BMC Complementary Medicine and Therapies

RESEARCH

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Network pharmacology mechanism of *Rosmarinus officinalis* L.(Rosemary) to improve cell viability and reduces apoptosis in treating Alzheimer's disease

Jingzhi Zhao[1†], Zhejian Li[1†], Rongping Zhang[2], Haofei Yu[1*] and Lanchun Zhang[1*]

Abstract

Background Alzheimer's disease (AD) is a neurodegenerative disorder characterized by neurodegeneration, nerve loss, neurofibrillary tangles and Aβ plaques. Different process of the AD pathology present more opportunities for treatment. In addition, the holistic approaches involving network pharmacology with traditional Chinese medicine (TCM) may be viable options for AD treatment, and lead to an effective cure for AD in the future. Therefore, this study explored the therapeutic effect and mechanism of *Rosmarinus officinalis* L(rosemary) in the treatment of Alzheimer's disease on basis of cell experiments, network pharmacology and molecular docking.

Methods We performed cell experiments, to investigate the therapeutic effects of *Rosmarinus officinalis* L on AD in vitro using CCK8 assay, flow cytometry and TMRE Kit. In addition, carnosic acid is a major antioxidant diterpenoid in *Rosmarinus officinalis* L. We performed cell experiments, to investigate the neuroprotective effects of carnosic acid on AD in virto using CCK8 assay and flow cytometry. Furthermore, the main antioxidant compounds of rosemary ware collected through literature reviews, PharmMapper and Swiss Target prediction ware used to identify their potential targets. Targets of AD were obtained from Genecards and OMIM. The intersection targets of the main active components of rosemary and the therapeutic targets of Alzheimer's disease were subsequently obtained by using online Venn diagram. Protein–protein interaction, Cytoscape, Gene Ontology, and Kyoto Encyclopedia of Genes were used to analyze potential targets and key pathways of rosemary in AD. Besides, through molecular docking, the interactions of the main active components of rosemary, and the predicted candidate targets were verified. Finally, quantitative Real-Time PCR (RT-qPCR) was performed to confirm the effectiveness of the genes.

Results It was found that rosemary could reversed Aβ25–35 induced damage to mouse hippocampal neuron HT22 cells, significantly improved the viability of damaged cells, and reduced apoptosis. The results of fluorescent staining with Hoechst 33,342 and TMRE suggested that rosemary inhibited the reduction of mitochondrial

†Jingzhi Zhao and Zhejian Li contributed equally to this work.

*Correspondence:
Haofei Yu
yufei5322032@163.com
Lanchun Zhang
zhanglanchun@kmmu.edu.cn

Full list of author information is available at the end of the article

membrane potential levels induced by Aβ25–35, which had a specific protective effect on AD in vitro. Additionally, a main antioxidant compound in rosemary, carnosic acid, also has neuroprotective effects. Eight main antioxidant compounds of rosemary ware collected. Network pharmacology and molecular docking, revealed that rosemary plays a therapeutic role in the treatment of Alzheimer's disease through the main active carnosic acid, carnosol, rosmarinol, rosmadial, genkwanin, cirsimaritin, rosmarinic acid and caffeic acid in *Rosmarinus officinalis* L, which affects the gene regulation of HRAS, ESR1, RHOA, IGF1, SRC, ANXA5, MMP9, MAPK14, NOS3, and PIK3R1, and participates in the PI3K-Akt, Rap1, MAPK, and estrogen signaling pathways. RT-qPCR indicated that rosemary could elevated expression of IGF1, MMP9 and decreased mRNA levels of SRC, MAPK14, compared with the control group.

Conclusions Rosemary is an important economic plant with multi-component, multi-target and multi-pathway synergistic effects.The findings highlight the effectiveness of rosemary in helping to increase cell viability and reduce apoptosis when treating mouse hippocampal neuron HT22 cells, thereby supporting its therapeutic potential in treating Alzheimer's disease.

Keywords *Rosmarinus officinalis* L., Alzheimer's disease, Network pharmacology, Molecular docking

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and the most prominent cause of dementia [1].The pathogenesis of AD is complex and consists mainly of amyloid β (Aβ) deposition and hyperphosphorylation of tau protein [2, 3]. Aβ protein is derived from the proteolytic hydrolysis of Aβ precursor proteins, and the gradual accumulation of Aβ leads to the formation of Aβ oligomers and protofibrils, which aggregate to form plaques [4]. This aggregation leads to the activation of kinases, which results in the hyperphosphorylation of microtubule-associated τ proteins and their polymerization into insoluble neurogenic fiber tangles (NFTs). The aggregation of plaques and tangles promotes microglia activation and localized inflammatory responses, leading to neurotoxicity [5]. The main component of NFT is tau, a microtubule-associated protein expressed predominantly in neurons. Tau phosphorylation has been strongly implicated in AD, mainly because NFT purified from AD brains is enriched in highly phosphorylated tau [6].Currently, the primary drugs used to treat AD include cholinesterase inhibitors and N-methyl-D-aspartate receptor antagonists, which can relieve AD but fail to treat AD at its root [7, 8] .Therefore, there is a need to develop safe and effective drugs to combat Alzheimer's disease, which is a long-term threat to human life and health.

Rosmarinus officinalis L.(rosemary), a plant of the genus Rosmarinus in the family Labiatae, originated in Europe and the Mediterranean coast of North Africa, where its uses include culinary spices, medicinal plants and food preservatives [9]. Rosemary has been used to relieve headaches, dysmenorrhea, stomach pains, epilepsy, rheumatic pains, spasms, nervous agitation, improvement of memory, hysteria, depression, and physical and mental fatigue [10] Moreover, rosemary has antioxidant and anti-inflammatory properties, which inhibits acetylcholinesterase and amyloid aggregation in AD, and acts as a therapeutic agent for Alzheimer's disease [11]. However, the potential mechanism of rosemary in the treatment of Alzheimer's disease is not clear.

Diseases occur involves pathways with many diverse targets. With the advancement of bioinformatics, network pharmacology and molecular docking have become approaches for discovering effective drugs. Network pharmacology is an approach predicting the targets of diseases and drugs, and reflecting the mechanism of drugs in diseases, which includes systems biology, network analysis, connectivity, redundancy and pleiotropy [12]. Molecular docking is based on computerized structures that predict ligand-target interactions at the molecular level [13].Using network pharmacology and molecular docking techniques can reveal the molecular mechanisms of drug therapy for disease. What's more, the holistic approaches of network pharmacology with traditional Chinese medicine may be viable options for AD treatment, and lead to an effective cure for AD in the future. Hence, our study combines network pharmacology and molecular docking to explore the targets and pathways of rosemary associated with Alzheimer's disease.

In the present study, we mainly verified that rosemary can ameliorate damage in AD cell models. Network pharmacology and molecular docking techniques were applied to screen out and validate the core chemical components of rosemary as well as the core targets of action that are common to Alzheimer's disease, clarify the mechanism of *Rosmarinus officinalis* L in the treatment of AD. It may provide a theoretical basis for the use of *Rosmarinus officinalis* L. for the treatment of Alzheimer's disease.

Materials and methods

Vitro experiments

Materials and reagents

Rosemary medicine and carnosic acid were provided by the Department of Pharmaceutical Chemistry, School of

Pharmacy, Kunming Medical University. β-Amyloid (25–35) (236838) was purchased from MedChemExpress, 0.25% Trypsin (G4001), PBS (G4201), DMEM high glucose (G4510), and Penicillin/Streptomycin (G4003) were from Servicebio, Fetal Bovine Serum FBS (FBSRY0901) was from Ozfan, cell grade DMSO (D8371) was from Solarbio, Cell Counting Kit-8 was obtained from MCE (Cat. No.: HY-K0301), Annexin V-FITC Apoptosis Detection Kit (C1062S), Hoechst 33,342 (P0133-25) and TMRE Kit (C2001S) were purchased from Beyotime. Fluorescence microscope was performed using NIKON (ECLIPSE Ti), microplate reader was from Thermo Scientific (Varioskan LUX). Quantitative Real-Time PCR experiments were performed on ABI's QuantStudio 3 instrument.

Cell culture

HT22 (Mouse hippocampal neuron cells) were provided by Department of Laboratory Animal Science, Kunming medical University. Cells were cultured in DMEM high glucose(DMEM) with 10% fetal bovine serum(FBS) and 1% penicillin-streptomycin and placed in a 37 °C, 5% CO_2 incubator.

Cell viability assay

In order to detect the safe concentration of rosemary and the appropriate administration concentration of Aβ25–35, hippocampal neuron HT22 cells were inoculated in 96-well plates at a density of 5×10^3/well and placed in a 37 °C, 5% CO_2 incubator for cultivation. Rosemary was first dissolved with cell-grade DMSO, and then diluted with different concentrations (0.01875, 0.0375, 0.075, 0.15, 0.3, 0.6 and 0.8 μg/mL) of the drug solution using the culture medium, DMSO content in the medium was not more than 1‰. Aβ25–35 was diluted to 1 mM with sterilized saline water and then incubated at 37 °C for 7 days before use. And then, Aβ25–35 was diluted with medium to different concentrations (100,80,40,20,10 μM). Cells were treated with different concentrations of rosemary after Aβ25–35 affected for 24 h, then 10 μL of CCK-8 solution was added to each well and the plates were incubated for 2 h. Absorbance values were measured at a wavelength 450 nm with a microplate reader, thus the cell viability was calculated to get the suitable concentration of rosemary and Aβ25–35 administration. Finally, HT22 cells were randomly divided into normal group, Aβ group (Aβ25–35) and rosemary group (Aβ25–35 + rosemary); Aβ and rosemary groups were affected with Aβ25–35 for 24 h, then rosemary group was given rosemary for treated. Meanwhile, the neuroprotective effect of the main antioxidant compound carnosic acid in rosemary was evaluated via the same method.

Cell apoptosis assay

Hippocampal neuron HT22 cells were inoculated in 6-well plates at a density of 5×10^5/mL. HT22 cells were divided into normal group, Aβ group (Aβ25–35) and rosemary group (Aβ25–35 + rosemary). In the beginning, Aβ25–35 affected on Hippocampal neuron HT22 for 24 h, and the rosemary was added for treatment 24 h followed; After that, the cells were collected to tubes and washed twice with PBS, then 195 μL of Annexin V- FITC conjugate solution, 5 μL of Annexin V-FITC and 10 μL of PI staining solution were added to the tubes one by one. Lastly, gently mixed and incubated at 20–25 °C for 10 min, followed by placed in an ice bath and immediately detected by flow cytometry.

The test of mitochondrial membrane potential

HT22 cells were divided into normal group, Aβ group (Aβ25–35), CCCP group and rosemary group (Aβ25–35 + rosemary). The cells were treated with positive drug CCCP(10 μM) in a cell incubator at 37 ℃ for 40 min, and then incubated with TMRE(1X) staining solution for 35 min. After incubation, the cell morphology was observed by fluorescence microscope.

