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Identification of Key Pathways and Targets of Kai Xin San in The Treatment of Alzheimer's Disease Based On a Network Pharmacology Approach and Experimental Validation

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Abstract

Background: Alzheimer's disease (AD) is the commonest neurodegenerative disease characterized with a progressive loss of cognitive functions and memory decline. Kai Xin San (KXS), a traditional Chinese herbal classic prescription, has been used to ameliorate cognitive dysfunction for thousands of years. However, its specific pharmacological molecular mechanisms have not been fully clarified.

Methods: The ingredients of KXS and their corresponding targets were firstly screened from ETCM database. AD-related target proteins were obtained from Malacards database and DisGeNet database. Venn diagram was used to intersect the common targets between KXS and AD. Then, key ingredients and key targets were identified from compound-target-disease network and protein-protein interaction (PPI) network analysis respectively. Moreover, the binding affinity between the key ingredients and targets were verified by molecular docking. KEGG enrichment analysis further predicted the potential key signaling pathway involved in the treatment of KXS on AD, and the predicted signaling pathway was validated via experimental approach.

Results: A total of 38 ingredients and 469 corresponding targets were screened, and 264 target proteins associated with AD were obtained. Compound-target-disease network and PPI identified the key active ingredients and targets, which correlate with the treatment of KXS on AD. Molecular docking revealed a good binding affinity between key ingredients and targets. KEGG pathway analysis suggested the potential effect of KXS in treatment of AD via Aβ-GSK3β-Tau pathway. $A\beta_{1\text{-}42}$-injected induced a decline in spatial learning and memory and upregulated the expression of GSK3β and CDK5 along with the downregulated PP1 and PP2 expression. However, KXS significantly improve the cognitive deficits induced by $A\beta_{1\text{-}42}$, decrease the GSK3β and CDK5 levels and increase the expression of PP1 and PP2.

Conclusions: Our research elucidated that KXS exerted neuroprotective effects through regulating the Aβ-GSK3β-Tau signaling pathway, which provided a novel insight into the therapeutic mechanism of KXS in treatment of AD.

1. Introduction

Alzheimer’s disease (AD) is among the most frequent neurodegenerative brain disease with insidious onset, characterized by progressive memory decline and intellectual impairment such as memory impairment, aphasia, apraxia, agnosia, impairment of visual spatial skills, executive dysfunction, and personality and behavior changes in clinic [1–2]. With the aging of the population, the prevalence of AD is high and increasing annually. The number of AD patients worldwide is estimated to reach 130 million by 2050 [3–4], which has become one of the main diseases that do harm to the health of the elderly[5] and result in a notable increase in healthcare needs. Currently, here is no cure for Alzheimer's disease, even though the U.S. Food and Drug Administration (FDA) approved only four cholinesterase inhibitors (donepezil, galantamine, remifendamine, tacrine) and one NMDAR antagonist (memantine) for AD treatment, compensating for the decline of brain function [6–7]. However, the drugs cannot prevent or

delay the underlying progress of AD and the adverse effect including nausea, diarrhea, insomnia and a slower heart rate, cannot be neglected [8–9].

Histopathologically, AD features extracellular amyloid plaques containing the aggregated amyloid precursor protein (APP) peptide fragment Amyloid β (Aβ) and Neurofibrillary tangles (NFTs), which consists of phosphorylated tau proteins in paired helical or straight filaments [10–11]. Tau, a microtubule related protein, is responsible for promoting microtubule assembly and stabilizing microtubules [12]. When exposed to pathogenic conditions, Tau aggregates and toxic oligomers form, leading to intraneuronal NFTs [13]. Tau is assigned to the major phosphoproteins in the brain and hyperphosphorylated tau at several serine and threonine residues regulated by protein kinase and protein phosphatase causes microtubule depolymerization, axon degeneration, the disruption of neuronal transport and cell loss of neurons [14–15]. And the process of tau hyperphosphorylation was accelerated by aberrant activation of serine/threonine protein kinases including glycogen synthase kinase (GSK)-3β and cyclin-dependent kinase 5 (CDK5) [16]. Serine/Threonine protein phosphatase can be divided into protein phosphatase-1 (PP1) and protein phosphatase-2 (PP2), and PP2A accounts for approximately 71% of the total tau phosphatase activity, followed by PP1 accounting for about 11% [17]. Research found that the over-activation of CDK5 caused the hyperphosphorylation of tau protein and neurons loss [18], which suggested a close relationship between CDK5 and tau protein abnormal phosphorylation. Besides, the CDK5 could synergistically regulate the GSK3β-mediated phosphorylated tau [19]. Therefore, inhibiting hyperphosphorylation of tau or reducing the expression of potentially toxic tau species is critical for alleviating AD[20].

Traditional Chinese medicine (TCM) have been applied for treating dementia and forgetfulness for hundreds of years in Asian countries[21]. TCM exerts anti-dementia effects through a variety of mechanisms including cholinesterase inhibitory activity, anti-oxidation, anti-apoptosis, anti-neuro-inflammation, neurogenic activity, and regulation of Aβ and p-tau metabolism[22–23]. Kai Xin San (KXS) formula, a traditional Chinese herbal prescription, was first utilized to treat dementia in "*Beiji Qianjin Yao Fang*", known as the earliest clinical encyclopedia in China. It is comprised of four herbs: *Panax ginseng C. A. Mey* (Chinese name: Renshen, RS), *Polygala tenuifolia Willd* (Chinese name: Yuanzhi, YZ), *Acorus tatarinowii Schott* (Chinese name: Changpu, CP) and *Poria cocos (Schw.) Wolf* (Chinese name: Fuling, FL) in a ratio of 3:3:2:2. Previous studies revealed that KXS could improve the impairment of cognition in clinical, and increase both the synthesis and the release of neurotrophic factors in cultured astrocytes [24–25]. However, the specific molecular mechanism underlying the KXS in the treatment of AD, and whether it can inhibit hyperphosphorylation of tau or reduce the expression of potentially toxic tau species still remain unclear.

Network pharmacology is capable of systematically explaining complexities among biological systems, drugs, and diseases, which is consistent with the holistic idea of TCM [26]. It has been widely used to identify the biological functions of TCM herbs and active compounds, and clarify the mechanisms of TCM formula[27–30]. In this study, network pharmacology approaches combined with experimental validation were employed to explore the pharmacological and molecular mechanisms of the KXS in

treatment of AD (Figure 1). The chemical components of KXS were screened out and AD-related genes were collected, then the potential targets were predicted from the selected compounds. Combining the KXS-related targets with the AD-related genes, the intersection targets was obtained and the ingredient-target-disease network was constructed. Then the key ingredients in KXS were recognized according to the degree value. Moreover, the protein-protein interaction (PPI) network was constructed to identify the most potential key targets. Molecular docking was next employed to identify the binding affinity between key ingredients and targets. The pathway enrichment analysis was applied on the intersection key targets to predict the involved pathways and potential mechanisms of KXS on AD. Finally, the most key target and signaling pathway were validated by in vivo experiments. Taken together, our research investigated the therapeutic mechanisms of KXS on AD based on network pharmacology approach combined with experimental validation.

2. Material And Method

2.1 Data preparation

2.1.1 Screening of the chemical component and compound-related targets of KXS

The chemical ingredients of KXS were collected from the Encyclopedia of Traditional Chinese Medicine (ETCM, updated on 2018 Oct 26, doi:10.1093/nar/gky987, http://www.nrc.ac.cn:9090/ETCM/index.php) database, which includes comprehensive and standardized information for the ingredients and target of TCM [31]. The active constituents of KXS are retained according to ADME in pharmacokinetic including absorption, distribution, metabolism as well as excretion. In this study, four ADME-related parameters including oral bioavailability (OB)≥30%, drug-like (DL)≥0.18, blood brain barrier (BBB)≥-0.3 and Caco-2 permeability >0 were considered as selection criteria of bioactive compounds in KXS. And the compound-related targets of KXS were also obtained from ETCM based on the above principles.

2.1.2 Collection of AD-related targets

The AD-related target proteins were obtained from two sources: 1) Malacards (https://www.malacards.org/) database, which is an integrated database of human maladies and their annotations; and 2) DisGeNet (https://www.disgenet.org/) database, which is a discovery platform including one of the largest publicly available collections of genes associated to human diseases [32–33]. On October 14, 2019, the target proteins related to AD were acquired through the two disease databases Malacards and DisGeNet with the keyword “Alzheimer’s disease”.

2.1.3 Network construction of herb-compound-target and compound-target-disease interaction

To characterize the multi-compound therapeutic features of KXS, the herb-compound-target network of RS, YZ, CP and FL were constructed respectively (RS-target, YZ-target, CP-target and FL-target). Next, the

ingredient-target network of KXS was built after removing the duplicate targets among the four herbs to better elaborate the holistic mechanism of the complex relationships among chemical constituents, targets, and diseases. The network was visualized and analyzed in Cytoscape 3.7.1 software. Furthermore, the Venn diagram (http://bioinformatics.psb.ugent be/webtools/ Venn/) was employed to intersect the targets of KXS with the AD-related proteins. Then the ingredient-target network was constructed based on the common targets between KXS and AD, which implicated in the AD treatment using KXS. According to the degree value, the key ingredients were mainly screened by degree that represents the number of edges adjacent to a node.

