# 2023 S.T. Yau High School Science Award

## **Research Report**

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# **Title of Research Report**

Exploring the Effect of *Polygonum multiflorum* Thunb on  $A\beta_{1-42}$  Oligomer-Burdened Microglia Model for Alzheimer's Disease Based on Network Pharmacology and Experiments *in vitro* 

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**Research Report** 

# Exploring the Effect of *Polygonum multiflorum* Thunb on Aβ<sub>1-42</sub> Oligomer-Burdened Microglia Model for Alzheimer's Disease Based on Network Pharmacology and Experiments *in vitro*

Yifei Zuo & Jonathan Yang

#### Abstract

Alzheimer's disease (AD) is the most common degenerative disease of the central nervous system, with an increasing death rate but no effective treatment strategy. Traditional Chinese medicine (TCM) is becoming an attractive alternative approach for the understanding and cleaning of amyloid  $\beta$  (A $\beta$ ) accumulation in AD. *Polygonum multiflorum* Thunb (Heshouwu) has been widely reported to be effective for AD via meta-analysis and network pharmacology. Rhein in Polygonum multiflorum Thunb was isolated as the most promising chemical compound with the highest potential in producing anti-oxidative results. Microglia cell lines were utilized in trials to assess mitochondrial availability. To explore the effect and mechanism of Rhein on AD treatment,  $A\beta_{1-42}$  oligomer-burdened microglia cell line model was constructed. Rhein was found effective in reducing the inflammatory activation of microglia caused by  $A\beta_{1-}$ <sup>42</sup> oligomer incubation and alleviating microglial mitochondrial dysfunction resulting from Aβ<sub>1</sub>-42 oligomer incubation. Our study is the first to demonstrate Rhein's therapeutic effects on neuroinflammation caused by microglia. This finding further supports the feasibility of Polygonum Multiflorum Thunb, a TCM, as a potential drug for AD treatment. These results broaden our knowledge of improving mitochondrial function as an approach for relieving microglia oxidative and over-activation stress in AD.

Keywords: Alzheimer's disease, Traditional Chinese medicine, Network pharmacology, Microglia

#### Declaration of Academic Integrity

The participating team declares that the paper submitted is comprised of original research and results obtained under the guidance of the instructor. To the team's best knowledge, the paper does not contain research results, published or not, from a person who is not a team member, except for the content listed in the references and the acknowledgement. If there is any misinformation, we are willing to take all the related responsibilities.

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# 1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects a person's memory, cognitive ability, and behavior. It is the most common type of dementia, accounting for 60-80% of all cases (Kumar et al., 2023). While the disease typically affects individuals over 65 years old, early-onset Alzheimer's can occur in people as young as their 30s or 40s (Kumar et al., 2023). It's quickly becoming one of the most expensive, lethal, and burdening diseases of this century.

AD is characterized by the accumulation of amyloid  $\beta$  (A $\beta$ ) peptides, a class of abnormally folding proteins in the brain. This accumulation leads to the death of neuronal cells and the shrinkage of brain tissue (Jack et al., 2016). Currently, scientists use various biomarkers in an attempt to track the process of the development of Alzheimer's based on the ATN framework: A (amyloid), T (phosphorylated tau), and N(neurodegeneration). These biomarkers potentially provide the chance of individualized risk modeling (Ittner & Gotz, 2023). As the disease progresses, individuals may experience difficulties with daily activities, have trouble in communication, and undergo noticeable changes in mood and behavior. Currently there is no cure for AD – there are only some treatments that can help manage the symptoms and improve quality of life. Ongoing research on AD aims to understand the underlying mechanisms of the disease better and develop new therapies. Recently, traditional Chinese medicine (TCM) has made excellent progress and is expected to provide a new possibility for AD treatment.

There is no clear concept of Alzheimer's in TCM theory. Instead, it is classified under the broader category of dementia, and various treatments that have been identified as effective in treating dementia offer new insights for AD treatment (Pei et al., 2020). The concept of "treatment based on syndrome differentiation" in TCM allows the compound prescription to act across the entire set of symptoms, having a more holistic effect. However, due to the variations and differences in TCM prescriptions and acupoint selections, the clinical efficacy of combined interventions can be unstable and inconsistent. The underlying therapeutic mechanism of TCM is not clear, either. Studying the phytochemistry and interpretation of TCM formula can help to understand the core pharmacological activities of TCM in AD treatment.

Meta-analysis and network pharmacology were adopted in this study. Massive amounts of information from multiple databases were used to produce accurate results. Meta-analysis uses statistical methods and screenings to produce reliable sets of scientific papers. This approach is

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especially useful in determining which studies are promising for future research, as well as reaffirming the reliability of past research. Network pharmacology, a recent development in drug discovery, utilizes advancements in computing technology to revolutionize the one-to-one drug-to-target approach. It allows for enhanced drug target determination, as well as multi-compound and multi-target approaches.

To date, there is no cure for Alzheimer's. TCM is an attractive approach for understanding and cleaning the A $\beta$  accumulation in AD. In order to determine whether TCM is effective in treating AD, a meta-analysis was conducted to select the best herb for experimentation. Once selected, we used network pharmacology to select a core chemical component of the herb and designed the experiments to test its effects. An *in vitro* experiment was designed to observe the effects of the chemical components on microglial inflammation and oxidation characteristics of Alzheimer's. Ideally, the data obtained could provide a deeper understanding on TCM in AD treatment. Rhein, a component to be discussed later in the study, could be a promising component on the path towards a drug to combat AD.

# 2. Method

#### 2.1. Meta analysis

#### 2.1.1. Search Strategy

The PubMed database was searched independently by researchers until May 24, 2023. There were no date limits regarding the publication date of the included studies. The search was carried out by combining subject terms and free words. All Clinical trials of TCM for treating AD were collected. Search terms included "traditional Chinese medicine" and "Alzheimer's Disease"

The retrieval strategy is shown in Fig. 1.



