

Original Research Article

Ameliorative effects of olibanum essential oil on learning and memory in A β 1-42-induced Alzheimer's disease mouse model

Zhenzhen Zhang^{1*}, Wenhua Chen², Jie Luan¹, Dagui Chen³, Lina Liu¹, Xu Feng¹

¹Naval Medical Research Institute, Second Military Medical University, 880 Xiangyin Road, Shanghai 200433, ²School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, ³Institute of Translational Medicine, Shanghai University, Shanghai 200444, China

*For correspondence: **Email:** zz_jane@126.com; **Tel:** (+86) 021-81883318

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Abstract

Purpose: To study the effect of olibanum essential oil (OEO) on learning and memory in Alzheimer's disease (AD) mouse.

Methods: Mice were administered the 42-amino acid form of amyloid β -peptide (A β 1-42) to induce AD and then treated with OEO at 150, 300, and 600 mg/kg, p.o. for two weeks. Following treatment, the AD mice were assessed by step-down test (SDT), dark avoidance test (DAT), and Morris water maze test (MWM). Blood and brain tissues were collected for biochemical assessments. Gas chromatography-mass spectroscopy was used to analyze the main constituents of OEO.

Results: The main constituents of OEO were limonene, α -pinene, and 4-terpineol. Treatment with OEO prolonged t latency in SDT and DAT, but decreased error times. Escape latency decreased and crossing times were rose in the MWM following OEO treatment ($p < 0.5$). Treatment with OEO also enhanced the acetylcholine levels and decreased the acetylcholinesterase levels in serum and brain tissue ($p < 0.5$). Additionally, OEO reduced amyloid plaques in the hippocampus and protected hippocampal neurons from damage. Furthermore, OEO decreased c-fos expression in hippocampus tissues from AD mice ($p < 0.5$).

Conclusion: OEO has significant ameliorative effect AD-induced deterioration in learning and memory in AD mouse induced by A β 1-42. The mechanisms of these effects are related to increased acetylcholine contents, reduction of amyloid plaques, protection of hippocampal neurons, and down-regulation of c-fos in brain tissues. The results justify the need for further investigation of candidate drugs derived from OEO for the management of AD.

Keywords: Olibanum, Essential oil, Learning, Memory, AD

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INTRODUCTION

Alzheimer's disease (AD), a intractable senile diseases with multifactorial etiology, is currently

considered the predominant factor of dementia [1,2]. Epidemiology investigations have shown that AD currently affects nearly 50,000,000 people in the world, and the number of AD

patients is estimated to triple during the next 30 years because of an increase in the older adult population [3,4]. More importantly, although the diagnostic technologies for AD have improved considerably, there is still no decisive treatment or effective drug for AD patients. The only available course of action is to delay the development of the pathologic process during its early stages [5,6].

Consequently, finding more candidate agents for curing AD is both important and urgent. Increasing numbers of research studies are focusing on the potential effects of natural monomers from plants or herbs to treat various intractable diseases; natural agents are precious resources for identifying novel monomers/extracts with promising pharmacological activities [7,8]. For examples, the known antitumor drug Taxol is a natural extract isolated from *Taxus chinensis*, and the promising antimalarial drug artemisinin is a sesquiterpene lactone extracted from the *Artemisia annua*.

Olibanum, the resin of the *Boswellia serrata* (Burseraceae), mainly distributed in India and Eastern Africa, and is a known folk medicine used for treating pain, inflammation, and swelling [9]. Furthermore, previous work indicates that agents or extracts of olibanum can effectively improve memory deficit in rats tested in a Morris water maze, which reflects the level of memory [9-11]. In addition, previous evidence revealed that the constituents of olibanum are mainly triterpenes, essential oils, and resin. The gum resin of olibanum combined with beta-boswellic acid (BBA) has long been used in Ayurveda (India's traditional medicine) for preventing amnesia by enhancing memory. In addition, studies have indicated that this natural essential oil is beneficial for improvement of learning and memory potentials of experimental AD rats [9-11]. So far, no previous investigation has focused on the effects of olibanum essential oil (OEO) on learning and memory abilities. The aims of this work were to research the beneficial effects of OEO on learning and memory in a β -amyloid peptide-induced AD mouse model and to determine if there is significant value for further investigation of candidate drugs derived from OEO to treat AD in clinical.

