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ORIGINAL ARTICLE

The extraction, antioxidant and against β-amyloid induced toxicity of polyphenols from *Alsophila spinulosa* leaves

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KEYWORDS

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P13K/AKT

Abstract *Alsophila spinulosa* is a tree-like fern, and many evidences suggested that plant polyphenols had the potential therapeutic for Alzheimer s disease (AD). Herein, polyphenols (ASP) was isolated from *A. spinulosa* leaves and its major constituent were isoorientin and vitexin. ASP displayed excellent antioxidant activity and obvious anti-lipid peroxidation capacity *in vitro*. ASP improved the survival rate of *C. elegans* under high temperature by enhancing the antioxidant enzymes activities and decreasing the lipid peroxidation level. Moreover, ASP alleviated β-amyloid (Aβ) induced paralysis and reduced Aβ deposition, decreased reactive oxygen species (ROS) accumulation and improved the level of *skn-1* mRNA. In addition, ASP decreased the levels of *pdk-1* and *akt-1* mRNA in *P13K/AKT* signaling pathway. In conclusion, ASP may be a potential ingredient for the alleviation of AD.

Abbreviations: AD, Alzheimer's disease; ASP, *A. spinulosa* leaves polyphenols; Aβ, β-amyloid; APP, Amyloid precursor protein; ROS, Reactive oxygen species; UAE, Ultrasonic assisted extraction; RSM, Response surface methodology; BBD, Box-Behnken Design; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); SOD, Superoxide dismutase; CAT, Catalase; MDA, Malondialdehyde; NGM, Nematode growth medium; HPLC, High performance liquid chromatography; DCFH-DA, 2',7'- Dichlorodihydrofluorescein diacetate; GSH, Glutathione

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1. Introduction

AD, representing 60%-80% of all dementia, occurred mainly over the age of 65, and it was a neurodegenerative disease related to memory and cognitive impairment (Grontvedt et al., 2018). Although its exact pathogenesis is uncertain, the hypothesis about Aβ cascade is considered the major pathological cause (Yang et al., 2019). The aggregation of Aβ will trigger neurotoxicity and oxidation (Shankar et al., 2008). One of the major features for AD is oxidative damage. Aβ entered mitochondria, which would promote the accumulation of ROS and induced the damage to the integrity of the cell structure in early course of AD (Pratico, 2008). Antioxidants might play a crucial part in prevention or mitigation of

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the symptom of AD (Petrovic et al., 2020). Some studies indicated that natural polyphenols had great potential in treatment of AD (Zeng et al., 2015; Freyssin et al., 2018).

Alsophila spinulosa (Hook.) Tryon (*Cyatheaceae*) is a tree-like fern (Morton, 1971). The stem of *A. spinulosa* is known as "Caulis Alsophilae" in China, and its a Chinese traditional medicine for the cough, asthma, cold and rheumatism. But its leaf is neglected and discarded. Polyphenols is a secondary metabolic product widely existing in plants and received considerable attention due to their health-promoting properties in neurodegeneration diseases, including AD and Parkinson's disease (PD) (Abbas et al., 2016). Unfortunately, there are many polyphenols, such as vitexin, isoorientin, apigenin and luteolin in *A. spinulosa* leaves (Chen et al., 2008), but its mitigation effect of AD has not been reported.

The ultrasonic-assisted extraction (UAE) is considered as a green method for polyphenols extraction from the natural material because it could save energy, time, and solvents (Ameer et al., 2017). Therefore, UAE may be an efficient and energy-saving way to extract polyphenols from the leaves of *A. spinulosa*. Response surface methodology (RMS) is a scientific optimization method, and the Box–Behnken design (BBD) is a good design for RSM. They are widely used to optimize the extraction of polyphenols. Unfortunately, there was no report that extraction of polyphenols from *A. spinulosa* leaves by UAE and optimization with RSM.

In this work, the extraction polyphenols from *A. spinulosa* leaves was optimized with RSM and purified by AB-8 macroporous resin. The major constituent was analyzed by HPLC. In addition, their antioxidant activities and their potential effects on AD were investigated.

2. Materials and methods

2.1. 1 Materials and chemicals

Sample leaf of *A. spinulosa* was gifted by Hongya Qingyuan Sci-tech Co., LTD (Hongya county, Sichuan Province, China) and identified by professor Ming Yuan. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Folin-Ciocalteu were purchased from Solarbio Sci-tech Co., Ltd. (Beijing, China). The test kits of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). The kits of RNAisoPlus reagent, RNA reverse transcription and fluorescent qPCR were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The phenolic standards (HPLC $\geq$ 98%) were obtained from Plant Origin Biological Co., Ltd. (Nanjing, China). Other reagents were purchased from Chengdu Kelong Chemical Industry. All reagents are analytically pure.

The *C. elegans* strains used in this study are N2 (wild type) and CL4176 (dvIs27 [myo-3p::A-Beta (1–42)::let-851 3′UTR) + rol-6(su1006)] X). The *C. elegans* CL4176, expressing human Aβ peptide in muscle cell, was gifted by Prof. Yunjiao Chen (South China Agricultural University, China). *C. elegans* was cultured on nematode growth medium (NGM) and seeded with *Escherichia coli* OP50 at 20 °C (N2) or 16 °C (CL4176). For experiments, the synchronous populations of CL4176 worms had cultured to the L1 stage and transferred to with or without ASP plates and incubated at 16 °C for 36 h, then moved to 25 °C for promoting the expression of Aβ.

2.2. RSM for the extraction of polyphenols

On the basis of single factors experiments, BBD with four key parameters (ultrasonic power, liquid–solid ratio, ethanol concentration and extraction time) at three levels was employed to optimize extraction parameters. Table 1 showed the 29 experiments which were designed by Design-Expert 8. The data of BBD were explained by the following second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{j<i}^{4} \sum_{i=1}^{4} \beta_{ij} X_i X_j$$

Where Y is the yield of polyphenols, Xi and Xj represents independent variable, and $\beta 0$, βi, βii and βij represents regression coefficient.

2.3. Extraction and purification of polyphenols

The leaves of *A. spinulosa* was washed and dried at 50 °C, then crushed and sifted through 100 mesh sieves and pre-treated with petroleum ether to remove pigments and lipids. The samples were extracted with 40% (v/v) ethanol (1:25, w/v) through an ultrasonic-assisted technique (240 W) for 20 min (Savic and Gajic., 2020). The supernatant was obtained by centrifugation 4000 rpm for 10 min, and then dried by freeze dryer.

The crude polyphenols was dissolved with ethanol, and purified by a macroporous resin column of AB-8 (Xi et al., 2015). 70% ethanol worked as eluent. The elution curve was drawn by the Folin-Ciocalteu method and the major peak was collected. Purified polyphenols were still named ASP and lyophilized.

