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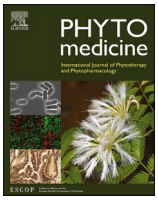

Magnoflorine improves cognitive deficits and pathology of Alzheimer's disease via inhibiting of JNK signaling pathway

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ABSTRACT

Background: Cognitive deficit is the main clinical feature of Alzheimer's disease (AD), and the massive death of neuronal cells is the leading cause of cognitive deficits. So, there is an urgent clinical need to discover effective drugs to protect brain neurons from damage in order to treat AD. Naturally-derived compounds have always been an important source of new drug discovery because of their diverse pharmacological activities, reliable efficacy and low toxicity. Magnoflorine is a quaternary aporphine alkaloid, which naturally exist in some commonly used herbal medicines, and has good anti-inflammatory and antioxidant effects. However, magnoflorine has not been reported in AD.

Hypothesis/purpose: To investigate the therapeutic effect and mechanism of magnoflorine on AD.

Methods: Neuronal damage was detected by flow cytometry, immunofluorescence and western blotting. Oxidative stress was measured by detection of SOD and MDA, as well as JC-1 and reactive oxygen species (ROS) staining. The APP/PS1 mice were given drugs by intraperitoneal injection (I.P.) every day for one month, and then the new object recognition and Morris water maze were used to detect the cognitive ability of the mice.

Results: We demonstrated that magnoflorine reduced Aβ-induced PC12 cell apoptosis and intracellular ROS generation. Further studies found that magnoflorine significantly improved cognitive deficits and AD-type pathology. Most interestingly, the efficacy of magnoflorine was better than that of the clinical control drug donepezil. Mechanistically, based on RNA-sequencing analysis, we found that magnoflorine significantly inhibited phosphorylated c-Jun N-terminal kinase (JNK) in AD models. This result was further validated using a JNK inhibitor.

Conclusion: Our results indicate that magnoflorine improves cognitive deficits and pathology of AD through inhibiting of JNK signaling pathway. Thus, magnoflorine may be a potential therapeutic candidate for AD.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP/PS1, APPswe/PSEN1dE9; DAPI, 4',6-diamidino-2'-phenylindole; DCFH-DA, dichlorodihydrofluorescein diacetate; DMEM, dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FITC, fluorescein Isothiocyanate; FBS, fetal bovine serum; HRP, horseradish peroxidase; IF, immunofluorescence; JNK, c-Jun N-terminal kinase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine; MAPK, mitogen-activated protein kinase; MDA, malonaldehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MWM, Morris water maze; NFT, neurofibrillary tangle; NOR, novel object recognition; PI, propidium iodide; PBS, phosphate buffered saline; PVDF, poly vinylidene fluoride; RT-qPCR, real time quantitative polymerase chain reaction; RIPA, radio-immunoprecipitation assay; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of mean; SOD, superoxide dismutase; TBST, Tris-buffered saline containing Tween; WT, Wild-type.

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Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disorder that mainly occurs in elderly people (Sharma et al., 2020). At present, AD patients account for about 60% to 80% of all dementia patients, which has become a serious social problem (Cass, 2017). However, the etiology of AD is unknown and there are currently no effective treatments. With the rapid aging of the world population, how to prevent and treat AD and the screening of therapeutic drugs have become the focus of social attention.

The loss of neurons is the most basic pathological change of AD and the main cause of cognitive impairment (Jacobsen et al., 2005). The detection of hippocampal neuron apoptosis in AD patients and aged controls suggested that the apoptosis of hippocampal neuronal cells in AD group was significantly increased, indicating that apoptosis may be involved in the degeneration of AD hippocampal neurons (Stadelmann et al., 1999). It is well known that AD has two main pathogenic hypotheses: one is neurofibrillary tangle (NFT), which is mainly caused by the hyperphosphorylation of tau protein; the other is the aggregation of β-amyloid (Aβ), eventually leading to the neuron damage (Maccioni et al., 2001). In order to reduce the extensive neuronal damage, researchers have designed a series of small molecule compounds, but most of them failed to achieve the desired effect (Weggen et al., 2007). Recent years, researchers have become more and more interested in natural compounds derived from plants because of their diverse pharmacological activities, low toxicity, and good neuroprotective effects, which provide new ideas for the discovery of new drugs for the prevention and treatment of AD (Atanasov et al., 2015; Cragg and Newman, 2013). For example, polyphenols have anti-inflammatory and antioxidant effects, and alkaloids have the activity of inhibiting cholinesterase, both of which have been reported for many times in preclinical studies to have the potential to become AD therapeutics (Sharifi-Rad et al., 2020; Wu et al., 2010).

In this study, we screened 160 plant-derived natural compounds and found that magnoflorine had the best protective effect on Aβ1–42-induced PC12 cell injury. Magnoflorine, isolated from some commonly used Chinese herbal medicines, has anti-inflammatory, antimicrobial and immune-enhancing effects (Alagu Lakshmi et al., 2021; Yates et al., 2022). Toxicity studies have shown that magnoflorine is nontoxic to most cells, and given its good pharmacological activity (Xu et al., 2020a). However, its role in AD has not been reported. Therefore, we tested the protective effect of magnoflorine on neuronal injury and AD pathology *in vivo* and *in vitro*, and the effect was compared with that of the marketed positive drug donepezil. The molecular mechanism was detected by RNA sequencing. Our study suggests that magnoflorine has good potential in the treatment of AD, and the effect is better than the current clinical control drug donepezil.

Materials and methods

Reagents

Magnoflorine (2141–09–5) was ordered from Chengdu DeSiTe Biological Technology (Chengdu, China). Donepezil (110,119–84–1) were bought from Chengdu Alfa Biotechnology (Chengdu, China). 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT; M2128), Dimethyl sulfoxide (DMSO, D2650), High Glucose Dulbecco's modified Eagle's medium (DMEM, D1152), bovine serum albumin (BSA; A8020) and Fetal bovine serum (FBS, F7524) were bought from Sigma (St. Louis, MO, USA). Phosphatase inhibitor cocktail (78,441) were bought from Thermo Fisher Scientific (Rockford, IL, USA). 0.25% Trypsin (25,200,056) and Penicillin/Streptomycin (15,140–122) was ordered from Gibco (Carlsbad, CA, USA). Annexin V-FITC/PI Apoptosis Detection Kit (BD556547) was ordered from BD Biosciences (San Diego, CA, USA). Triton X-100 (P1080), 4, 6-diamidino-2-phenylindole (DAPI; C0065), DCFH-DA reagent (S0033), MDA assay kit (MDA; S0131S), SOD assay kit (SOD; S0101s), JC-1 assay (C2005) and RIPA lysis buffer (P0013B) were got from Beyotime Institute of Biotechnology (Shanghai, China). Aβ 1–42 (PA4391), NH2-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA–COOH ($C_{203}H_{311}N_{55}O_{60}S$; Molecular Mass: 4514.10), was ordered from Ontores Biotechnologies (Zhejiang, China). The antibodies used in this research were presented in Table S1. 160 natural products used in this study were purchased from TargetMol (Boston, USA). The catalog number of natural compounds were shown in Table S2.

Cell culture

PC12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% streptomycin/penicillin (100 mg/ml) and 10% fetal bovine serum (FBS). Cells were placed in a 37 °C cell culture incubator with a humidified 5% CO_2 atmosphere. The cells were replaced with fresh DMED medium every two days. PC12 cells reaching 80% to 90% confluence were digested with 0.25% trypsin, centrifuge at 1000 rpm for 5 min to collect cells, then re-suspended in fresh 10% FBS DMEM medium and seeded into cell culture dishes (Zhao et al., 2022).

MTT kit

The effect of magnoflorine on cell viability were analyzed by MTT assay as described previously (Chong and Zheng, 2016). Briefly, PC12 cells were seeded in 96- well plate at the density 8×10^3 cells per well. Then the cells were exposed to various concentration of Aβ for 24h After 24 h of incubation 10 μl MTT (0.5 mg/ml, dissolved in blank DMEM medium without FBS) reagent was added into each well of treated PC12 cells and incubated for 3–4 h. Then, discarded medium with MTT and added 100 μl DMSO to each well to dissolve formazan formed by living cells. Shaked the plate for 10 min and the absorbance was tested through a microplate reader at 570 nm. Living cell viability were determined by being compared to the control group.