Fig 1. Search process depicted by the PRISMA flowchart

#### 2.1.2. Inclusion and Exclusion Criteria

Inclusion and exclusion criteria were formulated based on the principle of PICOS (P-population; I-intervention; C-comparison; O-outcome; S-study design):

- Study design: published RCT clinical trials. The language of materials was limited to English or Chinese.
- 2) Population: patients who were diagnosed with AD or met the diagnostic criteria, such as "AD was diagnosed according to the American Diagnostic and Statistical Manual of Mental Disorders And the standard diagnosis criteria of the National Institute of Neurological Disorders and Stroke"(Chen et al., 2015), there was no limitation in patients' gender, age, nationality, race, occupation, education level, course, and severity of the disease. The baseline of the same RCT was balanced (P > 0.05). Participants suffering from hypertension, diabetes, hyperlipidemia, and other underlying diseases were not considered.
- Intervention and comparison: the experimental group was treated with TCM alone. The control group was treated with a western medicine, such as donepezil, which had proven positive effects in treating AD.

 Outcome: The outcome of effect for treating AD was measured by Mini-mental state examination (MMSE) scores.

## 2.2. Network pharmacology

TCM patients were examined through Mini-mental state examination (MMSE) score, ability of daily living scale (ADL), and other neurophysiological data to determine a suitable drug for experimentation. *Polygonum multiflorum* Thumb (Heshouwu) was selected due to its high relevance, low statistical error, and positive neurophysiological effect (Tekleab et al., 2021).

Chemical components of *polygonum multiflorum* were screened using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (https://tcmsp-Encyclopedia e.com/tcmsp.php) and the of Traditional Chinese Medicine (http://www.tcmip.cn/ETCM/) database. The screening progress utilized evaluations of molecular weight, alogP, hydrogen donor & acceptors, blood brain barrier permeance, and other characteristics. Target genes of chemical components were determined using the Encyclopedia of Traditional Chinese Medicine database in order to determine relevance in regards to AD; target genes regarding AD in humans were determined using GeneCards: The Human Gene Database (https://www.genecards.org/). JVenn (https://www.bioinformatics.com.cn/static/ others/jvenn\_en/usermanual.html) was utilized in order to determine the intersection between the two sets of gene data. All intersection genes were imputed into the PPI network (STRING: functional protein association networks (string-db.org)), and Cytoscape 3.10.0 software was used to visualize and determine degree in order to find core target genes with high degrees. Upon core target gene determination, the gene was matched to chemical components accordingly one by one in excel. The core target gene list was then imputed into SRplot (https://www.bioinformatics.com.cn) to perform Gene Ontology (GO) and Pathway Enrichment analysis to obtain results regarding the function of core components. Rhein was then selected as our target component based on its chemical characteristics, molecular functions, and relevance in past studies.

### 2.3. Cell culture

BV2 Cells, a microglial sub-line derived from C57/BL6 murine brain, were purchased from Procell Life Science Technology Co., Ltd. (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100U/ml penicillin, 100 mg/ml streptomycin, and 100 mg/ml amphotericin in a 37°C, 5% CO<sub>2</sub>/95% air incubator. The cell medium was changed every two days.

### 2.4. Rhein treatment and cell viability assay

Rhein, a lipophilic anthraquinone, was purchased from MedChemExpress (HY-N0105) as a yellow-brown powder. 14 mg of Rhein was dissolved in 12.31 mL DMSO at 50 °C for 4 hours with a final concentration of 4 mM.

Cells were seeded into 96-well plates at a concentration of  $5-6\times10^4$  cells/ml and treated with different concentrations of Rhein: 0 µM, 1 µM, 2 µM, 4 µM, 10 µM, 15 µM, 20 µM, and 50 µM. The outermost wells received additional PBS to avoid evaporation. Cell viability was evaluated via Cell Counting Kit-8 (CCK-8) assay, followed by incubation at 37°C for 1 h. Absorbance was measured at 450 nm using an automated microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was tested by absorbance and the data was transformed into a percentage of the cell viability of the control culture value.

#### 2.5. Aβ<sub>1-42</sub> Oligomer-burdened microglia model for AD

A $\beta$  oligomers were grown from synthetic A $\beta_{1-42}$  peptides. Peptide was added to fresh anhydrous 100% DMSO to form a peptide film, which was then diluted with culture solution at 4°C for 24 h with a final order of 50  $\mu$ M. BV cells were incubated with A $\beta_{1-42}$  oligomers (2  $\mu$ M) for 24 h to construct the A $\beta_{1-42}$  oligomer-burdened microglia model for AD.

### 2.6. Phalloidin staining to observe the morphology

Cells were seeded into 12-well plates at a concentration of  $5-6\times10^4$  cells/ml. After A $\beta_{1-42}$  oligomers and/or Rhein treatment, cells were rinsed with PBS and subsequently treated with 3.7% PFA for 10 min at room temperature. PBS washing was performed 3 times for 5 minutes every wash. After using PBS with 0.1% Triton X for cell permeabilization, the cells were then washed with PBS again. The Actin-Tracker Green-488 was added to the film at a ratio of 200  $\mu$ L per film and incubated at room temperature for 30-60 minutes away from light. DAPI (1  $\mu$ g/mL) was used to stain the cell nucleus.

#### 2.7. RNA extraction and real-time PCR

RNA was then extracted from cells using standard extraction methods with Trizol, dissolving in deionized water, and quantification with spectrophotometer. RNA was reverse transcribed into DNA through the use of reverse transcriptase. Primers were designed and

Tgfb1-FP	TGATACGCCTGAGTGGCTGTCT
Tgfb1-RP	CACAAGAGCAGTGAGCGCTGAA
II10 -FP	CGGGAAGACAATAACTGCACCC
II10 -RP	CGGTTAGCAGTATGTTGTCCAGC
CD206-FP	GTTCACCTGGAGTGATGGTTCTC
CD206-RP	AGGACATGCCAGGGTCACCTTT
TNF-a-FP	GGTGCCTATGTCTCAGCCTCTT
TNF-a-RP	GCCATAGAACTGATGAGAGGGA G
II1b -FP	TGGACCTTCCAGGATGAGGACA
II1b -RP	GTTCATCTCGGAGCCTGTAGTG
Cd86-FP	ACGTATTGGAAGGAGATTACAG CT
Cd86-RP	TCTGTCAGCGTTACTATCCCGC
iNos -FP	GAGACAGGGAAGTCTGAAGCAC
iNos -RP	CCAGCAGTAGTTGCTCCTCTTC
Arg1-FP	CATTGGCTTGCGAGACGTAGAC
Arg1-RP	GCTGAAGGTCTCTTCCATCACC

synthesized for the target gene (Table 1). New cDNA was amplified with real-time PCR, and fluorescence was measured. The changed ratio was analyzed with the  $2^{-\Delta\Delta ct}$  method.

