



Research Article

# Anti-aging and metabolic intervention of Glabridin and Tatarol combination

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**Abstract:** Inspired by the similar antioxidant effect functions of Glabridin and Tatarol, we investigate their synergistic anti-aging effects and the underlying metabolic intervention in *Caenorhabditis elegans* (*C. elegans*). Various concentrations of Glabridin and Tatarol, individually and in combination, were administered to *C. elegans*, and their impacts on lifespan, body development, motility, oxidative stress resistance, reactive oxygen species (ROS) levels, lipofuscin accumulation, antioxidant enzyme levels, and gene expression were evaluated. The combined treatment significantly extended the lifespan by 37.3% and showed enhanced effects over individual treatments on *C. elegans*. Metabolomic profiling using LC-MS revealed that the combination modulated aging-related metabolic pathways, particularly those involving NAD<sup>+</sup>/NADH balance and insulin signaling. The synergistic action elevated antioxidant enzyme activities, reduced ROS levels, improved oxidative stress tolerance, and upregulated key genes like *daf-16*. These findings suggest that the combined administration of Glabridin and Tatarol exerts profound anti-aging effects through synergistic interactions, enhancing metabolic regulation and stress resistance. This study provides new insights into their mechanisms of action and highlights their potential as a novel strategy for developing products for anti-aging.

**Keywords:** Glabridin; Tatarol; synergistic effect; anti-aging; metabolic intervention

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## 1 Introduction

The concept of "medicine-food homology" emphasizes the dual role of certain herbs as both food and medicine, rooted in traditional Chinese medicine (TCM). The increasing global demand for effective healthcare products has led to the exploration of nutritional supplements, particularly those containing lycopene and grape extract, for their anti-aging properties.

Glabridin, a typical flavone found in the food and medicinal homological herb, *Licorice* (*Glycyrrhiza glabra*), known as "whitening gold" in the cosmetics industry. The compound is highly esteemed for its outstanding skin-lightening effect<sup>[1]</sup>. It also has many biological activities, such as antioxidant, anti-inflammatory and antimicrobial properties<sup>[2]</sup>. These attributes render it a compelling candidate for cosmetic applications, particularly in formulations seeking antioxidant and skin-lightening benefits<sup>[3]</sup>.

Tatarol, a unique natural phenolic diterpene compound, has garnered significant attention from the scientific community. This patented active ingredient, extracted from the core of the Tatarol pine utilizing supercritical CO<sub>2</sub> technology, possesses a subtle aroma and demonstrates remarkable biological activity<sup>[4]</sup>. Its

robust bioactivity, human safety profile, and compatibility with various product formulations have been thoroughly validated by several independent laboratories, garnering widespread recognition. Due to these attributes, Tatarol has been classified as a cosmeceutical ingredient in Europe and the United States. It is extensively utilized as an antimicrobial agent in everyday consumer products<sup>[5]</sup>. Recently, the beauty and skincare industry has shown keen interest in Tatarol's application in anti-aging, antioxidant, and free radical scavenging formulations, which undoubtedly expands its usage spectrum and highlights its immense developmental potential.

The use of combination therapies involving multiple active substances to enhance treatment efficacy in diseases is a focal point in recent researches<sup>[6]</sup>. Combination drugs play a crucial role in disease management and prevention, with significant benefits observed in clinical trials. For example, in chemotherapy, combining paclitaxel with other cytotoxic drugs such as antimetabolites has shown promising results. Studies indicate that the sequence of administering methotrexate (MTX) and paclitaxel can influence their synergistic effects, with pre-treatment of MTX enhancing efficacy while the reverse order leads to antagonism<sup>[7]</sup>. This synergy has been observed in both chemo-sensitive and multidrug-resistant ovarian cancer cells, as well as in ovarian



tumor mice, highlighting the potential for developing novel drugs and therapeutic approaches.

Inspired by the similarities in biological functions of the two compounds, Glabridin and Tatarol, we examined the synergistic effect of their combined use on anti-aging efficacy, as well as the changes in internal metabolism after their joint application. We also aims to elucidate the roles of aging-related metabolic regulatory pathways and gene factors.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Juglone was procured from Chengdu Phytostandard Chemical Co., Ltd. (Chengdu, China). The necessary reagents, comprising agar, peptone, yeast extract, NaCl, CaCl<sub>2</sub>, MgSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and K<sub>2</sub>HPO<sub>4</sub>, as well as dimethyl sulfoxide (DMSO), for preparing the growth medium for *Caenorhabditis elegans* (*C. elegans*) and the Luria-Bertani (LB) medium for *Escherichia coli* OP50, were sourced from Beijing Solarbio Biotechnology Co., Ltd. (Beijing, China). Additionally, 5-Fluorouracil and cholesterol were acquired from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China). Levamisole was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Furthermore, Shaanxi Huatai Biofine Chemical Co., Ltd. (Xi'an, China) supplied Glabridin with 40% purity and Tatarol with 80% purity. The nicotinamide mononucleotide required for our experiments was procured from Shanghai Taitan Science and Technology Co., Ltd. Regarding the organic solvents, they were all purchased from Shanghai Energy Chemical Co., Ltd., with methanol being of LC-MS grade and the remaining solvents being of analytical grade. Glabridin and Tatarol were kindly provided by Huatai (Shaanxi Huatai Bio-Fine Chemical Co., Ltd., Xi'an, China), and purified with a C<sub>18</sub> semi-preparation column on an Agilent 1100 equipment (Agilent Technologies, Inc., CA, United States) before use.

### 2.2 Lifespan, body development and motility assays on *C. elegans* strains

The phenotypic assays on nematodes were conducted following the methods we previously reported<sup>[8]</sup>. The difference in the current investigation is that the nematodes were exposed to a mixture of two compounds, Glabridin and Tatarol, in specific ratios, in addition to being exposed to the compounds individually. In the lifespan investigation, synchronized *C. elegans* at the L4 developmental stage were exposed to Glabridin (20, 40, and 80 µmol/L), Tatarol (20, 40, and 80 µmol/L) or the combinations of Glabridin-Tatarol mixtures at 40–40 µmol/L, 40–80 µmol/L, 80–40 µmol/L, and 80–80 µmol/L.

