

Neuroprotective effect of emodin against Alzheimer's disease via Nrf2 signaling in U251 cells and APP/PS1 mice

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Abstract. Emodin is a naturally-occurring medicinal herbal ingredient that possesses numerous pharmacological properties, including anti-inflammatory and antioxidant effects. In the present study, potential neuroprotective effects associated with the antioxidant activity of emodin were assessed in U251 cells that were subjected to β -amyloid peptide ($A\beta$)-induced apoptosis and in amyloid precursor protein (APP)/presenilin-1 (PS1) double-transgenic mice. U251 is a type of human astrogloma cell line (cat. no. BNCC337874; BeNa Culture Collection). In apoptotic U251 cells, 3-h emodin pre-treatment prior to 24-h $A\beta$ co-exposure improved cell viability, suppressed lactate dehydrogenase leakage and caspase-3, -8 and -9 activation to inhibit apoptosis. Compared with those after $A\beta$ exposure alone, emodin ameliorated the dissipation of the mitochondrial membrane potential, inhibited the over-accumulation of reactive oxygen species, enhanced the expression levels of nuclear factor-erythroid-2-related factor 2 (Nrf2), haemeoxygenase-1,

superoxide dismutase 1, Bcl-2 and catalase in addition to decreasing the expression levels of Bax. In APP/PS1 mice, an 8-week course of emodin administration improved spatial memory and learning ability and decreased anxiety. Emodin was also found to regulate key components in the Nrf2 pathway and decreased the deposition of $A\beta$, phosphorylated- τ and 4-hydroxy-2-nonenal in APP/PS1 mice. Taken together, the present data suggest that emodin may serve as a promising candidate for the treatment of Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is characterised by behavioural impairment and cognitive dysfunction (1) and is associated with age, where the prevalence may be as high as 50% among humans aged >95 years (2). The pathological cause of AD has not been fully elucidated. Previous research has indicated that the accumulation of β -amyloid peptide ($A\beta$) in the brain may be an important contributor to the development of AD (3). In patients with AD, $A\beta$ aggregates that form around neurons not only induce neuronal apoptosis but also trigger a cascade of cellular damage, including τ hyperphosphorylation and mitochondrial reactive oxygen species (ROS) production (4). Previous reports have indicated that symptoms of AD are attributable to the occurrence of oxidative stress (2,5,6). In particular, oxidative stress induces mitochondrial dysfunction and excessive ROS accumulation, ultimately leading to neuronal cell death (7).

Mitochondria are the primary source of intracellular ROS and are key targets of $A\beta$ toxicity during the AD pathological process (8). In addition to increasing the levels of $A\beta$ in the brain, overaccumulation of ROS and subsequent collapse of mitochondrial membrane potential (MMP) lead to reductions in ATP levels (9). Therefore, the function and integrity of the mitochondria must be maintained to protect nerve cells. During the occurrence and development of oxidative stress, the transcription factor nuclear factor E2-related factor 2 (Nrf2) dissociates from Kelch-like ECH-associated protein 1 (Keap-1) to activate numerous downstream proteins and detoxification enzymes, including superoxide dismutase 1 (SOD 1) and haemeoxygenase-1 (HO-1) (10,11).

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Abbreviations: APP, amyloid precursor protein; PS1, presenilin-1; LDH, lactate dehydrogenase; ROS, reactive oxygen species; AD, Alzheimer's disease; MMP, mitochondrial membrane potential; Keap-1, Kelch-like ECH-associated protein-1; 4-HNE, 4-hydroxy-2-nonenal; SOD, superoxide dismutase; CAT, catalase; HO-1, heme oxygenase-1; MWM, Morris water maze

Key words: emodin, Alzheimer's disease, β -amyloid peptide, nuclear factor-erythroid-2-related factor 2, oxidative stress

Since activation of the Nrf2 pathway has been documented to ameliorate oxidative stress and decreases accumulation of A β (12,13), rendering Nrf2 to be a potential therapeutic target for AD (14).

Emodin, which is structurally known as 1,3,8-trihydroxy-6-methylanthraquinone (Fig. 1), is a naturally occurring anthraquinone derivative. This compound can be isolated from medicinal herbs, including *Rheum palmatum* (15), *Cassia obtusifolia* (16) and *Polygonum multiflorum* (17), and has been used as a laxative in eastern Asia for 2,000 years (18). Previous studies have indicated that emodin is multifunctional. For example, evidence suggests that emodin enhances nerve cell survival by upregulating Bcl-2 expression levels and blocking A β -induced autophagy (19). In addition, emodin exhibits antioxidative effects: In viral myocarditis, emodin alleviates oxidative stress by increasing myocardial SOD expression levels whilst decreasing malondialdehyde expression levels (20). Emodin has also been shown to decrease collagen overproduction and inflammation by stimulating the Nrf2-antioxidant signalling pathway in pulmonary fibrosis (21), and to serve important roles in neurological disorders (22). For example, emodin protects neurons from A β ₂₅₋₃₅-induced neurotoxicity and inhibits excess τ accumulation in cortical neurons (19,23). To the best of our knowledge, however, no reports have yet described the systematic protective effects of emodin against AD or A β deposition in either *in vitro* or *in vivo* mouse models.

In the present study, amyloid precursor protein (APP)/presenilin 1 (PS1) double-transgenic mice and U251 cells subjected to A β ₁₋₄₂-induced apoptosis were used to determine the neuroprotective effects of emodin against AD. Emodin exerted protective effects against A β toxicity in U251 cells and ameliorated behavioural effects in the double-transgenic mouse model. The experimental data indicated that emodin protected neurons against neurodegeneration in AD.

