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Linarin improves the dyskinesia recovery in Alzheimer's disease zebrafish by inhibiting the acetylcholinesterase activity

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ABSTRACT

Background: Due to complex pathogenesis of Alzheimer's disease (AD), currently there is no effective disease-modifying treatment. Acetylcholinesterase (AChE) has introduced itself as an important target for AD therapy. Linarin as the representative active ingredient of flavonoid glycoside in *Flos chrysanthemi indici* has been found to have anti-acetylcholinesterase effect.

Aims: The present study intended to explore the potential effect of linarin for treatment of AD.

Main methods: In this study, molecular docking simulation was used to evaluate whether linarin could dock with AChE and decipher the mechanism of linarin as an AChE inhibitor. After molecular docking simulation, $AlCl_3$-induced Alzheimer's disease zebrafish model was established. Effects of linarin on treating AD zebrafish dyskinesia and AChE inhibition were compared with donepezil (DPZ) which was used as a positive control drug.

Key findings: Molecular docking simulation showed that linarin plays a critical role in AChE inhibition by binding AChE active sites. The experiments illustrated that the dyskinesia recovery rate of AD zebrafish could be significantly improved by linarin. The dyskinesia recovery and AChE inhibition rate were 88.0% and 74.5% respectively, while those of DPZ were 79.3% and 43.6%.

Significance: These findings provide evidences for supporting linarin to be developed into an AD drug by inhibiting the activity of AChE.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease associated with dementia. AD is characterized by a progressive loss of cognitive abilities and diminished executive function, associated with various degrees of behavioral disturbances. To our knowledge, the pathogenesis of AD is still not clear. There are various hypothesis about the pathogenesis of AD, such as amyloid-β (Aβ) hypothesis, tau protein hypothesis, chronic inflammation hypothesis [1–3]. There are various types of drugs based on different strategies, including Aβ aggregation inhibitor, anti-tau, anti-inflammatory and cholinergic enhancing drugs [4]. Among them, those aiming at Aβ and tau hypotheses have been investigated extensively. However, these candidates drugs could not be clinical drugs because of serious side effects represented by tacrine, donepezil, galanthamine and rivastigmine, have been approved by the US FDA. These drugs can increase the availability of acetylcholine by inhibiting AChE, thus enhancing the cholinergic neurotransmission. Although these AD drugs are already available in clinical practice, the number of drugs to be used in AD is still limited. In light of this, the development of AD drugs without serious clinical side effects is urgently needed. On the other side, the natural compound is a rich source for human beings to discover novel effective drugs [5–7].

Linarin [$C_{28}H_{32}O_{14}$, CASRN 480-36-4, molecular structure shown as Fig.1, a representative active ingredient of flavonoid glycoside in *Flos chrysanthemi indici*, previously proved to have antibacterial, anti-inflammation, sedative, hypnosis, coagulation, anticancer activities and so on [8]. Recently, linarin has also been reported to have anti-inflammation, AChE inhibitory and neuroprotection activities, which seem to have some connections with AD [9–11].Thus, despite of these therapeutic effects some potential functions of linarin remaining to be explored, especially in the field of AD. Besides, compared with the existing natural compound huperzine A, linarin is extracted from *Flos chrysanthemi indici*, which is more economical and accessible in large quantities. It is very meaningful to perform a further study on the treatment of Alzheimer's disease with linarin.

