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Ganoderma lucidum methyl ganoderate E extends lifespan and modulates other aging-linked indicators in *Caenorhabditis elegans*

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Supplementary materials and methods

Isolation and structure determination of MGE

The fruiting bodies of *G. lucidium* from Sichuan province were extracted with ethyl alcohol. The organic solvent was filtered and evaporated to dryness under vacuum to obtain the crude extract (53.5 g). The residue was dissolved in H_2O (500 mL) and partitioned with ethyl acetate (500 mL) to yield an ethyl acetate-soluble fraction (25.3 g). The extract was subjected to silica gel CC using dichloromethane-methanol elution (100, 100/1, 100/2, 100/5, 100/10, 0/100, *v*/*v*) to yield 8 fractions (fr.1 to fr.8). Methyl Ganoderate E (MGE) (18.4 mg, t_R 29.7 min) was purified as colorless powder from fr.3 (586.3 mg) by RP-HPLC using 36% acetonitrile in acid water. Its molecular formula was determined as $C_{31}H_{43}O_7$ by HRTOFMS data at *m/z* 527.3012 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.68 (s), 1.64 (s), 1.28 (s), 1.19 (d, *J* = 7.2 Hz), 1.14 (s), 1.12 (s), 0.98 (d, *J* = 6.4 Hz), 0.88 (s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 215.5, 207.7, 206.9, 199.5, 199.4, 176.2, 149.8, 146.8, 57.2, 52.0, 50.8, 49.0, 48.8, 47.0, 46.7, 44.4, 43.9, 39.8, 39.3, 37.3, 34.6, 34.6, 33.8, 32.0, 27.6, 20.9, 20.3, 19.8, 18.6, 17.1, 16.0. ^{1}H NMR and ^{13}C NMR data were consistent with the literature value [1].

***C. elegans* strains and maintenance**

C. elegans strains used in this study include: N2-Bristol (wild type); GR1307 (*daf-16 (mgDF50)I*); PS3551 (*hsf-1(sy441)I*); EU1 (*skn-1(zu67) IV/nT1 (IV;V)*); MQD54 *(hqIs9[pDYH5(pDAF-16::DAF-16::his::gfp)+pRF4(rol-6)]I)*; SJ4005*(zcIs4 [hsp-4::GFP]V)*; CL4176*(smg-1(cc546)I;dvIs27X)*; NL5901 *(pkIs2386[unc54p::alphasynuclein::YFP+ unc-119(+)])*. *Escherichia coli* (OP50) was obtained from the Caenorhabditis Genetics Centre (CGC) (Minneapolis, MN, USA) and used as food source. The strains were cultivated, maintained and assayed at 20°C (unless otherwise stated) on nematode growth medium (NGM) seeded with *E. coli* (OP50) bacteria using the standard protocol [2,3] . All worm strains used for the assay were cultured in the presence of sufficient food to avoid starvation for at least three generations before use. Synchronized populations of worms for all experiments were obtained by filtering a mixed population of properly maintained worms through an 11 µm Nylon Net filter (Merck Millipore) using M9 buffer to obtain the L1 larvae. L1 larvae were cultured to grow to L4 stage before use in assays.

Lifespan assay

Synchronized populations of N2 worms at the L4 stage were transferred to NGM plates containing OP50 with either 10 or 20 µg/ml concentrations of MGE. During the reproductive period, worms were transferred to fresh NGM plates every 2 days to avoid progeny interference and thereafter every 3 days to ensure the availability of fresh food. Alive and dead worms were counted daily, starting on day 1 of adulthood, until all the nematodes were dead. Worms that did not respond when gently stimulated several times with a worm pick were recorded as dead [4]. Worms that crawled off and desiccated on the side of the petri dish were censored. The same procedure was applied to the lifespan assay with *daf-16 (mgdf50)*, *hsf-1(sy441)* and *skn-1(zu67)* mutants to determine the pathways implicated. Three independent trials were carried out for each assay.

DAF-16::GFP localization assay

Synchronized L1 worms of MQD54 were grown with or without 10 µg/ml MGE. On day 1 of adulthood, fluorescence images of DAF-16::GFP localization were captured [5], and the fluorescence intensity was quantified using ImageJ software [6]. A positive control group that was not pretreated with MGE was heat-shocked at 35°C for 1 hour [7]. Three independent trials were conducted with 60 worms per trial for each treatment.

