Ethyl acetate extract of *Gastrodia elata* protects *Caenorhabditis elegans* from oxidative stress and amyloid β peptide toxicity

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Abstract. *Gastrodia elata* Blume is a traditional Chinese medicine with a long history, which has numerous pharmacological activities, such as anti-inflammation, anti-oxidation and protection of nerves. The present study investigated the regulatory effect of ethyl acetate extract of *Gastrodia elata* (EEGE) on the β-amyloid (Aβ) toxicity of *Caenorhabditis elegans* (*C. elegans*). First, the main components of EEGE were analyzed using high-performance liquid chromatography, and the total phenols, total flavonoids and total antioxidant capacity of EEGE were determined. Next, the regulation effect of EEGE on Aβ-induced toxicity of *C. elegans* was evaluated through experiments on nematode paralysis, lifespan, oxidative and heat stress, locomotor ability, reproductive ability, reactive oxygen species (ROS) level, Aβ aggregation test, malondialdehyde (MDA) level, catalase (CAT) activity and superoxide dismutase (SOD) activity. Finally, the mechanism of EEGE was elucidated using RNA sequencing (RNA-Seq) and the expression levels of related genes were verified using quantitative PCR. The present study revealed that the main components of EEGE included phosphorylated (p)-hydroxybenzyl alcohol, p-hydroxybenzaldehyde and 4,4'-dihydroxydiphenylmethane, possessing strong *in vitro* free radical scavenging and reducing abilities. In addition, after the intervention of EEGE, the paralysis of nematodes could be delayed, the survival time of the nematodes was prolonged, the survival rate of the nematodes under stress (high temperature and oxidation) conditions was improved, the activity capacity and reproductive capacity of the nematodes were improved, the activities of SOD and CAT were improved and the levels of ROS and MDA were reduced. Notably, EEGE directly inhibited Aβ plaque aggregation in nematodes. RNA-Seq analysis showed that EEGE regulated metabolism and longevity-related genes, and these genes were regulated by the insulin/IGF-1 signaling (IIS) pathway. Therefore, the present study hypothesized that the regulatory mechanism of EEGE was significantly related to the IIS pathway. The present research results demonstrated that the protective effect of EEGE on transgenic *C. elegans* was to reduce Aβ protein aggregation, improve the *in vivo* antioxidant level, effectively remove free radicals and to regulate the expression of genes related to IIS pathway, thereby reducing Aβ-induced toxicity and delaying nematode paralysis.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that causes the most common form of dementia worldwide (1). Clinical manifestations of AD include progressive memory loss, language and intellectual disorders, and inability to take care of oneself (2). Amyloid β (Aβ) deposition is one of the main pathological mechanisms of AD. Aβ aggregation can have a direct toxic effect on nerve cells, and oligomers formed by Aβ aggregation can stimulate the graded activation of inflammation and oxidative stress, which in turn promote further Aβ aggregation in a positive feedback loop (3,4). Therefore, the mechanism of Aβ deposition in AD has been increasingly investigated in recent years (5-8). The drugs currently used to treat AD are symptomatic and have very limited effects on improving the course of the disease (9). According to estimations in the World Alzheimer's Disease Report 2021, there will be >78 million AD patients worldwide by 2030 (https://www.alzint.org/resource/world-alzheimer-report-2021/). Therefore, the development of drugs to prevent and treat AD is urgently needed.

Traditional Chinese medicine and other natural plant-based therapies have been used to prevent and treat AD. For example, Liuwei Dihuang, as a representative prescription for nourishing yin and tonifying kidneys, has a long history of use. Liuwei Dihuang increases antioxidant activity in nematodes by increasing the expression of heat shock proteins (HSPs), and decreasing reactive oxygen species (ROS) levels to alleviate Aβ protein toxicity (10). Polysaccharides from *Coptis chinensis* Franch can regulate the expression of HSPs, thereby delaying aging in nematodes, and can inhibit Aβ deposition and

thus reduce its toxicity (11). This demonstrates that traditional Chinese medicine has the potential to play an increasingly important role in the treatment of diseases.

The dried tuber of the orchid, *Gastrodia elata* Bl., is one of the most valuable Chinese medicines. Pharmacological research shows that *Gastrodia elata* can improve cognitive function, protect nerves and delay aging, and has the potential to treat AD (12-15). The active ingredients of ethyl acetate *Gastrodia elata* extract (EEGE) include phosphorylated (p)-hydroxybenzyl alcohol and p-hydroxybenzaldehyde, which have anti-aging and anti-oxidative stress effects and regulate Aβ protein, which may be key in the treatment of AD (16). However, the mechanism by which EEGE regulates Aβ is not clear.

Transgenic *Caenorhabditis elegans* (*C. elegans*) can express human Aβ in muscle cells and neurons. This is a powerful model for elucidating the mechanisms of AD and for studying AD-related drugs (17,18). Therefore, the present study used a transgenic *C. elegans* model of AD to study how EEGE affects Aβ toxicity.

Materials and methods

Chemicals and reagents. 4-Hydroxybenzyl alcohol, 4-hydroxybenzaldehyde and 4,4-dihydroxydiphenylmethane were purchased from Chengdu Alfa Biotechnology Co., Ltd.; trolox and 2,4,6-tripyridine-s-triazine (TPTZ) were purchased from Macklin Biochemical Co., Ltd.; 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Shanghai Yi En Chemical Technology Co., Ltd.; and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd.

Strains and maintenance. The *C. elegans* strains employed in the present study were: Wild-type N2 (Bristol), the transgenic strains CL2006 {dvls2 [pCL12(unc-54/human Aβ1-42) + rol-6 (su1006)]}, CL4176 {dvIs27 [myo-3p::Aβ (1-42)::let-851 3'UTR] + rol-6(su1006) X}. *Escherichia coli* OP50 was obtained from the Caenorhabditis Genetics Center (University of Minnesota). CL4176 is a temperature-sensitive transgenic model in which the Serine/threonine-protein kinase 1 system is inactivated when the temperature increases from 16 to 25˚C, which leads to overexpression of Aβ in muscles and a paralysis phenotype. All *C. elegans* were cultured on solid nematode growth medium (NGM; consisting of 300 ml deionized water including 0.82 g peptone, Oxoid Limited; Thermo Fisher Scientific; 1.2 g NaCl, Tianjin Fengchuan Chemical Reagent Co., Ltd.; 5.15 g agar, Beijing Solarbio Science & Technology Co., Ltd.; autoclaved and supplemented 1 mM $MgSO_4$, 1 mM $CaCl_2$, Tianjin Fengchuan Chemical Reagent Co., Ltd.; 12.9 mM cholesterol solution, Macklin, Inc.) containing *E. coli* OP50. CL4176 was maintained at 16˚C, while N2 and CL2006 were maintained at 20˚C.