Table 1. Primers for real time PCR

## 2.8. Mitochondrial function assay

The disruption of mitochondria function can be detected using a variety of immunochemistry-based assays including measurements of enzyme activity, superoxide, and membrane potential. There are 4 groups settled: control,  $A\beta_{1-42}$  oligomers,  $A\beta_{1-42}$  oligomers+Rhein (4  $\mu$ M),  $A\beta_{1-42}$  oligomers+Rhein (20 $\mu$ M).

Cytochrome P-450 activity was first measured using a sandwich ELISA kit according to manufacturer instructions. Post-treatment BV2 cells were washed briefly with cold PBS and lysed in an extraction solution. The standard well and sample well were then set by adding different concentrations of 50  $\mu$ L standard solutions to every standard well. Add sample diluent 40  $\mu$ L after adding 10  $\mu$ L sample to the sample well, and leave the blank well blank. HRP

labeled detecting antibodies were added to every well except for blanks, which was followed by incubation at 37°C for 1 h. The absorbance was then measured at 450 nm using an Epoch Microplate Spectrophotometer.

Mitochondrial membrane potential in BV2 cells was detected using a TMRE (tetramethylrhodamine, ethyl ester) probe. Cells were cultured in 12-well plates and stained with the TMRE staining kit according to manufacturer instructions. TMRE staining solution was added quickly to 6-well plates followed by culture at 37°C for 20 min. Cells were washed twice to completely remove excessive staining solution. Preheated cell culture medium was added and fluorescence images were taken under a fluorescence microscope.

Reactive Oxygen Species (ROS) in BV2 cells were detected using a H<sub>2</sub>DCFDA probe. BV2 cells were cultured and treated in 96-well plates. H<sub>2</sub>DCFDA (5  $\mu$ M) was added to wells. After incubation for 30 min at 37 °C, cells were washed to remove the excessive probes. Fresh culture medium was added and fluorescence density (488 nm) was taken under a Fluorescent Microplate Spectrophotometer.

# 3. Results

#### 3.1. Meta study selections and the characteristics of included studies

The literature search and selection process for systematic review is shown in PRISMA flowchart (Fig. 1). A total of 37 records were retrieved from the preliminary search. After excluding 27 records, 10 records remained for screening based on the titles and abstracts. The full text of 10 studies were examined to determine eligibility and were included in the final review (Table 2).

Among all the studies, a total of 772 AD patients were included in the population, with little variance among the studies. MMSE is commonly used to examine the cognitive ability of the AD patients, and the score before and after the treatment is an efficient way to measure the effect of the trial. ADL is also often used to measure the ability of daily living of AD patients. Details of the study characteristics are shown in Table 1.

The control group of the studies commonly used donepezil as the positive western medicine. Other drugs such as Aricept and Piracetam were also used in some studies. The formula of TCM decoction used in every study contains more than three types of herbs. For herbs in the formula of TCM decoction, Heshouwu (*Polygonum multiflorum* Thunb) was the most commonly used herb in all studies, appearing 4 times.

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1st Author (year)	Intervention (N)	Outcome	Herbal Formula
Shui- Miao Lin (2003)	Tianxin Recipe (20), Bushen Recipe (20), Donepezil (20)	MMSE Score, ADL Score, Neuropsychology	Tiaoxin Recipe: Dangshen (Codonopsis pilosula), Yuanzhi (Radix Polygalae), Fuling (Sclerotum Poriae Cocos), Guizhi (Ramulus Cinnamomi), Shichangpu (Acorus gramineus), Zhigancao (Acorus gramineus), et al. Bushen Recipe: Shudihuang (Radix Rehmanniae), Gouqizi (Fructus Lycii), Shanyurou (Corni Fructus), Maidong (Ophiopogon japonicus), et al.
Zhi-Lin Zhou (2007)	Reinhardt and Sea Cucumber Capsule (22), Donepezil (22), Combined Treatment (22)	MMSE Score, ADAS-Cog Score, ADL Score, level of thyroid hormones	Reinhardt and sea cucumber capsule: Haishen ( <i>Stichopus</i> <i>japonicus</i> ), Yuanzhi ( <i>Radix</i> <i>Polygalae</i> ), Shichangpu ( <i>Acorus</i> <i>gramineus</i> )
Lie Chen (2010)	Compound Polygonum Multiflorum Extract (120), Pure Polygonum Multiflorum Extract (60), Naofukang (29)	MMSE Score, ADL Score, Therapeutic effect	Compound Polygonum Multiflorum Extract: Huangjing ( <i>Polygonum</i> <i>multiflorum</i> ), Bajitian ( <i>Morinda</i> <i>officinalis How</i> ), Danggui ( <i>Angelica</i> <i>sinensis (Oliv.)Diels</i> ), Yizhiren ( <i>Alpinia oxyphylla</i> ), Yinxingye ( <i>Ginkgo biloba</i> ), Shichangpu ( <i>Acorus gramineus</i> ), et al.
Lu Yu (2012)	Tiaoxin / Bushen Recipe (66), Donepezil (65)	MMSE Score, FOM, DS, BD, fMRI	Tiaoxin Recipe: Dangshen (Codonopsis pilosula), Yuanzhi (Radix Polygalae), Fuling (Sclerotum Poriae Cocos), Guizhi (Ramulus Cinnamomi), Shichangpu (Acorus gramineus), Zhigancao (Acorus gramineus), et al. Bushen Recipe: Shudihuang (Radix Rehmanniae), Gouqizi (Fructus Lycii), Shanyurou (Corni Fructus), Maidong (Ophiopogon japonicus), et al.
Ping Liu (2013)	Bushenhuatanyizhi (30), Piracetam (30)	MMSE Score, ADL Score, Clinical effect, SOD activity, content of lipid peroxides, Triglyceride levels	Bushenhuatanyizhi: Heshouwu (Polygonum multiflorum Thunb), Zhujieshen (Rhizoma Panacis Japonici),