EXPERIMENTAL

Plant material

Olibanum was purchased from the *Tongrentang* drug store (Shanghai, China) and authenticated by Dr. Ting Han (Department of Pharmacognosy,

2nd Military Medical University (SMMU), Shanghai, China). A voucher specimen (no. S20180904) was kept in the specimen laboratory of the Naval Medical Research Institute, SMMU.

Animals and ethics statement

Experimental groups consisted of male ICR mice (25 – 30 g) were acquired from the CAVENS Animal Centre (Changzhou, China). Mice were housed under normal conditions (22 ± 2 °C and 40 – 60 % humidity) and had free access to water and diet. All animal protocols were strictly obeying the NIH Guide concerning the Care and Use of Laboratory Animals [12], and approved by the Ethics Committee of SMMU (approval no. 20190217an-1#).

Extraction of essential oil

The OEO was extracted referenced the recorded method [13]. The olibanum powder (100g) was boiled in 15 times its volume of water for 8 h. The distillate was extracted by n-hexane and subsequently dried using anhydrous sodium sulfate. The yield of OEO was 4.52% (v/w). The essential oil was stored under -10 °C until used in experimental assays.

Gas chromatography-mass spectroscopy (GC-MS) analysis

The OEO was dissolved in n-hexane to determine the constituents using an Agilent 7890 gas chromatograph combined with the Agilent 5975 mass spectrometer. The essential oil samples (1.0 μ L) were determined using a capillary column (DB-5MS, 30 m \times 0.25 mm \times 0.25 μ m). The temperature program of the column used was bellow: for starting, 60 °C for 2 min, and subsequently increased to 100 °C (2 °C/min), and then to 260 °C (10 °C/min); 260 °C was kept for 3 min. The flow rate of carrier gas (helium) was 1.0 mL/min.

The split ratio used was set at 10:1, and the electron impact ionization was 70 eV and the mass scanning range was 50 - 450 amu. Compounds were analyzed based on comparing the retention indices (RI) to n-alkanes (C₈–C₄₀), and further identified by searching the NIST database (USA).

Protocols and grouping of animals

A total of 60 ICR mice were divided into 5 groups randomly: normal, control, and three OEO testing groups (low dose, middle dose, and high dose). Each group consisted of 12 animals. The AD mouse model was induced by injection of A β ₁₋₄₂

(Sigma-Aldrich, Shanghai, China) intracerebroventricularly (i.c.v.) according to a previously published method [13,14]. Briefly, the mice were anesthetized by sodium pentobarbital (Sigma-Aldrich) (50 mg/kg, i.p.). Subsequently, mice were fixed with a stereotaxic device (Yuyan Instruments, Shanghai, China). The $A\beta_{1-42}$ was dissolved in sterile normal saline to achieve a concentration of 1. Next, 5 μ L of the $A\beta_{1-42}$ solution (400 pmol/ μ L) was injected into the bilateral ventricles using a Hamilton microsyringe. Normal mice received an equal volume of sterile saline using the same surgical procedure. Behavioral tests [step-down test (SDT), dark avoidance test (DAT), and Morris water maze test (MWM)] were carried out 2 weeks after $A\beta_{1-42}$ injection. During the 2 weeks, the AD mice in the OEO treatment groups were administered OEO at 150, 300, or 600 mg/kg. All drugs were administered orally, and doses were expressed as mg/kg. After the last treatment and the MWM test, under anesthetization (sodium pentobarbital, 40 mg/kg, i.m.), blood samples were collected from the aorta abdominalis of mice anesthetized by sodium pentobarbital (50 mg/kg, i.p.). Following blood collection, mice were sacrificed by cervical dislocation, and the hippocampi were precisely separated from the brains for further biochemical assays.

Step-down test (SDT)

The SDT was performed following the reported method [15]. ICR mice were kept in a dark room for 40 min before the test. Then, the mice underwent the following adaptive training: mice were placed on a platform (5.0 \times 5.0 \times 4.5 cm), if the animal stepped down from the platform, the animal suffered an electric foot shock (36 V, AC). Then, the mice were placed back in the animal cage. After 24 h, the formal experiment was carried out, and the latency of step down (latency) with the maximum latency time of 300 s and the step-down times (error times) were recorded.