Preparation of EEGE and analysis of its main components by high-performance liquid chromatography (HPLC). *Gastrodia elata* Bl. was purchased from Yunnan Huide Pharmaceutical Co., Ltd. The samples were collected from artificially cultivated *Gastrodia elata* in Xiaocaoba (Yiliang County, Zhaotong, Yunnan, China), and analyzed by Associate Professor Zili Yin, Yunnan University of Chinese Medicine (Kunming, China). Drug extraction was performed as previously described (19). The specified amount of *Gastrodia elata* Bl. was extracted three times with 95% ethanol under heating reflux at 85˚C. The organic solvent was evaporated under reduced pressure and the remaining extract was dissolved in deionized water and extracted with ethyl acetate three times. Samples were concentrated in a rotary evaporator at 60˚C under reduced pressure, and dried. The obtained EEGE was analyzed by HPLC (Agilent 1290 InfinityIIHPLC system; Agilent Technologies, Inc.). The gradient elution process was performed as follows on a ZORBAX SB-C18 column (4.6x250 mm; 5 μm; Agilent Technologies, Inc.). Mobile phase A was acetonitrile, and mobile phase B was water (0-25 min, from 13% A to 58% A; 25-26 min, from 58% A to 100% A). The detection wavelength was 221 nm. EEGE and standards were dissolved in methanol, the flow rate was 1.0 ml/min, and the column was at room temperature, with a sample quantity of 10 μl.

Determination of total flavonoid and total phenol amount and total antioxidant capacity. Total phenolic content of EEGE was measured by the Folin-Ciocalteu method (20). Briefly, gallic acid of known concentration was used as a standard and Folin-Ciocalteu reagent was allowed to react with different concentrations of standard and sample for 5 min, Na_2CO_3 was then added, mixed thoroughly, and incubated at room temperature in the dark for 2 h. Absorbance at 760 nm was then measured. The total flavonoid content was determined with reference to Navarro-Hortal *et al* (21). Rutin was used as a standard. The samples were reacted with $NaNO_2$ for 6 min and then incubated with $AlCl_3$ for 5 min (at room temperature). Finally, 4 ml NaOH was added, thoroughly mixed and reacted at room temperature for 5 min. Absorbance was determined at 510 nm. The results for total phenolic and flavonoid content are expressed as mg gallic acid equivalent/g dry extract and mg rutin equivalent/g dry extract, respectively. The total antioxidant capacity of EEGE was evaluated by three methods including ferric reducing antioxidant power (FRAP), DPPH and ABTS. In the FRAP method (22), when iron and TPTZ are complexed in sodium acetate solution, the color changes and the absorbance is measured at 593 nm, which can be used to evaluate the ability of samples to reduce Fe^{3+} to Fe^{2+}. DPPH detection was performed according to Qadir *et al* (23). At a wavelength of 517 nm, the stronger the reducing power of the compound in the sample, the faster the color elimination of DPPH. Finally, oxidation of ABTS by $K_2S_2O_8$ results in green $ABTS^+$ free radicals (24). At a wavelength of 734 nm, the stronger the anti-oxidation ability of the sample, the faster the color elimination of $ABTS^+$ radicals. Absorbance was measured using a Varioskan Flash instrument (Thermo Fisher Scientific, Inc.). Each experiment was repeated three times. The results are expressed as mM trolox equivalent/g dry extract.

Paralysis assays. CL4176 worms synchronized to the L1 stage were transferred to 35 mm culture plates with or without EEGE (0.125, 0.25, 0.5, 1 and 2 mg/ml) and cultured at 16˚C for 36 h. The temperature was then raised to 25˚C for transgene induction. After culture at 25˚C for 24 h the paralyzed

C. elegans were observed every 2 h. Worms were considered paralyzed when they did not move and did not respond to platinum wire stimulation.

Lifespan assay. L4 stage CL4176 nematodes were transferred to NGM plates with or without EEGE. Three replica plates and no less than 70 nematodes for each group were prepared. The nematodes were incubated at 16˚C. To prevent the influence of egg and larval development on the nematode counts, oviposition was inhibited by adding 12 mM fluorouracil to the NGM medium. The number of nematodes surviving on each culture plate was counted every 2 days until all nematodes died. *C. elegans* death was determined by the absence of movement and swallowing, and no reaction after being touched by a platinum wire. Nematodes that burrowed into the agar or climbed the wall of the plate and died of desiccation were excluded from the statistics. The experiment was repeated independently three times.

Heat stress resistance assays. L4 stage N2 nematodes were transferred to NGM plates with or without EEGE. Three replica plates and no less than 70 nematodes for each group were prepared. The nematodes were incubated at 20˚C for 48 h. The temperature was then changed to 35˚C, and the number of nematodes surviving in each culture plate was counted every hour until all nematodes died. *C. elegans* death was determined by the absence of movement and swallowing, and no reaction after being touched by a platinum wire. The experiment was repeated independently three times.

Juglone induction of stress in wild-type N2. The effect of EEGE on juglone-induced oxidative stress was evaluated using wild-type N2 worms (10). N2 nematodes were cultured to the L1 stage after synchronization, and transferred to NGM plates with or without EEGE. A total of three replica plates for each group were prepared. The nematodes developed to the L4 stage at 20˚C and were then transferred to NGM plates supplemented with juglone (300 μM) (Shanghai Yuanye Biotechnology Co., Ltd.). The survival of the nematodes was observed every hour until all had died. Worms that were rigid and unresponsive to light and slight vibrations were recorded as dead.

Locomotion assay and reproduction assay. The reproductive and locomotor abilities of *C. elegans* are physiological markers related to senescence (25). After synchronization, N2 nematodes were cultured at 20˚C to the L4 stage and transferred to NGM medium with or without EEGE (0.5 or 1 mg/ml), with three parallel plates in each group and at least 15 nematodes in each plate. After culture for 48 h, nematodes were transferred to blank NGM medium to observe the number of sinusoidal movements within 20 sec. A reproduction assay was performed according to Meng *et al* (26); two N2 nematodes at the L4 stage were selected from each group and fed separately at 20˚C (three replica plates were prepared for each group). This was recorded as the first day of the reproduction assay. They were transferred to new plates every 24 h until the reproductive capacity of the nematodes was lost. The egg-laying boards were incubated at 20˚C for 48 h and the number of offspring was counted (in this experiment, the number of nematode offspring indirectly reflected the number of eggs laid).

Cytosolic ROS measurement. Cytoplasmic ROS were detected as reported (27) after nematodes were incubated for 4 h with a fluorescent probe, 2,7-dichlorofluorescein diacetate at 25 μM. The probe becomes fluorescent after combining with reactive oxygen species in the cytoplasm. N2 nematodes were synchronized and cultured to the L1 stage, then transferred to an NGM culture plate with or without EEGE, and developed to the L4 stage at 20˚C. Then, 5 mM paraquat (Aladdin) was added to the NGM plate and the nematodes incubated for 4 h. The nematodes were then rinsed with M9 buffer solution, placed on a slide and covered with a cap. The nematodes were observed using a positive fluorescence microscope (Axio Scope A1; Carl Zeiss AG). The fluorescence intensity was quantified using ImageJ v1.8.0. software (National Institutes of Health).

Fluorescent staining of Aβ deposits. Transgenic *C. elegans* strain CL2006 synchronized to the L4 stage (early adult stage) was inoculated on NGM plates with or without EEGE. N2 worms were used as a negative control for Aβ deposition (28). After incubation at 20˚C for 48 h, worms were collected with M9 buffer and fixed in 4% paraformaldehyde/PBS (pH 7.4) (cat. no. BL539A; Biosharp Life Sciences) at 4˚C for 24 h. The worms were then incubated in 5% β-mercaptoethanol (cat. no. M828395; Macklin, Inc.), 1% Triton X-100 (cat. no. MB2486; meilunbio) and 125 mM Tris (pH 7.4) (cat. no. T8060; Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 24 h. The worms were then stained with 0.125% thioflavin S (cat. no. S19293; Shanghai Yuanye Biotechnology Co., Ltd.) in 50% ethanol at room temperature for 2 min, and then rinsed in 50% ethanol 2-3 times. The worms were then placed on a glass slide for observation under a laser scanning confocal microscope (LSM900; Carl Zeiss AG). The amount of thioflavin S deposition in the prepharyngeal region of each nematode was scored to quantify amyloid deposits.