Quantitative real-time PCR(RT-qPCR)

Total RNA of HT22 cells were isolated by TRIzol® reagent (Thermo Fisher Scientific), according to the manufacturer's protocols and reverse transcribed into cDNA using the RevertAid cDNA Synthesis kit (Thermo Fisher Scientific). RT-qPCR was performed at 95 ˚C for 2 min, followed by 40 cycles at 95 ˚C for 20 s, 52˚C for 30 s and 60 ˚C for 40 s. The calculation of relative gene expression was operated with the $2^{-\Delta\Delta Cq}$. Sequences of the primers used in this study are listed in Table 1.

Statistical analysis

All the statistical analyses were performed using R software (version 4.2.1) and SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Data between the two groups were analysed using the Student's *T*-test. Data are presented as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$. LSD (L) test was used when homogeneity of variance was assumed, and Dunnett's T3 test was used when the variance was not homogeneous.

Network pharmacological analysis

Software and databases

Software of Cytoscape 3.9.0, CytoHubba plug-in, R4.2.0, ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 were used for network pharmacological analysis. Databases of PubChem (https://pubchem.ncbi.nlm.nih.gov/), Swisstargetprediction (http://www.swisstargetprediction.ch/), PharmMapper (http://lilab-ecust.cn/pharmmapp

Table 1 Primers for RT–qPCR

Genes	Primer: Sequence (5'->3')
GAPDH	Forward primer: GCATCTTCTTGTGCAGTGCC Reverse primer: GATGGTGATGGGTTTCCCGT Product length: 262
ESR1	Forward primer: AAGACGCTCTTGAACCAGCA Reverse primer: AGGCTTTGGTGTGAAGGGTC Product length: 121
IGF1	Forward primer: TCCCAGATCTCAGCATAGCCT Reverse primer: CCCTTGTCCTAGTTGCCAGG Product length: 138
MMP9	Forward primer: AAACCTCCAACCTCACGGAC Reverse primer: CTGAAGCATCAGCAAAGCCG Product length:122
NOS3	Forward primer: AGATGCCCAACCCAAACCTT Reverse primer: AGGTGTCTGGGACTCACTGT Product length:114
SRC	Forward primer: AGGCCACATTTGAAAACCCG Reverse primer: TAAGGAGGTAGTGTGGCCCT Product length:139
MAPK14	Forward primer: GCGCCCCTCAAGATCAAGAA Reverse primer: GGTAGGTCCTTTTGGCGTGA Product length:113

er/index.html), GeneCards (https://www.genecards.org/), OMIM database (https://www.omim.org/), Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/), String database (https://cn.string-db.org/), DAVID database (https://david.ncifcrf.gov/), online microbioconductor mapping tool (http://www.bioinformatics.com.cn/) and Bioconductdatabase (http://www.bioconductor.org/) were used for Gene acquisition and gene analysis. PDB database (https://www.rcsb.org/) can provide protein data bank and structure models.

Screening of active ingredients and their targets of Rosmarinus officinalis L

The main active ingredients of rosemary were obtained through literature reviews, and the names of the active ingredients were entered into the PubChem database to search for their "Canonical SMILES". In the Swiss database, the species were set as "Homo sapiens", the SMILES numbers of the obtained compounds were inputted, the obtained genes were screened according to the "Probability" value of ≥ 0.3, and the corresponding targets were obtained. In the PharmMapper database, the names of the active ingredients were entered. The genes obtained were screened according to the "zscore" value greater than or equal to two times the median. Finally, the results from the PharmMapper and Swiss databases were integrated to identify the relevant targets of the drug ' s active ingredients.

Alzheimer's disease target screening

"Alzheimer's Disease" was entered as a keyword into the GeneCards database, and the obtained targets were screened according to the "Relevance Score" values, which were filtered based on a median ≥ 2.47. WE searched for "Alzheimer's Disease" in the OMIM database to obtain the corresponding target. Integrate data from GeneCards and OMIM to obtain the relevant targets for the disease.

Drug-disease intersection targets

The obtained targets of the drug's active ingredients and disease-related action targets are intersected by an online Venn Diagram to get the intersecting targets of the drug and disease.

Construct protein interaction network

Enter the intersecting genes into the "Multiple Proteins" column in the String database, and set "Organisms" to "Homo sapiens", remove all isolated genes and obtain the PPI network diagram of the intersected targets. Using R4.2.0 software, the top ten genes were calculated based on the degree value and exported as a histogram. Export the obtained PPI network as a tsv file in the String database. Cytoscape 3.9.0 software for visualization, and calculated the top ten genes in terms of Degree value by CytoHubba, PPI analysis was carried out in Cytoscape to yield the top 10 potential core genes based on the Maximal Clique Centrality (MCC) algorithm [14].

Constructing "drug-component-target gene-disease" network

The obtained list of main active ingredients and intersecting genes of rosemary was imported into Cytoscape 3.9.0 software, and the size, shape and colour of the nodes were modified to create a "drug-ingredient-target-gene-disease" network for network pharmacology visualization.

GO functional enrichment analysis and KEGG pathway enrichment

Gene ontology (GO) functional enrichment analysis was performed using the DAVID database. The top 10 biological processes (BP), cellular components (CC), and molecular functions (MF), as well as intersecting genes, were obtained by filtering according to $P < 0.05$. The top 10 BP, CC, MF, and intersecting genes were functionally enriched. The online microbioconductor mapping tool was utilized to export the BP, CC, and MF three-in-one GO functional analysis maps. Based on the Bioconductor database, the ClusterProfilerba package and Pathway package were installed using R4.2.0 software, and the Kyoto encyclopedia of genes and genomes (KEGG) Signaling pathway enrichment analysis was carried out, and the top genes were obtained according to the $P < 0.05$ filter, the top 20 pathways and the enrichment

of intersecting genes on the pathways were created, and bubble plots were exported for visualization.

Molecular docking

The 3D structures of the screened compounds were drawn using ChemDraw and Chem3D, and the genes obtained from the screening were inputted into the PDB database to search for proteins corresponding to ligands with similar structures to the core components. The protein structures obtained were imported into PyMOL. After performing operations such as de-watering and searching for ligands, the pdb files of the resulting proteins and ligands, as well as the mol2 files of the compounds, were the pdb files of the resulting protein and ligand and the mol2 files of the compounds were imported into AutoDock Tools for the format conversion of pbdqt. The molecular docking operation was carried out by utilizing AutoDock Vina. Finally, editing was carried out in PyMOL to obtain the molecular docking results. A molecular docking heatmap was produced by utilizing Excel.

Results

Rosmarinus officinalis L. improves cell viability and reduces apoptosis in vitro cell model of Aβ-induced AD

CCK-8 method was used to detect the affect of Aβ25–35 on the viability of hippocampal neuron HT22 cells. Compared with normal group, Aβ25-35 significantly decreased the cell viability ($P<0.001$), and when the cells were induce by 20 μM Aβ25–35 for 24 h, the cell viability was decreased to about 50% (Fig. 1A-B), which was used the concentration chosen for subsequent experiments. After pretreatment with different concentrations of rosemary for 24 h, there was no significant difference between the negative control and rosemary groups at the concentrations of 0.01875, 0.0375 and 0.15 μg/mL.

There were some pro-proliferative affects at 0.3 and 0.6 μg/mL concentrations. There was an affect on the cell viability at the concentration of 0.8 μg/mL ($P<0.05$) (Fig. 2A), and rosemary was used at 0.6 μg/mL as the concentration for subsequent experiments. As well, carnosic acid was used at 0.5 μg/mL as the concentration for subsequent experiments (Fig. 3A). CCK-8 assay results showed that compared with Aβ group, rosemary and carnosic acid significantly increased the cell viability($P<0.001$) (Fig. 2B) (Fig. 3B); apoptosis assay found that the apoptosis rate was significantly decreased in the rosemary-treated group and carnosic acid-treated, compared with the Aβ group ($P<0.001$) (Fig. 2C) (Fig. 3C).

Under normal conditions, there is a large amount of negative charge in mitochondria, and TMRE, as a cation probe, can accumulate in the mitochondrial matrix after entering the cell and emit bright orange fluorescence. When apoptosis occurs, mitochondrial membrane potential is lost, the mitochondrial permeability transition pore (MPTP) continues to open, TMRE is released into the cytoplasm, and the intensity of orange

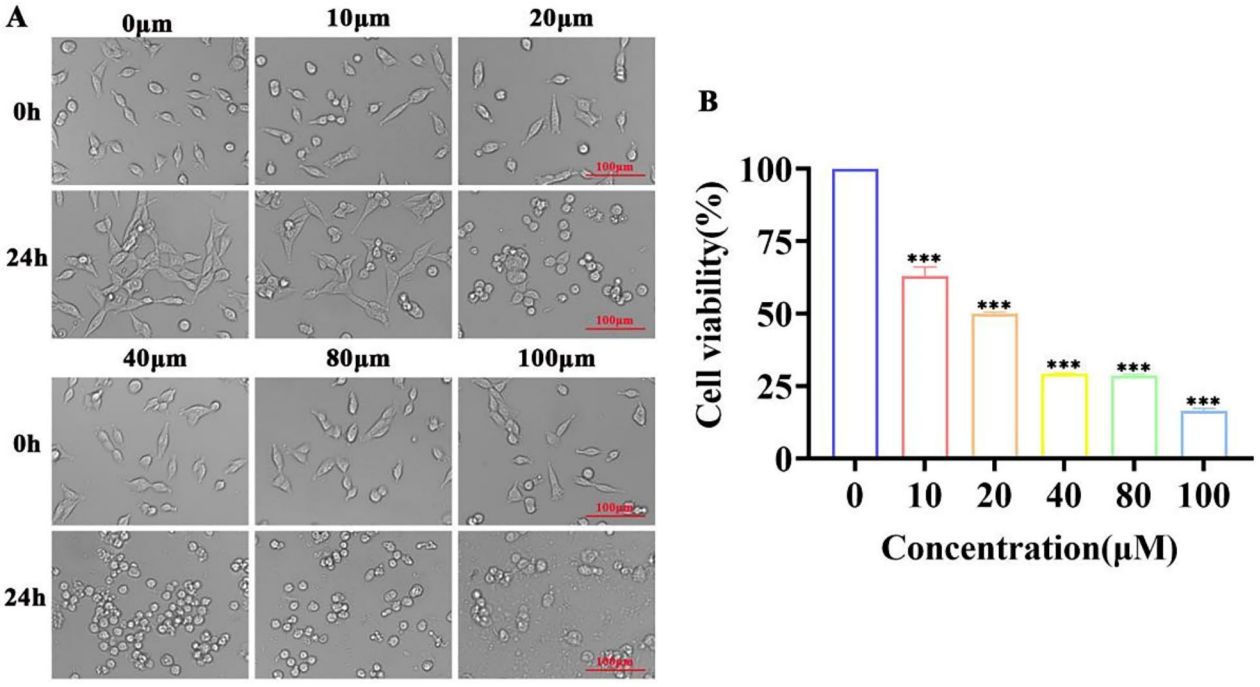

Fig. 1 (**A**) Morphology of Aβ25-35-induced injury in HT22 cells; (**B**) Viability of Aβ25-35-induced injury in HT22 cells. ***: $p<0.001$

