*Full Length Research Paper*

# Study on the anti-dementia and neural protection effects of ethamol soluble extract of Polygalae Radix

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Polygalae radix, the root of *Polygala tenuifolia* Willd, has been widely used as a drug for the treatment of amnesia, calm, convulsions, aging, physical weakness, abnormal intelligence, etc, in traditional Chinese medicine. Recent research indicated that Polygalae radix has the function of neural protection. Extractions and fractionation of Polygalae radix were used as effective compounds in this research, which mainly focused on the expression of Tau and BACE1, symbolic markers for Alzheimer's disease. The result indicated that Polygalae radix could reduce the expression of Tau and BACE1 after the treatment. This may improve and protect the normal function of the nervous system and reduce the harm of Alzheimer's disease. Amyloid plaques are characteristic features of Alzheimer's disease and believed to be neurotoxic, which could be stained with Thioflavin S. The result indicated that the amyloid plaques were reduced significantly, this suggested that the symptom of Alzheimer's disease was improved and the neurotoxic effect reduced. By Morris water maze tests, the study and memory ability of mice were improved after the treatment of Polygalae radix.

**Key words:** Polygalae radix, dementia, Morris water maze tests, BACE-1, Tau, amyloid plaques, Thioflavin S staining.

## INTRODUCTION

*Polygala tenuifolia* is a plant in the family of Polygalaceae. It is one of the fundamental herbs used in traditional Chinese medicine, and is considered a small medicinal herb. Recently, study on the extract of dried roots of *P. tenuifolia* has shown that it could enhance the memory of healthy adults and even elderly people (Zhang et al., 2008; Yun et al., 2007). Calm and anti-convulsion, anti-aging, prevent dementia and brain protection effects, physical strength and intelligence enhancing, dopamine receptor activity have been reported (Howes and Perry, 2011; Karakida et al., 2007; Lee et al., 2009). Furthermore, many *in vitro* experiments have tested the use of the herb in Alzheimer's disease, memory disorder, depression, cognitive defects, amnesia, neurotoxicity,

degenerative disease and dementia among others (da Rocha et al., 2011; Jesky and Hailong, 2011; Lee et al., 2004). However, there is little research for *in vivo* study on the neural protection and treatment. Whether Polygalae radix could provide some benefit for dementia, such as AD *in vivo*, still remains unknown.

Alzheimer's disease (AD) is the most common form of dementia, which occurs mainly in old people usually over 65 years. Symptoms of AD include confusion, mood swings, irritability, difficulty with language, aggression, long-term memory loss, and so on (Di Stefano et al., 2011). This disease will worsen as it progresses, and eventually leads to death (Green et al., 2011). This disease is identified as a protein misfolding disease, caused by accumulation of abnormally folded Aβ and tau proteins in the brain (Khairallah and Kassem, 2011; Götz et al., 2011). There is no effective cure for AD, so herbs may help us to find a new way to treat this disease. This research mainly studied the effects of ethanol soluble

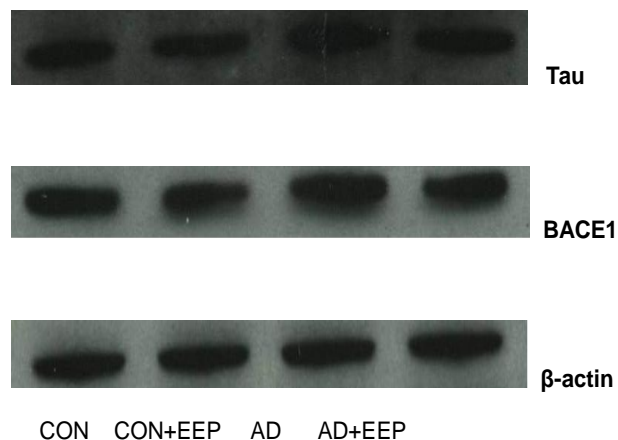*Corresponding author. E-mail: happyzhou@vip.163.com, yaoyunyiyyy@126.com.

**Figure 1.** The expression of Tau and BACE1. Tau and BACE1 in 4 groups (lane from left to right: CON, CON + EEP, AD, AD + EEP) were detected using Western Blot. β-actin was used as control.

extraction and fractionation of Polygalae radix on the treatment of AD mice.

