Optimal Formula of *Angelica sinensis* Ameliorates Memory Deficits in β-amyloid Protein-induced Alzheimer's Disease Rat Model*

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[Abstract] Objective: *Angelica* (*A.*) *sinensis* is used as a traditional medical herb for the treatment of neurodegeneration, aging, and inflammation in Asia. *A. sinensis* optimal formula (AOF) is the best combination in *A. sinensis* that has been screened to rescue the cognitive ability in β-amyloid peptide ($A\beta_{25-35}$)-treated Alzheimer's disease (AD) rats. The objective of this study was to investigate the effect of AOF on the learning and memory of AD rats as well as to explore the underlying mechanisms. **Methods:** Male Wistar rats were infused with $A\beta_{25-35}$ for AD model induction or saline (negative control). Five groups of AD rats were fed on AOF at 20, 40, or 80 mL/kg every day, donepezil at 0.9 mg/kg every day (positive control), or an equal volume of water (AD model) intragastrically once a day for 4 weeks, while the negative control rats were fed on water. The Morris water maze test was used to evaluate the cognitive function of the rats. The Aβ accumulation, cholinergic levels, and antioxidative ability were detected by ELISA. Additionally, the candidate mechanism was determined by gene sequencing and quantitative real-time polymerase chain reaction. **Results:** The results showed that AOF administration significantly ameliorated $A\beta_{25-35}$-induced memory impairment. AOF decreased the levels of amyloid-β precursor protein and Aβ in the hippocampus, rescued the cholinergic levels, increased the activity of superoxide dismutase, and decreased the malondialdehyde level. In addition, AOF inhibited the expression of *IL1b*, *Mpo*, and *Prkcg* in the hippocampus. **Conclusion:** These experimental findings illustrate that AOF prevents the decrease in cognitive function and Aβ deposits in $A\beta_{25-35}$-treated rats via modulating neuroinflammation and oxidative stress, thus highlighting a potential therapeutic avenue to promote the co-administration of formulas that act on different nodes to maximize beneficial effects and minimize negative side effects.
Key words: optimal formula of *Angelica sinensis*; Alzheimer's disease; amyloid β aggregation; oxidative stress; neuroinflammation

Alzheimer's disease (AD), the most prevalent type of dementia, is a neurodegenerative disorder prevalent among the older age group of the population. With an increasing aging population globally, AD is becoming a global health problem[1]. AD is characterized pathologically by the aggregation of extracellular β-amyloid peptide (Aβ) in the brain tissue as well as the neurofibrillary tangles and neuronal loss formed by the aggregation of intracellular hyperphosphorylation of tau protein, which cause broader loss of cognitive function, cholinergic dysfunction, oxidative stress, and neurodegeneration[2, 3]. Though many drugs have been researched and developed according to the above targets, there is still no effective intervention to prevent or reverse AD. Meanwhile, due to the complex pathogenesis of AD, the clinical treatment is not easy to be achieved by a single compound with a single target. Researching the drugs that have multiple targets may be the optimal scheme to prevent and treat AD in the future.

Traditional Chinese herbs and formulas can treat multiple targets and are safe and suitable for long-term use in the prevention and treatment of many chronic diseases, and they have been gradually accepted by doctors and patients[4]. Multiple herbs and formulas exhibit significant effects in treating AD, such as Herbal Formula Fo Shou San[5], Bushen-Yizhi Formula[6], and Dang gui shao yao san[7]. *Angelica* (*A.*) *sinensis* (Oliv.) Diels, known as Dang Gui (in Chinese), is an important component in the Herbal Formula Fo Shou San and Dang gui shao yao san. *A. sinensis* is a traditional medicinal

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and edible plant in Asia and Europe, containing three main bioactive types of compounds: polysaccharides, volatile oils, and ferulic acid[8]. Currently, it has been found that *A. sinensis* exerts neuroprotective, antioxidant, anticancer, and immunoregulatory effects, etc.[9]. Our previous works have shown that *A. sinensis* polysaccharide improves the learning and memory capabilities in AD rats via reducing the Aβ levels and plaque deposition[10]. The optimal formula among polysaccharides, volatile oils, and ferulic acid in *A. sinensis* to treat AD was screened[11]. However, the involved molecular mechanisms need further study.

In this study, we investigated the role of *A. sinensis* optimal formula (AOF) in improving cognition in AD rats with lateral ventricle injection of $A\beta_{25\text{-}35}$ by the Morris water maze test. The Aβ accumulation, cholinergic levels, and antioxidative ability were detected by ELISA. Additionally, the candidate mechanism was determined by gene sequencing and quantitative real-time polymerase chain reaction (RT-qPCR). This study presented a feasible way to develop multiple targets to the brain for treating AD.

