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Anti-aging Effects and Mechanisms of *Phyllanthus emblica Linn.* Polyphenols Extract Targeting Sirt6

Na Wu[1,2,3]; Ziyuan Li[1,3], Jiamin Tian[1,3], Fang Chen[1,3]; Zeyuan Deng[4]; Yao Pan[1,3*]

[1.] *School of Public Health, Jiangxi Medical College, Nanchang University, Nanchang, 330006, China.*

[2.] *Shanghai Pudong New Area Gaoqiao Community Health Service Center, Shanghai, 200135, China.*

[3.] *Jiangxi Provincial Key Laboratory of Disease Prevention and Public Health, Nanchang University, Nanchang, 330006, China.*

[4.] *State Key Laboratory of Food Science and Resources, Nanchang University, Nanchang,330000, China*

ABSTRACT: The anti-aging effects and possible molecular mechanisms of *Phyllanthus emblica Linn* (PE) were investigated in this study. Results revealed that PE extracts increased antioxidant enzyme activities, T-AOC levels, and decreased MDA content in the serum and tissues of D-galactose(D-gal) mice. PE also improved the motor and learning memory abilities of D-gal mice. In addition, PE regulated the abundance of *Firmicutes*, *Bacteroidota*, and other bacteria in the gut microbiota of D-gal mice, by affecting the TCA cycle based on functional predictive analysis. The molecular docking and cellular thermal shift assay revealed that chebulic acid (CA, binding energy of -183.6289 kcal/mol) might be the key active substance in PE extract. Sirt6 was an important protein that affects aging and energy metabolism. Circular dichroism spectrum and western blot analysis found that CA altered the secondary protein structure of Sirt6 and increased its deacetylase activity. Co-Immunoprecipitation assay results showed that Sirt6 and Nrf2 interacted in the presence of CA. Finally, western blotting analysis found that PE extracts up-regulated the expression of Sirt6, Nrf2, and HO-1 proteins in the tissues of D-gal mice. Overall, the phenolic acids of PE may regulate oxidative stress through the interaction of sirt6, and affect the gut microbiota of mice. This study provided theoretical guidance for the potential application and the mechanism of health effects from PE.

Keywords: *Phyllanthus emblica Linn*; polyphenols; anti-aging; gut microbiota; Sirt6

1. Introduction

Nowadays, in the worldwide, chronic diseases, such as aging, cancer, cardiovascular disease generate great economic burden (esp. for individuals, families and society) for human wellness. The free radical theory was widely recognized as one of the mechanisms of aging[1]. Although interventions such as drugs and dietary restrictions had some alleviating effects on oxidative stress-induced aging, these treatments still have major side effects such as loss of bone density and reduced physical endurance[2].

In recent years, active ingredients in natural products had attracted extensive attention from the nutritional community because of their characteristics (eg. multi-channel, multi-target, low toxicity and side effects)[3]. *Phyllanthus emblica Linn* (PE) was a kind of fruit belongs to "medicinal & food homology" with

***Corresponding author**
panyao@ncu.edu.cn

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various beneficial effects, including antioxidant, anti-aging, anti-obesity, anti-cancer, blood sugar regulation, and cardiovascular protection properties[4,5]. Modern researches had found that due to its powerful antioxidant capacity, PE extract could scavenge excess reactive oxygen species, reduce oxidative stress, and increase the activity of antioxidant enzymes thus providing an anti-aging effect[5-7]. Only a few studies[8,9] had found that gallic acid, gallocatechin, methyl gallate, ethyl gallate, and ellagic acid in PE extracts may be the active substances in maintaining blood glucose homeostasis and anti-tumor effects. However, the key substances responsible for its anti-aging effects and the possible molecular mechanisms were still unclear.

The gut microbiota was considered to be the host's “second genome” and played an important role in maintaining homeostasis[10]. Aging was often accompanied by disruption of the gut microbiota, which is critical for regulating health status and longevity[11]. In fact, the expression and activity of some proteins/genes are closely related to the abundance of gut microbiota. Sirt6 was a member of the Sirtuins protein family. It played an important role in stem cell senescence. Meanwhile, dysregulation of the gut microbiota in senescent mice induced oxidative stress and inhibited the expression level of Sirt6[12]. Sirt6 knockout mice exhibited disturbed gut microbiota and premature aging, and a prolonged lifespan was observed in Sirt6 knockout mice when transplanted with microbiota from WT mice[13]. Studies found that the expression of Sirt6 was significantly reduced in senescent bone mesenchymal stem cells (MSCs)[14]. Knockdown of Sirt6 impaired the proliferation, migration, and resistance to oxidative stress of MSCs, leading to their senescence[15]. Thus, aging might be associated with dysregulation of gut microbiota as well as the expression of Sirt6. In addition, Sirt6 acted as a transcriptional co-factor that binds Nrf2 and regulates the expression of its key downstream target gene HO-1[16]. However, there were no studies that have explored whether the polyphenols in PE exert their anti-aging effects by targeting the Sirt6 protein.

Therefore, this study aimed to target the Sirt6 protein to investigate the anti-aging effects and possible mechanisms of PE polyphenol extract as well as the regulation of gut microbiota. Our study provided new ideas for application of "medicinal & food homology" plants to prevent aging.

2. Materials and methods

2.1. Chemicals, reagents and antibodies.

D-galactose (D-gal) was from Nanjing Jiancheng Technology Co., Ltd. (Nanjing, Jiangsu, China; purity≥99%). Commercial assay kits, including malondialdehyde (MDA), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), catalase (CAT) were provided by the Nanjing Jian-cheng Bioengineering Institute (Nanjing, Jiangsu, China). All other solvents and chemicals were of analytical grade, from local suppliers.

BCA protein assay kit, radioimmunoprecipitation assay (RIPA) lysis buffer,phenylmethylsulfonyl fluoride (PMFS) were provided by Beyotime Biotech Inc (Shanghai,China). Sirt6, Nrf2, and HO-1 were from Abcam (USA).

2.2. Preparation of sample.

Preparation of PE polyphenol extract: Dried PE fruits were selected, crushed and sieved twice to obtain PE powder. Water was used as the solvent and the extraction parameters were material-liquid ratio 1:22, temperature 45℃ and time 53 min. The extract was concentrated to 0.1 g/mL using a rotary evaporator.

2.3. Experimental animals and treatments.

All C57BL/6J mice, weighing (20 ± 2) g, 6 weeks old, were obtained from SPF(Beijing) Biotechnology Co., Ltd. (Beijing, China). All procedures performed on animals were approved by the Institutional Animal Care and Use Committees of Nanchang Royo Biotech Co,.Ltd. (IACUS Issue NO:RYE2024030601). Number of the using of Laboratory Animal: No.SCXK(JING)2019-0010 (Beijing, China).

