

Characterization of the bioactive compounds with efficacy against epilepsy from the herb pairs *Polygala tenuifolia* - *Zizyphus jujuba* by modulating CHRNA4/CaMKII signaling pathway: LC-MS/MS combined with network pharmacology analysis and experimental evidence

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Abstract

Background

Epilepsy is a typical nervous system disorders identified by the spontaneous recurrence of seizures which injure periods of electroencephalographic activity and behavior. Traditional Chinese Medicine (TCM) herb pairs *Polygala tenuifolia* and *Zizyphus jujuba* have been used in treatment of epilepsy in China, while the mechanism of action still remains unclear. This article aims to disclose the substances and potential mechanisms of the anti-epilepsy activity of *P. tenuifolia* and *Z. jujuba* extract (*PZE*) using LC-MS/MS, network pharmacology, ethology and molecular biology methods.

Methods

With the help of the self-built components database, identification of the chemical parameters of *PZE* was possessed through LC-MS/MS method, and the "ingredient-target-pathway" network of *PZE* was established through online databases. Molecular docking was performed using Discovery Studio Visualizer. In the setting of the epilepsy model, pentylenetetrazol (PTZ, 10 mg/kg) was administered intraperitoneally injected for a period of 21 days. Mice were assessed for anxiety-like behavior by Elevated plus maze test, open field test, forced swimming test and tail suspension test. HE staining, western blotting, and immunofluorescence staining were used to detect morphological changes and signal pathway.

Results

Through network analysis, 37 active ingredients were obtained from *PZE*, SLC6A4, CHRNA4 and MAOA and were found to play a major role in the PPI network. GO and KEGG analyses that display their anti-epilepsy activity. The "Ingredient-target-pathway" network diagram consists of 99 targets, 24 kinds of constituents, and 20 signaling pathways. The values of **M15** and **M17** show the largest degree. Molecular docking analysis shows the key components screened by network pharmacology have a good interaction with the predicted targets. Animal experiments results showed that: 1) *PZE* effectively lengthened the latent time of PTZ-induced epilepsy in mice model. 2) PTZ-induced depression-like behavior was strikingly ameliorated by *PZE*. 3) Hippocampal neurons are significantly shielded by *PZE*. 4) *PZE* was shown to play a key role in modulating the CHRNA4/CaMK II signaling pathway in to show anti-epilepsy potency.

Conclusion

This study has successfully identified constituents of *PZE* through LC-MS/MS methods and predicted the potential targets and CHRNA4/CaMK II as potential signaling pathways of anti-epilepsy effects for *PZE*, which was proved by animal experiments. The results of this paper are conducive to the systematic elucidating of its mechanism of action and the development of TCM-based anti-epilepsy agents.

1. Introduction

Epilepsy is a severe and chronic neurological disorder of the brain, resulting in motor, consciousness, sensory and other disorders, its damage is persistent. The life and work of patients is greatly affected by the features of recurrent seizures and transient central nervous system dysfunction. The survey estimates that there are over 70 million patients with epilepsy worldwide, and the number of patients is on the rise every year [1, 2]. The current conventional treatment for epilepsy is antiepileptic drugs, approximately 40% of patients worldwide have achieved the best results, but there are still features of seizures and complications. And many antiepileptics have significant side effects, severely limiting the use of medications. All procedures used to date for fully diagnosed patients can only provide symptomatic relief, and there are no medications that can be used for prevention purposes [3]. Recent studies have demonstrated that the treatment of epilepsy by Chinese medicine. Traditional Chinese Medicine (TCM) offers a new perspective in this area [4, 5]. According to our previous studies [6–8], we have excavated anticonvulsant precursors TCM herb pairs *Polygala tenuifolia* and *Gastrodia elata*, which provided the evidence of the anti-epilepsy potency for *P. tenuifolia*.

For many decades, TCM has been used to effectively treat epilepsy in China. TCM is a treasure house provide invaluable therapia for diseases such as epilepsy. The aim of this paper is to investigate the anti-epilepsy potential of TCM herb pairs *P. tenuifolia* and *Zizyphus jujuba* (*PZ*) through the pharmacology of LC/MS-based networks. Pairs of herbs were recorded in multiple TCM prescriptions using to treating epilepsy including *Zaoren Yuanzhi Tang* () from *Zheng Yin Mai Zhi* (, 1700 AD), *Yangxue Qingxin Tang* () from *Wanbing Huichun* (, 1600 AD) and *Ding Xian Dan* () from *Yizong Jinjian* (, 1700 AD). In Chinese patent medicines, the herb pairs *P. tenuifolia* and *Z. jujuba* (*PZ*) were also widely recorded, such as *Ding Xin Wan* (, approved number: Z42021255), *Xianzheng Zhenxin Wan* (, approved number: Z33020108) and *Zhen Xian Pian* (, approved number: Z20063511). Traditionally, the roots of *P. tenuifolia* and the seeds of *Z. jujuba* were recorded as the medicinal parts for the treatment of insomnia, palpitation and epilepsy for their tonic effects on heart and sedative efficacy the brain. Contemporary pharmacological research has revealed the therapeutic potential of *PZ* on diseases of the central nervous system

diseases such as Alzheimer's disease, Parkinson's disease, neuroinflammation and epilepsy [9–12]. To date, however, the potential mechanism underlying the anti-epilepsy action of *PZ* remains unclear, and this is the major difficulty in restricting its standardized application.

Network pharmacology is a new system for drug research, particularly suited to medicinal herbs with multi-components like TCM [13]. According to the results of “disease-pathway-target-drug” interaction network, the intervention and influence of drugs on the disease mechanism network can be systematically observed. Therefore, in this article, we investigate the potential mechanism by which *PZ* exerts its effect on anti-epilepsy targets and pathway using this method. Comparing to other data mining methods, the liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS) identification of the chemical constitutions and molecular docking analysis of predicted targets make the results more reasonable. Figure 1 describes the workflow of LC/MS and network pharmacology parts of this article. In the present study, we characterized the chemical constitutions of extracts of *P. tenuifolia* and *Z. jujuba* (*PZE*) and summarized the constituents-targets-pathways relationships of *PZE*. We were able to construct the possible interaction between targets and *PZE*'s epilepsy-related target network. In addition, we analyzed relative pathways to recapitulate the related mechanism of action of *PZE* against epilepsy, and the CHRNA4/CaMKII signaling pathway was chosen and proven by molecular biological methods.

Insert Fig. 1.

2. Methods

2.1. Reagents and materials

The dried roots of *Polygala tenuifolia* Willd. (batch number: 20210328) and seeds of *Ziziphus jujuba* Mill. (batch number: 20210325) were purchased from Bozhou (Anhui province of China, a city famous for TCM herbs). The samples were authenticated by Professor Qiang Wang (Shaanxi University of Traditional Chinese Medicine). PTZ was purchased from Alfa Aesar, Shanghai, China. Lot: 10180463; Formic acid and methyl alcohol, used in HPLC-QTOF-MS analysis, were obtained from Fisher Sigma-Aldrich (SigmaAldrich, MO, USA), respectively.

For the preparation of extracts, coarse herbal crushing, 80 mesh sieve. Then, 10.0 g powder of *P. tenuifolia* and 10.0 g powder of *Z. jujuba* (according to the proportion of *Zaoren Yuanzhi Tang*) was added in 100.0 mL of 95% ethanol, then the extracts was possessed by reflux extraction for 2.0 h at 80 °C and the solution was concentrated in vacuo to obtain the extracts [14]. The extracts were diluted with methanol to a concentration of 10 mg/mL for the HPLC-QTOF-MS/MS analysis.

2.2. The condition of HPLC-QTOF-MS/MS and Data analysis

HPLC-QTOF-MS/MS analyses were possessed on a Triple TOF™ 5600+ system (AB SCIEX, CA, USA). Both the positive and negative ion modes were selected for MS data acquisition of *PZE*. The separation of the components in *PZE* were detailly recorded in the **Supporting information**.

The systematic information on ingredients from *PZE* was collected and organized by searching the published phytochemical literatures and databases including MassBank and SciFinder scholar. etc [15]. Therefore, a components database containing compound names was established, and chemical formulas of each compound was established to provide a basis for further structural identification.

