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Role of the alleviating Aβ toxicity in the anti-AD effect of brown wolfberry

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ABSTRACT

Wolfberry (*Lycium barbarum* L.) is a medicinal herb with anti-aging and neuroprotective effects. The purpose of this study was to explore the enhanced neuroprotective effects of red wolfberry water extract (RW), and brown wolfberry water extract (BBW). The reduced percentage of reducing sugar in BBW was shown to be 62.8%, 64.3%, 39.8%, and 11.6% for glucose, fructose, maltose, and lactose compared to those of RW, as well as free amino acids. And the contents of total phenols and total flavonoids were increased by 62.0% and 51.0%. Melanoidins (1.00%) were first isolated from BBW. RW and BBW increased anti-stress abilities (oxidative stress: 24.2% *vs.* 35.7%, and heat stress: 17.78% *vs.* 57.57%) while decreasing the reactive oxygen species (ROS) levels *in vivo*. RW and BBW reduced the rate of paralysis and odor cognitive deficits. At the same time, RW and BBW reduced the number of fluorescent spots of Aβ::GFP. The RW and BBW activated autophagy by upregulating the gene levels (*bec-1*, *lgg-1*, *lgg-2*, *unc-51*, *vps-34*, *atg-5*, *atg-18*, and *sqst-1*). Additionally, BBW promoted the nuclear-cytoplasmic ratio of DAF-16::GFP and fluorescence intensity of SOD-3::GFP by 39.4 and 1.16 fold. This study laid a new insight for exploring the Maillard reaction in improving the anti-Alzheimer's disease (AD) activity of wolfberry.

1. Introduction

Alzheimer's disease (AD) is one of the neurodegenerative diseases that most affects the quality of life of middle-aged and elderly people[1], and the disease is predicted to increase to 82 million people by 2030[2]. The current Food and Drug Administration (FDA)-approved drugs for the treatment of AD do not have significant effects[3]. The medicinal and dietary substances provided new ideas as drugs for the treatment of AD[4]. For example, the nanostructured lipid carriers of *Salvia officinalis* extract showed the potential for brain-targeted and controlled release in the treatment of AD through hCMEC/D3 cell culture experiments[5]. In addition, previous studies have shown that *Asparagus cochinchinensis* extract exerted its neuroprotective effects in Tg2576 mice for AD by reducing oxidative stress[6]. Current research suggested that the deposition of amyloid-β (Aβ) plaques in the brain triggers AD[7-8], such as *Ginkgo biloba* extract has been shown to improve memory deficits in TgCRND8 AD

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mice by reducing the number of Aβ positive signals[9]. In addition, wolfberry extract has been shown to reduce Aβ toxicity and improve microglia survival by increasing autophagy[10]. Meanwhile, the phenols in wolfberry (*Lycium barbarum* L.) might play an important role in attenuating autophagy dysregulation in AD[11]. These studies provided a theoretical basis for us to explore the anti-AD effect mechanism of wolfberry.

Wolfberry (*L. barbarum* L.) was documented in the Compendium of Materia Medica as an herbal medicine used to improve immunity and anti-aging[12]. In the traditional medicinal processing of wolfberry, the processes of concoction and decoction were accompanied by the Maillard reaction[13]. Previous studies have shown that Maillard reaction products from anchovy (*Coilia mystus*) improved the viability of PC12 cells and the memory ability of 3xTg-AD mice[14]. It was found that flavonoids in *L. barbarum* leaves attenuated the apoptosis of HUVEC cells induced by H_2O_2[15]. Meanwhile, it has been found that *L. barbarum* extract had a neuroprotective effect, and it improved the memory and cognition of APPswe/PSEN1dE9 transgenic (APP/PS1) mice[16]. Moreover, *L. barbarum* extract alleviated Aβ induced inflammatory reactions in microglial cells[10]. However, the difference of wolfberry with/without the Maillard reaction and the effect of anti-AD were unknown. This study aimed to explore the changes of active components and the difference in the anti-AD effect using red wolfberry water extract (RW) and brown wolfberry water extract (BBW).

In this study, the content changes of reducing sugar (RS) and free amino acids in RW/BBW were measured, as well as the effect of the Maillard reaction on the total phenolic content (TPC), total flavonoids content (TFC), and free radical scavenging ability of wolfberry. *In vivo*, Aβ transgenic *Caenorhabditis elegans* were used to explore the mechanism of the anti-AD effect of RW/BBW. Then, quantitative real-time polymerase chain reaction (qRT-PCR) technology was used to verify the correlation of the anti-AD mechanism of BBW, the autophagy pathway, and dauer formation family member 16 (DAF-16). The study would provide a new option to reduce the incidence of AD by wolfberry with Maillard reaction, further providing a new insight into the good anti-aging products in the future.

2. Materials and methods

2.1 Materials

H_2DCF-DA, cDNA, and SYBR Green kits were purchased from Beyotime Co. (Beijing, China). Fructose, glucose, maltose, lactose, NaN_3, and 30% H_2O_2 were purchased from Maclean's Co. (Shanghai, China). Triton X-100, *β*-mercaptoethanol, Tris-HCl, paraformaldehyde, and 3,5-dinitrosalicylic acid (DNS) were obtained from Dingguo Changsheng Biotechnology Co. (Beijing, China). Wolfberry was obtained from QI Tianxia Biotechnology Co. (NingXia, China). The wolfberry (*L. barbarum* L.), Ningqi No. 4 with Number of S-SC-LB-001-2005, was obtained from Zhongning County in Ningxia, China. N2, Aβ transgenic *C. elegans*, and *Escherichia coli* OP50 were purchased from the Caenorhabditis Genetics Center (CGC) (Minnesota, USA). Analytical-grade chemicals were used in this experiment.

2.2 Preparation of brown wolfberry

A total of 50 g red wolfberry was put into a glass evaporating dish filled with saturated potassium bromide (humidity: 79%), and stored at 70 ℃ for 72 h to get brown wolfberry[17]. Red wolfberry/brown wolfberry (50 g) was decocted in 500 mL water for 1 h at 90 ℃ and then pulped using a high shear homogenizer (2 500 r/min, 5 min) (Yuldor, Germany). The filtrate was collected using the rotary evaporator (80 ℃) (IKA, China) to remove excess water. Finally, RW and BBW were dried into solid powder by vacuum freeze-drying method.

2.3 Fundamental research on Maillard reaction

2.3.1 Determination of RS composition and content

The powder of RW/BBW (2.5 mg) was mixed with 100 mL of ultrapure water. All samples were filtered through aqueous phase microporous filter membrane (0.45 μm) (Dinguo, China). The determination of the RS composition of RW, BBW was measured by a high-performance liquid chromatography (HPLC) system equipped with Shimadzu LC-20A series apparatus (Shimadzu, Japan) and chromatographic columns (DAISOPAK SP-120-5-APS-P; 4.6 mm × 250 mm, 5 μm) at 40 ℃. The mobile phase consisted of A: 100% ultrapure water and B: 75% acetonitrile (acetonitrile:ultrapure water = 3:1, *V/V*) with an equal concentration gradient elution at a flow rate of 0.8 mL/min. The detected volume was 25 μL.