30 SPF-grade healthy male C57BL/6J mice were housed with free access to water and food at a temperature of (25 ± 2) ℃ and a 12 h light/dark cycle. After one week of adaptive feeding, the mice were equally divided into 5 groups according to weight (n = 6 per group): normal control (NC) group, model control (MC) group, low dose of PE extract (EL) group, high dose of PE extract (EH) group, positive control (PC) group. The aging model was established by intraperitoneal injection of D-gal (150 mg/kg/d) for 8 weeks. Mice in the NC and MC groups received gavage water. Mice in the EL and EH groups received gavage PE polyphenol extract(12 mg/20 g mice/d and 24 mg/20 g mice/d. Mice in the PC groups received gavage metformin (250 mg/kg/d).

The dose of D-gal was determined according to the literature[17]. The adult dose of dried PE recommended by the Chinese Pharmacopoeia (2020) was 3-9 g/d[18]. In this experiment, 4.5 g/d was taken as low dose,9 g/d was taken as high dose. The equivalent dose ratio was 0.0026 for humans and mice based on body surface area, therefore, the equivalent low dose for mice was 12 mg/20 g mice/d, high dose was 24 mg/20 g mice/ d.

At the end of the experiment, all mice were sacrificed and dissected. Mice were fasted the night before sacrifice. The blood supernatant was separated after centrifugation at 3000r for 10 min. The liver, kidney, heart, and spleen were taken to calculate their organ index (= organ weight (mg)/body weight (kg)). The tissue samples and serum were frozen at -80℃ and kept for analysis.

2.4. Cell culture and treatment.

Cell culture was conducted according to Pan [19]et al. with minor adjustments. H9c2 cells (Zhichenhui Biotechnology Co., Shanghai, China) were plated in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Shanghai, China) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Biological Industries, Shanghai, China), 100 U/mL penicillin, 100 mg/mL streptomycin (Solarbio Co., Beijing, China) and cultured in a humidified incubator at 37 ℃, 5% CO_2. Cells were plated in the suitable plates for 24 h before the appropriate treatments for the different assays. 1 mg/mL PE extract, 20 μmol chebulic acid(CA) and ellagic acid(EA) were added respectively for 12 h. After that, 200 μL H_2O_2 was added for one hour.

2.5. Measurement of content of MDA and activities of antioxidant enzymes.

Blood was centrifuged (3000r,10min) and the supernatant was taken to obtain serum. Each tissue was prepared as a tissue homogenate using saline. The T-SOD, GSH-Px, CAT activity and the levels of MDA and T-AOC in mice serum and tissue were determined using commercially available kits, following the manufacturer's instructions.

2.6. Behavioral experiments.

In this experiment, the learning and memory ability of aged mice was tested by open field test (OFT) and morris water maze (MWM) with camera recording system. The OFT and the MWM test was constructed by referring to the previous study. All mice were subjected to the OFT and the MWM at week 8. The entire experiment of MWM consisted of a 4-day localization navigation test and a 1-day spatial exploration test. The space exploration experiment was conducted on day 5.

2.7. gut microbiota 16S rRNA High-Throughput Sequencing.

Fresh mice feces were collected in sterile cryopreservation tubes. After quick-freezing in liquid nitrogen for 5-10 min, it was stored in -80℃ refrigerator. The samples were handed over to to Novogene Co., Ltd (Beijing,China) to perform the 16S rRNA sequencing and analysis. Amplified and sequenced the V4region using Illumina NovaSeq, followed by related data processing. After the total DNA of fecal samples was extracted, the samples were subjected to quality control and PCR amplification. After the amplification was completed, electrophoresis was performed, and the PCR products were qualified. The amplicons were ligated and the library was constructed and quality-controlled. The sequencing data were used for species identification analysis.

2.8. Western blotting (WB).

The heart, liver and kidney tissue samples were lysed into homogenate using RIPA lysis buffer and PMSF. A BCA protein assay kit was used to determine protein concentrations, and all of the samples in each experiment were adjusted to have the same protein content. SDS-PAGE gel electrophoresis was used to separate the protein samples. They were then transferred to a PVDF membrane and treated with the appropriate mixture of primary and secondary antibodies. The luminescent solution is added dropwise to the membrane, and exposed using a gel imaging system, and the grayscale values are analyzed using imaging software.

2.9. Cellular thermal shift assay (CETSA).

CETSA was conducted according to Jafari[20] and Chen[21] et al. with minor adjustments. A total of 1.0×10^6 H9c2 cells were cultured in 10 cm dishes to reach 100% confluency before treatment. Following pretreatment with 20 μmol/L CA, cells were washed and resuspended with PBS, divided into seven aliquots in an equal volume, and heated individually at different temperatures (37 ℃–72 ℃) for 5 min and then cooled at room temperature for 3 min. They were lysed with RIPA solution. Following centrifugation at 14000 g for 15 min at 4 ℃, the supernatants were transferred to new tubes and stored at − 80 ℃ until immunoblotting was performed.

2.10. Quantitative PCR (qPCR).

Referring to the method of Pan[22] et al. total mRNA of brain tissue was isolated with a TRIzol reagent and then purified using the mRNA Purification Kit (BersinBio). The purified mRNA was cleaved into 300 nt fragments with $ZnCl_2$ in an ice-water mixture for 10s. Next, 1/10 volume of the RNA fragments was saved as "10% input". Extracted RNA was analyzed by qPCR for P21 and P53 and standardized with β-actin. Specific primer sequences were as follows: P53: forward, CTCACACTGGGCTAAAGTTCTGA; reverse, CGACTGTGAATCCTCCATGAC. p21: forward, CTGTGTTTCTGATCCTGGCG; reverse, GACCCCGAAGTCCTACTCAG.

2.11. Molecular Docking.

Preparation of ligands: The ligand molecules involved in this study were obtained from PubChem database. These molecules were downloaded in Structural Data File (SDF) format and converted to Protein Data (PDB) format using Open Babel GUI software.

Preparation of the receptor: Retrieve the crystal structure of Sirt6 protein from the PDB. The molecule was downloaded in PDB format, removing water molecules as well as other heteroatoms from the protein molecules.

Docking: Discovery Studio software was used to process Sirt6 protein and polyphenol small molecule docking, calculate binding energies, and visualize results.

2.12. Protein Secondary Structure Detection.

The effect of polyphenols in PE on the secondary structure of Sirt6 protein was detected using circular dichroism. Sirt6 protein was configured with polyphenols to a suitable concentration (0.1 mg/mL) with a concentration ratio of 1:1. All data were averaged over three scans. The scanning range was 190 nm-250 nm and the scanning time was 0.5 s. The optical range of the quartz cuvette was 0.1 cm, the scanning speed was 3.3 nm/s, the sensitivity was 2 mdeg/cm, the resolution was 0.1 nm, and the spectral calibration was set to be on to eliminate the corresponding wavelengths of the grating and detector.