Dark avoidance test (DAT)

The DAT was conducted as previously described with minor modifications [16]. The apparatus was equipped with two identical light and dark square boxes (15 \times 15 \times 15 cm). Similar to the SDT, the DAT consisted of a training trial and a formal test trial. For the training trial, animals were placed in the light box and, if the animal entered the dark box, the animal suffered a 0.5-mA electric foot shock for 3 s. After 24 h, the formal experiment was carried out, and the darkness avoidance latency with a maximum latency time of 300 s and error times were recorded.

Morris water maze test (MWM)

The MWM test was performed using a previously reported method, with minor modifications [17]. The MWM apparatus consisted of a roundness pool (50 \times 120 cm) full of opaque water (23 \pm 1 $^{\circ}$ C) with a depth of 18 cm. The pool consisted of four equal quadrants, with a 5-cm diameter round a platform located 1.0 cm below the water surface. The MWM test consisted of four consecutive days of training sessions followed by a spatial probe test. During the training sessions to find the rounded platform, mice were trained in different quadrants four times each day for a maximum of 120 s. For the spatial probe test, the platform was removed on the fifth day, and the escape latency to the original platform site and crossing times of the original platform site were recorded within a maximum of 180 s.

Enzyme-linked immunosorbent assay (ELISA)

After the MWM test and the last treatment, mice were anesthetized (sodium pentobarbital, 50 mg/kg, i.m.) and blood samples were collected from the aorta abdominalis. Serum was prepared by centrifugation (2000 g, 4 $^{\circ}$ C, 10 min). Subsequently, brain was excised under ice and divided into two parts. One part was used to further separate the hippocampus tissue that was stored in 10% neutral formaldehyde solution (Solarbio Life Sci, Beijing, China) for further histopathological examination. The remaining brain part was homogenized, and the supernatants were collected for ELISA. The levels of acetylcholine (ACh) and acetylcholinesterase (AChE) in brain tissues and serum were determined using commercial ELISA assay kits using the standard processing (Multi Sci. Co., Hangzhou, China).

Histopathological examination

The hippocampus was fixed in neutral formaldehyde (10%), and embedded in paraffin. Subsequently, paraffin blocks were sectioned into 5- μ m slices, de-paraffinized, and stained with Congo Red (Solarbio Life Sci, Beijing, China) and hematoxylin-eosin (H&E) (Solarbio Life Sci, Beijing, China) or H&E alone. Finally, the hippocampus tissues sections were observed with an optical microscope (Olympus, Tokyo, Japan).

Immunohistochemical assay

The hippocampus sections (5 μ m) were routinely de-paraffinized, incubated with 3% H_2O_2 for 10 min, and boiled in 0.01 M citric acid buffer (5 min). After blocking by goat serum for 30 min,

sections were probed with antibodies of c-fos (Abcam Biotechnology, Cambridge, MA, USA; 1:2000) overnight (4 °C). Next, a secondary antibody (Abcam Biotechnology) was used and the peroxidase label was visualized by incubating with 3,3'-diaminobenzidine tetrachloride (Beyotime Biotechnology, Haimen, China). The immunohistochemical changes in c-fos in hippocampus were observed by optical microscope.

Statistical analysis

Data are presented as mean \pm standard deviation (SD), and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with the aid of SPSS. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Chemical composition of essential oil from Olibanum

The total ion chromatogram of OEO is shown in Figure 1. Ten constituents account for 87.44 % of the total constituents. The essential oils of olibanum were identified using GC-MS analysis. The identified constituents and their composition percentages are presented in Table 1. The main components of OEO are α -pinene (59.4 %), limonene (10.5 %), β -thujene (3.73 %), sabenene (2.92 %), *p*-cymene (2.76 %), and myrcene (2.25 %).