1 MATERIALS AND METHODS

1.1 Animals

Male Wistar rats (3 months), weighing 250–300 g, were supplied by the Lanzhou Veterinary Research Institute (China). Rats were maintained under the specific pathogen-free standard living conditions of the Animal Center of Gansu University of Chinese Medicine at room temperature (23±1°C), a humidity of (55±5)%, a 12-h light/dark cycle, and with free access to food and water. The rats were randomly divided into six groups (table 1). The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Gansu University of Chinese Medicine, with project identification code SCXK (Gan) 2015-0001.

Table 1 Experimental treatment groups

Group	Description	Treatment (i.c.v.)	Treatment (i.g.)
Ⅰ	Control	Physiological saline	Ultrapure water
Ⅱ	AD Model	1 μL $A\beta_{25\text{-}35}$	Ultrapure water
Ⅲ	Donepezil	1 μL $A\beta_{25\text{-}35}$	Donepezil 0.9 mg/kg
Ⅳ	AOF-L	1 μL $A\beta_{25\text{-}35}$	AOF 20 mL/kg
Ⅴ	AOF-M	1 μL $A\beta_{25\text{-}35}$	AOF 40 mL/kg
Ⅵ	AOF-H	1 μL $A\beta_{25\text{-}35}$	AOF 80 mL/kg

i.c.v.: intracerebroventricular injection; i.g.: intragastric gavage; AD: Alzheimer's disease; AOF-L, AOF-M, AOF-H: *A. sinensis* optimal formula-low, -moderate, -high concentrations, respectively

1.2 Drugs and Reagents

AOF consists of a mixture of three types of bioactive compounds, including polysaccharides, volatile oils, and ferulic acid (table 2). Donepezil hydrochloride (Eisai China Inc., China) was dissolved in sterile distilled water at a concentration of 9 μg/μL. $A\beta_{25\text{-}35}$ (ChinaPeptides Co., Ltd., China) was dissolved in 0.9% sterile physiological saline at a concentration of 10 μg/μL and incubated at 37°C for 4 days to induce aggregation before usage.

1.3 Modeling and Administration

Rats were implanted with an i.c.v. catheter. The procedure of i.c.v. cannula implantation was performed according to a previously described method[12]. The rats were anesthetized with 10% chloral hydrate (350 mg/kg) and placed in a stereotaxic instrument (Leica, Germany). A guide cannula (25-gauge) was implanted in the right lateral ventricle. Stereotaxic coordinates were 4 mm deep from the dural surface, 1 mm posterior to the bregma, and 1.5 mm right lateral to the midline. For the AD model rats, 1 μL of $A\beta_{25\text{-}35}$ was injected into the lateral ventricle. An equal volume of physiological saline was infused into the controls. The needle connected to a Hamilton syringe was retained for 5 min after $A\beta_{25\text{-}35}$ or physiological saline injection and then slowly retrieved. The guide cannula was covered with a medical gelatin sponge to prevent the cerebrospinal fluid and injection to drain out. Penicillin was daubed on the incision to prevent infection.

After $A\beta_{25\text{-}35}$ infusion, the rats were fed on three different concentrations of AOF (20, 40 and 80 mL/kg, serving as AOF-L, AOF-M, and AOF-H groups respectively). The positive control group (donepezil) was given donepezil at 0.9 mg/kg every day. The negative control group (control) and AD model group were given an equal volume of ultrapure water. All treatments were administered intragastrically once a day for 4 weeks. After administration, the Morris water maze was used to evaluate cognitive function.

1.4 Behavioral Test

The Morris water maze test was used to evaluate spatial learning and memory. The water maze equipment (Chengdu Taimeng Technology Co., Ltd., China) is comprised of a circular pool, a circular hidden escape platform, and a recording system. The tests were performed in a dark room at 24±1°C. The pool was spatially divided into four imaginary quadrants (first, second, third, and fourth), and the hidden escape platform was located in the center of the first quadrant. The appropriate nontoxic black ink was added into

Table 2 Components of AOF

Components	Concentration	Purity	Origin
Polysaccharides	8.8 mg/mL	98%	Ci Yuan Biotechnology Co., Ltd., China
Volatile oils	2.2 μL/mL	Steam distillation	Gansu Light Industry Research Institute, China
Ferulic acid	0.6 mg/mL	99%	Ziyi-reagent, China

AOF: *A. sinensis* optimal formula

the water to contrast sharply with the rat's white skin, helping capture the rat's trajectory easily. Escape latencies and the swimming pathways of the rats to find the hidden platform were recorded each day. If the rat failed to locate the platform within 120 s, it was guided to the platform and kept there for 10 s. On the fifth day of the probe trials, the escape platform was removed. The times of crossing the escape platform position and the times in the third quadrant were recorded by the apparatus attached to a computer, when the rat was allowed to swim freely in the pool for 120 s.

1.5 Enzyme Linked Immunosorbent Assay

After the behavioral experiment, blood was taken from the rat abdominal aorta and centrifuged at 10 000 r/min for 3 min at 4°C, and then the supernatant was aspirated and stored at –80°C. The hippocampus was collected on ice, rinsed, and stored at –80°C.