Zebrafish, with high homology to human, is becoming attractive

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; DPZ, donepezil; Aβ, amyloid-β

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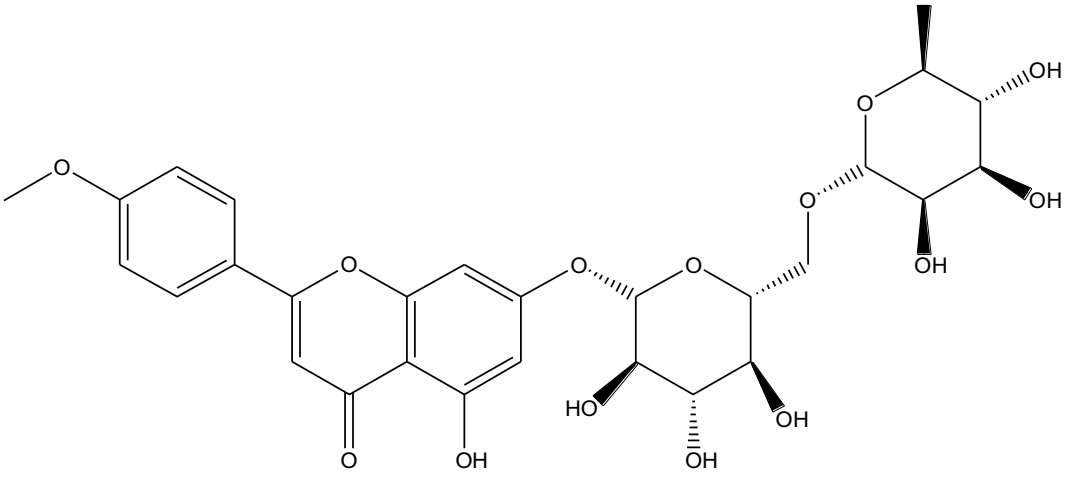

Fig. 1. Molecular structure of linarin.

and valuable model system for human disease [12,13]. The advantages of this model such as small size, short generation time, rapid development and large numbers of offspring make it superior to others. Moreover, the rich array of developmental, behavioral, and molecular benefits provided by the zebrafish has contributed to an increasing demand for the use of zebrafish in AD research [14,15].

In the present study, it is mainly investigated whether linarin can treat AD by inhibiting AChE activity. Firstly, the molecular docking simulation was used to demonstrate the possible mechanism of linarin as an AChE inhibitor. Futhermore, an $AlCl_3$-induced AD zebrafish model was established as in vivo animal model to quickly and reliably observe the therapeutic effect of linarin.

2. Material and methods

2.1. Chemicals

AChE inhibitor DPZ was purchased from TRC Canada. The linarin (92% pure) was prepared in our laboratory. Aluminum chloride was purchased from Aladdin Reagent Inc. DMSO and all the other reagents provided by Sinopharm Chemical (Shanghai, China) were of analytical grade. Amplite™ Fluorimetric Acetylcholinesterase Assay Kit was purchased from AAT Bioquest.

2.2. Molecular docking

As target protein, the crystal structure of AChE was obtained from Protein Data Bank; molecular structure of linarin was obtained from scifinder. Molecular docking process was carried out by AutoDock 4.0. In the process, AChE crystal structure, modified by clearing needless protein, non-polar hydrogens, water chain and adding hydrogen, kollman charges, is regarded as receptor, and linarin structure identified the torsion angles is regarded as ligand. A grid box of 48 × 38 × 58 points in the *x*, *y* and *z* directions were constructed with a grid spacing of 0.375 Å at the grid center (33.60, 66.36, 63.87). The Genetic Algorithm method was applied for finding of optimum binding position. After completion of docking and clustering analysis, the most favorable binding conformation with the lowest free energies was selected as the binding pose.

2.3. Animal maintenance and ethics

The wild-type zebrafishes of both sex were used in this study. Zebrafish embryos were generated by natural pairwise mating in our aquaculture facility. The developmental age of the embryos corresponded to hours post fertilization (hpf) was staged according to Kimmel et al. [16]. Only embryos that developed normally and reached appropriate stages were selected for experiments. The zebrafishes were kept at 28 °C in aerated water (200 mg instant ocean sea salt L^{-1}; conductivity 480-510 μS/cm; pH 6.9–7.2; hardness 53.7–71.6 mg/L $CaCO_3$), on a 14 h light and 10 h dark cycle. After the completion of the experiment, the various developmental stages of zebrafishes were killed by treating 0.25 mg/mL tricaine methanesulfonate. All the procedures of zebrafish experiments were carried out according to EU Directive 2010/63/EU for animal experiments.