Mitochondrial membrane potential (△ψm) measurement

The effect of magnoflorine on △ψm was assessed using the JC-1 staining. JC-1 staining is a common method used to detect changes in △ψm (Menke et al., 2003). Briefly, PC12 cells were seeded into 96-well plates (8×10^3 cells/well) and treated with appropriate drugs. Then, the cells were incubated with 10 μg/ml 1x JC-1staining solution (diluted in blank DMEM medium without FBS) at 37 °C for 30 min. Then, the cells were washed twice with 1x phosphate buffered saline (PBS) and detected the fluorescence intensity. Set the intensity of green fluorescence (excitation 485 nm, emission 535 nm) and red fluorescence (excitation 488 nm, emission 590 nm) and capture images by a multimode microplate reader fluorescence microscope (She et al., 2023). The Δψm was presented as the ratio of the red/green fluorescence intensity.

Reactive oxygen species (ROS) staining

The effect of magnoflorine on intracellular ROS levels was measured by the fluorescent probe DCFH-DA reagent according to the protocols provide by manufacturer. Briefly, PC12 cells were treated with appropriate drugs, then the cells were incubated with 10 μM DCFH-DA reagent in blank DMEM (without FBS) for 30 min in the dark in 37 °C cell incubator and washed twice with 1xPBS solution.Fluorescence intensity was measured using an Infinite M200 PRO multimode microplate with excitation at 488 nm and emission at 525 nm. The intensity of green fluorescence is proportional to the level of intracellular ROS.

Flow cytometry

The effect of magnoflorine on apoptosis was assessed by flow cytometry according to the manufacturer's protocols. PC12 cells in the

6-well plate (5×10^5 cells/well) were treated with appropriate drugs, then collected by centrifugation at 1000 rpm for 5 min and resuspended in 1x Annexin V-FITC/PI binding buffer (185 μl). Add 5 μl of annexin-V-FITC and 10 μl of propidium iodide (PI) solution successively to the cell suspension, and stain for 40 min. The percentage of apoptotic cells was determined using flow cytometry analyze. Each experiment was repeated three times.

Animals and treatment

APP/PS1 (APPswe/PSEN1dE9) double-transgenic mice (8 months, 30–32 g, female) were purchased from the Jackson Laboratory and kept in the animal facility of Hangzhou Medical College under the conditions of 25 °C, 12 h/dark light cycle, and free access to water and food. The mice study protocol was approved by Hangzhou Medical College Animal Ethics Committee (2022–001, 3rd March 2022). Totally 50 mice with similar body weight were randomly divided into five groups, 10 in each group: Wild-type (WT), APP/PS1, APP/PS1+1 mg/ml magnoflorine, APP/PS1+10 mg/ml magnoflorine groups and APP/PS1+ Donepezil groups. Among them, 1 mg/kg magnoflorine, 10 mg/kg magnoflorine and 5 mg/kg Donepezil were dissolved in 1xPBS containing 2% DMSO. Then, the APP/PS1 mice were intraperitoneally injected with the drugs according to their body weight every day. Mice from the WT and APP/PS1 groups were injected with equal volumes of 1xPBS (with 2% DMSO). The behavior of the mice was tested after continuous drug treatment for one month.

Morris water maze (MWM)

We performed the MWM to test the effect of magnoflorine on the spatial memory and learning skills of mice using previously published protocols (Zhao et al., 2020). Before the experiment, the mice were transferred to the behavior room for training for three days. Then, mice were tested for place navigation on four consecutive days then followed by a spatial probe test (without platform) on the fifth day. During the place navigation tests, the hidden platform was placed 1 cm below the water surface in the middle of the target quadrants. The time and trajectory of each mice to find the hidden platform were recorded by the device's image automatic camera system. Repeat the above operation every day from the 2nd to the 4th day. On the 5th day, the hidden platform was taken out and let the mice swim freely for 60 s for the spatial probe experiments. The number of crossings the original hidden platform and the time spent in the target quadrant were recorded. All data acquisition and processing were completed by image automatic monitoring and processing system (VisuTrack, Shanghai China).

Novel object recognition (NOR)

The effect of magnoflorine on cognitive function is further verified by NOR test. The bottom of the experimental device is a square of 25 × 25 cm, and the four walls are 40 cm high, with a video camera overhead. Before the test, mice of each group were moved to the test room to be accustomed to the new environment. On the first day, two identical objects A were placed in the experimental place. After the mice were put in, the record equipment was turned on to trace the mice, including the number of times they touched the two objects and the time spent for exploring them within 2–3 cm from the objects. The test duration was 5 min. After 24 h, substitute one of the original objects with B object, and record with video equipment for 5 min. Then analyze the performance of the mice: if the mice have poor cognitive ability, there is no difference in the exploration of new and old objects; if the mice have normal cognitive ability, they spend more time exploring the new object.

SOD and MDA assay

The superoxide dismutase (SOD) assays were performed according to the manufacturer's operating instructions. First, hippocampus tissue sample was extracted after mice were perfused with saline (0.9% NaCl) and added 100 μl of SOD sample preparation solution per 10 mg of tissue for homogenization. After centrifugation at 12,000 g for 3–5 min at 4 °C, the supernatant was taken out for detection. After adding the working solution and incubating for 30 min, measure the absorbance at 450 nm. Similarly, we detected the malonaldehyde (MDA) content in brain tissue according to the instructions provided by the manufacturer. Add freshly prepared MDA detection working solution to the different samples to be tested, mix well and heat in 100 °C water for 15 −20 min. Cool to the room temperature and centrifuge at 1500 rpm for 5 min. 200 μl of the supernatant was taken out and added to a new 96-well plate, and then the absorbance was measured at 532 nm using a microplate reader.

Immunofluorescence (IF)

After behavioral tests, all mice from different group were euthanized with 0.25 mg/ml chloral hydrate, and the animals were sacrificed by decapitation after cardiac perfusion with pre-cooled 0.9% saline. The brains of three mice in each group were taken out and fixed in 4% PFA (4% paraformaldehyde) at 4 °C for 24 h, a portion of the samples were dehydrated in a sucrose gradient and embedded in optimal cutting temperature compound (OCT). For tissue immunofluorescence, brain tissue brain tissue was sectioned at a thickness of 30 μm using a cryostat (Leica CM3050, Germany). Wash three times with 1xPBS to remove OCT on the tissue, blocked the non-specific binding with 10% BSA (in 1x PBS) for 60 min at room temperature. Then, the tissue sections were added with primary antibodies and incubated overnight at 4 °C. The next day, sections were rinsed 3 times with 1xPBS and added corresponding secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594) and incubated for 2 h at room temperature. Then washed three times with 1xPBS and mounted with DAPI-containing anti-fluorescence quencher. Images of each section were acquired using Nikon A1 confocal microscope.

Western blotting

The cultured PC12 cells or brain tissue samples under different treatments were lysed in ice-cold RIPA lysis buffer with a protease phosphatase inhibitor cocktail. The concentration of samples in each group was detected using BCA kit (Thermo Fisher, 23,225) and equal concentration of samples were loaded on 12% SDS-PAGE gels, then the proteins on the gel were transferred to a 0.22 μm PVDF membrane under the condition of 300 mA for 90 min. Then PVDF membranes were blocked with 3% BSA diluted in 1xTBST(Tris-buffered saline with 0.1% Tween20) for 1 h and incubated the membrane with primary antibody (1:1000) overnight at 4 °C. Following day, the PVDF membranes was washed with 1 × TBST thrice, and incubated with HRP (horseradish peroxidase)-conjugated secondary antibody for another 2 h at room temperature. Protein bands were visualized using the Bio-Rad Gel Doc XR system after addition of ECL luminescent liquid, and band intensities were quantified by Image J software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA). Error bars were presented as the mean ± SEM. The statistical significance between different groups was determined using one-way or two-way ANOVA followed by Tukey's post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Magnoflorine attenuates the cell injury induced by Aβ1–42 in PC12 cells