Materials and methods

Cell culture. U251 cells (cat. no. KCB200965Y), a human glioma cell line that was purchased from the Cell bank of Chinese Academy of Sciences, were cultured in high glucose DMEM containing 10% fetal bovine serum, 1% 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) to provide a humidified atmosphere. All reagents were purchased from Invitrogen, Thermo Fisher Scientific, Inc.

Measurement of cell viability. U251 cells (5x10³ cells/well) cultured in plates (96-well) were pre-treated with emodin (purity, \geq 90%; CAS no. 518-82-1; cat. no. E7881; Sigma-Aldrich; Merck KGaA) at doses of 25 and 50 μ M at 37°C for 3 h and then co-incubated with 15 μ M A β ₁₋₄₂ [cat. no. 87233; Gill Biochem (Shanghai) Co., Ltd.] or culture medium at 37°C for a further 24 h. Non-treated cells served as the control. Cell viability was analysed using MTT assay as described previously (24). Briefly, cells were exposed to 5 mg/ml (final concentration) MTT at 37°C for 4 h in the dark before the formazan precipitates were dissolved in 100 μ l DMSO (Sigma-Aldrich; Merck KGaA). Absorbance was analysed using a Synergy™ 4 Microplate Reader at 490 nm (BioTek Instruments, Inc.).

Measurement of lactate dehydrogenase (LDH) levels and activity of caspases-3, -8 and -9. U251 cells (2x10⁵ cells/well) were cultured in plates (6-well) and treated with emodin at doses of 25 and 50 μ M at 37°C for 3 h, then co-incubated with 15 μ M A β ₁₋₄₂ or culture medium at 37°C for another 24 h. Culture medium-only-treated U251 cells served as the control group. The intracellular levels of LDH and the activities of caspase-3, -8 and -9 were detected using LDH (cat. no. C0016), caspase-3 (cat. no. C1168M), -8 (cat. no. C1151) and -9 (cat. no. C1157) Activity Assay kits (Beyotime Institute of Biotechnology) according to manufacturer's protocols.

Flow cytometry assay. U251 cells (2x10⁵ cells/well) were cultured in 6-well plates with emodin at doses of 25 and 50 μ M at 37°C for 3 h before being co-incubated with 15 μ M A β ₁₋₄₂ or culture medium for a further 24 h. Non-treated cells served as the control. Cells (1x10⁵) were collected and incubated with Annexin V and propidium iodide (cat. no. APT750; EMD Millipore) for 15 min at 37°C in the dark, and detected using a Muse Cell Analyzer (EMD Millipore) and analyzed with FlowJo v10 (FlowJo LLC).

Measurement of MMP and ROS levels. U251 cells (2x10⁵ cells/well) cultured in plates (6-well) with emodin at doses of 25 and 50 μ M at 37°C for 3 h and then co-incubated with 15 μ M A β ₁₋₄₂ or culture medium at 37°C for a further 24 h. Culture medium-only-treated U251 cells served as the control group. MMP was analysed using a MMP Detection kit (cat. no. M8650; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The red fluorescence represented the higher membrane potential, and the green fluorescence represented the damaged or lower membrane potential. Intracellular ROS was analysed using a Reactive Oxygen Detection kit (cat. no. S0033; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. After the cells were washed, the fluorescence intensities were detected using a fluorescence microscope (magnification, x400; Eclipse TE 2000-S; Nikon Corporation). Quantitative data analysis was performed using ImageJ software version 1.46 (National Institutes of Health), where the data were presented as the fluorescence intensity.

Animals and treatment. For the animal model, 8-month old male (42–49 g) B6C3-Tg (genotype APP^{swe}, PSEN1^{De9/Nju}) APP/PS1 double-transgenic mice (n=30) and wild-type (WT) littermates (n=10) were purchased from Nanjing Biomedical Research Institute of Nanjing University [SCXK (Su) 2015-0001; Nanjing, China]. All mice were housed in a temperature-controlled environment with a standard 12-h light/dark cycle at 23 \pm 1°C and 40–60% humidity, with access to food and water *ad libitum*.

The present study was approved by the Animal Ethics Committee of School of Life Sciences, Jilin University (approval no. SY20171208; Changchun, China). APP/PS1 mice were randomly divided into model (n=10; orally received 10 ml/Kg normal saline) and emodin-treated groups [orally received 10 (n=10) or 20 mg/kg (n=10) emodin once per day for 8 continuous weeks]. WT mice (n=10) orally received normal saline once per day for 8 continuous weeks. On the last five days of agent administration, behavioural tests were

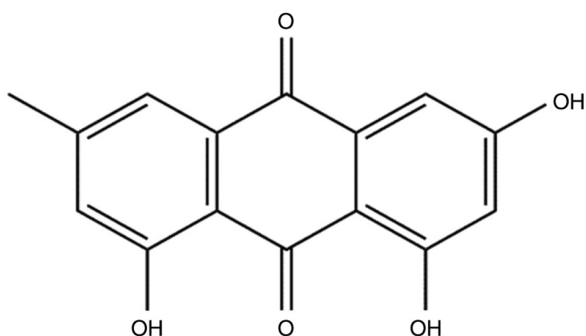


Figure 1. Chemical structure of emodin.

performed on each mouse, and blood was collected from the caudal vein of the mice under anaesthesia with isoflurane inhalation with an initial level of 4% and a maintenance level of 1.2%. Mice were then euthanized using a small animal euthanasia system (32x25x20 cm; cat. no. CL-1000-S2; Shanghai Yuyan Scientific Instrument Co., Ltd.). Briefly, mice were placed in the carbon dioxide tank for 2 min at a CO₂ concentration of 30%. The whole brain tissue was collected from each mouse for further investigation.