2.3.2 Determination of free amino acid content

The free amino acid content was determined by GB/T 5009.124−2003 *Determination of Amino Acids in Food*, and the analytical instrument was UK bio30+ series amino acid analyzer (Biochrom, UK). In short, the powder of RW/BBW (200 mg) was hydrolyzed by HCl (10 mL, 6 mol/L) at 115 ℃ for 24 h. Then the hydrolysate (2 mL) was mixed with ethanol (8 mL) for centrifugation (3 000 r/min, 25 min). The vacuum-dried supernatant was redissolved in HCl (2 mL, 0.01 mol/L) and filtered by a nylon filter membrane (0.22 μmol/L) for further use.

2.3.3 Melanoidins extraction and colorimetric analysis

The precipitation extraction was prepared concerning the previous method[18]. Briefly, RW and BBW aqueous solutions were prepared with RW/BBW freeze-dried powder (6 g) and 50 mL double distilled H_2O (ddH_2O) then anhydrous ethanol was added until the scale of 30% and stood for 30 min to obtain supernatant by centrifugation (10 ℃, 3 000 r/min, 30 min) using acetone (72 mL). The melanoidins were finally obtained by the mix of acetone (90 mL) and supernatant after completed evaporation.

The color parameters (lightness (*L**), red-green axis (*a**), and yellow-blue axis (*b**)) of RW/BBW were measured using a SC-10 portable colorimeter (3nh, Shenzhen, China).

2.3.4 Determination of TPC, TFC, and RS

The TPC was determined by the Folin-Ciocalteu method with gallic acid as the standard. Briefly, 100 μL of RW/BBW (1 mg/mL) was mixed with distilled water (2.8 mL) and Folin-Ciocalteu

reagent (100 μL), then added with Na_2CO_3 solution (7.5%, 300 μL) for incubation (2 h) in the dark. Finally, the optical density was measured at 765 nm using the microplate reader (Miu, Hangzhou, China)[19].

For TFC was determined referred by Yang et al.[20]. Similarly, RW/BBW (1 mg/mL, 100 μL) was diluted with distilled water (2.6 mL) and 5% $NaNO_2$ (150 μL), then added $Al(NO_3)_3$ (10%, 150 μL) within a wait of 6 min. Finally, 4% NaOH (2 mL) was added after another wait for 6 min. The absorbance was measured at 510 nm using a microplate reader (Miu, Hangzhou, China).

The DNS method was used for the determination of RS[21]. Simply, 1.5 mL of DNS reagent was reacted with 2 mL of sample solution by shaking in a boiling water bath for 5 min and cooled in an ice water bath immediately. Then 0.35 mL of the reaction solution was mixed with 2.15 mL of H_2O to measure the OD value at 540 nm.

The TPC, TFC, and RS of the RW/BBW were calculated by the regression equation of the gallic acid (y = 2.243 1x + 0.007 8; R^2 = 0.999), rutin (y = 0.996 4x + 0.008; R^2 = 0.995 1), and the glucose (y = 1.105 7x + 0.006 4; R^2 = 0.996 6).

2.4 Determination of antioxidant capacity in vitro

The free radical-scavenging activity was determined referred to Wang et al.[22]. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (200 μmol/L) was mixed with RW and BBW (100 μmol/L) to measure at 517 nm after the reaction was carried out in the dark for 30 min[23].

The calculation formula was as follows:

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

Where A_{control} and A_{sample} represented the absorbance of the control and the test samples.

The 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) cation radical ($ABTS^{+\cdot}$) assay was determined according to the study of Zhang et al.[24]. The $ABTS^{+\cdot}$ (7 mmol/L) with potassium persulfate (45 mmol/L) were mixed at the ratio of 1:2. The $ABTS^{+\cdot}$ was diluted with anhydrous ethanol until the absorbance to 0.70 ± 0.02 at 734 nm. The absorbance was measured at 734 nm for both $ABTS^{+\cdot}$ and RW/BBW (200 μL) after standing for 6 min in the dark.

The calculation formula was as follows:

$$ABTS^{+\cdot}\ \text{scavenging activity}\,(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

Where A_{control} and A_{sample} represented the absorbance of the control and test samples.

2.5 Anti-AD effect of RW, BBW on C. elegans

2.5.1 C. elegans culture and experiment groups

In this study, 7 kinds of *C. elegans* were selected and incubated with inactivated OP50 (65 ℃, 45 min). Of these, CL2006, CL4176, CL2355, CL2122, CL2331, and AM141 were cultured in an incubator at 15 ℃ and N2 at 20 ℃[25]. N2 was used for assays of lifespan and stress assays. CL4176 was used for reactive oxygen species (ROS) level determination and paralysis assays. The transgenic strains of CF1038, VC893, and CB369 were used for the mutant longevity experiment. The strains of TJ356, CF1553 CL2331, and AM141 were chosen to measure the fluorescence intensity of DAF-16::GFP, SOD-3::GFP, $A\beta_{3\text{-}42}$::GFP and polyQ40::YFP. The transgenic strains of CL2355 and CL2122 were selected for the chemotaxis assay.

2.5.2 Lifespan assay

The optimal safe concentration of RW and BBW was screened by pre-experiment. In short, N2 (L4 stage, 100 per group) were fed 2.5, 5 mg/mL RW and BBW. The survival rate of the *C. elegans* was calculated every 24 h. The experimental method of mutant longevity was the same as above[26].

2.5.3 ROS fluorescence intensity

For CL4176, L4 stage adults were cultivated at 20 ℃ for 3 days in NGM plates with/without RW and BBW (2.5, 5 mg/mL). After treatment, CL4176 were incubated in 1.0 mL M9 (pH 7.4, 4.4 mmol/L KH_2PO_4, 8.4 mmol/L $Na_2HPO_4{\cdot}12H_2O$, 20.5 mmol/L NaCl, 1.7 mmol/L $MgSO_4$) containing 100 μmol/L H_2DCF-DA for 1 h in the dark. *C. elegans* were centrifuged 3 times at 3 500 r/min for 10 min. The CL4176 anesthetized with NaN_3 were mounted on a microscope slide and fluorescence intensity was observed using fluorescence microscopy (Olympus, Tokyo). Fluorescence intensity was analyzed by ImageJ.

2.5.4 Stress assay

N2 (L4 adults) were cultured for 3 days after treatment with/without RW and BBW. For the oxidative stress test, the *C. elegans* were placed on the nematode growth medium (NGM) plate containing 30% H_2O_2 and the number of deaths was measured per hour. For the heat stress test, the *C. elegans* were placed at 35 ℃ and observed for heat stress every 1 h.

2.5.5 Aβ expression-phenotypic assays

Refer to Kalmankar et al.[27] for the method of paralysis experiment. The CL4176 (80/group) were cultured on NGM plates with/without RW and BBW (2.5 mg/mL, 5 mg/mL) at 16 ℃ for 48 h, and then *C. elegans* were transferred to 25 ℃ for 24 h. The paralysis rate of CL4176 was recorded every 2 h.