Measurement of ROS, malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) in nematodes. With reference to Song *et al* (6), CL4176 nematodes were synchronized at 16˚C for 48 h, cultured at 25˚C for 40 h then rinsed twice with M9 buffer to remove *E. coli*. The nematodes were then homogenized and protein abundance was measured by the bicinchoninic acid assay (Beyotime Institute of Biotechnology). The levels of MDA, SOD and CAT were determined using Total Superoxide Dismutase Assay (Beyotime Institute of Biotechnology), Catalase Assay (Beyotime Institute of Biotechnology) and Malondialdehyde Detection (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. ROS accumulation was determined with reference to Wang *et al* (29). Briefly, 50 μl of nematode supernatant was added to each well of a 96-well plate and 50 μl of 100 μM DCFH-DA solution (a fluorescent probe) (Beyotime Institute of Biotechnology) was added to give a final DCFH-DA concentration of 50 μM, The solutions were thoroughly mixed by shaking for 30 sec. Fluorescence detection was performed using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Detection was conducted once every 10 min, and ROS changes within 100 min were counted.

RNA-sequencing (RNA-seq) analysis. Gene expression in transgenic CL4176 nematodes treated with EEGE (1 mg/ml) and controls was analyzed by RNA-seq. Extraction and purification of total RNA, library construction and sequencing were performed at Beijing Fruit Shell Biotechnology Co., Ltd. using the Illumina Novaseq 6000 system (Illumina, Inc.). The quality of the data sets was evaluated using an Agilent bioanalyzer 2100 (Agilent Technologies, Inc.). Transcript levels were estimated using fragments per kilobase of transcript per million mapped reads values to allow different genes or samples to be compared. Settings: Two-fold change in expression levels and a false discovery rate with a P-value <0.05 were used to screen the RNA-seq data for differentially expressed genes (DEGs). All analyses were performed at Beijing Fruit Shell Biotechnology Co., Ltd. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs was carried out using the DAVID (https://david.ncifcrf.gov/) online platform. The R package ggplot2 (version 3.3.2) (30) was used to generate volcano, GO and KEGG bubble maps to visualize the distribution of DEGs.

Validation of the RNA-seq results via reverse transcription-quantitative PCR (RT-qPCR). To verify the RNA-seq results, synchronized L1 stage CL4176 nematodes were transferred onto NGM plates with or without EEGE (1 mg/ml) (~500 nematodes per plate) and cultured at 15˚C for 36 h. The temperature was then raised to 25˚C, and the culture continued for ~40 h. The nematodes were then collected with M9 buffer into an EP tube, and washed three times. Total RNA was extracted from nematodes using an RNA extraction kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. RNA concentration and purity were measured using an ultra-micro spectrophotometer (SMA6000; Merinton Instrument, Ltd.). cDNA (Promega Beijing Biotech Co., Ltd.) was generated by RT-PCR in a PCR instrument (Veriti™ 96-Well Fast Thermal Cycler; Applied Biosystems; Thermo Fisher Scientific, Inc.), and then the target mRNA was quantified (GoTaq® qPCR and RT-qPCR Systems, Promega Beijing Biotech Co., Ltd.) using a real-time PCR instrument (C1000 Touch PCR; Bio-Rad Laboratories, Inc.). RT-qPCR was performed with the following cycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 55˚C for 30 sec, 72˚C for 30 sec and then maintained at 4˚C. Relative gene expression was calculated by the $2^{-\Delta\Delta Cq}$ method, using β-actin as a housekeeping gene (31). The analysis was performed in triplicate for each group. Primer 3 Plus (https://www.primer3plus.com/) was used to design the primers and they were synthesized by TsingKe Biological Technology. The primers are listed in Table SI.

Statistical analysis. Using GraphPad prism 8.0.0 (GraphPad Software, Inc.) statistical software for analysis and processing, if the data conformd to the normal distribution and the variance was uniform (P>0.05), ANOVA followed by Bonferroni's multiple comparison test was used. For lifespan and paralysis assays, Kaplan Meier survival was utilized and P-values were calculated using the log-rank test. P<0.05 was considered to indicate a statistically significant difference, and all values were expressed as means ± standard deviation. All experiments were repeated three times.

Table I. Quality analysis of EEGE.

Parameter	Mean ± SEM
Total flavonoids content (mg rutin equivalent/g EEGE)	95.41±1.77
Total phenolic content (mg gallic acid equivalent/g EEGE)	3.47±0.06
FRAP (μM TE/g EEGE)	68.89±5.18
DPPH (mM TE/g EEGE)	7.51±0.01
ABTS (mM TE/g EEGE)	0.53±0.11
4-Hydroxybenzyl alcohol (mg/g EEGE)	32.40±0.07
4-hydroxybenzaldehyde (mg/g EEGE)	36.25±0.05
4,4-Dihydroxydiphenylmethane (mg/g EEGE)	11.18±0.05

ABTS, 2,2'-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; FRAP, ferric-reducing antioxidant power; TE, trolox equivalent; EEGE, ethyl acetate extract of *Gastrodia elata*; SEM, standard error of mean.

Results

Quality control of EEGE. As revealed by HPLC analysis, three main compounds were detected in EEGE, namely: 4-Hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4,4-Dihydroxydiphenylmethane (Fig. 1A and B). All three compounds have benzene rings in their structures (Fig. 1C). The total phenols, total flavonoids, total antioxidant capacity (DPPH, FRAP and ABTS) and relative contents of main compounds of EEGE are presented in Table I. These results indicate that EEGE has a strong antioxidant capacity *in vitro*.

Effects of EEGE on paralysis. The present study first studied the effect of different concentrations of EEGE (0.125, 0.25, 0.5, 1 and 2 mg/ml) on the Aβ-induced toxicity of the transgenic *C. elegans* strain CL4176. None of the concentrations were lethally toxic to CL4176 nematodes (Fig. 2A). Compared with the control group, 0.125 mg/ml EEGE had no significant effect on CL4176 nematodes (P>0.05). EEGE (0.25, 0.5, 1 and 2 mg/ml) could prolong the paralysis time of nematodes. The drug effect was dose-dependent within the concentration range of 1 mg/ml, and the efficacy was weakened when the drug concentration reached 2 mg/ml. Therefore, 1 mg/ml was the optimal concentration for EEGE. These results suggested that the potential of EEGE to protect CL4176 from Aβ-induced toxicity.

Effect of EEGE on lifespan. Senescence plays an important role in the development of AD. Survival analysis showed that EEGE treatment significantly shifted the survival curve of CL4176 to the right (Fig. 2B). Compared with the control group, 0.5 and 1 mg/ml EEGE increased the maximum lifespan of CL4176 nematodes by 10.0% (P<0.01), 20.0% (P<0.01), respectively. These data indicated that EEGE could delay the senescence of CL4176 nematodes.

