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ARTICLE

Polygala crotalarioides treatment improves cognitive function in mouse model of Alzheimer's disease

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Abstract

Background: Alzheimer's disease (AD) as a progressively neurodegenerative brain disease is a devastating pathology leading to disastrous cognitive impairments and dementia. *Polygala crotalarioides* (*P. crotalarioides*) has a good effect on AD, but there are few reports on the specific mechanism of action on AD.

Methods: The AD progression was assessed by pathological and behavioral observation to evaluate the therapeutic effect of *P. crotalarioides* on AD *in vivo* using APPswe/PS1dE9 male mice. Activated microglia in mouse brain tissues was analyzed by immunofluorescent staining. Aβ and Tau proteins levels were tested by enzyme linked immunosorbent assay (ELISA). Morris water maze test, balance beam test and new object recognition test were employed to evaluate the memory and learning after *P. crotalarioides* treatments in AD mice.

Results: Activated microglia increased in brain tissues of AD mice, whereas *P. crotalarioides* treatment reduced microglia activated. AD mice had enhanced Aβ and Tau clearance when treated with *P. crotalarioides*. Moreover, *P. crotalarioides* treatment improved cognition and mobility in AD mice.

Conclusions: Our results suggested that *P. crotalarioides* treatment ameliorated AD progression in mice.

Keywords: Alzheimer's disease, *Polygala crotalarioides*, cognition

Introduction

Alzheimer's disease is the most common neurodegenerative disease among the elderly, and its incidence increases with the age of the global population. The most typical clinical manifestation of AD is memory loss and cognitive decline, affecting more than 50 million people worldwide[1], and this

number is expected to grow rapidly to 90 million by 2050 due to population aging[2]. AD is an irreversible progressive brain dysfunction. In its most severe stage, people with AD are completely dependent on others for basic activities of daily living. Therefore, it is crucial to develop effective therapeutic drugs for AD.

Pathologically, AD is main characterized by amyloid-β (Aβ)-mediated extracellular senile plaques, neurofibrillary tangles in cells involved in Tau protein hyperphos-phorylation, and the absence of neurons[3]. The amyloid precursor protein (APPswe) gene is a human gene with a mutation associated with familial AD and the PS1 gene is a mouse presenilin 1 (PS1) gene which has a human mutation associated with familial AD inserted. The mice with transgenic of familial AD mutant forms of APPswe and PS1 are often used to study the mechanisms of the neuropathology of AD and the therapeutic effects of drugs on Alzheimer's disease.

Polygala crotalarioides is a perennial herb used as a rare Chinese herbal medicine by the Wa Minority of Yunnan. *P. crotalarioides* classified in the Polygalaceae, the whole plant can be used as medicine, and its roots have the best effect. Pharmacological research has shown that *P. crotalarioides* has excellent biological activity, and is widely used in folk to tonic the heart and calm the mind, anti-fatigue, anti-hypoxia, and improve the body's ability to adapt stress[4]. It has been reported that *P. crotalarioides* can improve cognitive function[5, 6]. In this study, we preliminarily explored the therapeutic effect of *P. crotalarioides* on AD, and verified and evaluated the administration of *P. crotalarioides* in the treatment of AD through behavioral and pathological observation in APPswe/PS1dE9 mice.

Materials and Methods

Preparation of *Polygala crotalarioides* and Huperzine A

The fresh plants of *P. crotalarioides* were provided by Yunnan Yihua Agrobiological Company (Lincang, China). After drying, the root was ground into powder for reserve, dissolved in distilled water before administration, and prepared into a suspension by adding sodium carboxymethyl cellulose. Huperzine A (purity > 98%, Yuanye Biotechnology, Shanghai, China) was dissolved in distilled water that contained 40% volume of 0.1 M hydrochloric acid at 5 mg/ml as a stock solution and was the diluted to the proper concentration with distilled water for administration.

Animal treatment

The 6.5-month-age male APPswe/PS1dE9 mice and wild-type C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University. All procedures for animal uses were in strict accordance with the guidelines of animal welfare set by the World Organization for Animal Health and the Chinese national guideline for animal experiments. All procedures involving animals and their care in this study were approved by the Institutional Animal Care and Use Committee of Yunnan Labreal Biotech CO.,LTD. The mice were totally set to four groups (ten in each group). The wild-type C57BL/6 mice served as negative control (WT group) and treated with normal saline intragastric administration for three days. The APPswe/PS1dE9 mice were randomly divided to three groups. The APPswe/PS1dE9 mice in the second group (AD group) were treated with normal saline intragastric administration, in the third group (AD + *P. crotalarioides* group) were treated with *P. crotalarioides* (5 mg/kg/day) intragastric administration, in the fourth group (AD + Huperzine A group) were treated with Huperzine A (0.1 mg/kg/day) intragastric administration for three days.