For the chemotactic test, the culture conditions were consistent with the paralysis experiment. CL2355 and CL2122 (35 per group) were washed by M9 buffer. The plate was divided into four quadrants, and a point was selected in each quadrant: A, B, C, and D. The distance from each point to the center of the plate was the same. Points A, D were added with 0.1% benzaldehyde, points B and C were added with 1 μL of absolute ethanol. Then 1 μL NaN_3 anesthetized *C. elegans* were added in all 4 points.

$$\text{Chemotactic index (CI)} = \frac{\text{The number of } C.\ elegans_{\text{A, D points}} - \text{The number of } C.\ elegans_{\text{B, C points}}}{\text{Total number of } C.\ elegans} \quad (3)$$

2.5.6 ThS staining of Aβ deposits assay

The CL2006 (L4 stage, 80 per group) were fed on NGM plates with or without RW and BBW (2.5, 5.0 mg/mL) for 96 h at 20 ℃. After washing and collection with M9 buffer, *C. elegans* were

permeabilized in the tissue fixator (4% polyformaldehyde, 125 mmol/L Tris-HCl, pH 7.4, 5% β-mercaptoethanol, and 1% Triton X-100) for 24 h at 37 ℃. The treated *C. elegans* were immersed in 0.125% Thioflavin S (ThS) for 2 min. The accumulation of Aβ spots in CL2006's head was observed by fluorescence microscopy (Olympus, Tokyo) after destained sequentially in ethanol (50%, 70%, and 90%).

2.5.7 Fluorometric assay

The *C. elegans* (L4 stage) expressing GFP were cultured for 3 days on NGM plates with or without BBW and RW. The *C. elegans* anesthetized by NaN_3 were observed under a fluorescence microscope (Olympus, Tokyo). Fluorescence intensity was analyzed using ImageJ.

2.5.8 qRT-PCR assay

RNA was extracted from *C. elegans* by TRIzol method and its purity was determined by spectrophotometer. Then, a cDNA synthesis kit (Biyuntian, Beijing, China) was used to convert RNA into cDNA. SYBR Green kit (Steinheim, Germany) was used for qRT-PCR analyses. The internal data were analyzed by comparing the $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 7 software and Origin (2021). The fluorescence intensity in *C. elegans* was analyzed by ImageJ software. All experimental data were analyzed by unpaired two-tailed Student's *t*-test (mean ± SEM). The asterisks (*) indicate significant differences.

3. Results

3.1 Decreased content of RS and free amino acid

The contents of glucose, fructose, maltose, and lactose in BBW were reduced by 62.8%, 64.3%, 39.8%, and 11.6% compared with RW (Figs. 1A-B, Table 1). The total RS was reduced by 49.7% (Fig. 1D). Another, 14 kinds of free amino acids were decreased in BBW compared to RW and the three decreased more were Arg, Glu, and Pro (Fig. 1C). Similarly, Paravisini et al.[28] found that the RS and amino acids were reduced during the non-enzymatic browning of orange juice. It was worth noting that the content of three amino acids increased slightly in BBW, which might be the heat treatment caused protein breakdown in wolfberry (Table 2). The content of Met, Tyr, and Phe was also found to be increased in sesame roasting at the same time[29].

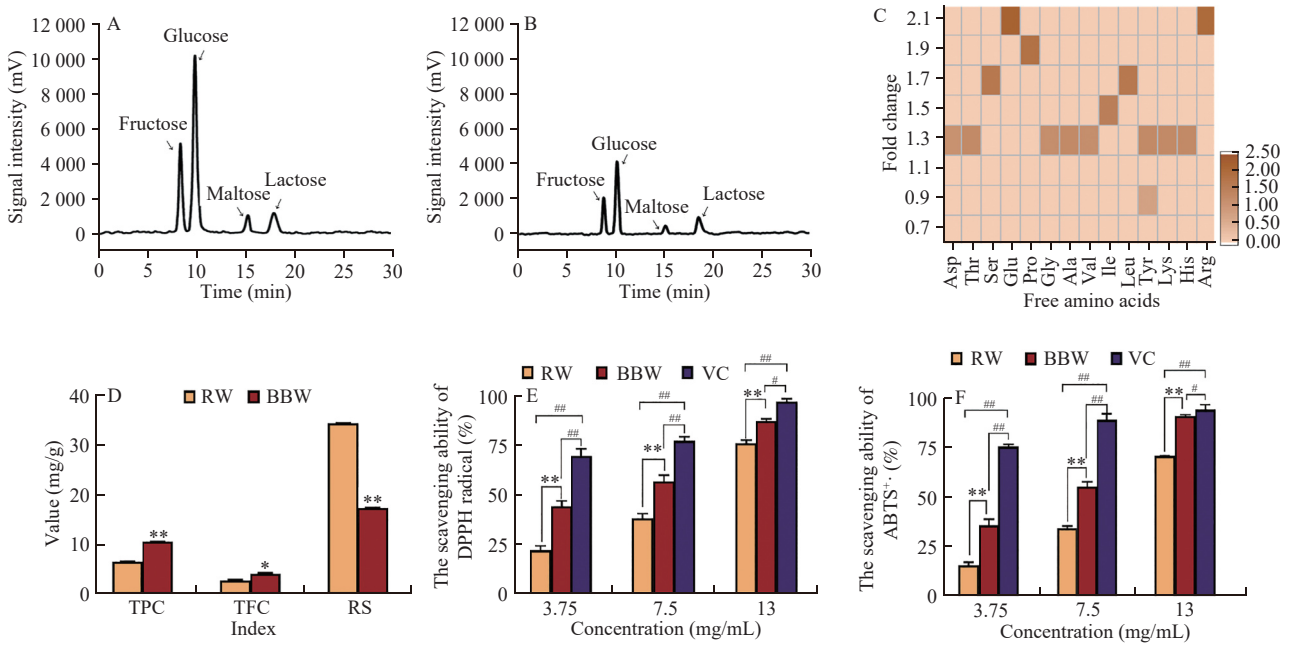

Fig. 1 (A) HPLC of the RS from RW. (B) HPLC of the RS from BBW. (C) The fold change of free amino acids in RW and BBW. (D) The content of TFC, TPC, and total RS. (E) DPPH free radical scavenging ability in RW, BBW and VC. (F) $ABTS^{+\cdot}$ scavenging ability in RW, BBW and VC. *Indicated significant difference (*$P < 0.05$ and **$P < 0.01$) between RW and BBW groups. #Indicated significant difference (#$P < 0.05$ and ##$P < 0.01$) between positive control (VC) and RW, BBW groups.

Table 1
Fructose, glucose, maltose, and lactose content in RW and BBW.