In our observations, we categorize the movement abilities of nematodes into three levels: A, B, and C. Level A includes nematodes that can swim steadily and at will. Level C comprises those that only initiate movement upon contact with a needle at either the head or tail. Nematodes displaying behaviors that fall between these two extremes are classified as level B.

### 2.3 Oxidative stress resistance, ROS and lipofuscin levels assay of *C. elegans*

In the acute oxidative stress simulation assay, *C. elegans* were exposed to Glabridin and Tatarol for 5 days before being transferred to a culture medium containing 100 µmol/L carnosine

quinone in NGM. The worms were maintained at 20 °C, and their survival rates were monitored hourly. For the heat shock experiment, after the same 5-day treatment with the test compounds, *C. elegans* were placed on extract-free NGM plates and incubated at 35 °C. Mortality rates were recorded hourly until all nematodes had died.

To assess the level of ROS in *C. elegans*, we used the DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate) method<sup>[9]</sup>. After incubating the worms in extracts of varying concentrations for 5 days, they were treated with 50 µmol/L DCFH-DA for staining. Following a 3-hour incubation, they were rinsed three times with M9 buffer and immobilized with levamisole. Fluorescence intensity was observed using an DMi8-fluorescence microscope (Leica Camera AG, Inc., Germany) and analyzed with ImageJ software (version 1.54, <https://imagej.net/downloads>). The experiment was repeated three times in parallel, with 25 nematodes per group.

To quantify lipofuscin content, *C. elegans* were cultured in various concentrations of extracts for 10 days. They were subsequently anesthetized with levamisole and mounted on slides. Fluorescence was visualized using an inverted fluorescence microscope at a wavelength range of 500–560 nm, and images were captured for documentation. Fluorescence intensity was precisely measured using ImageJ software. Each group consisted of at least 25 worms, and the procedure was independently repeated three times.

### 2.4 Assay for antioxidant enzymes

The maintenance method of *C. elegans* was the same as section 2.3. Rinse thoroughly with M9 buffer during collection to ensure at least 1,000 worms per group. After rinsing, a 0.9% saline solution was added to the samples. These samples were processed using a cell disruptor under an ice bath to obtain a fine homogenate. The homogenate was centrifuged at 4 °C, 12,000 r/min for 10 min, and the resulting supernatant was carefully collected. This supernatant was used to quantify protein levels including catalase (CAT), glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) activity, following the protocols provided by the manufacturer (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China).

### 2.5 Quantitative real-time PCR assay

After synchronization, L4 *C. elegans* were exposed to Glabridin, Tatarol and their combinations for 5 days at 20 °C. The worms were then collected by thoroughly washing them with M9 buffer. Total RNA extraction, cDNA was amplification, and real-time quantification a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, Hercules, California, USA) were carried out according to the instructions of AG RNAex Pro reagent (Accurate biology Co., Ltd., Changsha, China). The Primers used for qPCR analysis refer to Table S1 in Supplementary Materials.

### 2.6 Metabolic profiling by LC-MS and data analysis

To investigate the combined effects on the metabolome of nematodes *in vivo*, we conducted experiments with combination-treatment groups: Control (GT0), 40 µmol/L Glabridin + 40 µmol/L Tatarol (GT1), and 80 µmol/L Glabridin + 80 µmol/L Tatarol (GT2). Synchronized L4 *C. elegans* were distributed into the blank and various treatment groups. Each group was cultured in NGM Petri dishes with FUDR and incubated at 20 °C. On the 5th day, *C. elegans* were collected with M9 buffer into sterile,

enzyme-free centrifuge tubes and rinsed 3 times with sterile water to remove impurities. They were then frozen with liquid N<sub>2</sub> quickly and stored at -80 °C. Subsequently, samples were freeze-dried to eliminate residual water. An 80% methanol solvent (containing 10 µmol/L 2-bromophenylalanine as internal standard) was added to the dried samples with the ratio 200 µL/mg. Worm tissues were disrupted on ice using a cell disruptor for less than five minutes to preserve metabolite integrity. Samples were centrifuged at 4 °C, 12,000 r/min for 10 min, and the supernatant was collected for analysis.

A Vanquish UPLC system, integrated with a Q Exactive Focus mass spectrometer (Thermo Fisher Scientific), was used for metabolite analysis. Each sample (2 µL) was separated on an Accucore™ aQ C18 polar capped column (150 × 2.1 mm, 2.6 µm). Instrument parameter settings are based on the metabolomics analysis method we have previously reported<sup>[10]</sup>.

The raw data files were imported into MS-Dial version 4.90 for peak detection and alignment. An inhouse library of MS reference was used for metabolites<sup>[11]</sup>. Quantitative calibration was conducted using internal standard correction. The alignment data was exported and then loaded using R scripts to perform bioinformatic analysis. The Principal component analysis was performed using the mixOmics package<sup>[12]</sup> from Bioconductor to identify metabolomic variations. Differential Metabolites (DMs) were filtered with criteria: Fold Change (FC) > 1.2 and FDR correlated *P*-value < 0.05. KEGG pathways were enriched using FELLA package<sup>[13]</sup>.

## 2.7 Statistical analysis

Data analysis was conducted using R language with one-way ANOVA, followed by either Dunnett's multiple comparisons test or the student *t*-test, based on the data type. Results are presented as the mean ± standard error of the mean (SEM). Statistically significant differences were indicated using asterisks: \* for *P* < 0.05, \*\* for *P* < 0.01, \*\*\* for *P* < 0.001. Non-significant differences are denoted as ns.