# Table 2. Meta analysis of research in clinical treatment of AD using TCM

1st Author (year)	Intervention (N)	Outcome	Herbal Formula
Chen Songlin (2015)	Aricept (33), Shenfu & DCBI (18), Shenmai & DCBI (15)	MMSE <sup>a</sup> Score, ADL <sup>b</sup> Score, CDR <sup>c</sup>	Shenfu Injection: Hongshen ( <i>Radix</i> Ginseng Rubra), Fuzi ( <i>Radix Aconti</i> Lateralis Preparata) Shenmai Injection: Hongshen ( <i>Radix Ginseng Rubra</i> ), Maidong ( <i>Radix Ophiopogonis Japonici</i> )
Yulian Zhang (2015)	Yishen Huazhuo Decoction (72), Donepezil (72)	ADAS-Cog <sup>d</sup> , MMSE Score, ADL Score, Neuropsychiatric Inventory	Yishen Huaozhuo Decoction: Yinyanghuo ( <i>Epimedium</i> ), Nvzhenzi ( <i>Fructus Ligustri Lucidi</i> ), Buguzhi ( <i>Psoralea Fruit</i> ), Heshouwu ( <i>Polygonum multiflorum</i> <i>Thunb</i> ), Huangqi ( <i>Radix Astragali</i> ), Chuanxiong ( <i>Ligusticum wallichi</i> <i>franchat</i> ), Shichangpu ( <i>Acorus</i> <i>gramineus</i> )
Yang Yang (2019)	Huannao Yicong (28), Donepezil (24)	ADAS-Cog, Chinese Medicine Symptom Scale, MMSE Score, Montreal Cognitive Assessment, MMSE Score, Serum levels of acetylcholinesterase, Aβ42	Huannao Yicong: Zhi Shou Wu (Radix Polygoni Multiflori), Ren Shen (Radix Ginseng), Chuan Xiong (Rhizoma chuanxiong), Shi Chang Pu (Rhizoma Acori tatarinowii), Huang Lian (Rhizoma coptidis)
Wang Hui Chan (2020)	Jiannao Yizhi (30), Donepezil (30)	ADAS-Cog, Chinese Medicine Symptom Scale, MMSE Score, Montreal Cognitive Assessment, MMSE Score, ADL Score, Serum levels of acetylcholine, Aβ42, Tau	Jiannao Yizhi: Zhi Shou Wu ( <i>Radix</i> <i>Polygoni Multiflori</i> ), Ren Shen ( <i>Radix Ginseng</i> ), Chuan Xiong ( <i>Rhizoma chuanxiong</i> ), Shi Chang Pu ( <i>Rhizoma Acori tatarinowii</i> ), Huang Lian ( <i>Rhizoma coptidis</i> )
Tatsuya Nogami (2023)	Jia Wei Gui Pi Tang (33), Placebo (30)	Neuropsychiatric Inventory Nursing Home Version, Delightful Emotional Index	Jia Wei Gui Pi Tang: Huang Qi (Astragali Radix), Chai Hu (Bupleuri Radix), Suanzaoren (Zizyphi Semen), Cang Zhu (Atractylodis Lanceae Rhizoma), Hong Shen (Ginseng Radix), Fu Ling (Poria), Yuanzhi (Polygalae Radix), Zhi Zi (Gardeniae Fructus), Da Zao (Zizyphi Fructus), Du Huo (Angelicae Radix), Gan Cao (Glycyrrhizae Radix), Gan Jiang (Zingiberis Rhizoma), Yun Mu Xiang (Saussureae Radix), Long Yan Rou (Longan Arillus)

<sup>a</sup> MMSE, Mini-mental state examination
 <sup>b</sup> ADL, Ability of daily living scale
 <sup>c</sup> CDR, clinical dementia rating
 <sup>d</sup> ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale

## 3.2. Risk of bias and certainty of evidence

All included papers were discussed regarding the standard for evaluating every type of bias (Fig. 2). All studies divided patients into control groups and experimental groups randomly, resulting in no Random sequence generation bias. Most of the included studies were assessed as low risk overall. Only a portion of the studies did well in allocation concealment. In some earlier studies including Lin et al.(2003), Zhou et al. (2007), and Chen et al.(2010), no blind experiments were used, meaning there is a high risk of performance bias and detection bias. It is possible that the design of RCT blinding clinical trials was not as polished at the time.

In some of the studies, quite a large percentage of patients did not finish the study, including Yu et al.(2012) and Zhang et al.(2015) (high risk in attrition bias) and Wang et al.(2020), Yang et al.(2019) (unclear risk in attrition bias). There is no bias of selective reporting in any of the studies.



Fig 2. Summary of bias risks based on the 10 studies.

# **3.3.** TCM provided equivalent therapeutic effects compared to positive control for AD

A total of 10 RCTs focusing on MMSE scores with a total sample size of 772 participants were included in the current meta-analysis. Lin et al.(2003) however, divided the TCM group further into Tiaoxin Recipe and Bushen Recipe randomly, so the data of this study has 2 columns. Figure 3 displays the contribution of each direct comparison result.

According to the results of the heterogeneity test,  $I^2 = 97\%$  and P < 0.05 were regarded as high heterogeneity. Thus, the random effect model was applied. As the forest plot demonstrates, the total mean difference result (95% CI) for TCM is 0.87 [-1.71, 3.45] and the test for overall effect of Z=0.66 (P=0.51 >0.05), which means there is no convincing evidence that points towards TCM having a better effect than western medicines. However, our results prove that TCM is at least as effective as positive western medicines, such as donepezil, in treating AD, which reveals the great potential of TCM as an alternative approach. It has the advantage of a manipulatable formula in the TCM decoction depending on the specific case. Further analysis shows that the study conducted by Chen et al.(2015) has the best efficiency 10.00 [8.93, 11.07] using the method of Dialectical thought.