2.4. Determination of total polyphenols

The total polyphenols was quantified according to Canalis et al. (2020). 0.05 mL ASP was added to 0.05 mL Folin-Ciocalteu and 0.15 mL 10% sodium carbonate and reacted in dark for 2 h. The absorbance of polyphenols was at 760 nm and the total polyphenols content was expressed as milligrams gallic acid equivalents per gram (mg GAE/g) according to the standard curve of gallic acid.

2.5. HPLC analysis

The component of ASP was analyzed using HPLC system (Agilent 1260, Agilent, Santa Clara, CA, USA) with an Agilent C18 reverse-phase column (5 μm, 250 mm × 4.6 mm) and an ultraviolet detector at 320 nm. 0.2% phosphoric acid solution (A) and acetonitrile (B) worked as mobile phase, and the gradient elution was: 0–25 min, 14%-40% B, 25–35 min, 40%-16% B, with 0.5 mL/min of the flow rate.

2.6. Assays of antioxidant activity in vitro

The scavenging activities of DPPH radical, hydroxyl radical and ABTS radical were evaluated with the method of Li et al. (2020). Briefly, the 0.1 mL DPPH ethanol solution

Table 1 BBD experimental design and the yield of polyphenols.

Number	A_1 (W)	A_2 (mL/g)	A_3 (%)	A_4 (min)	Yield (%)
1	−1 (210)	−1 (20)	0 (40)	0 (20)	6.10
2	1 (270)	−1	0	0	5.86
3	−1	1 (30)	0	0	5.39
4	1	1	0	0	5.88
5	0 (240)	0 (25)	−1 (20)	−1 (15)	6.41
6	0	0	1 (60)	−1	6.88
7	0	0	−1	1 (25)	5.68
8	0	0	1	1	4.91
9	−1	0	0	−1	5.51
10	1	0	0	−1	8.19
11	−1	0	0	1	6.68
12	1	0	0	1	4.30
13	0	−1	−1	0	5.79
14	0	1	−1	0	5.55
15	0	−1	1	0	5.76
16	0	1	1	0	5.28
17	−1	0	−1	0	6.95
18	1	0	−1	0	6.35
19	−1	0	1	0	6.05
20	1	0	1	0	6.95
21	0	−1	0	−1	6.07
22	0	1	0	−1	5.67
23	0	−1	0	1	4.37
24	0	1	0	1	4.35
25	0	0	0	0	8.79
26	0	0	0	0	8.75
27	0	0	0	0	8.90
28	0	0	0	0	8.89
29	0	0	0	0	8.86

A_1 means ultrasonic power, A_2 means liquid–solid ratio, A_3 means ethanol concentration, and A_4 means extraction time.

(0.2 mmol/L) was added to 0.1 mL different concentrations of polyphenols solution (0–0.2 mg/mL) and the mixed solution was cultured at 37 °C for 30 min, the absorbance was measured at 517 nm. For hydroxyl radical scavenging assay. The 0.10 mL different concentrations of polyphenols solution (0–1.0 mg/mL) were successively mixed with 0.10 mL 9 mmol/L salicylic acid, 9 mmol/L $FeSO_4$ and 0.01% H_2O_2. The mixture was incubated at 37 °C for 30 min and the absorbance was measured at 520 nm. In addition, the 0.10 mL different concentrations of polyphenols solution (0–0.1 mg/mL) was added to 0.15 mL ABTS radical and incubated at room temperature for 6 min for ABTS radical assay. The absorbance was measured at 734 nm. Anti-lipid peroxidation capacity was measured according to Cheng et al. (2019). Briefly, 0.10 mL different concentrations of polyphenols solution (1.00–5.00 mg/mL) were successively mixed with 0.10 mL lecithin concentrations (10 mg/mL), 0.10 mL $FeSO_4$ (25 mmol/L) and 0.50 mL PBS (0.10 mol/L, pH 7.4), the mixture was incubated at 37 °C for 1 h, then added 0.5 mL 10% trichloroacetic acid and 0.5 mL 0.8% thiobarbituric acid, the mixture was boiled for 10 min and cooled to room temperature. The absorbance was measured at 532 nm.

2.7. Nematodes stress assay

The synchronized worms were prepared by allowing hermaphrodites to lay eggs 4 h at 20 °C (N2) or 16 °C (CL4176) and then removed the adult nematodes. Eggs were incubated on NGM plates seeded with *E. coli* OP50 and cultivated to L1 stage. Afterwards, 20 nematodes in L1 stage were placed in plates with or without ASP (0.8 and 1 mg/mL) and cultivated to L4 stage. Heat stress was at 35 °C, and the dead of worms was recorded at 2 h interval until all the worms died. For oxidative stress, the worms were treated with 50 μmol/L juglone in 96-well plates and incubation at 20 °C for 16 h.

2.8. Enzyme activity and MDA content assays in nematodes

The N2 worms (L4 stage) were transferred to 35 °C for 3 h and collected and washed with M9 buffer (0.3 g KH_2PO_4, 1.25 g $Na_2HPO_4{\cdot}12H_2O$, 0.5 g NaCl to 100 mL distilled water), then broken with an ultrasonic crusher (300 W). After centrifugation at 12,000 g for 10 min at 4 °C and ollected the supernatant. MDA and the activity of antioxidant enzymes (SOD, CAT) were evaluated complying with the instruction of the kits.

2.9. Paralysis assay

The paralysis assay was done according to Ma et. al. (2017). The *C. elegans* CL4176 in L1 stage was placed in fresh NGM plates with or without 1.0 mg/mL ASP and cultured at 16 °C for 36 h, and then, worms were moved to 25 °C to promote the production of Aβ peptide and induced paralysis. The paralyzed worms were numbered every 3 h until all nematodes paralyzed.

2.10. Aβ accumulation assay

The CL4176 transgenic nematodes were collected and fixed with 4% paraformaldehyde (pH 7.4) at 4 °C for 24 h, and then treated with permeable solution (5% β-mercaptoethanol, 1% Triton X-100, 125 mmol/L Tris, pH 7.4) for 24 h at 37 °C. Afterwards, the nematodes stained with 0.125% thioflavin T for 2 min, and distained with 50% ethanol. The fluorescence images were taken with a fluorescence microscope (Nikon DS-Ri1, Japan)). The number of Thioflavin T reactive deposits in the anterior of pharyngeal bulb was scored.

2.11. Determination of ROS level in worms

Th CL4176 transgenic nematodes were collected and washed with M9 buffer. Afterwards, the 50 nematodes were moved to a microtiter plate with M9 buffer and 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA), and incubated for 60 min at 37 °C. The fluorescence intensity at 535 nm was recorded with the excitation at 485 nm (Wan et al., 2021).

2.12. Gene expression assay

The CL4176 transgenic nematodes were collected and washed with M9 buffer. The total RNA was extracted using the acid-phenol (TRIzol) method and converted to cDNA using PrimeScript™ RT reagent kit following the prescribed protocol. The quantitative PCR was performed in triplicate with SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) and CFX96 Real-Time PCR Detection Systems (Bio-Rad, Chicago, USA). The primers used for PCR was shown in Table S1. The gene expression was normalized using the *Actin* as a housekeeping gene. The fold change in mRNA was calculated by $2^{-\Delta\Delta Ct}$ method.