2.13. Determination of deacetylase activity.

By the method of reference[23], 600 μL of polyphenol DMSO solution (100 μM) as well as 600 μL of DMSO blank solution mixed with GST-Sirt6 (3 μg/well) were placed in a 96-well plate and incubated at 37℃ for 30 min. The reaction initiator consisted of H3K9Ac (40 μM) as well as 500 μM (NAD+) dissolved in Tris Buffer (25 mmol/L, pH 8.0). It was ensured that the concentration of DMSO in the samples did not exceed 1%. No NAD+ or Sirt6 was added to the control. 30 min later, the deacetylation reaction was terminated by adding 6 μL of 10% formic acid and centrifugation at 1000 r/min for 15 min. The samples were analyzed by HPLC-QE-MS2. The formation of the H3K9Ac peak was monitored and then quantified by measuring the area under the curve, which was repeated twice for each sample.

2.14. UPLC-QE-MS/MS analysis.

Chromatographic separations were performed using an C18 column (4.6 mm × 50 mm, 1.8 μm). The mobile phase A was ultrapure water with 0.02% formic acid and B was acetonitrile with 0.02% formic acid.The gradient program was (0−2) min with 0% B, (2−10) min with (0−8)%B, (10−10.1) min with (8−80)% B, (10.1−12) min with 80% B, (12−12.1) min with (80-0)% B, 15 min with 0% B. The flow rate of the mobile phase was 0.3 mL/min, and the injection volume was 2μL. Positive Ion PRM Mode. Xcalibur software (version 3.1, Thermo Fisher Scientific, Waltham, MA, USA)was used to record and analyze data.

2.15. Co-Immunoprecipitation Assay (Co-IP).

Cells treated with ethanol and CA were lysed in RIPA lysis buffer containing a protease inhibitor. Then, lysates were immunoprecipitated (IP) with anti-Sirt6 and anti-Nrf2 antibodies or the mouse/rabbit lgG . The total lysates or corresponding IP samples were immunoblotted with anti-Sirt6 and anti-Nrf2.

2.16. Statistical analysis.

Graphpad prism 9.5 was used to do the data analysis, significance analysis, and graph production, and the results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for multiple comparisons. # represented a significant difference between the MC group and the NC group ($P < 0.05$), ## represented a significant difference between the MC group and the NC group ($P < 0.01$). * represented a significant difference between each group and MC group ($P < 0.05$), ** represented a significant difference between each group and MC group ($P < 0.01$). *** represented a significant difference between each group and MC group ($P < 0.001$).

3. Results

3.1. Effects of PE extract on body weight, organ indices, antioxidant capacity, aging markers and motor and learning memory abilities of D-gal-induced aging mice.

There was no significant difference in the initial body weight of mice in each group ($P > 0.05$). After 60 d of D-gal injection, the average body weight in the MC group was significantly lower than the NC group (Supplementary Fig. 1A), while there was no significant difference in body weight with the other groups ($P < 0.05$). The cardiac index was higher and all the remaining organ indices were significantly lower in the MC group compared to the NC group (Supplementary Fig. 1B-E). Gavage of PE extract and metformin restored the altered organ indices in aging mice.

Compared with the NC group (Fig. 1A, Supplementary Table 1), MDA levels were significantly elevated in D-gal mice. T-AOC and antioxidant enzyme activity were significantly decreased in the serum and tissues of D-gal mice. In EL, EH and PC group (Fig. 1B-D), T-AOC and antioxidant enzyme activity in serum and tissues were significantly higher and MDA content was considerably lower than the MC group. It indicated that treated by high and low doses of PE extract and metformin could reduce oxidative stress and increase the antioxidant capacity in the serum and tissues of D-gal mice.

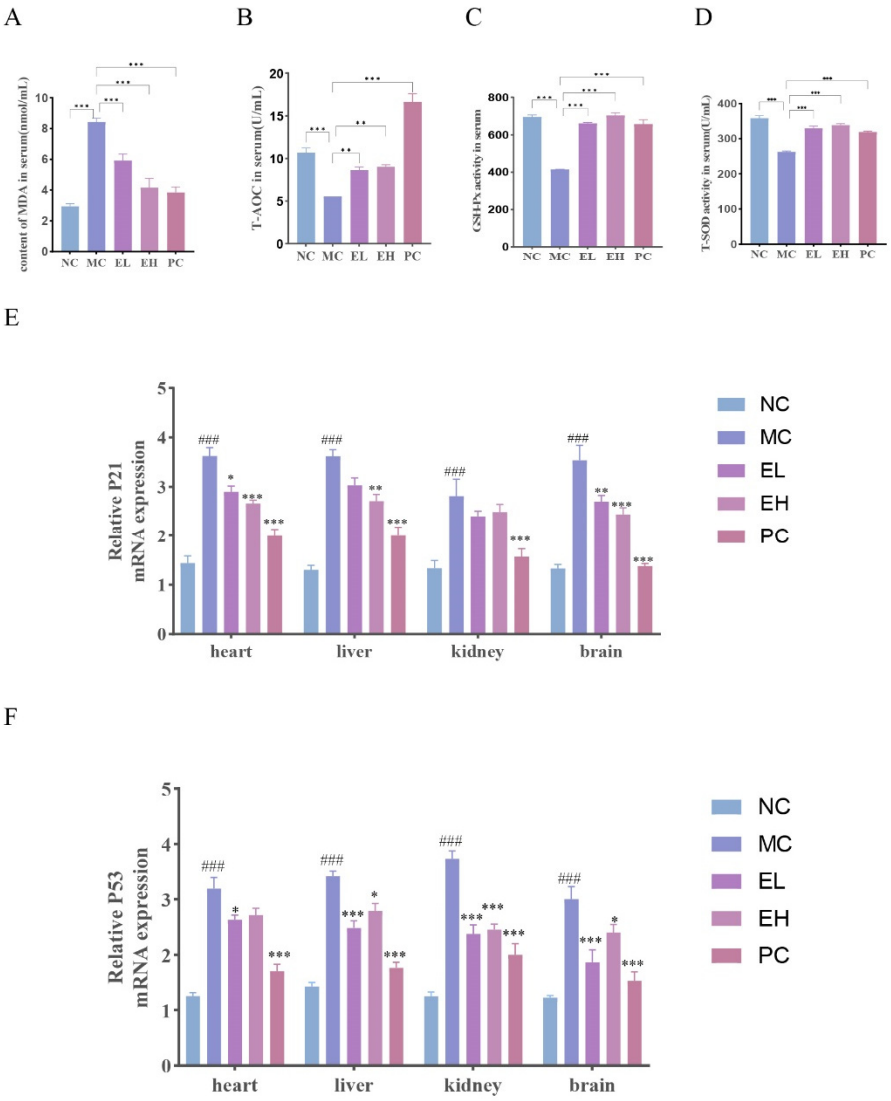


Fig 1. Effects of PE extract on antioxidant indices in serum and aging markers in brain of mice. A-D:Antioxidant indices in serum. E-F:Aging makers in brain.