The levels of amyloid-β precursor protein (APP) and $A\beta_{1-42}$ in the hippocampal tissue were measured by enzyme linked immunosorbent assay (ELISA), using APP and $A\beta_{1-42}$ EILSA kits (1069820 and 1069821, Shanghai Enzyme-linked Biotechnology Co., Ltd., China), according to the manufacturer's instructions. The protein levels were normalized by the BCA protein assay. The levels of APP and $A\beta_{1-42}$ in the hippocampus were determined from the standard curve expressed as pg/mg of tissue protein.

The level of acetylcholine (Ach) in the blood was assessed with an Ach EILSA kit (1069818, Shanghai Enzyme-linked Biotechnology Co., Ltd., China), according to the manufacturer's instructions. The choline acetyltransferease (ChAT) activity, acetylcholinesterase (AchE) activity, and superoxide dismutase (SOD) activity in the blood were determined individually, using a ChAT assay kit (1069819, Shanghai Enzyme-linked Biotechnology Co., Ltd., China), an AchE assay kit (20161218, Nanjing Jiancheng Bioengineering Institute, China), and a SOD ELISA kit (20161221, Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturers' instructions. Finally, the level of malondialdehyde (MDA) in the blood was measured using an MDA assay kit (20161220, Nanjing Jiancheng Bioengineering Institute, China), using an automatic chemical analyzer according to the manufacturer's instructions.

1.6 RT^2 Profiler PCR Array Analysis

RT^2 Profiler PCR Array Kits (QIAGEN, China) focused on genes and pathways associated with AD were employed to investigate the RNA profiles of the hippocampus in rats treated with AOF-M. The total RNA of the hippocampus was extracted and quantified using an RNeasy Mini kit, according to the manufacturer's instructions. The first strand of cDNA was synthesized based on the instructions of the RT^2 First Strand Kit. The final cDNA product was used for the RT^2 Profiler PCR Array using SYBR Green-based real-time PCR, according to the manufacturer's protocol. Each PCR array contains 84 genes relevant to a disease state or specific pathway. The software program determined the baseline for each plate automatically. All data were normalized to an average of five housekeeping genes, such as β-actin, β-2 microglobulin, hypoxanthine-guanine phosphoribosyl transferase 1, lactate dehydrogenase A, and ribosomal protein large P1. Qiagen's online web analysis tool was utilized to produce comparative heat maps, and fold change was obtained by determining the ratio of mRNA levels to control values.

1.7 Real-time Quantitative Polymerase Chain Reaction

To detect the results of RT2 Profiler PCR Arrays Analysis, real-time quantitative polymerase chain reaction (RT-qPCR) was performed. The primers for the genes of interest and reference genes were designed with Primer 5.6 software, according to the sequence in GenBank and manufactured by iDNA Technology (table 3). The reaction conditions were as follows: incubation at 95°C for 10 min, followed by 40 cycles for 95°C for 10 s and 60°C for 30 s. The relative lncRNA and mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Reactions were repeated three times for each sample to achieve linearity.

Table 3 Primer sequences

Gene		Primer sequence
IL1b	F	CCCTGAACTCAACTGTGAAATAGCA
	R	CCCAAGTCAAGGGCTTGGAA
Mpo	F	GGTCTTGACTTACCTGCCCTTAACA
	R	AAGCCCACAGAAGCGTCTCC
Prkcg	F	CACAGACTTCGGCATGTGTAAAGA
	R	CCATAGGGCTGATAGGCAATGA

1.8 Statistical Analysis

All data are expressed as the mean±standard error and were analyzed using SPSS 18.0 statistical software (SPSS Inc., USA). Comparison of data from multiple groups against one group was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or two-way repeated measures ANOVA with Tukey's multiple comparisons test. The level of significance was set at $P<0.05$.

2 RESULTS

2.1 AOF Rescues Cognitive Deficits in AD Rats

To build an *in vivo* AD-like spatial learning and memory deficits model, we infused $A\beta_{25-35}$ into the lateral ventricle of rats and tested their cognitive ability by the MWM test (fig. 1A). To evaluate the effects of AOF on curing AD, three different concentrations of AOF (20, 40, and 80 mL/kg, serving as AOF-L, AOF-M, and AOF-H groups, respectively) and donepezil were given to the $A\beta_{25-35}$-treated rats for 4 weeks. To study the role of AOF in $A\beta_{25-35}$-induced spatial memory