2.3. Construction and analysis of PZE compounds-disease target interaction network

The chemical formulas of the compounds in *PZE* were imported into the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) to know information about SMILES, and the potential targets were predicted by using SwissTargetPrediction database (www.swisstargetprediction.ch), the details were recorded in the **Supporting information**.

2.4. Acquisition of potential anti-epilepsy targets and the Protein-Protein interaction (PPI) network construction

The construction of PPI network mainly used database of DisGeNET (<http://210.107.182.61/geneSearch/>) and STRING (<https://string-db.org/>), the details were recorded in the **Supporting information**.

2.5. GO annotation and KEGG enrichment analysis

With the help of Metascape (metascape.org) web-based portal for comprehensive gene annotation and analysis resources [16], we annotated and analysed the Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, and the detailed information were recorded in the **Supporting information**.

2.6. Ingredien -target-pathway network construction

The ingredient-target-pathway network was constructed by introducing *PZE* targets, pathways and 24 kinds of active ingredients screened out in Cytoscape software. Nodes of different colors mean different types of clusters, while the edges represented the relationships between the nodes.

2.7. Molecular docking

The crystal structure of core target proteins for molecular docking in the PPI network was from PDB database (www.rcsb.org) [17]. The Discovery Studio Client software usage to add hydrogen and partial charges to the proteins and ligands [18]. The results of the docking computations were ranked by the binding affinity (-kcal/mol). We visualized conformations and interactions between the ligands and the target proteins by Discovery Studio Visualizer.

2.9. Animals and group

Our experiment was authorized by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University, fifty young adults male Kunming-strain mice (22–28 g). All mice were divided into five groups at random, and each group contains 10 animals: 1) normal saline group (NS); 2) pentylenetetrazol group (PTZ); 3) treatment group were divided into three group, included high (*PZEH* 300mg/kg), medium (*PZEM* 100 mg/kg) and low (*PZEL* 30mg/kg) dose group.

2.10 Establish mice epilepsy model and sc-PTZ-induced epilepsy

Epilepsy model was established by intraperitoneal (i.p.) injection of 10 mg/kg PTZ for 21 days [19]. PTZ (10 mg/kg) was injected intraperitoneally 60 min after oral administration of *PZE* in the treatment group. The NS group was only injected with saline. Subcutaneous (s.c.) injection of 85 mg/kg PTZ solution 60 min after the last treatment. The latent period and incidence of tonic convulsion were observed 30 min after PTZ injection. The latency of each seizure were recorded, and the average values were calculated for statistical analysis.

2.11. The behavioral animal tests

The fortieth day after the beginning of the experiment, four behavioral tests usually employed in studies on laboratory.

2.11.1 Elevated plus maze test (EPMT)

The EPMT experiment is applied to evaluated anxiety-like behavior and was conducted as described previously [20]. The details of the test were recorded in the **Supporting information**.

2.11.2. open field test (OFT)

OFT experiment is applied to evaluated locomotor activity and anxiety-like behavior [21]. The test was analysed for 5 min in a dimly lit room, as described previously [22–24]. The details of the test were recorded in the **Supporting information**.

2.11.3. Forced swimming test (FST)

FST was possessed referred to the previously research [25]. The details of the test were recorded in the **Supporting information**.

2.11.4. Tail suspension test (TST)

FST was possessed referred to the previously research [26]. The details of the test were recorded in the **Supporting information**.

2.12. Histological observation of brain tissue

2.12.1. Hematoxylin and eosin (HE) staining

HE staining was carried out to observe the changes of pathological tissue in brain. The details of the experiment was recorded in the **Supporting information**.

2.12.2. Immunofluorescence staining

Immunofluorescence staining was possessed to observe the levels of target proteins including CHRNA4 and CaMKII in brain. The details of the experiment was recorded in the **Supporting information**.

2.12.3. Western blot analysis

Western blotting methods was used to analyze the protein levels of target signaling pathway: CHRNA4/CaMKII/ERK1/2/CREB1-related proteins. The details of the experiment were recorded in the **Supporting information**.

2.13. Data analysis

The data of results were analyzed through GraphPad Prism 8.0.2 software and described as the mean $\pm$ SEM. The differences between control and BPA-treated groups were approximated using one-way ANOVA followed by a Tukey's test for multiple comparisons. $P < 0.05$ was deemed to have a statistical significance.

3. Results

3.1. Identification of compounds from PZE

The base peak chromatograms (BPC) of *PZE* in positive and negative modes are exhibited in Fig. 2. A total of 37 compounds were characterized using a self-constructed database analysis strategy, and the properties of their candidate compounds are shown in Fig. 2. It contained information on retention time, molecular formulas, mass weight, mass errors and fragments of major components, as shown in Table 1.

Insert Table 1. and Fig. 2.

3.2. Identification of compounds from PZE

To date, 37 kinds of constituents have been identified from the *PZE*, among them, xanthones, glycolipids and acids are the main types. All compounds are summarized and recorded in Table 1. Herein, we provide the analytical process of two kinds of representative constituents as the internal standard substances recorded in the Chinese Pharmacopoeia. As we can see from the Fig. 3, polygalaxanthone III (**M2**) was a classic xanthone from *P. tenuifolia* with molecular formula of $C_{25}H_{28}O_{15}$ as inferred from the $[M-H]^-$ (m/z : 567.1342). In the second stage mass spectrum detected, the daughter ions at m/z 59.0143 ($C_2H_3O_2^-$), 89.0248 ($C_3H_5O_3^-$), 271.0254 ($C_{14}H_7O_6^-$), 273.0415 ($C_{14}H_9O_6^-$), 315.0504 ($C_{16}H_{11}O_7^-$), 345.0604 ($C_{17}H_{13}O_8^-$), 417.0816 ($C_{20}H_{17}O_{10}^-$), 435.0924 ($C_{20}H_{19}O_{11}^-$), 447.0915 ($C_{21}H_{19}O_{11}^-$) in negative mass mode provided the evidence for the identification of the component.

Insert Fig. 3.

As for the constituents from *Z. jujuba*, typical compound spinosin (**M28**) recorded in the Chinese Pharmacopoeia as internal standard substance was detected with the chemical formula of $C_{28}H_{32}O_{15}$ based on the $[M+H]^+$ ion at m/z 609.1784 and $[M-H]^-$ ion at m/z 607.1675. In the second stage mass spectrum (Fig. 4), the daughter ions at m/z 297.0752 ($C_{17}H_{13}O_5^+$), 327.0854 ($C_{12}H_{23}O_{10}^+$), 351.0847 ($C_{16}H_{15}O_9^+$), 411.1058 ($C_{22}H_{19}O_8^+$), 429.1156 ($C_{22}H_{21}O_9^+$), 447.1262 ($C_{22}H_{23}O_{10}^+$) in positive mass mode and 427.1041 ($C_{22}H_{19}O_9^-$) in positive mass mode give the inspiration of the spectrum analysis.

Insert Fig. 4.

3.3. Acquisition of targets for PZE ingredients and anti-epilepsy

The results of the network construction showed: Screening of 24 core anti-epilepsy components from candidate ingredients by preliminary search. Generally, a total of 527 ingredient-related targets were identified from the SwissTargetPrediction databases. A total of 450 epilepsy-related targets were identified from DisGeNET (Score-gda ≥ 0.05) and GeneCards (Relevance score ≥ 9.21)

Insert Fig. 5.

3.4. Protein-protein interaction (PPI) network analysis results

To obtain the PPI function network, the top targets were entered into the STRING data platform. Of the 55 targets that were correlated, 48 targets had average interactions (with a confidence score ≥ 0.4) in the PPI map generated by the STRING database. The targets of CA5B CA5A CA6 PTPRF PTPN1 and ACP1 targets that were not bound to the lattice were dislodged, and the Cytoscape software was used to own the topology analysis to screen for significant central targets in the network. As shown in Fig. 6, the node size and color settings mirror the size, while the edge thickness and color settings reflect the combined score. When the color of the node closer to red, the corresponding value is higher. The results of the topological analysis displayed that the three targets with a higher degree value among the anti-epilepsy targets of neuronal acetylcholine receptor subunit α -4 (SLC6A4, degree = 25), neuronal acetylcholine receptor subunit α -4 (CHRNA4, degree = 22) and monoamine oxidase A (MAOA, degree = 21), which played a major role in the network.