Animal behaviour detection. For experimental mice, learning and memory ability were analysed using Morris water maze test (MWM), whilst anxiety was evaluated using the open field test as previously described (25). For the MWM test, water was mixed with titanium dioxide to form a white liquid to hide the platform and the mice were placed in the water maze (cat. no. MT-200; Techman Software Co., Ltd.) to locate a platform that was hidden in white water. The route taken by mice was then recorded using the watermaze software (version 2.0; Techman Software Co., Ltd.). In MWM, mice were trained for 7 days starting at week 7 to allow them to learn or remember the position of platform. Open field test was used to evaluate anxiety and performed on the day after the MWM test. An open field experimental video analyser was used to record the path taken by each mouse (cat. no. 1056306; Zhong Shi Di Chuang). The box was washed after each experiment to clear any remaining scents or traces that could affect the results of the next test.

Immunohistochemical procedures. Brain tissue was fixed with 4% formalin solution at 25°C for 24 h, and then dehydrated with 30, 50, 70, 80, 95 and 100% ethanol, washed in xylene, embedded in paraffin and cut into 5- μ m thick sections. All slides were gradient hydrated with 100, 95, 80, 70 and 50% ethanol and distilled water respectively (26). Following dewaxing and hydration, the brain slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min. After cooling, the slides were incubated with 3% hydrogen peroxide for 10 min and blocked using 10% goat serum for 30 min at 25°C. The slides were then incubated with primary antibodies against phosphorylated (p)- τ (1:100; cat. no. SC12414; Santa Cruz Biotechnology, Inc.), A β 1-42 (1: 500; cat. no. ab32136; Abcam) and 4-hydroxy-2-nonenal (4-HNE; 1:200; cat. no. ab46545; Abcam) overnight at 4°C, followed by 1-h incubation at 25°C with biotinylated horseradish peroxidase-conjugated

secondary antibody (anti-rabbit; 1:500; cat. no. sc-3836; Santa Cruz Biotechnology, Inc.). After visualization using 3,3'-diaminobenzidine and Mayer's hematoxylin and immunoperoxidase staining of A β 1-42, p- τ and 4-HNE at 25°C for 5 min, images were captured using light microscopy (magnification, x200; cat. no. IX73; Olympus Corporation).

Western blot analysis. U251 cells (2x10⁵ cells/well) were cultured at 37°C in 6-well plates with emodin at doses of 25 and 50 μ M for 3 h, and co-incubated with 15 μ M A β 1-42 or culture medium at 37°C for a further 24 h. Culture medium only-treated U251 cells served as the control group. Treated cells and brain tissues were lysed using radio immunoprecipitation assay buffer (cat. no. 89900, Thermo Fisher, Inc.) containing 1% protease inhibitor cocktail (Sigma Aldrich; Merck KGaA). After detecting the protein concentration using a bicinchoninic acid protein assay kit, 40 μ g protein/lane was separated by 12% SDS-PAGE and transferred onto 0.45 μ m nitrocellulose membranes (Bio Basic, Inc.). The membranes were then blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies against anti-SOD 1 (cat. no. sc-17767), anti-HO-1 (cat. no. sc-136960), anti-CAT (cat. no. sc-271803), anti-Nrf2 (cat. no. sc-365949), anti-Bax (cat. no. sc-7480), anti-Bcl-2 (cat. no. sc-7382) and GAPDH (cat. no. sc-47724; all from Santa Cruz Biotechnology, Inc.) at dilutions of 1:5,000 overnight at 4°C. The membranes were subsequently incubated with horseradish peroxidase-conjugated mouse anti rabbit secondary antibodies at 4°C for 4 h (dilution 1:5,000; cat. no. bs-0295G; Bioss). ECL Detection reagent (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.) was then used to visualise the specific bands, where the intensity was quantified by ImageJ software (v1.46r National Institutes of Health).

Statistical analysis. Data are expressed as the mean \pm SEM. Experimental repeats were n=5 in the slide staining experiments and n=6 in the other experiments, including apoptosis, ELISA and western blotting. One-way ANOVA followed by Tukey's post hoc test was used to determine statistical significance using SPSS 16.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Emodin protects U251 cells against A β 1-42-induced cell apoptosis. Exposure to cytotoxic A β 1-42 (15 μ M) for 24 h led to a 50.66% decrease in the viability of U251 cells (P<0.001). However, co-treatment with emodin at 25 and 50 μ M led to 16.4 and 18.9% increases in viability, respectively (both P<0.05; Fig. 2A). By contrast, incubation with emodin alone for 24 h had no effects on cell viability (Fig. 2A).

The cytotoxicity of A β 1-42 treatment was next evaluated by measuring the release of LDH. Notably, 3-h pre-treatment with 50 μ M emodin resulted in a 39.9% decrease of LDH in cells exposed to A β 1-42 for 24 h (P<0.01; Fig. 2B). Furthermore, co-treatment with emodin at 25 μ M and 50 μ M suppressed A β 1-42-induced apoptosis of U251 cells by 11.5% (P<0.05) and 17.3% separately (P<0.01 Fig. 2F and G). The activation of caspase -3, -8 and -9, which are classical markers of apoptosis (27), was also subsequently measured. It was

