methods. The locomotor capacity and body length of at least 10 nematodes in each dish were observed on the Day 8 of adults. The locomotor capacity of the nematode is determined by the frequency of its head moving from left to right within 10 s. UOPVIEW software was used to measure the relative length of worms in different GVO treatment groups and control group.

#### Spawning assay

For the spawning assay, this study was slightly improved according to the previous method (Yang et al. 2018). The synchronized L4-staged N2 worms was selected and pretreatment with different concentrations of GVO, with one in each plate and 10 plates in each group. Worms were transferred to fresh plates every 24 h, and the total number of eggs per

nematode in each group during the first 3 days of peak spawning was recorded.

#### Determination of SOD and MDA levels

Age-synchronized nematodes treated or not treated with GVO (12.5, 25, and 50 µg/mL) for 5 days were collected to measure SOD and MDA levels. Total protein contents were determined by the bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). SOD and MDA contents were measured following the instructions of the total superoxide dismutase kit and malondialdehyde determination kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Results were normalized by protein contents represented as U/mg protein, and nmol/mg protein, respectively.

#### Transcriptome sequencing

Age-synchronized nematodes were collected after growing for 10 days in the presence or absence of 50 µg/ mL GVO. The nematode transcriptome was sequenced by Novo Genomics (Beijing, China). Up- or down-regulated genes were identified by filtering the RNA-Seq data with the following cut-off: twofold change in expression level and a false discovery rate analogue of q-value less than 0.05.

#### Gene expression analysis by quantitative PCR

Total RNA was extracted from about 500 worms using Trizol reagent. Nucleic acid/protein analyzer (Du730, Beckman Coulter, Inc., Fullerton, California, USA) and gel electrophoresis were used to measure the quality and quantity of RNA. Complementary

DNA (cDNA) was obtained using the Thermo Scientific kit (Thermo Fisher Scientific, MA, USA) under the following conditions: 25 °C for 5 min, 42 °C for 60 s, and 70 °C for 5 min. Bio-Rad Minioption real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and SYBR Green Super Mix (Takara Biotechnology, Dalian, China) were used for real-time quantitative PCR analysis. The gene expression level was analyzed by  $2^{-\Delta\Delta C_t}$  method. Primer sequences for real-time PCR were shown in Table 1.

#### Determination of P62 protein accumulation in BC12921 strain

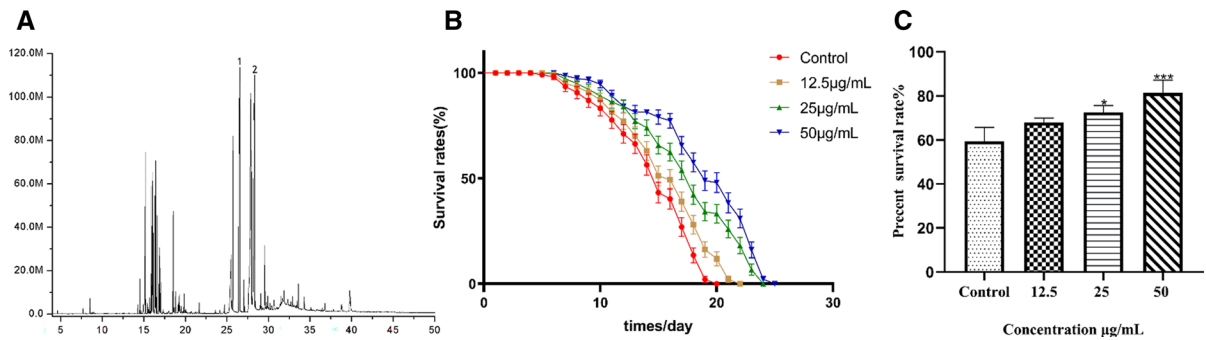
The accumulation of P62 protein in BC12921 strain was determined as described above (Wei et al. 2016). The worms were pretreated with or without GVO for 8 days. M9 buffer was used to collect and rinse the nematodes three times. The nematodes were anesthetized with levamisole and neatly placed on 2% agarose spacers. Fluorescence intensity was measured at the excitation wavelength and emission wavelength of 485 and 535 nm by using FV3000 confocal laser microscope (Olympus, Japan).