2.1.4 Protein-protein interaction (PPI) network construction

PPI network in human genome were gained from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version 11.0, https://string-db.org/) database, a weighted interaction platform with physical and functional interactions, which are integrated from multiple data sources including experimental data, computation-based prediction and literature mining [34]. The interaction targets were delivered to the STRING and filtered with a threshold of 0.4 to obtain PPI information with higher confidence. Only interactions with weight exceeding the set threshold were selected for the newly constructed PPI network. The TSV format of the results from SRING was further uploaded and analyzed through the Network Analyzer plug-in in Cytoscape software. Targets with the highest degree value was recognized as the key target proteins involved in the treatment on AD by KXS.

2.1.5 Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis

To further clarify the mechanism of KXS treating AD, KEGG pathway enrichment analysis was performed using the g: Profiler database (https://biit.cs.ut.ee/gprofiler/), which maps genes to known functional information sources. The intersected targets were inputted into the the g: Profiler and the species was limited to Homo sapiens, and the significance threshold was set as 0.05. The top 15 pathways of *P* value were then applied to OmicShare (https://www.omicshare.com/tools/Home/Soft) for further generating chart. The pathway map of AD was downloaded from KEGG (http://www.kegg.jp) and the key targets were marked in red.

2.1.6 Molecular docking

The CDOCKER module in Discovery Studio software was performed to calculate the binding affinity between the key ingredients and target proteins. CDOCKER is a grid-based molecular docking method based on CHARMm force field, which can generate the accurate docking results. Before docking, the 3D conformations of the ingredients were downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) and the structures of the proteins were obtained from RCSB PROTEIN DATA BANK (PDB) (https://www.rcsb.org). Then the CHARMm force filed was assigned for the molecules of the ingredients and they were full minimized. The proteins were prepared by deleting the water and the other redundant peptides and chains. Next, they were cleaned to add hydrogens and refine the incomplete amino acids. The docking sites were determined either from previous researches or the

reference of origin ligand (Supplementary Table S1). All the docking box was set as 20Å × 20Å × 20Å to cover the ligands. When performing the docking, the “Top Hits” was set to 10, the “Pose Cluster Radius” was set as 0.5, the “Maximum Bad Orientations” and the “Orientation vdW Energy Threshold” was set to 2000 and 500 respectively, and other parameters were set to default. The best binding energy and action mode of each ingredient-target complex was analyzed by Discovery Studio.

2.2 Experimental validation

2.2.1 Preparation of KXS

The four herbs of KXS (*Panax ginseng C. A. Mey*, *Polygala tenuifolia Willd*, *Acorus tatarinowii Schott* and *Poria cocos (Schw.) Wolf*) were purchased from the Guoyitang affiliated to Hubei University of Traditional Chinese Medicine (Hubei, China). The quality of these herbs was controlled by the Chinese Pharmacopoeia (2015). And the voucher specimen of each herb (D00085, D00303, D00646, D00506) was prepared and deposited in the Bank of China(Hong Kong)Chinese Medicines Centre of Hong Kong Baptist University. For KXS preparation, RS, YZ, CP, and FL were mixed at a 3:3:2:2 ratio and then subjected to a grinding machine and the extract has been described in detail in previous studies [24]. Briefly, plant materials were extracted with 95% ethanol and then filtrated by filter paper. The filtrate was collected and the residue was extracted repeatedly until the filtrate turned colorless. The filtrate was combined and concentrated in an evaporator to 0.5 g/ml. Then the concentrate solution was diluted to required mass concentration and stored in the refrigerator at 4 ℃.

2.2.2 Animals experiment

To verify the improvement effect of KXS on AD symptoms in vivo, three-month-old male SD rats, weighing 220±20g, were obtained from Liaoning Provincial Laboratory Animal Research Center. All the rats were housed in the SPF-level animal center of Experimental Animal Center of Hubei University of Traditional Chinese Medicine at an ambient temperature between 21 ℃ to 25 ℃ and a humidity between 50–60% with a 12 h light/dark cycle while food and water were offered sufficiently. All animal protocols were performed in accordance with institutional guidelines for the care and use of laboratory animals approved by the Animal Care and Use Committee of Hubei University of Traditional Chinese Medicine (No.211002300036812) and strictly complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All rats were given habituation for 7 days before experiments with free access to sterilized food and water and a preliminary screening was performed using the Y-maze test to exclude those with congenital dementia. To construct AD rat models, the $A\beta_{1-42}$ peptide (AOBOX, Beijing, China) dissolved in sterilized physiological saline, was injected into the hippocampus of the rat according to the brain stereotactic coordinates which were determined with reference to the Paxions map. Then the chosen rats were randomly divided into the following four groups: sham group (Saline), model group (Saline), donepezil hydrochloride (DH, 0.33 mg/kg/d) group and KXS group (5 g/kg/d). All rats were given once daily by gastric perfusion for 28 consecutive days. The sham and model groups received an equal amount of sterile saline, DH diluted with physiological saline was administered intragastrically 0.33 mg/kg once a day and KXS formula was given once daily by gastric perfusion at 5 g/kg/day. After

the last behavioral test, the rats were sacrificed and the hippocampus was completely separated after being decapitated, then frozen in liquid nitrogen and stored in -80 ℃ refrigerator for Western Blot analysis.

2.2.3 Morris water maze (MWM) test

To evaluate the spatial learning and memory deficits of animals, we conducted MWM test as previously described [7]. In brief, MWM consisted of a blank circular pool with 120 cm in diameter and 40 cm in depth and was filled with water maintained at a temperature between 22°C and 25°C. The apparatus was divided into four equal quadrants by four poles along the circumference of the basin. A round platform with 12 cm in diameter was fixed in one of the quadrants of the pool and immersed 1 cm below the water surface. Training of finding the platform were conducted 5 times per day (90s/time) and last for 5 days. Escape latency was calculated based on the time spent in finding a fixed platform. Twenty hours after spatial test, the platform was removed and the animals had to search for the platform in the apparatus; The swimming times across the platform area were recorded. The spatial acquisition test was performed between days 24 and 28 after $A\beta_{1\text{-}42}$ infusion to evaluate the spatial learning ability of the animals through a 5-day memory acquisition experiment. A subsequent spatial probe experiment was performed on day 29 to determine the spatial memory retention ability of rats. The movements of the animals were recorded with an overhead video recorder connected to the tracking system (XR-XM101, Shanghai, China). After the MWM test, all animals were sacrificed via decapitation under anesthesia. The hippocampal tissues were flash frozen and stored in liquid nitrogen before analysis.

2.2.4 Western blot analysis

The hippocampus tissues obtained from rat brains were rapidly dissected out on ice and were lysed in precooled RIPA buffer (89900, Thermo scientific) containing a protease inhibitor mixture (78433, Thermo scientific) for 30 minutes. After centrifugation at 12,000 rpm and 4℃ for 5 minutes, the protein concentration was measured with the bicinchoninic acid assay (B9643-1L, SIGMA). Total protein samples were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel for electrophoresis at 120V for 90 minutes. The proteins were then transferred onto PVDF membranes at 200 mA for 120 minutes. Blocked membranes with 5% non-fat milk were incubated with primary antibodies, including β-actin (AF7018, Affinity Biosciences), GSK3β (AF5016, Affinity), CDK5 (AF6371, Affinity), PP1 (DF2991, Affinity), PP2A (AF4753, Affinity), Tau (AF6141, Affinity) and p-Tau Ser404 (AF3144, Affinity) at 4℃ overnight. Then the membrane was rinsed in phosphate buffer saline with 0.05% Tween-20 three times and incubated with corresponding secondary anti-rabbit or anti-mouse from Affinity for 1 hours at room temperature. The protein bands were visualized using the enhanced chemiluminescence detection reagents (180-501, Tanon, Shanghai, China) and digitized by Image J software.

2.3 Statistical analysis

Experimental data were represented as the mean ± standard deviation (mean ± SD). Statistical analyses were performed with GraphPad Prism 7.0 and SPSS (version 19.0) software (Abbott Laboratories, Chicago, USA). Student’s t test was utilized for statistical comparison between the two groups.

Comparisons between multiple groups were performed using the one-way analysis of variance (ANOVA) and the post hoc test by Dunnett's. $P<0.05$ denoted statistical significance, while $P<0.01$ represented extreme statistical significance.