As illustrated in Fig. 1E-F, D-gal-induced aging mice had significantly higher mRNA levels of the aging markers P21 and P53 in each organ compared to the NC group. However, it was successfully decreased by gavage of PE extract and metformin. These results demonstrated that D-gal-induced aging may be efficiently slowed down by PE extract.

Aging could lead to declines in motor and learning memories[24]. As shown in the Fig. 2B-D and Supplementary Fig. 1F-G, the total distance, the average speed of movement, the number of times and time of entering the center area within 1 minute were significantly lower and the percentage of resting time was significantly higher in the MC group than the NC group. Compared with the MC group, the total distance, the average speed of movement, the number of times and time of entering the central area were significantly increased and the percentage of resting time was decreased in the EL, EH and PC group. Similar results could be drawn from the trajectory graphs of the mice (Supplementary Fig. 2). It showed that D-gal mice had reduced locomotion, decreased autonomic activity, and decreased desire for spatial exploration. In contrast,

gavage of PE extract and metformin significantly restored motor ability and reduced anxiety-like behavior in D-gal mice.

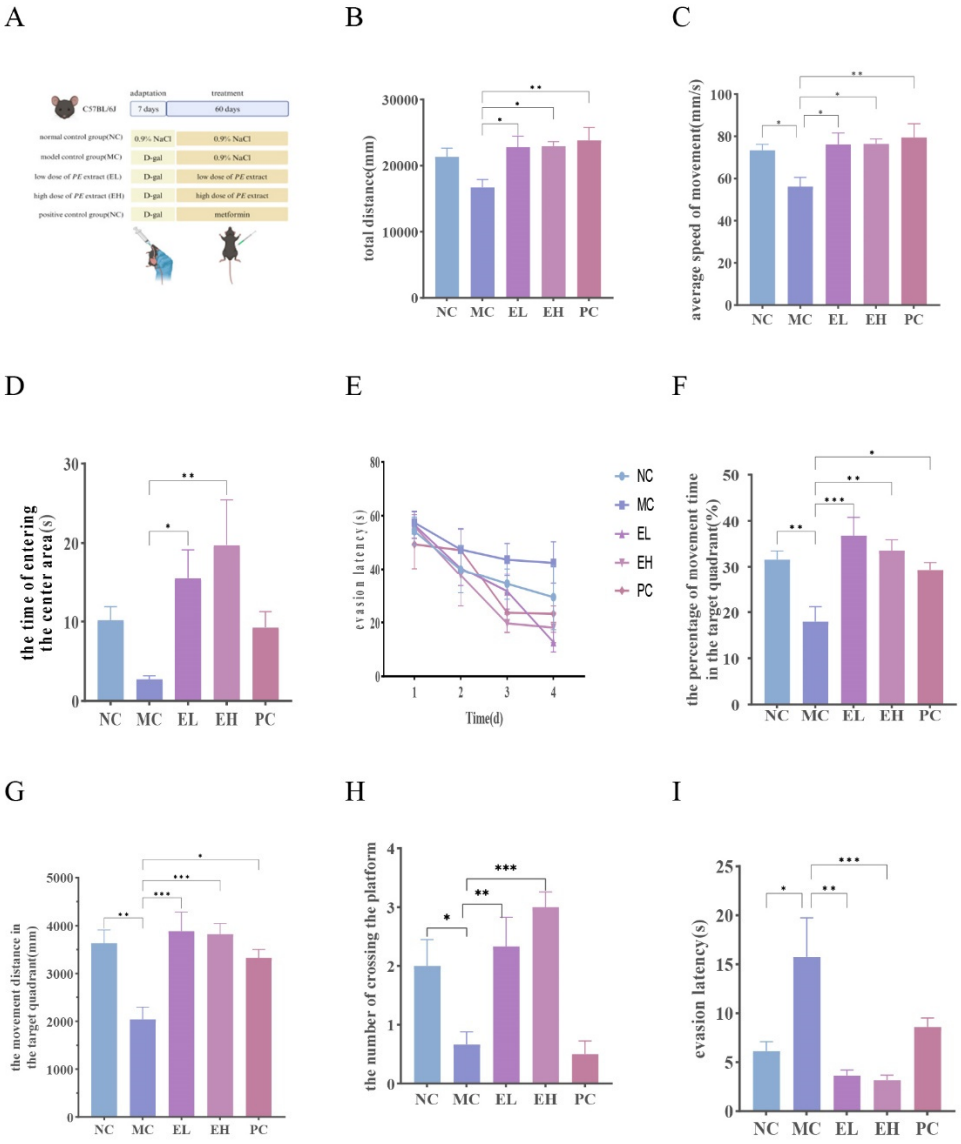


Fig. 2. Effects of PE extract on motor and learning memory capacity. A: Model diagram of animal experiment. B-D: Results of OFD. B:The total distance. C: The average speed of movement. D: The time of entering the center area. E: The escape latency of localization navigation test. F: The percentage of movement time in the target quadrant. G: The movement distance in the target quadrant. H: The number of crossing the platform. I: The evasion latency of spatial exploration test.

In the MWM test, the escape latency of the MC group was higher than the NC group from the third day of localization navigation test (Fig. 2E). The evasion latency of D-gal mice was significantly higher than that of normal control mice, while the percentage of movement time and the movement distance in the target quadrant, and the number of crossing the platform were significantly lower (Fig. 2F-I). Compared with the MC group, gavage of high and low doses of PE extract and metformin effectively reduced the evasion latency and increased the percentage of movement time and movement distance in the target quadrant in D-gal mice. Similar results could be drawn from the trajectory graphs of the mice (Supplementary Fig. 3). It was shown that the spatial memory capacity of senescent mice was significantly reduced. Gavage of PE extract and metformin restored the D-gal induced decline in learning memory capacity.

3.2. Effects of PE extract on gut microbiota and Sirt6 protein of D-gal-induced aging mice.

As shown in the Fig. 3A, there were 145 microbial species common to all groups, while the MC group had the lowest number of species-specific to each group, and both gavage of PE extract and metformin increased the number of gut microbiota species specific. As can be seen from the Fig. 3B, the low overlap of the samples in the NC and MC groups indicated that the similarity of the gut microbiota in these two groups was low, while the EL and the NC group overlapped completely, suggesting that the low-dose PE extract could rebuild the structure and composition of the gut microbiota of the D-gal mice.