2.13. Statistical analysis

Values were means ± SDs (standard deviations) from three separate operations. All data were analyzed with SPSS 25.0, and one-way ANOVA was employed to statistical analysis. $p < 0.05$ was regarded as significance.

3. Results

3.1. Effect of single factor on the ASP yield

With the increase of ultrasonic power (150 W, 180 W, 210 W, 240 W, 270 W), the yield of polyphenols gradually increased first, and then decreased when the ultrasonic power higher than 240 W (Fig. 1(A)). Ultrasonic improved the extraction of polyphenols, but the damage of polyphenols structure leads to the decrease of the yield when the ultrasonic power too high. Thus, ultrasonic power from 210 W to 270 W was used for the further experiments.

The polyphenols yield was improved first with liquid–solid ratio, and then decreased when more than 25:1 (Fig. 1(B)). The liquid–solid ratio ranges from 20:1 to 30:1 (mL/g) was selected to the further experiments.

With the increase of ethanol concentration (10%, 20%, 40%, 60%, 80%, 100%), the polyphenols yield displayed a trend with first rising and then declining (Fig. 1(C)). Polyphenols reached the highest yield when ethanol concentration was 40%. Therefore, the ethanol concentration from 20% to 60% was employed for further experiments.

The yield of polyphenols reached to the maximum (5.61%) at 20 min (Fig. 1(D)). When the extraction time exceeded 20 min, the yield declined. Therefore, 15 to 25 min was used for further experiments.

3.2. Model fitting and AVOVA

As shown in Table 1, the yield of polyphenols varied from 4.30% to 8.90% among the 29 groups of tests designed by BBD. The predicted model about response variables and tests variables could be expressed by a quadratic polynomial equation as follows:

$$Y = 8.84 + 0.07\ A_1\text{-}0.15\ A_2\text{-}0.075\ A_3\text{-}0.70\ A_4 + 0.18\ A_1A_2 + 0.38\ A_1A_3\text{-}1.26\ A_1A_4\text{-}0.06\ A_2A_3 + 0.095\ A_2A_4\text{-}0.31\ A_3A_4\text{-}1.01\ A_1^2\text{-}2.03\ A_2^2\text{-}1.22\ A_3^2\text{-}1.66\ A_4^2.$$

Where Y represents extraction yield, A_1 means ultrasonic power, A_2 means liquid–solid ratio, A_3 means ethanol concentration, and A_4 means extraction time.

The significance of model coefficients was evaluated with t-test and P-value (Table 2). The result ($P < 0.0001$) indicated the model was significant. The lack of fit ($F = 0.9155$ and $P = 0.5886$) was no significant. In addition, the determination coefficient R^2 implied 99.90% of the variations fitted this model. The adjusted determination coefficient (R^2adj) was 0.9980, suggesting this model was significant. In addition, linear coefficient (A_1, A_2, A_3 and A_4), the quadratic coefficients (A_1^2, A_2^2, A_3^2 and A_4^2), and the interaction coefficients (A_1A_2, A_1A_3, A_1A_4, A_2A_4 and A_3A_4) were very significant, implying these factors significantly affected on ASP yield.

3.3. Optimization for the extraction parameters with response surface

The three-dimensional response surface plots and contour plots for the model were produced according to the regression equation to evaluate the interactions of these any two variables (Fig. 2). The polyphenols yield (Y) increased with extraction variables, and it gradually declined after the yield reached the maximum. Among the four independent variables, extraction time showed the greatest influence on polyphenols yield, following by the ratio of liquid–solid, the concentration of ethanol, and the power of ultrasonic, in that order. According to the model, the optimal conditions for ultrasonic assisted extraction of ASP were 240.12 W (the power of ultrasonic), 25.01 (the ratio of liquid–solid, mL/g), 40.14% (the concentration of ethanol) and 20.03 min (extraction time). The theoretical yield of ASP was 8.83% under these conditions.

3.4. Verification of predictive model

For the convenience of operation, the extraction parameters were adjusted to extraction time 20 min, ultrasonic power 240 W, ethanol concentration 40%, and liquid–solid ratio 25 (mL/g). The experimental yield of ASP was 8.80 ± 0.04% ($n = 3$) under these optimal conditions, which are in good agreement with the predicted yield.

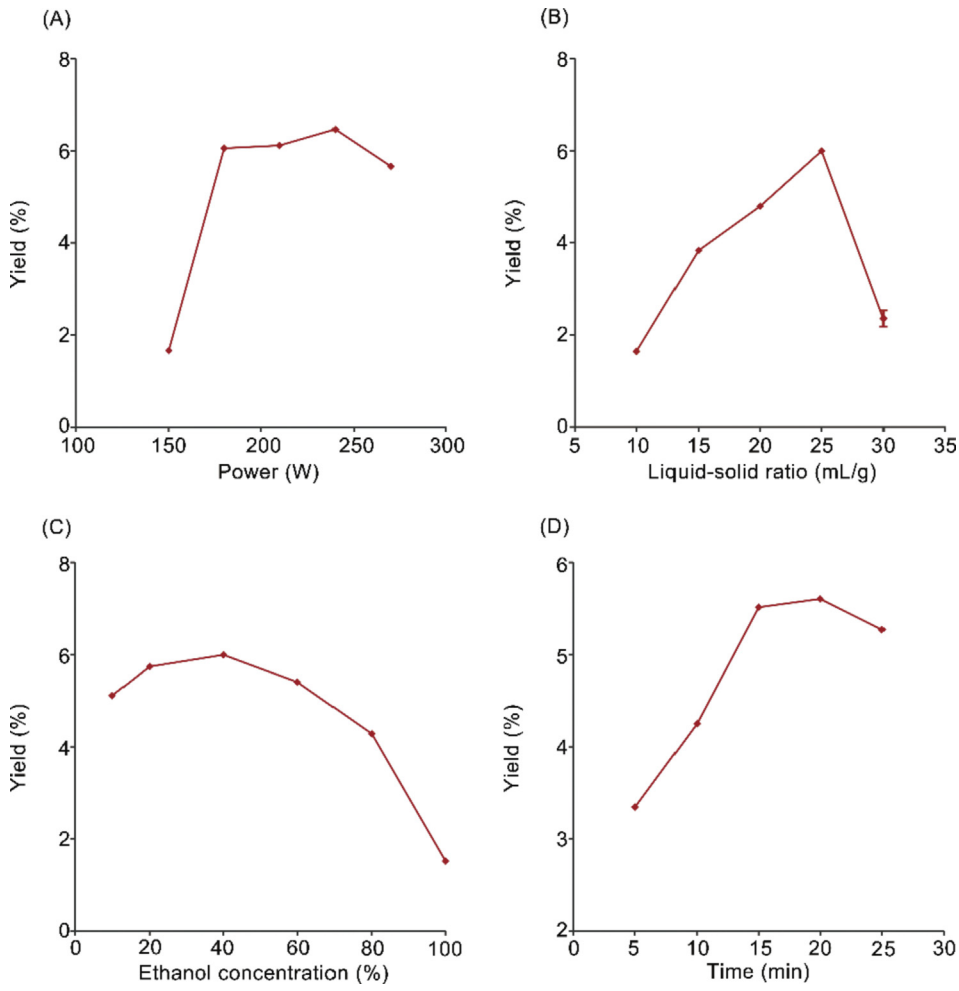

Fig. 1 Effect of singer factor on polyphenols yield. (A) the power of ultrasonic (W), (B) the ratio of liquid–solid (mL/g), (C) the concentration of ethanol (%), (D) time (min).