3. Results

3.1 Active ingredients and corresponding targets of KXS

There were 189 active components in RS, 35 active components in FL,43 active components in YZ and 28 active components in CP screened from ETCM database (Supplementary File S1). To further construct the active ingredient-target network of KXS, the candidate compounds were filtered according to (OB)≥30%, (DL)≥0.18, (BBB)≥-0.3 and Caco-2 permeability >0 in each herb. After the filtration, 27 chemical constitutes in RS, 6 active compounds in FL, 3 compounds in YZ and 2 chemical ingredients in CP were retained. It was found that the chemical constituents in RS, FL, YZ and CP targeted 379, 179, 18 and 38 gene target proteins, respectively (Supplementary File S2). Collectively, 38 chemical components of KXS and 469 compound-related targets after getting rid of the duplicates were remained from the databases (Supplementary File S3).

3.2 AD-related targets

To determine the pharmacological mechanisms of KXS treating AD, "Alzheimer's disease" was inputted into the disease-associated databases. There were 264 AD-related genes on the Malacards database and 303 on the DisGeNet database. Subsequently, a total of 264 target proteins of AD were obtained after removing overlapped targets (Supplementary File S4).

3.3 Construction of herb-compound-target network

The compound-target network of each of single herb in KXS was constructed and there 2 compounds in CP targeting 38 genes (Figure. 2A), 3 compounds in YZ targeting 18 genes (Figure. 2B), were 6 compounds in FL targeting 179 genes (Figure. 2C) and 27 compounds in RS targeting 379 genes (Figure. 2D). In order to reveal the multi-targets and holistic characteristics of KXS, the formula herb-compound-target network was further constructed (Figure. 3). It showed that KXS yield 38 candidate compounds and 469 potential targets after eliminating all duplicates. Moreover, the network included 507 nodes and 1277 edges, of which 38 candidate ingredients had a median of 7 target correlations, indicating the existence of complex relevancy among different compounds and targets.

3.4 Establishment of the compound-target-disease interactive network

469 target proteins corresponding to the active ingredients of KXS and 264 AD-related targets were uploaded to the Venn diagram for intersection, then 9 intersection targets were obtained (Figure. 4A). These intersected targets were thought to be hub target proteins including GSK3β (Glycogen synthase kinase-3 beta), TTR (Thyroid hormone-binding protein), GAPDH (Glyceraldehyde-3-phosphate

dehydrogenase), GRIN2B (Glutamate receptor ionotropic), PTGS2 (Prostaglandin G/H synthase 2), PTGS1 (Prostaglandin G/H synthase 1), S100B (Protein S100-B), ABCA1 (ATP-binding cassette sub-family A member 1) and PPARG (Peroxisome proliferator-activated receptor gamma). Then the 'compound-target-disease' interactive network was established in order to comprehensively reveal the possible chemical ingredients of KXS against AD (Figure. 4B). There were 17 nodes and 23 edges in the network representing the directive interaction between the active ingredients and core targets. The average degree of the ingredients was 2.88, and the ingredients whose degree value was more than 2.88 were undecanoic acid (degree=4), caprylic acid (degree=4), lauric acid (degree=4), dodecenoic acid (degree=3), salicylic acid (degree=3) and adenosine (degree=3) (Table 1). These 6 ingredients were regarded to be the key active constitutes for KXS to exert the therapeutic effect.

Table 1
Key ingredients obtained from compound-target-disease interactive network

Name	Formula	MW	CAS	PubChem_CID
Undecanoic acid	$C_{11}H_{22}O_2$	186.29	112-37-8	8180
Caprylic acid	$C_8H_{16}O_2$	144.21	124-07-2	379
Lauric acid	$C_{12}H_{24}O_2$	200.32	143-07-7	3893
Dodecenoic acid	$C_{12}H_{22}O_2$	198.30	65423-25-8	125207
Salicylic acid	$C_7H_6O_3$	138.12	69-72-7	338
Adenosine	$C_{10}H_{13}N_5O_4$	267.24	58-61-7	60961

3.5 Outcome of PPI and KEGG enrichment analysis

To further explore the underlying mechanism and the key protein target of KXS for the treatment of AD. The 9 intersected targets were searched for PPI analysis through SRTING, which combined them whose scores were greater than 0.95, illustrating that they have at least some biological connections (Figure. 5A). The network contained 9 nodes and 17 edges, and the average degree of nodes analyzed by Cytoscape was 3.56. Among the 9 targets, GAPDH (degree=8), PPARG (degree=5), GSK3β (degree=4) and PTGS2 (degree=4) showed a higher degree value than the average and were identified as the key targets. Then g: Profiler was employed to conduct the KEGG enrichment analysis of KXS for AD ($P < 0.05$). The top 15 pathways in KEGG enrichment was exhibited according to *P* value (Figure. 5B). It showed that Alzheimer disease (AD), IL-17 signaling pathway, PPAR signaling pathway, HIF-1 signaling pathway and NF-kappa B signaling pathway were concentrated in the pathways. Most significantly, AD showed the lowest *P* value in the top 15 pathways, indicating the exact potential therapeutic effect for KXS to treat AD. According to the critical pathway network of AD (Figure. 6), the activation of GSK3β induced by Aβ phosphorylates the tau protein to further aggravate the formation of NFTs, leading to the neuron cell death. Therefore, GSK3β played the key role in regulation of AD. Combined with the significant degree in PPI, GSK3β might be one of the most potential targets affecting regulatory network of KXS.

3.6 Binding affinity between the key ingredients and target proteins

3D conformations of the key proteins were obtained from PDB database and the PDB number were 2OW2 (GSK3β), 1U8F (GAPDH), 6FZY (PPARG) and 5JW1 (PTGS2). Then, the 6 key ingredients were docked into each of the 4 key target proteins and the 240 ingredient-target complexes were totally generated. The binding affinity was determined by the value of CDOCKER_Energy, and the lower of the value, the better of the combination between the ingredient and target. For each pair of ingredient-target protein, the best docking pose of the 10 hits was retained for further analysis. As the results showed (Table 2), the average value of docking energy between lauric acid and the 4 target proteins was the lowest followed by undecanoic acid and caprylic acid, while the adenosine was the highest among the 6 ingredients. Moreover, the lauric acid showed the best binding energy with both GSK3β and PPARG. The caprylic acid and undecanoic acid displayed the best binding energy with PTGS2 and GAPDH, respectively. The interaction between lauric acid, caprylic acid or undecanoic acid and the 4 targets were showed in Figure 7. It revealed the key role of lauric acid, caprylic acid and undecanoic acid in KXS, which was consistent with the results of the 'compound-target-disease' interactive network. It also indicates that the lauric acid was the most potential active ingredient in KXS formula to treat AD.

Table 2
CDOCKER_ENERGY between the key ingredients and targets (kcal/mol)

	GSK3β (2OW2)	GAPDH (1U8F)	PPARG (6FZY)	PTGS2 (5JW1)
Salicylic acid	-18.86	-18.35	-21.52	-17.53
Caprylic acid	-24.64	-29.15	-28.30	-29.09
Lauric acid	-33.99	-33.33	-31.37	-28.04
Undecanoic acid	-29.35	-34.61	-30.69	-28.59
Adenosine	-3.68	-7.21	-0.34	1.02
Dodecenoic acid	-25.80	-27.42	-23.38	-18.20

3.7 KXS improved cognitive impairment induced by $A\beta_{1-42}$ in rats

MWM test was performed to corroborate the treatment of KXS on cognitive function. There were significant differences between the $A\beta_{1-42}$ group and the control group in the escape latency and the time to cross the plateau area (P<0.01), suggesting that $A\beta_{1-42}$ injection into the hippocampus caused cognitive dysfunction in rats (Figure. 8A). However, the administration of KXS and DH significantly alleviated the memory deficit induced by $A\beta_{1-42}$. In the place navigation test, the escape latency of KXS

group was shorter than that of $A\beta_{1-42}$ group (P<0.01), which was similar in that to DH group (Figure. 8B). In probe test, as results shown in Figure. 8C, rats treated with KXS and DH greatly increased platform crossover in AD model rats (P<0.01) compared with $A\beta_{1-42}$ group. The results demonstrated that KXS could improve the learning and memory ability of AD rats.

3.8 Effect of KXS on the expressions of key proteins

To verify whether GSK3β is the key target of KXS on AD, the alterations of expression levels of related proteins were measured by western blotting. The next step is to elucidate the mechanism by which KXS improved cognitive impairment. The expression of Aβ in rats' hippocampus was increased after $A\beta_{1-42}$ administration (Figure. 9). Compared with the control group, the expressions of p-Tau Ser404, GSK3β and CDK5 in the $A\beta_{1-42}$ group were significantly increased, but the levels of PP1 and PP2A were down-regulated (P<0.01). Meanwhile, DH and KXS treatment could similarly reverse the $A\beta_{1-42}$-induced increase in Aβ, p-Tau Ser404, GSK3β as well as CDK5, and also reverse the decrease in PP1 and PP2A (P<0.01), indicating that Aβ/GSK3β/Tau signal could be modulated by KXS via inhibiting the phosphorylation of tau protein (Figure. 9).