#### Statistical analysis

All fluorescence images were taken at  $\times 10$  magnification and Image J software (NIH, Bethesda, MD, USA) was used to measure the relative fluorescence intensity of the worms. Comparisons between two groups were calculated using Student's t test. All statistical analyses were performed with GraphPad Prism8.0 software. Data were presented mean  $\pm$  SD of three independent experiments.  $P < 0.05$  indicates that the difference is statistically significant.

**Table 1** Oligonucleotide primers used in qRT-PCR studies

Gene	Forward primer	Reverse primer
<i>atg-4.2</i>	AGGCGAGGAACATGGAATGAAACG	AATCCGTGCTTGCCGCTTGTC
<i>atg-7</i>	CCAGCCAGCCACGACGATAATAC	GCAGCGATAGCGTCTCCACAAG
<i>lgg-2</i>	ACGCTCCAACCTCATCCACAAC	GCCATCTGGATCACGCTCTTGAC
<i>cyd-1</i>	CCTCTCCGAGCAAACCTCCATCAAC	TCCGACGACGCTTCTTCTCTGAG
<i>tax-6</i>	GAAGAAGCTCGCCGTCTCGATAAG	TGTGGTGGTTGAGGTGTCGTTTG
<i>sod-1</i>	TGGAAGCTGGAGCCGATGGAG	TCGCCGAGGTCGTCTTGTC



**Fig. 1** Ginseng volatile oil (GVO) chemical components and its effect on the lifespan and survival rate of N2 wild-type *C. elegans* after heat shock. **A** Chemical components in GVO analyzed by GC–MS. In chromatogram, 1 represents panaxynol, and 2 represents panaxydol, **B** GVO prolongs nematode lifespan in a dose-dependent manner, and **C** GVO supplementa-

tion significantly increased the survival rate of nematodes after heat shock compared with that of the control group. Statistical analysis was performed using Prism8.0, and p values were calculated using the Student's t test.  $p < 0.05$  was considered statistically significant. Data were presented mean  $\pm$  SD of three independent experiments

**Table 2** Top 10 components in GVO detected by GC–MS

Compound name	Molecular formula	CAS number	Relative content
Panaxynol	$C_{17}H_{26}O$	32768-90-4	17.51
Panaxydol	$C_{17}H_{24}O$	81203-57-8	17.15
Ethyl linoleate	$C_{20}H_{36}O_2$	7619/8/1	16.52
Palmitic acid	$C_{16}H_{32}O_2$	1957/10/3	4.57
Ethyl palmitate	$C_{18}H_{36}O_2$	628-97-7	4.11
(–)- $\alpha$ -Neoclovene	$C_{15}H_{24}$	4545-68-0	3.61
$\beta$ -Ginseng enol	$C_{15}H_{24}$	–	3.05
$\alpha$ -Humulene	$C_{15}H_{24}$	6753-98-6	2.84
Ethyl oleate	$C_{18}H_{31}ClO$	7459-33-8	2.72
Trans- $\beta$ -farnesene	$C_{15}H_{24}$	18794-84-8	2.51

## Results

### Identification and quantification of the active compounds in GVO

Seventy-three components were detected from GVO (Fig. 1A; Table 2). The main components in GVO are panaxynol (17.51%), panaxydol (17.15%), ethyl linoleate (16.52%), palmitic acid (4.57%), ethyl palmitate (4.11%), (–)- $\alpha$ -neoclovene (3.61),  $\beta$ -ginseng ene (3.05),  $\alpha$ -humulene (2.84%), ethyl oleate (2.72%), and trans- $\beta$ -farnesene (2.51%).

GVO extended the lifespan and thermal stress resistance of wild-type *C. elegans*

Different concentrations of GVO (12.5, 25, and 50  $\mu$ g/mL) were added to *C. elegans* diet to evaluate its life-extending activity. The results showed that different