Amyloid beta can produce a range of toxic effects, including

oxidative stress, apoptosis, and mitochondrial dysfunction, especially Aβ1–42 (Cline et al., 2018; Puzzo, 2019). So, in this study we used Aβ1–42 to induce a neuronal injury model. First, we checked the toxicity of Aβ1–42 in PC12 cells, the cells (7×10^3 per well) seeded in the 96-well plate were treated with different doses of Aβ1–42 for 24 h and the cell viability was determined by MTT assay. As showed in Fig. S1, compared with the control group, Aβ caused significant cytotoxicity in PC12 cells from 5 μM, and 10 μM Aβ1–42 was used in further experiments. Recently, several studies have shown that natural compounds have a positive effect on AD (Cao et al., 2020). Therefore, we next screened the 160 natural compounds (Table S2) to find the ones with the best protection against nerve injury. To do this, PC12 cells were pretreated with different compounds (10 μM) for 3 h, followed by Aβ1–42 (10 μM) stimulation for another 24h The cell viability was measured by MTT assay. Obtained result showed that magnoflorine had the best protective effect against Aβ1–42-induced neuronal cell damage (Fig. 1A). Then, we determined the effect of magnoflorine on Aβ1–42-induced oxidative stress by JC-1 staining and ROS staining. The result showed that magnoflorine pretreatment significantly reduced the intracellular ROS levels and restored the mitochondrial membrane potential (Fig. 1B). MTT result showed that decreased cell viability by Aβ can be reversed by magnoflorine treatment (Fig. 1C). Subsequently, we used flow cytometry to detect the effect of magnoflorine on apoptosis. The results showed that magnoflorine pretreatment significantly reduced the number of apoptotic cells compared with the Aβ1–42 group (Fig. 1D-1E). Meanwhile, results from western blot showed that after Aβ1–42 stimulated PC12 cells, the expression levels of bax and cleaved-caspase 3 significantly increased, while Bcl2 decreased. In contrast, magnoflorine pretreatment reversed these changes (Fig. 1F-1G), indicating that magnoflorine pretreatment could alleviate Aβ1–42-induced apoptosis in PC12 cells. Similarly, magnoflorine significantly increased mRNA level of bcl2, decreased the mRNA level of bax in PC12 cells (Fig. 1H). Furthermore, we verified the protective effect of magnoflorine in H_2O_2-induced cell injury model. The results suggest that magnoflorine pretreatment could increase PC12 cell vitality, restore mitochondrial membrane potential, and reduce oxidative stress damage (Fig. S2).

Magnoflorine conferred neuroprotection via inhibiting JNK signaling pathway

To explore the protect mechanism of magnoflorine in PC12 cells, we divided the samples into three groups (Each group of samples contained 3 replicates): Ctrl, Aβ and Mag+Aβ group for RNA sequencing. Obtained results identified 921 significantly altered genes (Fig. 2A). We next performed KEGG analysis on these genes and found that apoptosis-related pathway MAPK was at the top (Fig. 2B). Studies have shown that MAPKs are important mediators of signal transmission from the cell surface to the interior of the nucleus (Yue and López, 2020). In cells, there are mainly three mitogen-activated protein kinases closely related to cell growth and development, namely c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) (Kheiri et al., 2018). Then, we detected the changes of these three kinases by western blotting, results showed that magnoflorine had the greatest effect on JNK signaling pathway, and had little effect on ERK and P38MAPK (Fig. 2C-2D). Compared with control group, the expression of P- JNK was significantly increased after Aβ1–42 stimulation, while the P-JNK level was reversed after magnoflorine pretreatment. The above results suggest that magnoflorine may exert its protective effect mainly via JNK signaling pathway.

To verify this result, we pretreated cells with JNK inhibitor (SP600125) to block JNK and tested the protein expression of apoptosis-related genes. Obtained results showed no significant differences in the Bcl2/Bax ratio and cleaved caspase3 protein levels in the Aβ+SP600125, Mag+Aβ and Mag+Aβ+SP600125 groups suggest that magnoflorine protects neuronal cells from damage mainly by inhibiting JNK (Fig. 2E-2F). Similar results were obtained by Flow cytometry, compared with the control group, Aβ caused a significant increase in apoptotic cells, while groups Aβ+SP600125, Mag+Aβ and Mag+Aβ+SP600125 all improved apoptotic caused by Aβ, and there was no significant among them (Fig. 2G-2H).

Magnoflorine improved cognitive deficits

To determine the effect of magnoflorine on learning and memory skills in APP/PS1 model mice, we performed MWM test. The mice in each group were treated with the drug by intraperitoneal injection for one month, and then the MWM test was performed (Fig. 3A). Three days of training were given before the start of the formal experiment, followed by Place navigation test and Spatial probe test (Fig. 3B). The representative movement trajectory is shown in Fig. 3C. The results showed that compared with WT mice, APP/PS1 mice exhibited longer escape latency, fewer number of hidden platform crossings and less time to target quadrant. However, magnoflorine treatment greatly improved the above indicators and enhanced the spatial memory ability of APP/PS1 mice, and the therapeutic effect was better than that of clinical control drug donepezil (Fig. 3D-3F).

According to the nature of rodents like to explore novel things, Ennaceur and Delacour in 1988 reported NOR experiment (Ennaceur and Delacour, 1988), which was used to test the memory ability of rodents. The model evaluates the memory ability of the tested animals based on the length of time the animals spend exploring familiar and new objects they have seen (Antunes and Biala, 2012). To further validate our results, we performed NOR tests. The results showed that, the APP/PS1 model group had less exploration and shorter exploration time in the face of replaced new objects compared with the WT group. Conversely, after magnoflorine treatment group displayed a strong desire to explore new environments and showed a longer time to explore new objects (Fig. 4C-4F). The results above demonstrated that magnoflorine ameliorated cognitive deficits in APP/PS1 mice.

Magnoflorine reduced AD pathology

β-amyloid plaques, neurofibrillary tangles and neuroinflammation are the three histopathological hallmarks of AD (Cai et al., 2018). The effect of magnoflorine on AD pathology were detected by immunofluorescence. As expected, mice treated with magnoflorine or donepezil exhibited lower Aβ deposition, tau phosphorylation, and glial cell activation than APP/PS1 mice (Fig. 5A-5D). To further support these findings, the levels of APP/Aβ1–42 and P-tau in brain homogenates were measured via western blotting using the same antibodies. The results revealed that APP expression and tau phosphorylation were significantly reduced after treatment with magnoflorine or donepezil compared to the APP/PS1 group (Fig. 5E–5F).

Magnoflorine treatment reduces neuronal apoptosis

To observe the effect of magnoflorine on neuronal apoptosis in the hippocampus of APP/PS1 mice, we performed immunofluorescence staining of neurons (Fig. 6A). The results showed that the number of NeuN-positive cells increased after treatment with magnoflorine or donepezil. Next, we assessed the effect of magnoflorine on oxidative stress *in vivo*. As shown in Fig. 6B-6C, the level of MDA was increased and the level of SOD was decreased in brain extracts of APP/PS1-AD mice compared with WT mice, and these changes were significantly reversed after magnoflorine treatment. These findings further support the possibility that the antioxidant effects of magnoflorine inhibit brain apoptosis in the APP/PS1 mouse model. In addition, to verify whether the JNK signaling pathway was also inhibited *in vivo*, we measured the phosphorylation of JNK in mouse brain tissue by western blot. As shown in Fig. 6D-6E, phosphorylation of JNK was elevated in the hippocampus of APP/PS1 mice compared with WT mice, while magnoflorine-treated