Effect of EEGE on heat stress. Compared with the control group, 0.5 and 1 mg/ml EEGE could delay the survival rate of

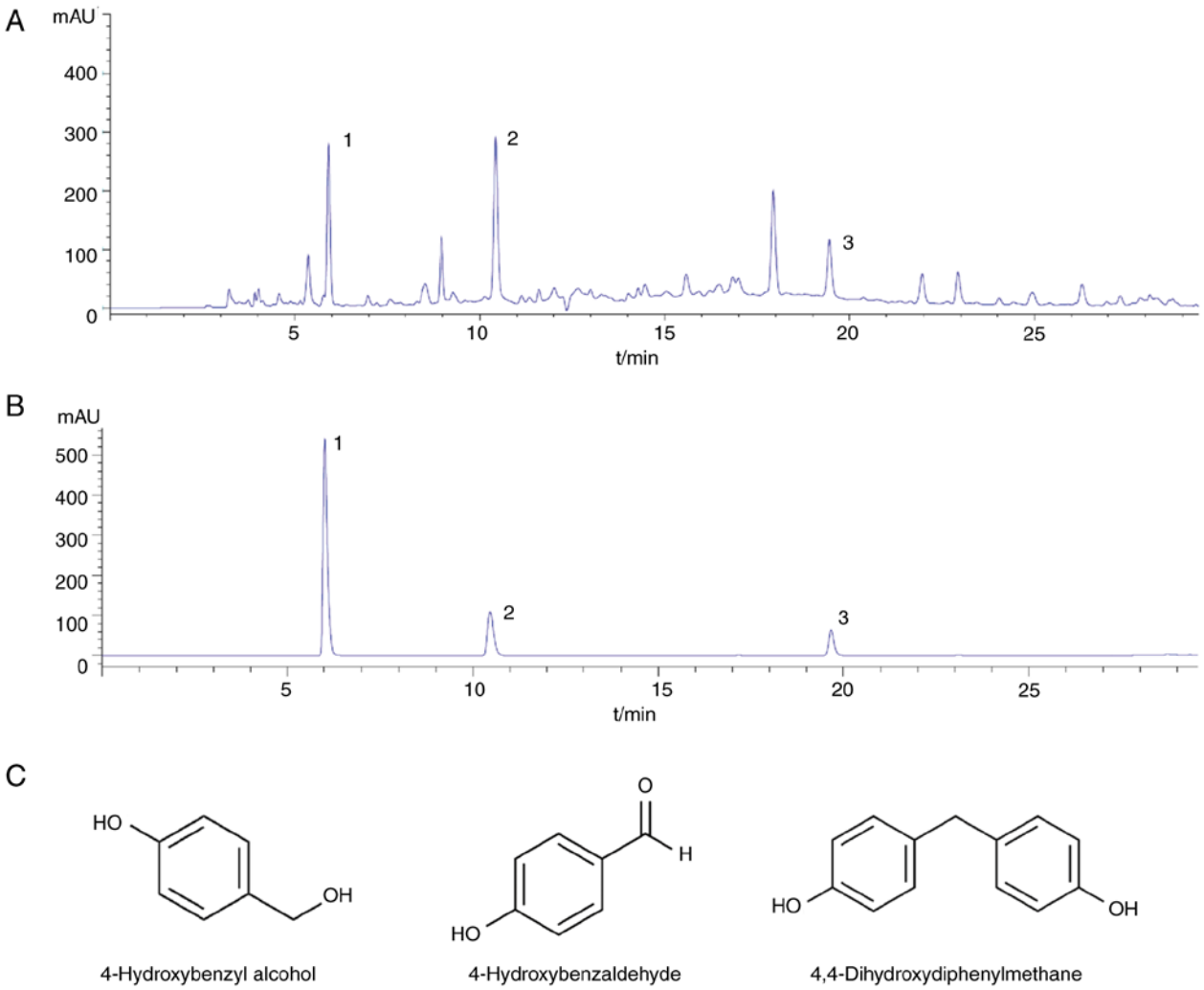

Figure 1. Composition analysis of EEGE. (A) Chromatogram of EEGE. (B) Chromatogram of standard. (C) Chemical structure of p-hydroxybenzyl alcohol, p-hydroxybenzaldehyde and 4,4'-dihydroxydiphenylmethane. EEGE, ethyl acetate extract of *Gastrodia elata*; p-, phosphorylated.

N2 nematodes under heat stress induced by high temperature (P<0.01; Fig. 2C), although the mortality time of *C. elegans* could not be prolonged, the overall survival rate of *C. elegans* was increased. The results showed that EEGE could improve the heat stress resistance of *C. elegans*.

EEGE reduces oxidative stress in C. elegans. The present study further revealed that EEGE could also protect wild-type N2 nematodes from oxidative stress produced by juglone, and after 6 h of juglone stress, all nematodes in the control group died, which was not significantly different compared with the control group with 0.5 mg/ml EEGE (P>0.05), and the maximum survival rate of the worms in the 1 mg/ml EEGE group was significantly improved by 16.7% (P<0.01; Fig. 2D). The results showed that the worms subjected to EEGE intervention exhibited significant protection against oxidative stress induced by juglone (300 μM).

Effect of EEGE on reproduction and locomotion. As the locomotor behavior of *C. elegans* decreased with age, the present study investigated whether EEGE affected the locomotor ability of *C. elegans*. Compared with the control group, EEGE intervention increased the activity of N2 wild-type nematodes (Fig. 2E), and the effect of 1 mg/ml EEGE was the most significant (P<0.01). Nematodes begin to lay eggs when they enter the adult stage. As the nematodes gradually aged, their egg-laying rate gradually decreased and reached the peak of growth on the 3rd day of adult life. Compared with the blank group, the egg laying rate of the EEGE group was not significantly different on Days 1 to 3 (P>0.05; Fig. 2F), and the egg laying rate was significantly increased on Days 4 to 6. 1 mg/ml EEGE could significantly increase the total oviposition of nematodes (P<0.05), while 0.5 mg/ml EEGE had no statistical significance (P>0.05). The results showed that EEGE could increase the number of nematode progeny and improve the reproductive ability of nematode.

Effects of EEGE on ROS production. Paraquat can induce the increase of free radicals in *C. elegans*, and EEGE has strong antioxidant capacity *in vitro* (27). The present study further determined the effect of EEGE on ROS in *C. elegans*. The higher the amount of green fluorescence in nematodes, the more ROS accumulation. Compared with the control group, the green fluorescence in EEGE group was decreased (Fig. 3A), and the fluorescence intensity of ROS per unit area was significantly decreased (P<0.01; Fig. 3B). The results