**Table 3** Effect of GVO on the lifespan of N2 wild-type *C. elegans* (mean  $\pm$  SD)

Group	Number	Maximum lifespan (days)	Median lifespan (days)
Control	99.67 $\pm$ 3.21	19.00 $\pm$ 1.00	13.67 $\pm$ 1.15
12.5 $\mu$ g/mL	98.33 $\pm$ 7.51	20.67 $\pm$ 1.15	15.00 $\pm$ 1.00
25.00 $\mu$ g/mL	100.67 $\pm$ 6.43	22.00 $\pm$ 1.73	16.67 $\pm$ 1.15
50.00 $\mu$ g/mL	102.33 $\pm$ 6.11	23.00 $\pm$ 1.73	17.67 $\pm$ 1.15

concentrations of GVO could prolong the lifespan of *C. elegans* (Fig. 1B). The median lifespan of the control group in the suitable growing environment was 13.67 days (Table 3), and that of the worms in 50  $\mu\text{g}/\text{mL}$  group was up to 17.67 days, which was 29.26% higher than that for the control group. In addition, the median lifespan of *C. elegans* in the 25  $\mu\text{g}/\text{mL}$  group was extended to 16.67 days, which was 21.94% higher than that of the control group. The median lifespan of nematodes in 12.5  $\mu\text{g}/\text{mL}$  group was 15.00 days, which was increased by 9.73% compared with that of the control group. These results indicated that GVO could significantly prolong the lifespan of nematode N2 in a concentration-dependent manner.

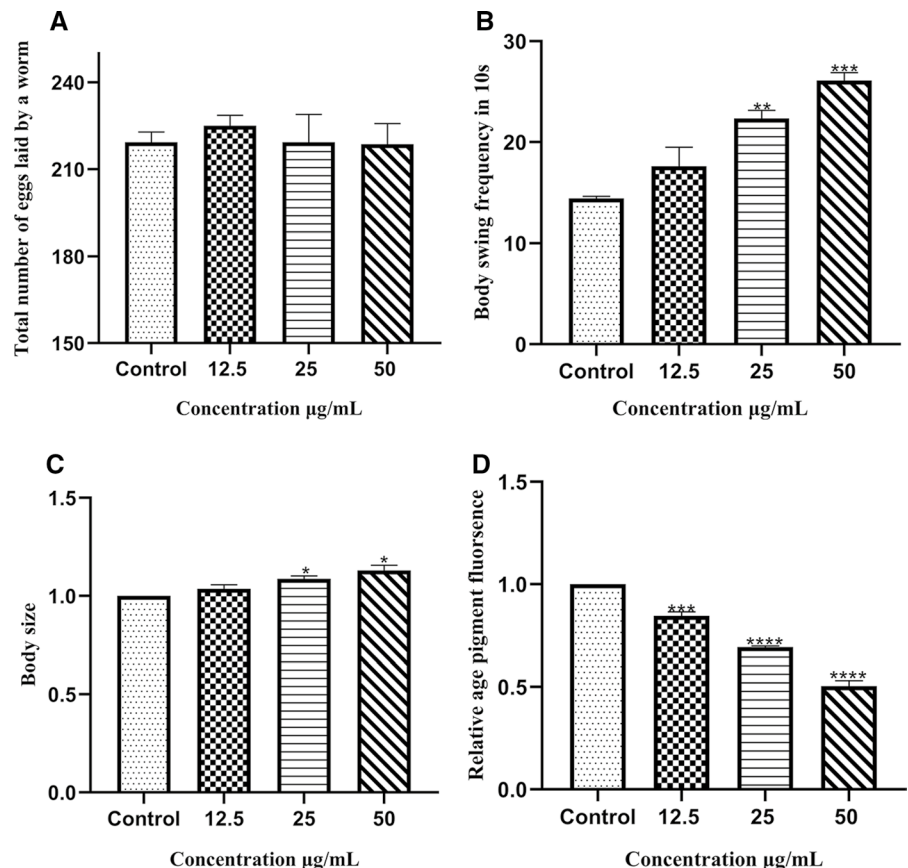
Aging is characterized by a reduced ability to withstand environmental pressures. In this study, the resistance to thermal stress of GVO-treated nematodes was investigated (Fig. 1C). The survival rate of *C. elegans* in the control group was 59.33%, and that of nematodes in 50 and 25  $\mu\text{g}/\text{mL}$  GVO groups were significantly higher than those in the control group

(81.3% and 72.67%, respectively). The survival rate of nematodes in 12.5  $\mu\text{g}/\text{mL}$  GVO group was slightly higher than that in control group, but the difference was not statistically significant. Thus, treatment with GVO significantly improved the thermal tolerance of nematodes.