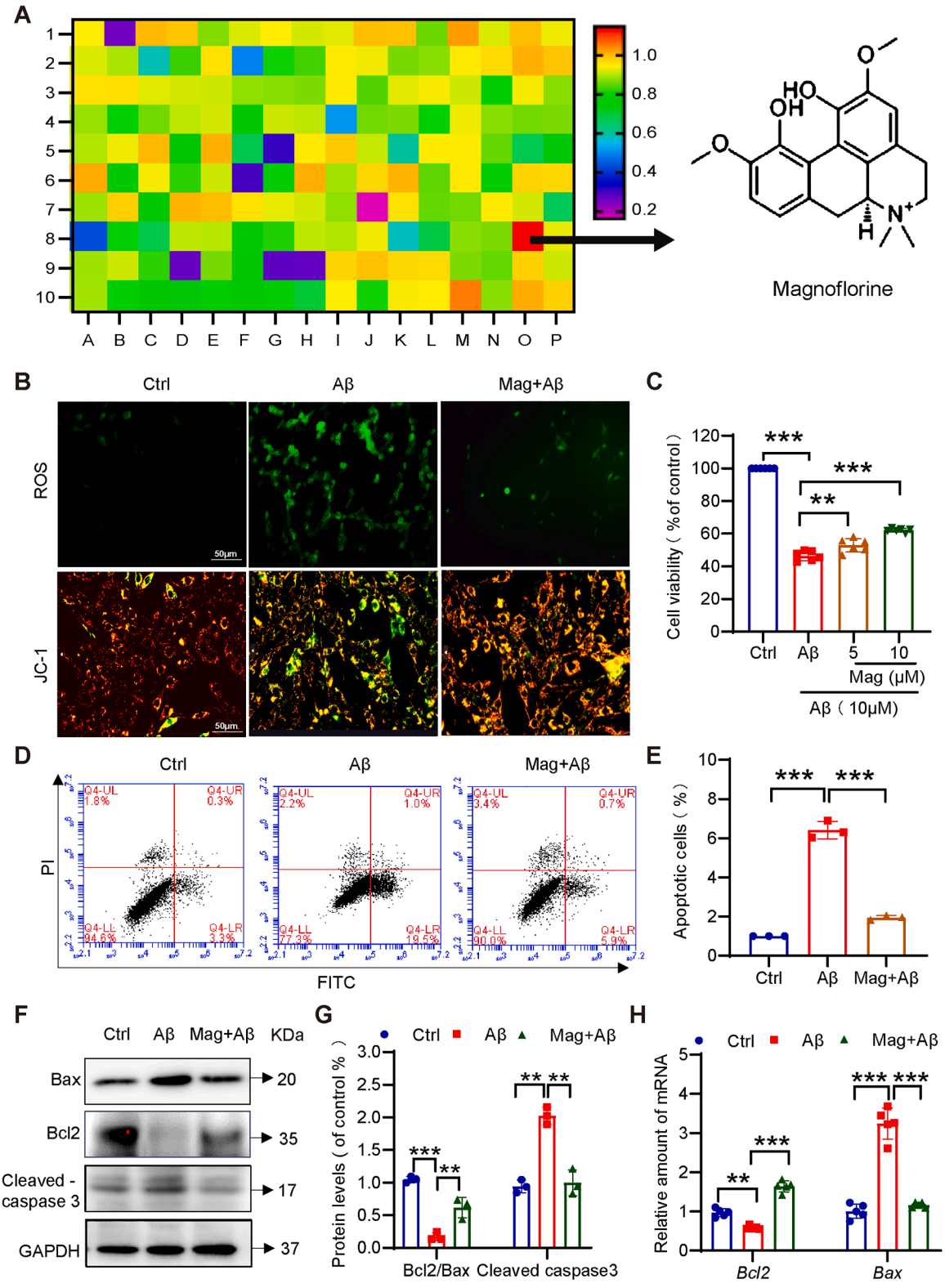

Fig. 1. Magnoflorine reduced Aβ1–42 induced cell injury in PC12 cells. (A) PC12 cells were pretreated with 160 natural compounds at 10 μM for 3 h, respectively, and then incubated with Aβ1–42 for 24 h; Cell viability was detected by MTT assay. The results showed that magnoflorine had the best protective effect. (B) PC12 cells were pretreated with 10 μM magnoflorine for 3 h before exposed to 10 μM Aβ1–42 for another 24 h Intracellular ROS levels was measured by ROS staining; △ψm was measured by JC-1 staining. (C) PC12 cells were cultured in 96-well plates and pretreated with magnoflorine of various concentrations for 3 h and then stimulated with or without 10 μM Aβ1–42 for another 24 h Cell viability was measured using the MTT assay. F (3, 20) = 505.3. (D) Cell apoptosis was measured by flow cytometry. (E) Quantitative data of apoptotic cells in D. F (2, 6) = 356.8. (F) Protein expression of Bax, Bcl2 and cleaved caspase-3 in PC12 cells were tested by western blot. (G) Quantitative analysis of the blot intensity ratio of Bcl-2/Bax and cleaved caspase-3 in F. (H) mRNA levels of Bax and Bcl2 detected by RT-qPCR in PC12 cells. *$p<0.05$, **$p<0.01$, or ***$p<0.001$ were considered significant.

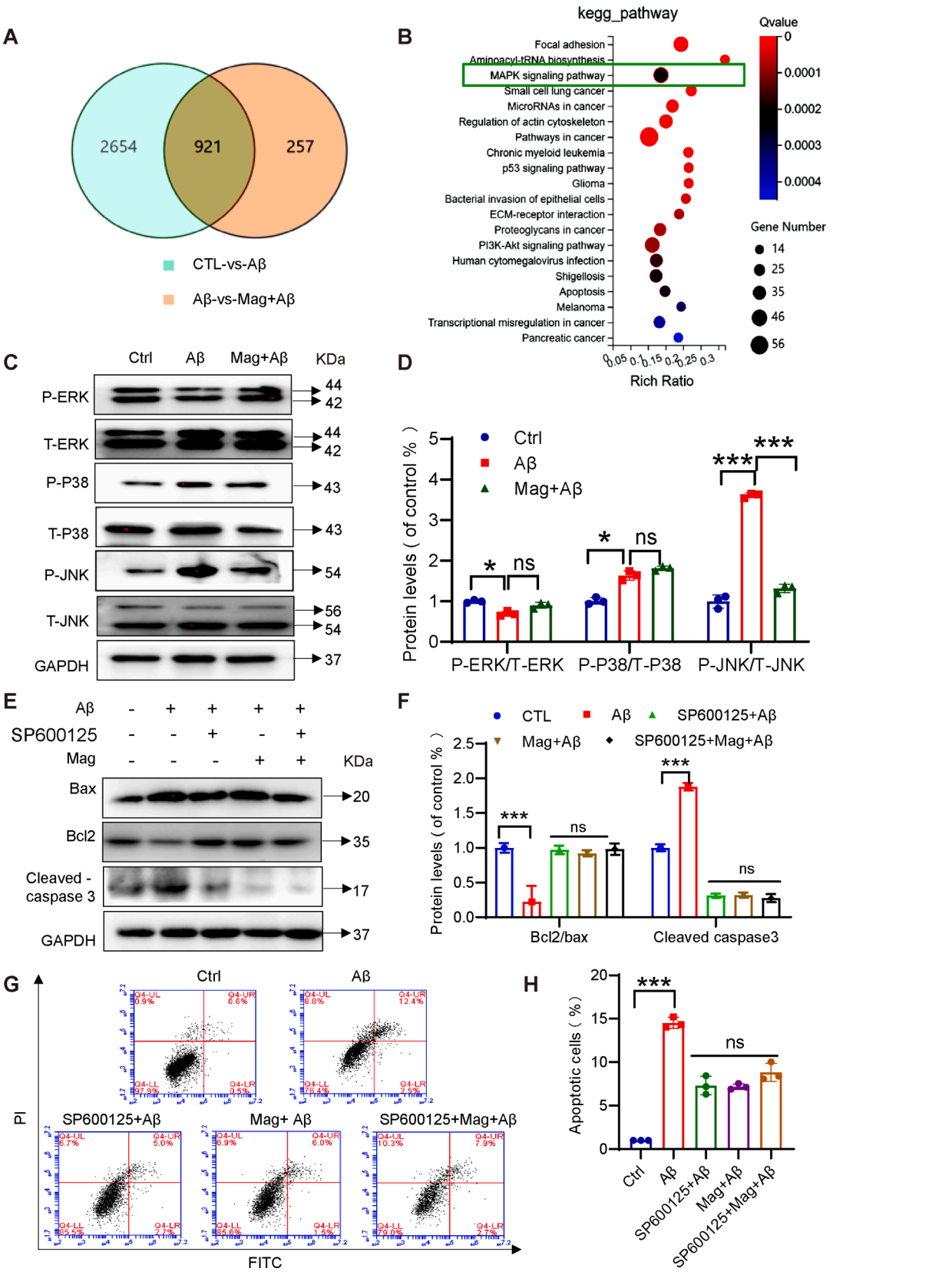

Fig. 2. Magnoflorine decreased apoptosis via inhibiting of JNK signaling pathway. PC12 cells were pretreated with or without 1.25 μM SP600125 for 30mins, and then treated with 10 μM magnoflorine for 3 h before exposing to 10 μM Aβ for 24h (A) Significant genes were analyzed via RNA-sequencing. (B) KEGG enrichment analysis in the potential signaling pathways of 921 genes and the signaling pathways. (C) Western blot results of the three main pathways of MAPKS (ERK, P38MAPK and JNK). (D) Quantitative statistics of the P-ERK, P-P38MAPK and P-JNK in C. (E) Western blot to test the expression of cleaved caspase-3, Bax and Bcl-2 in PC12 cells after block JNK signaling pathway. (F) Quantitative analysis of the blot intensity of caspase-3 and Bcl-2/bax in E. (G) Flow cytometry analysis of apoptosis in PC12 cells after block JNK signaling pathway. *$p<0.05$, **$p<0.01$, or ***$p<0.001$ were considered significant.