Morris water maze test

The assay was performed according to the published approach[7, 8]. During training trials, the visible platform (1.5 cm above the water surface) was placed in the circular pool (120 cm in diameter, 50 cm deep) with featureless inner surface. The circular pool was filled with white water dyed with nontoxic food-grade titanium dioxide and kept around 22 °C. The pool was divided into four quadrants of equal area. The mice were given 90 seconds to find the platform and allowed to stay at the platform for 10 seconds. If the mice failed to find the platform within 90 seconds, the mice were gently pulled from the water onto the platform and kept there for 10 seconds. On the test day, a probe trial was carried out and the platform was submerged 1 cm below the water surface so that it was invisible at the water level. The mice were placed into the water maze at one of four randomly quadrants and released, allowing the mice to find the hidden platform. After the mice found and climbed onto the platform, the trial was stopped, and the swimming distance and time was recorded.

Balance beam test

The ability of the mice to keep balance was evaluated in the balance beam test followed the methods of Chen et.al[9]. Mice were placed on the same starting point of a horizontal wooden bar (1 × 1 × 100 cm) 40 cm above the ground and a dark goal box on destination to attract the mice to run up to this dark and safe environment. The time taken to cross the beam was measured. Distance travelled in two minutes, speeds, number of foot slips, and latency to fall were recorded and analyzed[10].

New object recognition test

The test was performed in an open-field apparatus (28 × 28 × 20 cm^3). The task consisted of four sequential daily trials[11]. During the habituation trial (day 1 and 2), mice were placed in the center of the open-field apparatus and allowed to freely explore the space in the absence of any objects for 15 minutes. During the acquisition trial (day 3), mice were placed in the open-field apparatus again in the presence of two identical objects positioned near the two corners and allowed to freely explore space. Finally, the memory test was performed 24 hours later (day 4): one of the familiar objects was replaced by a novel object with difference in its shape, color, and texture and mice being left to explore both objects. The apparatus and objects were cleaned with ethanol (70%) before use and between each animal test. All data were collected for mice performance analysis by the discrimination index (DI) that is calculated as the difference between the time spent exploring the novel (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): DI = [TN-TF]/[TN+TF].

Immunofluorescent staining

After behavioral experiments, mouse brains were removed and fixed in 4% PFA at 4 ℃ for 48 hours, rinsed with PBS, and incubated in 30% sucrose at 4 ℃ for 48 hours, then frozen in a 2:1 mixture of 30% sucrose and optimal cutting temperature compound. The brain samples were sectioned on a cryostat (40 μm). Sections incubated at 4 ℃ overnight with anti-IBA1 antibody (ab178847; 1:100; Abcam, Shanghai). After washed in PBS for three times, the sections were applied with secondary antibody goat-anti-rabbit IgG Cy3 (ab6939; 1:200; Abcam, Shanghai) at room temperature for 1 hours, followed by three rinses in PBS. Nuclei were stained with DAPI (Abcam, Shanghai). Images were taken by confocal microscopy (Olympus Corporation, Tokyo, Japan).

ELISA

ELISA kits (Solarbio life science, Beijing, China) were used to quantify the concentrations of Aβ and Tau according to the manufacturer's instructions. A standard curve was generated in accordance with the protocol to obtain pg/ml concentration of each peptide.

Statistical analysis

Data were analyzed using Prism 9 (GraphPad Software). Results were presented as mean ± SD. The statistical significance between two various groups or treatments was measured by Student's *t* test, and the significance between multiple various groups was measured by one-way ANOVA. In all experiments, *P*-value < 0.05 was considered to be statistically significant, **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns means no significance $P \geq 0.05$.

Results

***P. crotalarioides* improved memory function in AD mice**

APPswe/PS1dE9 transgenic mice were utilized to imitate AD. After administrated with *P. crotalarioides*, the Morris water maze test was taken out to check spatial memory of AD mice. The swimming trace of mice in water maze was recorded and displayed in Figure 1A. AD mice performed impaired learning and spatial orientation in the submerged platform phase. However, the spatial deficits of AD mice were alleviated by *P. crotalarioides* or Huperzine A. The mice treated with *P. crotalarioides* or Huperzine A spent fewer travel to reach the platform compared to AD mice (Figure 1B). When AD mice were treated with *P. crotalarioides* or Huperzine A, the arrival time to platform reduced compared with AD group (Figure 1C). These observations supported *P. crotalarioides* could improve memory loss and cognitive decline in AD mice.