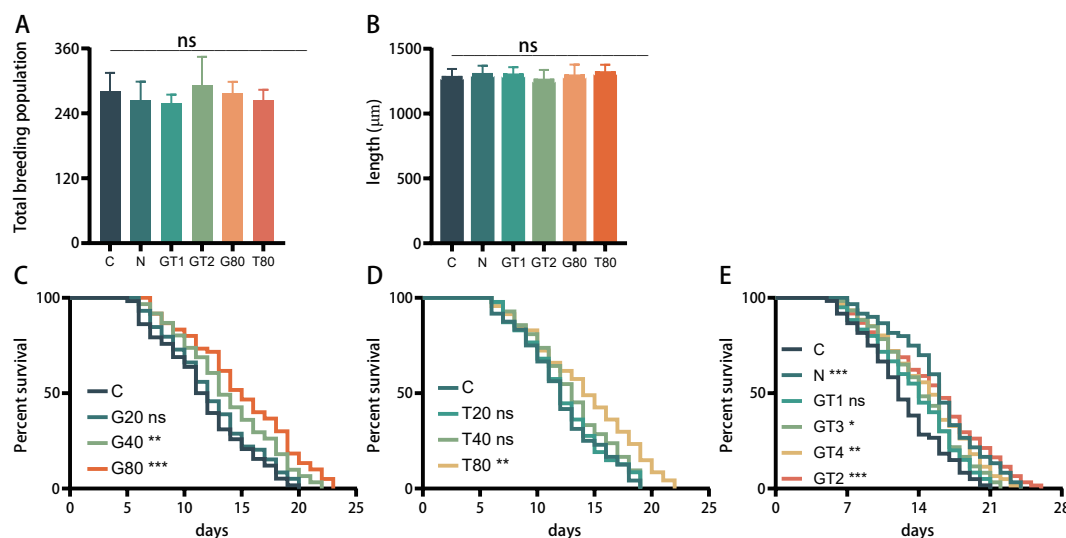
## 3 Results

### 3.1 Effect of combined action on body length and fecundity

A comprehensive analysis of body length and total egg production in *C. elegans*, as presented in Figs. 1A and 1B, revealed no significant alterations in body length following exposure to varying concentrations of Glabridin and Tatarol, individually or in combination. Furthermore, although a slight reduction in overall egg count was noted in the treatment groups, this decrease did not reach statistical significance. These findings suggest that the administered concentrations are safe, exhibiting no appreciable toxic effects on *C. elegans*.

### 3.2 Combined action extends *C. elegans* lifespan

The effects of varying concentrations of Glabridin and Tatarol on the lifespan of *C. elegans* under standardized laboratory conditions at 20 °C are depicted in Figs. 1C-E and Table S2. Exposure with Glabridin (20, 40, and 80 µmol/L) extended the mean lifespan to 12.38 ± 0.53, 13.80 ± 0.56, and 15.00 ± 0.61 days respectively, which increased 5.5%, 17.6%, and 27.8% above the blank control group. Similarly, exposure with 20, 40, and 80 µmol/L of Tatarol extended the mean lifespan to 12.34 ± 0.53, 13.07 ± 0.58, and 14.21 ± 0.68 days, showing improvements of 1.7%, 7.7%, and 17.0% over the blank control. It is evident that the combined application of 80 µmol/L Glabridin and 80 µmol/L Tatarol resulted in the most significant increase in lifespan, achieving an average extension of 24.6% compared to the control. In the current investigation, the effect of the combined use of Glabridin and Tatarol was comparable to the effect of nicotinamide mononucleotide (NMN), which is a natural molecule that has been proven to have significant anti-aging effects<sup>[14]</sup>. The findings revealed that the combination treatment exhibited a synergistic effect, notably enhancing the average survival and lifespan of *C. elegans*.



**Figure 1** Effects of the combined action of Glabridin and Tatarol on reproduction, body length, and life span. (A) Reproductive capacity. (B) Body length. (C) Effect of Glabridin on *C. elegans* life span. (D) Effect of Tatarol on *C. elegans* life span. (E) Combined effects on *C. elegans* lifespan. NMN (80 µmol/L) was used as positive control. Statistical significance: ns, no significance. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, compared to the control (log-rank test). Abbreviations of treatment groups: C, blank control; N, 80 µmol/L NMN; GT1, 40 µmol/L Glabridin + 40 µmol/L Tatarol; GT2, 80 µmol/L Glabridin + 80 µmol/L Tatarol; GT3, 40 µmol/L Glabridin + 80 µmol/L Tatarol; GT4, 80 µmol/L Glabridin + 40 µmol/L Tatarol; G20, 20 µmol/L Glabridin; G40, 40 µmol/L Glabridin; G80, 80 µmol/L Glabridin; T20, 20 µmol/L Tatarol; T40, 40 µmol/L Tatarol; T80, 80 µmol/L Tatarol.

### 3.3 Effect of combined action on mobility

To investigate the synergistic potential of Glabridin and Tatarol in enhancing health status and locomotor function, we carried out an investigation on the locomotory proficiency of *C. elegans*. As shown in Fig. 2A, most nematodes in the control group exhibited robust and unhindered movement (Motion A) in the first 5 days. After 10 days, a notable 21.0% nematodes reduced motility, falling into the Motion C movement. On day 15, this percentage escalated dramatically to 87.0%.

At the same time, the nematodes exposed to the combination of Glabridin and Tatarol demonstrated significant resilience. This combination effectively mitigated the decline in the locomotive ability of *C. elegans* in a dose-dependent manner. The combined concentrations, the reduction of nematodes with Motion C locomotive status compared to the control group was 8.0% and 55.0%, respectively. The effect was most pronounced with the co-treatment of 80  $\mu\text{mol/L}$  Glabridin and Tatarol, outperforming the treatment with NMN.