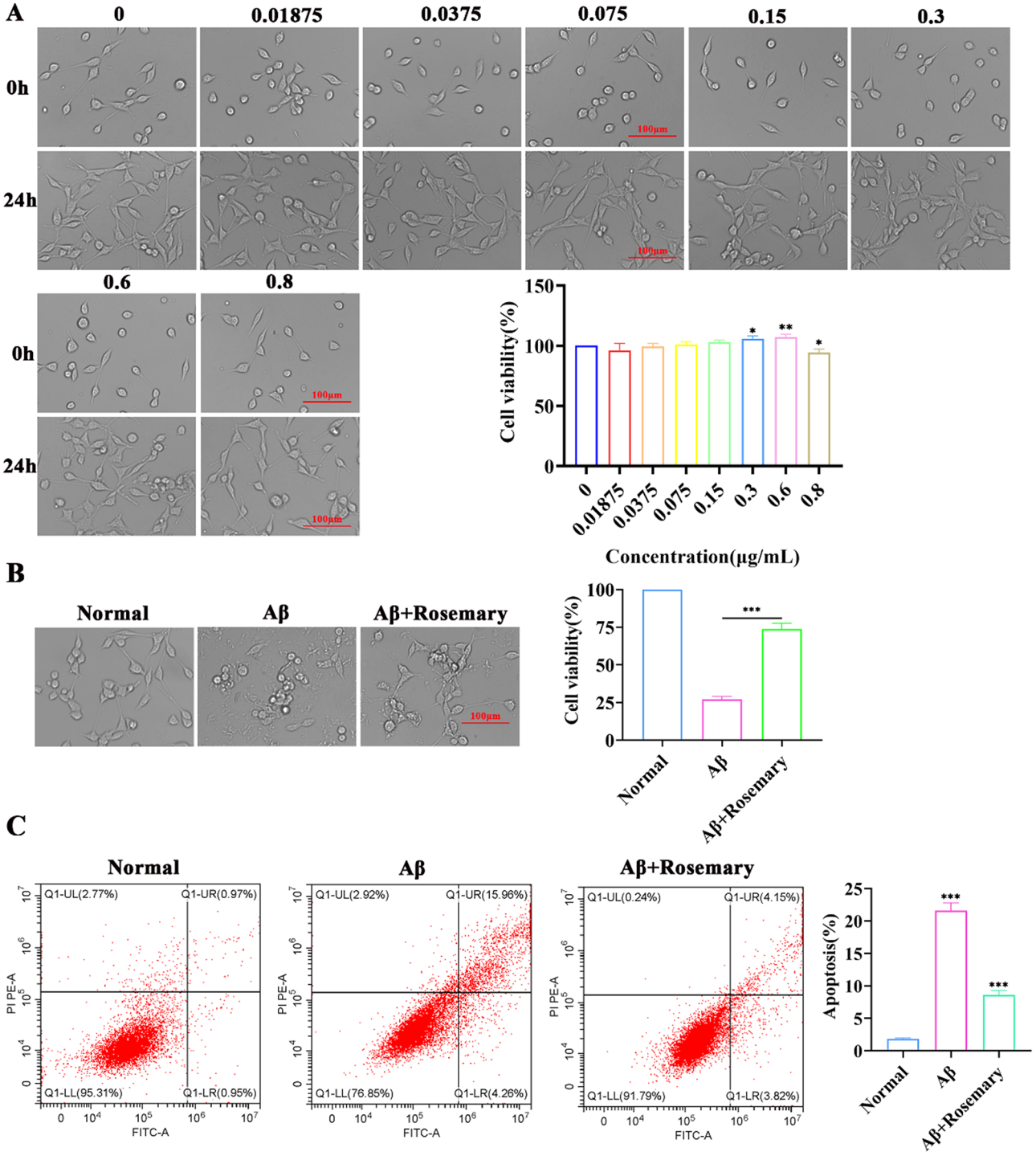

Fig. 2 (**A**) Treatment of different concentrations of rosemary on the morphology and viability of HT22 cells; (**B**) Rosemary on the viability of HT22 cells damaged by Aβ25–35;(**C**) Rosemary on the apoptosis rate of Aβ25-35-injured HT22 cells detected by flow cytometry. *: $p < 0.05$;**: $p < 0.01$;***: $p < 0.001$

fluorescence in mitochondria decreases significantly. Rosemary reduced Aβ-induced HT22 cell damage in AD models to enhance the mitochondrial membrane potential levels staining (Fig. 4).

Screening main active components of *Rosmarinus officinalis* L

The typical antioxidant constituents of rosemary are carnosic acid, carnosol, rosmarinol, rosmadial, genkwanin, and cirsimaritin [15]. Carnosic acid and carnosol are the major antioxidant components of rosemary extracts,

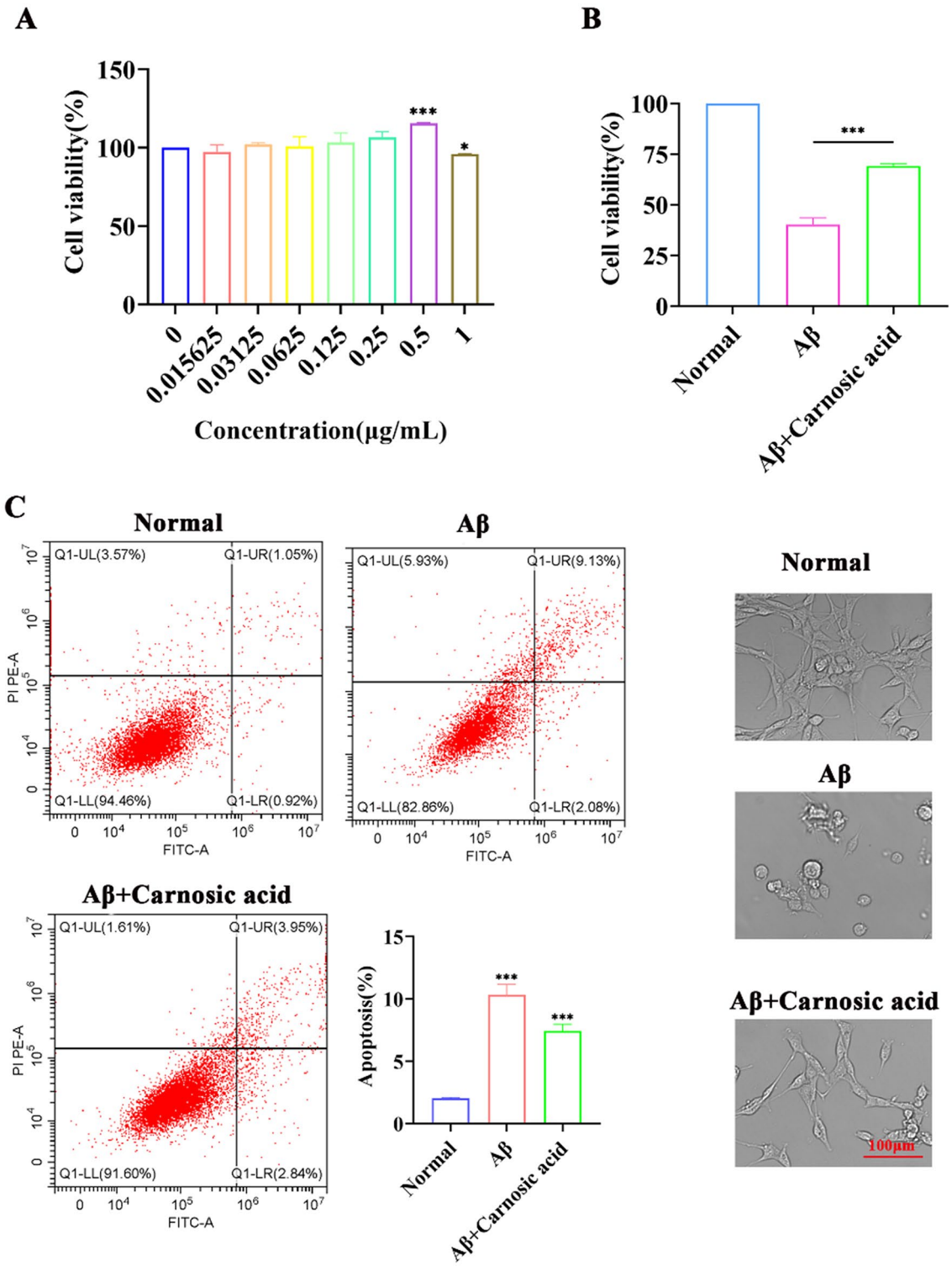

Fig. 3 (**A**) Treatment of different concentrations of carnosic acid on the viability of HT22 cells; (**B**) Carnosic acid on the viability of HT22 cells damaged by Aβ25–35; (**C**) Carnosic acid on the apoptosis rate of Aβ25-35-injured HT22 cells detected by flow cytometry. *: $p < 0.05$;. ***: $p < 0.001$

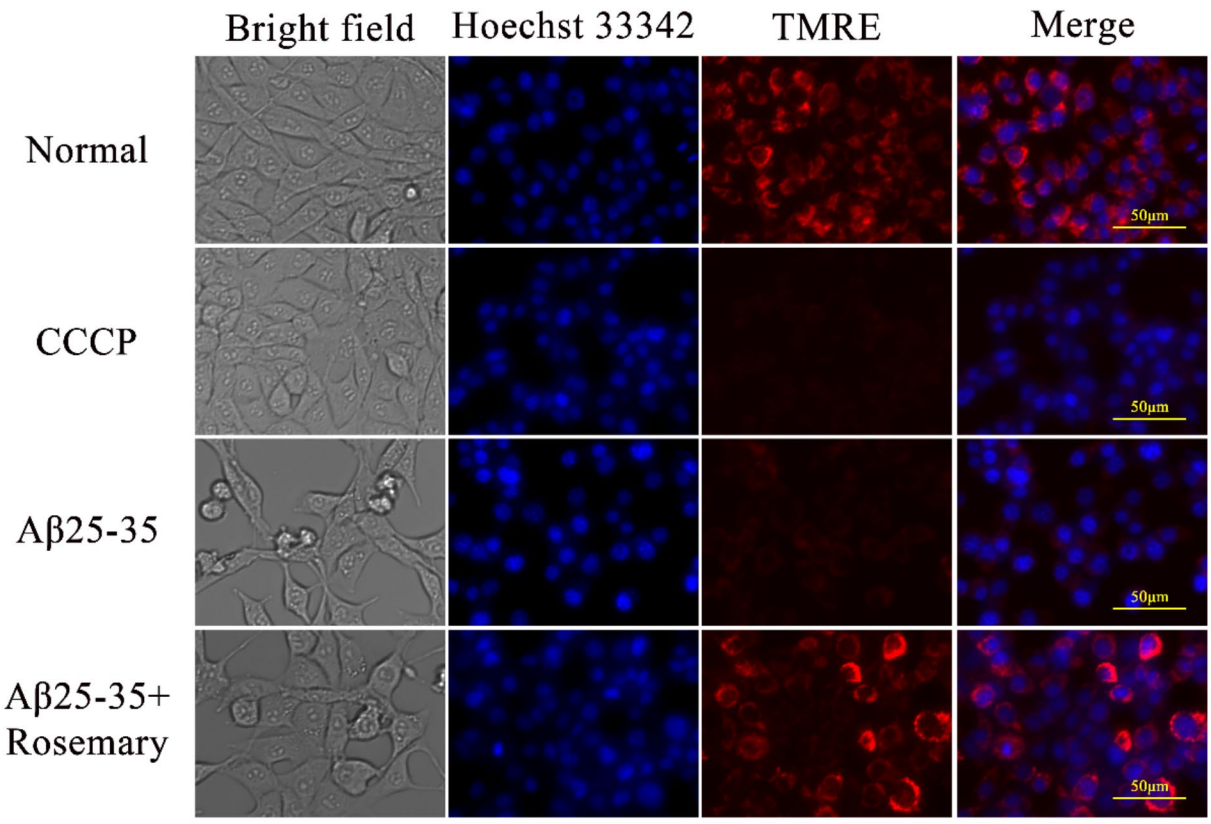

Fig. 4 Representative images of Hoechst 33,342 and TMRE staining of each group after Aβ25–35 and rosemary treatment