2.4. Establishment of AD zebrafish model

The well recognized AD zebrafish model has been used [17], briefly, zebrafishes were placed in a 6-wells microplate at a density of 30 zebrafishes per well and treated with 140 μM $AlCl_3$. For zebrafish, most developmental processes occur within the first day, and the endothelial blood-brain barrier begins to function at 3 day post fertilization (dpf). In lieu of this, 3 dpf zebrafishes were chosen as an optimal stage for the model development.

2.5. Model grouping

Zebrafishes were divided into four groups: vehicle group, model group, model + DPZ group and model + linarin group, each of which contained 30 zebrafishes. The vehicle group was maintained in the reverse osmosis water with 0.5% DMSO while the model group was treated with 140 μM $AlCl_3$ containing 0.5% DMSO from 3 dpf to 5 dpf. The model + DPZ group was co-treated with 140 μM $AlCl_3$ and 8 μM DPZ containing 0.5% DMSO from 3 dpf to 5 dpf. The model + linarin groups were co-treated with 140 μM $AlCl_3$ and different concentration of linarin containing 0.5% DMSO (5.6 μg/mL, 16.7 μg/mL and 50 μg/mL) from 3 dpf to 5 dpf.

2.6. Evaluation of dyskinesia rehabilitation effects in zebrafish

Three days after delivery, larvae movement were recorded by viewpoint behavior analyzer (Zebralab V3, ViewPoint Life Sciences Co., Ltd.) at 28 °C. All experiments were completed in 60 min, containing 3 cycles of light and dark phase (10 min each for light and dark). The distance traveled by zebrafish movement in 60 min was recorded for calculating the dyskinesia recovery rate (DRR).

$$DRR(\%) = \frac{D_{drug} - D_{model}}{D_{vehicle} - D_{model}} \times 100$$

where *D* is the movement distance of zebrafish during 60 min.

2.7. Determination of acetylcholinesterase activity

After dyskinesia rehabilitation assay, above zebrafishes were sacrificed. Then physiological saline was added (1: 9 Mass/volume) and mechanically homogenized under ice-cooling conditions. Homogenate was centrifuged at 2500 rpm for 10 min. Supernatants were taken for assay. Amplite™ Fluorimetric Acetylcholinesterase Assay Kit was used to determine the enzyme activity of AChE. Specific steps are as follows: (1) Preparation of acetylthiocholine reaction mixture (50 μL), (2) Add test samples addition (50 μL), (3) Incubation at room temperature for 20 min, (4) Monitor the fluorescence increase at Ex/Em = 490/520 nm. Fluorescence increase was recorded for Acetylcholinesterase inhibition rate (AIR) quantification.

$$AIR(\%) = \frac{S_{drug} - S_{\mathrm{mod}\,el}}{S_{vehicle} - S_{\mathrm{mod}\,el}} \times 100$$

where S is fluorescence intensity of AChE.

2.8. Data analyses and statistics

GraphPad Prism version 7.00 was used for descriptive statistical analyses. Data were expressed as means ± SD and analyzed by ANOVA and Dunnett's method to test for variability between each trial considering $P \leq .05$ as significant.