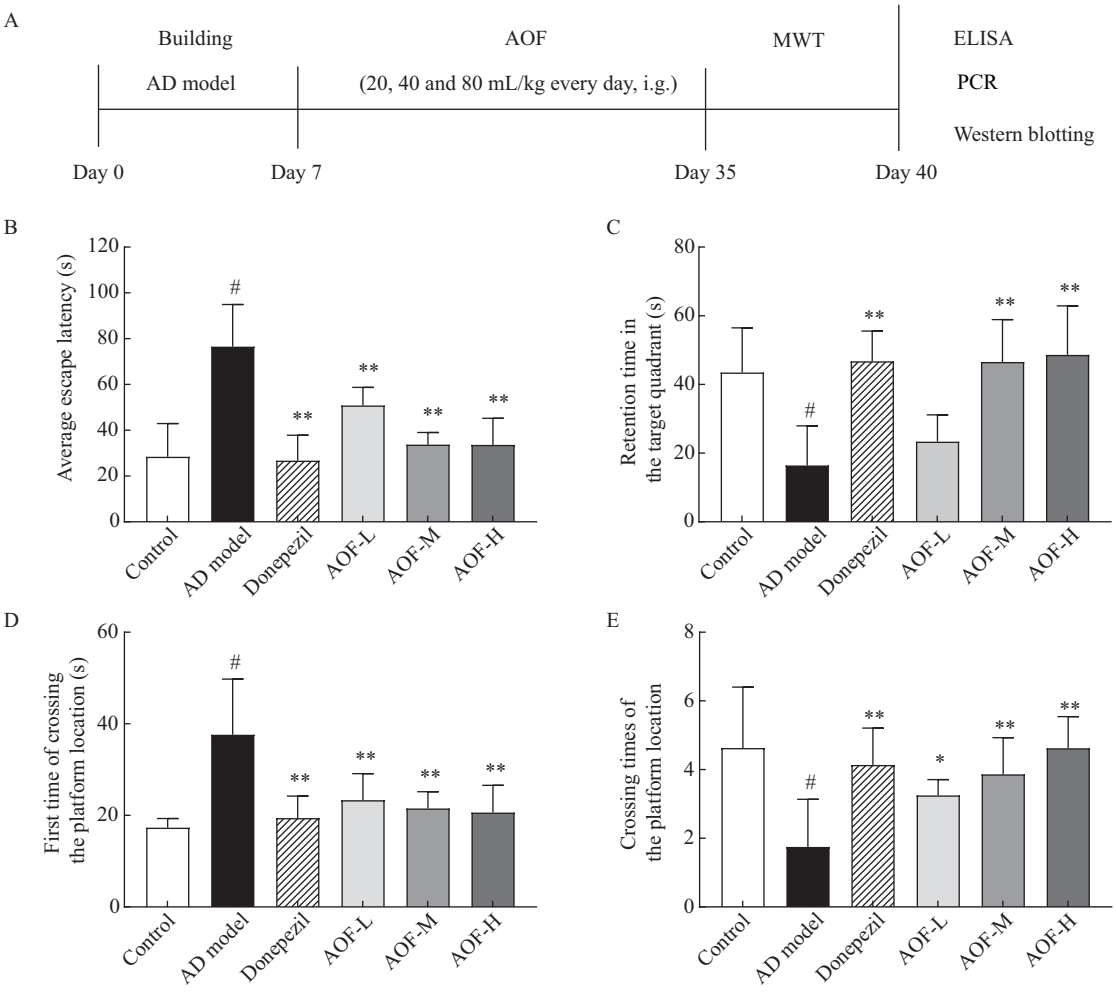

Fig. 1 Effect of AOF on $A\beta_{25-35}$-induced memory deficit in the Morris water maze test (MWT)
A: scheme of the experimental design shown as a pattern diagram; B: the average escape latency of first four days; C: the average retention time in the target quadrant of the first four days; D: first time of crossing the platform location quadrant on the fifth day; E: crossing times of the platform location on the fifth day. $^{\#}P<0.01$ *vs.* control; $^{*}P<0.05$, $^{**}P<0.01$ *vs.* AD model. n=7–8 rats/group. AD: Alzheimer's disease; AOF-L, AOF-M and AOF-H: *A. sinensis* optimal formulas-low, medium, high concentration; ELISA: enzyme-linked immunosorbent assay; i.g.: intragastrically; PCR: polymerase chain reaction

deficit, the rats were trained for 4 consecutive days to promote remembering the hidden platform in the water maze, and the hippocampus-dependent spatial learning and memory was measured by removing the platform on the 5th day (fig. 1).

The results showed that the average escape latency during the first 4 days in the AD model group was significantly longer than the escape latency in the control group, while there was a similar escape latency between the control group and the donepezil-treated group (fig. 1B). AOF and donepezil treatment significantly decreased the average escape latency ($P<0.01$). Meanwhile, the AD rats had a shorter retention time in the target quadrant than the control rats (fig. 1C). After treatment with donepezil, AOF-M, or AOF-H, the retention time in the target quadrant was remarkably increased ($P<0.01$). On the fifth day, the AD rats took the longest time to find the platform (fig. 1D), and AOF treatment at the low, middle, or high dose significantly decreased the first time of crossing the platform locations ($P<0.01$); a similar result was observed with donepezil treatment ($P<0.01$). In the probe trial, the $A\beta_{25-35}$-treated rats showed a fewer number of crossings than the control rats (fig. 1E), while AOF and donepezil treatment increased the number of crossings ($P<0.05$). Thus, these data suggest that AOF can dose-dependently rescue the cognitive ability in $A\beta_{25-35}$-induced AD rats.