RS	RW (mg/g)	BBW (mg/g)	Retention time (min)
Fructose	5.693 ± 0.22^{a}	2.032 ± 0.32^{b}	8.95 ± 0.06
Glucose	7.649 ± 0.16^{a}	2.846 ± 0.43^{b}	10.23 ± 0.19
Maltose	3.499 ± 0.08^{a}	2.107 ± 0.03^{b}	16.26 ± 0.27
Lactose	2.486 ± 0.17^{a}	2.198 ± 0.25^{b}	18.77 ± 0.06

Note: Different lowercase letters (a, b) indicated a significant difference between the RW group and the BBW group ($P < 0.05$). The same below.

Table 2
Content of free amino acids in RW and BBW.

Free amino acids	RW (mg/g)	BBW (mg/g)	Changes in BBW compared to RW (%)
Asp	0.075 ± 0.001[a]	0.066 ± 0.003[a]	−12.0
Thr	0.059 ± 0.001[a]	0.047 ± 0.002[b]	−20.3
Ser	0.215 ± 0.013[a]	0.125 ± 0.005[b]	−41.9
Glu	0.042 ± 0.001[a]	0.019 ± 0.001[b]	−54.8
Pro	1.087 ± 0.015[a]	0.576 ± 0.022[b]	−47.0
Gly	0.011 ± 0.001[a]	0.009 ± 0.001[a]	−18.2
Ala	0.150 ± 0.004[a]	0.113 ± 0.007[b]	−24.7
Val	0.037 ± 0.002[a]	0.030 ± 0.002[b]	−18.9
Ile	0.030 ± 0.001[a]	0.020 ± 0.002[b]	−33.3
Leu	0.030 ± 0.001[a]	0.018 ± 0.001[b]	−40.0
Tyr	0.071 ± 0.002[a]	0.056 ± 0.001[b]	−21.1
Lys	0.036 ± 0.001[a]	0.027 ± 0.001[a]	−25.0
His	0.015 ± 0.001[a]	0.012 ± 0.001[a]	−20.0
Arg	0.080 ± 0.001[a]	0.039 ± 0.001[a]	−51.3
Cys	0.044 ± 0.002[a]	0.045 ± 0.001[a]	2.3
Met	0.014 ± 0.006[a]	0.015 ± 0.001[a]	7.1
Phe	0.057 ± 0.002[b]	0.083 ± 0.001[a]	45.6

3.2 The content of active substances in BBW increased

The BBW contained (1.00 ± 0.05)% melanoidins, a Maillard reaction product, but RW contained almost none. The TPC increased 62.0% depending on the fact of (6.45 ± 0.01) mg/g for RW and (10.45 ± 0.03) mg/g for BBW as the previous studies of 6.38 mg/g[30]. The TFC also increased 51.0% with the contents of (2.67 ± 0.14) mg/g for RW and (4.03 ± 0.17) mg/g for BBW (Table 3). Similar to our study, Tian et al.[19] found the TFC of 1.67 RE mg/mL in *L. barbarum* fruit vinegar.

Table 3
Content of phytochemical components was in RW and BBW.

Phytochemicals	RW	BBW
Fat (mg/g)	8.55 ± 1.42[a]	8.58 ± 1.01[a]
TPC (mg/g)	6.45 ± 0.01[a]	10.45 ± 0.03[b]
TFC (mg/g)	2.67 ± 0.14[a]	4.03 ± 0.17[b]
RS (mg/g)	34.14 ± 0.09[a]	17.19 ± 0.11[b]
Total sugar (mg/g)	224.33 ± 6.02[a]	188.33 ± 12.01[b]
Total carotenoids (μg/g)	192.54 ± 6.75[a]	190.83 ± 10.10[a]
Moisture content (mg/g)	15.80 ± 0.90[a]	15.45 ± 0.99[a]
Ash content (mg/g)	20.14 ± 0.34[a]	19.64 ± 0.26[a]
Protein (mg/g)	90.63 ± 4.61[a]	65.82 ± 3.96[b]

3.3 New production of melanoidins and color accentuation for BBW

By measuring the colorimetric values of RW and BBW, the *L**, *a**, and *b** values were 26.43 ± 0.11, 2.14 ± 0.02, and 2.87 ± 0.02 in BBW while reduced by 16.5%, 86.9%, and 87.5% for RW, which indicated that the darker color of BBW than RW. The values of ΔEmpfindung (Δ*E*) in RW and BBW were 22.80 ± 0.32 and 29.12 ± 0.07, which increased by 27.72% for BBW (Table S1). The increase of Δ*E* value indicated that the color of wolfberry is deepened after browning[31].

3.4 Increased of free radical scavenging rate of BBW

The DPPH radical scavenging ability of 7.5 mg/mL RW and BBW was 38.3% and 56.9%, respectively. Similarly, red ginseng after the Maillard reaction had stronger DPPH clearance ability compared with white ginseng (5 mg/mL white ginseng: 26.67% *vs.* 5 mg/mL red ginseng: 42.06%) [32]. In addition, the $ABTS^{+\cdot}$ scavenging ability of 7.5 mg/mL RW and BBW was 33.9% and 55.0%. (Figs. 1E-F). VC showed the best free radical scavenging ability. As well as results of He et al.[33] , the oyster meat hydrolysate after the Maillard reaction showed better $ABTS^{+\cdot}$ scavenging power than the previous ones, which suggested that the Maillard reaction might increase the free radical scavenging activity of wolfberry (Figs. 1E-F).

3.5 The prolongment of the lifetime and anti-stress of C. elegans

The two concentrations with the best life-prolonging effect were selected for follow-up experiments (RW: 2.5, and 5 mg/mL *vs.* BBW: 2.5, and 5 mg/mL) (Figs. 2A-B). Compared with the control group, the maximum lifespan of *C. elegans* fed with 5 mg/mL RW, BBW was (13.64 ± 0.31) and (16.37 ± 0.26) days, the average lifespan increased by 21.5%, 32.3% (Fig. 2C), which indicated that BBW and RW could improve the survival rate of *C. elegans* with higher for BBW than that of RW.

For the oxidative stress test, the mean lifespan of *C. elegans* treated with RW/BBW (5 mg/mL) increased by 24.2%/35.7% after exposing to 30% H_2O_2. For the heat stress test, the lifespan increased by 17.78%/57.57% (Figs. 2D-E), while the ROS fluorescence intensity decreased by 40.7%/61.4% (Figs. 3A-C), which suggested the BBW could improve the anti-stress ability of *C. elegans.*

3.6 Inhibition of Aβ-induced toxicity by BBW

For the paralysis experiment, the addition of RW and BBW significantly prolonged the paralysis time of *C. elegans* (Fig. 3D). To

