

# Interaction of lobed kudzu vine root, rhizoma chuanxiong with both acetylcholinesterase and beta-amyloid (A $\beta$ 1-42)

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## ABSTRACT

**Background:** Lobed kudzu vine root and rhizoma chuanxiong are effective drugs in traditional Chinese medicine. **Objective:** Extracts of the two medicines were investigated for their *in vitro* of beta-amyloid (A $\beta$ 1-42)-aggregation and acetylcholinesterase (AChE)-inhibitory activities. **Materials and Methods:** The interaction of lobed kudzu vine root, rhizoma chuanxiong with both acetylcholinesterase and beta-amyloid (A $\beta$ 1-42) were studied by Michaelis–Menten equations, Thioflavin T (ThT) fluorescence analysis and transmission electron microscope (TEM). **Results:** Inhibition of acetylcholinesterase showed that 1-butanol fraction of the two medicines were noncompetitive inhibition, apparent inhibition constants were 9.947 and 7.1523. ThT fluorescence analysis and TEM results indicated that inhibition of the water fraction and 1-butanol fraction (both lobed kudzu vine root and rhizoma chuanxiong) was better. **Conclusion:** The result supported further research on chemical constituents and pharmacological mechanisms.

**Key words:** Acetylcholinesterase, Beta-amyloid, lobed kudzu vine root, rhizoma chuanxiong

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## INTRODUCTION

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder affecting millions of elderly individuals throughout the world. As the "baby boom" generation ages and medical advances enable people to live longer, the number of people afflicted by AD is expected to increase dramatically. Given these trends, there is a tremendous need to develop therapeutics that block or reverse this debilitating neurodegenerative disease.

Multiple factors for AD are thought to be the etiology of the disease, including oxidative stress, aluminum toxicity, cholinergic hypothesis,<sup>[1]</sup> amyloid cascade hypothesis.<sup>[2]</sup> In cholinergic hypothesis, function of acetylcholinesterase was damaged in AD, so some acetylcholinesterase inhibitors are developed as a drug. In A $\beta$  hypothesis, the conclusion of increasing and depositing abnormal A $\beta$  is to start the core of the event.<sup>[3]</sup>

Many kinds of active constituents such as alkaloids, flavonoids, saponins, coumarins, and lignanoids, etc., in Chinese materia medica aim directly at the pathogenesis of AD. And the alkaloids are usually contained in the water fraction. Flavonoids and coumarins are usually contained in 1-butanol fractions.

*Rhizoma Chuanxiong* is one of the most commonly used of the Chinese herbs; it has an excellent safety record and no evident toxicity.<sup>[4,5]</sup> The initial applications were based on traditional uses of the crude herb in decoctions and pills: for vitalizing blood circulation in the treatment of cardiovascular diseases and for treatment of headache and vertigo.<sup>[6,7]</sup> Lobed kudzu vine root was derived from Shen Nong Ben Cao Jing, which had a long history. Active ingredient is daidzein. It is commonly used for treatment of exogenous fever, head, and neck pain.

Therefore, in this work, we first prepared water, petroleum ether, ethyl acetate, and 1-butanol fractions (both lobed kudzu vine root and rhizoma chuanxiong). Then, the AChE-inhibitory activities of extracts of the two medicines were screened in detail. It was found that 1-butanol fraction showed typical noncompetitive inhibition of AChE. Finally,

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the interaction with Aβ1-42 was analyzed using Thioflavin T (ThT) and transmission electron microscope (TEM). The water fraction and 1-butanol fractions (both lobed kudzu vine root and rhizoma chuanxiong) were found to suppress the formation of amyloid fibrils.

## MATERIALS AND METHODS

### Materials and reagent

The dried leaves of lobed kudzu vine root and rhizoma chuanxiong were authenticated by Dr. Wan Dingrong, Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities. The dried leaves of lobed kudzu vine root and rhizoma chuanxiong were extracted with 70% aqueous ethyl alcohol for three times. It was refluxed for 2 h each time. The combined solution was filtered and concentrated under reduced pressure to afford the 70% ethanolic extract. The majority of the 70% ethanolic extract was suspended with water and successively extracted with petroleum ether, ethyl acetate, and 1-butanol.