Insert Fig. 6.

3.5. Molecular mechanism of anti-epilepsy the effect of PZE

We carried out a Metascape enrichment analysis of the 55 duplicate genes for GO and KEGG and achieved 583 biological processes (BP), 48 cellular ingredients (CC) and 68 molecular functions (MF) under the GO entry. The p -value determines the result of the correlation test [53], sort the top 20 of each category by -LogP value from large to small (Fig. 7). The top rank belonged to the vasoconstriction, regulation of tube diameter and blood vessel diameter maintenance enrichment in BP's, synaptic membrane, dendrite and integral component of synaptic membrane enrichment in CC's and G protein-coupled amine receptor activity, carbonate dehydratase activity, neurotransmitter receptor activity in MF's.

The KEGG enrichment analysis of potential anti-epilepsy targets produced 32 signal pathways, which were ranked on the basis of the $-\log_{10}P$ value from small to large, of which 20 pathways were relative to epilepsy (Fig. 8). Of the pathways related to epilepsy, Nitrogen metabolism has a high degree of enrichment, and can be considered as the main anti-epilepsy pathway. The significance of the count and the $-\log_{10}P$ value represent a highly correlated way in which active constituents display their antiepileptic activities.

Insert Fig. 7. and Fig. 8.

3.6. Construction of the ingredient-target-pathway network

We adopt a network of 99 nodes and 240 edges by Cytoscape software (Fig. 6). In particular, the nodes consisted of 24 kinds of puffball constituents, 55 kinds of potential PZE targets, and 20 signal paths. It can be seen that each active component corresponds to multiple targets in multiple pathways (Fig. 9), which fully mirrors the multi-ingredient, multi-target and multi-pathway mechanisms of the puffball anti-epilepsy activity. With respect to the component attribute nodes in the network graph, it has been reported that **M17** (Tetrahydrocolumbamine, degree value 12) and **M15** (*O*-hydroxybenzoic acid, degree value 11) had the highest degree value; each of these components can be thought of as the central nodes in the network with a major anti-epilepsy effect.

Insert Fig. 9.

3.7. Molecular docking analysis

Under the prediction of molecular simulation docking methods, it can be seen that the key components screened by the pharmacology of the network have a good interplay with the predicted targets. They have a high binding energy and can successfully penetrate into the binding pocket of the target molecule in order to interact with key handicaps in the form of intermolecular hydrogen bonds. The cinnamic acids showed a good interaction with monoamine oxidase A (MAOA) in terms of their Structure-Activity Relationship, xanthenes showed favorable interaction with nicotinic-acetylcholine receptor $\alpha 4$ (nAChR4), and saponins displayed preferable interaction with 5-HT transporter (SERT). Molecular construction and docking analysis between 2Z5Y and **M16**, **M18**, **M20**, and **M37** were performed; between 6AWN and **M17**, **M22**, **M25**, **M35**; and between 5KXI and **M18**, **M20**, **M31**, **M32**-all of which are listed below. The lowest energy predicted complexes are stable intermolecular hydrogen bonding and stacking interactions. Interactions in the complex are decided by steric hindrance and the functional group category. We can see from Table 2 that the docking results are basically in agreement with the structure-function relationship, the substituted cinnamyl group on the phenolic hydroxyl group of vanillyl moiety helped enormously in promoting the binding affinity of the enzyme-inhibitor complexes, the ester linkage played key roles in the interaction with the protein. In terms of the interaction energy, the result displayed that compound **M16** and **M20** outperformed **M18** and **M37** in 5Z5Y, **M22** and **M25** outperformed **M17** and **M35** in 6AWN, **M20** outperformed **M18**, **M31** and **M32** in 5KXI. Additionally, **M20** displayed the best interaction with the protein 5Z5Y, which was partially different with the result of the enzyme inhibition assay, in general, active 5Z5Y residues comprising TYR 444, ILE 335, ILE 180 can interact with **M16**, **M18**, and **M37** to a different extent (Fig. 10) [54–56]; in 6AWN that **M25** showed the best interaction with the protein than **M17**, **M22** and **M25**, the active residues of including ASP 98, SER 336, ALA 96, TYR 176, SER 438 can interact with **M17**, **M22** and **M25** in different degree (Fig. 10); in 5KXI that **M20** demonstrated the best interaction with the protein than **M18**, **M31** and **M32**, the active residues of including TRP 156, LEU 121, TYR 204, CYS 199 can interact with **M18**, **M31** and **M32** in different degree (Fig. 10).

Insert Fig. 10. and Fig. 11.

3.8. Anti-epilepsy Pathway

This study divulges that *PZE* activation of nAChRs can repair synaptic related protein levels. nAChRs belong to ligand gated ion channel coupled receptors, and form a centrally located ion channel, which primarily modulates Ca^{2+} plasma flow in and out of the cell. Calcium ions, acting as cytoplasmic second messengers, flow into cells via $\alpha 4$ nAChRs and connect with CaM to form an active CaM complex. The CaM complex may conjugate CaMK II, which regulates the spatial conformation of CaMK II and phosphorylates the CaMK II protein. Phosphorylated CaMK II enters the nucleus to catalyse the phosphorylation of Extracellular signal-regulated kinase (ERK) and cAMP responsive element binding protein (CREB). ERK and CREB are downstream of the $\alpha 4$ nAChR signal transduction pathway and serve as transcription factors that regulate neuron growth and development as well as the formation of long term memory (Fig. 12).

Insert Figure 12.

3.9. The efficacy and latency of PTZ

Convulsions were caused by an intraperitoneal (i.p.) injection of PTZ (10 mg/kg) dissolved in saline. Compared with PTZ group, after different degrees of *PZE* treatment, the latency time of *PZEH*, *PZEM* and *PZEL* was significantly prolonged in Fig. 13.

Insert Figure 13.

3.10. The behavioral animal tests

To assess anxiety-like and depression-like behaviors in PTZ-induced epilepsy mice using the EPMT, OFT, FST and TST. After 15 days of *PZE* intervention, the EPMT results showed that compared with NS group, although the number and timing of entry into the open arms of PTZ-induced epilepsy model mice was significantly lower than that of NS group mice. In comparison to the PTZ group, the number and timing of mice entering the open arm was increased in the *PZEH*, *PZEM*, and *PZEL* groups (Fig. 14C&D), the anxiety and depression-like behaviors were improved, it indicated that PTZ could induce anxiety and depression-like behavior in mice. The OFT result indicated that compared with the NS group, there showed striking differences in the total movement distance (Fig. 15E), the number of entering enter central entry (Fig. 15F), the residence time in the central area (Fig. 15G) and the number of feces (Fig. 15H) in mice with epilepsy induced by PTZ, the intervention of *PZEH* and *PZEM* group has significant improvement effect on the above indexes. FST result manifested that compared with NS group, the time of immobility of PTZ group was prolonged and the depression was obvious, when given *PZE*, the immobility time of *PZEH*, *PZEM*, and *PZEL* group decreased, the difference was statistically remarkable (Fig. 16I, $P < 0.05$). The difference in TST resting time of mice in each group was statistically significant, the resting time of PTZ group was higher than that of NS group. After *PZE* treatment, the immobility time of *PZEH*, *PZEM*, and *PZEL* group decreased (Fig. 16J). These findings suggested that *PZE* can ameliorate the depressive response in mice and has antidepressant effects.

Insert Figure 14., Figure 15. and Figure 16.

3.11. Histological observation of brain tissue

3.11.1. Hematoxylin and eosin (HE) staining

HE staining was used to determine the lesion effect of hippocampal neuros in mice exposed to BPA. The cells of the hippocampus cornuammon1 (CA1), and cornu ammon 3 (CA3), dentate gyrus (DG), and Temporal cortex (TeA) of mice in the NS group were arranged in a regular and compact manner, as shown in Fig. 17 by light microscopy. In the NS group, many pyramidal cells appeared smaller with small condensed nuclei compared to the mice. In contrast, the hippocampal structure of mice in the *PZEH* and *PZEM* groups was essentially normal, and nerve cell degeneration in the large hippocampal region was less than in the PTZ group.