Effect of OEO on behavior of AD mice in the step-down test

Results of the SDT are shown in Figure 2. Compared to normal mice, the step-down latency of AD model mice (Control) is shorter ($p < 0.01$), whereas the number of errors are increased ($p <$

0.01). However, compared to control mice, treatment with the OEO at the low, middle, and high doses significantly prolonged the step-down latency of AD mice ($p < 0.05$, $p < 0.01$), and decreased the number of errors ($p < 0.05$, $p < 0.01$, $p < 0.01$).

Effect of OEO on behavior of AD mice in the dark avoidance test

The results of the DAT are summarized in Figure 3. Overall, the results of the DAT are similar to those of the SDT. In the DAT, the latency of the AD mice (control mice) was significantly decreased compared to the normal mice ($p < 0.01$), whereas the number of errors were increased ($p < 0.01$). Interestingly, administration of OEO (low, middle, and high doses) prolonged the dark avoidance latency ($p < 0.05$, $p < 0.01$, $p < 0.01$) and decreased the number of errors ($p < 0.05$, $p < 0.01$, $p < 0.01$) compared to the AD mice.

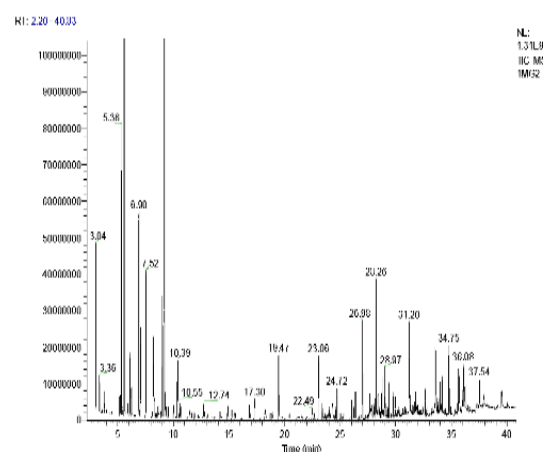


Figure 1: Total ion chromatogram of essential oil from olibanum

Table 1: Chemical composition of *Olibanum* essential oil

No.	Compound	t_R	Content (%)	RI
1	β -thujene	5.36	3.73	928
2	α -pinene	5.64	59.4	938
3	Camphene	6.11	1.65	951
4	Sabenene	6.90	2.92	975
5	β -Pinene	7.07	1.72	978
6	Myrcene	7.52	2.25	988
7	α -Phellandrene	8.2	1.54	1166
8	3-Carene	8.27	0.97	1014
9	<i>p</i> -Cymene	9.00	2.76	1027
10	Limonene	9.21	10.5	1031
Total identified (%)			87.44	

RI: retention indices relative to n-alkanes C₈-C₄₀ on HP-5MS column; Cont.: contents; t_R : retention time

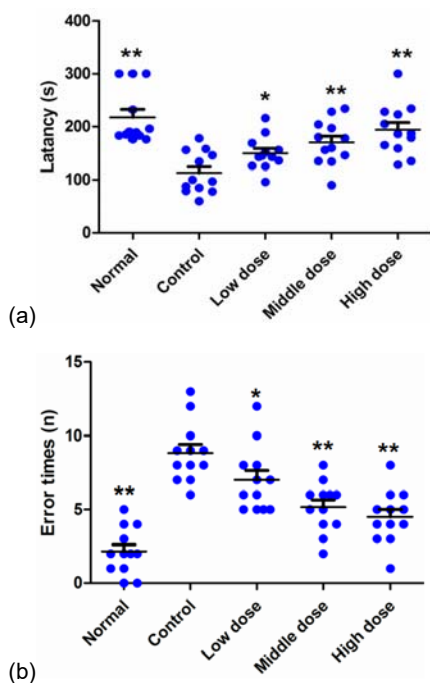


Figure 2: Effect of OEO on learning and memory impairment in AD mice during the step-down test: (a) Latency and (b) Error times. Data are expressed as mean \pm SD (n=12), * p <0.05, ** p <0.01, vs. control