#### Effects of GVO on the physiological responses of wild-type *C. elegans*

The spawning function of nematodes was first evaluated to determine whether GVO extends their healthy lifespan. N2 nematodes treated with GVO for 3 days showed no differences in spawning compared with the control group (Fig. 2A). The body swing frequency of N2 nematodes for 8 days in 10 s were then examined. In the control group, the average head swing frequency was 14.43 within 10 s. In the 12.5, 25, and 50  $\mu\text{g}/\text{mL}$  GVO groups, the head swing frequency was 17.6, 22.33, and 26.1 times higher than that of the control, respectively. The nematodes treated with

**Fig. 2** Effects of ginseng volatile oil on the physiological indices and antioxidant capacity of *C. elegans*. **A** GVO had no effect on the reproductive capacity of nematodes, **B** GVO improved the head swinging ability of nematodes, **C** GVO improved the body size of nematodes, and **D** GVO reduced lipofuscin levels in nematodes. Statistical analysis was performed using Prism8.0, and *p* values were calculated using the Student's *t* test. *p* < 0.05 was considered statistically significant. Data were presented mean  $\pm$  SD of three independent experiments





GVO showed higher body swing frequency than those in the control group (Fig. 2B).

Aging is often accompanied by a decrease in other physiological indicators. Hence, the body size of nematodes treated with different methods was evaluated at Day 8. The body size of N2 worms treated with GVO at 12.5, 25, and 50  $\mu\text{g}/\text{mL}$  was 3.62%, 8.89%, and 13.25% higher than that of the control group, respectively (Fig. 2C). The level of lipofuscin in nematodes accumulates with age can reflect the senescence level. The lipofuscin content in N2 worms treated with GVO at 12.5, 25, and 50  $\mu\text{g}/\text{mL}$  was 15%, 31%, and 50% lower than that in control group, respectively (Fig. 2D).

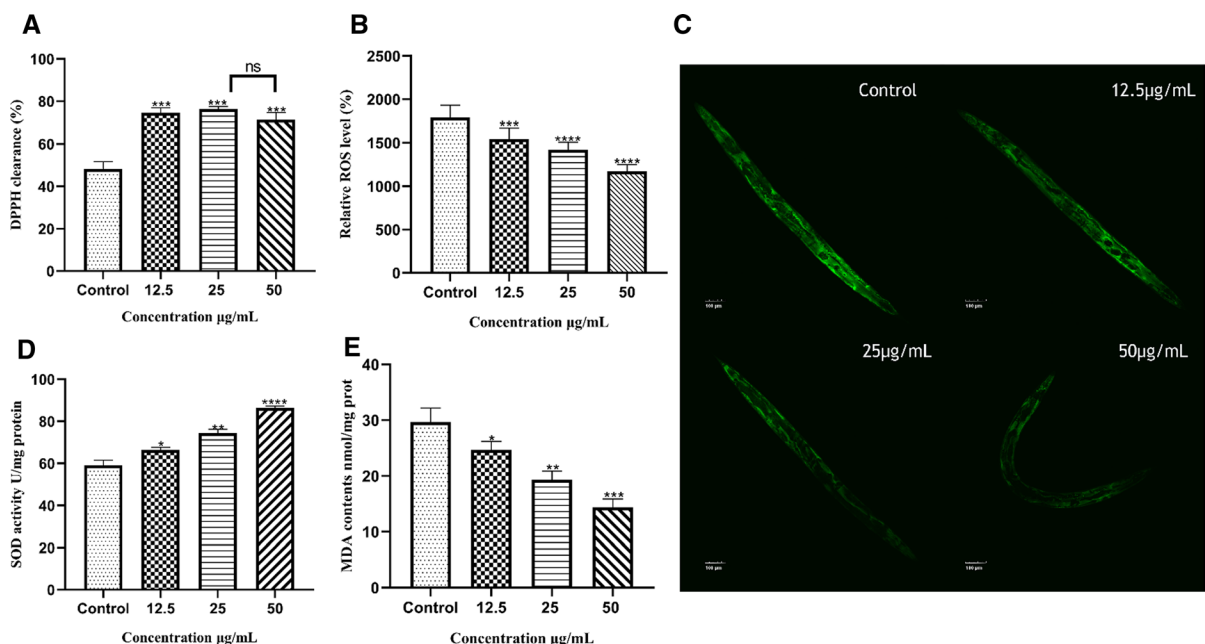
GVO increased the antioxidant capacity of wild-type *C. elegans*