4. Discussion

Traditional Chinese medicine (TCM), characterized by expressing cooperative effect through multiple targets, have been applied for treating AD and other cognitive impairment [35–36]. KXS formula was applied to treat AD for more than a thousand years in China. As one of the most common neurodegenerative disease, AD is clinically defined by progressive memory impairment, subjective cognitive decline, social dysfunction and even personality change [14–37]. Due to the complicated pathogenesis involved in the progression of AD and the muti-ingredients and -targets of KXS, it is difficult to uncover the underlying mechanism for KXS in the treatment of AD through the traditional way.

In the present study, network pharmacology combined with molecular docking was first applied to predict and identify the potential active ingredients and the key proteins of KXS in the treatment of AD. 6 key ingredients with highest degree value were obtained from compound-target-disease network. Moreover, PPI network was constructed based on the 9 intersection between KXS-related targets and AD-related genes, and GSK3β, GAPDH, PTGS2 and PPARG showed the significant importance due to their high degree. The binding affinity between targets and ingredients were further verified by molecular docking. The results revealed that among the 6 key ingredients, the average binding energy between lauric acid and the 4 targets were the lowest, indicating its crucial role in KXS. Lauric acid, a kind of middle chain fatty acids, has been suggested to have a therapeutic effect on mood disorders and cognitive dysfunctions [38]. In addition, research revealed that lauric acid could alleviate Aβ-induced enhancement of phagocytosis to attenuate the activation of microglial, exerting a therapeutic effect [39]. Furthermore, GSK3β was the target protein with the lowest binding energy (-33.99 kcal/mol) combined with lauric acid, indicating its essential role in treatment of AD by KXS. KEGG pathway enrichment analysis revealed that the intersection targets were significantly related to AD. According to the pathway of AD, Aβ induced

GSK3β and CDK5 facilitate the phosphorylation of Tau protein. Due to the definiteness of GSK3β in AD, we predicted that it might be responsible for KXS in the treatment of AD through Aβ-GSK3β-Tau signaling pathway.

GSK3β, as well as CDK5, promote the hyperphosphorylation of Tau protein, which accelerates its accumulation in brain and cerebrospinal fluid, directly promoting the formation of NFTs. NFTs is closely related to the accumulation of Aβ [40], which is composed of paired helical filament consisting of hyperphosphorylated or abnormally phosphorylated Tau proteins [41]. Tau is mainly located in the axons of central and peripheral nervous system, promoting the aggregation of tubulin and maintain the stability of microtubule by combining microtubule. When Tau is hyperphosphorylated, the axonal transport of neurons will be hindered, leading to neuronal dysfunction and death [42]. Moreover, the accumulation of Aβ occurs in the early stage of AD, which plays a key role in the progression of AD [24–43]. All these strongly suggest that the hyperphosphorylation of Tau plays an essential role in promoting the pathogenesis of AD. In addition to GSK3β and CDK5, PP2A can also facilitate the hyperphosphorylation of Tau through enhancing the activity of GSK3β by negatively regulation of Akt [44]. The abnormal hyperphosphorylation of Tau is partially due to the downregulation of PP2A activity in brain [45], which then causes microtubule destabilization, neuronal death and cognitive impairment [46]. Besides, the activity of PP1 also showed an obvious decrease in AD brain [47]. Therefore, the decrease of PP2A and PP1 activity may be the crucial cause of the hyperphosphorylation of Tau.

There are few phosphorylation sites of Tau in normal brain, but more than 40 phosphorylation sites of Tau protein exist in the brain of patients with AD, mainly including 181, 199, 202, 231, 396 and 404 poly-phosphorylation sites [48]. Phosphorylation site of Ser404 was selected for its previous research in AD [49]. In the experiment validation, MWM test was firstly performed. The results proved that KXS could improve the $A\beta_{1-42}$-induced cognitive deficit. Then, western blotting was utilized to detect the change of Tau phosphorylation at the site. The results showed that KXS could reduce the phosphorylated Tau of Ser404 in hippocampus of $A\beta_{1-42}$ injection rats, by increasing the level of GSK3β and CDk5 as well as decreasing the expression of PP1 and PP2A. Thus, it preventd the formation of NFTs, so as to control the development of AD. These results indicating the therapeutic effect of KXS in alleviating AD might due to the inhibition of Tau hyperphosphorylation and diminish the expression of potentially toxic tau species.

It was reported that *Jia Wei Kai Xin San* alleviated cognitive deficits and improved cholinergic neurotransmission function via modulating Aβ levels in hippocampus of Aβ-injection mice [50]. Moreover, *Na Wang et al* demonstrated that KXS restored the cognitive impairment and prevented the hippocampus neuronal damage induced by $A\beta_{42}$ in rats via increasing the level of neprilysin and accelerating the degradation of Aβ [51]. *Hang Chu et al* found that 36 metabolites associated with regulation of KXS on AD were identified through metabolomics analysis and KXS exerted an improvement in learning and memory in rats administrated with D-gal and AlCl3 [52]. These researches studied the therapeutic mechanisms of KXS on cognitive impairment related disease from diversities of aspects. However, the holistic characteristics of muti-compounds and corresponding acting targets of KXS urged the further decipher of the detailed molecular mechanisms of KXS in the treatment of AD. At present study, the

network pharmacology identified the most potential key protein GSK3β and its responsible signaling pathway of KXS effect on AD. *In vivo*, we found that KXS could not only reduce the amount of Aβ at protein level, but also inhibit the hyperphosphorylation of Tau by decreasing the CDK5 and GSK3β, and increase the expression of PP1 and PP2A.

Based on the findings above, our research demonstrated that KXS could significantly improve the cognitive function of AD and the therapeutic effect might due to the inhibition of Tau hyperphosphorylation via Aβ-GSK3β-Tau signaling pathway.

5. Conclusion

Taken together, network pharmacology approaches combined with molecular docking in this study were applied to forecast the key ingredients and target proteins, and the potential signaling pathway of KXS in treatment of AD was further identified, immediately following experiments *in vivo* verified the improvement effect of KXS on $A\beta_{1-42}$-induced cognitive impairment via Aβ-GSK3β-Tau signaling. Our results provided a novel strategy for the further research of KXS on AD and implied the development of KXS or its components as viable agents for treatment and prevention of AD.

Abbreviations

AD: Alzheimer's disease; Aβ: Amyloid β-protein; BBB: Blood Brain Barrier; CDK5: Cyclin-dependent Kinase5; DH: Donepezil Hydrochloride; DL: Drug-Like; ETCM: Encyclopedia of Traditional Chinese Medicine; GSK3β: Glycogen Synthase Kinase-3 beta; KEGG: Kyoto Encyclopedia of Genes and Genomes; KXS: Kai Xin San formula; NFTs: Neurofibrillary Tangle; OB: Oral Bioavailability; PDB: RCSB PROTEIN DATA BANK; PP1: Protein Phosphatase-1; PP2: Protein Phosphatase-2; PPI: Protein-protein Interactions; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; TCM: Traditional Chinese medicine

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

All data are available in the manuscript and they are showed in figures, tables and supplement file.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

KMW and LMM was responsible for the integrity of the work as a whole and conducted experiments design. YR and WK contributed to the drug preparation, the experiments and the writing of the manuscript. LT conducted network pharmacology analysis, analyzed and interpreted the data. All authors reviewed and approved the final version of the manuscript.