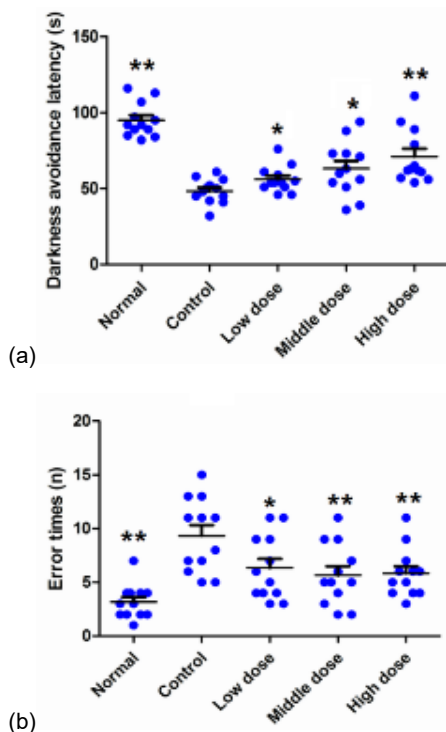


Figure 3: Effect of OEO on learning and memory impairment of AD mice in the dark avoidance test. (a) Darkness avoidance latency, (b) Error times. Data are expressed as mean \pm SD (n = 12), * p < 0.05, ** p < 0.01, vs. control

Effect of OEO on behavior of AD mice in morris water maze test

The MWM test was also used to determine the effects of OEO on learning and memory impairment in AD mice. After injection of $A\beta_{1-42}$, the escape latency of AD mice (control mice) was significantly prolonged ($p < 0.01$) compared to normal mice, whereas the number of crossings were decreased ($p < 0.01$). Interestingly, OEO treatment at low, middle, and high doses significantly reduced the escape latency ($p < 0.05$, $p < 0.01$, $p < 0.01$) and increased the number of crossings ($p < 0.05$, $p < 0.05$, $p < 0.05$) compared to AD mice (Figure 4).

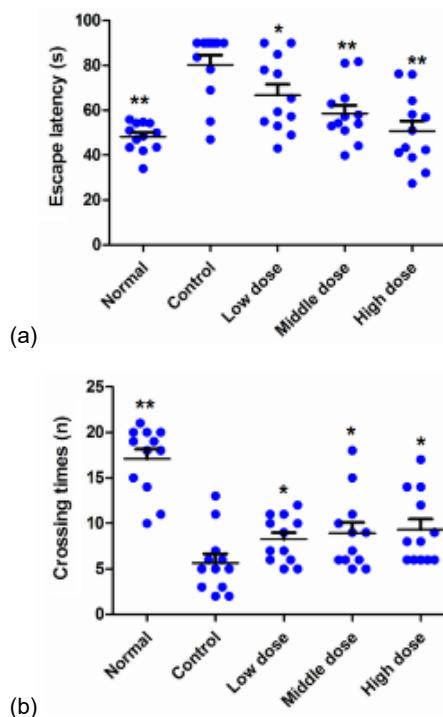


Figure 4: Effects of OEO on learning and memory impairment of AD mice in the Morris water-maze test. (a) Escape latency, (b) Crossing times. Data are expressed as mean \pm SD (n=12), * p < 0.05, ** p < 0.01, vs. control

Effect of OEO on Ach and AchE in serum and brain tissues of AD mice

Results of neurotransmitter (Ach and AchE) levels in serum and brain tissue are exhibited in Figure 5. After injection of $A\beta_{1-42}$, the levels of Ach in serum and brain tissues of AD mice were reduced ($p < 0.01$) and the AchE levels were increased ($p < 0.01$) compared to normal mice. Interestingly, administration of OEO at low, middle, and high doses ($p < 0.01$) enhanced the Ach levels in serum of AD mice and ($p < 0.01$) reduced the AchE levels in serum compared to

control mice. Treatment with OEO at middle and high doses also significantly increased the Ach levels ($p < 0.01$) and decreased the AchE levels in brain tissues of AD mice ($p < 0.05$ and $p < 0.01$) compared to control mice.