The free radical scavenging ability of GVO was first evaluated in vitro to investigate whether GVO enhances the antioxidant capacity of nematodes. The DPPH free radical scavenging rates of the control

samples was 48.25%, and the free radical scavenging rates of 12.5, 25, and 50  $\mu\text{g}/\text{mL}$  GVO were 74.67%, 76.3%, and 71.39%, respectively. These values are indicative of its strong free radical scavenging ability (Fig. 3A).

In another set of experiments, the effect of GVO on intracellular ROS levels in wild type worms was explored. Reactive oxygen indicator dichlorofluorescein diacetate ( $\text{H}_2\text{DCF-DA}$ ) was used to determine the reactive oxygen levels of *C. elegans* N2.  $\text{H}_2\text{DCF-DA}$  without fluorescence can be oxidized to 2',7'-dichlorofluorescein (DCF) only in the presence of intracellular ROS to determine the intracellular ROS level. The 8-day pre-treatment with GVO significantly reduced ROS at all concentrations. Compared with those of the control nematodes, the ROS levels were reduced by 13.69%, 20.57%, and 34.49% for the GVO-treated groups at 12.5, 25, and 50  $\mu\text{g}/\text{mL}$ , respectively (Fig. 2B, C).

The effects of GVO on SOD activity and MDA content in nematode are shown in Fig. 3D, E. After 8 days of treatment, the SOD activity in 12.5, 25,



**Fig. 3** GVO increased the antioxidant capacity of *C. elegans*. **A** In vitro evaluation of antioxidant activity using DPPH assay, **B**, **C** effect of GVO on ROS fluorescence in *C. elegans*, **D** SOD activity, and **E** MDA content. All fluorescence images were taken at  $\times 10$  magnification and Image J software (NIH, Bethesda, MD, USA) was used to measure the relative fluo-

rescence intensity of the worms. Statistical analysis was performed using Prism8.0, and p values were calculated using the Student's t test.  $p < 0.05$  was considered statistically significant. Data were presented mean  $\pm$  SD of three independent experiments

and 50  $\mu\text{g}/\text{mL}$  GVO groups was increased by 7.44%, 15.45%, and 27.37%, respectively, compared with that of the control group. Therefore, GVO can promote the clearance of superoxide anion free radical by up-regulating the expression of SOD in nematodes to protect the body from injury. The amount of MDA often reflects the degree of lipid peroxidation in the body and indirectly reflects the degree of cell damage. Given that GVO can improve the antioxidant capacity of N2 nematodes, their MDA levels were measured. Compared with those of the control, the MDA contents of 12.5, 25, and 50  $\mu\text{g}/\text{mL}$  GVO treat groups were decreased by 5%, 10.34%, and 15.34%, respectively.