Insert Figure 17.

3.11.2. Immunofluorescence staining

To investigate the role of CHRNA4/CaMK II in Epilepsy, we first examined the expression of CHRNA4/CaMK II in the hippocampus tissues through immunofluorescence (Fig. 18). Figure 18 is showing that the protein of CHRNA4 and CaMK II were mainly expressed within TeA, CA1 and CA3 regions. Compared with the NS group, the proportion of CHRNA4 positive cells decreased significantly in PTZ group, the level of CHRNA4 in hippocampus increased rapidly in *PZEH* and *PZEM* group after PTZ-induced epilepsy, which is approximate to control (Fig. 18K). Oppositely, compared with the NS group, the proportion of CaMK II positive cells increased significantly in PTZ group, The level of CaMK II in hippocampus decreased rapidly in *PZEH* and *PZEM* group after PTZ-induced epilepsy, which is approximate to control (Fig. 18L). These experimental results in vitro and in vivo showed that *PZE* could reduce the epilepsy response and reduce the loss of neuronal cells by inhibiting the CHRNA4/CaMK II signal pathway.

Insert Figure 18

3.11.3. Western blot analysis

To explore the effect of *PZE* on the CHRNA4/CaMKII signaling pathway, Western blot was used to determine the protein levels of CHRNA4, CaMKII, ERK_{1/2}, CREB1 in hippocampus tissues (Fig. 19). Further analysis of relative protein expression was performed using the optical density of the band. We found that the protein levels of CaMK II and CREB1 were significantly decreased in the PTZ group and that these increases were blunted by treatment with *PZE* (Fig. 19N & P, $p < 0.01$). Upon further comparison, it was found that the protein levels of CaMK II and CREB1 in the *PZEH* group were significantly higher than those in the *PZEL* group ($p < 0.01$). CHRNA4 and ERK_{1/2} expression levels in the PTZ group were both higher than those in the NS group, upon further comparison, we found that the protein levels of CHRNA4 and ERK_{1/2} in the *PZEH* group and in the *PZEM* group were strikingly lower than those in the PTZ group (Fig. 19M & O, $p < 0.01$). Together, these data

suggested that *PZE* may protect against neuronal injury. The results of western blot analysis reveal that *PZE* can regulate the expression of the CHRNA4/CaMKII signaling pathway on epilepsy induced by PTZ in mice.

Insert Fig. 19.

4. Discussion

Epilepsy is a chronic encephalopathy that is induced by ictal firing of neurons in the brain. Today, the pathogenesis of epilepsy remains poorly defined. Current antiepileptic drugs mainly suppress neuronal excitability via the regulation of neurotransmitters (like glutamate, γ -aminobutyric acid and acetylcholine) or ion channels [57–59] have been shown to play a role in the formation of ion channels. Pharmaceuticals extracted from botanical products have been considered as a therapy for epilepsy with few side-effects [60]. Studies in animal models of plant epilepsy over the last few decades have shown that medicinal plants have the potential to become a richer source of safer and more efficacious antiepileptic drugs. Botanical treatments can offer significant benefits to patients with drug resistant epilepsy by reducing adverse effects and improving quality of life [61].

The aim of this study was to evaluate the therapeutic power of *PZE* on epilepsy. The constituents of *PZE* were characterized by LC-MS/MS and the potential signaling pathway was predicted by network pharmacology methods, which were seen as a useful tool for connecting ingredients and potential targets. The power of *PZE* on epilepsy was proven by the PTZ-induced mouse model of epilepsy.

We measured spatial learning and memory after successful establishment of mouse models of epilepsy. Anxiety and depression-like behaviours were enhanced, it indicated that PTZ was able to induce anxiety and depression-like behaviour in mice [62, 63]. The hippocampus is a component of the limbic system and has been shown to play an important role in learning and memory [64]. Hippocampal sclerosis, hippocampal atrophy, and hippocampal sclerosis are the major pathological changes in epilepsy, primarily manifesting as neuronal loss and glial cell proliferation [65]. In the present study, we explored the effect of *PZE* on mice with epilepsy from hippocampal pathophysiological processes. In this study, HE staining showed that *PZE* could reduce the apoptosis of neurons and the loss of neurons in the CA1 region of the hippocampus, CA3 region, TeA region, and DG region of epileptic mice. In this experiment, *PZE* demonstrated both sedative and anticonvulsant effects.

PTZ is a commonly used experimental animal model of inflammation, which is an antagonist of the γ -aminobutyric acid (GABA) receptor and is easy to cross the blood brain barrier. This model is known to be a model for the histopathology and cytopathology of epilepsy [66, 67]. The most common and intuitive parameter for observing antiepileptic activity is latent time. Herein, we provided preliminary evidence for the anti-epilepsy activity of *PZE* by extending the latent period in the PTZ-induced mouse model to provide pharmacodynamic evidence.

Research on signaling pathways through molecular biology tools are widely used in the investigation of potential therapeutic mechanisms of medicinal herbs, herein, the CHRNA4/CaMKII/ERK_{1/2}/CREB1 pathway predicted by network pharmacology was chosen as a witness in this study. In this paper, the results of signaling pathway studies proved the reliability of network pharmacology based on LC-MS/MS as well. Acetylcholine (ACh) is a major neurotransmitter. Depending on the different sensitivities of the signal transducing receptors to muscarinic acid or nicotine, it mediates fast synaptic transmission within *ms*. According to the location can be divided into: Presynaptic nicotinic Acetylcholine receptor (nAChR), postsynaptic nAChR and non-synaptic nAChR. Activation of the pre-synaptic nAChRs can lead to increased neurotransmitter release, Postsynaptic nAChRs can rapidly transduce cellular excitability, however, non-synaptic nAChRs play an important role in neuronal excitability and neurotransmitter regulation [68].

nAChR is involved in many physiological functions such as neuronal excitability and neurotransmitter release, and plays a key role in learning and cognitive memory. The nAChRs are widely and patchily distributed in the brain and are closely linked to a range of central nervous system diseases including epilepsy, Alzheimer's disease, Parkinson's disease and schizophrenia [69]. Its permeability to sodium, potassium, calcium, and other ions, as well as its regulation of excitatory and inhibitory neurotransmitter release. This specific regulation is dependent upon the difference in nAChR expression in excitatory and inhibitory neurons. A large body of research has shown that mutation of nAChR-related coding genes is relative to the occurrence and development of epilepsy. Changes in the gene coding for nAChRs can lead to changes in nAChR-related structure and function, leading to changes in the local brain potential, resulting in the development of epilepsy [70, 71]. When the gene encoding nAChR changes, it may cause changes in nAChR-related structure and function, resulting in changes in brain local potential, resulting in epilepsy.

CHRNA4 consists of six exons, located on chromosome 20q13.33, approximately 17kb, it primarily encodes the α -4 subunit of the neuronal acetylcholine receptor, and its coding region is primarily located in exon 5. The first proven pathogenic ADSHE-related gene is CHRNA4 [72], which has been shown to be associated with cancer. Sporadic sleep-related high-exercise epilepsy previously known as sporadic nocturnal frontal lobe epilepsy previously known as sporadic nocturnal frontal lobe epilepsy has similar clinical features to ADSHE [73].

CaMK II is a multifunctional serine/threonine protein kinase that is found in nearly every subcellular region of the brain and in neurons [74]. CaMK II primarily exists in neuronal tissues, representing 2% of total protein in certain regions such as the hippocampus of rats. Calcium-sensitive CaMK II may be directly activated when Ca²⁺ in nerve cells is increased. The activated CaMK II can act on a variety of substrates, causing extensive biological effects, such as affecting synaptic reconstruction, neurotransmitter release, hippocampal formation and other plastic changes, which is essential in the occurrence and development of epilepsy.