2.2 AOF Decreases the Levels of APP and Aβ in the Hippocampus

The prevention of Aβ accumulation is considered an important part of AD treatment, and Aβ is produced by sequential cleavages of APP. The levels of APP and $A\beta_{1-42}$ in the hippocampus were examined among different groups (fig. 2). The levels of APP and $A\beta_{1-42}$ in the $A\beta_{25-35}$-treated group were significantly increased by 2.1-fold (fig. 2A; $P<0.01$) and 1.7-fold (fig. 2B; $P<0.01$), respectively, compared with the control group. After AOF-M, AOF-H, and donepezil treatment, the levels of APP and $A\beta_{1-42}$ were significantly reduced

in the hippocampus (P<0.01). In addition, AOF-L treatment decreased the content of APP, without affecting the level of $A\beta_{1\text{-}42}$. These findings indicate that AOF treatment inhibited Aβ accumulation in the rat hippocampus.

2.3 AOF Regulates Cholinergic Function by Activating Choline Acetyltransferase and Inhibiting Acetylcholinesterase Activity

To evaluate the potential anti-AD activity of AOF, the level of acetylcholine (ACh), the activity of ChAT, and the activity of the cholinergic lytic enzyme AchE were evaluated (fig. 3). The level of ACh (fig. 3A; P<0.01) and the activity of ChAT (fig. 3B) were significantly decreased in $A\beta_{25\text{-}35}$-treated rats, while the AChE activity (fig. 3C) was remarkably increased. AOF-H and donepezil treatment increased the level of Ach (P<0.01) and the activity of ChAT (P<0.01) as well as decreased the activity of AchE (P<0.05). AOF-M treatment significantly increased the level of Ach and decreased the AchE activity, without affecting the activity of ChAT. These data suggest that AOF dose-dependently improves AD by modulating the cholinergic system.

2.4 AOF Regulates the SOD Activity and MDA Level

Oxidative stress leads to oxidative damage of many cellular components in AD. To determine the effect of AOF on the oxidative stress status in $A\beta_{25\text{-}35}$-treated rats, we tested the activity of SOD and the level of MDA using assay kits individually. As shown in fig. 4, the activity of SOD was significantly decreased and the MDA content was remarkably increased in the blood of the $A\beta_{25\text{-}35}$-treated rats (fig. 4A and 4B). AOF (40 and 80 mL/kg) or donepezil treatment significantly increased the activity of SOD (P<0.01) and decreased the MDA level (P<0.01). Moreover, 20 mL/kg AOF administration in $A\beta_{25\text{-}35}$-treated rats only decreased the level of MDA. Thus, AOF exerted an antioxidant effect by increasing the SOD activity and decreasing the MDA level.

2.5 AOF Mediates the Transcriptional Expression of AD-related Genes

To further detect the mechanism of AOF in treating AD, the expression levels of AD-related genes in the hippocampus of the AD model were analyzed by an AD RT2 Profiler PCR Array assay. Fourteen of 84 examined genes demonstrated more than a 1.5-fold up- or downregulation in the control group rats compared with the AD rats (table 4), including 13 upregulated genes and 1 downregulated gene in the $A\beta_{25\text{-}35}$-treated rats. Amongst these genes, the expression levels of 12

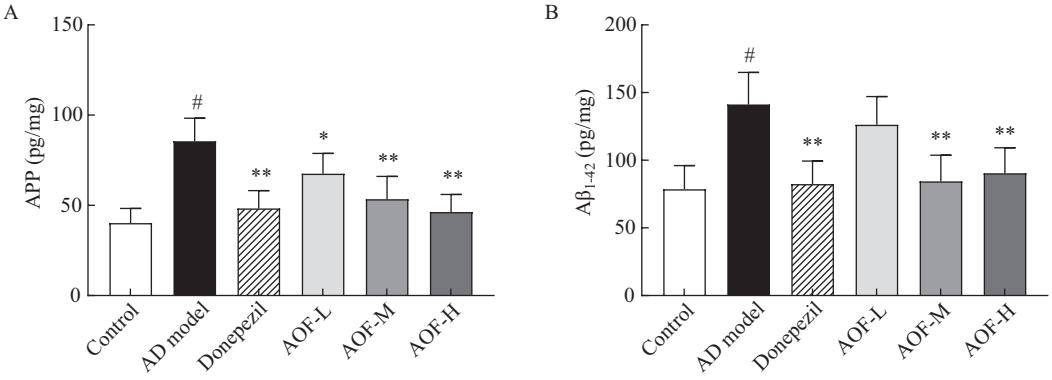

Fig. 2 Effects of AOF on the contents of APP and $A\beta_{1\text{-}42}$ in the hippocampus of $A\beta_{25\text{-}35}$-treated rats
A: the levels of APP in the hippocampus; B: the levels of $A\beta_{1\text{-}42}$ in the hippocampus. n=8. $^{\#}P$<0.01 *vs.* control; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* AD model. APP: amyloid-β precursor protein; AD: Alzheimer's disease; AOF-L, AOF-M, AOF-H: *A. sinensis* optimal formula-low, -moderate, -high concentrations, respectively