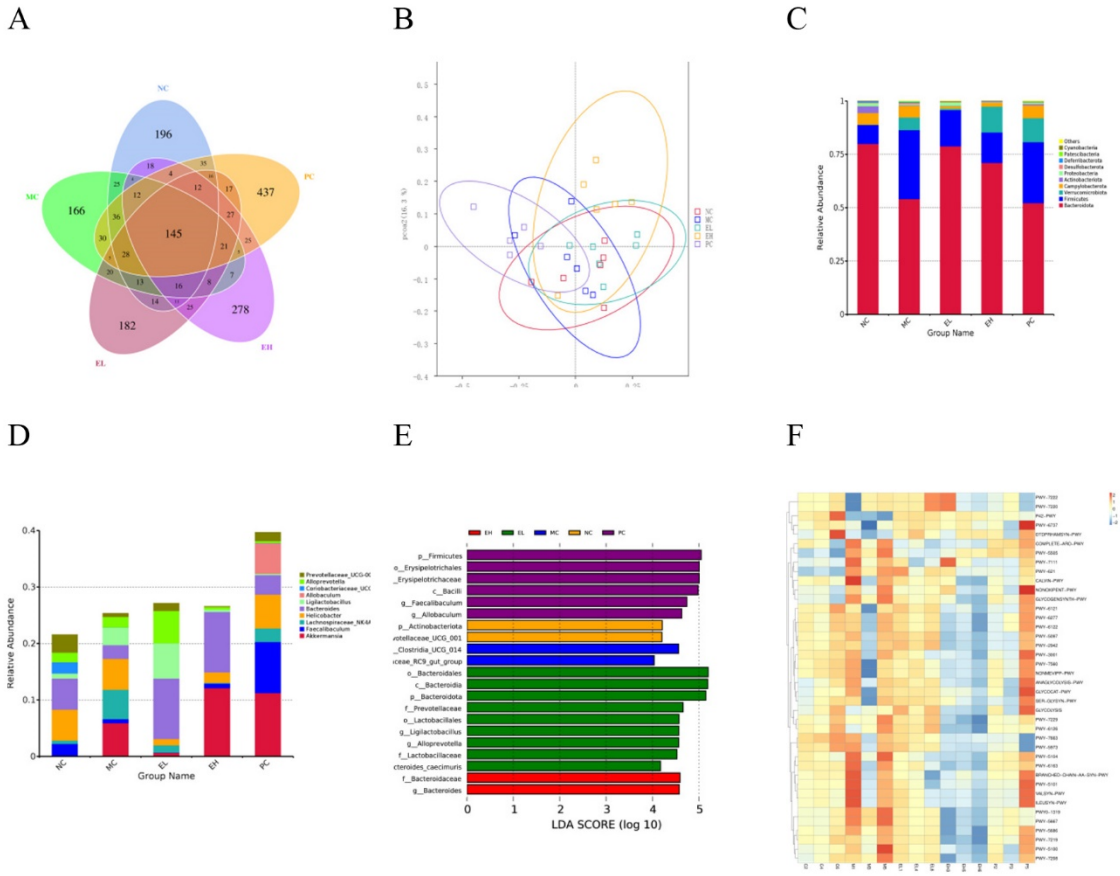


Fig. 3. Effects of PE extract on gut microbiota. A:Flower plot. B:PCoA. C:The top 10 highest relative abundance at the phylum level. D:F/B. E:The top 10 highest relative abundance at the genus level. F:Differential species of gut microbiota in groups of mice. H:functional prediction model. N=3.

As shown in Fig. 3C, the two species with the highest relative abundance at the phylum level in all groups were *Firmicutes* and *Bacteroidota*. The abundance of *Bacteroidota* in the MC group was significantly lower and the ratio of F/B was significantly higher than that of the NC group (Supplementary Fig. 1H). Compared with the MC group, the abundance of *Bacteroidota* was significantly higher and the values of F/B were significantly lower in EL and EH group. At the genus level, the abundance of *Prevotellaceae_UCG-001* and *Bacteroides* decreased and the abundance of *Lachnospiraceae_NK4A136_group* increased in the D-gal mice, as shown in the Fig. 3D. Low-dose PE extract and metformin treatment increased the abundance of *Prevotellaceae_UCG-001* significantly, and both low and high doses of PE extract and metformin greatly increased the abundance of *Bacteroides* and decreased the abundance of *Lachnospiraceae_NK4A136_group*.

As shown in Fig. 3E and Supplementary Fig. 1I, some species were significantly different between each group. It was found that 3 kinds of metabolic pathways were significantly down-regulated and 19 kinds of metabolic pathways were up-regulated in the model group compared with the NC group (Fig. 3F). The down-regulated metabolic pathways included P42-PWY, PWY-6737, and DTDPRHAMSYN-PWY. These pathways were incomplete reductive TCA cycle,starch degradation V and dTDP-L-rhamnose biosynthesis.

Next, WB experiments revealed that the expression levels of Sirt6, Nrf2, and HO-1 in the brain, liver, kidney, and heart of aging mice were lower than those of normal mice (Fig. 4A-C and Supplementary Fig. 4), although there was no significant difference in the heart. The expression levels of the Sirt6 protein in these four organs, the Nrf2 protein in the heart, kidney, and brain, and the HO-1 protein in the kidney and brain of aging mice were all markedly elevated by PE extract and metformin.