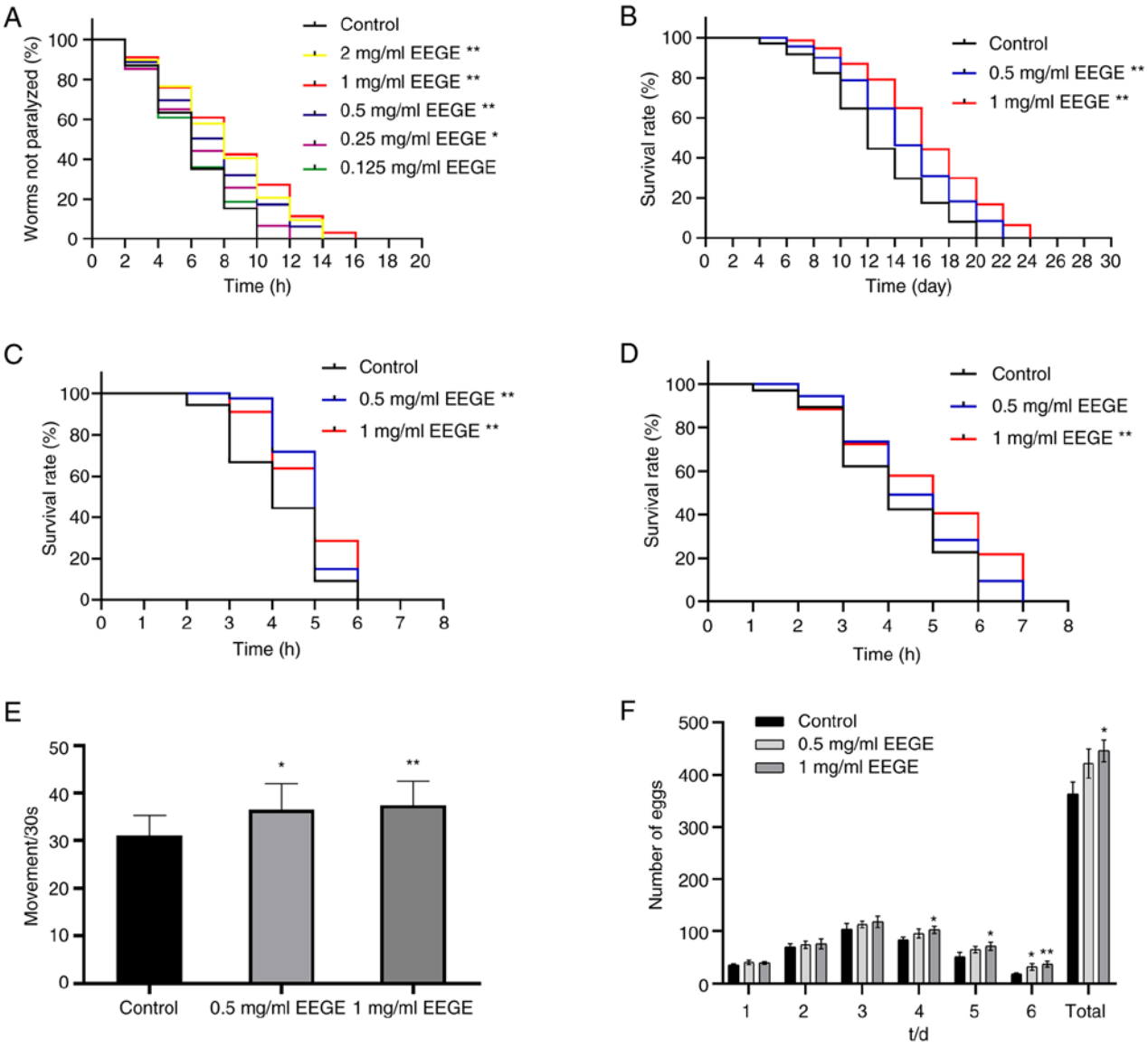

Figure 2. Effect of EEGE on *C. elegans*. Effects of EEGE on (A) paralysis, (B) life span, (C) heat stress, (D) Juglone-induced oxidative stress, (E) locomotor ability and (F) reproductive capacity of *C. elegans*. *P<0.05 and **P<0.01 vs. the Control. EEGE, Ethyl acetate extract of *Gastrodia elata*.

showed that EEGE could inhibit the generation of free radicals and improve the antioxidant capacity of *C. elegans*.

Effects of EEGE on Aβ aggregation. The present study observed the formation of amyloid fibrils using the thioflavin-S fluorescence method to study the effect of EEGE on the aggregation of Aβ. The transgenic *C. elegans* strain CL2006 used in the present study expressed Aβ protein fragments that are associated with the development of AD. *C. elegans* demonstrated that the expression and aggregation of Aβ in muscle led to progressive paralysis. CL2006 were stained with triterpenes for Aβ at the end of EEGE treatment (showing a green fluorescent spot). Fluorescence imaged of the heads of CL2006 nematodes demonstrated that, compared with untreated worms (negative control), Aβ deposition in *C. elegans* treated with EEGE was significantly decreased. Wild N2 strain has no Aβ deposition in the whole animal (Fig. 3C). EEGE (0.5 and 1 mg/ml) significantly reduced the number of Aβ oligomers (P<0.01; Fig. 3D), and these results indicated that EEGE directly inhibited the aggregation and deposition of Aβ in transgenic nematode muscle cells, thereby delaying nematode paralysis.

Effects of EEGE on SOD and CAT activities, and MDA and ROS levels. Oxidative stress has been shown to play an important role in Aβ-induced toxicity (32). The present study investigated the effects of EEGE on Aβ-induced SOD, CAT, MDA and ROS. As shown in Fig. 4, compared with the control group, EEGE was able to increase the CAT and SOD activities in nematodes (P<0.01; Fig. 4A and B), reduce the MDA level in nematodes (P<0.01; Fig. 4C), and inhibit the rising trend of ROS *in vivo* (P<0.01; Fig. 4D), indicating that after intervention with EEGE, the expression of antioxidant enzymes such as CAT and SOD in nematodes was increased, the accumulation of lipid peroxides was reduced, and the generation of free radicals was reduced to improve the antioxidant capacity of the body, thereby reversing the symptoms of AD to exert neuroprotective function.