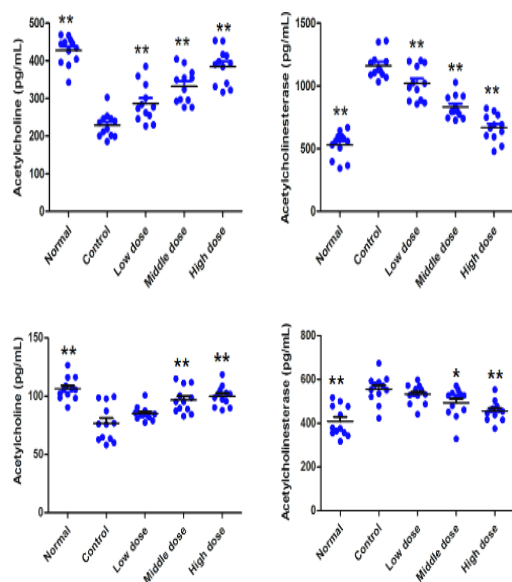


Figure 5: Effect of OEO on acetylcholine (Ach) and acetylcholinesterase (AChE) in serum (a) and brain tissues (b) of AD mice. Data are expressed as mean \pm SD ($n = 12$). * $p < 0.05$, ** $p < 0.01$, vs. control

Effect of OEO on histopathological changes of AD mice

The results of the histopathological examination are exhibited in Figure 6. Normal mice had intact hippocampal neurons with a clear structure that were arranged neatly with normal round cell nuclei. Obvious pathological changes were noted in the hippocampus of AD mice, such as wrinkled cells with irregular or abnormal nuclei and cells arranged in a disorderly manner. Interestingly, OEO treatment at low, middle, and high doses protected the hippocampal neurons from damage. In particular, no obvious pathological changes could be found in the high dose of OEO-treated mice compared to normal mice.

The results of Congo red staining, carried out to examine amyloid plaques in hippocampus tissues, are shown in Figure 7. No significant amyloid plaques were observed in the hippocampus tissues of normal mice. However, many obvious amyloid plaques (red staining) were found in hippocampus tissues of AD mice in the control group. After treatment with different doses of OEO, the amyloid plaques in hippocampus tissues were alleviated.

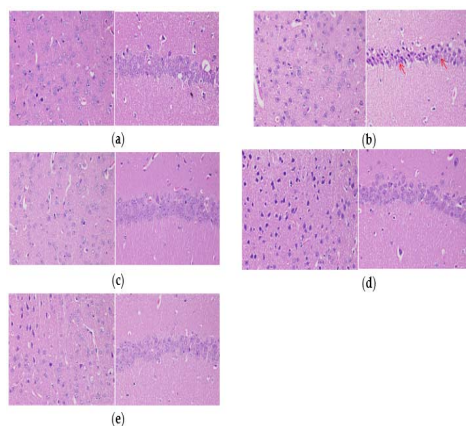


Figure 6: Morphology of H&E-stained hippocampus tissues in AD mice. a–e represent normal, control, and OEO (low, middle, and high dose) groups, respectively

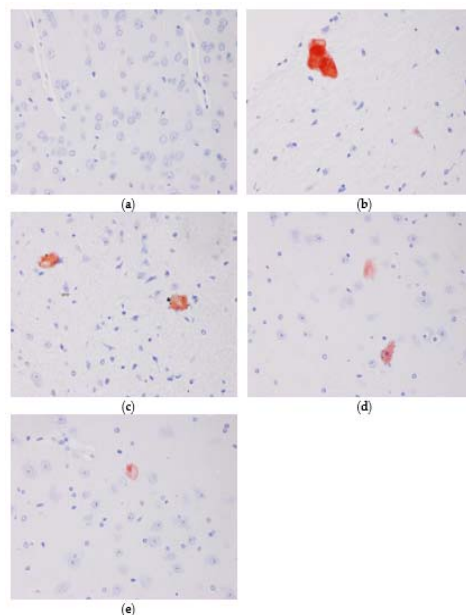


Figure 7: Morphology of Congo red-stained hippocampus tissues in AD mice. a–e represent the normal, control, and OEO (low, middle, and high dose) groups, respectively

Immunohistochemical results

To determine the possible mechanisms of action of OEO against AD, an immunohistochemical assay with anti-c-fos antibody was conducted (Figure 8). After injection of $A\beta_{1-42}$, the population of c-fos positive cells in hippocampus tissues of AD mice was increased compared to normal mice. Interestingly, the OEO treatment at low, middle, and high doses effectively decreased c-fos expression in hippocampus tissues of AD mice compared to control mice in a dose-dependent manner.