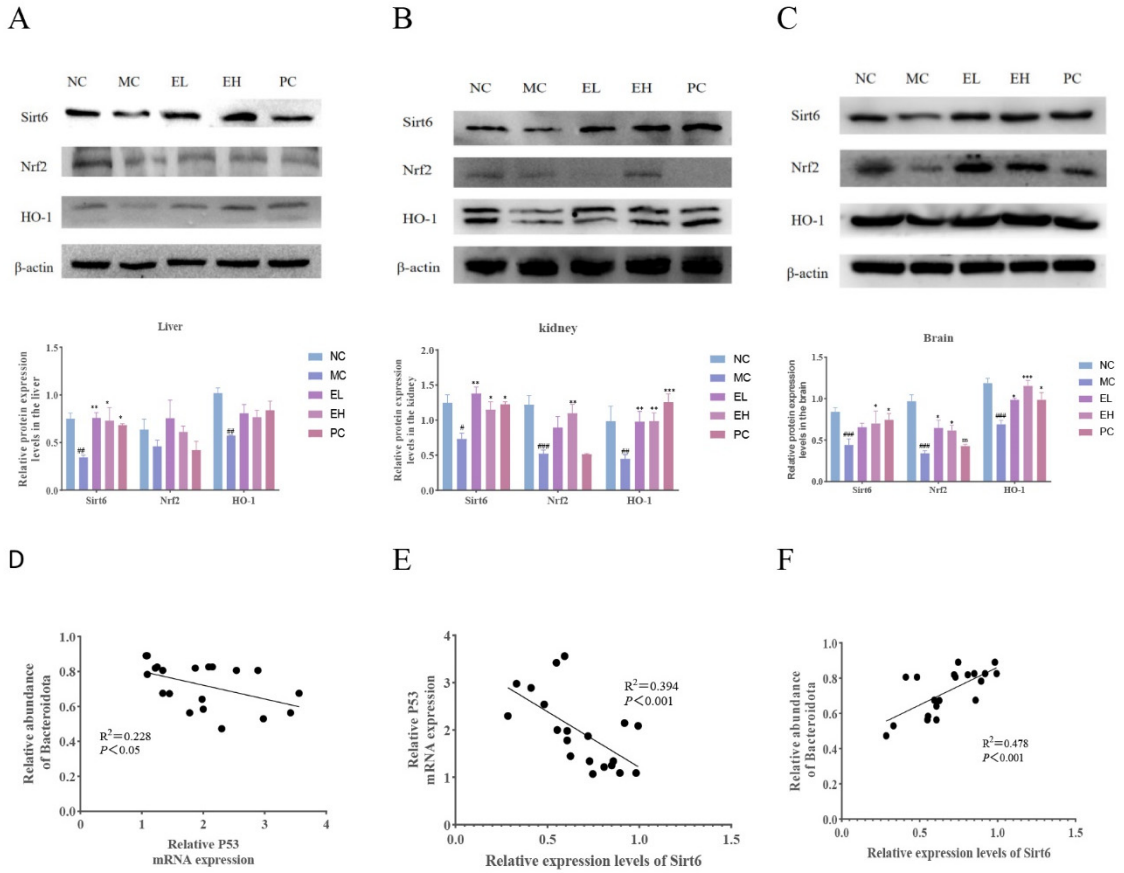
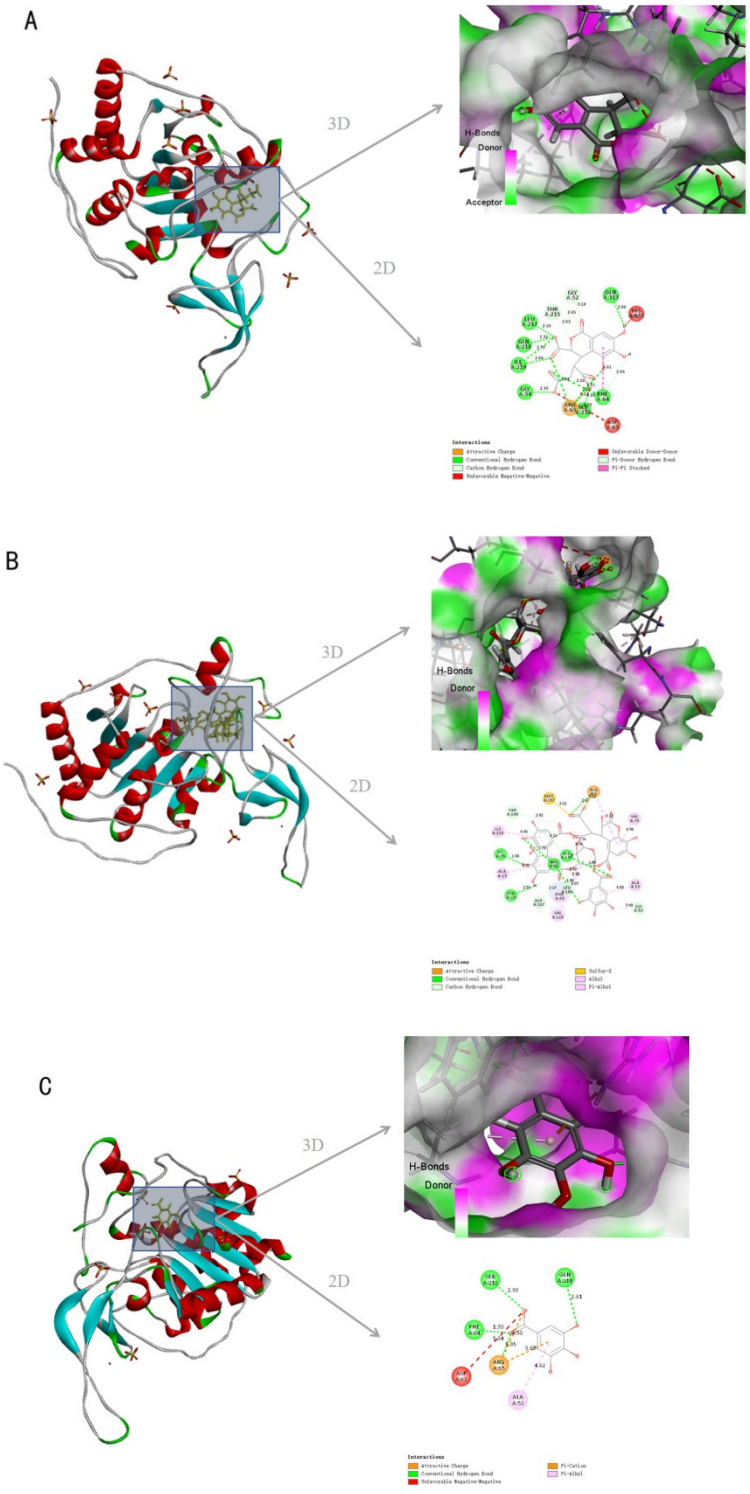


Fig. 4. A-C:Effect of PE extract on the relative expression of Sirt6, Nrf2 and HO-1 proteins in the liver, kidney, and brain. D-F:Correlation between the relative abundance of *Bacteroidota* in gut microbiota and the levels of the aging indicators P53 and sirt6 proteins in brain.

Finally, we used correlation analysis to confirm the relationship between the relative abundance of *Bacteroidota* in gut microbiota and the levels of the aging indicators P53 and sirt6 proteins in brain (Fig. 4D-F). The findings demonstrated a negative relationship between P53 and sirt6 protein levels, a positive relationship between sirt6 protein levels and relative abundance of *Bacteroidota*, and a negative relationship between P53 and relative abundance of *Bacteroidota*.

3.3. Key active substances and mechanism of anti-aging effects of PE extracts.

We tested whether the polyphenols identified from PE extract in our previous work[25]. It can be found that the mian active substances could interact with Sirt6 analysised by molecular docking (Fig. 5A-C and Supplementary Table 2). A total of 17 kinds of polyphenols might interact with Sirt6 proteins through hydrogen bonding, hydrophobic interaction, and electrostatic interaction. Chebulic acid (CA) has the lowest binding energy of -183.6289 kcal/mol. The CETSA found that the amount of Sirt6 protein broken down in CA-treated H9c2 cells at the same temperature was less than that in untreated cells. This suggested that the Sirt6 protein could bind to the CA (Fig. 5D). As illustrated in Fig. 6A, CA treatment significantly increased the H_2O_2-induced decreased expression of the Sirt6 protein.