3. Results

3.1. Molecular docking

AChE has introduced itself as an important target for AD therapy. AChE inhibitor is a recognized therapy for the AD in mild to moderate stages. To explore the possible combination of AChE and linarin, molecular docking simulation was used. The example of binding model between linarin and the crystallographic structures of AChE protein are shown in Fig. 2. The presence of hydroxy in linarin showed strong hydrogen bonding interaction with residues Tyr 130, Asn 85, Trp 84 and Asp 72. The oxygen of linarin in Benzopyran segment has hydrogen bonding interaction with residues Tyr 121. The methoxyflavone segment of linarin showed π-π interaction with residues Phe 331,Trp 279 and Phe 290. According to reports, the active site of AChE is found at the bottom of a deep and narrow gorge and consists of some domains. [18–20].The peripheral anionic site comprises residues Tyr 70, Tyr 121,Trp 279 and Asp 72. The anionic subsite, where Trp 84 is situated. The hydrophobic site for the alkoxy group of the substrate including residues Phe 330 and Phe 331. Linarin may play a role in inhibiting AChE by binding this hydrophobic active site. Based on the docking results, it's obvious that the interaction between linarin and AChE is strong enough to propose pharmacological effects, which could be the possible molecular mechanism to support linarin as a potent AChE inhibitor. These findings provide evidence for linarin to be developed as AD specific drugs.

Table 1
AD Zebrafish dyskinesia recovery rate %.

Groups	Concentration	Distance (mm) (mean ± SD)	Dyskinesia recovery rate (%)
Vehicle group	–	11,593 ± 1710	–
Model group	–	6870 ± 879###	–
DPZ	8 μM	10,615 ± 1539***	79.3***
Linarin	5.6 μg/mL	7939 ± 1180*	22.6*
	16.7 μg/mL	10,501 ± 1178***	76.8***
	50 μg/mL	11,028 ± 1312***	88.0***

p < .001 vs Vehicle group.
* p < .05 vs Model group.
*** p < .001 vs Model group.

3.2. Evaluation of dyskinesia rehabilitation effects in zebrafish

The dyskinesia recovery of zebrafish is listed in Table 1, Figs. 3 and 4. The results were highly significant (p value < .001) when distance value of model group (6870 mm), compared to vehicle group (11,593 mm) were measured indicating that establishment of model is successful. DPZ, a well-recognized drug for AD, was used as the positive control drug. The distance value of Model + DPZ group was 10,615 mm, compared with model group ($p < .001$), and dyskinesia recovery rate was 79.3%, which indicates the dyskinesia of AD zebrafish could be significantly improved by DPZ. The distance values of