Table 2 Coefficients and ANOVA analysis of the fitted regression model.

Source	Quadratic Sum	Degree of Freedom	Mean Square	*F*-Value	*P*-value; Prob > *F*	
Model	55.252	14	3.947	983.622	< 0.0001	significant
A_1	0.060	1	0.060	15.006	0.0017	
A_2	0.279	1	0.279	69.556	< 0.0001	
A_3	0.068	1	0.068	16.823	0.0011	
A_4	5.936	1	5.936	1479.498	< 0.0001	
A_1A_2	0.133	1	0.133	33.204	< 0.0001	
A_1A_3	0.562	1	0.563	140.195	< 0.0001	
A_1A_4	6.400	1	6.400	1595.335	< 0.0001	
A_2A_3	0.014	1	0.014	3.589	0.0790	
A_2A_4	0.036	1	0.036	8.997	0.0096	
A_3A_4	0.384	1	0.384	95.806	< 0.0001	
A_1^2	6.680	1	6.680	1664.983	< 0.0001	
A_2^2	26.792	1	26.792	6677.442	< 0.0001	
A_3^2	9.671	1	9.672	2410.521	< 0.0001	
A_4^2	17.951	1	17.951	4474.138	< 0.0001	
Residual	0.056	14	0.004			
Lack of Fit	0.0390	10	0.0039	0.9155	0.5886	not significant
Pure Error	0.0171	4	0.0043			
Cor Total	55.3080	28				
R^2	0.9990	R^2adj	0.9980			

A_1 means ultrasonic power, A_2 means liquid–solid ratio, A_3 means ethanol concentration, and A_4 means extraction time.

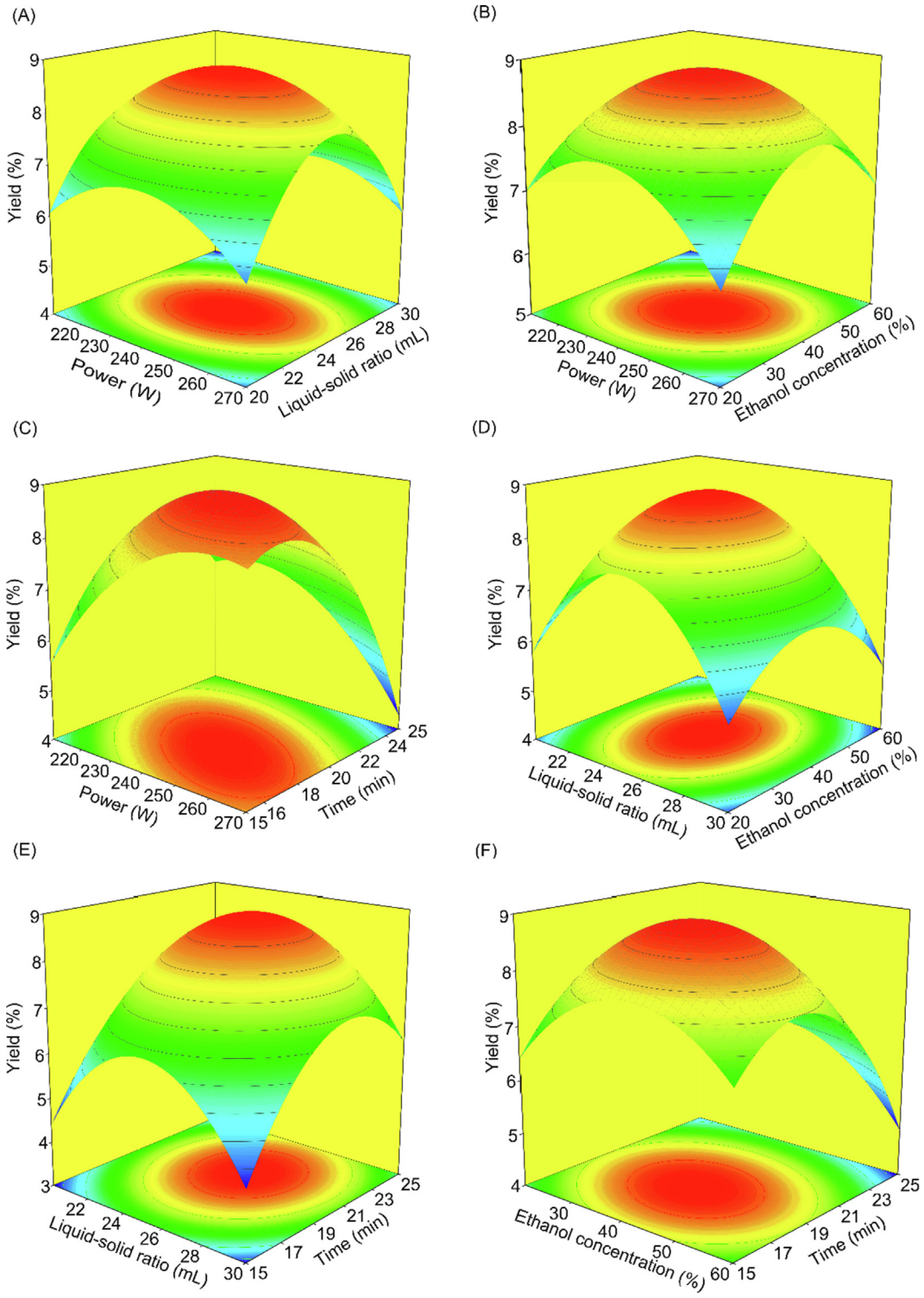

Fig. 2 Response surface plots for interaction of any two variables on ASP yield. (A) the power of ultrasonic and the ratio of liquid–solid, (B) the power of ultrasonic and the concentration of ethanol, (C) the power of ultrasonic and time, (D) the ratio of liquid–solid and the concentration of ethanol, (E) the ratio of liquid–solid and time, (F) the concentration of ethanol and time.