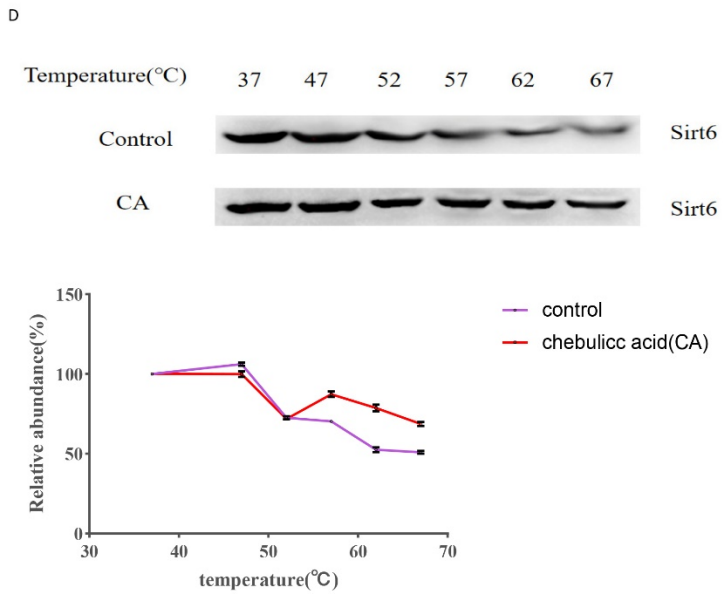


Fig. 5. Results of molecular docking and cellular thermal shift assay. A:Results of chebulic acid interact with Sirt6. B:Results of chebulagic acid interact with Sirt6. C: Results of gallic acid interact with Sirt6. D:CETSA to determine the binding ability of chebulic acid and Sirt6 proteins.

Next, we used circular dichroism to examine the secondary structure of the Sirt6 protein without and after adding polyphenols to explore the effect of PE extract on the secondary structure of the Sirt6 protein. The results were shown in Table 1. It can be seen that the α-helix content of the Sirt6 protein was higher and strand content was lower without polyphenols. After adding CA and other polyphenols, the α-helix content of Sirt6 protein was all significantly decreased and strand content was increased considerably. These results revealed that polyphenols identified from PE extract might interact with the sirt6 protein and alter its secondary protein structure.

Table 1. Secondary structure of the Sirt6 protein.

	α-Helix	Strand	Turns	Unordered	Total
control group	0.261±0.008	0.230±0.014	0.224±0.002	0.285±0.008	0.995±0.006
chebulic acid	0.020±0.002*	0.427±0.007*	0.216±0.003	0.326±0.004*	0.989±0.010
gallic acid	0.027±0.002*	0.492±0.002*	0.228±0.001	0.252±0.001*	0.998±0.002
chebulagic acid	0.027±0.001*	0.490±0.002*	0.228±0.001	0.251±0.001*	0.997±0.004

Note: * indicated a significant difference compared to the control group. $P<0.01$.

Sirt6 was a deacetylase, therefore we examined the effect of PE polyphenols on its deacetylase activity. From Fig. 6B，deacetylase activity increased in groups with the addition of polyphenols. Among them, the greatest increase was observed in the PE extract, followed by gallic acid, CA, and chebulagic acid. Then, by the WB experiment, we further demonstrated that CA and PE extract improved the deacetylase activity of the Sirt6 protein. This was shown by the results that the model group had a considerably higher amount of H3K9Ac than the control group, which was decreased by CA and PE extract treatment (Fig. 6C).

The functional interaction between Sirt6 and Nrf2 was confirmed by Co-IP assay using the endogenous proteins from H9c2 cells treated with or without CA for 24 h. Co-IP analysis showed that Sirt6 had the potential to interact with Nrf2, which was due to CA treatment (Fig. 6D).

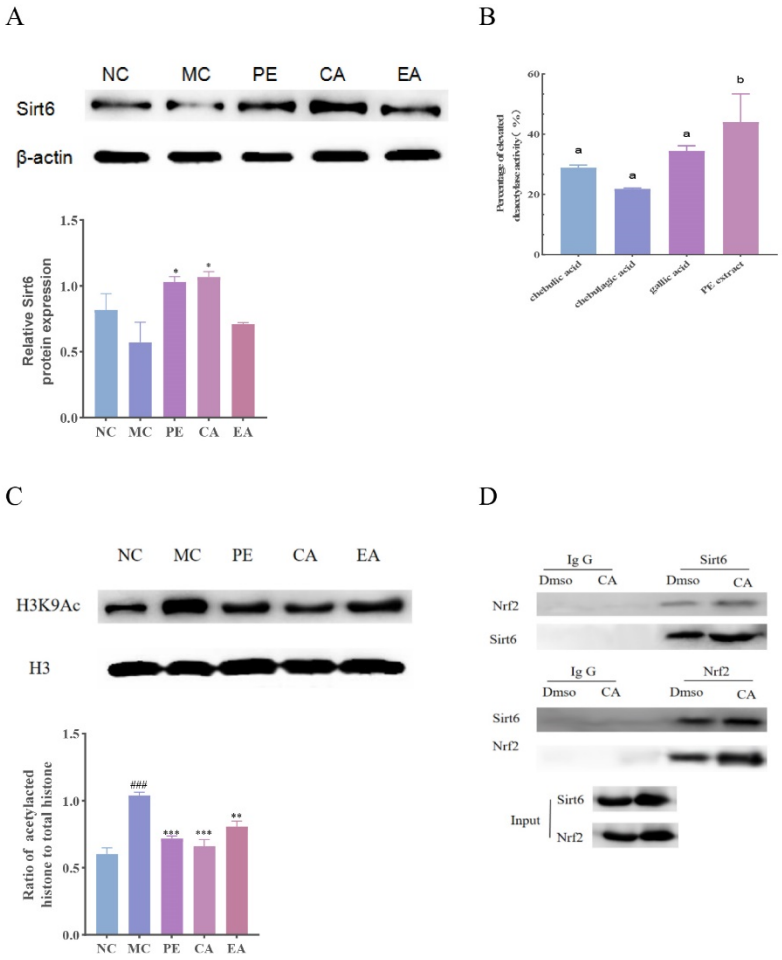


Fig. 6. A:Effect of CA on relative Sirt6 expression of H9c2 cells. B-C:Effect of polyphenols in PE extract on the deacetylase activity of the Sirt6 protein. D:Co-IP to identify the functional interaction between Sirt6 and Nrf2.

4. Discussion

D-gal induced mice serve as a well-established animal model for studying aging[26]. Long-term D-gal exposure altered redox-related indicators in mice, such as reduced GSH-Px, CAT, and T-SOD activity, along with increased levels of lipofuscin and MDA[26]. And studies had shown that chronic D-gal exposure could induce cognitive deficits and anxiety disorders. This study revealed that PE extract could reduce the level of oxidative stress and aging makers as well as improve the antioxidant capacity in serum and tissues. PE extract improved the deterioration of motor and learning memory abilities which showed anti-aging effects and was better than treated by metformin (Fig. 1-2). Therefore, as a kind of fruit with the same origin of medicine and food, PE has a potential effect of auxiliary prevention and treatment of aging, and has a good development prospect, and has greater benefits to human health. Nowadays, PE was only promoted as a health fruit, and the mechanism behind its antioxidant and anti-aging effects are still not explained.