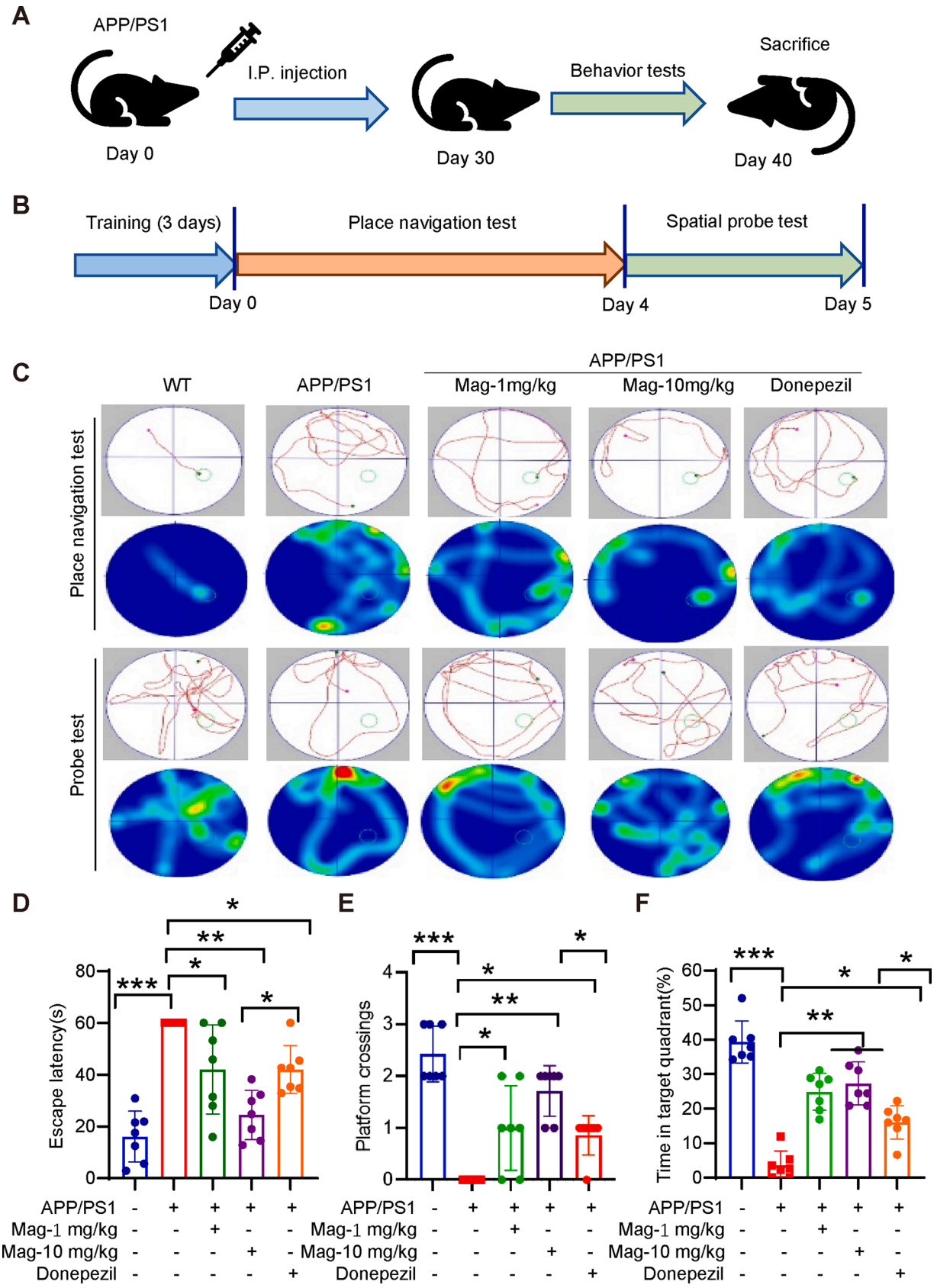

Fig. 3. Magnoflorine ameliorated learning and memory deficits in APP/PS1 mice. APP/PS1 mice were given magnoflorine or donepezil by intraperitoneal injection daily for one month, and behavioral tests were performed. (A) The operation process of animal experiments. (B) Timeline of MWM test. (C) The representative swimming trace of Place navigation test and probe test in MWM. (D) Time needed to find the hidden platform (escape latency) on day 4. F (4, 30) = 17.99. (E) The average crossing platform times of each group mice within 60 s in the fifth day. F (4, 30) = 22.13. (F) Time spent in the target quadrant where the platform had been located in the fifth day. F (4, 30) = 42.68. *p<0.05, **p<0.01, or ***p<0.001 were considered significant.

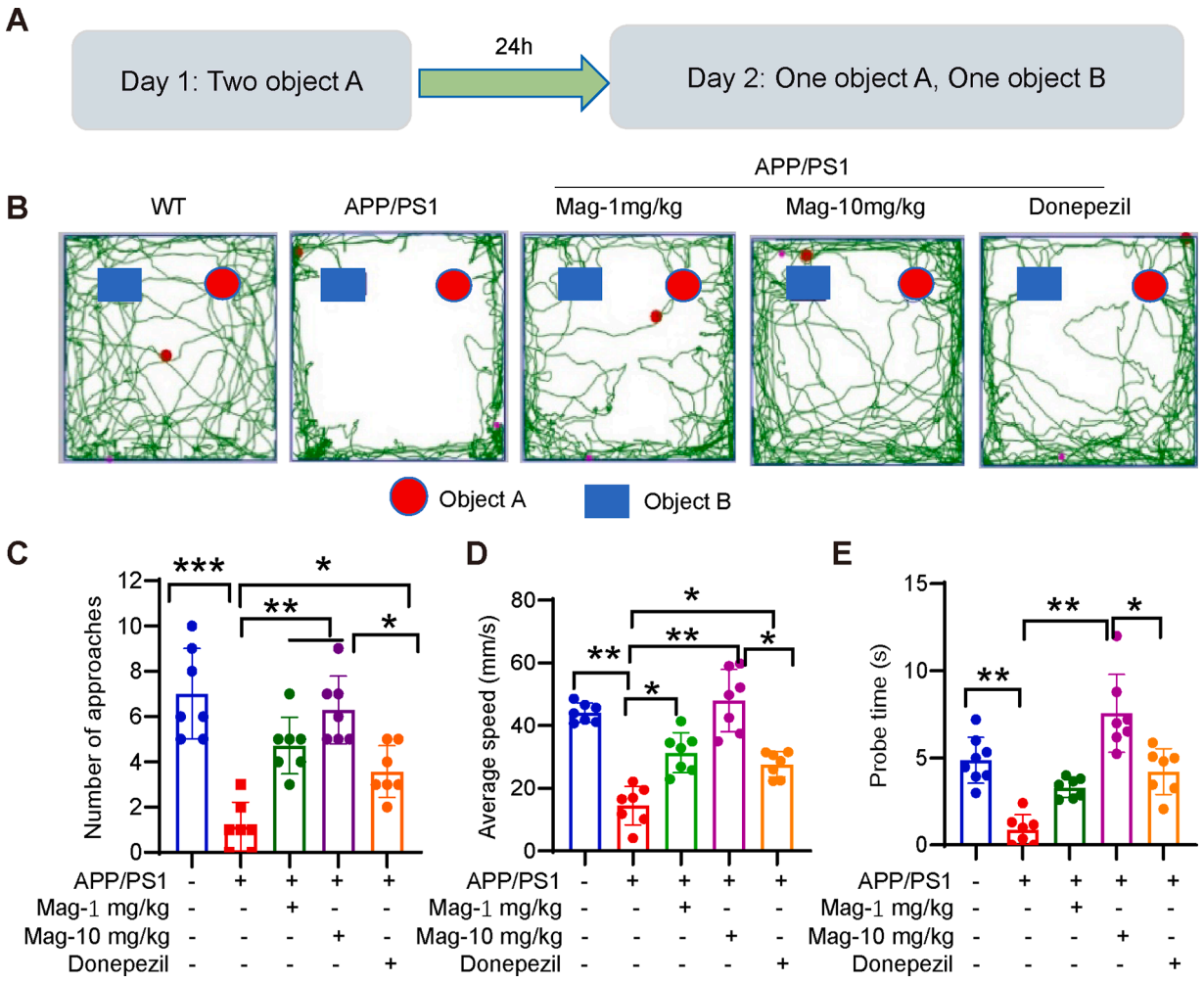

Fig. 4. Magnoflorine increased the exploration of novel objects in APP/PS1 mice. (A) Timeline of NOR test. (B) Representative trajectory of NOR experiments. (C) Number of novel objects approaches in the second day of the novel object recognition test. F (4, 30) = 18.47. (D) Average speed of NOR test on the second day. F (4, 30) = 31.14. (E) Probe time of the novel object approaches on the second day. F (4, 31) = 21.69. *p<0.05, **p<0.01, or ***p<0.001 were considered significant.

significantly reduced the expression of JNK phosphorylation. These findings are consistent with the *in vitro* results.