3.5. The composition of ASP

The crude polyphenols was separated by AB-8 macroporous resin and elution with 70% ethanol. The major fraction was collected and still named ASP for the following experiment. After purification, the polyphenols purity was increased from 17.34 ± 0.05% to 47.00 ± 0.04%.

The HPLC assay was performed to determine the component in ASP. The chromatogram of phenolic standards and ASP were shown in Fig. 3. Chlorogenic acid, isoorientin,

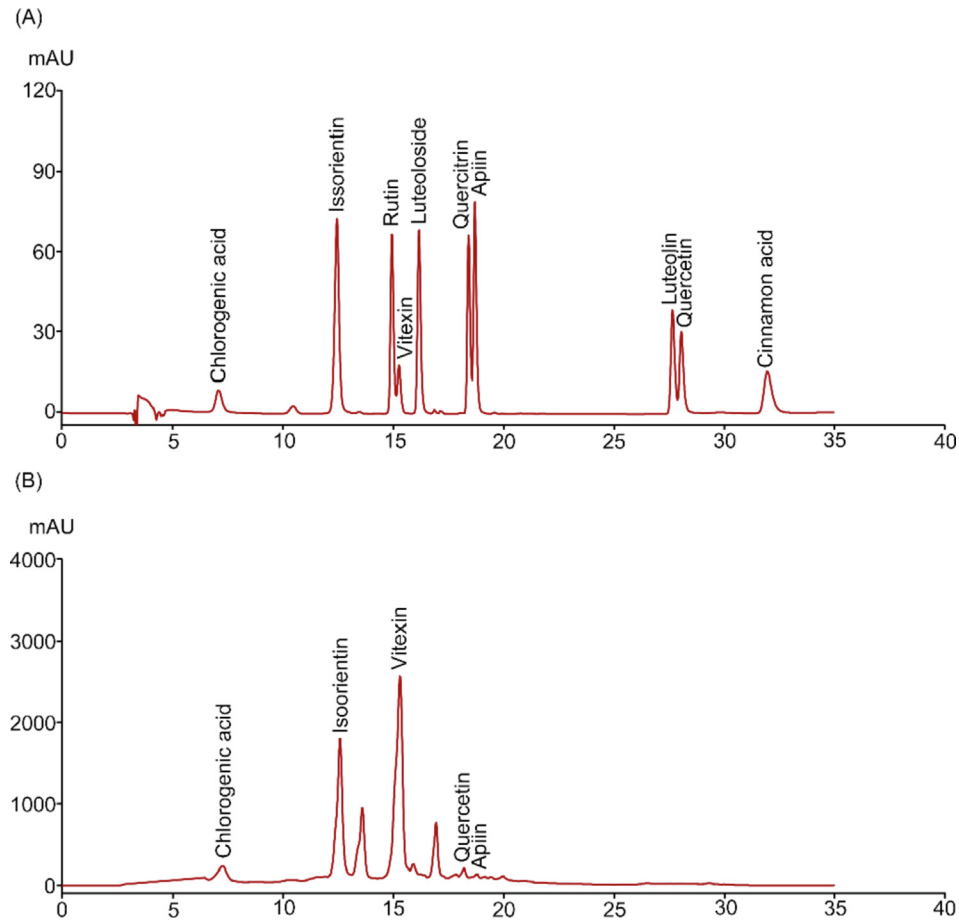

Fig. 3 The composition of ASP. (A) phenolic standards, (B) ASP.

vitexin, quercitrin and apiin were observed in ASP. Isoorientin and vitexin were the main compound in ASP, followed by chlorogenic acid, quercitrin, and apiin. Isoorientin and vitexin are natural *C*-glucosyl flavone with the robust antioxidant, neuroprotection and anti-inflammatory properties (Ziqubu et al., 2020; He et al., 2016), The results revealed the flavonoids was the main component of ASP.

3.6. Antioxidant activity of ASP in vitro

The scavenging activity of DPPH radical by ASP increased with its concentration (Fig. 4(A)). The scavenging rate of ASP reached 83.31% at 0.20 mg/mL, and its scavenging activity was almost as good as Vc (85.20%). The IC_{50} of ASP was 0.06 mg/mL. The scavenging ability of ASP to hydroxyl radical is dose-dependent (Fig. 4(B)). The scavenging rate reached the maximum (75.48%) at 1.00 mg/mL of ASP, and its IC_{50} was 0.72 mg/mL. ABTS was a non-physiological free radical that activated the chain reaction of free radical and destroyed the protein, DNA and lipid (Pu et al., 2019). ABTS radical scavenging capacity of ASP was positively related to its concentration (Fig. 4(C)). The ABTS radical scavenging rate of ASP achieved 86.97% at 0.10 mg/mL, and its IC_{50} value was 0.05 mg/mL. The anti-lipid-peroxidation capacity of ASP rose from 25.28% to 86.63% when its content increased from 1 to 5 mg/mL. Its IC_{50} was 2.03 mg/mL (Fig. 4(D)). These results suggested that ASP displayed favorable antioxidant activity *in vitro*.

3.7. ASP improved the tolerance of C. elegans N2 to heat stress

Heat stress disrupted the homeostasis of oxidation/antioxidant system and caused oxidative damage in body (Habashy et al., 2019; Yang et al., 2010). The treatment of 0.8 mg/mL and 1 mg/mL ASP improved the survival time of nematodes by 4 h and 7 h compared with the control (Fig. 5(A)) under heat stress. The PT50 (50% of the nematodes died) of worms increased by 4 h after treating with 0.8 mg/mL ASP, while it increased by 5.5 h for treating with 1 mg/mL ASP compared with the control. Therefore, we chose 1 mg/mL ASP for further study.

Heat stress caused oxidative damage, and it could be restored by improving the antioxidant system, which played a key part in maintaining cellular normal metabolism (Yang et al., 2010). SOD and CAT are key antioxidant enzymes in cell. SOD can convert harmful superoxide free radicals into oxygen and H_2O_2, and the latter was decomposed into harmless water by CAT, thus maintaining the dynamic balance of the oxidation/antioxidant system in the organism to prevent the body from oxidation damage (Lewandowski et al., 2018). SOD and CAT activities were significantly improved after treating with 1 mg/mL ASP (Fig. 5(B), (C)). Moreover, MDA is characteristic product of lipid peroxidation, which can bind to DNA and protein lead to tissue damage, and cause cytotoxicity (Rio et al., 2005; Ho et al., 2013). After treating with ASP, the level of MDA