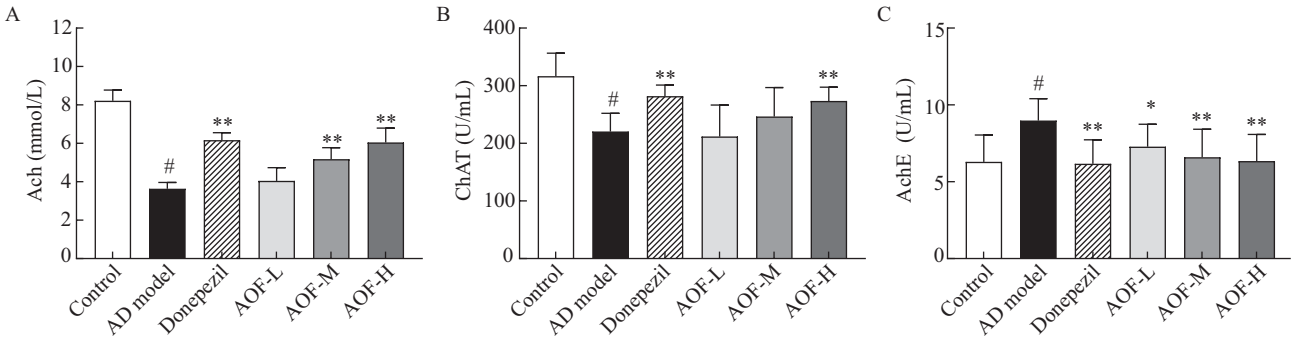

Fig. 3 Effects of AOF on the level of acetylcholine (Ach) and the activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AchE) in the blood of $A\beta_{25\text{-}35}$-treated rats
A: the levels of Ach; B: the activity of ChAT; C: the activity of AchE. n=7–8. $^{\#}P$<0.01 *vs.* control; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* AD model. AD: Alzheimer's disease; AOF-L, AOF-M, AOF-H: *A. sinensis* optimal formula-low, -moderate, -high concentrations, respectively

Table 4 RT² Profiler PCR array expression analysis in the control and AOF treatment groups with more than 1.5-fold up- or down-regulation compared with the AD group

Number	Gene symbol	Fold change	
		Control	AOF-M
1	*Apoa1*	–1.54	–6.03
2	*Casp4*	1.56	2.01
3	*Chat*	–1.59	–6.04
4	*Clu*	–1.95	–6.96
5	*Ctsg*	–2.60	–7.24
6	*Gng11*	–2.61	–2.11
7	*IL1b*	–2.43	–2.95
8	*Ins2*	–2.64	–5.77
9	*Mpo*	–2.62	–7.24
10	*Plau*	–1.69	–3.20
11	*Plg*	–1.92	–5.37
12	*Prkcg*	–1.68	–2.64
13	*Rgdc*	–2.60	–4.61
14	*Serpina3c*	–2.61	–6.69

Positive and negative fold changes stand for up- or down-regulation, respectively. AD: Alzheimer's disease; AOF-M: 40 mL/kg AOF

genes were downregulated in the AOF-M treatment group, which was consistent with the control group. The expression of caspase-4 (CASP4) was upregulated in the AOF treatment group.

The expression levels of these 14 genes were further validated individually in six groups by RT-qPCR (most data not shown). As shown in fig. 5, the expression of three genes, interleukin-1 beta (*IL1b*), myeloperoxidase (*Mpo*), and protein kinase C gamma type (*Prkcg*), was significantly increased in the $A\beta_{25\text{-}35}$-treated rats. Donepezil and AOF treatment remarkably decreased the expression of these genes. These results demonstrate that AOF mainly inhibits the expression of *IL1b*, *Mpo*, and *Prkcg* to treat AD.

3 DISCUSSION

In the present study, we found that AOF dose-dependently improved $A\beta_{25\text{-}35}$-induced cognitive impairment via various mechanisms, including preventing Aβ aggregation, rescuing the cholinergic levels, promoting the antioxidative ability, and inhibiting the expression of *IL1b*, *Mpo*, and *Prkcg* in the hippocampus.