## MATERIALS AND METHODS

### Extraction and fractionation of Polygalae radix

Dried Polygalae radix, purchased from Huaxi Hospital, Chengdu, China, were pulverized and extracted three times with EtOH at room temperature. The combined crude extract was dissolved in ddH$_2$O and extracted with n-hexane, EtOAc, and n-BuOH. The n-BuOH solution was concentrated to produce ethanol soluble extract of Polygalae radix (EEP) by centrifugation (300 g × 5 min). Separation of EEP from the fraction was chromatographed with increasing concentrations of EtOH in ddH$_2$O (0% → 25% → 50% → 75% → 100%) respectively. The EEP was dissolved in 0.9% sterile normal saline. 50% EEP at the concentration of 8 g/kg was used (p.o) to treat both the normal (CON group) and Alzheimer's disease mice (AD group) according to the former reports for totally 1 month.

### Generation of animal model

Male Kunming mice at the age of 8-month were selected and raised at the room temperature (24 ± 2°C) and humidity (55 ± 15%). 120 mg/kg D-Galactopyranose and 90 mg/kg sodium nitrite were intraperitoneally injected into these mice once per day for 2 months. Meanwhile, 2 µl poly-Aβ$_{5 \text{ to } 35}$ (Sigma, USA) was injected into left lateral ventricle of mice at the age of 9-month, one time for ideal model generation. These mice were used at the age of 10-month as Alzheimer's disease mice (AD mice). Half of these mice were treated with EEP at the concentration of 8 g/kg (AD + EEP group) according to the former research at the age of 9-month for totally 1 month treatment of EEP (p.o). 10-month normal mice were selected as negative control (CON group); while half of these normal mice were treated with EEP at the concentration of 8 g/kg (CON + EEP group) at the age of 9-month for 1 month (p.o) completely.

### Western blot

Cortex in hippocampus region of the mouse was collected and total protein was isolated in each group. The protein concentration was measured by BCA (Invitrogen, USA). Total protein (15 µg) was boiled at 100°C for 5 min and then subjected to 12.5% SDS-PAGE (Invitrogen, USA). After electrophoresis, gel was transferred onto NE membrane in transfer buffer (70 V, 1 h). After transfer and blocking in 5% nonfat milk for 1 h, membrane was incubated overnight at 4°C with 1$^{st}$ Ab (Tau and BACE-1, rabbit polyclonal IgG, Millipore, USA) in blocking buffer. After washing with 1X TPBS for 3 times, the membrane was incubated with 2$^{nd}$ Ab for 1 h at room temperature, washed again with 1X TPBS for 3 times, photos were taken by films and analyzed according to the density.

### Morris water maze tests

Morris water maze tests were performed according to the protocol. After the mice were familiar with the testing environment, the normal training was performed the next day. The experiments were divided into 2 parts: The first one is the orientation test. Mice were trained twice per day, one time in the morning and one time in the afternoon. Each training lasted 120 s and the gap time was 30 s. This training lasted for 4 days. Start area was randomly selected and the times of mice touching the platform in 120 s were recorded. Another test is space exploration. The platform was removed and the mice were put into water at the opposite side of platform. The percent of residence time in the central area and times for transferring the platform in 120 s were recorded.

### Thioflavin-S staining

Brains of mice in 4 groups were embedded in OTC and quickly removed on dry ice. Coronal sections were dissected at the thickness of 20 µm. Slides were deparaffinized in 4% PFA (Sigma, USA), hydrated to distilled water, washed in running water for 5 min and then rinsed in distilled water. Slides were placed in 1% Thioflavine S solution (Sigma, USA) for 10 min and then differentiated in 70% alcohol for 15 min. Finally, slides were rinsed in distilled water, two changes, mounted in glycerin jelly. Pictures were taken using confocal microscope (Leica, Germany) and analyzed by Image J (NIH, USA).

### Statistical analysis

All data are expressed as mean ± SD. Two-way ANOVA followed by Tukey's test was used to evaluate differences among groups of more than three. Student's test was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $p < 0.05$, while $p < 0.01$ was considered as obviously significant.