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Figures

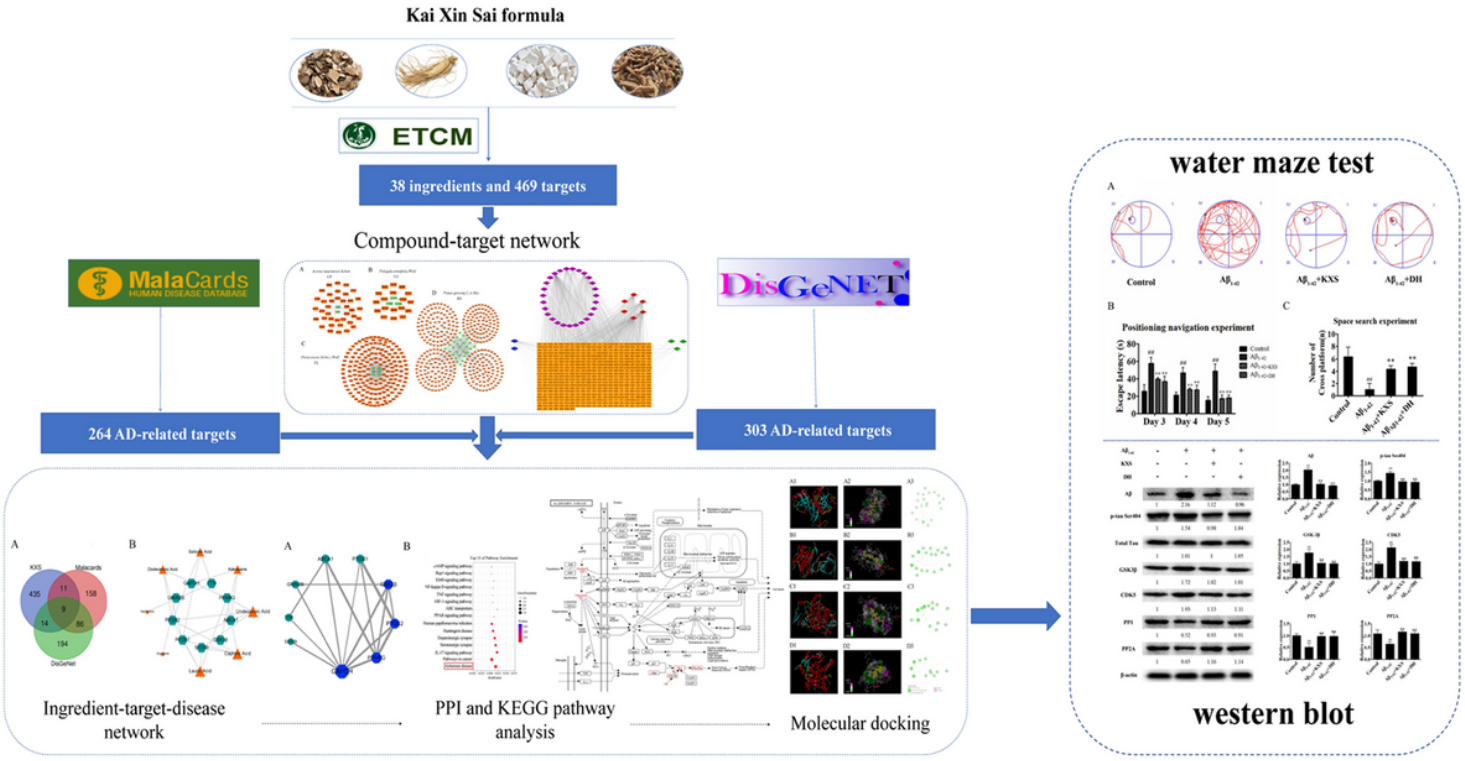

Figure 1

Workflow of Kai Xin San in treatment of AD based on network pharmacology and experimental validation.

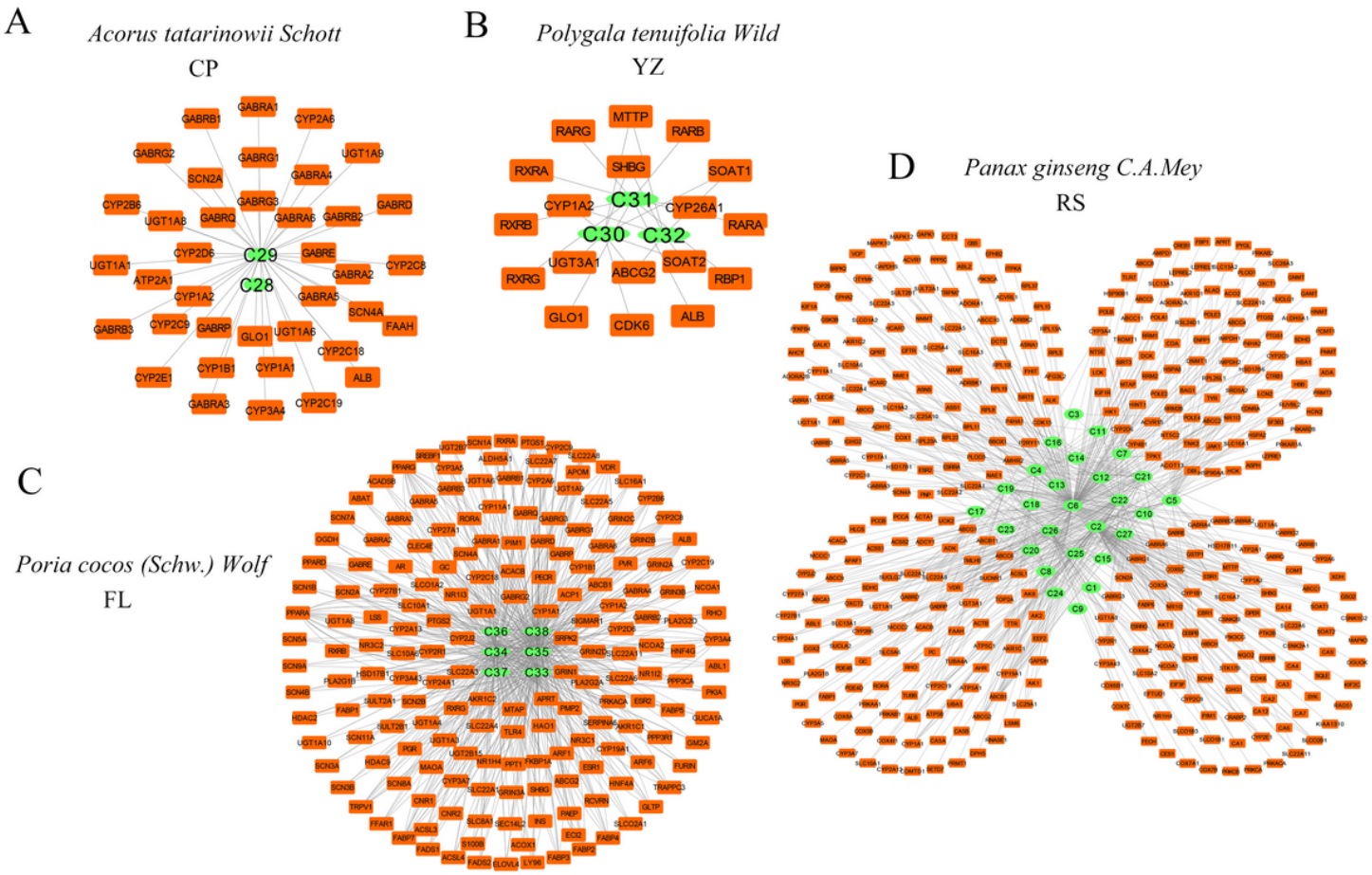

Figure 2

The ingredient-target network of single herb in Kai Xin San. (A) FL-targets, (B) YZ-targets, (C) CP-targets, and (D) RS-targets. The green oval nodes represent ingredients, and the orange rectangle nodes represent targets.