AChE was obtained from Sigma (St Louis, MO, US), phosphate buffer (PBS) pH=8.0, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 10 mM) and acetylthiocholine iodide (ATCI, 2 mM) were purchased from A Johnson Matthey Company and reconstituted in 50 mM aq. phosphate buffer (pH=8.0), ThT was obtained from Sigma-Aldrich, other reagents are analyzed pure. UV-Visible spectrophotometer (UV-1800PC) was supplied from Shanghai Mapada Instruments Co. Ltd.

Aβ 1-42 was purchased from Shanghai Apeptide Co. Ltd., purity was higher than 95% (weight). Aβ 1-42 was dissolved in 100% PBS and stored at -20°C. Fractions of Lobed kudzu vine root and rhizoma chuanxiong were dissolved in dimethylsulfoxide (DMSO) and diluted to 2.5 g/l in PBS.

### Inhibition of AChE

All tests were conducted according to reported standard procedures.<sup>[8,9]</sup> The components were added in the following order: 1000 μL of DTNB (5 mM), different concentrations of ATCI 500 μL (0.25, 0.4, and 0.5 mM), testing samples (inhibitor) 20 μL (20 μL DMSO in control) were mixed and then adjusted to 2000 μL by addition of buffer. AChE solution (40 μL, 10 U/ml) was added to initiate the reaction at room temperature. Reaction kinetics were analyzed by UV-VIS spectrophotometry, following the absorbance of the thioate product at 412 nm (measuring time was 90 s). Kinetics data were then analyzed by double-reciprocal plots using standard Michaelis–Menten equations<sup>[10]</sup> for noncompetitive and competitive inhibition, respectively. The actual type of inhibition was determined by analyzing the graphs of double-reciprocal plots. Parameters  $K_m$  and

$V_m$  were calculated in the absence of inhibitor (I), and apparent inhibition constants  $K_i$  were obtained from  $K_m/V_m$  as a function of inhibitor concentration [I] at a given substrate concentration [S] [Figure 1].

### ThT fluorescence assay

ThT dye was used to determine the presence of amyloid-like aggregates. The fluorescence emission of ThT is changed when ThT binds to β-sheet aggregate structures.<sup>[11]</sup> A solution of Ab1-42 (10 mM) with/without fractions of lobed kudzu vine root and rhizoma chuanxiong was incubated at 37°C for 48 h. To determine amyloid fibril formation, the solutions containing Aβ1-42 with/without fractions of lobed kudzu vine root and rhizoma chuanxiong were added to 50 mM glycine–NaOH buffer, pH 8.5, containing 25 mM ThT 180 μL, 50 mM PBS 200 μL to a final volume of 420 μL. Each assay was run in triplicate and fluorescence intensities were measured at 440 nm (excitation) and 490 nm (emission).<sup>[12]</sup>

### TEM imaging

To prepare specimens for TEM imaging, 10 mM Aβ1-42 and preformed Aβ1-42 were incubated in the presence or absence of fractions of lobed kudzu vine root and rhizoma chuanxiong for 48 h at 37°C, and then a 10 mL aliquot of each sample was spotted onto a glow-discharged, carbon-coated grid, and incubated for 20 min. The droplet then was displaced with an equal volume of 2.5% glutaraldehyde (v/v) and incubated for an additional 5 min. Finally, the grid was stained with 10 μL uranyl acetate twice, and the solution was wicked off and then the grid was air-dried.<sup>[13,14]</sup> Samples were examined using a JEOL JEM-2100 (HR) transmission electron microscope. All images were captured at voltage of 200 kV at instrumental magnification of 6000×.

$$V_0 = \frac{V_{\max} \cdot [S]}{(K_m + [S]) \cdot \left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

$$\frac{1}{V_0} = \frac{K_m}{V_m} \cdot \left(1 + \frac{[I]}{K_i}\right) \cdot \frac{1}{[S]} + \frac{1}{V_m} \cdot \left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

$$V_0 = \frac{V_{\max} \cdot [S]}{\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (3)$$

$$\frac{1}{V_0} = \frac{K_m}{V_m} \cdot \left(1 + \frac{[I]}{K_i}\right) + \frac{1}{[S]} + \frac{1}{V_m} \quad (4)$$