In summary, PZE significantly extended the latency time of PTZ-induced seizure, simultaneously modulated the expression of proteins related to the nAChR/CaMK II signaling pathway, and ameliorated the impaired learning and memory caused by hippocampal neuronal damage in epileptic mice.

5. Conclusions

In short, *PZE* decreased the intensity and duration of PTZ-induced seizures, restrained the activation of microglia and astrocytes, and inhibited the expression of CHRNA4/CaMK II pathways, while activated ERK_{1/2}/CREB1 pathway, and promoted the ratio of neuronal survival in relative encephalic region. This research uncovers the potential protective potency and mechanisms of *PZE* on brain damage after developmental epilepsy through the network pharmacology, ethology and molecular biology studies, with the efficacy substances characterized by LC-MS/MS methods. This research proposes a theoretical basis for the development for anti-epileptic agents excavated from TCM.

Abbreviations

TCM

Traditional Chinese Medicine

PZ

P. tenuifolia and *Zizyphus jujuba*

LC-MS/MS

liquidchromatography-massspectrometry/massspectrometry

PZE

the extracts of *P. tenuifolia* and *Z. jujuba*

CHRNA4

Cholinergic receptor nicotinic alpha 4

CaMK II

Calcium CaM-dependent protein kinase II

GO

Gene Ontology

KEGG

Kyoto Encyclopedia of Genes and Genomes

NS

Normal saline group

PTZ

Pentylenetetrazol group

PZEH

the high dose extracts of *P. tenuifolia* and *Z. jujuba*

PZEM

the medium dose extracts of *P. tenuifolia* and *Z. jujuba*

PZEL

the low dose extracts of *P. tenuifolia* and *Z. jujuba*

EPMT

Elevated plus maze test

OFT

open field test

FST

Forced swimming test

TST

Tail suspension test

HE

Hematoxylin and eosin
BPC
The base peak chromatograms
PPI
Protein-protein interaction
SLC6A4
Neuronal acetylcholine receptor subunit α -4
CHRNA4
Neuronal acetylcholine receptor subunit α -4
MAOA
Monoamine oxidase A
BP
Biological processes
CC
Cellular ingredients
MF
Molecular functions
SERT
Saponins displayed preferable interaction with 5-HT transporter
ERK
Extracellular signal-regulated kinase
CREB
cAMP responsive element binding protein
CA1
Cornuammon 1
CA3
Cornu ammon 3
DG
Dentate gyrus
TeA
Temporal cortex
GABA
antagonist of the γ -aminobutyric acid
Ach
Acetylcholine
nAChR
nicotinic Acetylcholine receptor

Declarations

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Author contributions

MN writing and editing the manuscript. ZZ design and revise manuscript. NM and YW do the experiments. JC, MQ, GL and AL guide the experiments. FQ and XY review and editing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets presented in this review can be found in online repositories

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Tables

Table 1
Identified compound composition of PZE.

Peak	Predicted	Formula	$t_{R(\text{min})}$	Ion Species	Measured Mass (Da)	δP	MS ² Ions	Source	Ref.
M1	Lancerin	C ₁₉ H ₁₈ O ₁₀	8.743	[M-H] ⁻	405.0822	-0.50772	59.0144 (C ₂ H ₃ O ₂ ⁻), 71.0143 (C ₃ H ₃ O ₂ ⁻), 89.0249 (C ₃ H ₅ O ₃ ⁻), 243.0306 (C ₁₃ H ₇ O ₅ ⁻), 255.0305 (C ₁₄ H ₇ O ₅ ⁻), 257.0462 (C ₁₄ H ₉ O ₅ ⁻), 267.0297 (C ₁₂ H ₁₁ O ₇ ⁻), 269.0459 (C ₁₂ H ₁₃ O ₇ ⁻), 285.0403 (C ₁₅ H ₉ O ₆ ⁻), 297.0408 (C ₁₃ H ₁₃ O ₈ ⁻), 315.0504 (C ₁₆ H ₁₁ O ₇ ⁻), 329.0699 (C ₁₆ H ₉ O ₈ ⁻), 345.0624 (C ₁₇ H ₁₃ O ₈ ⁻), 369.0620 (C ₁₉ H ₁₃ O ₈ ⁻), 387.0716 (C ₁₉ H ₁₅ O ₉ ⁻)	A	[27]
M2	Polygalaxanthone	C ₂₅ H ₂₈ O ₁₅	9.630	[M-H] ⁻	567.1341	0.04351	59.0143 (C ₂ H ₃ O ₂ ⁻), 89.0248 (C ₃ H ₅ O ₃ ⁻), 271.0254 (C ₁₄ H ₇ O ₆ ⁻), 273.0415 (C ₁₄ H ₉ O ₆ ⁻), 315.0504 (C ₁₆ H ₁₁ O ₇ ⁻), 345.0604 (C ₁₇ H ₁₃ O ₈ ⁻), 417.0816 (C ₂₀ H ₁₇ O ₁₀ ⁻), 435.0924 (C ₂₀ H ₁₉ O ₁₁ ⁻), 447.0915 (C ₂₁ H ₁₉ O ₁₁ ⁻)	A	[28]
M3	Polygalaxanthone V	C ₂₆ H ₃₀ O ₁₅	11.078	[M-H] ⁻	581.1537	-1.33884	273.0417 (C ₁₄ H ₉ O ₆ ⁻)	A	[29]
M4	1,7-Dihydroxyxanthone	C ₁₃ H ₈ O ₄	13.259	[M-H] ⁻	227.0373	3.83102	65.0048 89.0247 (C ₃ H ₅ O ₃ ⁻), 121.0315 (C ₄ H ₉ O ₄ ⁻), 243.0705 (C ₁₃ H ₇ O ₅ ⁻)	A	[30]
M5	1,3-Dihydroxy-7-methoxyxanthone	C ₁₄ H ₁₀ O ₅	14.791	[M + H] ⁺	259.0603	-2.37162	/	A	[31]
			14.038	[M-H] ⁻	257.0472	1.36305	/	A	[31]
M6	1,3,7-Trihydroxy-2-methoxyxanthone	C ₁₄ H ₁₀ O ₆	11.078	[M-H] ⁻	273.0417	0.57909	/	A	[31]