Discussion

In the present study, we demonstrated that the natural compound magnoflorine has a good therapeutic effect on AD model mice, and its therapeutic effect is better than that of the clinical control drug donepezil. Our research shows that magnoflorine pretreatment significantly reduced PC12 cell damage, restored mitochondrial membrane potential and reduced intracellular ROS production. The results of RNA-sequencing showed that MAPK signaling pathway involved in the protection of magnoflorine. Further studies revealed that magnoflorine inhibits JNK signaling, but not ERK and P38MAPK. At the *in vivo* level, the results showed that magnoflorine improved cognitive deficits and reduce Aβ plaques, hyperphosphorylated tau protein and activation of microglia cells in correlation with the inhibition of JNK pathway. Taken together, these results indicated that magnoflorine is expected to be an effective drug candidate for AD treatment.

Alzheimer's disease is caused by a variety of mechanisms, and the pathogenesis has not been fully elucidated, leading to unsatisfactory clinical drug treatment effects. It is urgent to find new clinical drugs to treat AD. Natural products have the advantages of novel structure, low toxicity and diverse activities, and have been an important source of new drug discovery (Balandrin et al., 1993; Zhang et al., 2020). For example, paclitaxel and artemisinin have received more and more attention because of their reliable curative effect and low toxicity (Hui et al., 2020). In addition, both preclinical and clinical studies have shown that extracts of ginkgo biloba leaves have good anti-AD effects and are well tolerated (Liu et al., 2015; Nowak et al., 2021). Studies have shown that in a variety of AD animal models, the active components of ginsenosides Rg1 and Rb1 can prevent memory loss and improve cognitive dysfunction (Fang et al., 2012; Li et al., 2015). Curcumin, the active ingredient of turmeric, can reduce cell apoptosis and improve cognitive dysfunction in model animals through a variety of pathways (Kou et al., 2021). Natural products have long-lasting effects and few side effects, which may open up a new situation for the treatment of AD.

In this study, we screened 160 natural products and found that magnoflorine has a good protective effect on Aβ-induced PC12 cell injury. Magnoflorine is an important quaternary aporphine alkaloid that has attracted extensive attention due to its various pharmacological activities. The pharmacological effects of magnoflorine include anti-inflammatory, antidiabetic, neuropsychopharmacology and antioxidant activities (Xu et al., 2020b). Toxicity studies have shown that magnoflorine is non-toxic to most cells and organs (Wu et al., 2020). These suggest that magnoflorine holds promise as a potential drug for the treatment of neurological disorders, including AD.

We showed that magnoflorine can significantly improve the pathology of AD, and its effect is better than the clinical control drug donepezil. Next, we need to explore the protection mechanism of

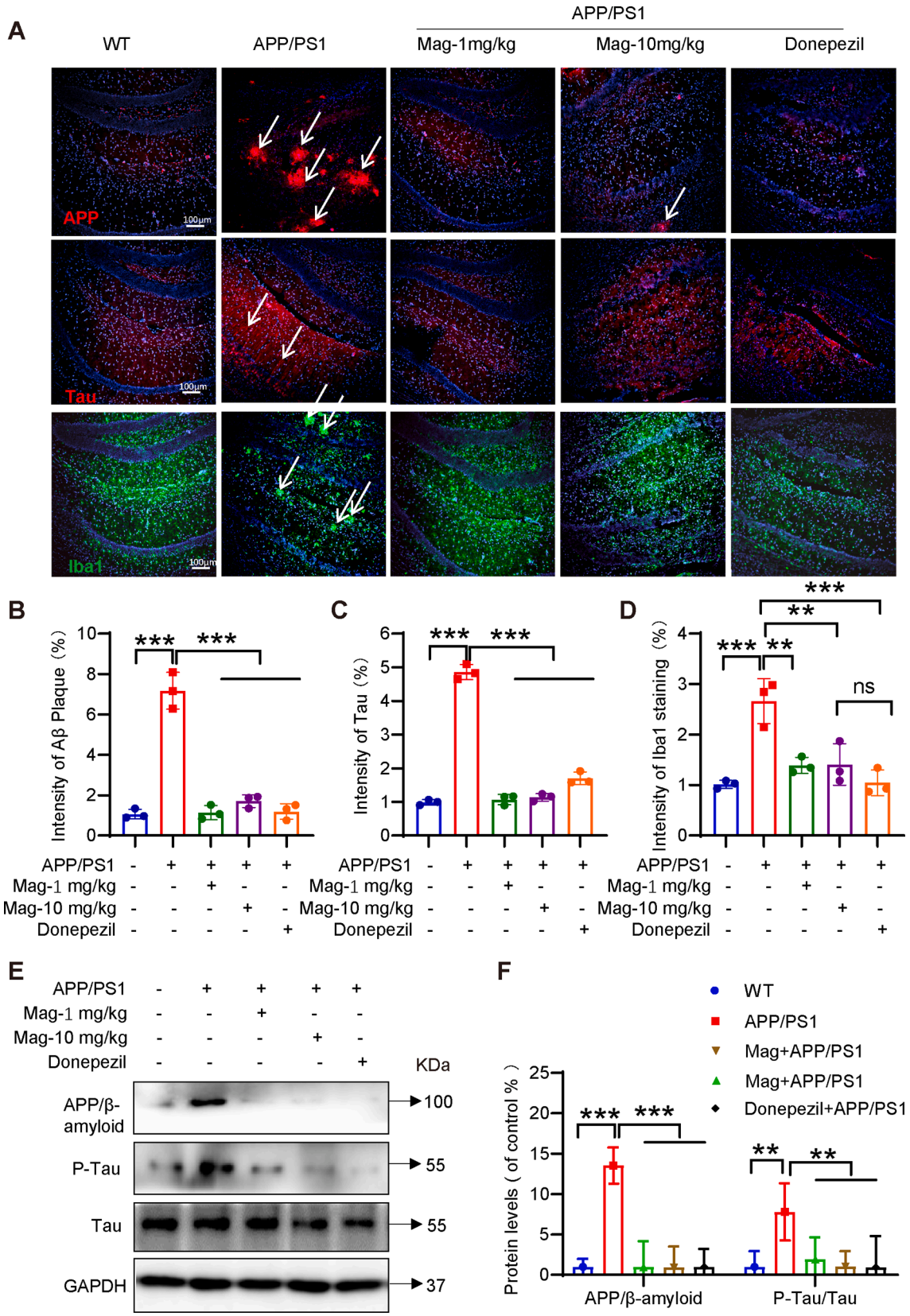

Fig. 5. Magnoflorine can alleviate AD pathology of APP/PS1 mice. (A) Representative images of APP/β-amyloid, Tau and Iba1 immunofluorescence in mouse hippocampus. (B-D) Fluorescence quantification of APP/β-amyloid [F (4, 10) = 84.37], Tau [F (4, 10) = 322.7] and Iba1 [F (4, 10) = 14.61] in A. (E) Representative western blot analysis of APP/β-amyloid, p-Tau, Tau, in the hippocampal homogenate. (F) Quantitative analysis of the blot intensity of APP/β-amyloid, p-Tau in E. *$p<0.05$, **$p<0.01$, or ***$p<0.001$ were considered significant.