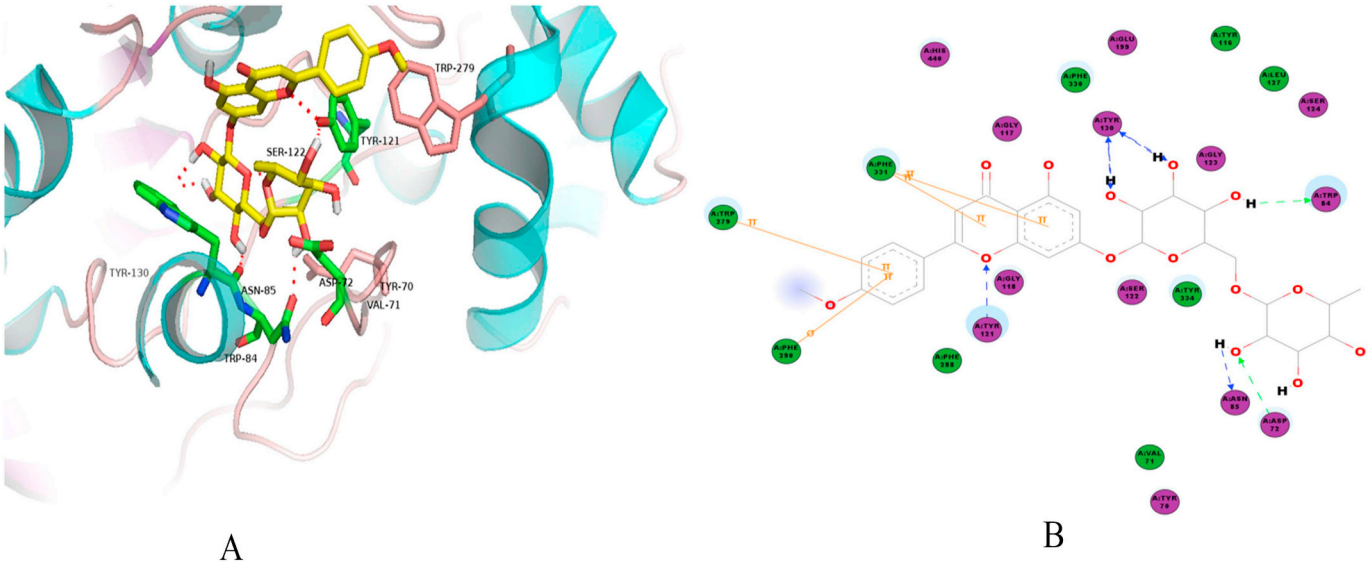

Fig. 2. Binding model between linarin and AChE.
A) The 3D view of the binding model and position of AChE and linarin. The structure of ligand molecule was highlighted by stick model. Interacting residues shown in line and stick model. Red solid lines represent hydrogen bonding B). The 2D view of the interaction models of AChE and linarin, hydrogen bonds were shown as dotted arrows; side-chain hydrogen bond interactions were represented by green dotted arrows; backbone hydrogen bond interactions were represented by blue dotted arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

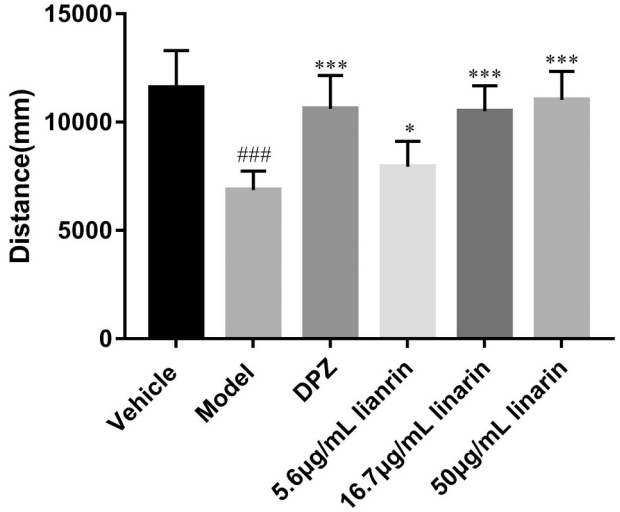

Fig. 3. The effect of linarin on distance of AD zebrafish.
p < .001 vs Vehicle group, *p < .05 vs Model group, *** p < .001 vs Model group. Results are shown as means ± SD, n = 10.

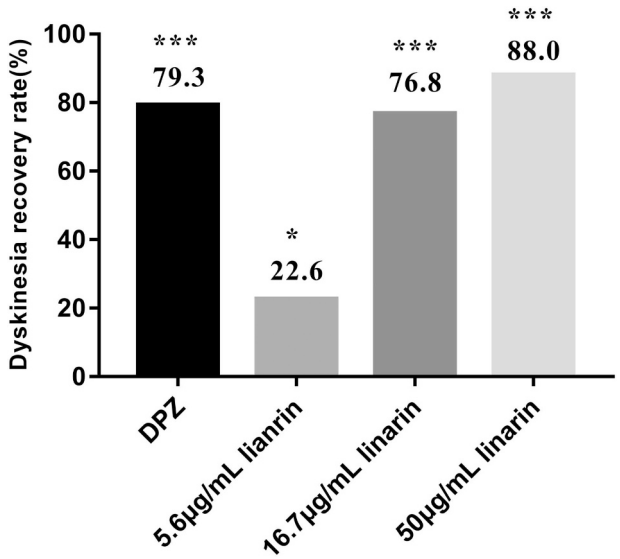

Fig. 4. The effect of different concentrations of linarin on DRR of zebrafish.
*p < .05 vs model group, *** p < .001 vs model group.

different concentrations i.e. 5.6 μg/mL, 16.7 μg/mL and 50 μg/mL Model + linarin compared with model group were 7939 mm (p > .05), 10,501 mm (p > .05) and 11,028 mm (p < .001) while dyskinesia recovery rate were 22.6%,76.8% and 88.0%, respectively, proving that as dose increases, the therapeutic effect became more and more significant. Over 16.7 μg/mL dose, linarin can play a role in cure of AD zebrafish. These results demonstrated that linarin can significantly improve the dyskinesia recovery rate of zebrafish in dose and time dependent manner.