Peak	Predicted	Formula	$t_{R(min)}$	Ion Species	Measured Mass (Da)	δP	MS ² Ions	Source	Ref.
M7	Irisxanthone	C ₂₀ H ₂₀ O ₁₁	9.630	[M-H] ⁻	435.0925	-1.58721	59.0143 (C ₂ H ₃ O ₂ ⁻), 89.0248 (C ₃ H ₅ O ₃ ⁻), 271.0253 (C ₁₄ H ₇ O ₆ ⁻), 273.0415 (C ₁₄ H ₉ O ₆ ⁻), 297.0397 (C ₁₃ H ₁₃ O ₈ ⁻), 315.0504 (C ₁₆ H ₁₁ O ₇ ⁻), 339.0510 (C ₁₅ H ₁₅ O ₉ ⁻), 345.0604 (C ₁₇ H ₁₃ O ₈ ⁻), 417.0816 (C ₂₀ H ₁₇ O ₁₀ ⁻)	A	[32]
M8	Polygalaxanthone	C ₂₅ H ₂₈ O ₁₅	9.630	[M-H] ⁻	567.1341	0.04351	59.0143 (C ₂ H ₃ O ₂ ⁻), 89.0248 (C ₃ H ₅ O ₃ ⁻), 271.0253 (C ₁₄ H ₇ O ₆ ⁻), 273.0415 (C ₁₄ H ₉ O ₆ ⁻), 345.0604 (C ₁₇ H ₁₃ O ₈ ⁻), 417.0814 (C ₂₀ H ₁₇ O ₁₀ ⁻), 435.0925 (C ₂₀ H ₁₉ O ₁₁ ⁻), 447.0919 (C ₂₁ H ₁₉ O ₁₁ ⁻)	A	[33]
M9	Tenuiphenone C	C ₃₆ H ₆₂ O ₄	30.755	[M + H] ⁺	559.4661	-8.99791	127.0410 (C ₆ H ₇ O ₃ ⁺), 139.0409 (C ₇ H ₇ O ₃ ⁺), 153.0201 (C ₇ H ₅ O ₄ ⁺), 181.0516 (C ₉ H ₉ O ₄ ⁺), 433.4381 (C ₃₀ H ₅₇ O ⁺)	A	[34]
			30.737	[M-H] ⁻	557.4577	-0.45866	437.4357 (C ₃₂ H ₅₇ O ₂ ⁻), 513.4667 (C ₃₃ H ₅₃ O ₄ ⁻)	A	[34]
M10	Sibiricose A1	C ₂₃ H ₃₂ O ₁₅	7.395	[M-H] ⁻	547.1695	-0.35158	149.0254 (C ₅ H ₉ O ₅ ⁻), 179.0712 (C ₆ H ₁₁ O ₆ ⁻), 205.0517 (C ₁₁ H ₉ O ₄ ⁻), 223.0639 (C ₁₁ H ₁₁ O ₅ ⁻), 265.0734 (C ₃ H ₁₃ O ₆ ⁻), 295.0835 (C ₁₄ H ₁₅ O ₇ ⁻), 325.0944 (C ₁₂ H ₂₁ O ₁₀ ⁻), 367.1051 (C ₁₇ H ₁₉ O ₉ ⁻),	A	[28]
M11	Tenuifoliside A	C ₃₁ H ₃₈ O ₁₇	11.401	[M-H] ⁻	681.2032	-0.96218	137.0247 (C ₇ H ₅ O ₃ ⁻), 179.0359 (C ₉ H ₇ O ₄ ⁻), 205.0516 (C ₁₁ H ₉ O ₄ ⁻), 223.0619 (C ₁₁ H ₁₁ O ₅ ⁻), 237.0777 (C ₁₂ H ₁₃ O ₅ ⁻), 239.0567 (C ₁₁ H ₁₁ O ₆ ⁻), 263.0568 (C ₁₁ H ₁₃ O ₆ ⁻), 281.0672 (C ₁₃ H ₁₃ O ₇ ⁻), 443.1181 (C ₁₉ H ₂₃ O ₁₂ ⁻)	A	[35, 36]

Peak	Predicted	Formula	$t_{R(min)}$	Ion Species	Measured Mass (Da)	δP	MS ² Ions	Source	Ref.
M12	Tenuifoliside B	C ₃₀ H ₃₆ O ₁₇	9.189	[M-H] ⁻	667.1879	-1.06376	137.0247 (C ₇ H ₅ O ₃ ⁻), 191.0356 (C ₁₀ H ₇ O ₄ ⁻), 205.0508 (C ₁₁ H ₉ O ₄ ⁻), 239.0570 (C ₁₁ H ₁₁ O ₆ ⁻), 367.1037 (C ₁₇ H ₁₉ O ₉ ⁻), 443.1193 (C ₁₉ H ₂₃ O ₁₂ ⁻), 461.1287 (C ₁₉ H ₂₅ O ₁₃ ⁻)	A	[35]
M13	Benzoic acid	C ₇ H ₆ O ₂	16.100	[M + H] ⁺	123.0450	-5.3472	77.0391 (C ₆ H ₅ ⁺), 105.0347 (C ₇ H ₅ O ⁺)	A	[37]
			6.308	[M-H] ⁻	121.0302	1.59343	77.0401 (C ₆ H ₅ O ⁻)	A	[37]
M14	1,5-Anhydro-D-sorbitol	C ₆ H ₁₂ O ₅	6.464	[M + H] ⁺	165.0693	-2.55948	/	A	[38]
			8.629	[M-H] ⁻	163.0616	-0.55827	71.0141 (C ₃ H ₃ O ₂ ⁻), 101.0232 (C ₄ H ₅ O ₃ ⁻), 147.0073 (C ₆ H ₁₁ O ₄ ⁻), 149.0263 (C ₅ H ₉ O ₅ ⁻), 163.0405 (C ₆ H ₁₁ O ₅ ⁻), 163.0734 (C ₆ H ₁₁ O ₅ ⁻)	A	[38]
M15	O-Hydroxybenzoic acid	C ₇ H ₆ O ₃	30.653	[M + H] ⁺	139.0400	-2.24204	/	A	[39]
			9.188	[M-H] ⁻	137.0247	3.80504	93.0352 (C ₆ H ₅ O ⁻)	A	[39]
M16	4-Hydroxy-3,5-dimethoxycinnamate	C ₁₂ H ₁₄ O ₅	8.106	[M-H] ⁻	237.0772	1.47702	/	A	[40]
M17	Tetrahydrocolumbamine	C ₂₀ H ₂₃ NO ₄	6.427	[M + H] ⁺	342.1683	-2.24204	/	A	[41]
			5.590	[M-H] ⁻	340.1561	0.65858	252.0437 (C ₁₆ H ₁₄ NO ₂ ⁻), 282.1145 (C ₁₇ H ₁₆ NO ₃ ⁻), 310.1093 (C ₁₈ H ₁₆ NO ₄ ⁻)	A	[41]
M18	3,4,5-Trimethoxycinnamic acid	C ₁₂ H ₁₄ O ₅	8.106	[M-H] ⁻	237.0772	1.47702	161.0463 (C ₁₀ H ₉ O ₂ ⁻)	A	[42]
M19	1,3-Dihydroxy-4,5-dimethoxyxanthone	C ₁₅ H ₁₂ O ₆	18.408	[M + H] ⁺	289.0712	-1.74459	217.0503 (C ₁₂ H ₉ O ₄ ⁺), 229.0515 (C ₁₃ H ₉ O ₄ ⁺)	A	[42]
			12.501	[M-H] ⁻	287.0567	1.62827	/	A	[42]
M20	1,6-Dihydroxy-3,5,7-Trimethoxyxanthone	C ₁₆ H ₁₄ O ₇	15.485	[M + H] ⁺	319.0812	-1.03534	/	A	[42]
			14.677	[M-H] ⁻	317.0682	0.51318	287.0205 (C ₁₇ H ₇ O ₇ ⁻)	A	[42]