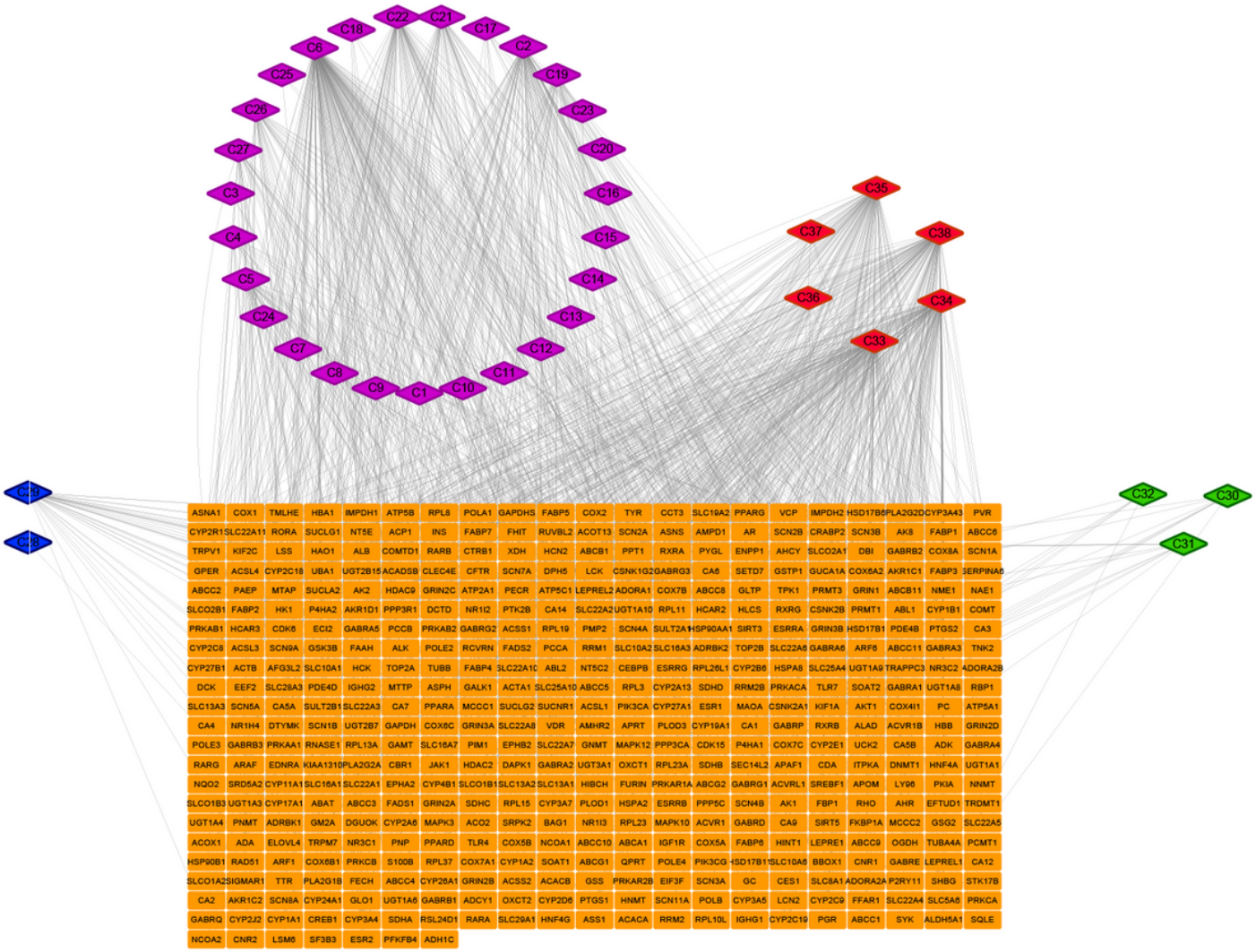

Figure 3

The ingredient-target network of Kai Xin San. The compound-target network was constructed by linking the herbs, candidate compounds and their potential targets. The nodes represent ingredients of herbs (blue for CP, purple for RS, red for FL and green for YZ), and the orange rectangle nodes represent the shared targets of these four herbs.

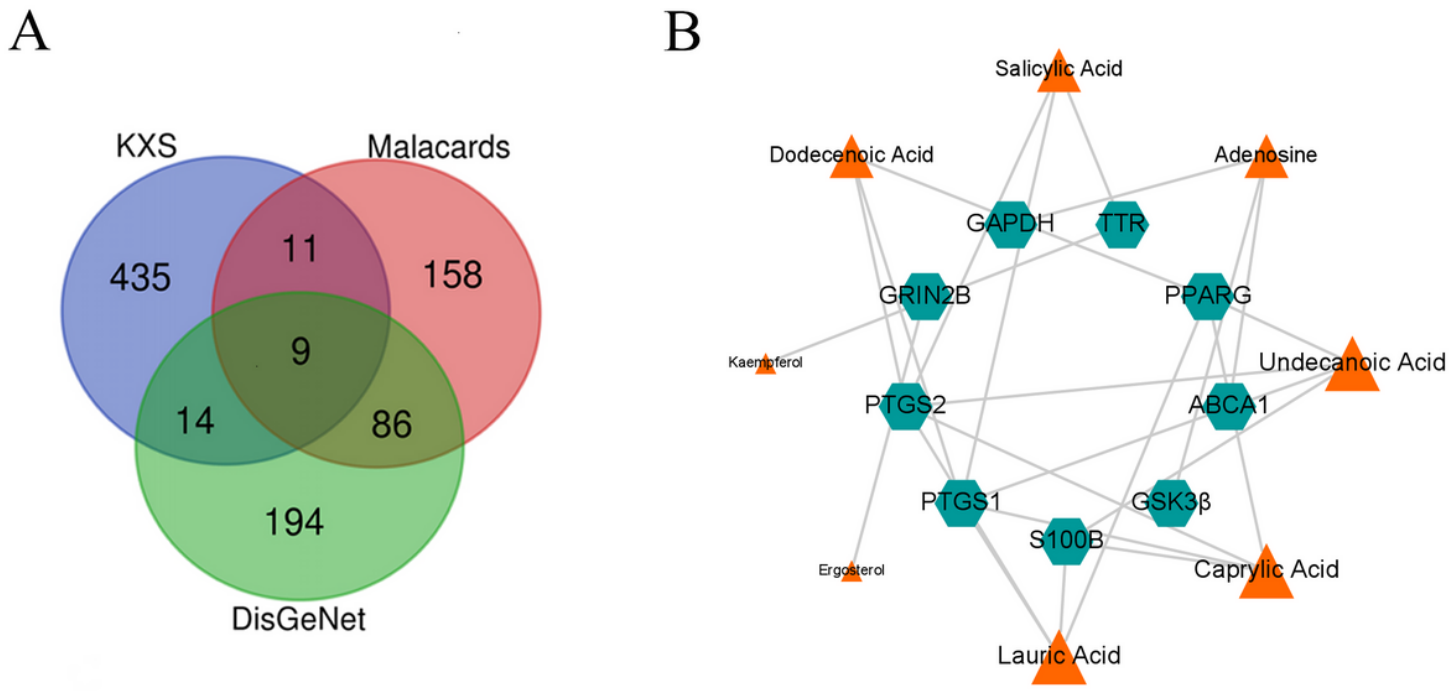

Figure 4

Compound–target-disease network implicated in the AD treatment using Kai Xin San. (A) The Venn diagram analysis showed the intersection of KXS-related targets and AD-related genes from Malacards and DisGeNet database. (B) The compound-target-disease network based on the intersection targets revealed the key ingredients in KXS. Blue hexagons represent target proteins and the orange triangle nodes represent the KXS-derived active compound. The gray edges showed the interaction between the compounds and targets, and the node size of compounds is proportional to the degree of interaction.

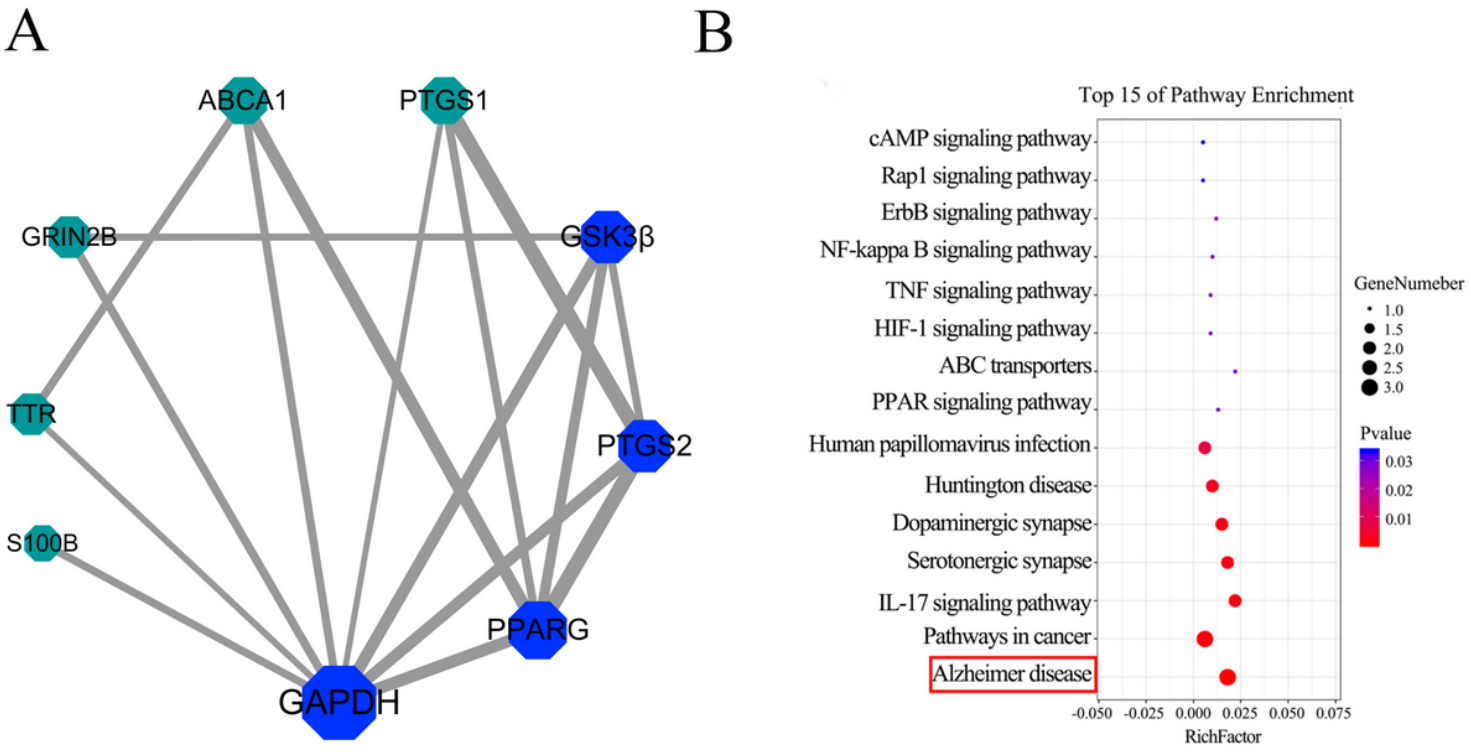

Figure 5

PPI network and KEGG pathway enrichment analysis. (A) The PPI network of potential targets of Kai Xin San for the treatment of AD. The hexagons represent intersection hub targets and their node size is proportional to the degree value in the network. Key targets with the largest node size according to “Degree” were highlighted in blue. (B) Top 15 of KEGG pathway enrichment analysis of the 9 hub genes.