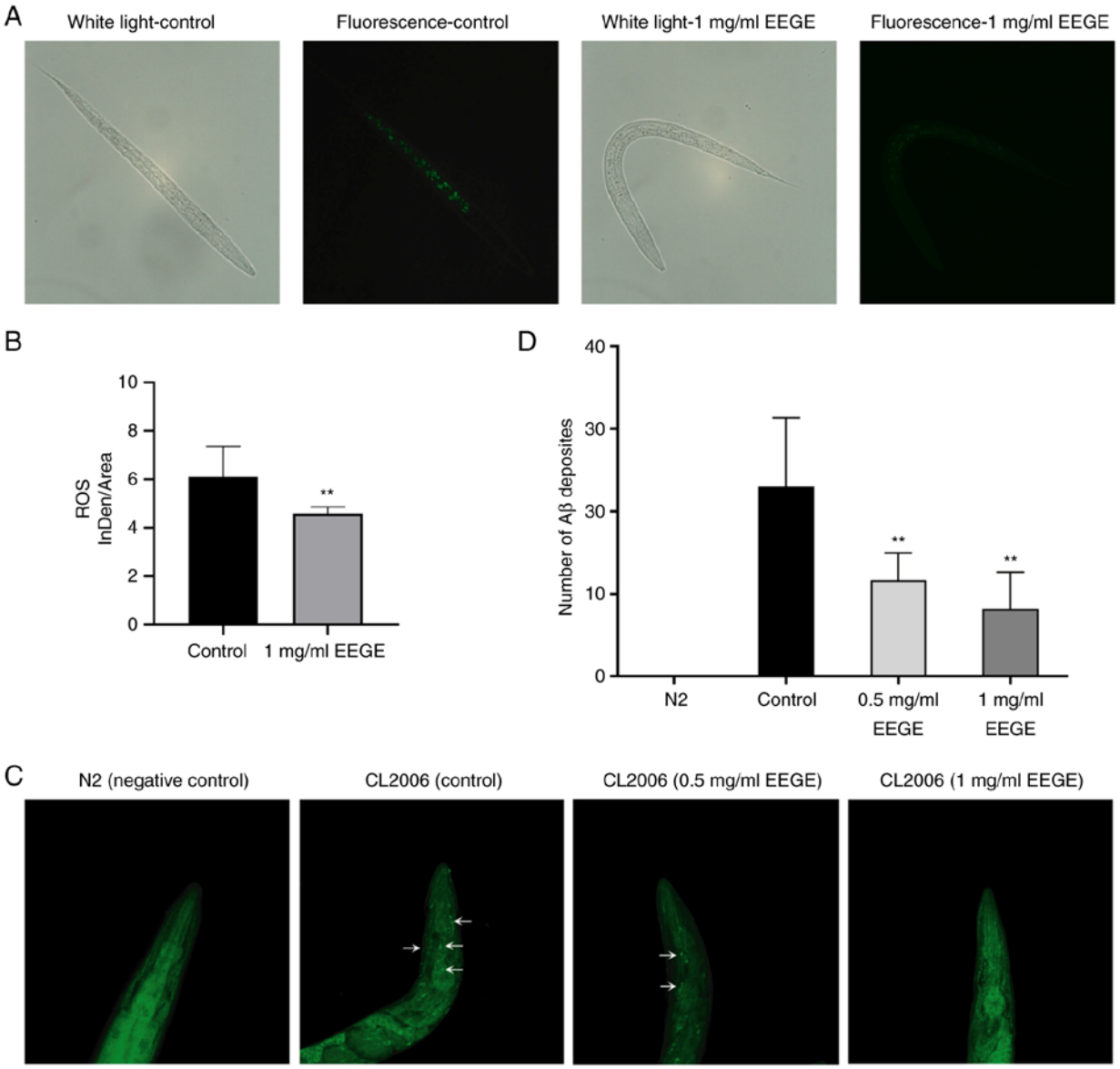

Figure 3. EEGE decreases ROS and Aβ accumulation in *C. elegans*. (A) ROS accumulation in *C. elegans*. (B) ROS fluorescence value per unit area. (C) Effect of EEGE on Aβ aggregation in N2 and CL2006 *C. elegans*. (D) Deposition of Aβ in *C. elegans*. The white light and fluorescence images of ROS in N2 *C. elegans* were observed under a fluorescence microscope (magnification, x100). The fluorescence images were observed in N2 and CL2006 *C. elegans* after Thioflavin S staining by a laser scanning confocal microscope (magnification, x200). **P<0.01 vs. the Control. EEGE, Ethyl acetate extract of *Gastrodia elata*; Aβ, β-amyloid; ROS, reactive oxygen species.

Genome-wide transcriptional profiling of transgenic C. elegans treated with EEGE. A total of 763 DEGs were identified by RNA-Seq analysis, including 145 upregulated genes and 618 downregulated genes (Fig. 5A). The present study demonstrated the biological process in GO analysis. GO analysis showed that the regulatory mechanisms of EEGE involved 25 biological processes such as 'innate immune response', 'transmembrane transport' and 'lipid metabolic process' (Fig. 5B). In addition, nine pathways were identified by KEGG enrichment analysis, which were related to, for example, 'metabolic pathways', 'lysosome' and 'longevity regulating pathway-worm' (Fig. 5C). The results demonstrated that the treatment of diseases with complex pathogenesis by a single target is limited, highlighting the advantages of multi-component and multi-target treatment of diseases by traditional Chinese medicine.

Validation of DEGs using qPCR. To verify the results of RNA-Seq, qPCR analyses were performed. A total of 11 genes involved in nematode longevity, oxidative stress, immunity, aging and regulation of Aβ protein were selected for verification. These 11 genes and their functions were considered to be closely related to AD pathogenesis (Table II). Compared with the control group, the expression levels of gst-4, gst-25, hsp-12.3, hsp-12.6, ugt-37 and ugt-63 genes were upregulated (Fig. 6A) and the expression levels of fat-7, ins-7, ins-23, rgba-1 and dod-22 genes were downregulated (P<0.05) (Fig. 6B). These 11 genes each showed the same tendency as observed in the RNA-Seq experiments, and the present study inferred that the EEGE regulation mechanism was likely related to the insulin pathway based on the function and characteristics of the genes.

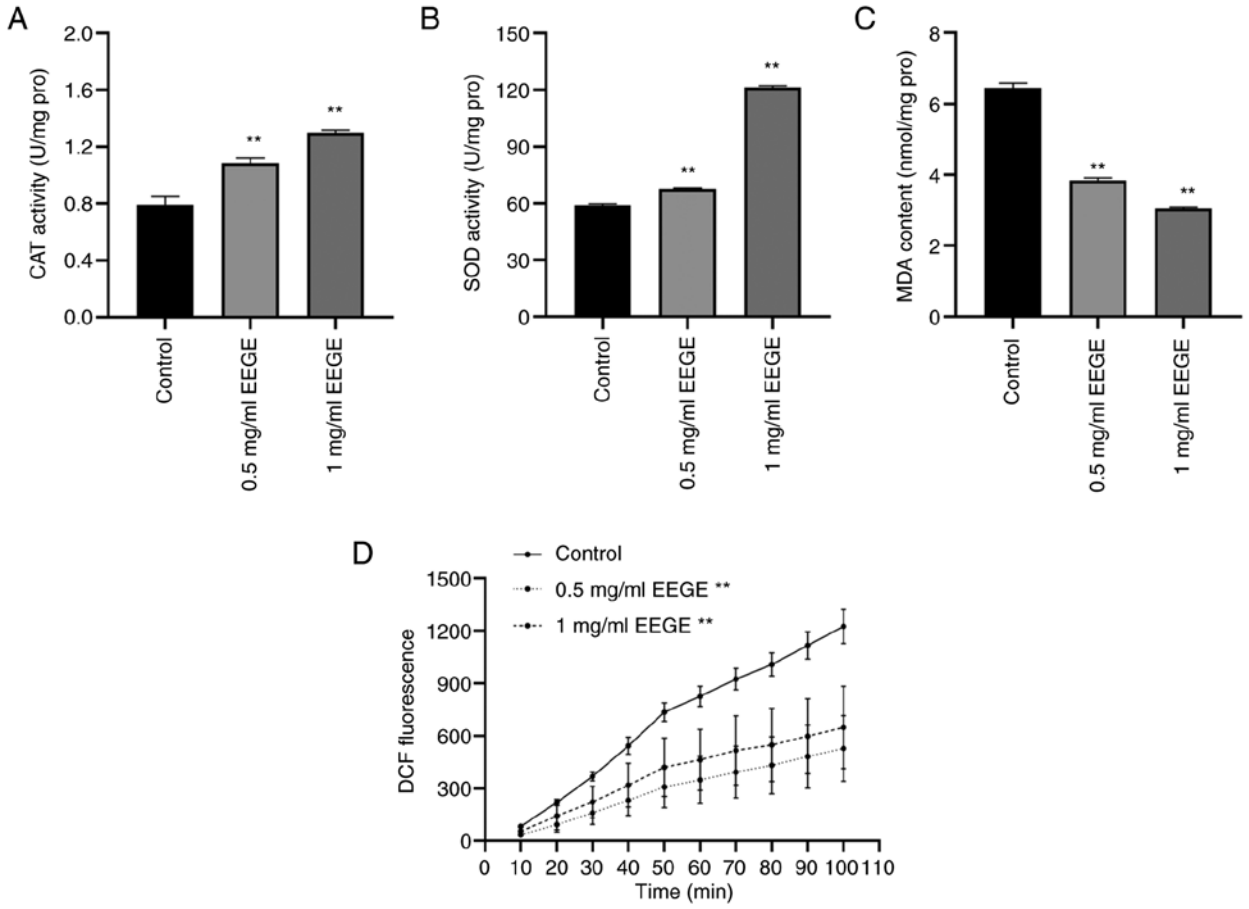

Figure 4. Effects of EEGE on antioxidant enzymes and ROS levels in *C. elegans*. (A) CAT activity. (B) SOD activity. (C) MDA content. (D) Continuous changes of ROS fluorescence in *C. elegans*. **P<0.01 vs. the Control. ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; EEGE, Ethyl acetate extract of *Gastrodia elata*.