Hydrogen peroxide (H_2O_2)-induced Stress tolerance assay

The oxidative stress tolerance assay was carried out using age-synchronized L4 worms grown in the presence or absence of 10 µg/ml MGE. On day 5 of adulthood, the worms were transferred to NGM plates containing 5 mM hydrogen peroxide. The worms were incubated at 20°C and scored for viability every 1 hour until all the animals were dead [8]. The assay was carried out in triplicates with 30 worms per plate in 3 independent trials.

Measurement of reactive oxygen species (ROS)

Synchronized N2 L4 worms were cultured for 10 days at 20°C with either 10 µg/ml or 20 µg/ml MGE. The worms were then stained with 50 µM 2'-7'-dichlorofluorescein diacetate (H2DCF-DA) and incubated in the dark at 20°C for an hour [9]. Fluorescence images were captured using a DM6B fluorescence microscope (Leica, Germany). Each experiment used forty worms, and the procedure was repeated independently three times. ImageJ software was employed to analyze the fluorescence intensity.

Lipofuscin assay

To assess intestinal autofluorescence resulting from lipofuscin accumulation, day 8 adult worms thant had been treated with either 10 µg/ml or 20 µg/ml MGE from the L4 stage were used. In each trial, forty worms were mounted on a glass slide with a 2% agarose pad, immobilized with levamisole (5 µM) and covered with a coverslip. Images were captured with a DM6B fluorescence microscope (Leica, Wetzlar, Germany) using DAPI filter set (Ex/Em 350/460 nm). The level of lipofuscin was quantified by determining the average pixel intensity in each worm using ImageJ software [9,10]. Three independent trials were performed.

Heat stress tolerance assay

To evaluate heat stress tolerance, synchronized L4 worms were cultured in the presence or absence of 10 µg/ml MGE. On day 5 of adulthood, the worms were subjected to a temperature of 35°C on NGM plates. The worms were scored as alive, dead, or censored at 2-hour intervals until all the worms were dead [8]. This assay was conducted in triplicates with 50 worms per plate, and the experiment was independently repeated three times.

Endoplasmic reticulum stress response assay

Endoplasmic reticulum (ER) stress in *C. elegans* was induced using tunicamycin, a chemical agent that blocks N-linked glycosylation, leading to the accumulation of misfolded proteins in the ER [11]. Synchronized L1 larvae of the SJ4005 (*hsp-4::gfp*) strain were grown under the conditions specified in the results. On various days of adulthood as indicated, 50 worms were transferred to a 25 ng/µl solution of tunicamycin in M9 buffer and incubated for 4 hours at 20°C [12]. There were 4 treatment groups: tunicamycin treated and untreated control groups, as well as the tunicamycin treated and untreated groups with 10 µg/ml MGE. Subsequently, the worms were placed onto a 2% agarose pad on a glass slide, immobilized with 5 µM levamisole, and covered with a coverslip. Images were captured with a DM6B fluorescence microscope GFP filter (Leica, Germany) and quantified using the ImageJ software. Three independent trials were performed.

Analysis of α-synuclein Protein aggregation

The aggregation of the Parkinson's disease associated protein α-synuclein was analyzed using the *C. elegans* NL5901*(pkIs2386)* strain expressing α-synuclein::YFP in the muscles [13]. Worms were cultured from the L4 stage on 10 µg/ml MGE for 72 hours. Forty adult worms were mounted on 2% agarose pads and immobilized using 5 µM levamisole. Images were captured using a DM6B fluorescence microscope (Leica, Germany) with a YFP filter (Ex/Em 350/460 nm) , and the quantification of fluorescence was performed using ImageJ software [14]. Three independent trials were performed.

Amyloid-β-induced paralysis assay

Synchronized L1 worms of the CL4176 transgenic strain (carrying the human amyloid-β protein) were raised in the presence or absence of 10 μg/ml MGE at 15°C until L3 stage and then upshifted to 25°C for induction of Aβ1-42 expression. Paralyzed worms were scored at 2-hour intervals until all the animals were paralyzed [15]. Three independent trials were performed.

Determination of pharyngeal pumping rate

Synchronized N2 L4 worms were transferred to OP50 seeded NGM plates with or without 10 μg/ml MGE. The rhythmic pharyngeal contractions per minute of day 5 and 10 adult worms were recorded as pharyngeal pumping rate using a MVX10 dissecting microscope (Olympus, Japan) [16]. Three independent trials were carried out with 10 worms for each treatment per trial.

Locomotion assay

Synchronized N2 L4 worms were transferred to OP50 seeded NGM plates with or without 10 μg/ml MGE. The locomotion rate was evaluated on days 5 and 10 of adulthood by counting the number of body bends in a coordinated sinusoidal manner per minute using a SMZ168 stereo microscope (Motic, China) [17]. This was further validated by counting the number of crests or troughs in the wave-like track created on the OP50 lawn by the worm's motion. Three independent trials were carried out with 10 worms per trial.