Table 2 The main active compounds of rosemary and their sources

Compound	Literature resources
carnosic acid	(15, 16)
carnosol	(15)
rosmanol	(15)
rosmadial	(15)
genkwanin	(15)
cirsimaritin	(15)
rosmarinic acid	(17, 18)
caffeic acid	(18)

and their presence and distribution during the growth of rosemary plants have been described [16]. Among all the rosemary extracts, rosmarinic acid was found in higher concentrations and exhibited better antioxidant capacity [17]. Rosemary extract has better antioxidant activity than α tocopherol as it contains caffeic and rosmarinic acid [18]. The above results are presented in Table 2.

Main active components of *Rosmarinus officinalis* L. and their targets

The chemical structures of the main active compounds in rosemary were carnosic acid, carnosol, rosmarinol, rosmadial, genkwanin, cirsimaritin, rosmarinic acid and caffeic acid (Fig. 5).

The targets of these eight active compounds were searched using a Swiss database, and the corresponding genes were screened based on the "Probability" value of 0.3 or more. The PharmMapper database was used to search for the targets of these eight compounds, and the z-score values were screened according to the median value of two times or more. Finally, the data obtained from the Swiss database were integrated, resulting in a total of 334 genes (Table 3).

Targets for Alzheimer's disease

Using the GeneCards database, the relevant targets of Alzheimer's disease were obtained, and the "Relevance Score" value was filtered based on the median ≥ 2.47. Using the OMIM database, the corresponding targets of action were obtained. Integrating the Alzheimer's disease therapeutic targets obtained from GeneCards and OMIM databases, 5670 genes were obtained after de-emphasis.

Alzheimer's disease- *Rosmarinus officinalis* L. PPI network

The active targets of rosemary and the action targets of Alzheimer's disease were analyzed by Venn Diagram.

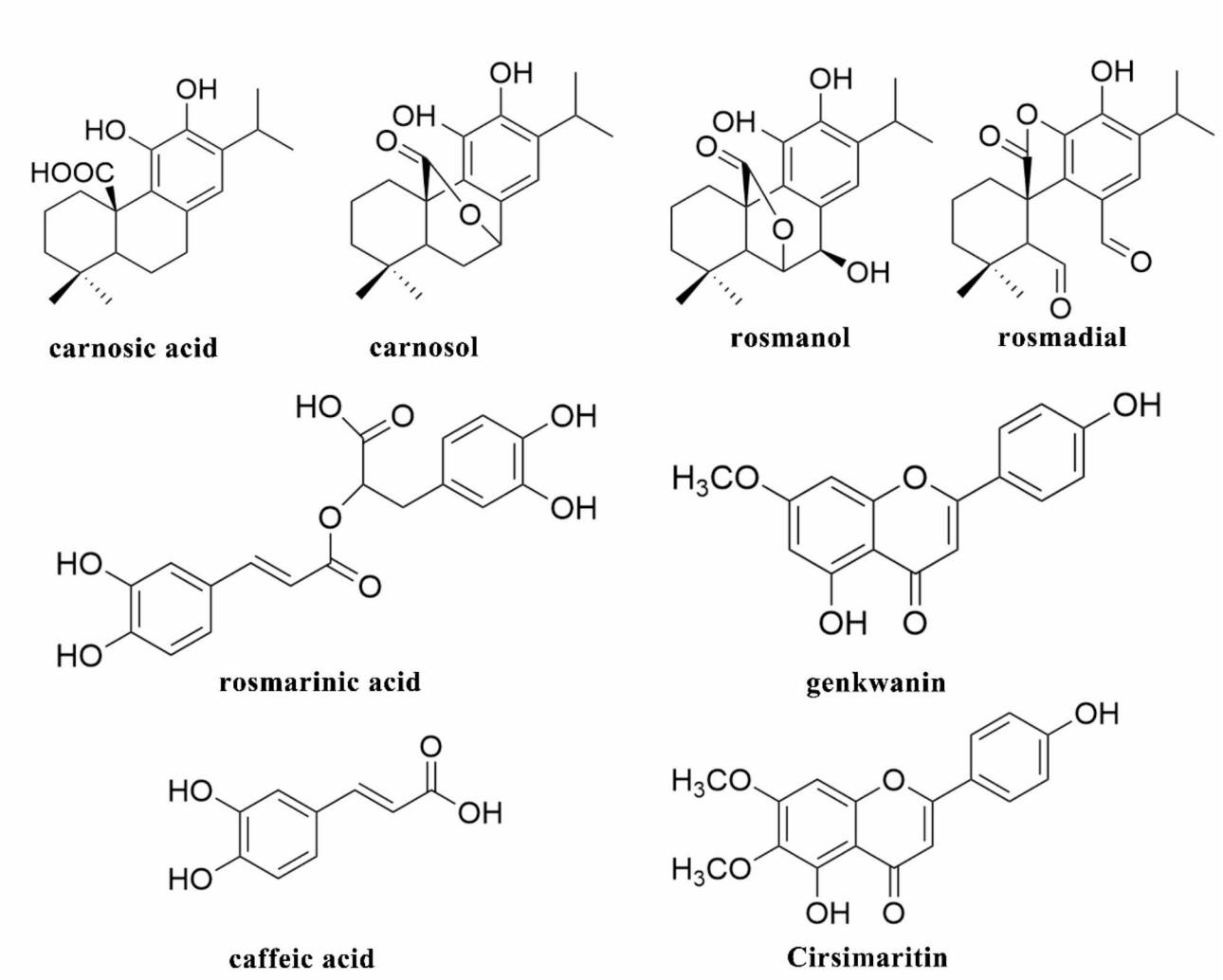

Fig. 5 The main active compounds of rosemary

Results of Venn analysis suggested 127 common targets of rosemary and Alzheimer's disease were obtained (Fig. 6A).Protein mutualism analysis of 127 common targets of rosemary and Alzheimer's disease was carried out via the String database. The PPI network mutualism graph was obtained (Fig. 6B). The protein connectivity node graph in the protein mutualism network was exported using R4.2.0 (Fig. 6C), and the top ten genes of HRAS, ESR1, RHOA, IGF1, SRC, ANXA5, MMP9, MAPK14, NOS3, PIK3R1 were obtained using the CytoHubba plugin in Cytoscape 3.9.0, which have the top ten degree values, respectively, (Fig. 6D). The larger the size of the circle where the gene is located, the higher the core of the gene.

Construction of "drug-component-target gene-disease" network

Cytoscape 3.9.0 was used to visualize network pharmacology and construct the "drug-ingredient-target-gene-disease" network diagram of rosemary and Alzheimer's disease (Fig. 7), which contains 137 nodes representing AD, rosemary, the main active ingredients of rosemary, and the common effects of rosemary and AD targets, and 334 edges, representing target-target interactions.

GO functional enrichment and KEGG pathway-enrichment analysis

GO function analysis was performed using the DAVID database, and graph was exported using the online microbiome mapping tool (Fig. 8). The horizontal coordinate in the graph indicates the function, and the vertical coordinate indicates the ratio of genes occupied. The larger the vertical coordinate, the more genes are enriched in