Figure 1: Standard Michaelis–Menten equations

## RESULTS

### Inhibition of AChE

In Figures 2a and b, a plot of  $1/V_0$  vs.  $1/[ATCI]$  is shown for 1-butanol fraction (rhizoma chuanxiong) together with a plot of  $K_m/V_m$  as inhibitor concentration ( $[I]$ )<sup>2</sup>. The findings show that, at the drug concentrations tested (0.25, 0.4, and 0.5 mM), water saturated 1-butanol fraction showed typical noncompetitive inhibition of AChE, that is, the saturation parameter  $V_m$  was decreasing with increasing inhibitor concentration, while  $K_m$  changed only slightly. Figures 2c and d present the kinetics analysis and double-reciprocal plot of 1-butanol fraction (lobed kudzu vine root). The 1-butanol fraction of both Rhizoma chuanxiong and lobed kudzu vine root showed typical noncompetitive inhibition of AChE [Figure 2].

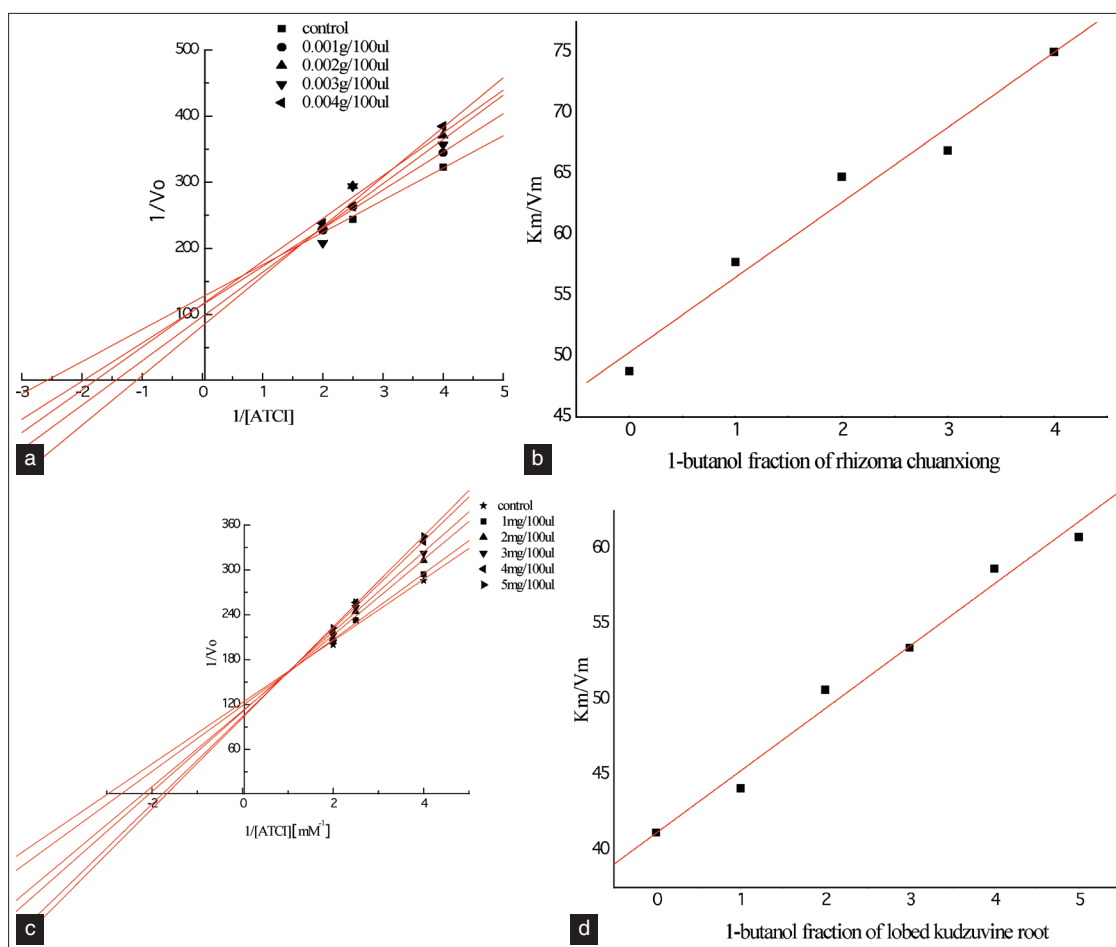
### Analysis of Thioflavin T fluorescence intensity