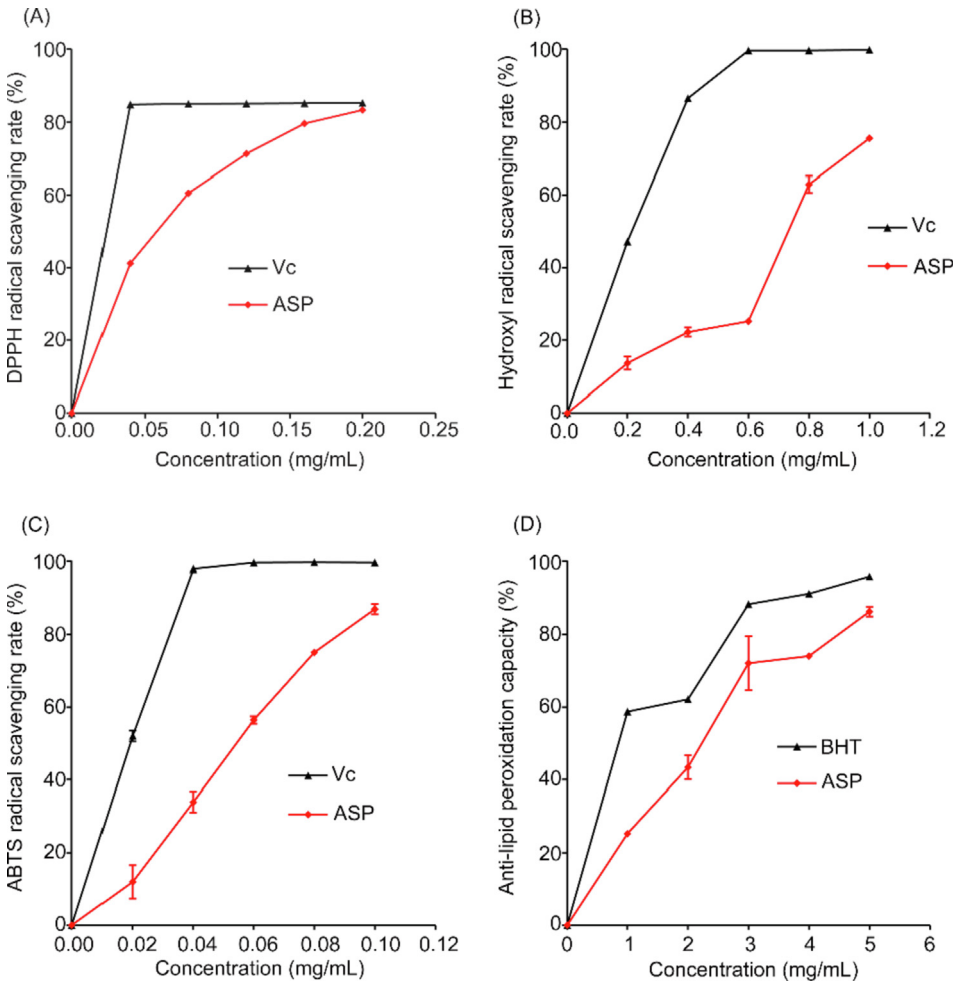

Fig. 4 Antioxidant activity of ASP *in vitro*. (A) the scavenging ability to DPPH radical, (B) the scavenging ability to hydroxyl radical, (C) the scavenging ability to ABTS radical, (D) anti-lipid peroxidation capacity. Each point represent mean ± SD of three independent experiments.

remarkably decreased (Fig. 5(D)). These results indicated that ASP could improve the capacities of antioxidant enzymes and relieving the level of lipid peroxidation in *C. elegans* N2 under heat stress.

3.8. ASP relieved the paralysis and reduced Aβ deposition in C. elegans CL4176

The heat stress and oxidative stress were used to evaluate the protection effect of ASP from the oxidative damage in AD model of *C. elegans*. The survival time of the nematodes treated with 1 mg/mL ASP increased by 5 h, and the PT50 is up to 13 h under heat stress (Fig. 6(A)). In addition, the average survival rate of the nematodes treated with 1 mg/mL ASP increased by 38.89% under oxidative stress (Fig. 6(B)).

Expression of the Aβ gene in muscle of *C. elegans* CL4176 is associated with age-progressive paralysis, and it is widely used as AD models for evaluating the toxicity of Aβ to screening potential drugs. Therefore, we investigated the paralysis of *C. elegans* CL4176 treated with 1 mg/mL ASP after incubation for 46 h at 25 °C. The paralysis rate of control group was 89.32%, while it was only 40.74 ± 3.21% for the ASP treated group (Fig. 6(C)). The result indicated that the ASP protected *C. elegans* CL4176 from the Aβ-induced paralysis. Next, we measured the Aβ deposition in the AD model of *C. elegans* CL4176, we observed ASP significant decrease the number of Aβ deposition in the head area of *C. elegans* CL4176 compared with the control group. ASP treatment was significantly decreased by the Aβ deposition in *C. elegans* CL4176 (Fig. 6 (D), (E)).

3.9. ASP reduced oxidative stress through P13K/AKT signal pathway in C. elegans CL4176

The accumulation of Aβ will increase the level of ROS and causing oxidative stress (Pratico, 2008). Therefore, we investigated whether the accumulation of Aβ-induced paralysis was related to the levels of ROS in *C. elegans* CL4176. The level of ROS was obviously decreased by ASP (Fig. 7(A)), which preliminary indicated that ASP relieved A*β*-induced paralysis probably by reducing the level of ROS.

The transcription factor skinhead-1 (*SKN-1*) was involved in oxidative stress (Li et al., 2019). Besides, oxidative stress can damage the *P13K/AKT* signaling pathway that causes neuron damage and AD (Jiang et al., 2016). AD can be alleviated by the *P13K/AKT* signaling pathway (Sun et al., 2020). ASP