***P. crotalarioides* improved the ability of AD mice to maintain balance**

The balance beam test was performed to examine the ability of motor balance and coordination in mice. As shown in Figure 2A, the latency time of fall in AD mice was shorter than that in WT group, but prolonged after *P. crotalarioides* or Huperzine A treatment. The moving distance (Figure 2B) of AD mice within 2 minutes was increased after *P. crotalarioides* or Huperzine A intragastric administration, and the average speed of movement throughout was also enhanced. In addition, the number of slipping footsteps was reduced in *P. crotalarioides* or Huperzine A treated AD mice. These observations suggested that *P. crotalarioides* could improve balance in AD mice.

***P. crotalarioides* attenuated cognition decline in AD mice**

The New object recognition test were conducted to examine mice cognitive ability. AD mice exhibited impaired novel object recognition compared to control WT mice as shown by the amount of time spent in exploring the familiar and the new objects (Figure 3A-B). Cognitive ability assessed by discrimination index [Discrimination Index = (the time spent exploring the novel object − the time spent exploring the novel object)/ (the time spent exploring the novel object + the time spent exploring the novel object)] was improved in *P. crotalarioides* or Huperzine A treated AD mice compared with AD mice. These results showed that *P. crotalarioides* treatment ameliorated the cognitive status of AD mice.

***P. crotalarioides* ameliorated microgliosis in AD mice**

Microgliosis is one of the evidence of AD histopathologically. Staining with IBA1 detected the activation status of microglia

in treated mice. The fluorescence intensity of IBA used to label microglia in AD group was much greater than that in the WT group (Figure 4A). Compared with the AD group, AD mice treated with *P. crotalarioides* or Huperzine A showed lower fluorescence intenstiy of activated microglia marked by IBA1. These data implied that *P. crotalarioides* might reduce microgliosis in AD mice.

***P. crotalarioides* enhanced Aβ and Tau elimination in AD mice**

Structural changes such as the accumulation of plaques in the AD brain have been classified as "positive" lesions, and the senile plaques were determined to be largely composed of the Aβ peptide. Many neurites surrounding plaques exhibit swollen, dystrophic morphologies and often contain aggregates of phospho-Tau and multiple cellular components. Therefore, both Aβ and Tau contribute to neurodegenerative process in AD[12]. We scrutinized whether *P. crotalarioides* or Huperzine A treatment affects deposition of Aβ and Tau in AD mice. Figure 5A showed that the accumulation of Aβ in AD mice treated with *P. crotalarioides* or Huperzine A were significantly lower than that in the untreated group, and Tau had the same trend (Figure 5B). These results indicated that *P. crotalarioides* could promote Aβ elimination in AD mice.

Discussion

AD is the fifth leading cause of death, and it robs people of their independence[1]. Despite large gains in our understanding of AD pathogenesis and how the disease is conceptualized, there are still no drugs that can slow the progression of AD, let alone offer a cure. This study investigated a folk herbal medicine *P. crotalarioides* for the treatment of AD, and verified the effect of *P. crotalarioides* on AD mice in pathological and behavioral aspects.

It is well established that individuals with AD show multifaceted cognitive impairment that progressively interfere with their day-to-day functioning. Along with progressive memory loss occurring early in the disease, AD patients exhibit psychotic and emotional psychological symptoms, and attention deficits[13]. A variety of animal models have been developed to observe behavioral performance such as cognition and memory in AD research[14-16]. The therapeutic efficacy of *P. crotalarioides* was further validated by behavioral observations in transgenic AD model mice. This study found that *P. crotalarioides* treatment improved the memory and cognitive function, and elucidated the potential mechanisms of *P. crotalarioides* action, including reduction of Aβ and Tau deposition and microgliosis.