Discussion

At the forefront of AD research is the mechanism of Aβ deposition (33). *Gastrodia elata* Bl. can improve the memory of rats given bilateral hippocampal injections of Aβ25-35 by reducing Aβ deposition in the hippocampus, and can have a protective effect in this AD rat model (34). However, its anti-Aβ effect has not been systematically studied. Therefore, the present study investigated the effect of EEGE on Aβ using transgenic *C. elegans* expressing the human Aβ gene (35,36). This revealed that that EEGE intervention delayed the paralysis of *C. elegans*. However, the current study observed a weaker potency of 2 mg/ml EEGE compared with 1 mg/ml EEGE, which may be related to the drug metabolism pattern *in vivo*. Some drugs need transporters to metabolize in the body, and when the dose is too high, there will be overload (25). CL4176 is a strain obtained from wild-type N2 by transgenic technology. The present study did not find a higher mortality rate in the EEGE group compared with in the control group with CL4176. Therefore, the present study considered the concentrations in the experiment to be safe for *C. elegans*. In addition, EEGE not only promoted the movement and reproduction of *C. elegans*, but also extended the life span of *C. elegans*.

The present study then explored the underlying mechanism by which EEGE functions. The activities of SOD and CAT increased, and the levels of ROS and lipid peroxide MDA decreased, indicating that the antioxidant level of *C. elegans* increased. Accumulation of Aβ in the wireworm head was significantly reduced by EEGE (37), which might be key for EEGE reversing the paralytic phenotype of nematodes. These data indicate that EEGE has a protective effect against Aβ-induced neurotoxicity, and that this effect delays senescence in *C. elegans*. AD can be a pathological manifestation of the aging process (38) and the present results indicated that EEGE had an anti-aging effect on the AD model *C. elegans*. Therefore, anti-aging may play an important role in the prevention of AD. The anti-AD effect of EEGE is closely related to its antioxidant properties (39).

The present study determined the main components of EEGE by HPLC. The main constituents of EEGE are p-hydroxybenzyl alcohol, p-hydroxybenzaldehyde and 4,4'-dihydroxydiphenylmethane, all of which are phenolic components of *Gastrodia elata* and have strong antioxidant capacity (19). The present EEGE extraction method yielded higher total phenol and total flavonoids contents compared with that used by Song *et al* (40). P-Hydroxybenzyl alcohol, the active ingredient of *Gastrodia elata*, can reduce ROS accumulation and inhibit Aβ mRNA by regulating the transcription factor FOXO/DAF-16, thereby delaying nematode paralysis and playing a neuroprotective role (41). However, studies separately analyzing p-hydroxybenzaldehyde and 4,4'-dihydroxydiphenylmethane in AD have not been reported

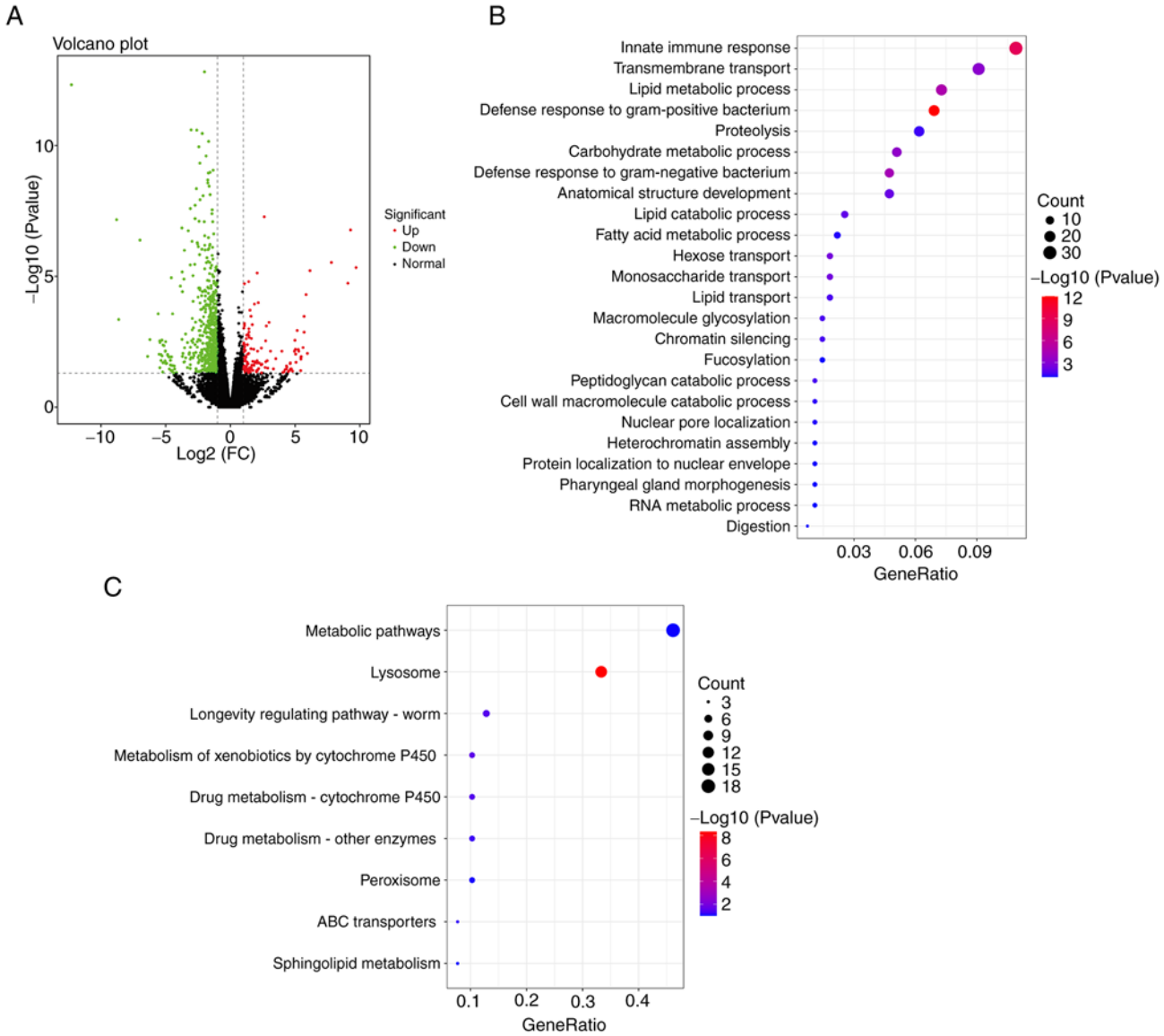

Figure 5. Distribution and enrichment analysis of DEGs of RNA-sequencing. (A) Volcano map of DEGs. Red dots represent upregulated genes, green dots downregulated genes and gray dots represent genes with no significant differences. (B) GO analysis for DEGs. (C) KEGG analysis for DEGs. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

yet. It is well known that traditional Chinese medicine functions through synergistic effects of multiple components on multiple targets, and that the active substances may be a group of components with similar structures. Therefore, the more purified the active substance, the more its biological activity is lost (42,43). The large number of compounds contained in EEGE means that there may be other types of chemical besides phenolic compounds that have anti-AD effects. The present study hypothesizes that p-hydroxybenzaldehyde and 4,4'-dihydroxydiphenyl methane have potential anti-AD activity; therefore, in future studies, the authors will investigate the biological activity of these two components against AD.