### 3.4 Effects of Glabridin-Tatarol combination against ROS

To assess the impact of the Glabridin-Tatarol combination on ROS levels in *C. elegans* and their potential role in mitigating nematode senescence and oxidative stress, we conducted a further investigation. As shown in Fig. 2B, the ROS expression levels reduced remarkably in 5 days after being treated with the Glabridin-Tatarol individuals or combination. Compared to the control group, the Glabridin-treated group exhibited a 30.3% decrease in ROS levels, while the Tatarol-treated group showed a 20.7% reduction. Notably, 80  $\mu\text{mol/L}$  Glabridin and 80  $\mu\text{mol/L}$  Tatarol led to an even more significant decline, reducing ROS levels by 29.3% compared to the control. The combined use of the two compounds yielded a better effect than Tatarol individual use at the same concentration, indicating the presence of a synergistic effect.

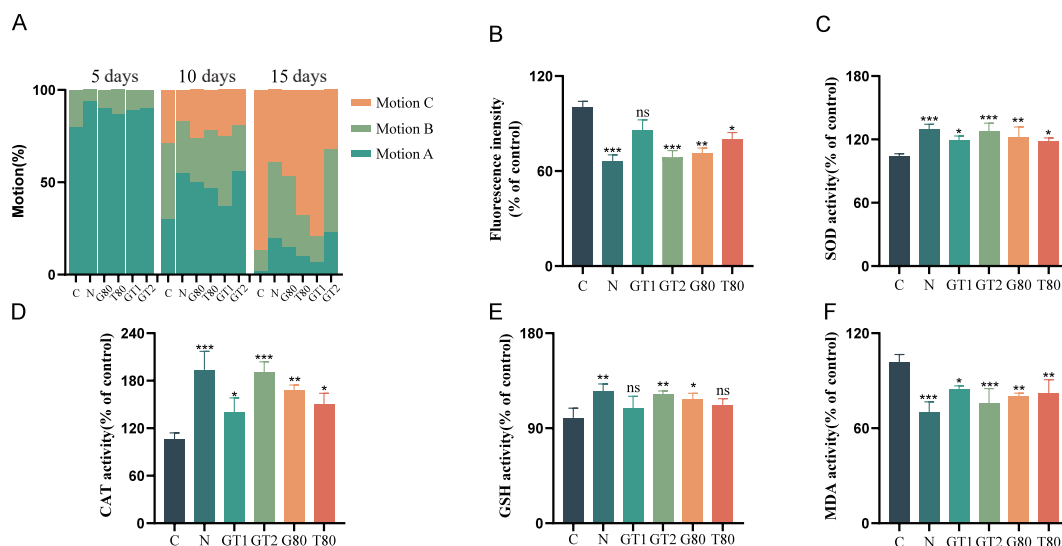
CAT, MDA, SOD and GSH serve as crucial biomarkers for

evaluating oxidative stress activities. As shown in Figs. 2C-2F, the combined application of Glabridin and Tatarol markedly augmented the activity of SOD in *C. elegans*, ranging from 1.2- to 1.5-fold compared to the control. Similarly, CAT activity was significantly enhanced, spanning 1.4- to 2.0-fold higher. Furthermore, GSH activity increased by 1.2- to 1.5-fold. Notably, the MDA content in the co-treated group underwent a significant reduction, ranging from 16.4% to 25.7% lower than that of the control.

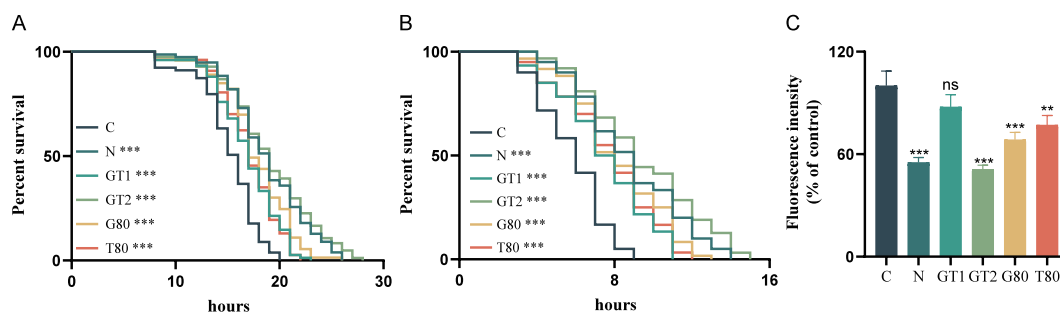
### 3.5 Effect of combined action on the resistance of *C. elegans*

The prolonging of lifespan is often linked to improved resilience, including the ability to resist oxidative stress, which is a key indicator of overall health. To better understand the impact of these compounds on the ability to handle stress in *C. elegans*, we examined important factors such as thermotolerance and resistance to sudden oxidative stress. We investigated the effects of Glabridin and Tatarol on the lifespan of *C. elegans* under heat stress, as presented in Fig. 3A and Table S3. The nematodes treated with Glabridin showed a 17.1% increase in survival time compared to the untreated group, while the group treated with Tatarol exhibited an 12.5% extension in lifespan. Additionally, the group treated with NMN, used as a positive control, increased the lifespan of *C. elegans* by 24.0%. The combination of Glabridin-Tatarol (80  $\mu\text{mol/L}$ ) prolonged the lifespan by 25.9%, surpassing the effects of the single-component treatments.