The test results of the MWM task showed that the $A\beta_{25\text{-}35}$-treated rats displayed increased escape latency, decreased average retention times in the target quadrant during the training sessions, and increased the first time of crossing the platform location quadrant in the probe trial. However, AOF (20, 40, and 80 mL/

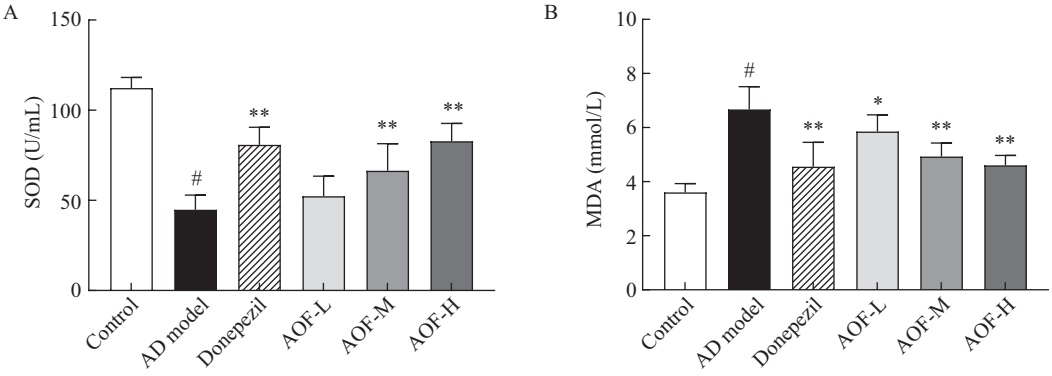

Fig. 4 Effects of AOF on the superoxide dismutase (SOD) activity and the malondialdehyde (MDA) level in $A\beta_{25\text{-}35}$-treated rats
A: the activity of SOD in the blood; B: the MDA level in the blood. n=7. $^{\#}P$<0.01 *vs.* control; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* AD model. AD: Alzheimer's disease; AOF-L, AOF-M, AOF-H: *A. sinensis* optimal formula-low, -moderate, -high concentrations, respectively

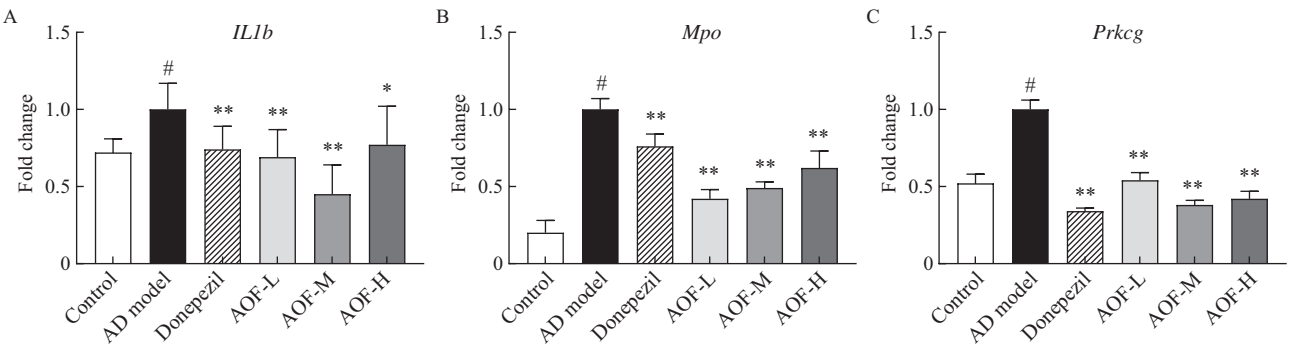

Fig. 5 Verification of the mRNA expression of three differentially altered genes identified from the AD arrays
A: the mRNA levels of *Il1b*. B: the mRNA levels of *Mpo*. C: the mRNA levels of *Prkcg*. All data are expressed in fold changes compared to the AD model group. n=3. $^{\#}P$<0.01 *vs.* control; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* AD model. AD: Alzheimer's disease; AOF-L, AOF-M, AOF-H: *A. sinensis* optimal formula-low, -moderate, -high concentrations, respectively

kg) and donepezil remarkably decreased the escape latency during the training sessions and increased the crossing times of the platform location in the probe trial. Thus, AOF treatments significantly improved the spatial learning and memory in the $A\beta_{25-35}$-treated rats. We previously found that AOF is the optimal formula among polysaccharides, volatile oils, and ferulic acid in *A. sinensis* (Dang Gui), which has the best function to treat AD in rats[11]. *A. sinensis* is one of two materials in Danghui Buxue Tang that has been proved to protect against Aβ-induced cell death by reducing the apoptosis rate in cultured cortical neurons[13]. Ferulic acid in *A. sinensis* exhibits antioxidative functions by suppressing hydroxyl and peroxyl radical generators, lipid peroxidation, and ROS formation, and it has a role in protecting against cognitive impairment-induced learning and memory deficits[14]. In addition, Z-ligustilide, the primary component of volatile oils, has been reported to diminish mortality, neurobehavioral deficits, and cerebral vasospasm in an AD rat model[15]. Therefore, these data suggest that AOF could be a drug development candidate for AD therapy.