## RESULTS

### Data analysis for western blot

The expression of Tau and BACE1 were shown in Figure 1. The expression of Tau and BACE1 were obviously increased in AD mice group compared with CON group, the same as an earlier report. As shown in Figure 1, the expression of Tau and BACE1 were down-regulated after treatment with EEP compared with the AD group, however, still higher than CON group. No significant changes were found between CON and CON + EEP
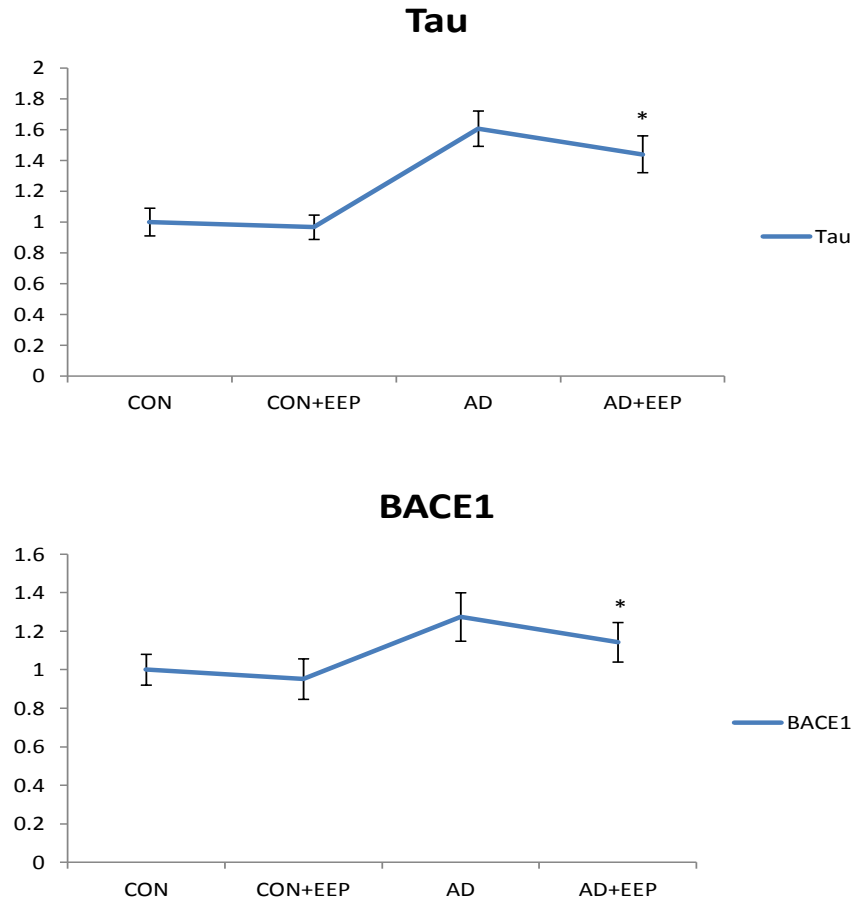
## Tau



## BACE1



**Figure 2.** Analysis on the expression of Tau and BACE1. The value in group CON was set as 1 while the relative value in other groups was compared with CON. $p < 0.05$ was considered as statistic significance.

**Table 1.** Morris water maze tests; latent period for escape and the percent of crossing the central area were measured in 4 groups (CON, CON + EEP, AD, AD + EEP).

| Variable | CON | CON + EEP | AD | AD + EEP |
|---|---|---|---|---|
| Latent period for escape (t/s) | 19.7 ± 3.1 | 17.3 ± 4.3 | 40.0 ± 4.2 | 26.3 ± 4.4* |
| Central area (%) | 31.5 ± 3.9 | 31.1 ± 4.2 | 18.8 ± 2.8 | 22.1 ± 3.0* |

* Significant change compared with AD group ($p < 0.05$).

groups. The value of CON group was set as 1 and the values of other groups were compared with CON group by density analysis. All data were analyzed in Figure 2.

### Comparison of study and memory ability

As shown in Table 1, the latent period of escape in the swimming orientation test in AD group was much longer than CON group, the same as an previous report. It was much shorter in AD + EEP group compared with AD group after EEP treatment, however, still longer than

CON group. In the space searching test, compared with CON group, the percent of residence time and the times passing platform were decreased obviously in AD group, while increased in AD + EEP group compared with AD group. For the normal groups, there were no significant changes between CON and CON + EEP.