Table 3 The targets of the main active ingredients of Rosemary

Compound	Targets
carnosic acid	CHEK1,CHK1,SETD7,KIAA1717,KMT7,SET7,SET9,EIF4E, EIF4EL1,EIF4F, PGR, NR3C3,BCAT2,BCATM, BCT2,ECA40,NR3C1,GRL, NT5M, DNT2,CTSS, PGF, PGFL, PLGF, MTHFD1,MTHFC, MTHFD, PPP1CC, VDR, NR1I1,ANXA5,ANX5,ENX2,PP4,HSD11B1,HSD11,HSD11L, SDR26C1,KAT2B, PCAF, GSTT2B, GSTT2,EPHB4,HTK, MYK1,TYRO11,PIK3R1,GRB1,FKBP1A, FKBP1,FKBP12,ADH5,ADHX, FDH, ITK, EMT, LYK, KIT, SCFR, TRDMT1,DNMT2,ABL1,ABL, JTK7,MME, EPN, RARB, HAP, NR1B2,DAPK1,DAPK, HNMT, PTGES
carnosol	PTGES, HSD11B1,HSD11,HSD11L, SDR26C1,NR1H3,LXRA, ELANE, ELA2,THRB, ERBA2,NR1A2,THR1,HDAC8,HDACL1,CDA07,CHEK1,CHK 1,ABO, NR1I2,PXR, HSD17B1,E17KSR, EDH17B1,EDH17B2,EDHB17,SDR28C1,AKR1C3,DDH1,HSD17B5,KIAA0119,PGFS, CTNNA1,PDE4D, DPDE3,PTPN1,PTP1B, PTPN11,PTP2C, SHPTP2,HNF4G, NR2A2,CDK2,CDKN2,SULT2A1,HST, STD, TRAPPC3,BET3,CDABP0066,PRKACA, CD1A, CES1,CES2,SES1,RARA, NR1B1,NR1I3,CAR, MAP2K1,MEK1,PRKMK1,RARB, HAP, NR1B2,IGF1,IBP1,PPP5C, PPP5,RARG, NR1B3
rosmanol	PDE4D, DPDE3,ESR1,ESR, NR3A1,GSK3B, PGF, PGFL, PLGF, NR1H3,LXRA, ME2,PAH, HSD11B1,HSD11,HSD11L, SDR26C1,FKBP1A, FKBP1,FKBP12,SOD2,ABO, THRB, ERBA2,NR1A2,THR1,CTNNA1,MTHFD1,MTHFC, MTHFD, MTAP, MSAP, SYK, PPP5C, PPP5,ANXA5,ANX5, ENX2,PP4,PTPN1,PTP1B, SORD, PDE4B, DPDE4,ITK, EMT, LYK, CHEK1,CHK1,NR1I2,PXR, EIF4E, GSTA1,ABL1,ABL, JTK7,KAT2B, PCAF
rosmadial	CYP2C9,CYP2C10,FKBP1A, FKBP1,FKBP12,PIK3R1,GRB1,GSK3B, HSD11B1,HSD11,HSD11L, SDR26C1,SHBG, RXRA, NR2B1,PCK1,PEPCK1,RARB, HAP, NR1B2,RARA, NR1B1,DHODH, SYK, ELANE, ELA2,LTA4H, LTA4,HNMT, ANXA5,ANX5,ENX2,PP4,TEK, TIE2,VMCM, VMCM1,NQO1,DIA4,NMOR1,NR3C1,GRL, HCK, LCK, RXRB, NR2B2,CASP1,IL1BC, IL1BCE, JAK3,SULT1E1,STE, ABL1,ABL, JTK7
genkwanin	CDK6,CDKN6,PDE4D, DPDE3,PDE5A, PDE5,CHEK1,CHK1,SRC, SRC1,HSD17B1,E17KSR, EDH17B1,EDH17B2,EDHB17,SDR28C1,NQO2, NMOR2,DCK, FKBP1A, FKBP1,FKBP12,ARL5A, ARFLP5,ARL5,TRDMT1,DNMT2,NR1I2,PXR, GPI, PLA2G2A, PLA2B, PLA2L, RASF-A, GART, PGFT, PRGS, UCK2,UMPK, FKBP3,FKBP25,ANG, RNASE5,HCK, NOS3,Esr2,Erbeta, Nr3a2,BHMT, ADORA1,ADORA2A, ESR2,PIM1,ESR1
cirsimaritin	CDK6,CDKN6,PDE5A, PDE5,CHEK1,CHK1,ESR2,ESTRB, NR3A2,FGFR1,BFGFR, CEK, FGFBR, FLG, FLT2,HBGFR, HSD17B1,E17KSR, ED H17B1,EDH17B2,EDHB17,SDR28C1,GSTZ1,MAAI, NR1I2,PXR, NR3C1,GRL, AMD1,AMD, NQO2,NMOR2,GART, PGFT, PRGS, ANG, RNASE5,MMP12,HME, MMP13,FKBP3,FKBP25,BHMT, F10,GSR, GLUR, GRD1,GPI, MAPK14,CSBP, CSBP1,CSBP2,CSPB1,MXI2,SAPK2A, FKBP1A, FKBP1,FKBP12,ARL5A, ARFLP5,ARL5,GSTM2,GST4,ABO, NMNAT1,NMNAT, DCK, PTPN1,PTP1B, AKR1B1,ADORA1,ADORA2A, ADORA3,OPRD1,ABCG2,OPRM1
rosmarinic acid	DTYMK, CDC8,TMPK, TYMK, PIM1,NDST1,HSST, HSST1,RND3,ARHE, RHO8,RHOE, AGXT, AGT1,SPAT, WARS1,IFI53,WARS, WRS, SRC, SRC1,BST1,IMPDH2,IMPD2,HSPA1A, HSP72,HSPA1,HSX70,HSPA1B, HSP72,CBS, APRT, ARG2,KIT, SCFR, ACAT1,ACAT, MAT, GART, PGFT, PRGS, CLK1,CLK, GSTA3,GP1BA, FGFR1,BFGFR, CEK, FGFBR, FLG, FLT2,HBGFR, AKT2,PKLR, PK1,PKL, RAC2,INSR, FYN, AKR1B1,TTR, MMP1
caffeic acid	AKT2,KIT, SCFR, AMD1,AMD, TPI1,TPI, DCK, GCK, KDR, FLK1,VEGFR2,PTK2,FAK, FAK1,ITPKA, AGXT, AGT1,SPAT, GSTA3,ABL1,ABL, JTK7,KAT2B, PCAF, PDPK1,PDK1,BST1,FAP, NDST1,HSST, HSST1,CDK2,CDKN2,ARG2,EPHA2,ECK, HRAS, HRAS1,PAH, LCN2,HNL, NGAL, CBS, DCXR, SDR20C1,SOD2,BACE1,BACE, KIAA1149,RHOA, ARH12,ARHA, RHO12,RAF1,RAF, CA2,ALOX5,CA7,CA1,CA6,MMP9,CA12,MM P1,MMP2,PTPN1,CA14,CA9,CA5B, CA5A

the function and the more critical the function is. A total of 341 biological processes were obtained from the GO analysis, including signal transduction, positive regulation of RNA polymerase II promoter transcription, protein phosphorylation, negative regulation of apoptotic process, cell differentiation, negative regulation of cell proliferation, protein autophosphorylation, negative regulation of RNA polymerase II promoter transcription, peptidyl tyrosine phosphorylation, positive regulation of gene expression, and other processes. 56 cellular components, including cytoplasmic lysate, cytoplasm, nucleus, plasma membrane, nucleoplasm, extracellular exocytosis, extracellular compartment, mitochondria, membranes, extracellular space, and other components. 99 molecular functions, including protein-binding, ATP-binding, identical-protein-binding, zinc-ion-binding, enzyme-binding, DNA-binding, and RNA polymerase II Transcription factor activity and ligand-activated sequence-specific DNA binding, transmembrane receptor protein tyrosine kinase activity, sequence-specific DNA binding, protein kinase activity, and other functions.

KEGG signaling pathway analysis was performed using R 4.2.0 software, and bubble plots were generated for visualization (Fig. 9). In the graph, the color from blue to red indicates the P value from large to small, and the size of the circle represents the number of enriched targets; the larger the size, the more genes are enriched in the pathway and the more critical the pathway. The top 20 pathways are shown in the figure. After ranking the pathways according to their importance, we searched the literature in PubMed. We found that the pathways related to Alzheimer's disease were the PI3K-Akt signaling pathway, Ras signaling pathway, estrogen signaling pathway. The relationship between the above pathways and the regulation of AD was then further verified by molecular docking. Among them, Hub genes HRAS, ESR1, RHOA, IGF1, SRC, MMP9, MAPK14, NOS3, and PIK3R1 were found among the genes related to the above pathways.

Molecular docking verification

The eight active components of rosemary, carnosic acid, carnosol, rosmarinol, rosmadial, genkwanin, cirsimaritin, rosmarinic acid and caffeic acid were molecularly docked with the Hub genes obtained from the Cytoscape screen, respectively. The binding was demonstrated as a list (Table 4). The corresponding molecular docking heatmap was generated (Fig. 10). The groups with better docking situations were selected for visualization (Fig. 11). The larger the negative value, the higher the binding energy, the more stable the binding, and the easier the interaction between the ligand and receptor. Binding energies less than −5 kcal/mol indicated a better docking

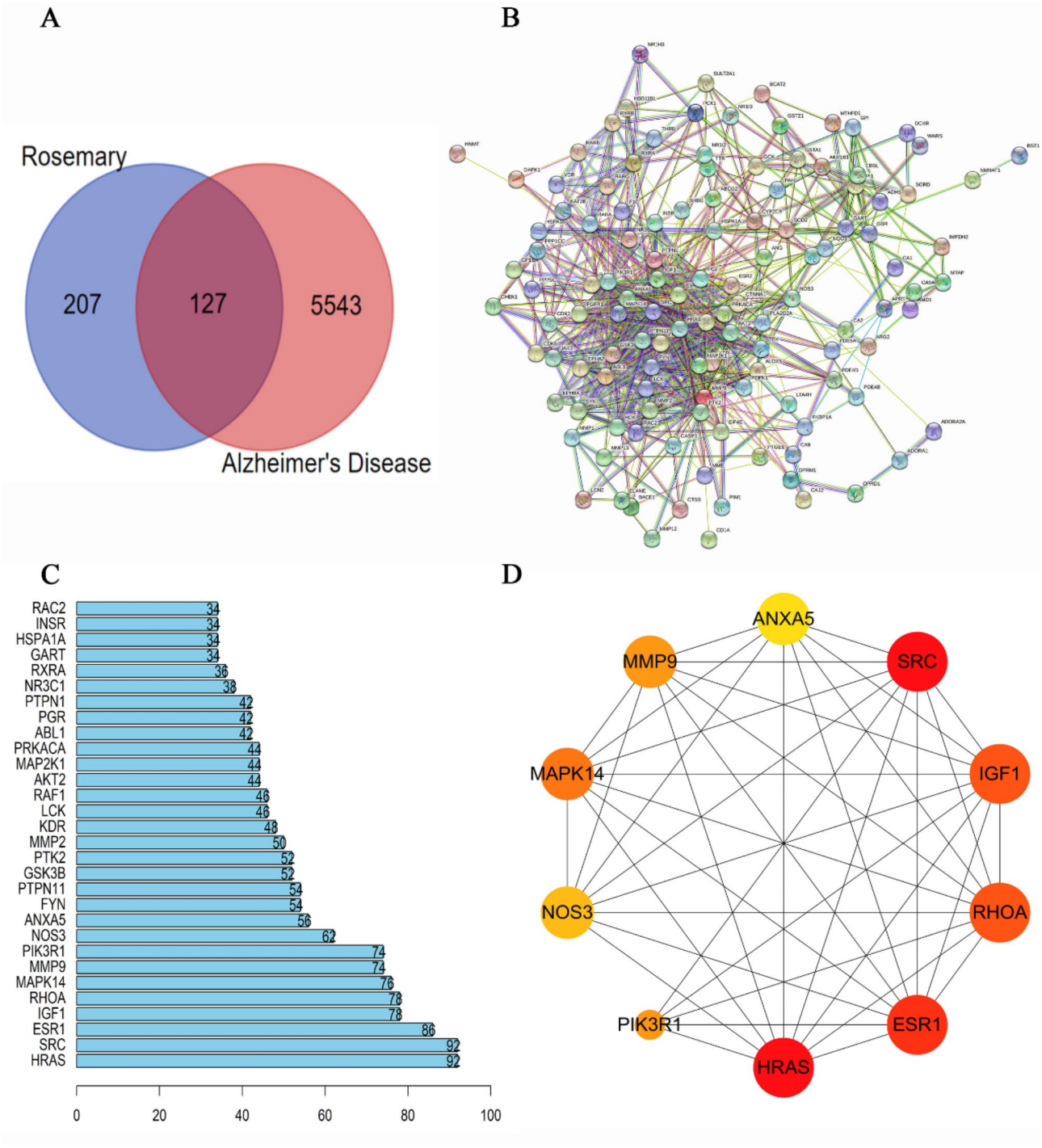

Fig. 6 (**A**) The intersection target of rosemary and AD; (**B**) PPI network map of rosemary therapy for AD; (**C**) Count and list of the top 30 genes of PPI network map; (**D**) The top 10 Hub genes by degree value

situation. In all molecular docking results, the docking binding energies were less than −5 kcal/mol, indicating that the eight active constituents of rosemary, carnosic acid, carnosol, rosmarinol, rosmadial, genkwanin, cirsimaritin, rosmarinic acid and caffeic acid, interacted with the Hub genes HRAS, ESR1, RHOA, IGF1, SRC, ANXA5, MMP9, MAPK14, NOS3 and PIK3R1. In the results of genkwanin, cirsimaritin, and rosemarinic acid with ESR1, SRC, MMP9, and NOS3, the results with binding energies all less than −8 kcal/mol occupied 91.6%, indicating that the binding activities of genkwanin, cirsimaritin, and