Figure 3 shows the ThT fluorescence spectra of A $\beta$  (control) with/without fractions of lobed kudzu vine root [Figure 3a] and rhizoma chuanxiong (Figure 3b, incubated for 48 h). Figures 3c and d displays the ThT

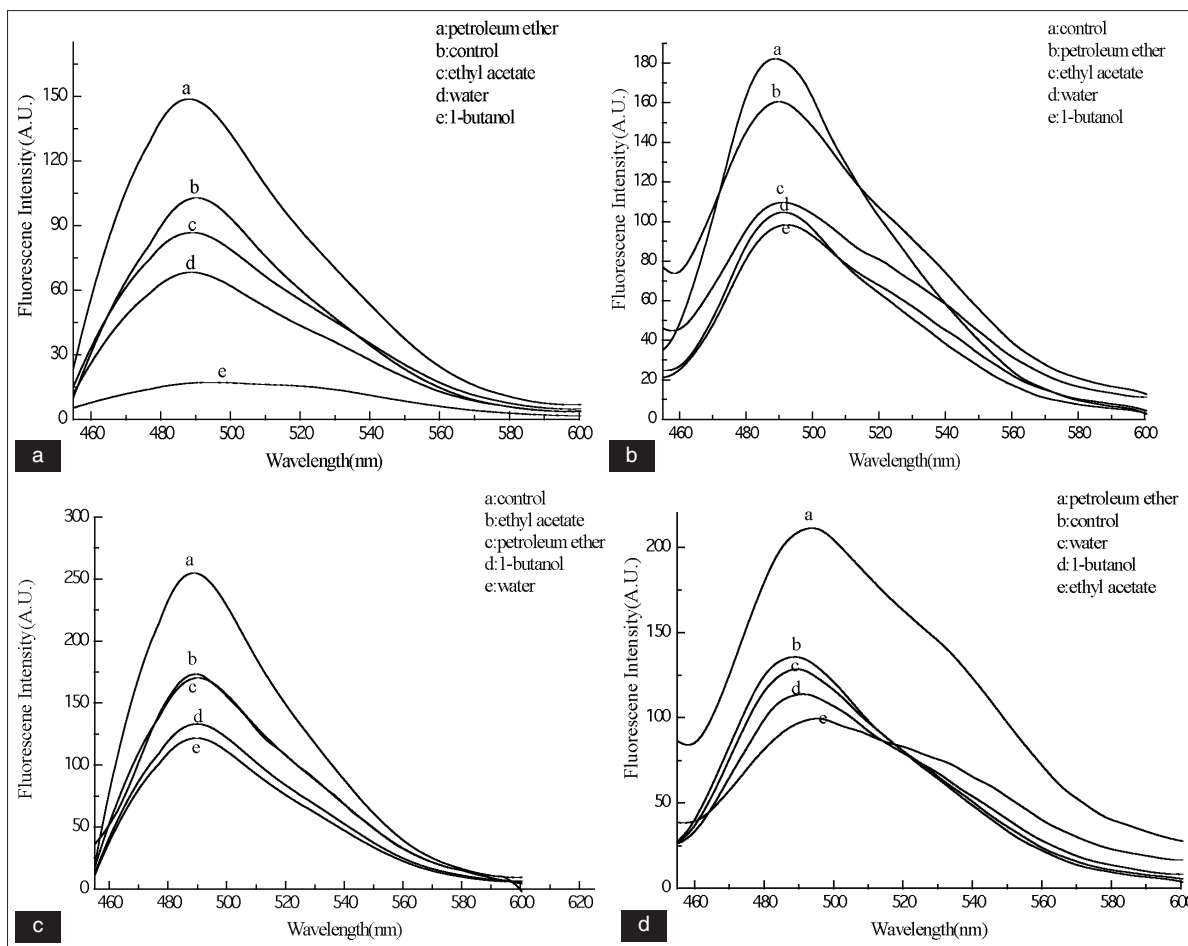
fluorescence spectra of elderly A $\beta$  with/without those fractions. The fluorescence intensity of untreated A $\beta$  (control) was relatively strong. When fractions, especially the water fraction, 1-butanol fraction and ethyl acetate fraction (lobed kudzu vine root and rhizoma chuanxiong), were added into A $\beta$ , the fluorescence intensity decreased. The inhibitory effect of 1-butanol fraction (rhizoma chuanxiong and lobed kudzu vine root) was much stronger than that of other fractions. The fluorescence intensity of the 1-butanol fraction (lobed kudzu vine root and rhizoma chuanxiong) is about 13% and 50% compared with the control, respectively. As seen in Figures 3c and d, four fractions (lobed kudzu vine root) and three fractions (rhizoma chuanxiong) inhibit the aggregated A $\beta$  [Figure 3].