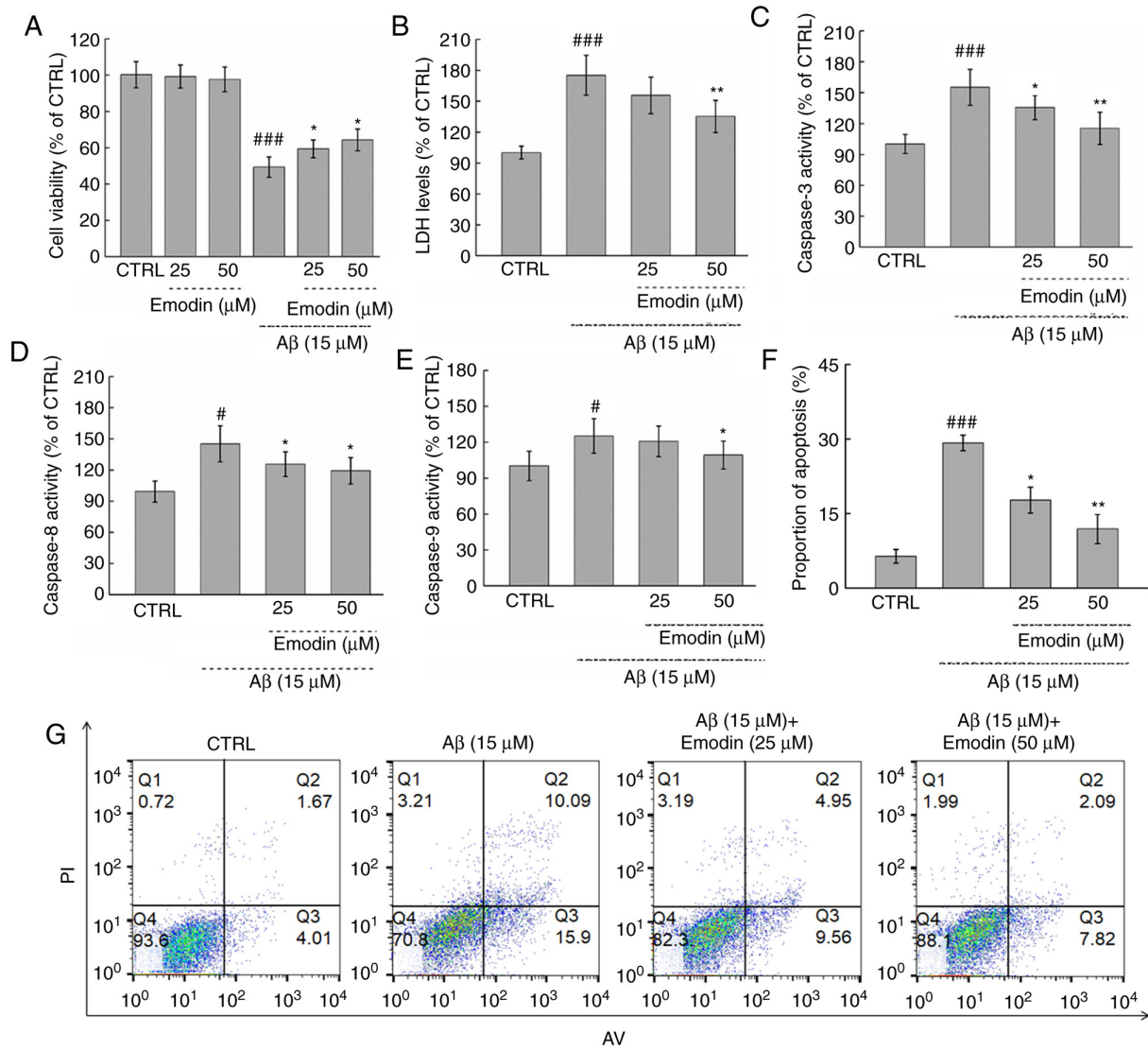


Figure 2. Emodin exerts neuroprotective effects against A β 1-42-induced apoptosis in U251 cells. (A) Emodin did not have toxic effects on U251 cells and enhanced the viability of cells exposed to 15 μ M A β 1-42 for 24 h. Emodin suppressed (B) leakage of LDH and the activity of (C) caspases-3, (D) -8 and (E) -9 in U251 cells that were exposed to A β 1-42 for 24 h. (F) Emodin suppressed the apoptosis of A β 1-42-exposed U251 cells. (G) Representative flow cytometry dot plots of the quantifications presented in (F). Data are expressed as the mean \pm SEM (n=6 experiments). *P<0.05 and ***P<0.001 vs. CTRL. #P<0.05 and **P<0.01 vs. A β 1-42 only. A β , β -amyloid peptide; LDH, lactate dehydrogenase; CTRL, control; AV, Annexin V.

found that 50 μ M emodin treatment led to decreases in the activity of caspase-3, -8 and -9 by 39.9% (P<0.01; Fig. 2C), 25.9% (P<0.05; Fig. 2D) and 15.9% (P<0.05; Fig. 2E), respectively, in A β 1-42-exposed U251 cells.

Emodin ameliorates A β 1-42-induced mitochondrial dysfunction and activates the Nrf2 pathway. Mitochondrial injury one of the early events of cell apoptosis (28). MMP was observed to be significantly dissipated in A β 1-42-exposed U251 cells. However, co-incubation with emodin, particularly at 50 μ M, restored the MMP, as indicated by decreased green and increased red fluorescence (Fig. 3A). The mitochondria are the major site of ROS production in cells, such that accumulation of ROS may cause further mitochondrial dysfunction (29,30). In U251 cells exposed to A β 1-42 for 24 h, 3-h pre-treatment with 50 μ M emodin successfully suppressed the overaccumulation of intracellular ROS, as indicated by the decreased green fluorescence intensity (Fig. 3B).

Both anti- and pro-apoptotic members of the Bcl-2 protein family serve key roles in modulating mitochondrial cell apoptosis (31). Compared with U251 cells exposed to A β 1-42 alone, those co-treated with emodin exhibited significantly increased Bcl-2 (P<0.001) and decreased Bax expression levels (P<0.01; Fig. 3C).