Peak	Predicted	Formula	t _R (min)	Ion Species	Measured Mass (Da)	δP	MS ² Ions	Source	Ref.
M21	5,6,7-Trimethoxycoumarin	C ₁₂ H ₁₂ O ₅	6.478	[M + H] ⁺	237.0895	16.2122	165.0489 (C ₉ H ₉ O ₃ ⁺), 191.0843 (C ₁₀ H ₇ O ₄ ⁺), 205.0642 (C ₁₁ H ₉ O ₄ ⁺), 207.0794 (C ₁₁ H ₁₁ O ₄ ⁺), 209.0948 (C ₁₁ H ₁₃ O ₄ ⁺), 219.0795 (C ₁₂ H ₁₁ O ₄ ⁺)	A	[42]
			11.648	[M-H] ⁻	235.0625	-0.33897	/	A	[42]
M22	Alphitolic acid	C ₃₀ H ₄₈ O ₄	22.786	[M-H] ⁻	471.3471	-1.48703	/	B	[43]
M23	Maslinic acid	C ₃₀ H ₄₈ O ₄	22.826	[M-H] ⁻	471.3471	-1.48703	/	B	[43]
M24	Epiceanothic acid	C ₃₀ H ₄₆ O ₅	21.523	[M-H] ⁻	485.3274	-0.81051	423.3265 (C ₂₉ H ₄₃ O ₂ ⁻)	B	[43]
M25	Zizyberanalic acid	C ₃₀ H ₄₆ O ₄	22.560	[M-H] ⁻	469.3316	-1.21452	/	B	[43]
M26	Betulinic acid	C ₃₀ H ₄₈ O ₃	24.445	[M-H] ⁻	455.3520	-1.55475	/	B	[43]
M27	Vicenin-2	C ₂₇ H ₃₀ O ₁₅	11.418	[M-H] ⁻	593.1517	-1.48353	/	B	[44]
M28	Spinosin	C ₂₈ H ₃₂ O ₁₅	10.722	[M + H] ⁺	609.1784	-3.10585	297.0752 (C ₁₇ H ₁₃ O ₅ ⁺), 327.0854 (C ₁₂ H ₂₃ O ₁₀ ⁺), 351.0847 (C ₁₆ H ₁₅ O ₉ ⁺), 411.1058 (C ₂₂ H ₁₉ O ₈ ⁺), 429.1156 (C ₂₂ H ₂₁ O ₉ ⁺), 447.1262 (C ₂₂ H ₂₃ O ₁₀ ⁺)	B	[44]
			9.921	[M-H] ⁻	607.1675	-0.34371	427.1041 (C ₂₂ H ₁₉ O ₉ ⁻)	B	[44]
M29	6-Feruloylspinosin	C ₃₈ H ₄₀ O ₁₈	12.477	[M + H] ⁺	785.2228	-3.61507	/	B	[44]
			11.648	[M-H] ⁻	783.2139	-0.30584	235.0625 (C ₁₂ H ₁₁ O ₅ ⁻), 427.1026 (C ₂₂ H ₁₉ O ₉ ⁻)	B	[44]
M30	1,7-Dimethoxyxanthone	C ₁₅ H ₁₂ O ₄	17.609	[M + H] ⁺	257.0812	0.93414	199.0399 (C ₁₂ H ₇ O ₃ ⁺), 213.0557 (C ₁₃ H ₉ O ₃ ⁺), 227.0350 (C ₁₃ H ₇ O ₄ ⁺), 241.0505 (C ₁₄ H ₉ O ₄ ⁺)	A	[45]
M31	1-Hydroxy-3,7-dimethoxyxanthone	C ₁₅ H ₁₂ O ₅	17.687	[M + H] ⁺	273.0781	-53.21667	/	A	[46]
M32	1-Hydroxy-3,6,7-trimethoxyxanthone	C ₁₆ H ₁₄ O ₆	19.264	[M + H] ⁺	303.0862	-0.6369	259.0607 (C ₁₄ H ₁₁ O ₅ ⁺), 271.0599 (C ₁₅ H ₁₁ O ₅ ⁺), 287.0554 (C ₁₅ H ₁₁ O ₅ ⁺)	A	[47]
M33	Propyl benzoate	C ₁₀ H ₁₂ O ₂	27.557	[M + H] ⁺	165.0915	2.75397	149.0975 (C ₉ H ₉ O ₅ ⁺)	A	[48]
M34	Stigmasterol	C ₂₉ H ₄₈ O	28.541	[M + H] ⁺	413.3748	-6.14861	/	A	[49]
M35	α-Spinasterol	C ₂₉ H ₄₈ O	28.541	[M + H] ⁺	413.338	-6.14861	/	A	[50]

Peak	Predicted	Formula	t _R (min)	Ion Species	Measured Mass (Da)	δP	MS ² Ions	Source	Ref.
M36	1,2,3,6,7-Pentamethoxyxanthone	C ₁₈ H ₁₈ O ₇	16.325	[M + H] ⁺	347.1111	-2.78277	285.0758 (C ₁₆ H ₁₃ O ₅ ⁺), 289.0708 (C ₁₅ H ₁₃ O ₅ ⁺), 303.0863 (C ₁₆ H ₁₅ O ₆ ⁺), 317.0651 (C ₁₆ H ₁₃ O ₇ ⁺), 331.0800 (C ₁₇ H ₁₅ O ₇ ⁺)	A	[51]
M37	N-Normuciferine	C ₁₈ H ₁₉ NO ₂	1.838	[M + H] ⁺	282.1525	-0.98118	236.1473 (C ₁₆ H ₁₄ NO ⁺)	B	[52]

Notes: A: *P. tenuifolia*; B: *Z. jujuba*.

Table 2
Molecular docking score of the key active compounds.

Target (PDB)	Com.	CDOCKER interaction energy (Kcal/mol)	Key interactions
MAOA (2z5y)	M16	-44.7752	ASN 181 (H-bond), ALA 68 (H-bond), PHE 208 (van der Waals), LEU 337 (van der Waals), ILE 335 (van der Waals), ILE 180 (van der Waals), TYR 444 (van der Waals), TYR 407 (Pi-Pi-Stacked)
	M18	-40.7385	TYR 69 (H-bond), ALA 68 (H-bond), PHE 208 (van der Waals), LEU 337 (van der Waals), ILE 335 (van der Waals), ILE 180 (van der Waals), TYR 444 (van der Waals)
	M20	-46.7966	TYR 444 (H-bond), ASN 181 (H-bond), CYS 323 (Pi-Sulfur), PHE 208 (van der Waals), LEU 337 (van der Waals), ILE 335 (pi-alkyl), ILE 180 (pi-alkyl), TYR 407 (Pi-Pi-Stacked)
	M37	-34.5098	GLA 215 (H-bond), ILE 207 (H-bond), ASN 181 (H-bond), PHE 208 (van der Waals), LEU 337 (van der Waals), ILE 335 (van der Waals), ILE 180 (van der Waals), TYR 444 (Pi-Pi-Stacked), TYR 407 (Pi-Pi-Stacked)
SERT (6awn)	M17	-49.3710	THR 439 (H-bond), ASN 177 (H-bond), ALA 169 (van der Waals), ILE 172 (Amide-Pi-Stacked), ALA 173 (Carbon H-bond), VAL 501 (van der Waals), SER 336 (Carbon H-bond), NA 707 (van der Waals), ALA 96 (Carbon H-bond), ASP 98 (Pi-Anion), TYR 176 (Pi-Sigma), SER 438 (van der Waals)
	M22	-50.4302	TYR 95 (H-bond), GLU 493 (H-bond), ALA 169 (van der Waals), ILE 172 (pi-alkyl), ASP 98 (pi-alkyl), TYR 176 (pi-alkyl), SER 438 (van der Waals)
	M25	-58.9826	GLA 100 (H-bond), LEU 99 (H-bond), ASP 98 (H-bond), ILE 172 (pi-alkyl), VAL 501 (van der Waals), SER 336 (van der Waals), NA 707 (van der Waals), ALA 96 (van der Waals), TYR 176 (pi-alkyl), SER 438 (van der Waals)
	M35	-56.7521	ARG 104 (H-bond), GLU 494 (H-bond), ILE 172 (pi-alkyl), ALA 169 (pi-alkyl), ALA 173 (pi-alkyl), VAL 501 (van der Waals), ASP 98 (pi-alkyl), TYR 176 (Pi-Sigma), SER 438 (pi-alkyl)
nAChR4 (5kxi)	M18	-27.5261	LYS 79 (H-bond), TRP 156 (van der Waals), LEU 12 (van der Waals), THR 157 (Carbon H-bond), CYS 200 (Pi-Sulfur), TYR 204 (Pi-Anion), CYS 199 (van der Waals)
	M20	-39.0269	GLU 202 (H-bond), ALA 201 (H-bond), TRP 156 (Carbon H-bond), LEU 121 (pi-alkyl), THR 157 (Carbon H-bond), CYS 200 (Pi-Anion), TYR 204 (Pi-Long pair), CYS 199 (Sulfur-X), TRP 57 (van der Waals)
	M31	31.0103	GLU 202 (H-bond), LYS 79 (H-bond), TRP 156 (van der Waals), LEU 121 (van der Waals), THR 157 (Carbon H-bond), CYS 200 (Pi-Sulfur), TYR 204 (Pi-Long pair), CYS 199 (van der Waals)
	M32	-37.7832	GLU 202 (H-bond), LYS 79 (H-bond), TRP 156 (Carbon H-bond), LEU 121 (van der Waals), THR 157 (Carbon H-bond), CYS 200 (Pi-Sulfur), TYR 204 (Pi-Long pair), CYS 199 (van der Waals)