The current study used RNA-seq technology to analyze AD-related gene expression changes and to explore the molecular mechanism of EEGE against AD. After querying gene function, 11 genes were identified that might be related to the inhibition of Aβ toxicity by EEGE. They were *gst-4*, *gst-25*, *hsp-12.3*, *hsp-12.6*, *dod-22*, *fat-7*, *ins-7*, *ins-23*, *rgba-1*, *ugt-37* and *ugt-63*. These genes are involved in the regulation of nematode longevity and Aβ protein expression. qPCR showed that the relative expression of all 11 genes to have the same trend as that observed by RNA-seq. Among these genes, *hsp-12.3* and *hsp-12.6* were further studied. *Hsp-12.3* and *hsp-12.6* belong to the HSP family and are regulated by the insulin/insulin-like growth factor-1 signaling (IIS) pathway (44). HSPs are stress-reactive proteins that are expressed in the majority of organisms under heat and oxidative stress. The production of HSPs contributes to longevity extension and stress resistance of nematodes (11). In addition, the IIS pathway also regulates antioxidant genes such as *gst-4* and *gst-25* (45), which helps to increase oxidation in *C. elegans*, thereby prolonging lifespan. *Rgba-1* regulates behavior and aging and can be activated by the mitochondrial unfolded protein reaction regulated by SIR-2.1 (46). *Fat-7* is related to lipogenesis, and can regulate the lipid metabolism pathway of *C. elegans* to inhibit Aβ deposition (47,48). *Ins-7* can reduce oxidative stress to reduce

Table II. Background information of 11 genes.

Gene symbol	Gene name	Gene function
Gst-4	Glutathione S-transferase-4	Enhances the activity of glutathione transferase and participates in the glutathione metabolism. It is an important detoxification enzyme that regulates the oxidation level and lifespan of *C. elegans*
Gst-25	Glutathione S-transferase-25	
Hsp-12.3	Heat shock protein 12.3	Improving the stress resistance of *C. elegans*
Hsp-12.6	Heat shock protein 12.6	
Dod-22	Downstream of DAF-16 protein DOD-22	Involved in the defense response to gram-negative bacteria and innate immune response
Fat-7	δ(9)-fatty-acid desaturase fat-7	Regulating the dysregulation of lipid metabolism associated with Aβ toxicity
Ins-7	Insulin-like peptide 7	It is expected to enhance the hormone activity of *C. elegans* and participate in the regulation of olfaction and learning of *C. elegans*
Ins-23	Insulin-like peptide 23	
Rgba-1	Regulatory gene for behavioral aging-1	Regulating life span and reproduction of *C. elegans*
Ugt-37	UDP-glucuronosyltransferase-37	It can enhance the activity of glucuronosyltransferase and the expression of UGTs can be used to increase antioxidant stress and aging

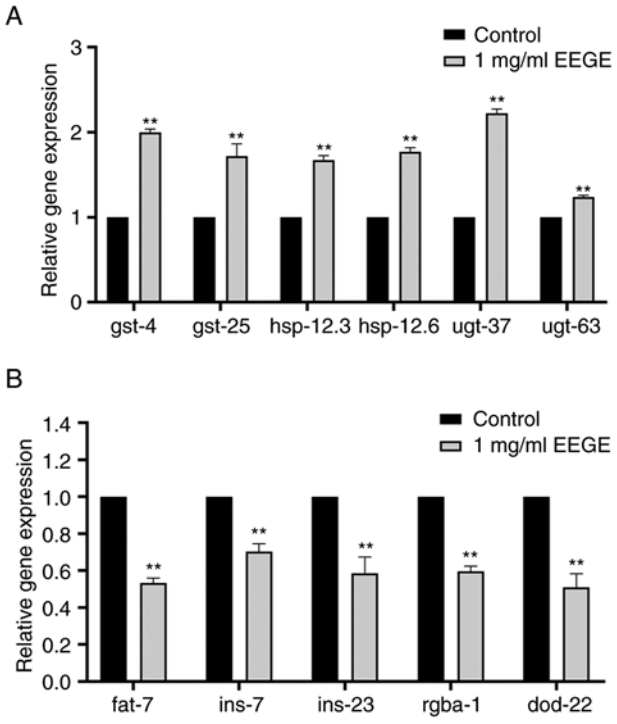

Figure 6. The result verification of RNA-Seq. (A) Validation of up-regulated genes in DEGs. (B) Validation of down-regulated genes in DEGs. **P<0.0 vs. the Control. RNA-Seq, RNA Sequencing; EEGE, Ethyl acetate extract of *Gastrodia elata*.

ROS production, improve neuronal damage and prolong the lifespan of nematodes (49). *Dod-22* is regulated by *daf-2* and *daf-16*, and participates in the regulation of nematode life-span (50).

There are three transcription factors in the IIS pathway: SKN-1, DAF-16 and HSF-1. Insulin signaling plays a central role in regulating metabolism and senescence in nematodes and a number of other species (51-53). KEGG analysis showed DEGS to be significantly related to the regulation of nematode metabolism and longevity. The present study therefore deduced that the regulation of Aβ toxicity by EEGE was likely to occur through the IIS pathway, but this needs verification.

In the IUCN red list, *Gastrodia elata* is listed as a vulnerable species, but, as it is a completely heterotrophic plant, it has been artificially cultivated in China (54). In addition, *Gastrodia elata* has gastrodin, p-hydroxybenzyl alcohol and other components that reduce inflammatory factors, reduce Aβ deposition and other pharmacological activities, which have unique advantages for neuroprotection (12,55). It can be seen that *Gastrodia elata* has great development value in the treatment of neurodegenerative diseases and is a hope for the treatment of dementia (15). However, there are some limitations to the present study. Although *C. elegans* is transferred into human Aβ gene to form Aβ deposition, the specific mechanism of Aβ formation cannot be completely simulated. In addition, *C. elegans* is only a low organism, and the pathogenesis of AD is complex. In the future, we will study the mechanism of action of EEGE in animal models such as rats and mice, in order to obtain data that may be clinically used.

In conclusion, the present study investigated the effect of EEGE on alleviating Aβ toxicity in a nematode AD model. The protective effect of EEGE on transgenic *C. elegans* was to reduce the aggregation of Aβ protein, improve antioxidant levels, effectively remove free radicals and regulate the expression of genes related to the IIS pathway, thereby reducing the toxicity induced by Aβ and delaying the paralysis of *C. elegans*. This study reveals the potential for EEGE to have a positive effect in preventing AD, and also provides a theoretical basis for the prevention and treatment of aging-related diseases by EEGE. It is necessary to further clarify the active compounds in EEGE and to verify their pharmacodynamics using AD models in more complex animals, such as rats.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The sequence data from this study have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra), with accession number SRP440005.

Authors' contributions

XS, XY, LY and XD made considerable contributions to the experimental design, statistical data analysis and English language editing. XD and LY are responsible for drafting the manuscript and revising it for important intellectual content. XS and XY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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