### Genome-wide transcriptional profiling of N2 worms

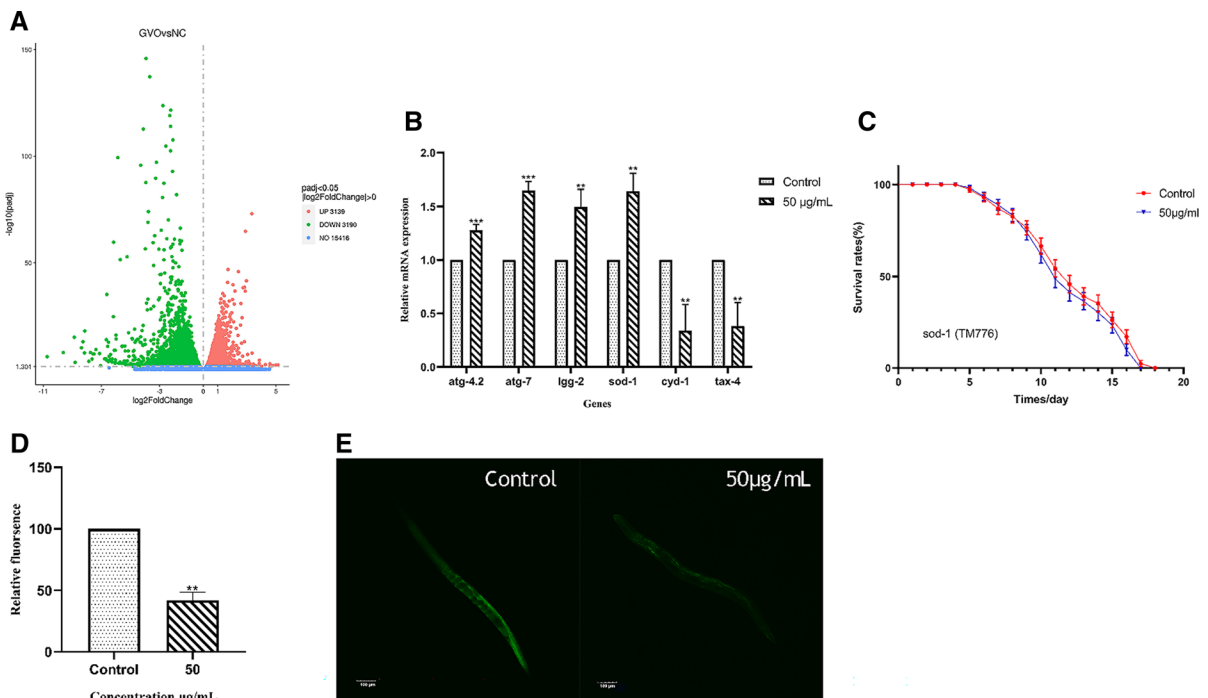
RNA sequencing analysis was performed on the 10th to compare the transcriptional profiles of N2 worms

in the 50  $\mu\text{g}/\text{mL}$  GVO-treated and control groups to broadly identify genetic contributions to GVO-induced longevity extension. After GVO treatment for 10 days, 3139 upregulated and 3190 downregulated genes were found in the treated worms (Fig. 4A, Supplementary Material, Table 1).

Six differentially expressed genes, namely, *atg-4.2*, *atg-7*, *sod-1*, *lgg-2*, *cyd-1* and *tax-4*, were selected to determine their expression changes by qPCR to verify the RNA-Seq results. The expression of all these genes was consistent with the results of RNA-Seq assay (Fig. 4B).

### Lifespan extension of *C. elegans* by GVO is related to SOD-1

GVO can improve the antioxidant capacity of nematodes and increase their SOD enzyme activity. The level of *sod-1* gene, a member of superoxide



**Fig. 4** Potential mechanism of GVO extending lifespan of *C. elegans*. **A** A total of 3139 genes upregulated and 3190 genes downregulated were found in GVO-treated nematodes compared with those of untreated worms at Day 10, **B** validation of the differentially expressed genes screened by RNA-Seq, **C** 50  $\mu\text{g}/\text{mL}$  GVO did not extend lifespan in SOD-1 mutant worms; and **D**, **E** expression of P62 protein in BC12921 strain treated with 50  $\mu\text{g}/\text{mL}$  GVO was > 50% lower than that in the

control group. All fluorescence images were taken at 10 $\times$  magnification and Image J software (NIH, Bethesda, MD, USA) was used to measure the relative fluorescence intensity of the worms. Statistical analysis was performed using Prism8.0, and p values were calculated using the Student's t test.  $p < 0.05$  was considered statistically significant. Data were presented mean  $\pm$  SD of three independent experiments



dismutase family, was increased as indicated by transcriptome sequencing. GVO may prolong the lifespan of nematodes by influencing the antioxidant pathway. For hypothesis testing, SOD-1 mutant nematodes were treated with or without 50 µg/mL GVO to observe changes in their lifespan. The results showed that after 50 µg/mL GVO treatment, the longevity of SOD-1 mutants had no significant difference compared with that of the control group (Fig. 4C). The life-prolonging activity of GVO may be affected by SOD-1.