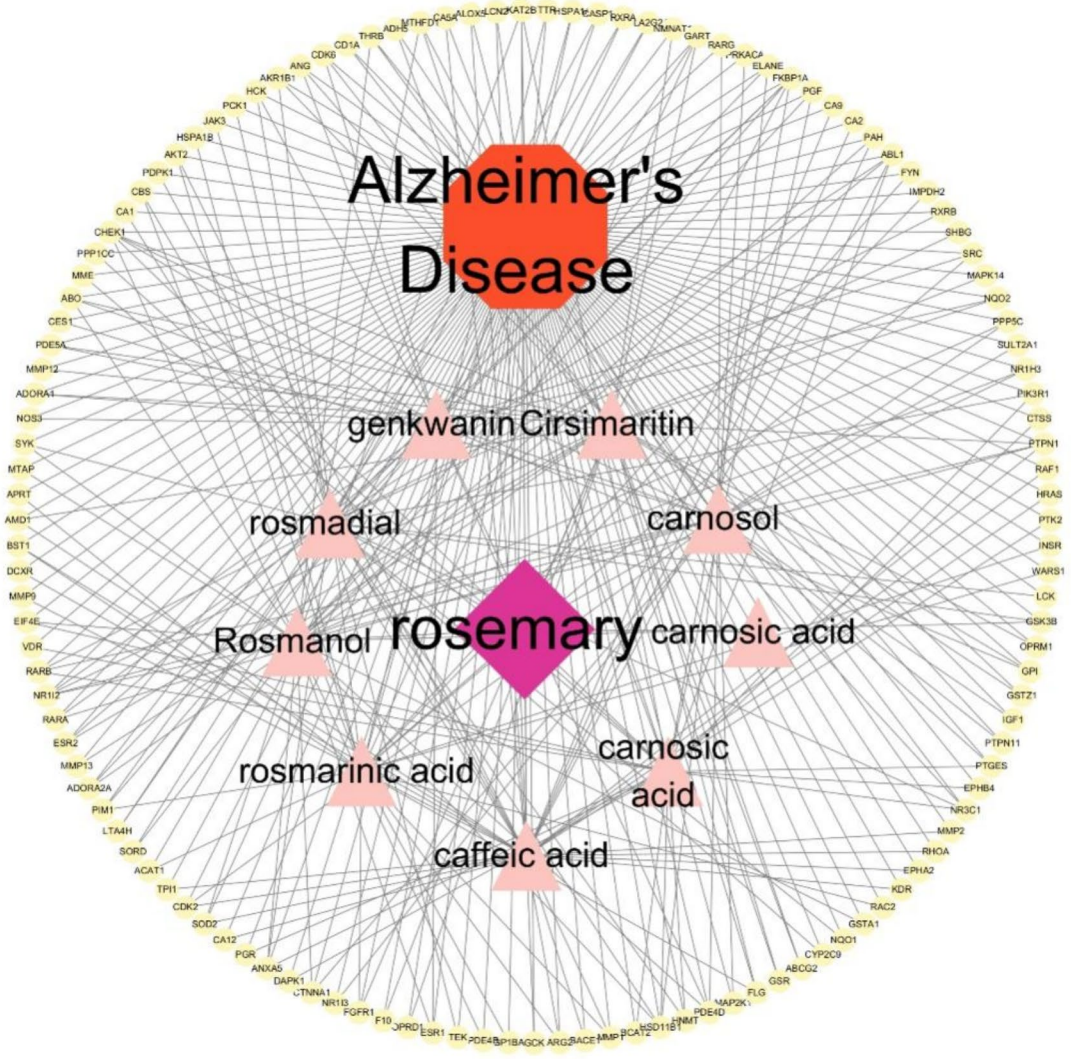

Fig. 7 Drug-compounds-genes-disease network

rosemarinic with ESR1, SRC, MMP9, and NOS3 were better among all the molecular docking results obtained.

Expression of target genes

To further dissect the neuroprotective effects of rosemary on AD by upregulating or repressing the genes. The ESR1, MMP9, NOS3, SRC and MAPK14 mRNA levels were determined using RT-qPCR (Fig. 12). The results demonstrated that mRNA expression of ESR1, IGF1, MMP9 and NOS3 decreased after Aβ25-35-induced injury, expression of IGF1 and MMP9 were evidently elevated by rosemary-treated ($P < 0.05$). Whereas, Aβ25–35 significantly increased mRNA levels of SRC and MAPK14, rosemary effectively decreased mRNA levels of SRC and MAPK14 ($P < 0.001$).

Discussion

Despite the complexity of the pathogenesis of AD, the presence of extracellular plaques of insoluble β-amyloid peptide (Aβ) and P-tau neurogenic fibril tangles (NFTs) in the cytoplasm of neurons is a hallmark of AD in the known pathogenesis [19]. Whereas extensive studies have shown that oxidative stress is an early factor in the AD process, antioxidants can minimize its harmful effects [20]. Rosemary, as an antioxidant, has a good effect on the treatment of Alzheimer's disease.

In vitro experiments, rosemary and its main antioxidant component carnosic acid had potential neuroprotective effects on Aβ25-35-induced hippocampal neuronal injury and significantly improved the cell viability and reduced the apoptosis of injured cells.

The gene with the highest degree value obtained via Cytoscape software analysis was HRAS. HRAS is involved in regulating the expression of further

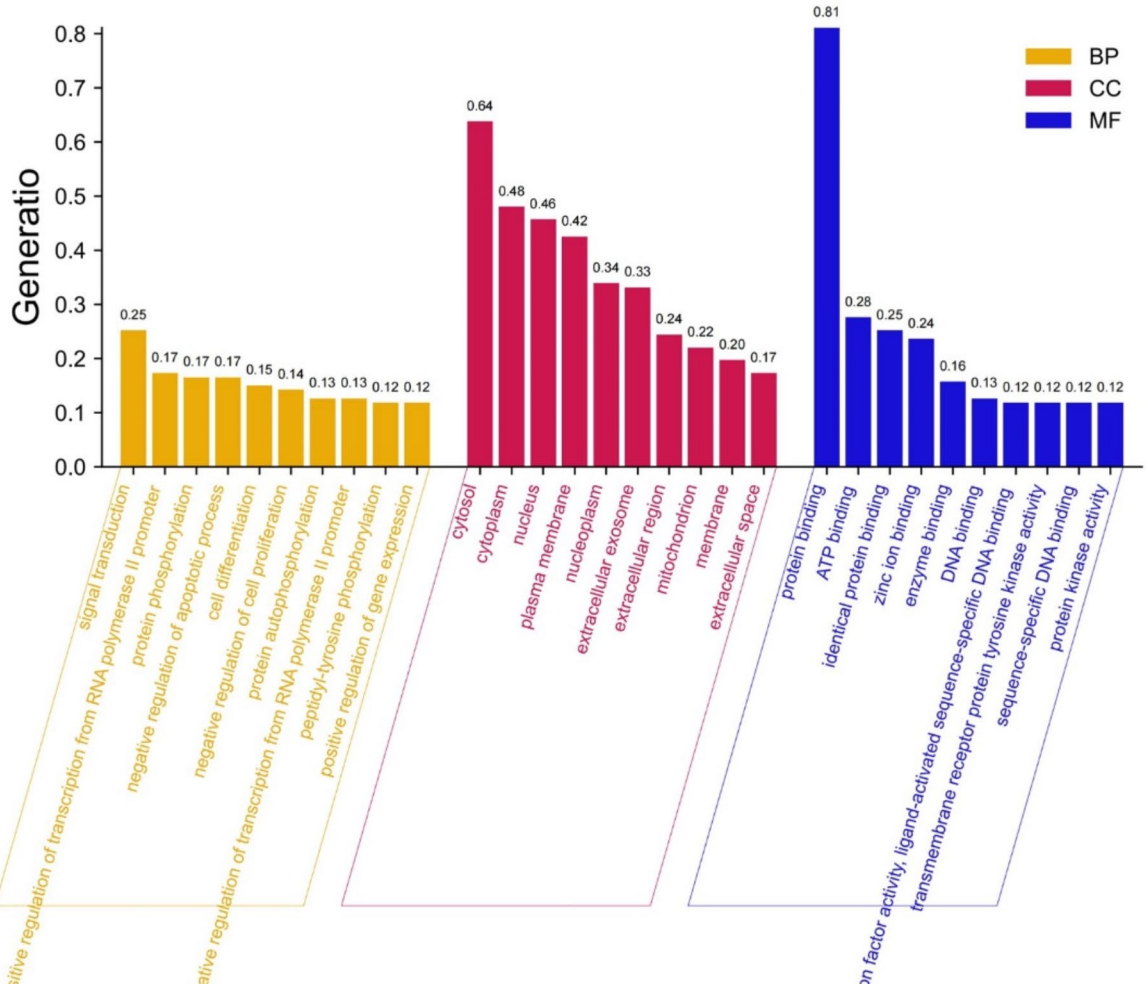

Fig. 8 GO functional analysis bubble chart

downstream signaling components of proliferation and differentiation, and the mechanisms of growth abnormality and proliferation regulation may be seriously involved in neurodegeneration in Alzheimer's disease (AD) [21]. Therefore, it is speculated that HRAS may be a potential target of rosemary in the treatment of AD. Among the Hub genes with high degree values are RHOA and IGF1. Reduced dendritic spine density due to the activation of RhoA / ROCK2 in neurons has been reported to increase cognitive decline in AD patients [22]. Insulin-like growth factors (IGF-1 and IGF-II) play critical roles in neurogenesis and neurodevelopment and exert neuroprotective effects against amyloid-induced neurotoxicity [23, 24]. Related studies have shown that the upregulation of IGF-1 may have neuroprotective effects in AD [25]. The molecular docking results showed IGF-1 was better bound to rosemary dialdehyde. RT-qPCR results showed that IGF1 mRNA levels were significantly increased in the rosemary treatment group, compared with the model group, which conjectures IGF-1 may be a potential AD action target.

GO analysis elucidated the hub genes associated with rosemary and AD were enriched mainly in signal transduction, positive regulation of RNA polymerase II promoter transcription, protein phosphorylation and other processes, with the involvement of multiple substances such as cytoplasmic lysate, cytoplasm, nucleus, plasma membrane, and nucleoplasm, and multiple functions such as protein binding, ATP binding, and identical protein binding. KEGG analysis discovered the common targets were enriched mainly in the PI3K-Akt signaling pathway, Ras signaling pathway, estrogen signaling pathway, Rap1 signaling pathway and MAPK signaling pathway, indicating that the abovementioned pathways may be play an essential role in the treatment of AD by rosemary.