Juglone can accelerate the aging process. Under the influence of juglone, the lifespan of nematodes is significantly reduced. As shown in Fig. 3B and Table S4, Glabridin alone augmented the lifespan of juglone-treated *C. elegans* by 39.8% compared to the control group. Tatarol alone extended the lifespan by 32.1%. The Glabridin-Tatarol (80  $\mu\text{mol/L}$ ) combination prolonged the lifespan by 62.3%. This synergistic effect significantly surpassed the individual contributions of Glabridin and Tatarol. These results strongly suggest that the combined use of Glabridin and Tatarol effectively protected nematodes from heat stress-induced damage,



**Figure 2** Effect of combined action on mobility, ROS, MDA, and antioxidant enzymes. (A) Effect of combined action on oscillations on days 5, 10, and 15. Motion A *C. elegans* were able to autonomously perform sinusoidal trajectory locomotion, showing strong locomotor ability; Motion B *C. elegans* had locomotor ability between Motion A and Motion C; and Motion C *C. elegans* showed only weak locomotor responses after slight stimulation. (B) Relative change of ROS. (C, D, E, F) relative levels of CAT, GSH, and MDA antioxidant enzymes. NMN (80  $\mu\text{mol/L}$ ) was used as the positive control. Statistical significance: ns, no significance. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , compared to the control (log-rank test). Abbreviations of treatment groups: C, blank control; N, 80  $\mu\text{mol/L}$  NMN; GT1, 40  $\mu\text{mol/L}$  Glabridin + 40  $\mu\text{mol/L}$  Tatarol; GT2, 80  $\mu\text{mol/L}$  Glabridin + 80  $\mu\text{mol/L}$  Tatarol; G80, 80  $\mu\text{mol/L}$  Glabridin; T80, 80  $\mu\text{mol/L}$  Tatarol.



**Figure 3** Effect of combined action on resistance and lipofuscinations. (A) Heat shock. (B) Juglone stimulation. (C) Fluorescence quantification of lipofuscin in *C. elegans*. NMN (80  $\mu\text{mol/L}$ ) was used as the positive control. Statistical significance: ns, no significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared to the control (log-rank test). Abbreviations of treatment groups: C, blank control; N, 80  $\mu\text{mol/L}$  NMN; GT1, 40  $\mu\text{mol/L}$  Glabridin + 40  $\mu\text{mol/L}$  Totarol; GT2, 80  $\mu\text{mol/L}$  Glabridin + 80  $\mu\text{mol/L}$  Totarol; G80, 80  $\mu\text{mol/L}$  Glabridin; T80, 80  $\mu\text{mol/L}$  Totarol.

strengthening their defenses against thermal stress and enhancing the overall well-being of *C. elegans*.

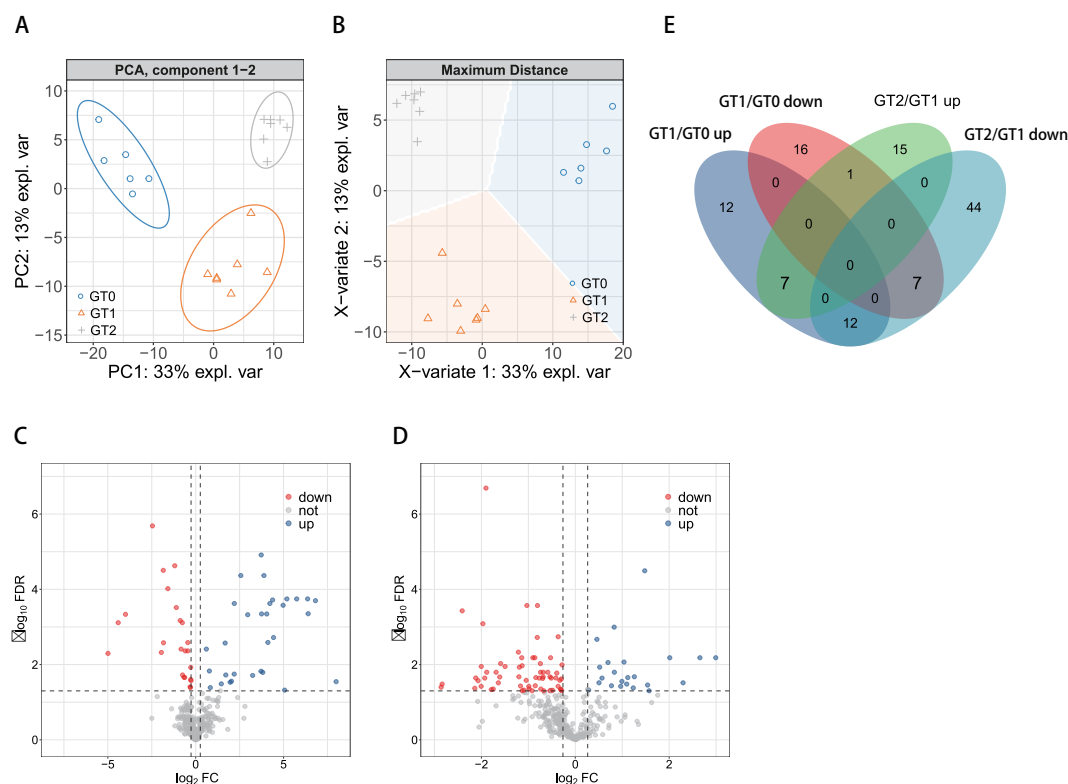
### 3.6 Glabridin-Totarol combined effect on lipofuscin in *C. elegans*

Lipofuscin, a lysosomal polymerized substance exhibiting autofluorescence at specific wavelengths, is an indicator of aging in *C. elegans*. We investigated the effects of the combined Glabridin-Totarol on lipofuscin accumulation in *C. elegans*. As shown in Fig. 3C, treatment with the Glabridin-Totarol combination led to a gradual decrease (by 45.6%) in autofluorescence intensity in the body of *C. elegans*, indicating a significant reduction in lipofuscin accumulation. Treatment with Glabridin and Totarol individually resulted in a 29.7% and 22.7% reduction in lipofuscin levels, respectively, compared to the untreated group. This synergistic effect was significantly more potent than the individual treatments. These findings highlight the potential of the Glabridin-

Totarol combination in reducing lipofuscin accumulation, potentially enhancing the overall health of *C. elegans*.