Future studies will explore the fundamental components of different TCM herbs in order to improve the efficiency of treatment and develop new therapies.

		тсм			Control			Mean difference	Mean difference	
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI	L
Chen lie 2010	24.3	3.69	120	22.93	3.71	29	9.3%	1.37 [-0.13 , 2.87]		
Chen songlin 2015	27.7	2	33	17.7	2.4	33	9.5%	10.00 [8.93 , 11.07]		-
Lin shuimiao 2003 (BSR)	17.5	2.59	20	19.6	3.39	20	9.2%	-2.10 [-3.97 , -0.23]		
Lin shuimiao 2003 (TXR)	17.9	2.59	20	19.6	3.39	20	9.2%	-1.70 [-3.57 , 0.17]		
Liu ping 2013	20.7	1.6	30	18.7	2.1	30	9.5%	2.00 [1.06 , 2.94]	-	
Tatsuya Nogami 2023	11.4	8	29	13.5	6.1	30	8.1%	-2.10 [-5.74 , 1.54]		
Wang huichan 2020	20.93	2.39	27	21.96	2.65	24	9.4%	-1.03 [-2.42 , 0.36]		
Yang yang 2019	21.64	2.47	28	21.96	2.65	24	9.4%	-0.32 [-1.72 , 1.08]		
Yu lu 2012	20.71	5.7	55	18.62	6.55	52	8.9%	2.09 [-0.24 , 4.42]		
Zhang yulian 2015	22.37	5.31	54	20.6	4.52	50	9.2%	1.77 [-0.12 , 3.66]		
Zhou zhilin 2007	20.3	5.6	23	21.5	5.6	21	8.3%	-1.20 [-4.51 , 2.11]		
Total (95% CI)			439			333	100.0%	0.87 [-1.71 , 3.45]		
Heterogeneity: $Tau^2 = 17.92$ ; Test for overall effect: $Z = 0.6$	Chi <sup>2</sup> = 286. 66 (P = 0.51	.80, df = 1 )	0 (P < 0.0	00001); l²	= 97%				-10 -5 0 5	10
Test for subgroup differences	s: Not applic	able						Favour	s [experimental] Favour	s [contro

Fig 3. The forest plot for efficacy of TMC on MMSE score with highlighted 3 studies using *Polygonum multiflorum* Thunb as core treating herb

# 3.4. *Polygonum multiflorum* Thunb was selected to pharmacological evaluation

*Polygonum multiflorum* Thunb was the most utilized herb in TCM treatments, appearing in 40% of our included studies (Chen et al.(2010); Liu et al.(2013); Yang et al.(2019); Wang et al.(2020)) and playing the role of core treating herb in three studies ((Chen et al.(2010); Liu et al.(2013); Yang et al.(2019)). Moreover, based on the results in the forest plot (Fig. 3) we can see that the three studies using *Polygonum multiflorum* Thunb as the core treating herb had a relatively better effect in treating AD.

The other core treating herbs aside from *Polygonum multiflorum* Thunb are mostly in the Ginseng class, which already has a significant amount of research done. Based on the frequency of use in TCM herbal formulas, results from clinical trials and references, we selected *Polygonum multiflorum* Thunb for further pharmacological evaluation in the treatment of AD.

Using ETCM, we imported all chemical components and compiled detailed data for each chemical component of *Polygonum multiflorum* Thunb (Table 3). There are 19 different chemical components in total. The data is drawn from the ETCAM and TCMSP databases. Certain components have incomplete data with absence of formal CAS number.

Chemical components with an OB% less than 30% or a DL value less than 0.18 were eliminated, leaving Sitosterol, $\hat{I}$ '-Sitosterol, $\gamma$ -sitosterol, Rhein, and N-Trans-Feruloyltyramine.

Chemical Component	CAS No.	MW	AlogP	Hdon	Hacc	OB (%)	Caco-2	BBB	DL	FASA-	TPSA	RB N	HL
Sitosterol,Î'-Sitosterol,γ- sitosterol	83-47-6	414.79	8.08	1	1	36.91	1.33	0.88	0.75	0.22	20.23	6	5.05
Physcion	521-61-9	284.28	2.74	2	5	22.29	0.52	-0.4	0.27	0	83.83	1	-
Emodin	518-82-1	270.25	2.49	3	5	24.4	0.22	-0.66	0.24	0	94.83	0	-
Chrysophanol	481-74-3	254.25	2.76	2	4	18.64	0.62	-0.2	0.21	0.44	74.6	0	-
Chrysarobin, Chrysophanol-9- Anthrone	491-58-7	240.25397	3.381	2	3	-	-	-0.042	-	-	-	-	-
Piceid		390.42	1.11	6	8	21.44	-0.9	-1.81	-1.81	0.41	139.84	5	
Procyanidin B1 3'-O-Gallate	N/A	730.62449	4.882	12	16	-	-	Undefined	-	-	-	-	-
Rhein	478-43-3	284.23	1.88	3	6	47.07	-0.2	-0.99	0.28	0.47	111.9	1	32.1 9
1,3-Dihydroxy-6,7- Dimethylxanthone-1-O-Î'-D- Glucoside	N/A	4.882	1.522	5	9	-	-	Undefined	-	-	-	-	-
N-Trans-Feruloyltyramine	66648-43-9	313.38	2.86	3	5	86.71	0.55	-0.51	0.26	0.33	78.79	6	3.71
Guaijaverin	22255-13-6	434.38	-0.08	7	11	29.65	-1.34	-1.34	0.7	0.34	190.28	3	-

 Table 3. Complete chemical component and data for Heshouwu, not mentioned
 (Polygonum multiflorum Thunb)