3.3. Effects of linarin on AChE activity

The results of inhibitory effect of AChE are shown in Figs. 5 and 6. The fluorescence intensity of model group (29156) compared to vehicle group (21198), p < .001 indicates that the model was highly significant and the establishment of model was successful. The fluorescence intensity and AChE inhibition rate of Model + DPZ group

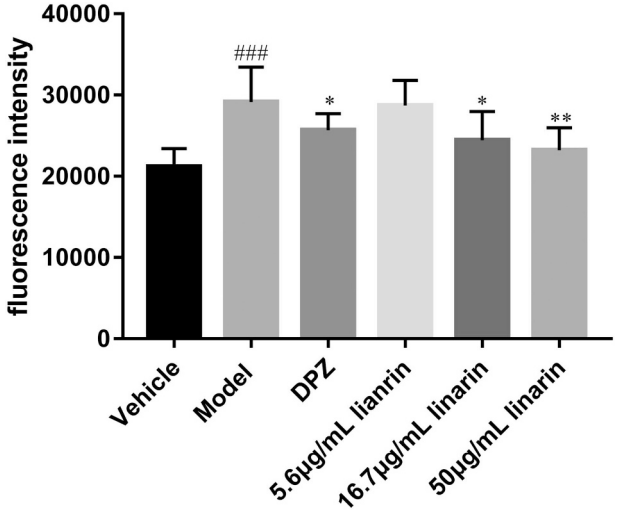

Fig. 5. The impact of different concentration of linarin as AChE inhibitor on zebrafish.
p < .001 vs vehicle group, *p < .05 vs model group, ** p < .01 vs model group. Results are shown as means ± SD, n = 10.

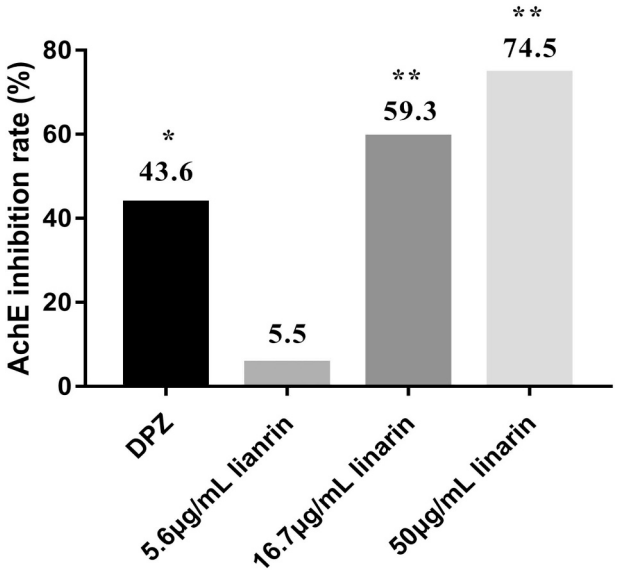

Fig. 6. The effect of linarin on AIR of zebrafish.
*p < .05 vs model group, ** p < .01 vs model group.

compared with model group, were 25,682.9 (p < .05) and 43.6% respectively which demonstrated that AChE activity of AD zebrafish could be inhibited by DPZ. The fluorescence intensity and AChE inhibition rate of Model +5.6 μg/mL linarin were 28,722 (p > .05) and 5.5%, while Model +16.7 μg/mL linarin were 24,438.4 (p > .05) and 59.3%, and Model +50 μg/mL linarin were 23,230 (p < .05) and 74.5%, respectively, compared with model group. These results suggested that 16.7 μg/mL and 50 μg/mL linarin concentration had prominent AChE inhibition. Meanwhile, with the dose increasing the effect became more and more significant. In low concentration DPZ has the better efficacy than linarin. However, Linarin has less side effect than DPZ in high concentration, it is meaningful to consider linarin as a AChE inhibitor. These results were consistent with the results of evaluation of dyskinesia rehabilitation effects of linarin. In conclusion, linarin may become a prominant anti-Alzheimer's disease agent by inhibiting the activity of AChE.