### 3.7 Metabolomic analysis

We employed Principal Component Analysis (PCA, Fig. 4A) and Partial Least Squares Discriminant Analysis (PLS-DA, Fig. 4B) to investigate the trends within our metabolomic data. PCA reveals how samples are distributed across the first two principal components, emphasizing the variance among treatment groups. The clustering, represented by distinct ellipses in blue, orange or grey, signified that samples within each group share similar characteristics. This effective segregation highlighted the differences between groups, as the first principal component accounted for a considerable amount of the total variance. In Fig. 4B, PLS-DA results, further enhanced our understanding of the dataset by focusing on the predictive relationships between variables. The plot demonstrates a clear separation among the



**Figure 4** Metabolomic variations of *C. elegans* exposed with Glabridin-Totarol combination. (A) Score plot of PCA. (B) Score plot of OPLS-DA. (C) Metabolites variations of GT1/GT0. (D) Metabolites variations of GT2/GT1. (E) Numbers of upregulated and downregulated metabolites in the GT0, GT1, and GT2 groups. FDR corrected  $P$ -value  $< 0.05$ ,  $FC > 1.2$ . Control (GT0), 40  $\mu\text{mol/L}$  Glabridin + 40  $\mu\text{mol/L}$  Totarol (GT1), and 80  $\mu\text{mol/L}$  Glabridin + 80  $\mu\text{mol/L}$  Totarol (GT2).

treatment conditions (GT0, GT1, GT2), indicating that the PLS-DA model successfully discriminated between these groups. The significant distances measured between clusters suggest a strong classification capability of the PLS-DA model, allowing for effective identification of differences among treatments. These results proved essential in advancing our insights into the data and guiding future investigations within the field.

In this metabolomic analysis, a total of 352 metabolites were identified. Figs. 4C and 4D illustrate the overall changes in metabolite levels across different group comparisons. Specifically, Fig. 4C depicts the changes between GT1 and GT0, while Fig. 4D shows the changes between GT2 and GT1. From GT0 to GT1, 31 metabolites exhibited an increase in levels, whereas 24 metabolites showed a decrease. Conversely, from GT1 to GT2, 23 metabolites were upregulated, and 63 metabolites were downregulated. Notably, the treatment in GT2 induced a more significant downregulation of metabolite levels.

To understand the changes in metabolite levels under the influence of GT, we filtered the data to identify metabolites that consistently increased or decreased in both comparisons (GT1/GT0 and GT2/GT1). We found that 7 metabolites were consistently upregulated, and another 7 metabolites were consistently downregulated in both comparisons (Fig. 5). These findings suggest that the GT treatment has a substantial impact on the metabolomic profile, with a notable trend towards downregulation in the GT2 treatment phase. The identification of consistently upregulated and downregulated metabolites provides valuable insights into the metabolic pathways affected by GT treatment and could serve as potential biomarkers for further investigation.

Using the differentially expressed metabolites acquired above, we conducted a KEGG pathway analysis with the FELLA toolkit, resulting in the pathway network (Fig. 6). The analysis revealed significant involvement of several metabolic pathways: Biosynthesis of unsaturated fatty acids, Phenylalanine metabolism, Fatty acid degradation, and Butanoate metabolism. The result indicated their critical roles in the metabolic changes interfered with Glabridin and Tatarol. The network diagram also highlights specific enzymes and reactions involved, providing a detailed map of the metabolic shifts. At the same time, we also observed that

NAD<sup>+</sup> and NADH are positioned as network hubs. This suggests that the combined intervention of glabridin and totarol primarily acts on the biochemical conversion between NAD<sup>+</sup> and NADH, which is closely related to the antioxidant capabilities of these two substances.

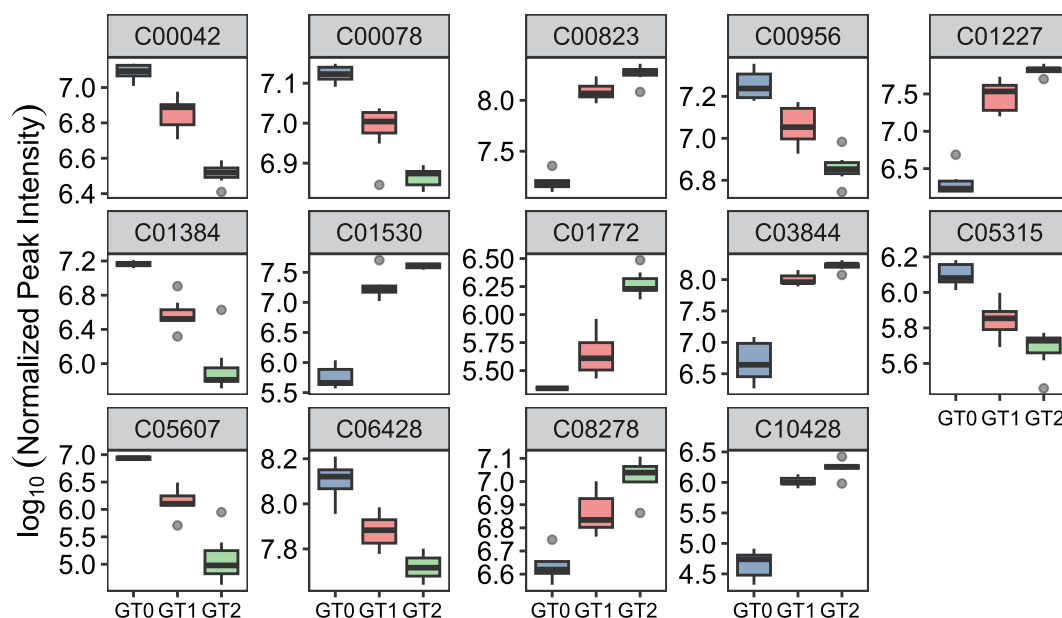
### 3.8 Gene expression levels

We selected out the genes integral to antioxidant defense mechanisms and the regulation of NAD<sup>+</sup>/NADH balance for further examination, which are critical for mitigating oxidative stress and promoting cellular longevity<sup>[8,15,16]</sup>. These genes are *age-1*, *daf-2*, *daf-16*, *sod-3*, *skn-1*, *mtl-1*, *hsp-16.2*. *age-1* and *daf-2* are key components of the insulin/IGF-1 signaling pathway, which influences metabolic processes and oxidative stress responses. *daf-16*, a FOXO transcription factor, regulates the expression of various antioxidant genes, including *sod-3* and *skn-1* thereby enhancing the organism's ability to manage ROS. Additionally, *mtl-1* and *hsp-16.2* are involved in metal ion homeostasis and protein folding, respectively, contributing to cellular resilience.