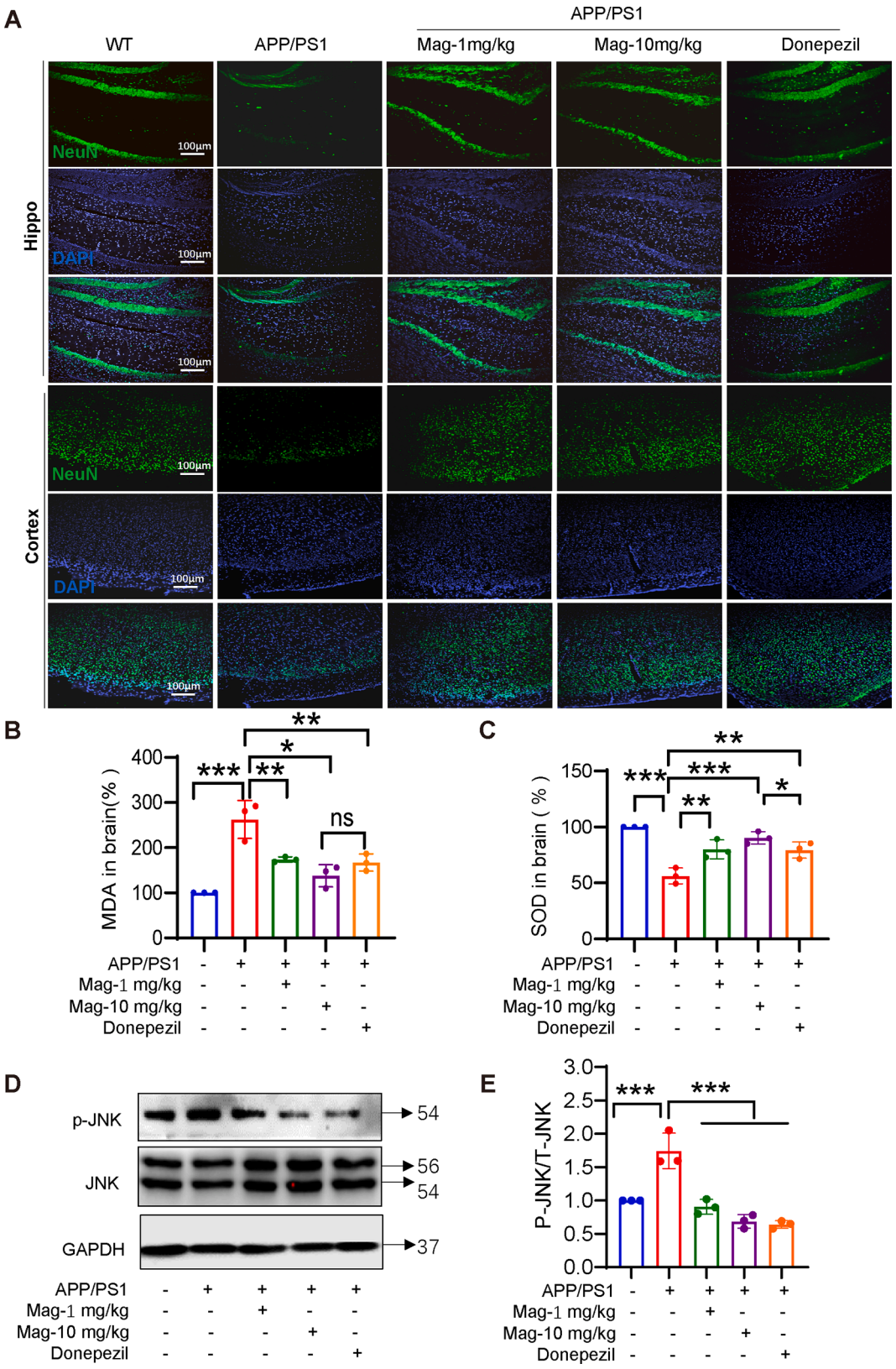

Fig. 6. Magnoflorine treatment reduces neuronal apoptosis in APP/PS1 mice. (A) Representative images of NeuN immunofluorescence in mouse hippocampus. (B-C) Levels of SOD [F (4, 10) = 19.79] and MDA [F (4, 10) = 19.67] in hippocampal lysates were tested using MDA and SOD kits, respectively. (D) The protein expression of JNK and p-JNK in hippocampus was detected by western blot. (E) Quantitative analysis of P-JNK/JNK protein expression in D. F (4, 10) = 30.84. *p<0.05, **p<0.01, or ***p<0.001 were considered significant.

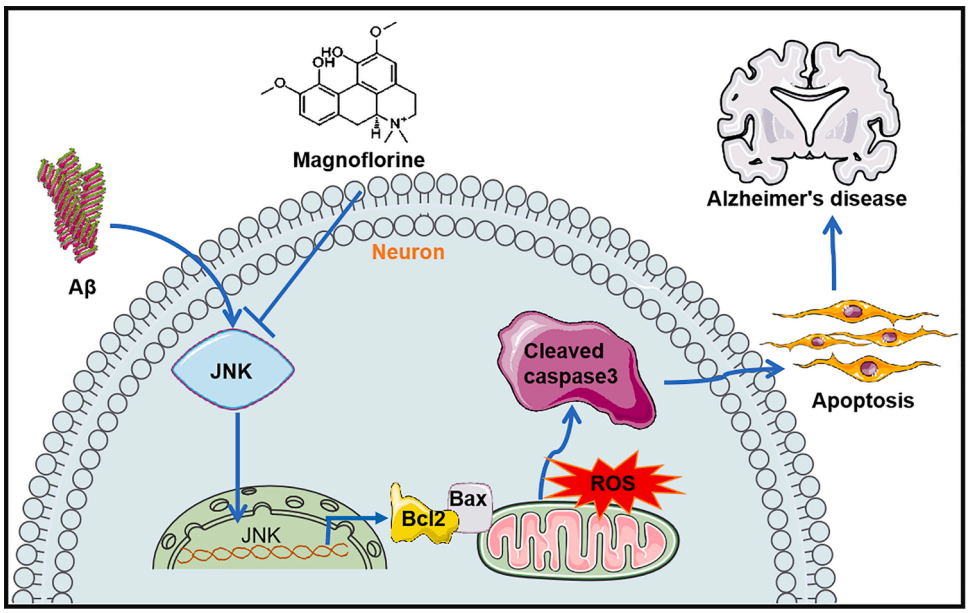

Fig. 7. The possible mechanism of Magnoflorine confer neuroprotection in AD. Aβ1–42 stimulated the JNK pathway signaling pathway, resulting in the massive expression of apoptosis genes. While magnoflorine pretreatment suppressed the activation of the JNK pathway and reduced the expression of apoptosis factors, which in turn protects neuronal cells from damage, and finally improving the cognitive deficits of APP/PS1 mice.

magnoflorine. Therefore, we performed RNA-sequencing, from the results we found the protective effect of magnoflorine may through JNK signaling. We further verified this result using the specific JNK inhibitor SP600125. JNK plays a pivotal role in stress response-related signaling pathways regulated by neuronal regeneration, synaptic plasticity, cell injury, and cellular senescence (Yao et al., 2017). It has been reported that the JNK pathway is significantly activated after exposure to different oxidative stressors including growth factors, cytokines, Aβ peptides or unfolded protein response signals (Espinet et al., 2015). Furthermore, the activation of JNK signaling pathway has been identified as a key factor in the regulation of apoptotic signaling, and thus, it is critical for the regulation of pathological neuronal cell death associated with neurodegenerative diseases, including AD (Vila and Przedborski, 2003). *In vitro* and *in vivo* studies have reported regulation of JNK pathway may be involved in AD pathogenesis and neuronal death (Yarza et al., 2016). Activation of JNK not only enhances the generation of Aβ plaques, but also plays an important role in promoting the generation and development of neurofibrillary tangles (Park and Bowers, 2010). Therefore, inhibitors of JNK may be considered as potential important targets for the treatment of neurodegenerative disease mechanisms associated with AD. In this experiment, we found that magnoflorine had a significant inhibitory effect on the JNK pathway, thus further supporting the possibility of magnoflorine as a potential therapeutic drug for AD. However, whether magnoflorine directly or indirectly inhibits JNK phosphorylation remains to be explored.

In conclusion, this study demonstrates that magnoflorine confers neuroprotection by correcting mitochondrial membrane potential and reducing ROS, thereby inhibiting apoptosis. In APP/PS1 mice, magnoflorine effectively attenuated AD pathology and the therapeutic effect is better than the clinical control drug donepezil. Moreover, we showed that the therapeutic effect of magnoflorine via inhibiting of the JNK signaling pathway (Fig. 7). Magnoflorine is a natural compound isolated from common herbs with low toxicity, high absorption and removal, and is expected to be a potential therapeutic drug for AD.

Author contributions

X.Z. and G.L. contributed to the literature search and study design. X. Z. participated in the drafting of the manuscript. G.L. contributed to manuscript modification. J.Z., W.W. contributed to methodology. X.Z., L.Z., Y. Q., M.L., J.S., H.T., Y. Z. carried out the experiments.

Author statement

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Supplementary material

Supplemental information includes 2 figures and 2 tables. All the other data are available from the authors on request.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2023.154714.

References

Alagu Lakshmi, S., Shafreen, R.M.B., Priya, A., Shunmugiah, K.P., 2021. Ethnomedicines of Indian origin for combating COVID-19 infection by hampering the viral replication: using structure-based drug discovery approach. J. Biomol. Struct. Dyn. 39, 4594–4609.

Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn. Process. 13, 93–110.