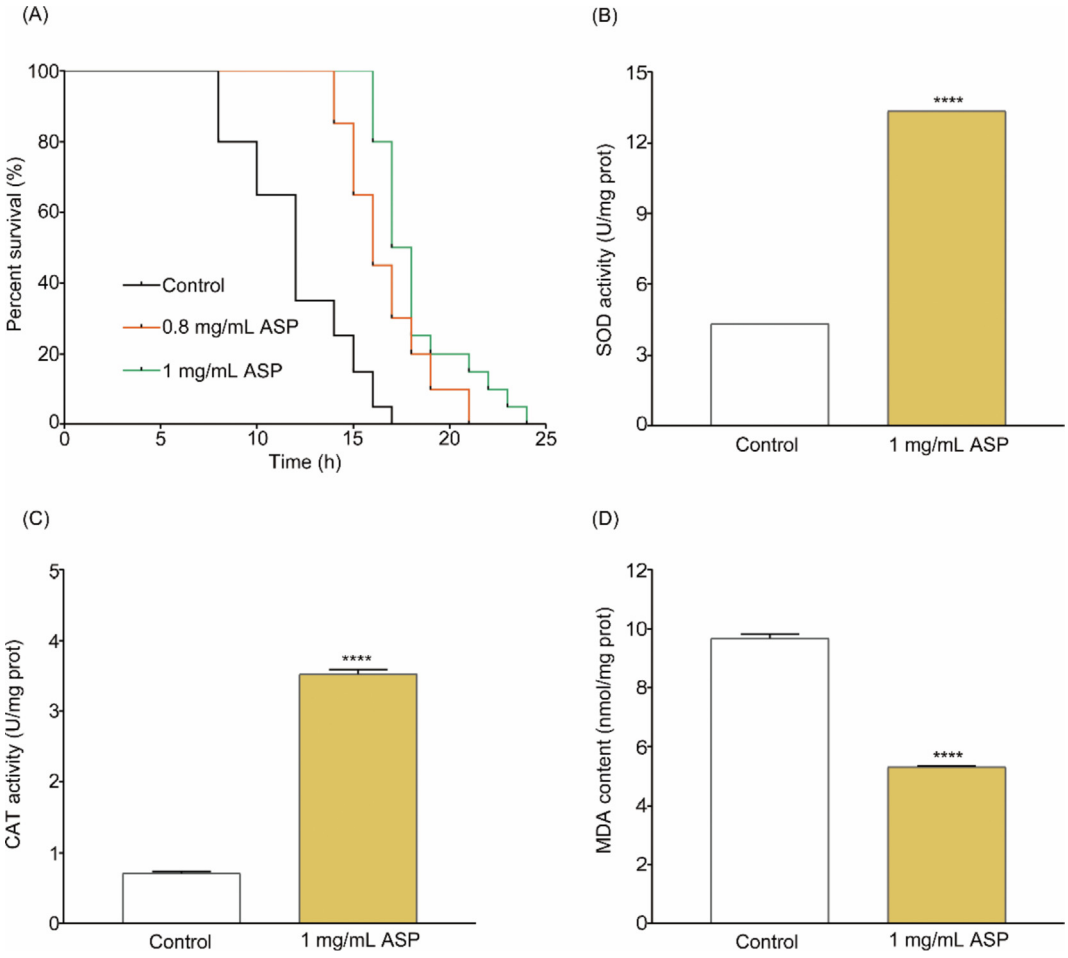

Fig. 5 Activity of ASP under heat stress in N2. (A) the survival curves of *C. elegans* N2 after treatment with 1 mg/mL ASP at 35 °C, (B) the activity of SOD, (C) CAT and (D) the content of MDA of *C. elegans* N2 after treatment with 1 mg/mL ASP at 35 °C for 3 h. Each value is the average ± SD. **** $p < 0.0001$ compared with the control group.

improved the expression of *skn-1* and down-regulated the transcription of *akt-1* and *pdk-1* (Fig. 7(B), (C)). These results revealed that ASP might alleviate AD by down-regulation of *P13K/AKT* signaling pathway.

4. Discussion and conclusion

Polyphenols as a type of secondary metabolic product have been drawn more and more attentions. UAE is considered as a green method for polyphenols extraction from the natural material. Compared with the traditional extraction techniques, ultrasonic assisted extraction saved energy, time, and solvents (Ameer et al., 2017). We optimized the process of ultrasonic assisted extraction polyphenols from the leaves of *A. spinulosa* and the extraction yield was 8.80 ± 0.04%. Detti et al. (2020) extracted the polyphenols from *Pistacia lentiscus* L. leaves with UAE, the yield of polyphenols was 5.13 ± 0.18%. In addition, Chmelova et al. (2020) found that the polyphenols from *Picea abies* bark with UAE was higher than solvent extraction.

The antioxidant activity of polyphenols is responsible for protecting cells. There are two main mechanisms for the antioxidant activity of polyphenols. Firstly, the functional group of phenol can provide free radical $R^{\bullet}$ with hydrogen atom. In this case, the polyphenols act as chainbreaker. Secondly, polyphenols form a radical cation through transferring a single electron to the free radical (Quideau et al., 2011). In addition, polyphenols can reduce the cell oxidative damage through the antioxidant enzyme system. We observed that ASP had good scavenging abilities to DPPH radical, hydroxyl radical and ABTS radical and good anti-lipid-peroxidation capacity in vitro. Further, ASP can confer *C. elegans* N2 with higher tolerance to heat stress, improved the capacities of SOD and CAT, and reduced the content of MDA. The polyphenols of apple leaves had good scavenging capacity of DPPH radical, and reduced the H_2O_2-induced oxidant damage in rat hippocampal neurons by improved SOD and lactate dehydrogenase (LDH) activities (Lu et al., 2019). Vitexin relieved oxidative damage by decreasing the content of MDA, enhancing the activity of SOD and CAT (Li et al., 2021).

Abnormal Aβ accumulation is one of the main etiologies in AD (Ashrafian et al., 2020). There is evidence suggesting that Aβ accumulation will trigger neurotoxicity and oxidation (Shankar et al., 2008). The antioxidant could inhibit the aggregation of Aβ and contribute to preventing the occurrence or progression of AD (Petrovic et al., 2020). 1 mg/mL ASP significantly relieved the Aβ-induced paralysis and reduced the Aβ plaques in *C. elegans* CL4176. The transcription factor *SKN-*