### TEM result

Figure 4a shows the typical amyloid fibril formation from untreated aged (incubated for 48 h) A $\beta$  1-42.<sup>[15]</sup> The filaments are several microns in length. However, when A $\beta$  1-42 was coincubated with the water fraction and 1-butanol fraction (both lobed kudzu vine root and rhizoma chuanxiong), only less fibrils and a few short fibrils were observed [Figures 4b-e].



**Figure 2:** Kinetics analysis (a) and double-reciprocal plot (b) of acetylcholinesterase inhibition by 1-butanol fraction of rhizoma chuanxiong. Kinetics analysis (c) and double-reciprocal plot (d) of acetylcholinesterase inhibition by 1-butanol fraction of lobed kudzu vine root



**Figure 3:** ThT fluorescence emission spectra profiles in the presence of A $\beta$ 1-42 (control) with or without lobed kudzu vine root (a, c), rhizoma chuanxiong (b, d). Results of the elderly group were c and d

## DISCUSSION

Acetylthiocholine iodide (ATCI) was used as substrate to screen two medicines' AChE activities.<sup>[16,17]</sup> From Figure 2, we could calculate that the values of the apparent inhibition constants ( $K_i$ ) calculated for 1-butanol fraction (rhizoma chuanxiong and lobed kudzu vine root) were 9.947 and 7.1523, respectively.