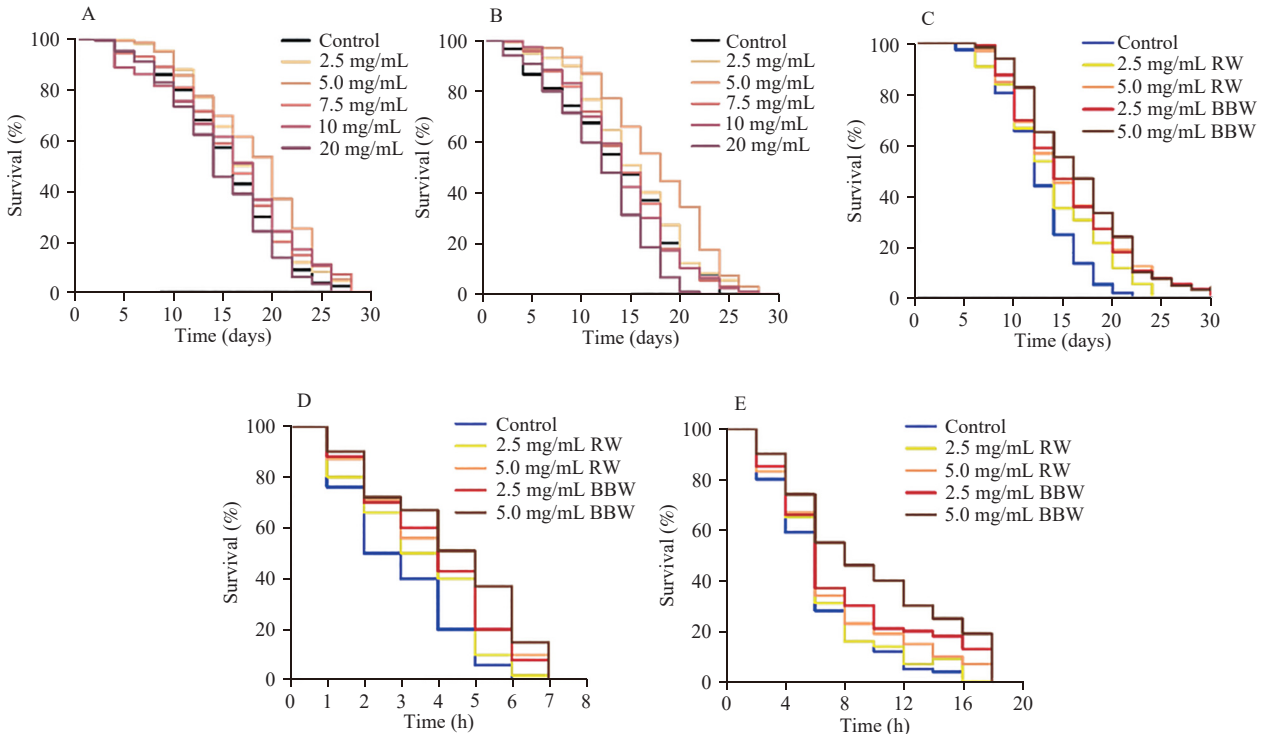

Fig. 2 (A) Effects of different concentrations of RW on longevity of *C. elegans*. (B) Effects of different concentrations of BBW on longevity of *C. elegans*. (C) Effect of RW and BBW (2.5, 5 mg/mL) on longevity of *C. elegans*. (D-E) Effect of RW, BBW on *C. elegans*' lifespan under H_2O_2, heat stress conditions. Compare with the control group, $^*P < 0.05$, $^{**}P < 0.01$.

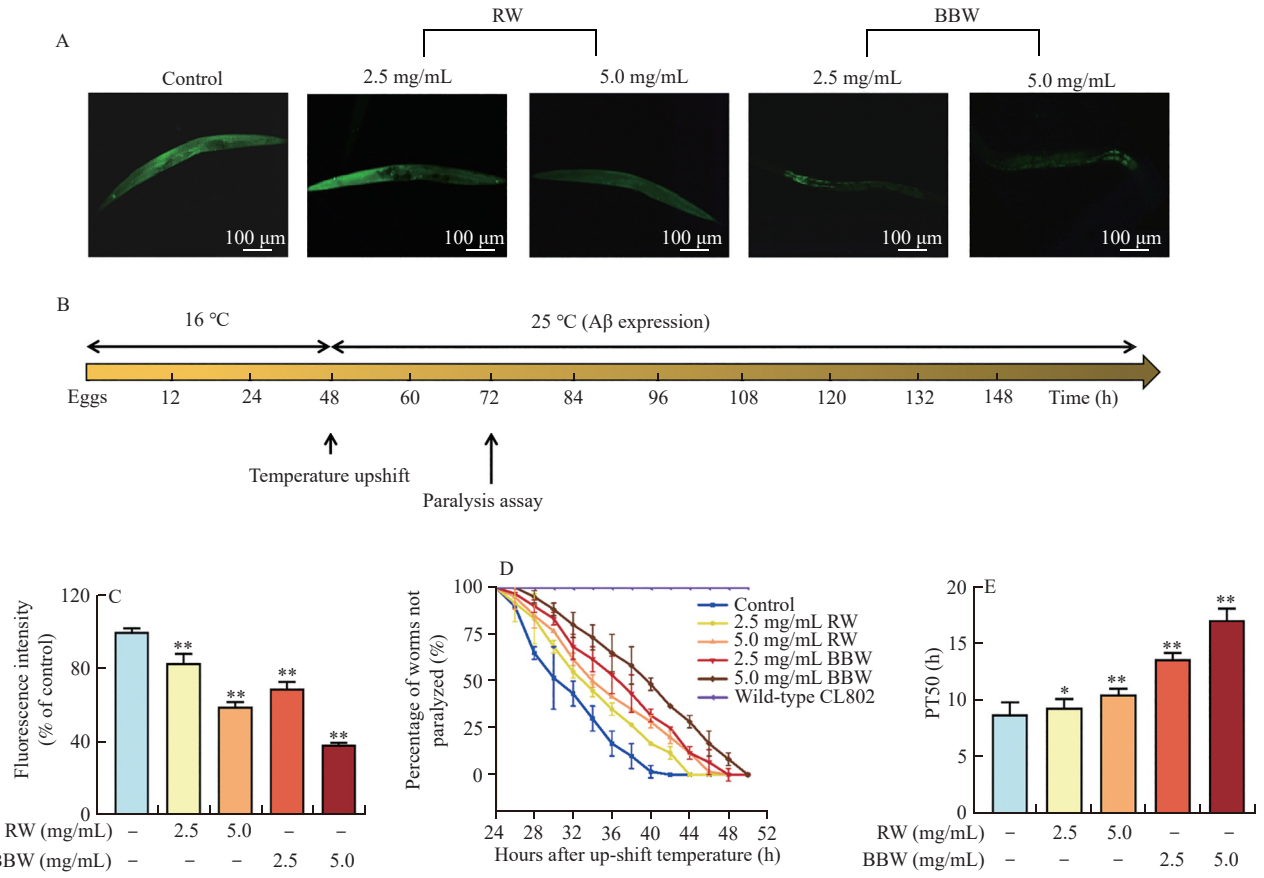

Fig. 3 (A) Representative ROS fluorescence picture in *C. elegans*. (B) Schematic diagram of paralysis experiment procedure. (C) The relative fluorescence intensity of ROS was compared with that of control group. (D) Effect of RW and BBW on the nonparalytic rate of CL4176. (E) Effects of RW and BBW on 50% *C. elegans* to paralyse (PT50). (F) Schematic representation of the chemotaxis assay. (G) Effect of RW and BBW on CI of CL2355.