Autophagy is involved in the lifespan extension of *C. elegans* by GVO

Autophagy is the main degradation system in cells. Through this process, the metabolic needs of cells and the renewal of organelles can be completed. Genetic and pharmacological experiments on *C. elegans* showed that all the life-extension mechanisms ranging from reduced insulin/IGF-1 signaling to spermidine supplementation, required the assistance of autophagy genes. In this study, BC12921 nematode was used to evaluate the effect of GVO on autophagy activity. The fluorescence intensity of BC12921 was decreased by 57.95% after 50 µg/mL GVO treatment (Fig. 4D, E). These results indicated that GVO treatment increased the autophagy activity of nematodes.

## Discussion

Aging is the loss and degeneration of body from tissue structure to physiological function, however, this process is not fully understood. Studies on humans and model organisms revealed that GVO has various health benefits, including antioxidant, anti-tumor, and liver protection functions (Jiang et al. 2014; Wang et al. 1992; Reyes et al. 2017; Bak et al. 2012). However, its effect on biological lifespan has not been reported.

GC–MS showed that the main components of GVO are panaxynol and panaxydol. Panaxynol, the most abundant substance in the GVO, is a compound of polyacetylenes with main carbon chain of C that protects from liver injury (Lee et al. 2019), alleviates anxiety and depression-based behaviors (Sun et al. 2020), and inhibits cardiac hypertrophy (Qu et al. 2015). Panaxydol is the second most abundant

substance in GVO and has significant properties of anti-inflammatory, anti-platelet agglutination, and inhibition of cell oxidase, leukemia cell growth (Yan et al. 2011) and thrombosis. In addition, panaxydol has potential anticancer activity, especially against EGFR cancers (Wang et al. 2011b; Kim et al. 2016). The synergistic action between compounds maximizes the anti-aging effect of the whole substance (Wang et al. 2018). Treatment with 75% ethanol extract of *L. japonica* extends the average lifespan of nematodes to 21.87%, and its life-prolonging activity is greater than that of main compounds, such as chlorogenic acid, 1,5-dicaffeoylquinic acid, and 1,3-dicaffeoylquinic acid (Yang et al. 2018). Panaxynol and panaxydol may also play a synergistic role in the anti-aging activity of GVO.

The effect of GVO on longevity was studied using *C. elegans*. The results showed that GVO extended the lifespan of nematodes in a dose-dependent manner. In the preliminary experiment, all the GVO concentrations used in this study did not inhibit the growth of *E. coli* OP50. Therefore, GVO did not prolong the lifespan of nematodes by restricting their diet. This needs to be further studied.

Aging is a decline in the body's ability to adapt to the environment. In addition to genetic makeup that plays a dominant role, the environment is crucial in determining longevity (Chen et al. 2019; Wang et al. 2008). Here, different pretreated worms were exposed to heat shock. The survival rate of *C. elegans* treated with GVO was significantly increased under heat stress. This result indicated that GVO could improve the resistance of nematodes to heat and other harsh environments. *Coix seed oil* (Chen et al. 2020), *L. japonica* (Yang et al. 2018), and *glycyrrhizae radix* (Ruan et al. 2016) can prolong the lifespan and survival rate of nematodes after heat shock. These results are consistent with the finding that GVO can prolong the lifespan and improve the survival rate of nematodes after heat shock.

In the reproductive capacity test, GVO did not affect the total progeny yield of nematodes, indicating that this substance did not prolong the lifespan of nematodes at the cost of damaging their reproductive capacity (Meng et al. 2018). Motor ability and muscle structure will gradually decline with age (Glenn et al. 2004). Hence, the head swing frequency and body length of the worms were measured. The results showed that the head swing frequency and

body length of *C. elegans* increased significantly after treatment with GVO compared with those of the control group. Lipofuscin is a fluorescent compound that accumulates with age and can be found in everything from nematodes to humans and even their retinal pigment epithelium (Wolf 1993; Wang et al. 2020). In line with other studies, GVO has the same effect as orange and apple extracts in reducing lipofuscin levels in nematodes (Wang et al. 2020; Vayndorf et al. 2013). The inhibiting effect of GVO on age pigment accumulation was dose-dependent and was most significant at 50 µg/mL (50% lower than that of the control). These results indicate that GVO can promote the health of *C. elegans*.