Nrf2 is activated to regulate the functions of mitochondria during the initiation and progression of oxidative stress (32). Compared with A β 1-42-exposed U251 cells, emodin-treated cells exhibited increased expression levels of Nrf2 (P<0.05), SOD 1 (P<0.01), HO-1 (P<0.001) and the antioxidant enzyme CAT (P<0.01; Fig. 3C). Taken together, these results suggest that emodin restored MMP and decreased ROS in cells whilst activating the Nrf2 pathway.

Emodin improves AD-like behaviour and suppresses A β 1-42, p- τ and 4-HNE deposition in APP/PS1 mice. Cognitive impairment and anxiety are the primary clinical manifestations of

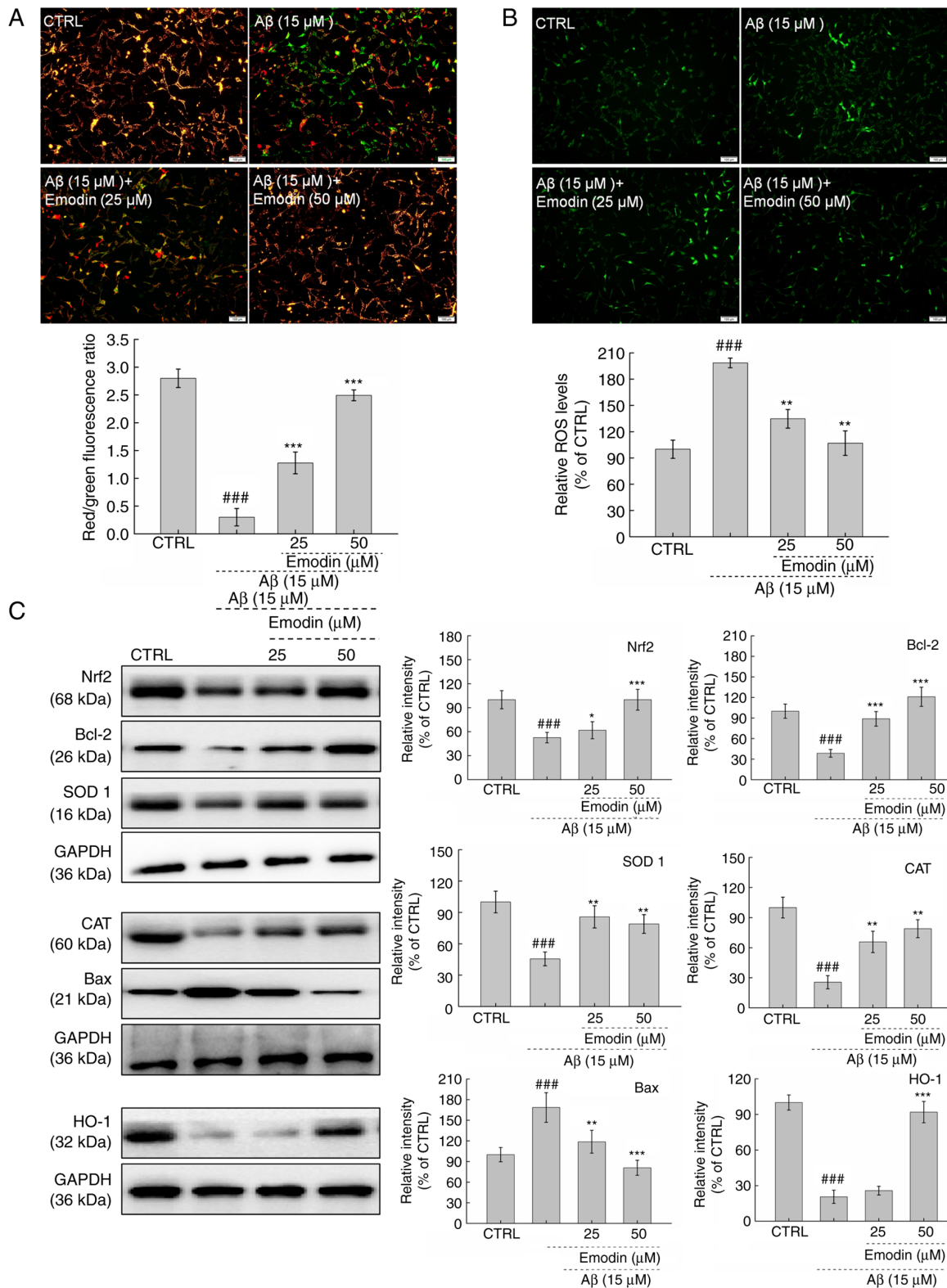


Figure 3. Emodin suppresses oxidative stress by increasing the expression levels of Nrf2 in U251 cells undergoing Aβ₁₋₄₂-induced apoptosis. A 3-h emodin pre-treatment (A) ameliorated dissipation of mitochondrial membrane potential and (B) inhibited excessive ROS accumulation in U251 cells exposed to Aβ for 24 h (magnification, x10; scale bar, 100 μm). (C) Levels of oxidative stress- and apoptosis-associated proteins in Aβ₁₋₄₂-exposed U251 cells were examined by western blotting. Quantitative protein expression levels were normalised to those of GAPDH. Data are presented as the mean ± SEM (n=6 experiments). ###P<0.001 vs. CTRL. *P<0.05, **P<0.01 and ***P<0.001 vs. Aβ₁₋₄₂ only. Nrf2, nuclear factor E2-related factor 2; Aβ, β-amyloid peptide; ROS, reactive oxygen species; CTRL, control; SOD, superoxide dismutase; CAT, catalase; HO, heme oxygenase.