In recent years, increasing number of natural products have been shown to effect the PI3K/AKT signaling pathway to protect dopaminergic neurons, hippocampal

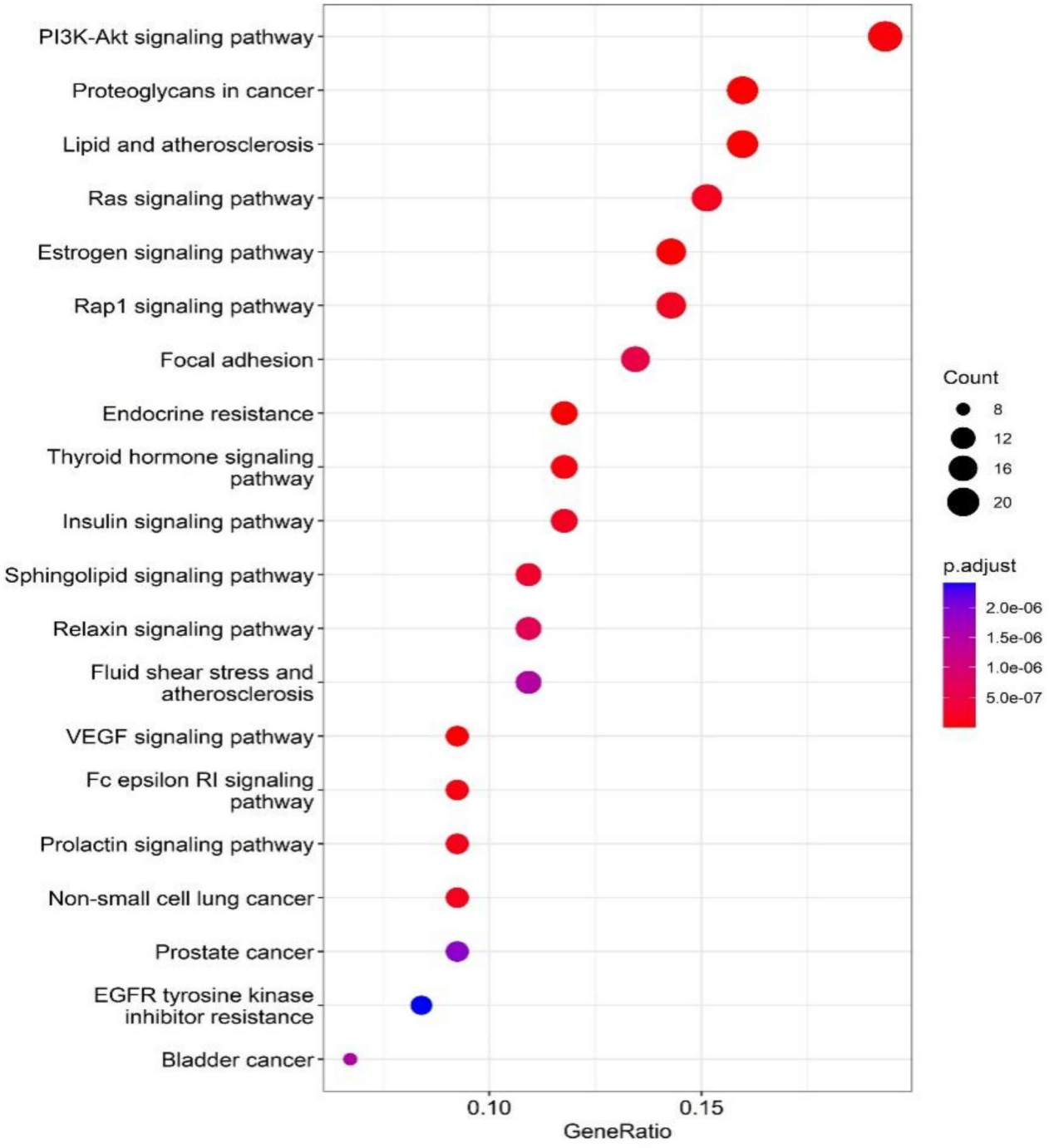

Fig. 9 KEGG pathway analysis bubble chart

neurons, and cortical neurons, which plays a role in the prevention and treatment of AD [26]. Among the KEGG pathway analysis, NOS3 is closely related to the PI3K/AKT signaling pathway, and among all the compounds that bind to NOS3, rosemarinic acid has the best binding status. RT-qPCR revealed Rosemary slightly increased the decreased NOS3 mRNA expression induced by Aβ25–35. It is speculated that rosemary may play a role in preventing and controlling AD by binding rosemarinic acid to NOS3, thereby modulating the PI3K/AKT signaling pathway.

Estrogen binding to the estrogen receptor in neuronal cells reduces amyloid β protein deposition. It antagonizes its cytotoxic effects in Alzheimer's disease, which has been

Table 4 The binding energy of molecular docking

Binding energy (kcal/mol)	HRAS	ESR1	RHOA	IGF1	SRC	ANXA5	MMP9	MAPK14	NOS3	PIK3R1
carnosic acid	-6	-7.4	-6.6	-7	-7.5	-6.6	-6.3	-8	-8.3	-5.8
carnosol	-6.5	-8	-6.9	-6.8	-8.2	-7.5	-7.1	-8	-7.9	-6
rosmanol	-5.9	-8	-6.8	-6.2	-7.1	-6.6	-7.2	-8.2	-8	-6.1
rosmadial	-6.3	-7.3	-6.5	-6.9	-7.3	-7.2	-6.5	-7.8	-8.8	-6.3
Rosmarinic acid	-6.9	-8.1	-8.2	-7.6	-9.3	-7.6	-9.7	-7.5	-9	-8.1
genkwanin	-7.1	-8.4	-7.4	-7.4	-9.7	-6.8	-9.7	-7.6	-8.3	-8.3
caffeic acid	-6.3	-6	-6.5	-5.9	-6.9	-5.7	-7.6	-6.7	-7.8	-6.8
cirsimaritin	-6.8	-7.1	-7.4	-7.3	-9.2	-6.6	-9.5	-7.5	-8.1	-7.3

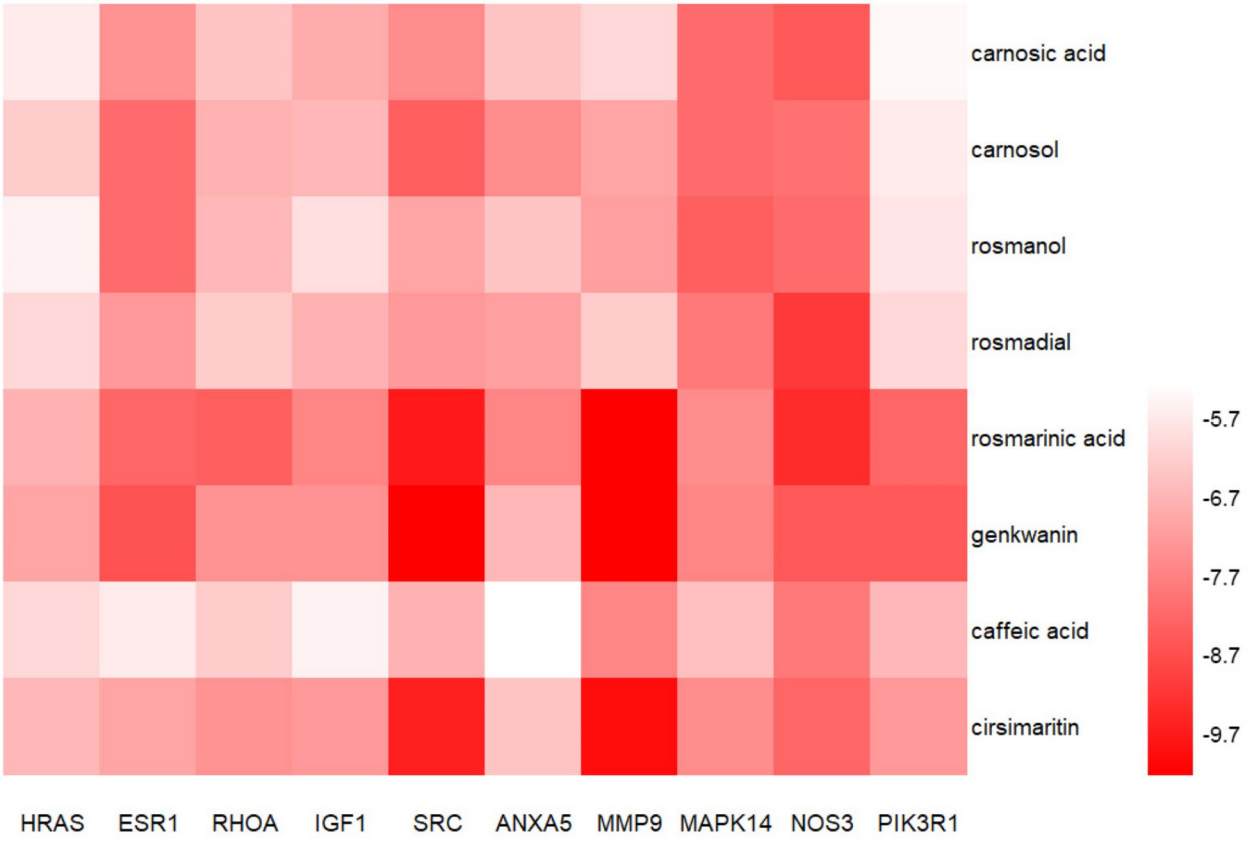

Fig. 10 The Heatmap of the molecular docking

implicated in treating Alzheimer's disease [27]. KEGG pathway analysis showed that the estrogen signaling pathways associated with estrogen are MMP9 and ESR1. ESR1, or estrogen receptor α. Estrogen can regulate β-amyloid precursor protein (β-amyloid precursor protein). Protein hydrolytic processing of the amyloid precursor protein (β-APP), which plays a role in normal and abnormal brain metabolism, and potentially hormonal interventions can reduce or prevent the formation of amyloid deposits in AD [28, 29]. The MMP-9-TIMP-1 pathway promotes glial cell proliferation to self-defensively eliminate amyloid deposits in AD brains [30].The best binding to MMP9 and ESR1 were genkwanin and rosmarinic acid, cirsimaritin, caffeic acid, rosmarinol, and carnosol. RT-qPCR indicated rosemary was helpful in the reduction of MMP9 and ESR1 mRNA induced by Aβ25–35. It is hypothesized that rosemary may regulate the estrogen signaling pathway mainly through the binding of genkwanin, rosmarinic acid, caffeic acid, cirsimaritin, rosmarinol, and carnosol to MMP9 and ESR1, reduce amyloid deposition in the brains of AD patients, and antagonize cytotoxicity, thus preventing and controlling AD. The Rap1 signaling pathway may play a neuroprotective role, thus having a specific link with Alzheimer's disease [31]. In the KEGG pathway analysis, it can be obtained that the Rap1 signaling pathway is associated with SRC, and among all the compounds that bind to SRC, the best binding is genkwanin,