Chemical Component	CAS No.	MW	AlogP	Hdon	Насс	OB (%)	Caco-2	BBB	DL	FASA-	TPSA	RB N	HL
6-Methoxy-2-Acetyl-3- Methyl-1,4-Naphthoquinone- 8-O-Î'-D-Glucopyranoside	N/A	422.38268	-0.294	4	10	-	-	Undefined	-	-	-	-	-
Polygoacetophenoside	N/A	346.28672	-1.328	7	10	-	-	Undefined	-	-	-	-	-
Questin	3774-64-9	N/A	2.74	2	5	20.44	0.43	-0.16	0.27	0.36	83.83	1	-
Resveratrol	501-36-0	228.26	3.01	3	3	19.07	0.8	-0.01	0.11	0.49	60.69	2	-
2,3,5,4'-Tetrahydroxystilbene- 2-O-(6"-O-Acetyl)-Î'-D- Glucopyranoside	N/A	448.41996	1.297	6	10	-	-	Undefined	-	-	-	-	-
2,3,5,4'-Tetrahydroxystilbene- 2-O-(6"-O-Î'-D- Glucopyranosyl)-Î'-D- Glucopyranoside	N/A	568.52387	-0.829	10	14	-	-	Undefined	-	-	-	-	-
2,3,5,4'-Tetrahydroxystilbene- 2-O-Î'-D-Glucoside	55327-45-2	406.38328	0.918	7	9	-	-	Undefined	-	-	-	-	-
2,3,5,4'-Tetrahydroxystilbene- 2,3-O-Î'-D-Glucoside	N/A	568.52387	-1.012	10	14	-	-	Undefined	-	-	-	-	-

# **3.5.** AD pathogenesis-associated target genes in *Polygonum multiflorum* Thunb's main bioactive targets

All 154 target genes of *Polygonum multiflorum* Thunb from ETCM and all 14407 target genes for Alzhelmer's Disease in humans were sourced from Genecard. The jVenn chart was used in order to find intersection genes among the two sets, and 111 intersection genes were found (Fig. 4 and supplementary table 1). These bioactive targets of *Polygonum multiflorum* Thunb were related to AD pathogenesis. The components involve over 100 target genes, including APP, COMT, PTGS2, CYP2D6, GRIN2B and PPARG.



Fig 4. The jVenn chart shown the intersection of target genes of AD and Polygonum multiflorum Thunb

# **3.6.** Protein–Protein Interaction network construction to find the core target genes

All 111 genes from previous steps were imputed into the PPI network with the selected organism Homo sapiens. The result was then downloaded and imported into Cytoscape (Fig. 5).

The string intersection chart was exported to Cytoscape for degree (the connection of genes to others) visualization of every gene. 47 core target genes with degree  $\geq 10$  (avg=10.7) were selected due to their high connectivity with other genes.



Fig 5. Protein–Protein Interaction network for *Polygonum multiflorum Thunb* therapeutic target related to AD pathogenesis.

#### **3.7.** Rhein was selected as the core chemical component.

The 47 core target genes were then matched with the chemical components one by one to obtain the core chemical components. The relationship between them was then analyzed with Cytoscape (Fig. 6). The chemical components which are linked to the most target genes are shown in figure 6. The interactions of the core chemical components were visualized in Cytoscape, and 8 outstanding core chemical components were selected for their high connectivity.

From the complete chemical components and data for Heshouwu (Table 2), rhein has a molecular weight 284.23, which falls in the range of 180-500 and is considered suitable for usage in drug formulation. Rhein has an AlogP value of 1.88, which falls in the range of 0-3, meaning it is easily absorbed. Rhein also has an OB(%) of 47.07, which is higher than 30, proving its high oral bioavailability. In addition, the Druglike data for Rhein is 0.28, which is



greater than 0.18, meaning its different characteristics are quite suitable for drug development. Based on the data above, Rhein was selected to be the final core component for this study.

Fig 6. Target genes and core chemical components interaction network of Polygonum multiflorum Thunb

# **3.8.** Core chemical components were mainly involved in the mitochondriamediated cellular function.

The core target gene list of Rhein was imputed into SRplot. GO pathway Enrichment Analysis was done to obtain the biological process (BP), cellular component (CC), and molecular function (MF) of the core genes (Fig. 7). The dot size shows the number of target genes that are involved in their corresponding entry, and the dot color represents the p-value of enrichment score, which indicates the importance of the genes' participation. Red indicates the most significant genes while purple indicates the least significant ones. The enrichment score in the BP, CC, and MF graph reflects the level of importance in the biological process of cell component or molecular functions.

In the BP graph (Fig.7A), it is clear that the target genes of Rhein mainly play a role in biosynthetic and metabolic processes, such as long-chain fatty acid biosynthetic and metabolic processes. As we know, the base of most synthetic and metabolic processes is the energy transform of the mitochondria. This provides a reason for us to analyze mitochondrial function in later experiments.

In the CC graph (Fig.7B), the expression of the target gene of Rhein is mainly located in granule and lumen, such as ficolin-1-rich granule and granule lumen. Ficolin-1-rich granules are highly exocytosable granules found in neutrophils; they are secretory granules that generally become associated with the surface membrane of cells. In mitochondria, the lumen (named 'matrix'), which is the site of the Krebs cycle, mitochondrial DNA replication, and protein

biosynthesis, is surrounded by two membranes. Biological energy conversion in the mitochondria is carried out by membrane protein complexes. Thus, we speculate that Rhein preferentially acts on mitochondrial function in monocytes.





(A) Biological Process analysis from GO analysis of the target genes of Rhein;

(B) Cellular Component enrichment from GO analysis of the target genes of Rhein;

(C) Molecular function analysis from GO analysis of the target genes of Rhein.

In the MF figure (Fig.7C), the molecular function of Rhein's target gene is mainly enriched in oxidative processes and binding of the enzyme related to oxidation, especially for oxygen donor and some oxidoreductase activity. Overall, GO enrichment of the target genes of Rhein showed a high score in mitochondria related biological processes, cellular components, and molecular function, which indicates that Rhein is likely involved in mitochondrial function regulation.