AD (33). Therefore, MWM and open field tests were used to evaluate these manifestations in APP/PS1 mice. Compared with untreated mice, those treated with emodin at 20 mg/kg

exhibited a 14.3% decrease in time required to locate the platform during the MWM test, indicating that emodin enhanced the spatial memory and learning ability of the mice (P<0.05;

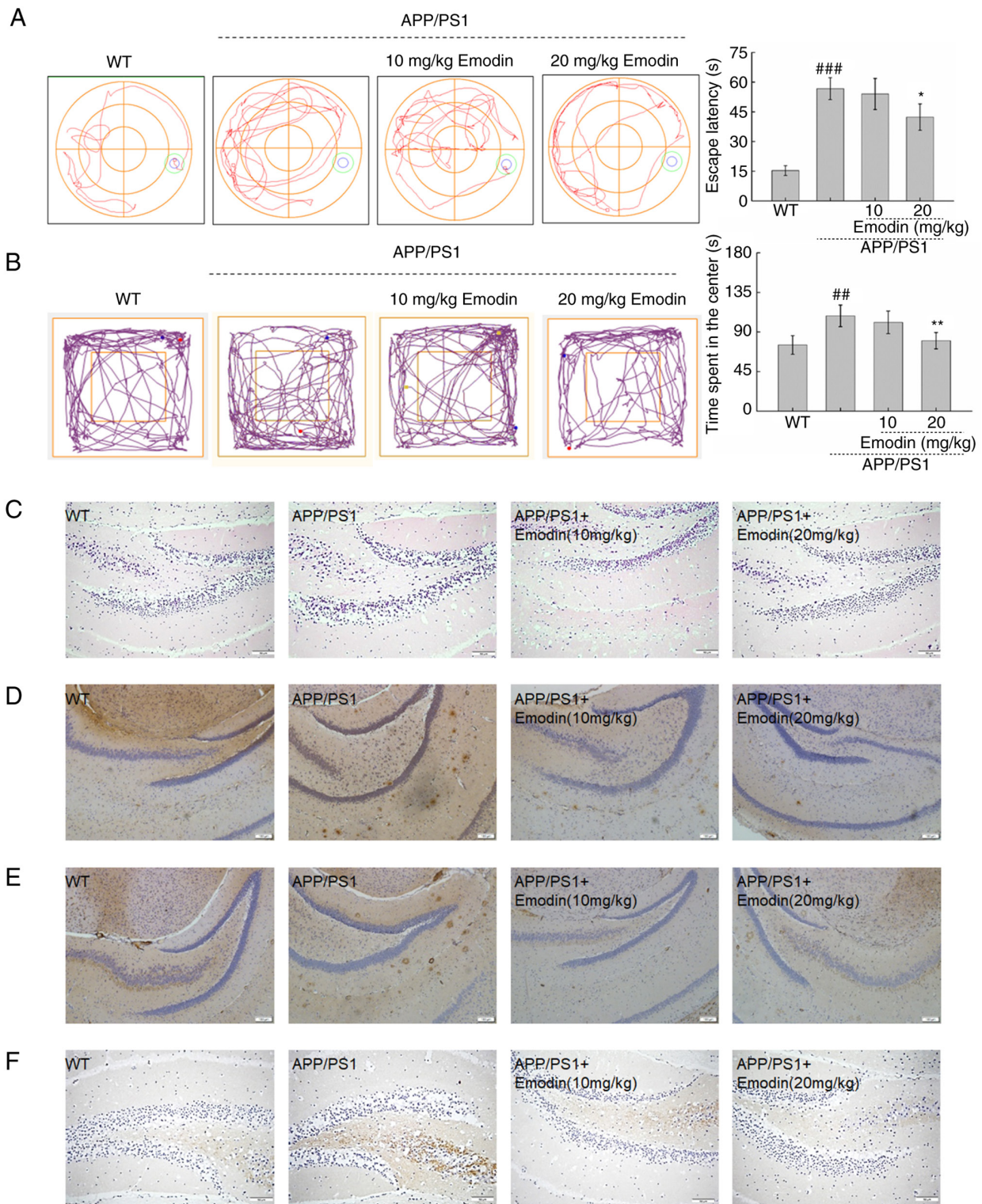


Figure 4. Emodin improves behavioural performance of APP/PS1 mice by decreasing deposition of A β 1-42, p- τ and 4-HNE. (A) Emodin decreased the escape latency time of APP/PS1 mice during the Morris water maze test. (B) Emodin decreased the time spent by APP/PS1 mice in the central area during the open field test. Data are presented as the mean \pm SEM (n=10). ^{##}P<0.01 and ^{###}P<0.001 vs. WT mice. ^{*}P<0.05 and ^{**}P<0.01 vs. untreated APP/PS1 mice. (C) Haematoxylin and eosin staining of brain tissue (scale bar, 150 μ m; n=5 experiments). (D) Emodin markedly suppressed deposition of A β 1-42. (E) Overaccumulation of p- τ in the brain of APP/PS1 mice (scale bar, 1100 μ m; n=5 experiments). (F) High expression levels of 4-HNE (scale bar, 150 μ m; n=5 experiments) in the brain of APP/PS1 mice detected by immunohistochemistry. APP, amyloid precursor protein; PS1, presenilin-1; A β , β -amyloid peptide; p-, phosphorylated; 4-HNE, 4-hydroxy-2-nonenal; WT, wild-type.

Fig. 4A). In the open field test, mice treated with emodin at 20 mg/kg spent significantly less time in the centre of the field, which indicated that this agent ameliorated anxiety in the mouse model (P<0.01; Fig. 4B).