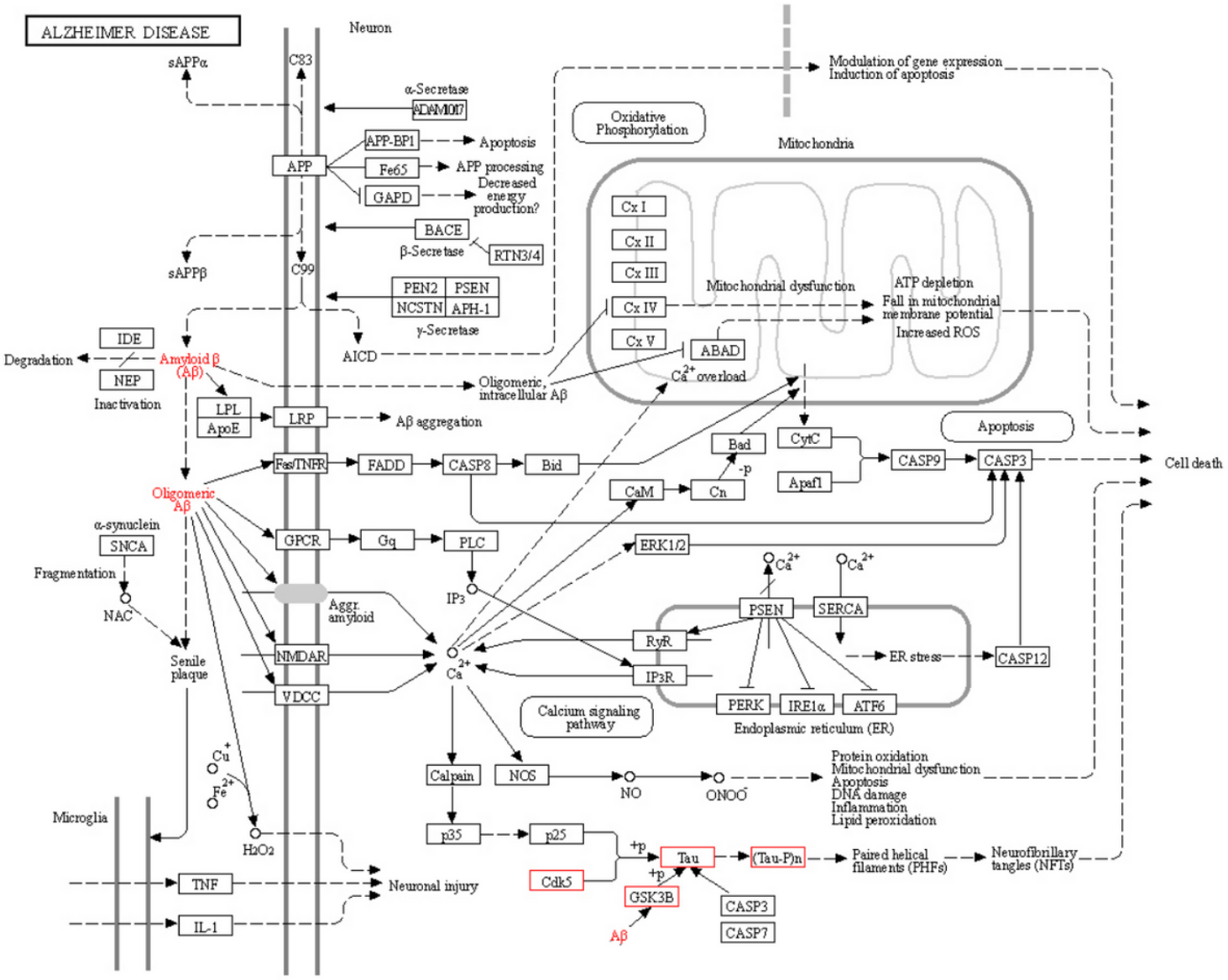

Figure 6

KEGG pathway suggested that various targets including GSK3β in AD were tightly associated with Kai Xin San pharmacological actions. Representative compounds and targets that were involved in AD-related pathways. The red rectangle nodes represent the most significant genes or biological pathways associated with pharmacological actions of KXS (P=0.0000789).

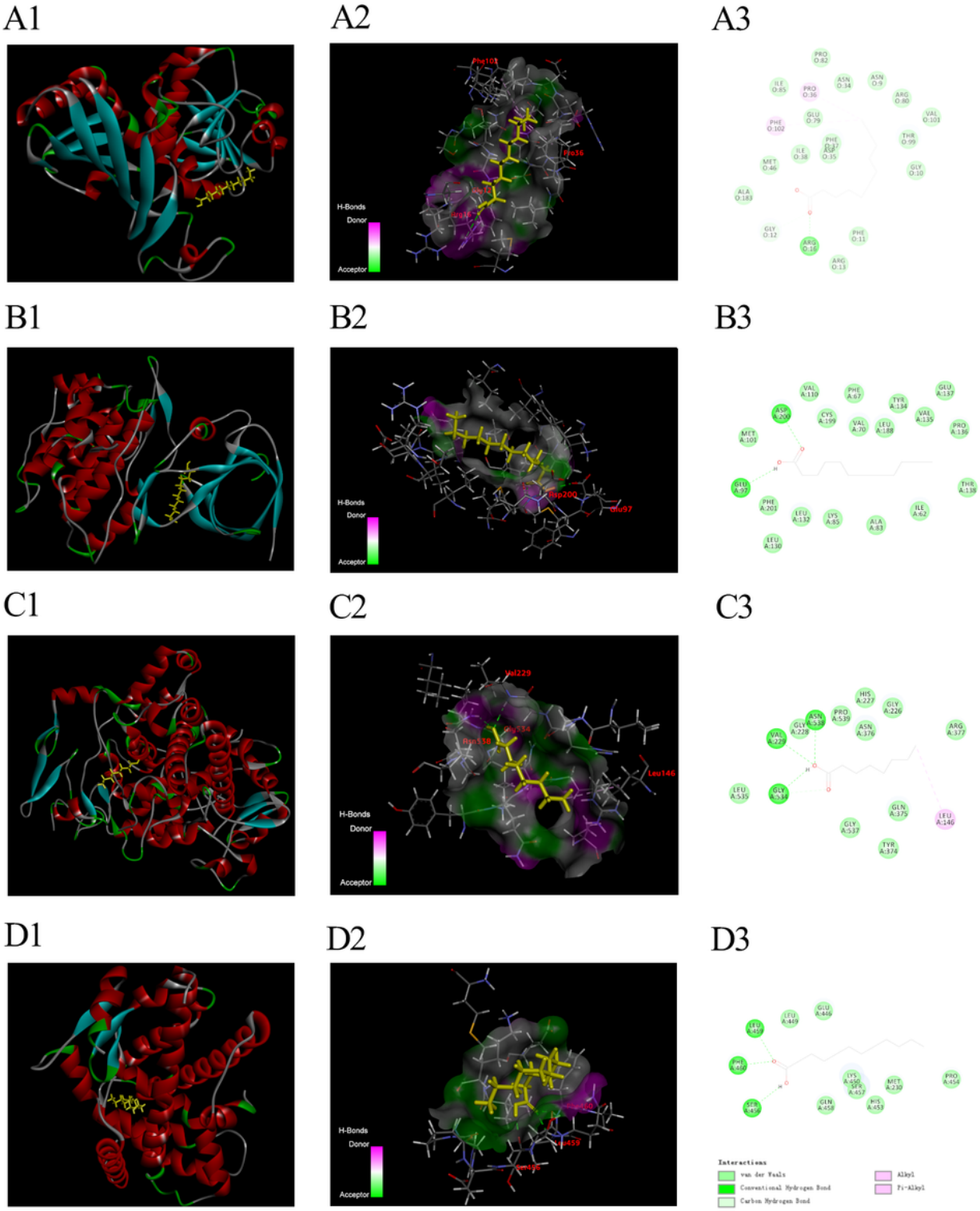

Figure 7

Interaction diagram between key ingredients and target proteins. (A1-A3) Docking between GAPDH and undecanoic acid, (B1-B3) docking between GSK3β and lauric acid, (C1-C3) docking between PTGS2 and caprylic acid, and (D1-D3) docking between PPARG and lauric acid. The key compound molecules are shown in yellow sticks, the key target protein is displayed in solid ribbon and the interacting residues around the compound are displayed as sticks.