Aβ is one of the primary toxic elements in AD that is generated by sequential proteolysis of APP[16]. Astrocytes and microglia are activated by the accumulation of Aβ plaques, and the inflammation and persistent activation in the central nervous system may lead to the progression of AD[17]. Preventing the accumulation of Aβ is an effective treatment for improving AD-related cognitive decline[18]. In the present study, we found that AOF dose-dependently decreased the level of APP and prevented Aβ aggregation in the hippocampus. These findings indicate that the effect of AOF in improving learning and memory in AD rats might be partly through preventing Aβ aggregation.

The cholinergic hypothesis suggests that AD is caused by a deficiency in the level of the cerebral neurotransmitter ACh, which is hydrolyzed by AChE[19]. ChAT is an enzyme that synthesizes ACh in cholinergic neurons[20]. In the $A\beta_{25-35}$-treated rats, the level of ACh and the activity of ChAT were significantly decreased, while the AChE level was increased. AOF treatment increased the ACh level and the ChAT activity as well as reversed the increase of the AChE level by $A\beta_{25-35}$ injection. Thus, AOF might rescue the cognitive ability in the AD model via modifying the cholinergic system.

Oxidative stress is typically characterized by an imbalance in antioxidant systems, like the increased levels of ROS and MDA as well as a decrease in SOD activity[21]. The elevation of oxidative stress is considered as a typical contributing risk factor in the progression of AD, and Aβ induces high levels of ROS[22]. Our study showed that AD rats induced by $A\beta_{25-35}$ presented a decreased SOD activity and increased MDA levels as compared with the control rats. AOF administration in AD rats dose-dependently ameliorated these differences. Therefore, it is expected that AOF can effectively prevent the decrease in cognitive function and Aβ deposits via modulating oxidative stress.

In order to further dissect the underlying molecular mechanism of the memory-improving effects of AOF in the $A\beta_{25-35}$-treated rats, we analyzed the expression of AD-related genes in the hippocampus of the AD model by AD RT2 profiler PCR array assay. Compared with the AD rats, 13 genes belonging to different functional classifications, including lipid and lipoprotein metabolism (*Apoa1* and *Glu*), hormone and hormone processing gene (*Ins2*), clearance and degradation (*Plg*), synaptic formation (*Chat*), apoptosis (*IL1b* and *Mpo*), protein kinases (*Prkcg* and *Prkcd*), cell signaling molecules (*Gng11* and *Plau*), other AD-related genes (*Ctsg* and *Serpina3c*), were significantly downregulated in the control rats and the AOF-M-treated rats. In addition, the expression of Casp4, which promotes apoptosis, was upregulated in the AOF-M-treated rats and control rats compared with the AD rats. These results confirmed that AOF could affect multiple molecular signaling pathways in the treatment of AD rats induced by $A\beta_{25-35}$ injection.

The expression levels of these genes were further validated individually in the control group, AD model group, donepezil group, and three different AOF concentration groups by RT-qPCR. We found that compared with the AD model group, AOF (20, 40, and 80 mL/kg) treatment significantly decreased the expression of three genes: *IL1b*, *Mpo*, and *Prkcg*. IL1b and Mpo are inflammatory genes that are strongly upregulated in AD[23]. Additionally, increased serum levels of IL1b are used as a stage marker of the ongoing brain neurodegeneration between normal aging and AD[24, 25]. Aβ fibrils also promote the release of the inflammatory cytokine IL1b, which can damage neurons and promote AD progression[26]. Mpo is an oxidant-generating enzyme that is not present in the normal aged brain, but it is abundant in the AD brain[27, 28]. Mpo-generated oxidants lead to lipid peroxidation, which is likely to contribute to neurological dysfunction in AD[29]. In addition, overexpression of Mpo in the APP mouse model leads to greater memory deficits[30]. Moreover, *Prkcg* has been identified as the disease-causing gene for multiple neurodegenerative disorders, including AD[31]; however, the mechanism regarding how *Prkcg* affects AD is not clear and needs to be verified in further studies. Thus, based on the findings of the current study, AOF improved spatial learning and memory in $A\beta_{25-35}$-treated rats via decreasing the expression of inflammatory genes to prevent Aβ aggregation.

This study evaluated the efficacy of AOF treatment on alleviating cognitive impairment in $A\beta_{25-35}$-treated rats

and detected multiple mechanisms. Taken together, this investigation showed that AOF may prevent the decrease in cognitive function and Aβ deposits in $A\beta_{25-35}$-treated rats via modulating neuroinflammation and oxidative stress. These findings demonstrate that AOF has a potential therapeutic benefit in AD patients.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization, corporation, or individual that can inappropriately influence this work.

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