Several criteria have been proposed for the pathological diagnosis of AD, including amyloid plaques and neurofibrillary tangles. The amyloid hypothesis is the prevalent theory of AD pathogenesis, which suggests that accumulation of pathological forms of Aβ in the brain is the primary pathological process, driven through an imbalance between Aβ production and Aβ clearance. The formation of Neurofibrillary tangles and subsequent neuronal dysfunction and neurodegeneration are thought to be downstream processes[17]. Neurofibrillary tangles are primarily composed of paired helical filaments consisting of hyperphosphorylated Tau[18]. Tau pathology typically begins in the allocortex of the medial temporal lobe (entorhinal cortex and hippocampus) before spreading to the associative isocortex. In this experiment, we used APPswe/PS1dE9 mice as a model of AD and observed Aβ and Tau deposition in their brain tissues, while *P. crotalarioides* gavage administration treatment substantially reduced Aβ and Tau deposition, demonstrating the efficacy of *P. crotalarioides* in the pathology.

Microglial cells are primary cell of the central nervous system and the principal mediators of inflammation in response to Aβ accumulation, their contribution to neuroinflammation is highly correlated to the progression of neurodegeneration and synaptic dysfunction, particularly with respect to AD[19]. In AD mice, we observed microglial cells proliferation and *P. crotalarioides* demonstrated its reversal effect of microgliosis.

Huperzine A is a novel Lycopodium alkaloid originally extracted from the Chinese herb moss *Huperzia serrata* used as a potent, specific, selective and reversible inhibitor of acetylcholinesterase (AChE)[20]. Huperzine A can improve cognitive dysfunction, attenuate behavioral disturbance, and slow the progression of AD in a broad range of animal models as well as in AD patients[21]. Studies have confirmed that Huperzine A protects neuron from harm in AD by reducing the loss of acetylcholine and preventing Aβ-induced apoptosis. In this research, Huperzine A was used as the positive drug control, and the results from each part of the experiment showed that *P. crotalarioides* was comparable to Huperzine A in efficacy. The composition analysis study proved that *P. crotalarioides* plant contained a variety of bioactive ketones such as 3-hydroxy-1,2,6,7,8-pentamethoxy xanthone and 6-O-β-d-glucopy-ranosyl-1,7-dimethoxy xanthone[22, 23]. As a muti-active ingredient drug, *P. crotalarioides* may have a muti-target AD therapeutic effect like Huperzine A, and the deeper therapeutic mechanism needs to be further explored.

In conclusion, the results of our study demonstrated that *P. crotalarioides* treatment can restrain AD disorder through ameliorating microgliosis, inhibiting the accumulation of Aβ

and Tau, reducing the memory and cognitive decline, and improving the ability to maintain balance in AD mice, suggesting it is effective for AD therapy.

Declaration of competing interest

On behalf of the authors, no conflict of interest is present in this work.

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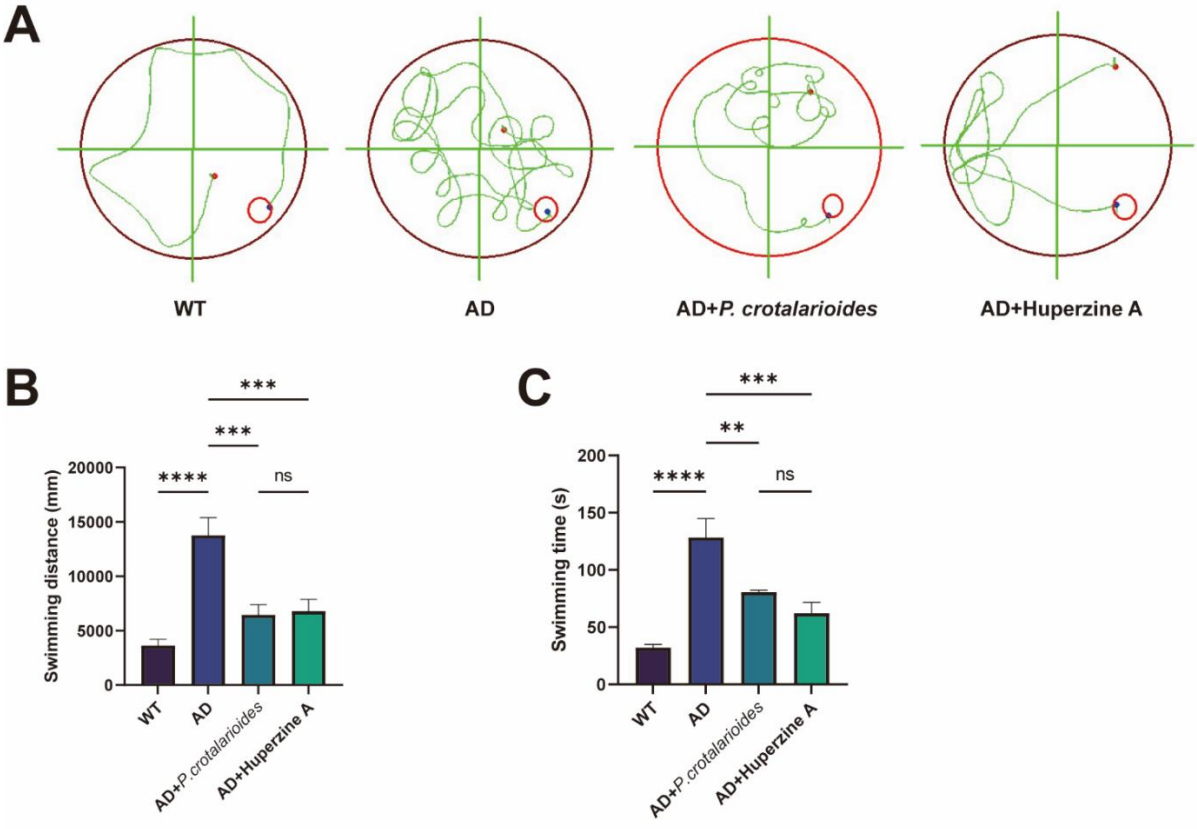