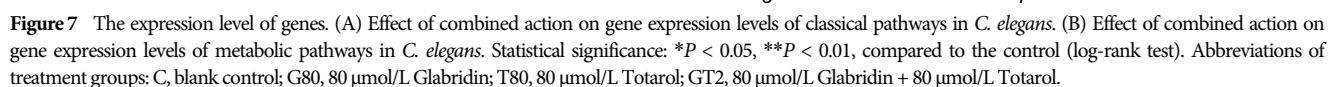
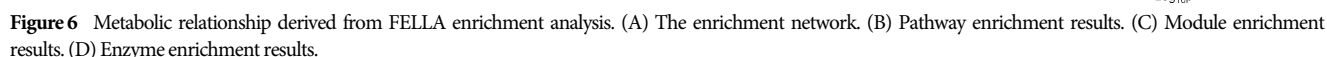
Our experimental analysis demonstrated a significant extension in the average lifespan of *C. elegans* treated with a combination of Glabridin and Tatarol. This treatment was also associated with improvements in healthspan indicators and increased stress tolerance. To elucidate the anti-aging mechanisms underlying this combined therapy, we analyzed the expression patterns of longevity-associated genes, as illustrated in Fig. 7. Co-treatment with Glabridin and Tatarol resulted in a marked repression of *age-1* and *daf-2* gene expression, decreasing to 0.53-fold and 0.58-fold of control levels, respectively. In contrast, there was substantial upregulation of *daf-16*, *sod-3*, *skn-1*, and *mtl-1*, with expression levels increasing to 3.12-fold, 2.82-fold, 2.62-fold, and 1.87-fold, respectively. Additionally, *hsp-16.2* expression was elevated by 2.75-fold compared to controls.

## 4 Discussion

ROS plays a central role in the aging process by inducing oxidative stress, which damages cellular components and impairs mitochondrial function. As organisms age, ROS levels accumulate, overwhelming antioxidant defenses and accelerating cellular



**Figure 5** Level variations of differentially expressed metabolites in *C. elegans* interfered with Glabridin and Tatarol. FDR corrected *P*-value < 0.05, FC > 1.2.

conditions<sup>[17]</sup>.

In this study, we employed *C. elegans* as a model organism to investigate the anti-aging effects of Glabridin and Totarol.

*C. elegans* is advantageous for aging research due to its short lifespan, well-characterized genetics, and conserved molecular pathways that mirror those in humans. Its transparency allows for real-time observation of physiological changes, and its genetic tractability facilitates precise manipulation of genes involved in longevity and stress responses.

#### 4.1 GT-combination extends life span and improves health indicators

The global elderly population is expanding at an unprecedented rate, surpassing other demographic groups. Age is a significant predictor of various chronic diseases, multimorbidity, geriatric syndromes, and reduced recovery capabilities following illnesses or accidents. Rather than merely extending life expectancy with prolonged morbidity, enhancing the duration of health and independence—termed a healthy lifespan—is highly desirable. Throughout history, humanity has sought to uncover the secrets of longevity, with numerous contemporary natural products recognized for their substantial anti-aging properties. Natural products present several advantages over conventional pharmaceuticals, including a diverse array of compounds, multi-target mechanisms, and minimal toxicity.

For instance, research indicates that goji berry extract extends the lifespan of *C. elegans* by targeting the deacetylase sir-2.1 and prolongs the life of neurodegenerative *C. elegans* deficient in hsf-1 while reducing toxic protein aggregation<sup>[18]</sup>. Similarly, mulberry extract enhances the nuclear translocation of DAF-16 and its downstream targets SOD-3 and GST-4, alleviating amyloid- $\beta$  (A $\beta$ )-induced paralysis by approximately 55.6% and significantly decreasing A $\beta$  accumulation by over 50.0%<sup>[19]</sup>.

Glabridin and Tatarol, both recognized for their diverse biological activities, have emerged as promising agents in preventing chronic diseases such as diabetes and inflammation. However, research exploring the synergistic effects of Glabridin and Tatarol, particularly at the genetic level, remains scarce.

The combined treatment of Glabridin and Tatarol significantly enhanced the lifespan of *C. elegans* compared to their individual applications. This synergistic effect was accompanied by substantial improvements in health parameters, including increased fertility, enhanced motility, greater resistance to heat stress, and reduced lipofuscin accumulation. These results highlight the superior anti-aging benefits of the Glabridin and Tatarol combination over their individual use, providing critical insights for future research in the field of anti-aging therapies.

#### 4.2 GT-combination enhances the antioxidant capacity in *C. elegans*

The free radical hypothesis, or oxidative stress theory, posits that free radicals can oxidize various biomolecules, significantly contributing to the aging process<sup>[20]</sup>. When organisms encounter adverse stimuli, they produce ROS<sup>[21]</sup>. Excessive ROS can overwhelm cellular defenses, leading to oxidative damage and accelerated aging. The body's antioxidant defense system, including enzymes like SOD, CAT, and glutathione peroxidase (GPx), plays a crucial role in mitigating this damage<sup>[22]</sup>.