It is notable that some researchers believe that oxidative stresses are primary causes for microglia activation that drive the onset of AD (Huang et al., 2016). Accumulating studies have supported that Rhein is involved in mitochondria function regulation. Rhein is unique in its interactions with certain members of the cytochrome p-450 family, and can lead to many beneficial effects regarding the treatment of Alzheimer's. Rhein is capable of inhibiting mitochondrial oxidative stress-induced apoptosis. This is due to its incredibly strong antioxidant activities. Rhein decreases the amount of cyto c, an apoptotic factor, in the cell. Furthermore, Rhein increases the activities of key enzymes SOD and CytOx (Yin et al., 2021). These enzymes are heavily involved in the regulation of reactive oxygen species in the mitochondria, and are observed to have lower activity in AD patients. Notably, rhein is also capable of repairing the mitochondrial electron transport chain through its antioxidant activities. This, combined with the aforementioned improvement in defense against reactive oxygen species, makes for a chemical component with great therapeutic potential.

Previous studies have demonstrated that rhein could relieve oxidative stress and inflammatory response in an Alzheimer's neuron model and mouse model via several pathways. It is well known how microglia play an important role in the regulation of neuroinflammation in the brain. Suppression and stimulation of microglial function has been shown to affect the development of Alzheimer's disease, specifically through an increase in A $\beta$  plaques & tau interference. However, the influence of Rhein on beta-amyloid plaques burdened microglia and the underlying pharmacological mechanisms is not clear.

Hence Rhein was selected to further explore the mechanism of Heshouwu's treatment for AD. Experiments in vitro were performed to search for the effect of Rhein on the mitochondria function in the AD microglia model.

### 3.9. Rhein had a dose-dependent effect on microglia cell availability.

As Rhein was selected as the targeting molecule based on suspicions that it could affect mitochondria function and release oxidative stress, it is necessary to explore the mitochondrial function in our BV2 cell line. Therefore, the cell line was treated with different concentrations

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of Rhein, and CCK-8 was used to check cell availability. Concentrations of Rhein that did not affect cell survival were selected.

Through ANOVA test, we compared every treatment group with the control. The treatment groups did not produce any significant results aside from the 50  $\mu$ M group, which saw P value less than 0.0001 (F (7, 49) = 13.19), which meant more than 50  $\mu$ M Rhein would impair cell viability. The data indicated that high concentrations of Rhein (50  $\mu$ M) decreased the cell viability significantly. This prompted the selection of 4  $\mu$ M and 20  $\mu$ M as the medium and high concentrations for the latter experiment.



Fig 8. CCK8 assay to evaluate the cell viability in BV2 cells with Rhein treatment at various doses ( $\mu$ M). Control group is normalized as 1.0. Data are presented by Mean ±SEM. ANOVA test with Dunnett's multiple comparisons test.

#### 3.10. Rhein decreased the pro-inflammatory activation in BV2 cells

BV2 cells grow as monolayers with a predominant shape of unipolar or bipolar adherent cells. Cell morphology is expected to change into amoeboid shape upon exposure to  $A\beta$  oligomers and different concentrations of Rhein (Fig. 9).

We observed a decrease in cell density upon the introduction of  $A\beta$  oligomers. The length of cell protrusions increased as well, which can be observed in both brightfield and phalloidin staining. There is also a greater percentage of cells with protrusions, as well as an increase in cells with amoeboid shape in the  $A\beta$  oligomers group. Upon the addition of Rhein, cell density remained similar to the  $A\beta$  group, but the length of cell protrusions was decreased, which is especially noticeable in the brightfield image of the 20  $\mu$ M group. However, the proportion of cells with protrusions remained constant in each group. Very rarely were amoeboid-shaped cells observed. These data suggested that the activated microglia model in vitro to mimic AD brains was constructed successfully with  $A\beta$  oligomer treatment. Furthermore, Rhein had no significant effect on cell density and activated percentage. BV2 cells with  $A\beta$  oligomers and Rhein remained in a broadly active state.



Fig 9. Cell Morphology in control, Aβ oligomer, and Aβ oligomer + Rhein groups. Shown in both brightfield (top) and phalloidin staining (bottom).

Active microglia's function can be divided into two major categories: M1 and M2. M1 denotes pro-inflammatory and neurotoxicity-inducing function, whereas M2 functionality is characterized by anti-inflammatory and A $\beta$  cleaning processes. Real time PCR was performed to assess relative mRNA concentrations of a set of 8 separate microglial genes, which were regarded as the cell marker for individual activated types. Each trial had four groups: control, A $\beta$  oligomer, A $\beta$  oligomer & Rhein (4  $\mu$ M and 20  $\mu$ M). The four M2 (top) genes showcased little significance, with both CD206 and Arg1 showing no significant decrease in relative mRNA. TGF- $\beta$ 1 showed a significant decrease from A $\beta$  oligomer on its own to the addition of Rhein at 20  $\mu$ M, but did not show a significant decrease at the 4  $\mu$ M level. IL10 saw a significant increase between the A $\beta$  oligomer and the 20  $\mu$ M Rhein group. The M1 group (bottom) saw greater significant differences between the experimental groups. All four genes saw a significant decrease in relative mRNA levels in the 20  $\mu$ M Rhein groups when compared to the A $\beta$  oligomer group. Only TNF-a and iNOS observed significant decreases in the 4  $\mu$ M Rhein group. This suggests that Rhein treatment had a greater effect on M2 type microglia activation, especially the cytokine of TNF- $\alpha$  and iNOS.



Fig 10. The relative mRNA levels of cytokines in BV2 cells detected by real time PCR. ANOVA test, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. no significance.

# 3.11.Rhein relieved stress of mitochondria function induced by Aβ<sub>1-42</sub> oligomers in BV2 cells

Within the mitochondria, cytochrome p-450 is involved in the metabolism of a plethora of drugs and xenobiotics. The family also plays a role in regulating apoptosis and other cellular processes (McDonnell & Dang, 2013). Specifically, apoptosis chains can lead to the loss of neuronal synapses, further increasing the severity of the onset of AD. For this reason, relieving cellular mitochondria of oxidative stress is a promising avenue in combating Alzheimer's.