Figure 1. *P. crotalarioides* improved memory function in AD mice. (A) Representative search traces of each group, and the swimming distance (B) and time of mice to the hidden platform in Morris water maze. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, ns means no significance $P \geq 0.05$.

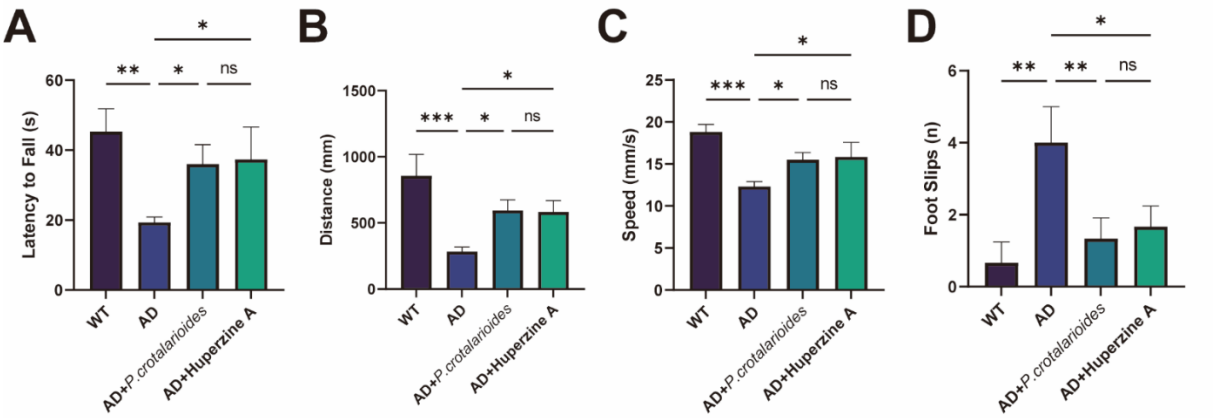

Figure 2. P. crotalarioides improved the ability of AD mice to maintain balance. (A)Latency to fall, (B)moving distance within 2 minutes, (C)the average speed, (D)and the number of foot slips in balance beam test. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns means no significance $P \geq 0.05$.