### Thioflavin-S staining

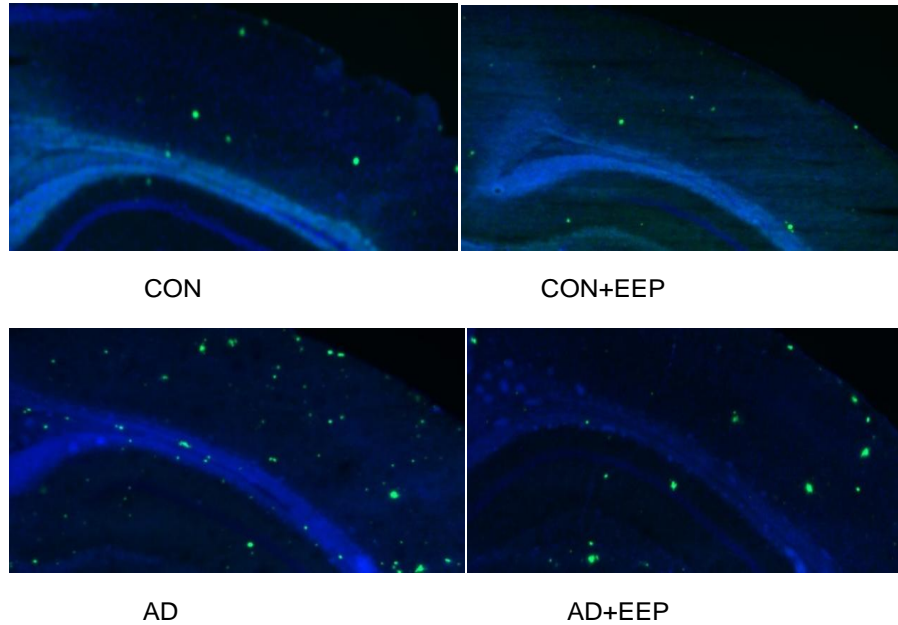As shown in Figure 3, compared with CON group, the amyloid plaques, stained in green colour with Thioflavin S

**Figure 3.** Amyloid plaques with Thioflavin S staining. Amyloid plaques in the cortex of hippocampus were stained using 1% Thioflavin S solution.
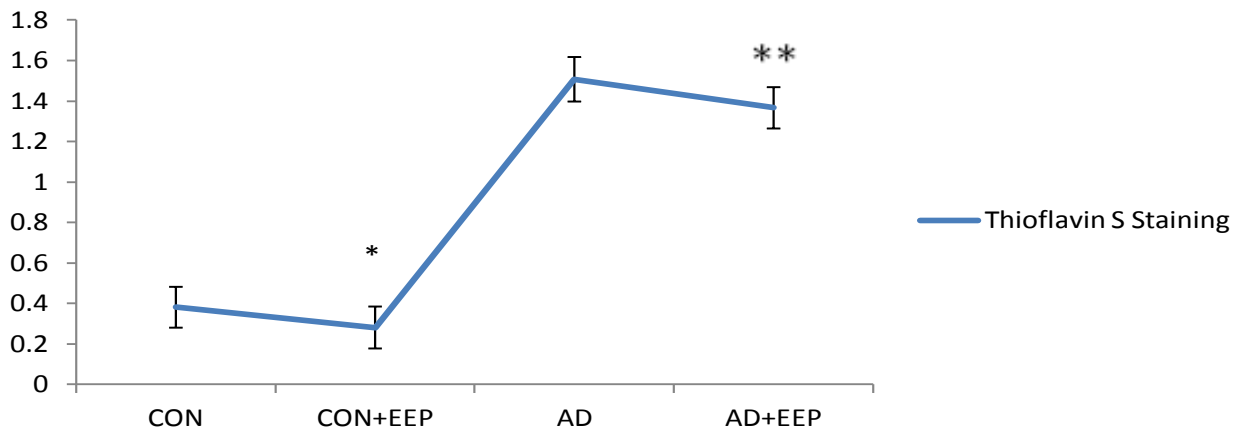