Some previous studies had shown that D-gal induced aging changed microecological characteristics of the gut in mice, including a decrease in diversity, a decrease in the *Bacteroidota*, and an increase in the *Firmicutes*[27,28]. Analogously, in our study, PE extract restored these alterations in the gut microbiota of D-gal mice. One of the characteristic bacterial groups of the MC group, *o_Clostridia_UCG_014*, was a pro-inflammatory bacterium whose increased abundance leads to an inflammatory response and

up-regulation of lipid-associated pro-inflammatory metabolites[29]. Oxidative stress was often accompanied by pro-inflammatory microorganisms in the gut, which lead to the development of chronic dieases[30]. Characteristic bacterial in the EL group, such as *g_Alloprevotella*, *o_Lactobacillales*, and *f_Prevotellaceae*, were beneficial bacteria that improved memory and cognitive performance in D-gal mice[31-33]. Characteristic bacterial in the EH group included *Bacteroides*, a bacterium whose elevated relative abundance was associated with improved cognitive performance and negatively correlated with depression[34,35]. Its increased abundance also prevented metabolic disorder of lipid and glucose[36]. The above results indicated that PE extract could reduce the abundance of harmful bacteria and increase the level of beneficial bacteria in D-gal induced aging mice (Fig. 3).

Aging was often accompanied by metabolic dysfunction, primarily in the form of a decline in the ability to produce energy, such as the TCA cycle[37]. Using a functional prediction model of gut bacteria, it found that PE extract could up-regulate the TCA metabolic pathway in D-gal mice. Sirt6 over-expression was found to maintain the energy homeostasis of normal mice, and to improve the blockage of the cycle due to oxidative mice, which prolonged the healthy lifespan of mice[37]. Previous studies have shown that aging might be associated with dysregulation of gut microbiota as well as the expression of Sirt6[12,13]. We confirmed a correlation between aging, Sirt6 levels and gut microbiota by correlation analysis (Fig. 4D-F). In addition, PE extract was rich in polyphenols, and Sirt6 was an important site for different polyphenols to regulate antioxidant proteins and their downstream pathways[38,39]. The Sirt6 protein might be a target for its anti-aging effects.

Polyphenols were the main active components of PE. Hence, we explored the main active substances in PE polyphenols that interacted with Sirt6 proteins by molecular docking. The results revealed that many of the polyphenols in PE may interact with the Sirt6 protein. Among them, CA had the lowest docking binding energy, and chebulagic acid had the most similar site of action to known Sirt6 agonists[38]. Therefore, we suggested that these substances may be the key substances in the anti-aging effect of PE extract. CETSA further confirmed that the Sirt6 protein was able to bind to CA. And CA treatment significantly increased the Sirt6 protein expression in H9c2 cells. Therefore, CA might be the main active substance in PE extract (Fig. 5-6).

However, how polyphenols in PE extract affect the Sirt6 protein was unclear. We investigated the possible mechanism of PE extract exerting antiaging effects. Sirt6, as a co-transcription factor, could bind Nrf2 and deacetylates specific histones (H3K9) at the HO-1 promoter, thereby recruiting the RNAP II complex and enabling solid transcription of HO-1, which in turn up-regulated expression of HO-1[16]. The antioxidant enzyme HO-1 played an important role in maintaining redox, and its up-regulation could diminish D-gal-induced behavioral dysfunction and neurological deficits[40]. It also could maintain redox homeostasis[41]. Thus, the expression and deacetylation of Sirt6 were crucial for HO-1 expression and its antioxidant effect. Studies[38,39] found that some polyphenols could up-regulate Sirt6 protein expression, and

others were regulators of Sirt6 deacetylase activity. Therefore, we explored whether polyphenols in PE extract affect the expression of Sirt6 protein and its deacetylase activity.

WB experiments based both on animal and cells model revealed that the expression levels of the Sirt6 protein in the heart, liver, kidney, and brain of aging mice and aging cells were markedly elevated by PE extract and CA (the main active ingredient of PE). From circular dichroism, we found that CA and other polyphenols from PE extract changed the secondary protein structure of the Sirt6 protein. Changes in the secondary structure of proteins were associated with protease activity. The change of proteins' secondary structures from other structures to α-Helix might increase protein structural stability and hence decrease enzyme activity[42]. In contrast, in the present study, the addition of CA and other polyphenols from PE extract changed the secondary structure of the Sirt6 protein from α-Helix to other structure, which may elevate its enzymatic activity. Thus, we proceeded to measure the deacetylase activity of Sirt6 and H3K9Ac expression. The results found that CA and PE decreased the level of H3K9Ac in H_2O_2-indcued cells. The findings demonstrated that the deacetylase activity of Sirt6 may be increased by CA and PE extract.

Generally speaking, both PE and CA (the main active component of PE), could significantly improve the expression of Sirt6 protein. CA could significantly affect the secondary structure and enzyme activity of Sirt6 protein (Fig. 5-6).

To further verify the effect of CA on the interaction of Sirt6 with Nrf2, we performed Co-IP experiments. The results showed that the interaction between Sirt6 and Nrf2 was more significant in the presence of CA (Fig. 6D). Previous study revealed that Sirt6 could bind Nrf2 at the HO-1 promoter, thereby recruiting the RNAP II complex and enabling solid transcription of HO-1, which in turn up-regulated expression of HO-1[16]. Our WB results indicated that PE indeed increased the expression of HO-1 (Fig. 4). Thus, CA might be influencing the expression of downstream genes by affecting the binding of Sirt6 and Nrf2.

Therefore, the mechanism of PE extract exerting anti-aging effects may be acting on the Sirt6 protein, changing its secondary structure, increasing its deacetylation ability, and binding with Nrf2, thus increasing the expression level of the downstream antioxidant enzyme HO-1.

In general, our study demonstrated the anti-aging effects of PE extract, the key active substance and the molecular mechanisms. It was conducive to developing local characteristics of the "medicinal & food homology" economy. Also, it provided a new direction and ideas for the development and utilization of PE.

5. Conclusions

PE extract was rich in polyphenols and showed anti-aging effects. The main bioactive substance from PE extract were CA. PE extracts activated the Sirt6 protein by altering its secondary structure as well as increasing its deacetylase activity, thus increased the expression level of the antioxidant enzyme HO-1. This study provided thoughts and directions for the exploitation and utilization of PE and the development of anti-aging products.

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Conflict of interest

The authors declare no conflict of interest.

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