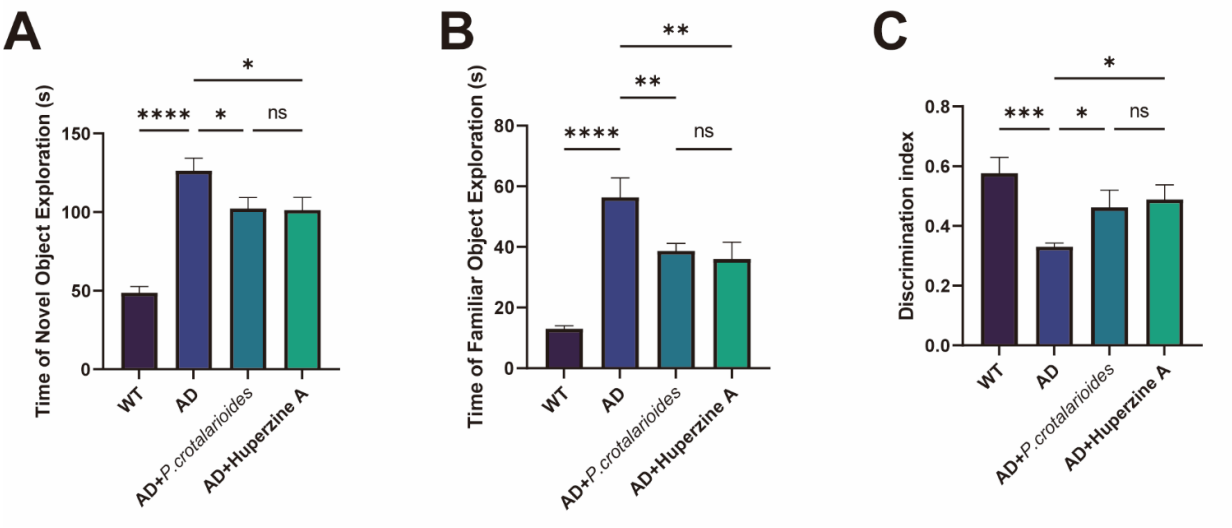

Figure 3. P. crotalarioides attenuated cognition decline in AD mice. (A)Time of novel and familiar (B) object exploration and (C)the discrimination index. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns means no significance $P \geq 0.05$.

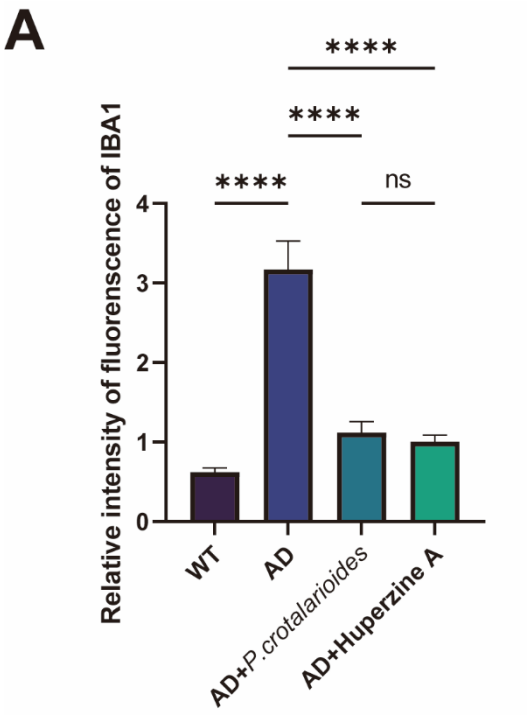

Figure 4. P. crotalarioides ameliorated microgliosis in AD mice. (A) Relative intensity of immunofluorescence of IBA1. **** $P < 0.0001$, ns means no significance $P \geq 0.05$.

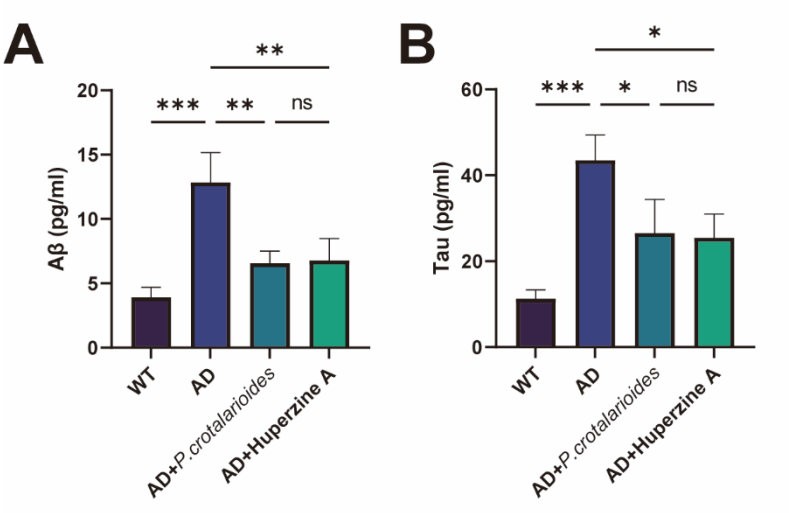

Figure 5. P. crotalarioides enhanced Aβ and Tau elimination in AD mice. (A)The concentration of Aβ and (B)Tau measured by ELISA. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns means no significance $P \geq 0.05$.