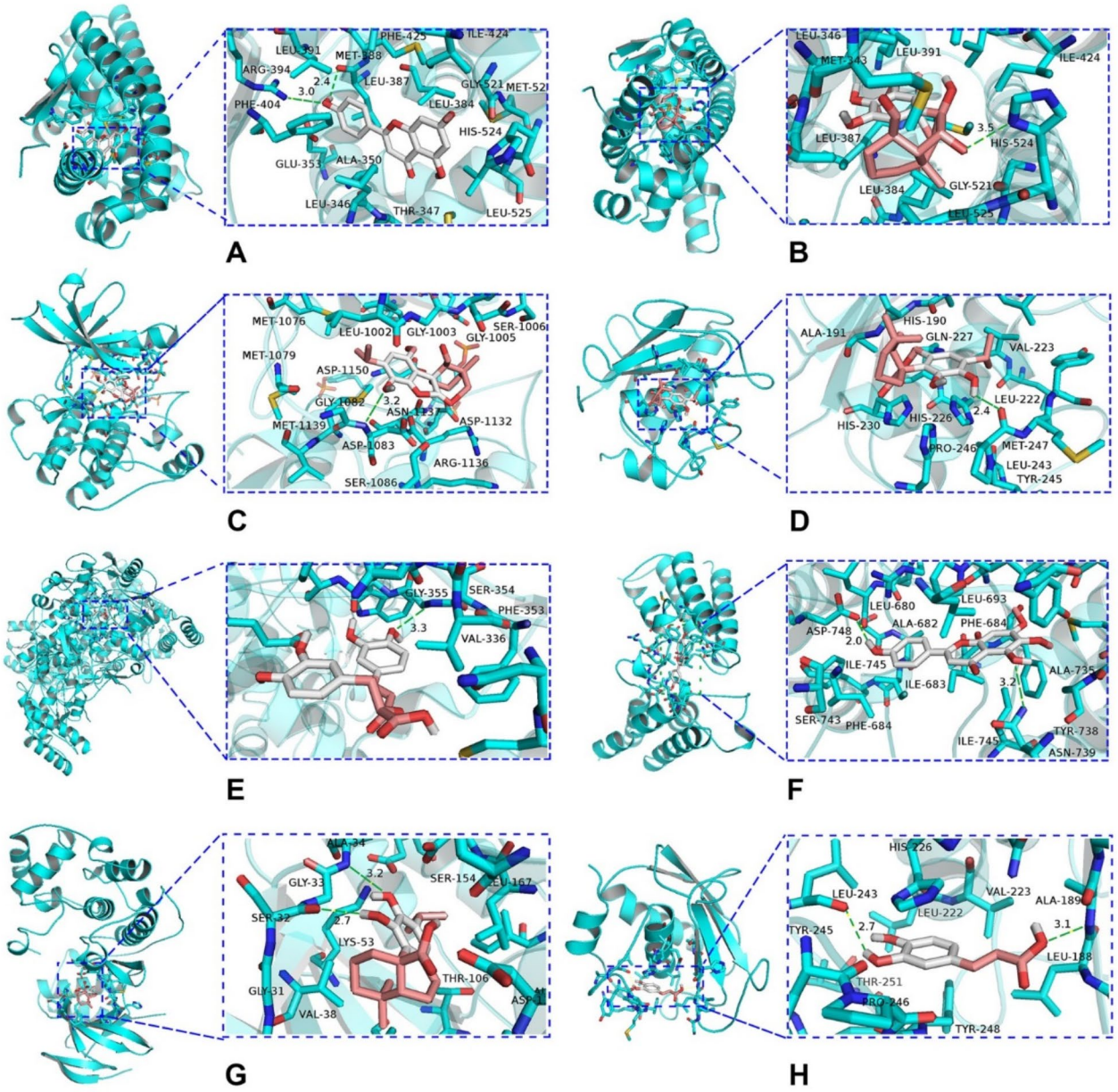

Fig. 11 The 3D map of the molecular docking. **(A)** ESR1-genkwanin; **(B)** ESR1-rosmanol; **(C)** IGF1-rosmadial; **(D)** MMP9-carnosol; **(E)** NOS3-rosmarinic acid; **(F)** SRC-cirsimaritin; **(G)** MAPK14-carnosic acid; **(H)** MMP9-caffeic acid

followed by rosemarinic acid and cirsimaritin. The result of RT-qPCR suggested rosemary could increase the Aβ25-35-induced reduction of SRC mRNA levels. It is hypothesized that rosemary may be able to treat AD mainly by modulating the Rap1 signaling pathway through the binding of genkwanin, rosmarinic acid, and cirsimaritin to the SRC and thus acting as a neuroprotective agent. p38 mitogen-activated protein kinase (MAPK) family of serine-threonine protein kinases, in particular p38αMAPK (MAPK14), is a crucial regulator of pro-inflammatory cytokine production in the brain [32]. Activation of p38αMAPK in neurons occurs in response to various CNS disease-associated stressors, and inhibition of neuronal p38αMAPK can be neuroprotective [33, 34]. In KEGG pathway analysis, closely related to the MAPK signaling pathway was MAPK14, and molecular docking results showed that the best binding to MAPK14 was rosemarinol, carnosol, and carnosic acid, followed by rosmadial and genkwanin. RT-qPCR demonstrated rosemary could decrease the expression of NOS3 mRNA induced by Aβ25–35. It is hypothesized that rosemary may regulate the MAPK signaling pathway mainly through the binding of rosemarinol, carnosol, carnosic acid, rosmadial, and genkwanin to MAPK14, and thus play a therapeutic role in treating AD.

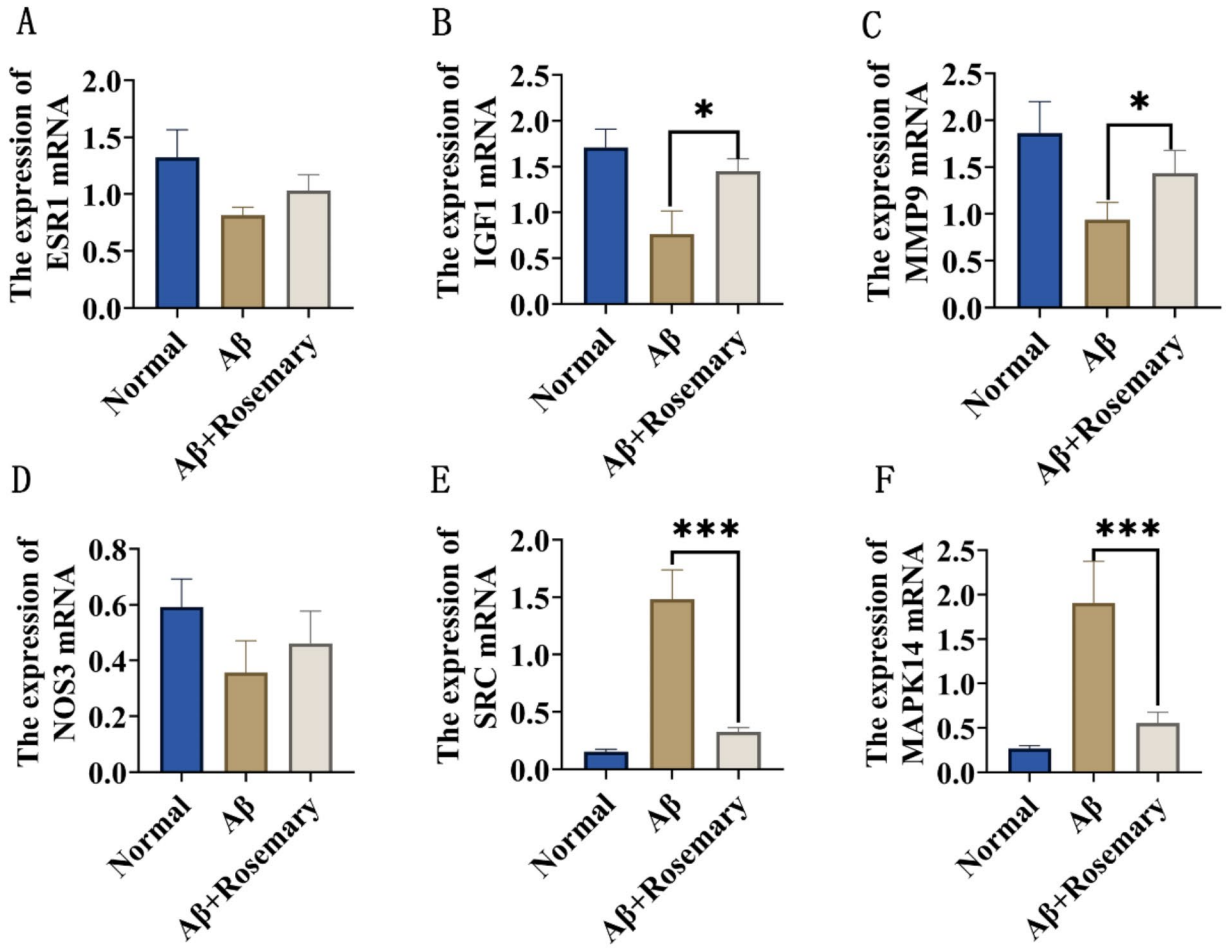

Fig. 12 The expression levels of ESR1, MMP9, NOS3, SRC, and MAPK14 mRNA were detected using RT-qPCR.*:$p < 0.05$;***:$p < 0.001$

Conclusion

Using KEGG pathway analysis, the molecular mechanism of rosemary in the treatment of Alzheimer's disease was elucidated, that rosemary might mainly bind to ESR1, SRC, MMP9, NOS3, and other targets through genkwanin, rosmarinic acid, cirsimaritin and other active ingredients, and regulate PI3K-Akt signaling pathway, estrogen signaling pathway, Rap1 signaling pathway, MAPK signaling pathway and other pathways. This study demonstrated the characteristics of rosemary in treating AD by combining multiple components with multiple targets and regulating multiple pathways and provided a relevant basis for further research.

Abbreviations

Rosemary	Rosmarinus officinalis L.
AD	Alzheimer's disease
NFTs	Neurogenic fiber tangles
FBS	Fetal bovine serum
DMEM	DMEM high glucose
GO	Gene ontology
BP	Biological processes
CC	Cellular components
MF	Molecular functions
KEGG	Kyoto encyclopedia of genes and genomes

Acknowledgements

Not applicable.

Author contributions

ZLC and YHF designed and supervised the study. ZRP collects data and final revision. ZJZ and LZJ perform data analysis and manuscript writing. All authors are responsible for reviewing data. All authors read and approved the final manuscript.

Funding

This work was supported by Special Fund of the Applied Basic Research Programs of Yunnan Province associated with Kunming Medical University in China (Grant No 2017FE467(-024) and 202201AY070001-034) from the Yunnan Provincial Science and Technology Department. This study was supported by Natural Medicine Synthetic Biology and Translational Pharmacy Team (2024XKTDTS14) and Medical Experimental Zoology team (2024XKTDPY17) of Kunming Medical University.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request, unless there are legal or ethical reasons for not doing so.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Laboratory Animal Science, School of Pharmaceutical Science & Yunnan Key Laboratory of Pharmacology for Natural Products, Kunming Medical University, Kunming, Yunnan 650500, China
[2]Yunnan Key Laboratory of Southern Medicinal Resources, Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan 650500, China

Received: 5 September 2024 / Accepted: 20 January 2025
Published online: 07 March 2025

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