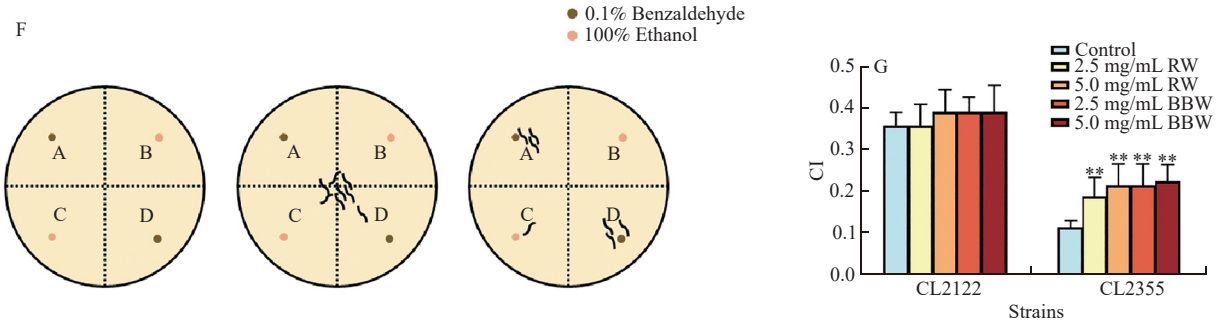

Fig. 3 (*Continued*)

see the difference more clearly, the time required for 50% *C. elegans* to paralysis (PT50) was calculated, and it was found that 5 mg/mL RW/BBW extended PT50 by 56.1%/95.9% (Fig. 3E). For chemotactic test, the CI value of CL2355 was increased 90.9% for RW and 118.2% for BBW under treatment of 5 mg/mL (Figs. 3F-G). Similar to the caffeic acid which reduced Aβ toxicity by the same way[34].

The number of Aβ deposits was reduced by 27.3%, and 68.0% after treatment with 5 mg/mL RW and BBW in CL2006 (Figs. 4A-B). Similarly, CL2331 reduced the number of Aβ in body wall muscles by 84.0% when treated with 5 mg/mL BBW (Figs. 4C-D), as well as the accumulation of Aβ spots in the brain and body wall muscles, which was similar to the anti-AD effect of disulfide-rich cyclic peptides by observing the number of spots in CL2006[27]. The fluorescent aggregation sites of AM141 gradually decreased during the growth of the *C. elegans* (Figs. 4E-F), which also reflected the good neuroprotective effect of BBW. Similarly, black rice anthocyanin extract reduced fluorescent spot aggregation in AM141[35].

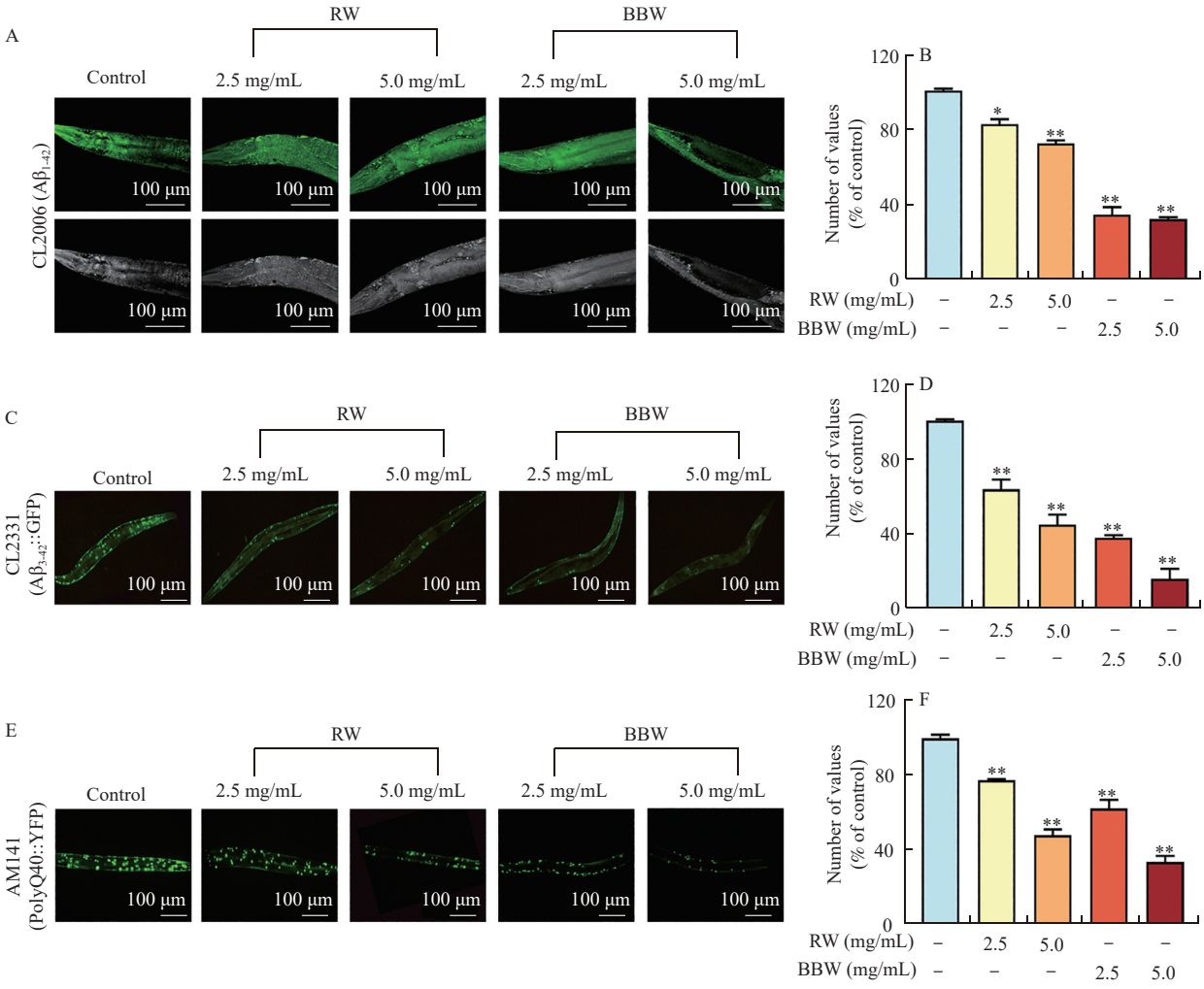

Fig. 4 (A) The ThS-stained Aβ deposition spots of CL2006 in the control group and RW, BBW treatment group. (B) Quantitative analysis of Aβ fluorescence spots in CL2006. (C) The Aβ deposition spots of CL2331 in the control group and RW, BBW treatment group. (D) Quantitative analysis of Aβ fluorescence spots in CL2331. (E) The polyQ40::YFP of AM141 in the control group and RW, BBW treatment group. (F) Quantitative analysis of fluorescence spots in AM141.