Body length and size assay

N2 L4 worms were cultured on OP50 treated with 10 μg/ml MGE to adult stage. Images of day 5 and 10 adult worms were captured using a DM6B fluorescence microscope (Leica, Germany) with DIC filter, and the body length and size of each individual animal were analysed using the ImageJ software [18]. Body length was determined by measuring the sagittal distance between the tip of the mouth and the tip of the tail. Similarly, the body size was determined by measuring round the entire outer boundary of the worm body. Three independent trials were carried out with 20 worms per trial.

Brood size and progeny viability assay

Brood-size was measured by plating one L4 worm per OP50 seeded NGM plate treated with 10 μg/ml or 20 μg/ml MGE. The worms were transferred into freshly seeded NGM plates at a 12-hour interval, and the total eggs laid in the previous plate were counted. This process was repeated for each worm until egg-laying ceased, and the total number of fertilized eggs laid throughout the fertile cycle was analyzed [19]. Following 24 hours after eggs were laid, the percentage of hatched eggs relative to the brood-size was used to quantify the progeny viability. Twenty worms were scored for each group.

Sample preparation and RNA-seq

Synchronized N2 L4 worms were cultured on fresh NGM plates containing 50 μM FuDR, seeded with OP50 containing 0 μg/ml or 10 μg/ml MGE. On day 5 of adulthood, worms were harvested and washed several times with M9 buffer to remove the OP50. Worms were then quickly frozen in liquid nitrogen and stored in -80^{o}C freezer prior to RNA extraction. RNA extraction and sequencing analysis were carried out by a sequencing company in the following steps: first, the concentration, purity, and integrity of the samples were measured prior to RNA sequencing. mRNA was enriched and cDNA libraries were constructed after mRNA random interruption and inverse transcription. The effective concentration of the library was quantified by Q-PCR to ensure the quality of the library. Sequencing was performed using an Illumina high-throughput sequencing platform.

Transcriptomic data analysis

Gene function was annotated based on several genomic databases, including non-redundant protein sequences, non-redundant nucleotide sequences, Pfam, KOG, Swiss-Prot, Gene Ontology, and KEGG databases. Quantification of gene expression levels was estimated using the metric "fragments per kilobase of transcript per million fragments mapped" (FPKM). The analysis of differential gene expression between two groups was conducted using the edgeR package, which is based on the model of negative binomial distribution. A significance threshold was set with a *P* value < 0.05 and a Fold Change ≥1.5, indicating that genes meeting these criteria were considered significantly differential expressed.

Q-PCR assay

For cDNA synthesis, 900 ng of total RNA extracted from RNA sequencing company was utilized, following the manufacturer's instructions for HiScript® Ⅲ RT SurperMix for qPCR (Vazyme). Each Q-PCR reaction was conducted in a final volume of 20 μL, containing 10 μL of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme), 0.4 μL each of 10 μM forward and reverse primers, 2.0 μL of dilute cDNA, and 7.2 μL of sterile water. The qPCR procedure consisted of an initial denaturation strp for 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Subsequently, a melting curve program was executed, involving steps at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The primer sequences of target genes are as follows: CGAACCCGAGATTACTGCT and CGTTTGTCCTTTGTCGCTTA for R09E10.1, ATGCCACTCCCTCCTGTT and TGCTCCATTACTCCCTTGC for F44D12.8, ACAACAAGTCGGACAAGGAT and GAAGCGGAAGTCTCAACG for Y47D7A.13, and for the reference gene *tbg-1*, the primer is AAGATCTATTGTTCTACCAGGC and CTTGAACTTCTTGTCCTTGAC. The relative expression levels of the tested genes were calculated using $2^{-\triangle\triangle CT}$ method.

Statistical analysis

Data handling and statistical analysis were conducted using GraphPad Prism 8.0. All the experiments were independently performed three times, and the results are presented as Mean±S.E.M, unless specified otherwise. The log-rank test of the Kaplan–Meier survival analysis was employed to assess the statistical significance of difference between groups in lifespan assays, oxidative and heat stress resistance assays, and Aβ-induce paralysis assays. For other datasets, statistical analyses were performed using one-way ANOVA, two-way ANOVA or Student's t tests, as appropriate. Multiple comparisons in ANOVA were assessed using Turkey's post-hoc tests. The levels of statistical significance are denoted as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, and ^{ns}P > 0.05.

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