Atanasov, A.G., Waltenberger, B., Pferschy-Wenzig, E.M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E.H., Rollinger, J.M., Schuster, D., Breuss, J.M., Bochkov, V., Mihovilovic, M.D., Kopp, B., Bauer, R.,

Dirsch, V.M., Stuppner, H., 2015. Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol. Adv. 33, 1582–1614.
Balandrin, M.F., Kinghorn, A.D., Farnsworth, N.R., 1993. Plant-derived natural products in drug discovery and development: an overview.
Cai, H.Y., Yang, J.T., Wang, Z.J., Zhang, J., Yang, W., Wu, M.N., Qi, J.S., 2018. Lixisenatide reduces amyloid plaques, neurofibrillary tangles and neuroinflammation in an APP/PS1/tau mouse model of Alzheimer's disease. Biochem. Biophys. Res. Commun. 495, 1034–1040.
Cao, D., Jiang, D., Zhou, D., Yu, H., Li, J., 2020. A Comparative STUDY ON 5hmC targeting regulation of neurons in AD mice by several natural compounds. Biomed. Res. Int. 2020.
Cass, S.P., 2017. Alzheimer's disease and exercise: a literature review. Curr. Sports Med. Rep. 16, 19–22.
Chong, C.-.M., Zheng, W., 2016. Artemisinin protects human retinal pigment epithelial cells from hydrogen peroxide-induced oxidative damage through activation of ERK/CREB signaling. Redox. Biol. 9, 50–56.
Cline, E.N., Bicca, M.A., Viola, K.L., Klein, W.L., 2018. The amyloid-β oligomer hypothesis: beginning of the third decade. J. Alzheimers Dis. 64, S567–s610.
Cragg, G.M., Newman, D.J., 2013. Natural products: a continuing source of novel drug leads. Biochim. Biophys. Acta 1830, 3670–3695.
Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. Behav. Brain Res. 31, 47–59.
Espinet, C., Gonzalo, H., Fleitas, C., Menal, M.J., Egea, J., 2015. Oxidative stress and neurodegenerative diseases: a neurotrophic approach. Curr. Drug Targets 16, 20–30.
Fang, F., Chen, X., Huang, T., Lue, L.F., Luddy, J.S., Yan, S.S., 2012. Multi-faced neuroprotective effects of Ginsenoside Rg1 in an Alzheimer mouse model. Biochim. Biophys. Acta 1822, 286–292.
Hui, Y., Wang, X., Yu, Z., Fan, X., Cui, B., Zhao, T., Mao, L., Feng, H., Lin, L., Yu, Q., 2020. Scoparone as a therapeutic drug in liver diseases: pharmacology, pharmacokinetics and molecular mechanisms of action. Pharmacol. Res. 160, 105170.
Jacobsen, J.S., Reinhart, P., Pangalos, M.N., 2005. Current concepts in therapeutic strategies targeting cognitive decline and disease modification in Alzheimer's disease. NeuroRx 2, 612–626.
Kheiri, G., Dolatshahi, M., Rahmani, F., Rezaei, N., 2018. Role of p38/MAPKs in Alzheimer's disease: implications for amyloid beta toxicity targeted therapy. Rev. Neurosci. 30, 9–30.
Kou, J., Wang, M., Shi, J., Zhang, H., Pu, X., Song, S., Yang, C., Yan, Y., Doring, Y., Xie, X., 2021. Curcumin reduces cognitive deficits by inhibiting neuroinflammation through the endoplasmic reticulum stress pathway in apolipoprotein E4 transgenic mice. ACS Omega 6, 6654–6662.
Li, N., Zhou, L., Li, W., Liu, Y., Wang, J., He, P., 2015. Protective effects of ginsenosides Rg1 and Rb1 on an Alzheimer's disease mouse model: a metabolomics study. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 985, 54–61.
Liu, X., Hao, W., Qin, Y., Decker, Y., Wang, X., Burkart, M., Schötz, K., Menger, M.D., Fassbender, K., Liu, Y., 2015. Long-term treatment with Ginkgo biloba extract EGb 761 improves symptoms and pathology in a transgenic mouse model of Alzheimer's disease. Brain Behav. Immun. 46, 121–131.
Maccioni, R.B., Munoz, J.P., Barbeito, L., 2001. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch. Med. Res. 32, 367–381.
Menke, T., Gille, G., Reber, F., Janetzky, B., Andler, W., Funk, R.H., Reichmann, H., 2003. Coenzyme Q10 reduces the toxicity of rotenone in neuronal cultures by preserving the mitochondrial membrane potential. Biofactors 18, 65–72.
Nowak, A., Kojder, K., Zielonka-Brzezicka, J., Wróbel, J., Bosiacki, M., Fabiańska, M., Wróbel, M., Sołek-Pastuszka, J., Klimowicz, A., 2021. The use of Ginkgo biloba L. as a neuroprotective agent in the Alzheimer's disease. Front. Pharmacol. 3136.
Park, K.M., Bowers, W.J., 2010. Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. Cell Signal 22, 977–983.
Puzzo, D., 2019. Aβ oligomers: role at the synapse. Aging 11, 1077–1078 (Albany NY).
Sharifi-Rad, M., Lankatillake, C., Dias, D.A., Docea, A.O., Mahomoodally, M.F., Lobine, D., Chazot, P.L., Kurt, B., Tumer, T.B., Moreira, A.C., Sharopov, F., Martorell, M., Martins, N., Cho, W.C., Calina, D., Sharifi-Rad, J., 2020. Impact of natural compounds on neurodegenerative disorders: from preclinical to pharmacotherapeutics. J. Clin. Med. 9, 1061.
Sharma, P., Sharma, A., Fayaz, F., Wakode, S., Pottoo, F.H., 2020. Biological signatures of Alzheimer's disease. Curr. Top. Med. Chem. 20, 770–781.
She, L., Xiong, L., Li, L., Zhang, J., Sun, J., Wu, H., Ren, J., Wang, W., Zhao, X., Liang, G., 2023. Ginsenoside Rk3 ameliorates Aβ-induced neurotoxicity in APP/PS1 model mice via AMPK signaling pathway. Biomed. Pharmacother. 158, 114192.
Stadelmann, C., Deckwerth, T.L., Srinivasan, A., Bancher, C., Bruck, W., Jellinger, K., Lassmann, H., 1999. Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. Am. J. Pathol. 155, 1459–1466.
Vila, M., Przedborski, S., 2003. Targeting programmed cell death in neurodegenerative diseases. Nat. Rev. Neurosci. 4, 365–375.
Weggen, S., Rogers, M., Eriksen, J., 2007. NSAIDs: small molecules for prevention of Alzheimer's disease or precursors for future drug development? Trends Pharmacol. Sci. 28, 536–543.
Wu, P.F., Zhang, Z., Wang, F., Chen, J.G., 2010. Natural compounds from traditional medicinal herbs in the treatment of cerebral ischemia/reperfusion injury. Acta Pharmacol. Sin. 31, 1523–1531.
Wu, S., Yu, D., Liu, W., Zhang, J., Liu, X., Wang, J., Yu, M., Li, Z., Chen, Q., Li, X., 2020. Magnoflorine from Coptis chinese has the potential to treat DNCB-induced Atopic dermatits by inhibiting apoptosis of keratinocyte. Bioorg. Med. Chem. 28, 115093.
Xu, T., Kuang, T., Du, H., Li, Q., Feng, T., Zhang, Y., Fan, G., 2020a. Magnoflorine: a review of its pharmacology, pharmacokinetics and toxicity. Pharmacol. Res. 152, 104632.
Xu, T., Kuang, T., Du, H., Li, Q., Feng, T., Zhang, Y., Fan, G., 2020b. Magnoflorine: a review of its pharmacology, pharmacokinetics and toxicity. Pharmacol. Res. 152, 104632.
Yao, Z., Yang, W., Gao, Z., Jia, P., 2017. Nicotinamide mononucleotide inhibits JNK activation to reverse Alzheimer disease. Neurosci. Lett. 647, 133–140.
Yarza, R., Vela, S., Solas, M., Ramirez, M.J., 2016. c-Jun N-terminal kinase (JNK) signaling as a therapeutic target for Alzheimer's disease. Front. Pharmacol. 6, 321.
Yates, C.R., Bruno, E.J., Yates, M.E.D., 2022. Tinospora Cordifolia: a review of its immunomodulatory properties. J. Diet Suppl. 19, 271–285.
Yue, J., López, J.M., 2020. Understanding MAPK signaling pathways in apoptosis. Int. J. Mol. Sci. 21.
Zhang, L., Song, J., Kong, L., Yuan, T., Li, W., Zhang, W., Hou, B., Lu, Y., Du, G., 2020. The strategies and techniques of drug discovery from natural products. Pharmacol. Ther. 216, 107686.
Zhao, X., Huang, X., Yang, C., Jiang, Y., Zhou, W., Zheng, W., 2022. Artemisinin attenuates amyloid-induced brain inflammation and memory impairments by modulating TLR4/NF-κB signaling. Int. J. Mol. Sci. 23, 6354.
Zhao, X., Li, S., Gaur, U., Zheng, W., 2020. Artemisinin improved neuronal functions in Alzheimer's disease animal model 3xtg mice and neuronal cells via stimulating the ERK/CREB signaling pathway. Aging Dis. 11, 801.