3.7 Relationship of the autophagy pathway and the anti-AD effect

After DA2123 (LGG-1::GFP) was treated with 5 mg/mL RW and BBW, the fluorescence intensity in the gut was increased by 11.0% and 35.7% (Figs. 5A-B). In addition, BBW enhanced the nuclear-cytoplasmic ratio of DAF-16 (RW:BBW = 14.08:34.43 times in 5 mg/mL) (Figs. 5E-F). Meanwhile, RW and BBW increased the fluorescence intensities of SOD-3::GFP by 63.2% and 115.7% by 5 mg/mL (Figs. 5C-D). Both RW and BBW activated key genes *lgg-1* through the autophagy pathway, *daf-16*, and *sod-3*, similar to phlorizin, which exerted an anti-aging effect by activating oxidative stress and autophagy pathway[36].

The BBW and RW could increase the mRNA levels of autophagy-related genes of *bec-1* (0.33 *vs.* 1.63), *lgg-1* (0.42 *vs.* 0.83), *lgg-2* (0.52 *vs.* 1.00), *unc-51* (0.33 *vs.* 0.74), *vps-34* (0.31 *vs.* 0.53), *atg-5* (0.46 *vs.* 0.90), *atg-18* (0.45 *vs.* 0.74), and *sqst-1* (0.72 *vs.* 0.74), as well as the key genes of oxidative stress pathway: *daf-16* (1.35 *vs.* 2.94) and *sod-3* (0.58 *vs.* 1.63) by BBW. The chlorogenic acid also showed a neuroprotective effect in the same autophagy pathway[37].

3.8 The reliant of BBW-mediated longevity on autophagy and DAF-16

The results in Fig. 6 showed that without autophagy-related genes (*unc-51* and *atg-18*), BBW and RW could not improve the lifespan of related *C. elegans*, as well as the lack of *daf-16* gene, which suggested that there might be a link between oxidative stress and autophagy (Fig. 6).

4. Discussion

In this study, the product after the Maillard reaction of melanoidins, produced by RS and free amino acids, could clear reactive oxygen species and decompose H_2O_2 (Fig. 1)[38]. Similarly, the healthy Maillard product of melanonids in novel coffee cascara beverage could improve antioxidant capacity[39]. In addition, Çelik et al.[40] found that melanoidins could resist oxidative stress and maintain the stability of human intestinal microflora. At the same time, the reaction products of fructose and Cys, Val, and/or Glu could improve the oxidation capacity and reduce the anti-proliferation ability of HCT116 cells[41]. As the result of the Maillard reaction, BBW had been shown to enhance DPPH and $ABTS^{+\cdot}$ radical scavenging abilities higher than that of RW (Fig. 1), which was also observed during sesame roasting[42] and cooking of *Polygonum multiflorum* root[43]. The increased TPC and TFC in BBW further revealed the relationship of the polyphenols in *Lycium ruthenicum* Murr with antioxidant ability (Fig. 1)[44], which was connected with the decomposed and released of macromolecules produced more phenolic hydroxyl groups during the Maillard reaction, similar as other previous research[33]. As a result, this study hypothesized the Maillard reaction could boost the free radical scavenging potential of *L. barbarum*, providing a foundation for future analysis of the

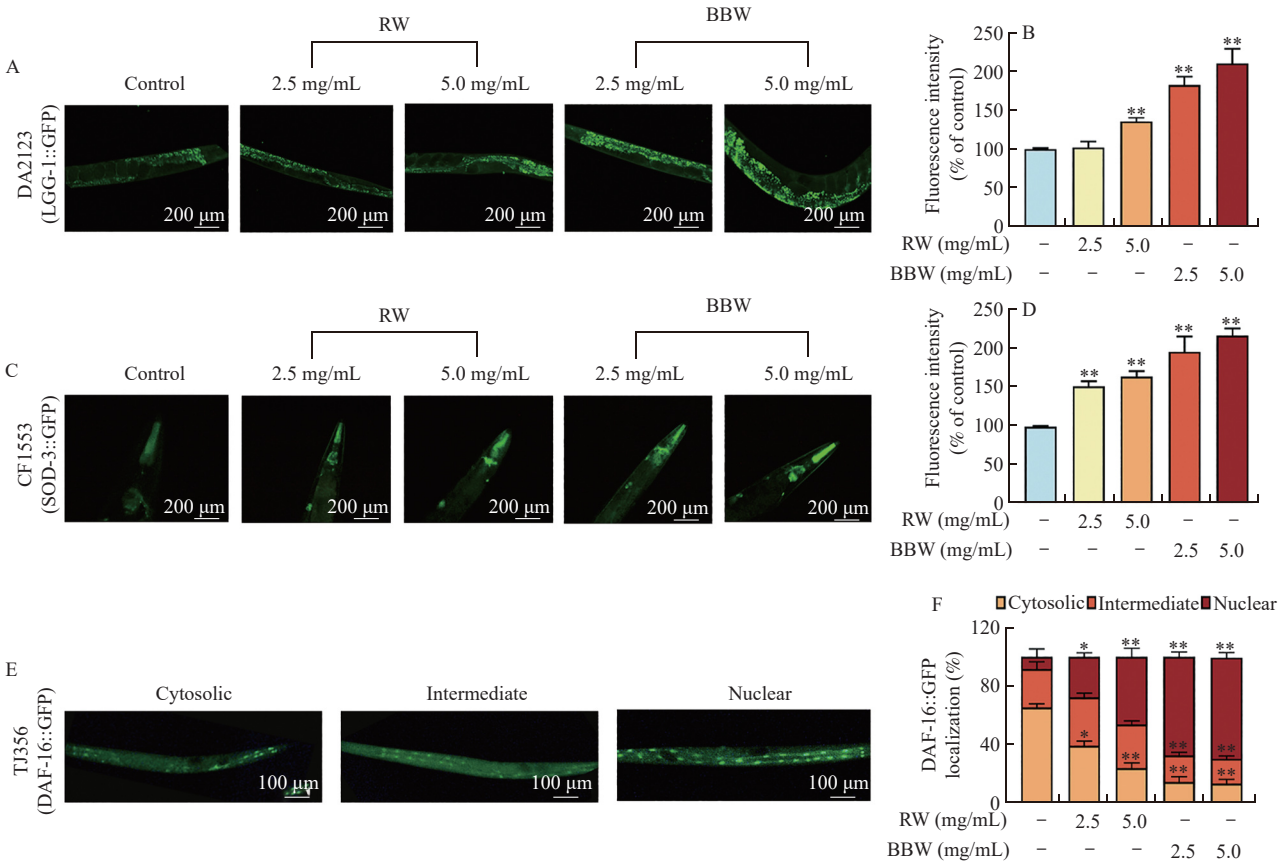

Fig. 5 (A) Representative fluorescence images of LGG-1::GFP. (B) The fluorescence intensity was quantitatively analyzed in DA2123. (C) Representative fluorescence images of SOD-3::GFP. (D) The fluorescence intensity was quantitatively analyzed in CF1553. (E) Representative fluorescence images of DAF-16::GFP. (F) The proportion of DAF-16::GFP localizing in the nucleus, cytoplasm, and intermediate.