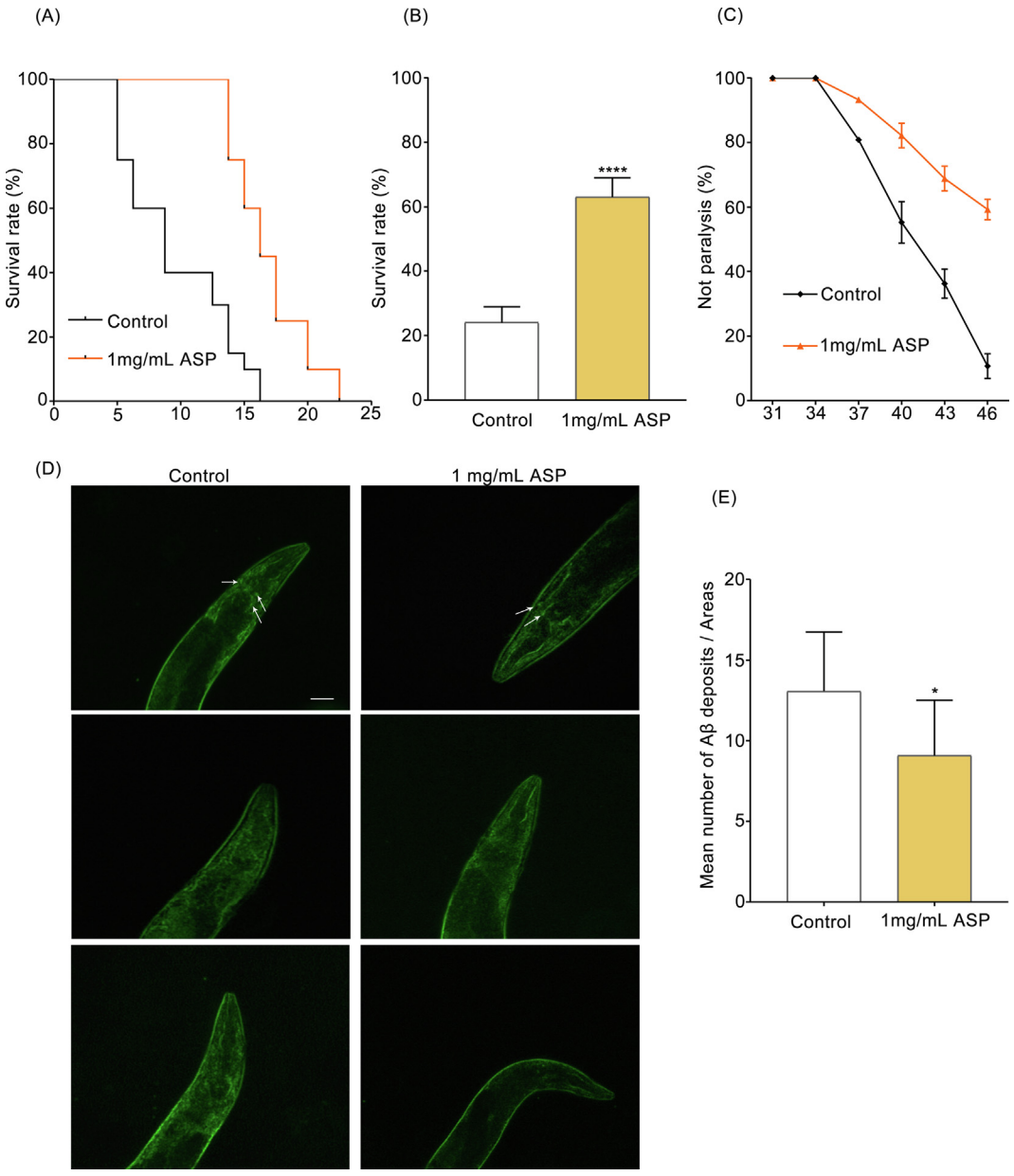

Fig. 6 ASP treatment significantly reduces paralysis and Aβ plaques in *C. elegans* CL4176. (A) survival curves in *C. elegans* CL4176 treated with 1 mg/mL ASP at 35 °C, (B), survival rate in *C. elegans* CL4176 treated with 1 mg/mL ASP exposed to 50 μmol/L juglone for 16 h, (C) the effect of ASP on the paralysis of *C. elegans* CL4176 at 25 °C, (D) the fluorescence images of Aβ deposition in *C. elegans* CL4176 stained with thioflavin T, the white arrows pointing to Aβ deposits (E) The quantity is expressed as mean number of Aβ deposits in the head region/anterior area of the worm. Each value is the average ± SD. * $p < 0.05$, **** $p < 0.0001$ compared with the control group.

1 was orthologous gene with *Nrf-2* (NF-E2-related factor 2) of human, and it was crucial in defending against oxidative damage in *C. elegans* (Li et al., 2017). Reduction of Aβ-induced toxicity through lessening oxidative damage in AD patients is important for mitigating the AD process. In present studies, we observed ASP decreased ROS level and enhanced the expression of *SKN-1* in AD model of *C. elegans* CL4176. In addition, ASP down-regulated the expression of *pdk-1* and *akt-1* in *P13K*/*AKT* signaling pathway in *C. elegans* CL4176. Our results indicated that ASP might alleviate the Aβ-induced oxidative damage through the *P13K*/*AKT* signaling pathway, thus relieving the paralysis symptom of *C. elegans* CL4176. In H_2O_2-treated APPswe cells, rutin and quercetin attenuated and reversed $A\beta_{25-35}$ fibril formation and improved intracellular GSH level and maintained the redox homeostasis (Jimenez-Aliaga et al., 2011). In addition, flavone, apigenin, luteolin, kaempferol and quercetin reduced the level of ROS which was induced by $A\beta_{1-42}$ oligomer (Pate et al., 2017).

In summary, the polyphenols was extracted from the leaves of *A. spinulosa* (ASP) with ultrasonic-assisted extraction. The

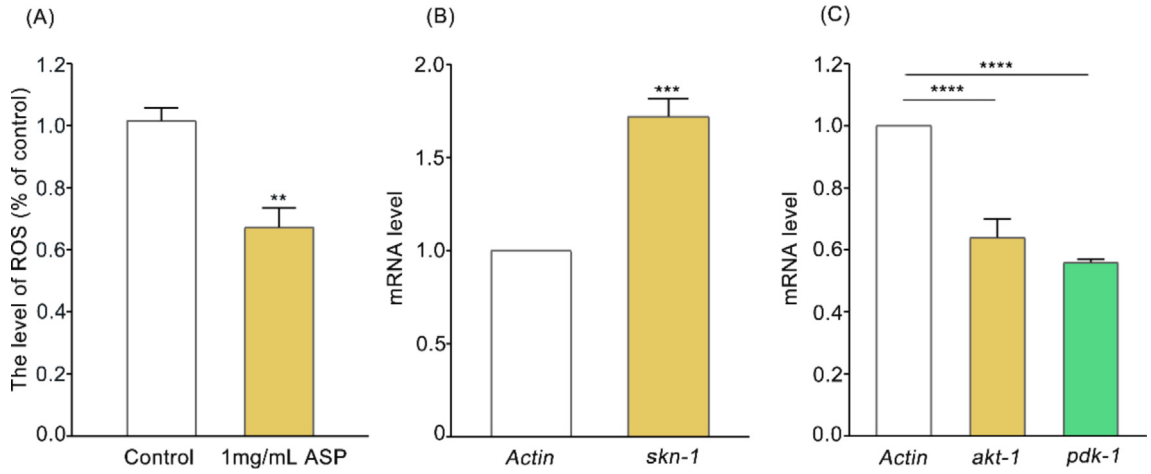

Fig. 7 ASP treatment reduced oxidative damage through *P13K*/*AKT* signal pathway in *C. elegans* CL4176, (A) the effect of ROS level in *C. elegans* CL4176 after treated with ASP at 25 °C for 36 h, the mRNA expression levels of *skn-1* (B) *akt-1*, *pdk-1* (C) in *C. elegans* CL4176 after treated with ASP at 25 °C for 36 h. Each value is the average ± SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared with vehicle control group.

extract yield of ASP reached 8.80 ± 0.04% under 240 W of ultrasonic power, 40% of ethanol concentration, 25 (mL/g) of liquid–solid ratio and 20 min of extraction time. ASP was composed of Chlorogenic acid, isoorientin, vitexin, quercitrin and apiin. ASP displayed strong antioxidant activity *in vitro*, and it can alleviate Aβ-induced toxicity and oxidative damage through down-regulated of *P13K*/*AKT* signaling pathway in AD model of *C. elegans* CL4176. ASP might contribute to treat or prevent the AD and other neurodegenerative disease in humans. However, our present study only preliminarily demonstrated the bioactivities of ASP, but the relationship between the bioactivities and structure of ASP is unclear. Therefore, we should research the structure and explore the direct target and detail regulation pathway network of ASP for AD in further work, and analyze the conformational relationship between structure and bioactivities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2022.103707.

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