H&E staining was used to detect neuronal damage in brain tissue. However, there was no obvious pathological reaction in the brain (Fig. 4C). The presence of extracellular A β and intracellular neurofibrillary tangles in the brain are

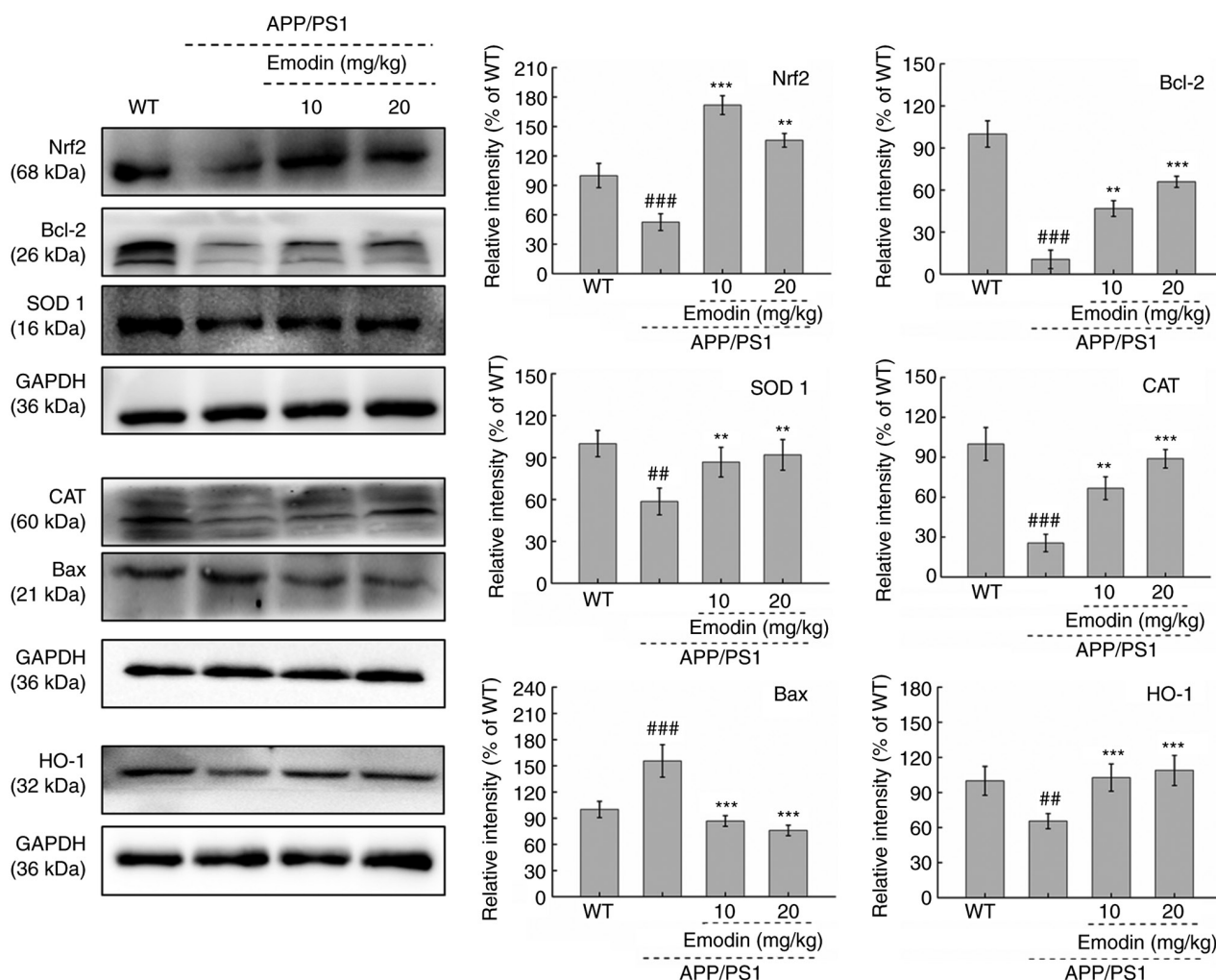


Figure 5. Emodin regulates the expression levels of oxidative stress- and apoptosis-associated proteins. Whole-brain lysates from APP/PS1 mice were analysed and quantitative protein expression level data were normalised to those of GAPDH. Data are presented as the mean \pm SEM (n=6). ##P<0.01 and ###P<0.001 vs. WT mice. **P<0.01 and ***P<0.001 vs. untreated APP/PS1 mice. APP, amyloid precursor protein; PS1, presenilin-1; WT, wild-type; Nrf2, nuclear factor E2-related factor 2; SOD, superoxide dismutase; CAT, catalase; HO, heme oxygenase.

two defining aetiological characteristics of AD (34). Compared with untreated APP/PS1 mice, emodin-treated mice, particularly those that received a dose of 20 mg/kg, exhibited lower expression levels of A β 1-42 (Fig. 4D) and p- τ (Fig. 4E) in the brain. Tissue was also stained for 4-HNE, a biomarker of oxidative stress, to further elucidate the function of emodin in this process. Notably, emodin decreased expression levels of 4-HNE in the brain compared with those in untreated APP/PS1 mice (Fig. 4F).

Emodin regulates Nrf2 signalling in the brain of mice. To further investigate the possible mechanism by which emodin exerts its effects in APP/PS1 mice, the expression levels of proteins associated with the Bcl-2 family and the Nrf2 pathway, in addition to the antioxidant enzyme CAT, were evaluated. Compared with those in untreated APP/PS1 mice, 20 mg/kg emodin exhibited significantly increased expression levels of Bcl-2 (P<0.01) and significantly decreased expression levels of Bax in the brain tissue of mice (P<0.001; Fig. 5). Furthermore, an 8-week course of 20 mg/kg emodin administration led to significant increases in Nrf2 (P<0.01), SOD 1 (P<0.01) and HO-1 expression (P<0.001) and a significant decrease in

CAT expression in the brain tissue, even at an emodin dose of 10 mg/kg (P<0.01; Fig. 5). These results demonstrated the ability of emodin to regulate Nrf2 pathway activity in APP/PS1 mice and its potential as an antioxidant.