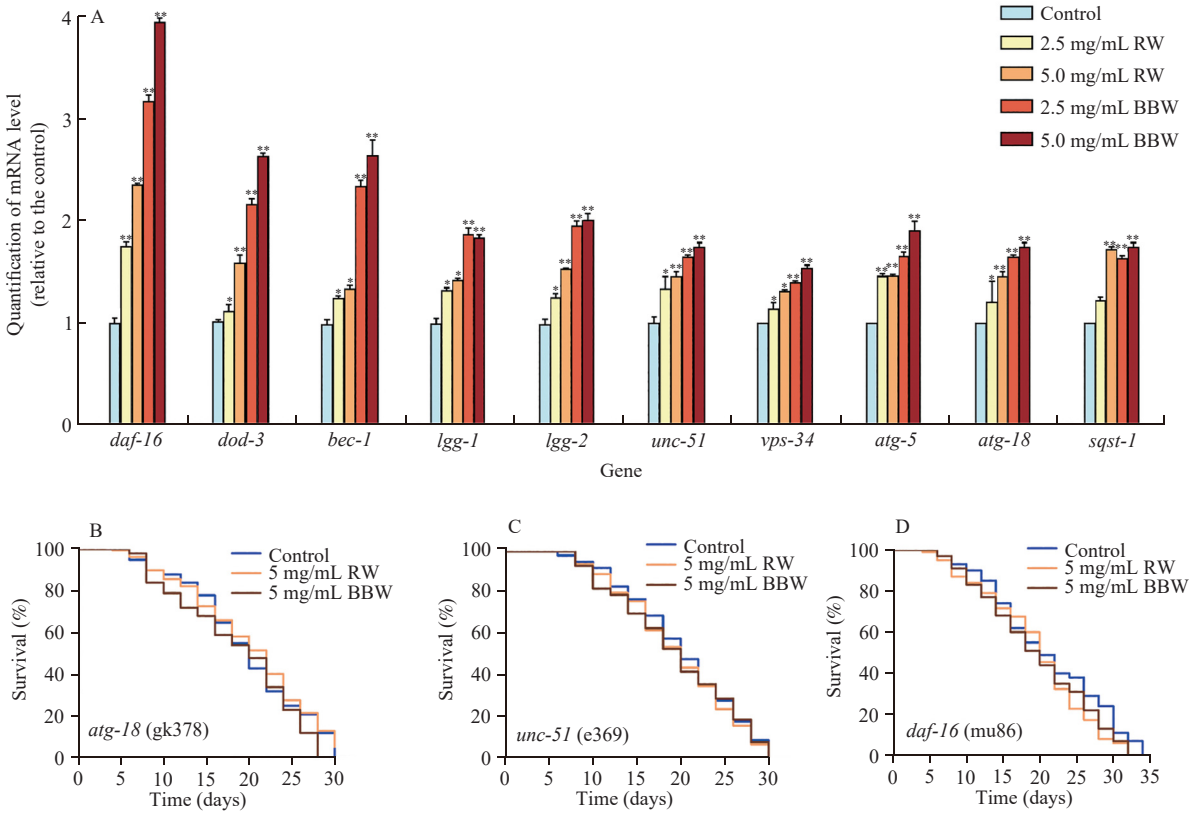

Fig. 6 (A) The expression of mRNA levels of autophagy genes. (B-D) Impacts of RW and BBW on the survival time of mutant strains including, *atg-18* (gk378), *unc-51* (e369), and *daf-16* (mu86).

antioxidant and anti-AD effects for BBW. The phenolic components in almonds (*Prunus dulcis*) gradually increased during baking with the increasing antioxidant activity of the Maillard reaction[45]. The dietary flavonoids could attenuate neuroinflammation in AD rats by relieving advanced glycation end products[46].

In vivo, ROS, a cause of many neurological illnesses, could cause oxidative stress in the body further aging[47]. The RW and BBW reduced ROS levels and improved anti-stress ability (Fig. 2), consistent with previous findings[26]. Observing the effect of Aβ accumulation was an important means[48]. The results showed that BBW reduced the paralysis rate of CL4176 and increased the sensitivity of CL2355 to odors by alleviating Aβ toxicity (Fig. 3). Similarly, Meng et al.[26] found that *L. barbarum* extract prolonged life by reducing the accumulation of toxic proteins. The extract of Gouqi could also improve memory and reduce $A\beta_{1\text{-}42}$ levels in APP/PS1 mice[49], which was consistent with the stronger anti-Aβ effect of BBW. The decreased fluorescence number of Aβ::GFP after BBW and RW treatment (Fig. 4) was also confirmed in Antler extract[50]. Similarly, Shin et al.[51] found that ginseng extract after Maillard reaction improved Aβ pathological behavior in 5XFAD AD transgenic mice. In addition, Browning ginseng showed stronger anti-aging activity than that of ginseng[52], as well as the comparation of the hot processed *Polygonum sibiricum* with *P. sibiricum*[53].

Age-related misfolding and aggregation of disease-causing proteins were responsible for a variety of neurodegenerative diseases[54]. At the same time, BBW was found to inhibit neurotoxicity by the reduction of fluorescent aggregations in AM141 (Fig. 4), which was similar to that of resveratrol in AM141 *C. elegans*[55]. The enhanced fluorescence spot number of LGG-1::GFP reflected the therapeutic effect on AD through the autophagy pathway (Fig. 5), which was also observed in the increasing number of LGG-1 spots in DA2123 *C. elegans* β-amyrin through activating autophagy and inhibiting Aβ accumulation[56].

The BBW and RW enhanced DAF-16 entry into the nucleus (Fig. 5) as sulforaphane improved antioxidant capacity through *daf-16*[57-58]. The mRNA levels of genes in the autophagy pathway were increased by RW and BBW (Fig. 6), which was similar to the prolonged lifespan of ALS *C. elegans* by up-regulating genes of *lgg-1*, *vps-34*, and *sqst-1* in the autophagy pathway[59-60]. Similarly, curcumin[61], astragalin[62] and the blueberry extract could alleviate cognitive impairment and neuronal damage by up-regulating autophagy-related proteins[63], which reflected the key role of the autophagy pathway in anti-AD effect of BBW[64].

5. Conclusion

AD has become a prominent issue currently. The increase of active substances (TPC, and TFC, and melanoidins) improved the scavenging ability of BBW on free radicals. *In vivo*, the relief of the Aβ aggregation in Aβ transgenic *C. elegans* was also observed. In addition, the RW and BBW could resist AD by activating autophagy and DAF-16. This study demonstrated that BBW could be a potential drug for neurodegenerative diseases and further provided strong support for the application of the Maillard reaction in Chinese wolfberry to alleviate AD disease. The comparison of the melanoidins obtained by Maillard reaction would be also expected since the predominant production.

Conflict of interest

Hao Wang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declared that no conflicts of interest to this work.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250256.

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