Free radicals are chemical entities that contain unpaired electrons and are usually highly reactive (Ionita 2021). High concentrations of free radicals can damage cellular structures and cause harm to organisms (Dröge 2002). DPPH is a stable free radical in nitrogen center and is often used for antioxidant evaluation in vitro (Sirivibulkovit et al. 2018). This study showed that GVO at different concentrations had significant scavenging ability for DPPH free radical in vitro. Variations among different concentrations were not statistically significant. The body generates oxygen free radicals through enzymatic and non-enzymatic systems that attack polyunsaturated fatty acids in biofilms and trigger lipid peroxidation, thus forming lipid peroxides, such as MDA (Doroshov 2020; Gawel et al. 2004). Lipid peroxidation converts ROS into active chemicals and amplifies the effects of ROS through chain or chain branched-chain reactions. Therefore, an initial ROS can lead to the formation of many lipidolytic products, some of which are harmless, and some can cause cell metabolism, dysfunction, or even death (Prie et al. 2016). Oxygen free radicals cause cell damage by peroxidizing polyunsaturated fatty acids in biofilms and decomposing the products of adihydroperoxides. Therefore, the amount of MDA can often reflect the degree of lipid peroxidation in the body and indirectly reflect the degree of oxidative damage. MDA and SOD are often simultaneously determined. The level of SOD activity indirectly reflects the ability of the organism to remove oxygen free radicals. In this study, the SOD activity of nematodes significantly increased after treatment with GVO, and the intracellular ROS level and MDA content significantly decreased. This may be due to the phenolic substances contained in GVO, which are

absorbed by nematodes and thus exhibit strong antioxidant activity.

Transcriptomic results showed that *atg-4.2*, *atg-7* and *lgg-2* genes were up-regulated. These genes play important roles in the autophagy pathway, and their expression level is proportional to the autophagy activity (Manil-Ségalen et al. 2014; Yang and Hekimi 2010; Hars et al. 2007). The up-regulated expression of these genes suggests that GVO may affect autophagy activity in *C. elegans*. Given the important role of autophagy in aging and protein homeostasis, we speculate that the beneficial effects of GVO might involve autophagy (Wong et al. 2020). For hypothesis testing, the effect of GVO on P62 protein expression in BC12921 nematodes was evaluated. BC12921 nematode was labeled with SQST-1-GFP, a homologue of P62 in mammals. With the increase in autophagy activity, the degradation degree of SQST-1-GFP also increased, and the fluorescence intensity of BC12921 decreased (Wei et al. 2016). Therefore, the expression of P62 in BC12921 strain significantly decreased by 56.54% after GVO treatment, suggesting that autophagy is a necessary pathway for GVO to prolong the lifespan of *C. elegans*. In addition, the expression of *sod-1*, a member of the superoxide dismutase family, was up-regulated. This finding further verified that GVO could improve the antioxidant capacity of nematodes and might prolong the lifespan of nematodes through the antioxidant pathway. Furthermore, GVO had no effect on the lifespan of SOD-1 mutant nematodes. Indicated that life-prolonging activity of GVO may be related to autophagy and antioxidant pathways.

Hormesis in aging is defined as the beneficial life-sustaining effects of cells in response to one or more rounds of mild stress (Rattan 2001, 2004). Studies have shown that dietary components such as vitamins, antioxidants, micronutrients and minerals, as well as ethanol and even herbicides and pesticides, show classic hormesis responses (Calabrese and Blain 2005). The essence of hormetins is that these compounds have biologically beneficial effects through one or more maintenance and repair as well as stress responses (Ali and Rattan 2006). Many TCMS have been reported to have anti-aging effects, which may be achieved through hormesis (Rattan and Ali 2007). In our study, all the nematodes treated with GVO showed high autophagy activity and antioxidant activity. SOD activity and autophagy were activated,

this is a clear expression of mild stress-induced phenomenon of hormesis by the activation of one or more stress responses. It is speculated that GVO may activate the hormesis pathway to exert its life-prolonging activity, which needs further research verification.

In summary, the main components of GVO are panaxynol and panaxydol. Adding GVO to the nematode diet may increase the health, lifespan, and antioxidant capacity of N2 wild-type *C. elegans* in a dose-dependent manner. GVO may exert its anti-aging function through hormesis pathway. Enhanced antioxidant activity and autophagy activation as expression of hormesis may be involved in GVO-induced longevity and health life extension in nematode worms. GVO has the potential to be developed as an anti-aging drug.

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## Declarations

**Conflict of interest** The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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