Our findings highlights the superior antioxidant effects of Glabridin and Tatarol when used in combination, particularly in reducing ROS levels. The combined treatment significantly upregulated the expression of antioxidant enzymes such as SOD, CAT, and glutathione in *C. elegans*, enhancing their ability to combat oxidative stress. Notably, the treatment also modulated the

expression of antioxidant genes *sod-3* and *skn-1*, further reinforcing the organism's defense mechanisms. In contrast, treatments with Glabridin or Tatarol alone did not achieve the same level of efficacy in reducing ROS and MDA levels, a toxic byproduct of lipid peroxidation. The combination therapy led to a marked decrease in ROS levels, thereby improving the resilience of *C. elegans* against oxidative stress more effectively than either compound used individually. These findings underscore the potential of Glabridin and Tatarol as synergistic agents in enhancing antioxidant defenses, suggesting that their combined application may be a promising strategy for delaying aging processes by more effectively mitigating oxidative stress.

#### 4.3 GT-combination regulates metabolic pathways around NAD<sup>+</sup> in *C. elegans*

The degradation of fatty acids and the accumulation of lipids play a crucial role in the aging process of the body. Aging leads to an imbalance in the body's metabolic homeostasis, which in turn leads to abnormalities in lipid metabolism. This is directly manifested by a decrease in the metabolic efficiency of intracellular fatty acids and a gradual weakening of lipid degradation pathways, leading to abnormal accumulation of lipids in tissues. The lipid accumulation not only triggers metabolic disorders but may also promote an increase in oxidative stress, exacerbating cellular damage<sup>[23]</sup>. For example, the excessive accumulation of fatty acids increases the oxidative risk of cell membranes, thereby affecting the function of membrane proteins and mitochondria, leading to a disorder of energy metabolism<sup>[24]</sup>. Our research found that the combined use of Glabridin and Tatarol targets fatty acid metabolism as a key regulatory pathway, indicating that the combined use of Glabridin and Tatarol can delay lifespan by reshaping the function of fat metabolism. It has been reported that unsaturated fatty acids, Omega-3, can slow down organ aging by promoting energy metabolism<sup>[25]</sup>. Our enrichment results also confirm that the combined use of Glabridin and Tatarol may trigger the interaction between lipid metabolism and energy homeostasis in the body during the aging process.

We also found that the combination affected the butyrate metabolism pathway in *C. elegans*. Butyrate, as a short-chain fatty acid present in the gut, is mainly produced through the fermentation of dietary fiber and supplies energy through fatty acid oxidation<sup>[26]</sup>. This further illustrates the potential effect of Glabridin and Tatarol on lipid metabolism. The phenylalanine metabolism pathway was also affected after the combination. There is evidence that during the aging of the heart, the level of phenylalanine increases, leading to an imbalance in phenylalanine catabolism through the liver-heart axis, becoming a new characteristic of heart aging<sup>[27]</sup>. Our research supports the characteristic role of phenylalanine metabolism in the aging process and describes various regulatory patterns of aging after the combined use of Glabridin and Tatarol.

#### 4.4 GT-combination regulates gene expression related to ROS or NAD<sup>+</sup> in *C. elegans*

The combined administration of Glabridin and Tatarol elicited higher expression levels of these genes than when administered individually, suggesting a synergistic interaction in promoting longevity. The enhanced expression of *daf-16*, a pivotal transcription factor, indicates its central role in mediating the lifespan extension and enhanced resilience observed in *C. elegans*. *daf-16* likely acts as a regulatory hub within the insulin signaling

pathway, orchestrating the upregulation of antioxidant defenses and metabolic adjustments essential for longevity.

These findings indicate that Glabridin and Totarol exert their anti-aging effects by modulating the insulin signaling pathway, leading to decreased *age-1* and *daf-2* activity and increased expression of *daf-16* and its downstream targets. This modulation enhances antioxidant defenses and optimizes NAD<sup>+</sup>/NADH balance, thereby reducing oxidative stress and promoting cellular homeostasis. The synergistic effect of Glabridin and Totarol presents a promising strategy for developing novel anti-aging therapeutics or functional foods. Future studies should focus on delineating the precise molecular interactions and exploring the efficacy of this combination therapy in other model organisms to further validate its potential applications in aging and age-related diseases.

## 5 Conclusion

In summary, our investigation confirms that the combined administration of Glabridin and Totarol exerts significant anti-aging effects in *C. elegans*. The combination exposure notably extended the lifespan of the nematodes by up to 37.3%, surpassing the effects of individual treatments. It enhanced aging indicators, improved motility, reduced ROS levels, and increased oxidative stress resistance. The synergistic effect was linked to the stimulation of the insulin signaling pathway and the activation of the *daf-16* transcription factor, leading to elevated antioxidant enzyme activities. Additionally, the combination modulated aging-related metabolic pathways, providing deeper insights into its mechanism of action. These findings highlight the potential of Glabridin and Totarol as a promising strategy for developing novel anti-aging therapeutics or functional products.

## Electronic Supplementary Material (ESM)

The online version contains supplementary material available at <https://doi.org/10.26599/FMH.2027.9420160>.

## Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

## Conflicts of interest

The authors declare that there are no conflicts of interest in this work. Qiang Zhang is an Editorial Board Member of Food & Medicine Homology, but was not involved in the processing, peer review or decision making of this article.

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## Author contribution statement

Zhi-Chao Zhang: Formal analysis, methodology, visualization,

data curation. Xiao-Yue Zhang: Visualization, preparation of materials, writing—original draft preparation. Lu-Qi Li: Forma investigation, methodology. Qiang Zhang: Conceptualization, supervision and funding acquisition, writing—editing and review. All authors have read and agreed to the published version of the manuscript.

## Informed consent

Not applicable.

## Ethics statement

The animal experiments were carried out following the guidelines of the animal ethics committee of Northwest A&F University for the Care and Use of Laboratory Animal (Ethical code: XN2024-0403).

## Use of AI statement

None.

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