Discussion

The majority of symptoms of AD can be attributed to oxidative stress (35,36). Therefore, the elimination of excess ROS or induction of endogenous antioxidant activity may be a potential strategy for the treatment of AD (37). Emodin is a biologically active compound that can be found in a number of herbal laxatives and exhibits pharmacological activity due to its strong antioxidative effects (38). The present study confirmed the neuroprotective effects of emodin against AD by using U251 cells subjected to A β 1-42-induced apoptosis and in APP/PS1 double-transgenic mice.

The U251 human astrocyte cell line has previously been used to investigate AD (39). In the present study, U251 cells were treated with A β 1-42 to induce cell injury. A β , a peptide containing 39-43 amino acids, has been shown to exert numerous toxic effects both *in vitro* and *in vivo* (40). For

example, A β accumulation is neurotoxic and can depolarise the cell membrane, decrease mitochondrial potential and increase the production of ROS, induce synaptic dysfunction and cause oxidative stress to mediate mitochondrial damage (41). The present study demonstrated that emodin improved cell viability and suppressed apoptosis in cells exposed to A β 1-42. Apoptotic neuronal death causes fatal injury to the brain tissue (42). Caspases-3, -8 and -9 are major components of the classical apoptosis pathway (43). Caspase-8 is located primarily in the mitochondria and is activated to form the apoptosome, which recruits pro-caspase-9 and induces pro-caspase-3 cleavage, ultimately leading to mitochondrial apoptosis (44).

The mitochondria are energy-producing organelles that serve as the primary source of ROS (45). A β accumulation directly exhibits negative effects on mitochondrial energy metabolism, specifically on α -ketoglutarate dehydrogenase and pyruvate dehydrogenase activity, which ultimately results in cell apoptosis (46). The Bcl-2 family member of proteins, which include the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2, have also been reported to be involved in mitochondrial injury (47). The pro-apoptotic protein Bax is activated in response to apoptotic stimuli (48), which leads to mitochondrial oxidative respiratory chain damage and decreased MMP (49).

In the present study, emodin also enhanced the activity of the antioxidant enzyme CAT and activated the transcription factor Nrf2. The latter is known to regulate a number of cytoprotective and detoxification genes. For example, Nrf2 binds with Keap-1 in the cytoplasm, which represses Nrf2 nuclear translocation (50). However, this interaction is disrupted by oxidative stress, which allows Nrf2 to translocate into the nucleus to interact with the antioxidant response element, thus activating an effector cascade involving HO-1 and SOD1 (10). Both HO-1 and SOD1 are powerful antioxidative reagents that can neutralize toxic superoxide radicals produced in AD (51,52). In addition to oxidative stress, Nrf2 controls the expression of nuclear genes that encode mitochondrial proteins, which affect mitochondrial biological function. For example, Nrf2 deficiency affects mitochondrial electron transport chain activity, fatty acid oxidation and availability of substrates (NADH and FADH₂/succinate) for respiration, and ATP synthesis (53), furthermore, it can also exacerbate APP and τ pathology (54). In the present study, emodin strongly decreased activity of caspase-3, -8 and -9, improved mitochondrial function, decreased ROS accumulation, enhanced the Bcl-2/Bax ratio and activated the Nrf2 pathway in both U251 cells subjected to A β 1-42-induced apoptosis and APP/PS1 mice, suggesting that emodin exerts both antioxidant and neuroprotective effects.

In the present study, double transgenic APP/PS1 mice were used to generate a model of AD with severe pathology. In this transgenic model, overexpression of the gene encoding APP and a mutant form of PS1 have previously been demonstrated to impair the processing of amyloid proteins and increase levels of A β (55). Accumulation of A β exacerbates cognitive impairment and induces anxiety in humans with AD (56). Therefore, the manifestations in APP/PS1 mice were similar to AD-like symptoms (57). The present study used a MWM test to evaluate the spatial learning ability (58) of mice and the open field test to evaluate anxiety (59). In

neurons, aggregation of A β affects the activity of kinases and phosphatases, leading to the hyperphosphorylation of τ protein and formation of neurofibrillary tangles (60). An increase in the number of neuroinflammatory plaques caused by excess p- τ is another pathological feature of AD that has previously been observed in the brain of APP/PS1 mice (61). In turn, excess p- τ inhibits kinesin-dependent transportation and blocks APP transport into axons and dendrites, which increases accumulation of A β in the neurons (62). In the present study, emodin treatment led to significant improvements in AD-like behaviour of APP/PS1 mice and decreased the levels of aggregated A β , pathogenic τ and the peroxidation product 4-HNE.

Increases in the production of H₂O₂ and other oxidative products lead to accumulation of A β and thus contribute to the initiation and progression of AD (35,63,64). An effective antioxidant therapy is necessary to relieve the symptoms of AD. The present study demonstrated that emodin improved AD-associated behaviour in APP/PS1 mice, ameliorated severe oxidative stress and activated the Nrf2 pathway both *in vivo* and *in vitro*.

In conclusion, emodin was demonstrated to exert protective effects against A β 1-42-induced cell apoptosis *in vitro* and A β deposition *in vivo* in APP/PS1 double-transgenic mice. These effects are likely due to the Nrf2-mediated anti-oxidative activity of emodin and suggest a potential role for this agent in AD protection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and XF designed the experiments, drafted and revised the manuscript. ZL, HB and HJ performed the experiments and analyzed the data. JS and QM revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by Animal Ethics Committee of School of Life Sciences, Jilin University (approval no. SY20171208).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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