4. Discussion

Although there is tremendous clinical research but the pathogenetic factors of AD are still complex and are not clear. The common characteristics of AD includes acetylcholine deficiency, inflammation, neurodegeneration, Aβ aggregation, tau hyperphosphorylation, neuritic plaques and neurofibrillary tangles. The most dramatic abnormalities noted in AD brains are associated with cholinergic system. One of the therapeutic approach to enhance cholinergic neurotransmission is to improve the availability of acetylcholine by inhibiting AChE, the enzyme that degrades acetylcholine in the synaptic cleft [21]. For instance, DPZ, acrine and galantamine are AChE inhibitors approved for AD treatment. In present study, main focus was on the inhibition of AChE by linarin. Molecular docking simulation was used to test its combination with AChE. Linarin can bind with AChE active sites through strong hydrogen bond and π-π interaction and take effect quickly, which indicates linarin could work as AChE inhibitor to ameliorate symptoms temporarily. To further validate the anti-AChE activity of lianrin, behavior assays of zebrafish were carried out. It is found that linarin can significantly improve dyskinesia in zebrafish. This suggests that linarin may have a therapeutic effect on AD zebrafish. Then further experiments were performed to detect the AChE activity of AD zebrafish and have found that linarin can indeed inhibit AChE activity, which was consistent with the results of the dyskinesia recovery experiment. All these experimental results suggested that linarin can be used as an AChE inhibitor for the treatment of Alzheimer's disease. It is noteworthy that zebrafish regenerative capabilities, particularly at this stage, represent a critical caveat in this model. Besides, although zebrafish shows high homology to human, it needs to be worried that the effects of linarin could be different between human and zebrafish. Mice model is necessary to further verify the test results.

Furthermore, *flos chrysanthemi indici*, with linarin as the main component, has been used as an anti-inflammatory drug in traditional Chinese medicine. Linarin has also been proved to have potent inhibitory effect on inflammatory via the inactivation of NF-κB, MAPKs, TNF and PI3K/Akt [10]. Thus, it may serve as a potential modulatory agent for the prevention and treatment of inflammatory relevant diseases. On the other hand, neuroprotective activity should be taken into consideration, as neurodegeneration of patients will lead to mild cognitive impairment, which is defined on the basis of subjective reports of memory loss that are verified by close personal informants and objective measures adjusted for age and education.

From the previous literature review and by this current research it is concluded that linarin has robust neuroprotective effect via the inactivation of NF-κB, MAPKs, TNF and PI3K/Akt and AChE inhibitor activity [11]. All of these indicates that linarin can not only be an AChE inhibitor but also a potential integrative drug for AD.

5. Conclusion

In this study, the molecular docking simulation was used to demonstrate the possible mechanism of linarin as an AChE inhibitor. Based on the docking result, it's obvious that the interaction between linarin and AChE active sites is strong enough to propose pharmacological effects, which could be the possible molecular mechanism to support linarin as a potent AChE inhibitor. A motility-based in vivo AD animal model using larval zebrafish was developed. Then dyskinesia recovery and AChE activity of zebrafish were investigated. The results showed that linarin can not only significantly improve the recovery of dyskinesia, but also dramatically inhibit AChE activity. These findings provide evidences for linarin to be developed into an AD drug by inhibiting the activity of AChE.

Conflicts of interest

The authors declare no conflict of interest.

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