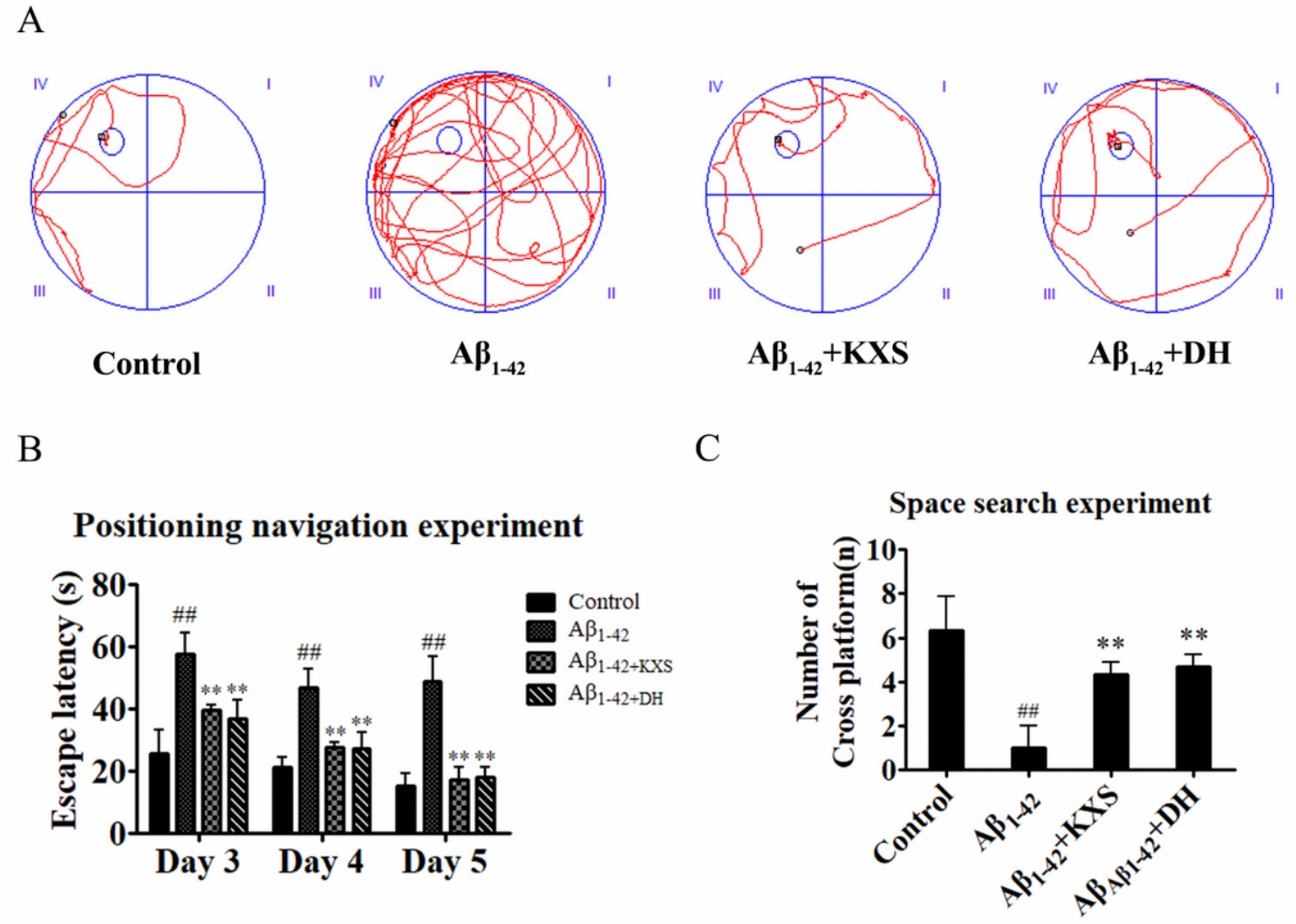

Figure 8

Kai Xin San ameliorated cognitive deficits on Aβ1-42 injection Rats. (A) Representative images of the swim paths. (B) In the directional navigation experiment, rats in the KXS and DH groups took less time to reach the platform on the training day compared with the Aβ1-42 group. During the test, the escape latency of rats in KXS and DH groups was significantly shorter than that of the Aβ1-42 group. (C) In the spatial search experiment, the numbers of platform crossings of the rats in the KXS and DH groups were higher than that of the Aβ1-42 group. All values represent the means ± SD (n=6, #P<0.05, ##P<0.01 vs. control group; *P<0.05, **P<0.01 vs. Aβ1-42 group).

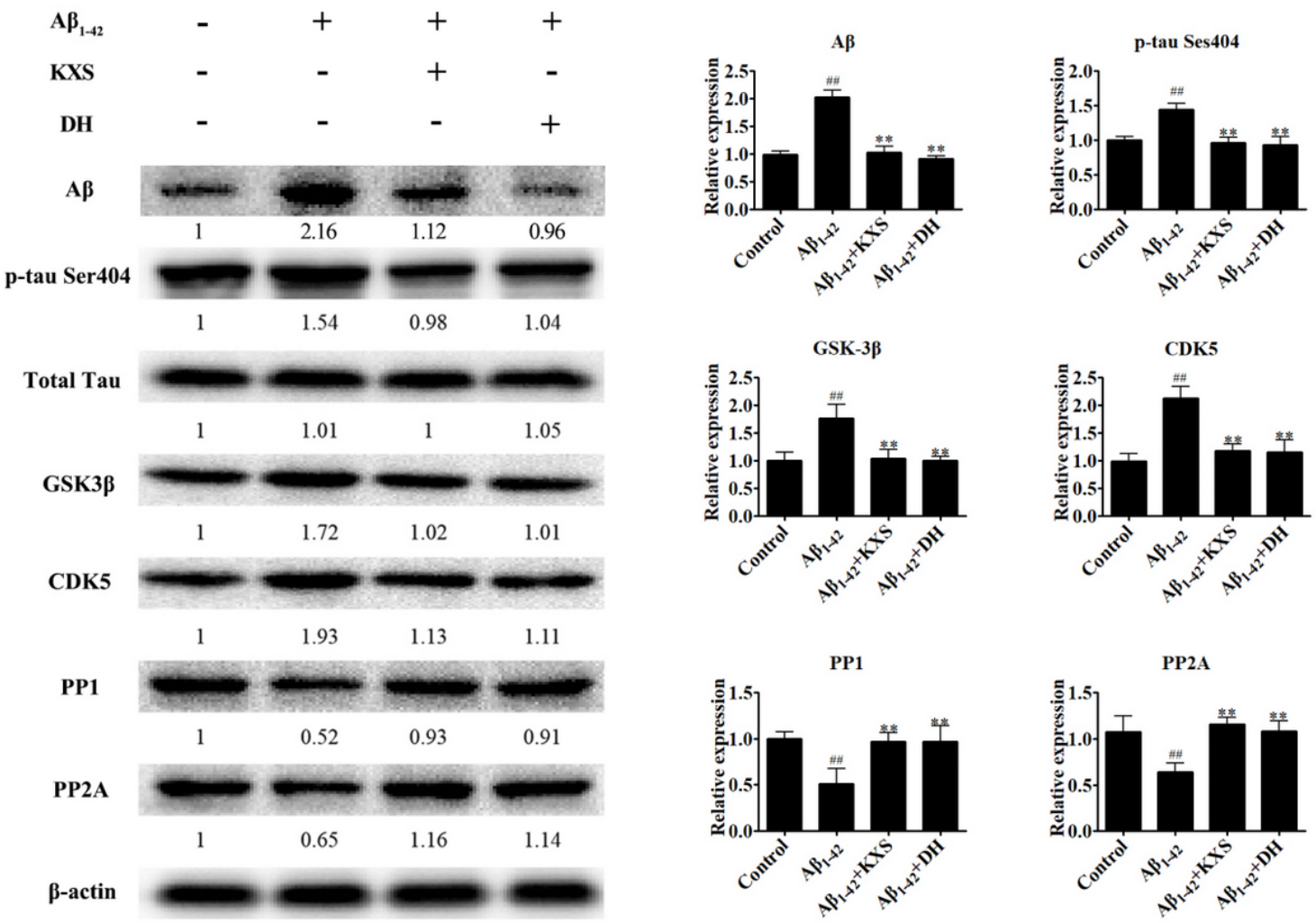

Figure 9

Kai Xin San administration attenuated Aβ, p-Tau Ser404, GSK3β as well as CDK5 levels, and upregulated PP1 and PP2A levels in hippocampus of Aβ1-42 injection Rats. Compared with the Aβ1-42 group, the levels of Aβ, p-Tau Ser404, GSK3β, as well as CDK5 decreased markedly in KXS and DH groups. Whereas, the expressions of PP1 and PP2A were increased obviously in KXS and DH groups. No significant change was found in the relative expression of Tau in each group. All values represent the means ± SD (n=6, #P<0.05, ##P<0.01 vs. control group; *P<0.05, **P<0.01 vs. Aβ1-42 group).

Supplementary Files

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- SupplementaryFile.rar