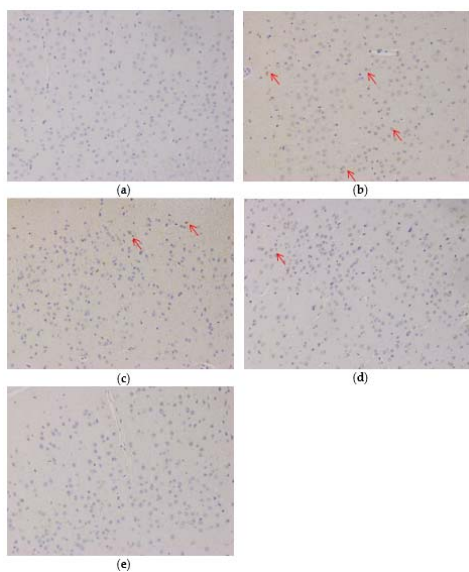


Figure 8: Immunohistochemical results for anti-c-fos antibody-stained hippocampus tissues in AD mice; **a–e** represent the normal, control, and OEO (low, middle, and high dose) groups, respectively. The arrows show the positive cells

DISCUSSION

Plant-derived natural extracts/monomers are precious resources for identifying novel chemical agents with potential medicinal properties [17-22]. As part of the continuous involvement in finding novel potential bioactive agents for treating AD from natural constituents, OEO was screened as a useful agent for reducing learning and memory impairment in AD mice. This study is the first systematic report regarding the beneficial effects of OEO on learning and memory in AD mouse resulted in the $A\beta_{1-42}$. Furthermore, the main constituents of OEO were identified as limonene, α -pinene, 4-terpineol, β -elemene, sabinene, p -cymene, γ -terpinene, β -pinene, (E)-caryophyllene, and calamenene.

Animal behavioral experiments are useful and reliable ways of evaluating the effects of candidate drugs on cognitive functions [23]. The neurodegenerative disease AD is characterized by cognitive dysfunction, which commonly results in deterioration of memory and difficulties in learning [6,24]. The SDT, DAT, and MWM tests are applied to study the curative effects of candidate drugs on AD-induced deterioration of memory and learning.

The results show that OEO treatment prolongs the latency in the SDT and DAT, but decreases the number of errors. In addition, OEO treatment reduced the escape latency and increased the number of crossings in the MWM test. These

results indicate that OEO could alleviate the impaired cognitive functions of AD mice. The deterioration of memory and learning is closely related to the reduction of Ach in the brain of AD patients compared to healthy people [25]. In addition, because AchE is the enzyme used for hydrolysis of Ach in the human body, a decrease in AchE content could be beneficial for increasing Ach levels in the brain [26]. Interestingly, the present results indicate that OEO could enhance the Ach levels and decrease the AchE levels in serum and brain tissues. Increasing investigations have suggested that $A\beta$ accumulation plays a crucial role in AD [5,6,27].

The results of this study show that OEO treatment reduces the amyloid plaque in hippocampal tissues. Furthermore, the pathological examination results indicate that OEO treatment could protect the hippocampal neurons from damage. Previous studies have revealed that c-fos is over-expressed in the brain tissues of AD patients and plays an important role in AD pathogenesis [28,29]. Importantly, the results also demonstrate that the OEO treatment could effectively decrease c-fos expression in hippocampus tissues of AD mice.

CONCLUSION

The findings of this study reveal that OEO has a significant beneficial effect on AD-induced deterioration of learning and memory in $A\beta_{1-42}$ -induced AD mice. The possible mechanisms are related to increase in Ach content, reduction of amyloid plaques in brain tissue, protection of hippocampal neurons, and down-regulation of c-fos in brain tissue. Thus, CEO has potentials for the management AD, but further investigations are required to ascertain this.

DECLARATIONS

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Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization, ZZ and XF; Data curation, WC, JL, and LL; Formal analysis, XF; Funding acquisition, XF; Investigation, ZZ, WC, JL, and DC; Methodology, ZZ; Project administration, DC and LL; Resources, XF; Writing – original draft, ZZ; Writing – review and editing, XF. All authors have read and approved the final manuscript.

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