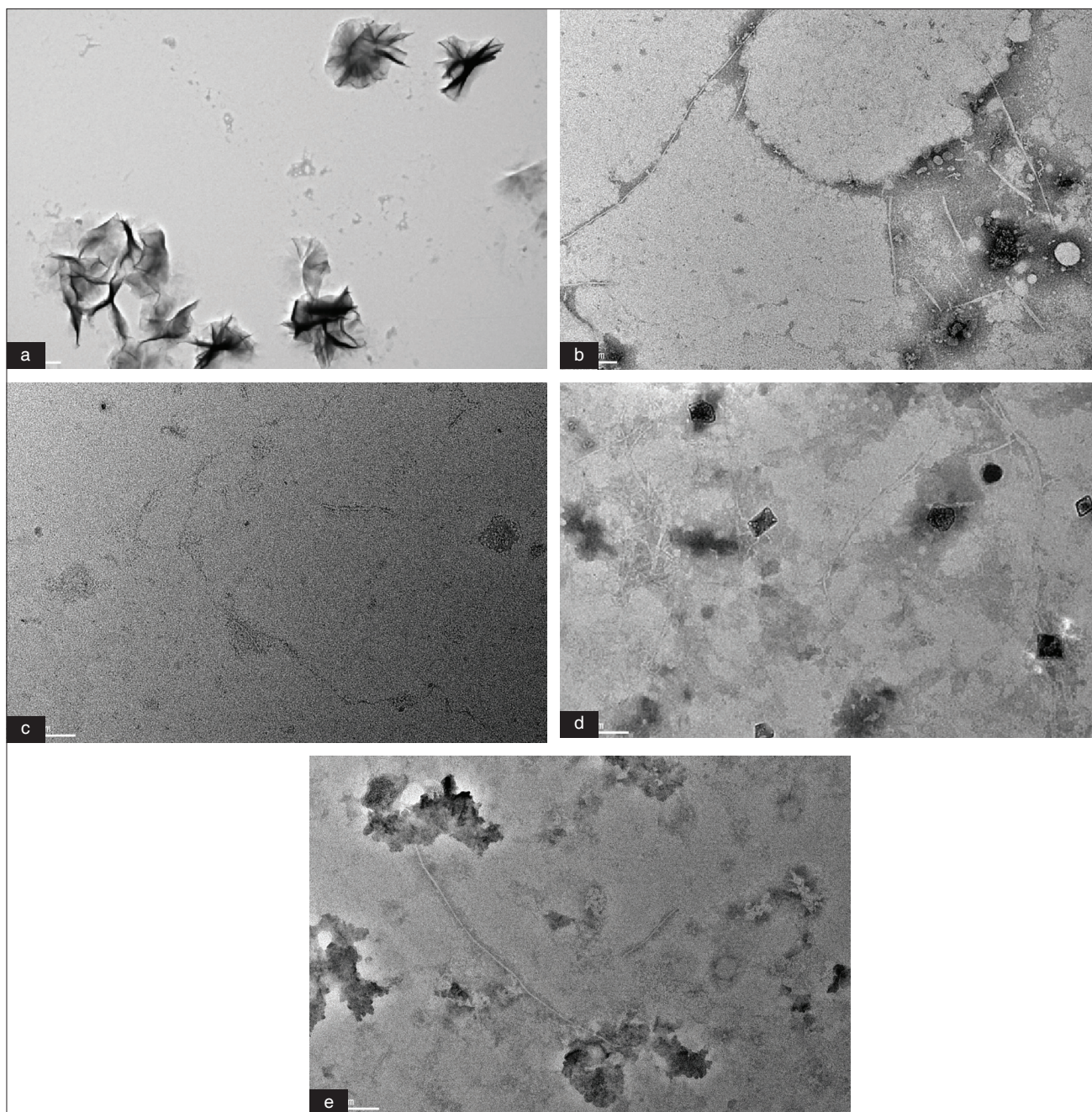
ThT binds specifically to amyloid and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the amount of amyloid formed. The method is more specific than other methods, such as turbidity or sedimentation, for the semi-quantitative determination of amyloid-like aggregates. Both the water fraction and ethyl acetate fraction (lobed kudzu vine root and rhizoma chuanxiong) significantly inhibit the aggregation of A $\beta$ /the aggregated A $\beta$ .

TEM imaging results are consistent with above fluorescence results. It suggests that the water fraction and 1-butanol fraction (both lobed kudzu vine root and rhizoma chuanxiong) can inhibit formation of A $\beta$  fibrils.

In the present study, the 70% ethanolic extract from lobed kudzu vine root (rhizoma chuanxiong) are separated into the four fractions of petroleum ether fraction, ethylacetate fraction, 1-butanol fraction, and water fraction by liquid-liquid extraction. The result indicates that extracts of lobed kudzu vine root and rhizoma chuanxiong can inhibit aggregation of A $\beta$ 1-42, especially the water fraction and 1-butanol fraction. The 1-butanol fraction (lobed kudzu vine root and rhizoma chuanxiong) shows the highest AChE-inhibition and A $\beta$ -aggregation-inhibition activities. However, due to the nature of multiple chemical constituents involved in the natural plants as well as the multifactorial condition of AD, it is very important to further separate chemical constituents from the 1-butanol fraction and water fraction. Further research will be conducted on chemical constituents and pharmacological mechanisms in the future.

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**Figure 4:** TEM of A $\beta$  1-42 aggregation with or without lobed kudzuvine root and rhizoma chuanxiong when incubated alone (a), or with rhizoma chuanxiong (water fraction, b, 1-butanol fraction, c) and lobed kudzuvine root (water fraction, d, 1-butanol fraction, e)

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