**Figure 4.** Analysis of Thioflavin S staining. Data were measured by comparing the area of amyloid plaques with total area (%). $p < 0.05$ and $< 0.01$ were considered as statistic significance.

staining using confocal laser microscope (Leica, Germany), were obviously increased in the cortex of the hippocampus of AD group, which suggested that the AD models were generated successfully. After the treatment of EEP, the amyloid plaques were decreased obviously, while still more than CON group. The amyloid plaques also decreased in CON + EEP compared with CON group. These results indicated that EEP could reduce the

formation of amyloid plaques both in normal or AD mice, thus reducing the damage of the cortex of the brain. Data analysis was shown in Figure 4.

**DISCUSSION**

Dementia means a person with serious loss of cognitive

ability, brain injury, and long-term decline due to damage or disease in the body. Loss of neurons and synapses in the cerebral cortex and other certain subcortical regions were found in AD patients (Sperling et al., 2011; Sokolowski and Mandell, 2011). There is no effective cure for AD, which worsens as it progresses, and finally leads to death. More and more evidence indicates that AD is associated with amyloid plaques, characteristic features of Alzheimer's disease and is believed to be neurotoxic in the brain (Sodhi et al., 2011).

β-Secretase 1 (BACE1), an aspartic-acid protease, plays an important role in the pathogenesis of AD (Griffiths et al., 2011). Tau proteins could stabilize microtubules (Iqbal et al., 2010). Abnormally high expression of tau and BACE1 proteins was found in Alzheimer's disease patients (Gozes, 2010; Bajda et al., 2011). Our research found out that the expression of tau and BACE1 were obviously increased in AD group, which was in accordance with the previous studies. After being treated with EEP, both of the expression of tau and BACE1 were attenuated significantly, while still higher than CON. For the normal mice, after being treated with EEP, the expression of tau and BACE1 were also down-regulated, however, without significant changes. These results indicated that EEP could reduce the expression of tau and BACE1 and thus may enhance the function of AD mice brain.

Thioflavin S is a homogenous mixture of compounds that results from the methylation of dehydrothiotoluidine with sulfonic acid. It is mainly used to stain amyloid plaques, which is considered to be a golden method (Lindgren and Hammarström, 2010). When stained with Thioflavine S, the amyloid plaques will fluoresce a green colour using the fluorescence microscope. Our research found out that there were a few amyloid plaques in normal mice. We considered these spots a normal senile phenomenon. The amyloid plaques obviously increased in AD mice group compared with control group. These plaques obviously decreased after the treatment of EEP, however, not better than CON group. Meanwhile, in the normal groups, we also found the decreased amyloid plaques in mice treated with EEP (CON + EEP). The results indicated that EEP could decrease the formation of amyloid plaques and thus reduced the harm and damage of these plaques to the brain, both in normal and AD mice brains.

Finally, we detected the effects of EEP on the memory and learning abilities of mice in all groups. The Morris water maze test is a behavioral procedure widely used in behavioral neuroscience to study spatial learning and memory (Gerlai, 2001). For Morris water maze test, the learning and memory abilities of AD mice were decreased compared with normal mice while obviously improved after the treatment of EEP. There were no significant changes between CON and CON + EEP groups. This test indicated that EEP could improve the memory, learning ability and function of brain in AD mice, but no obvious effects of improving the memory, learning ability and

function of brain were found in normal mice after the treatment of EEP. The concentration of EEP was high in hippocampus, which indicated that EEP could protect the CNS and promote the growth of neurons.

Polygalae radix has been well used in traditional Chinese medicine. Ethanol soluble extraction and fractionation of Polygalae radix were used as effective compounds in this research. We found out EEP could down-regulate the expression of Tau and BACE1, two symbolic markers for Alzheimer's disease. Also, EEP could eliminate the amyloid plaques deposited in the cortex in hippocampus region. All these suggested that the symptoms of Alzheimer's disease were improved and the neurotoxic effect reduced. By Morris water maze tests, the study and memory ability of mice were improved after the treatment of Polygalae radix. This research could help us to better understand and discover potential drugs to treat or prevent AD.

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