Here, we measured the relative level of Reactive Oxygen Species (ROS) in BV2 cells (Fig. 11A). The ROS level represents the function of mitochondria, high ROS level means the function of mitochondria got damaged. In Fig. 11(A) it's clear that  $A\beta_{1-42}$  oligomer damaged mitochondria function, which is consistent with the idea that oxidative stress is closely related to Alzhemer's. From this result and an ANOVA test we observe that  $A\beta_{1-42}$  oligomers severely damaged mitochondria function,  $A\beta_{1-42}$  oligomers+Rhein (4 µM) have no effect from  $A\beta_{1-42}$  oligomers, and  $A\beta_{1-42}$  oligomers+Rhein (20 µM) successfully decreased the ROS level, which suggests that a high dose of Rhein has a positive effect on relieving mitochondrial stress.

We also measured the Cytochrome P-450 activity (Fig. 11B). As one of the targets of Rhein's target gene, CYP-450 activity represented mitochondrial function. By the results and ANOVA test, it's clear that  $A\beta_{1-42}$  oligomer decreased mitochondria function,  $A\beta_{1-42}$  oligomers + Rhein (4  $\mu$ M) failed to rescue the mitochondria function and  $A\beta_{1-42}$  oligomers + Rhein (20  $\mu$ M) successfully rescued the mitochondria function. Although Rhein can relieve the functional stress brought by  $A\beta_{1-42}$  oligomers, there is still a gap to reach the CYP450 activity of the control group.

TMRE fluorescent probe was used to measure the mitochondrial membrane potential (Fig. 11C). From the graphs we can see that compared to the fluorescent intensity of the control group, the A $\beta$ 1-42 oligomer group is much darker, which points towards the decrease of mitochondrial membrane potential and the occurrence of cell apoptosis and necrosis. In the A $\beta$ <sub>1-42</sub> oligomers + Rhein (4  $\mu$ M) group and the A $\beta$ <sub>1-42</sub> oligomers + Rhein (20  $\mu$ M) group, we observed higher fluorescent intensity, proving that the mitochondrial membrane potential increased because of Rhein treatment. The effect of Rhein (20  $\mu$ M) is more significant than Rhein (4  $\mu$ M), but still cannot reach a level equal to the control group.



Fig 11. Mitochondria function alteration in BV2 cells treated with  $A\beta$  oligomer and Rhein.

(A) Relative level of reactive oxygen species (ROS).

(B) Relative CYP450 activity illustrated by  $OD_{450 \text{ nm}}$ .

(C) Representative images of the mitochondrial membrane potential with tetramethylrhodamine, a fluorescent probe.

**Research Report** 

# 4. Discussion and conclusion

Recently, TCM's potential as promising AD treatment continues to draw researcher attention. Some bioactive components in TCM showed powerful anti-neuroinflammatory and A $\beta$  cleaning abilities. In this study, meta-analysis and network pharmacology were used to screen the potential core TCM and corresponding component for AD treatment, based on microglia-specific pathophysiological processes. Then, A $\beta_{1-42}$  oligomer-burdened microglia model for AD was built up, and the effect of Rhein on microglia activation and mitochondrial function were explored.

For the evaluation of bias in meta-analysis, 10 included papers were selected. Among that, some research was performed in early years ((Lin et al.(2003), Zhou et al.(2007), and Chen et al.(2010)), in which allocation and binding of participants were missed. When they conducted the objective evaluation system, Mini Mental State Examination (MMSE) test, higher risks of bias were shown.

In most of these clinical trials, western medicine, such as donepezil hydrochloride, is considered the mainstream treatment for AD. However, combinations of TCM therapies and western mainstream medicine are being developed. The clinical efficacy of this combination has also been confirmed. TCM and western medicine may share similar pharmacology methods for AD treatment, and the combination theory could achieve maximum benefits. Although these combination therapy studies were not included in the meta-analysis here, it remains a promising treatment approach to AD for future study.

In the clinical trials included in meta-analysis, Heshouwu (*Polygonum multiflorum* Thunb) was the most commonly used herb, appearing 4 times. More importantly, prescription with *Polygonum multiflorum* Thunb had a better therapeutic effect on the cognitive functions, such as MMSE score, of AD patients. Based on a network pharmacology approach, a total of 19 bioactive phytochemicals in *Polygonum multiflorum* Thunb that corresponded to 111 targets correlated with AD pathogenesis were identified as core targets that play an important role in the regulation of oxidative stress in microglia. Rhein was selected as the core component based on network pharmacology. Our real-time PCR results showed the significant effects of Rhein on microglia activation concentration dependently, as seen in TGF- $\beta$ 1, TNF- $\alpha$ , and iNOS, among other genes of interest. Here, we observed significant differences in relative mRNA as a result of changing Rhein concentration. The limitations of our study did not allow for the indepth exploration of the effect of different concentration in terms of an optimum amount. For

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future research, it is reasonable to determine the ideal concentration for maximum positive effects in animal experiments or clinic application.

It was also observed that M1 functionality was more affected by the introduction of Rhein than M2. This is reasonable, as Rhein largely affects mitochondrial activity, targeting genes more related to M1 functions. On the other hand, M2 activated microglia is more related to phagocytosis, which is mostly mediated by lysosomes. The reactive oxygen species that Rhein affects are also heavily involved in M1 function, further supporting the idea that rhein should target M1 function more than M2 function as a whole.

Cellular oxidative stress and over-activation in microglia induced by  $A\beta$  deposition is an important pathological feature of AD. Here, we firstly confirmed that Rhein from *Polygonum multiflorum* Thunb is indeed very promising for antioxidant and anti-neuroinflammatory therapy of AD. The results suggested that rhein reduced intracellular ROS levels and rescued enzyme activity and membrane potential to varying degrees in a dose-dependent manner. Introducing antioxidants to relieve oxidative stress and neuroinflammation in microglia is an effective therapeutic strategy for AD.

In summary, we proved that meta-analysis together with network pharmacology could work as an efficient and effective method in discovering therapeutic TCMs for Alzheimer's. Moreover, Rhein, one of the core chemical components of *Polygonum multiflorum* Thunb, was confirmed to be effective in treating BV2 A $\beta_{1-42}$  oligomer cell models. For the first time, it was proved that Rhein has an inhibitory effect on microglia over-activation, or neuroinflammation. This study contributes to the fundamental research for a novel TCM therapy for AD.

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