Figures

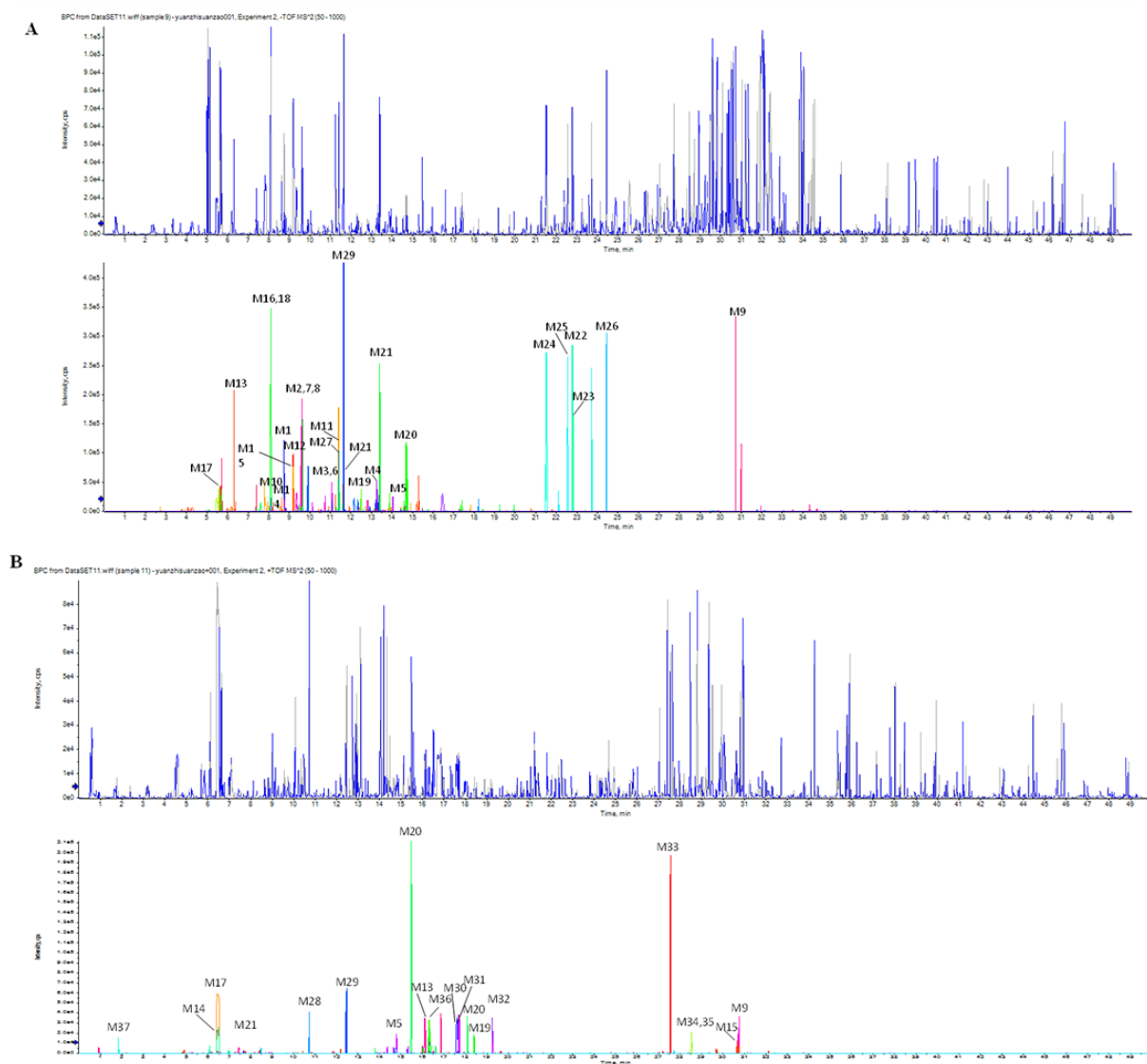
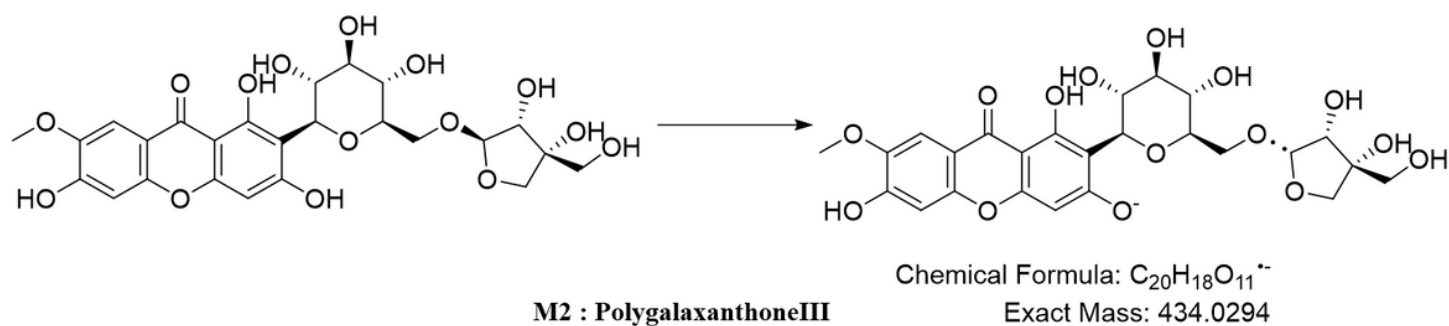


Figure 2

The BPC of *PZE* herb in negative (A) and positive (B) ion modes.



Spectrum from DataSE711.wiff (sample 9) - yuanchisruanzao001, Experiment 2, -TOF MS² (50 - 1000) from 9.602 to 9.647 min

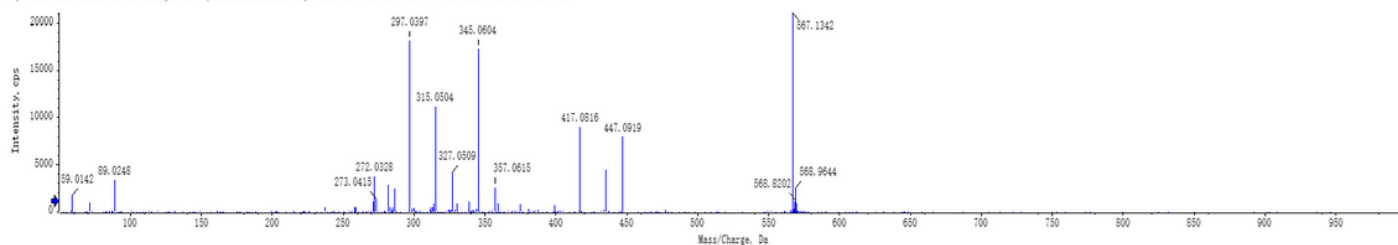
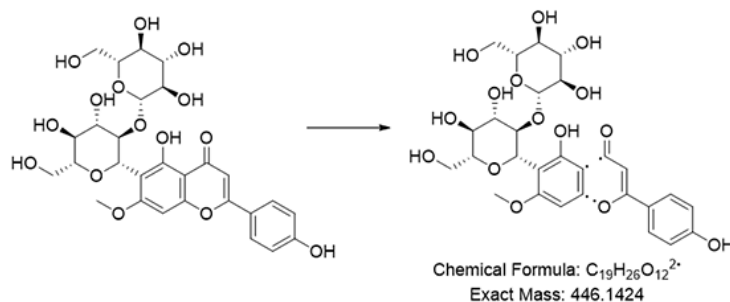
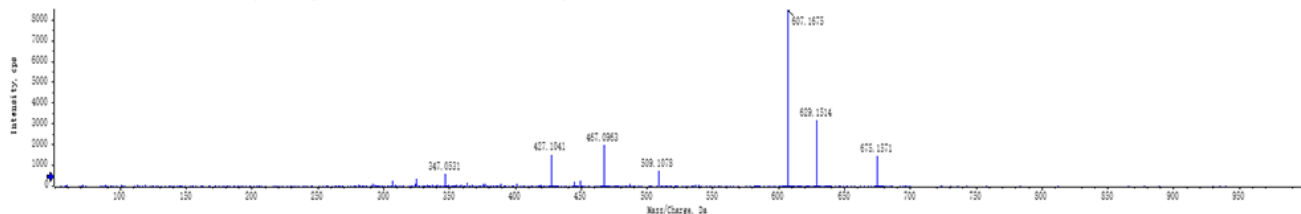


Figure 3

Fragmentation pathway and Negative mass model of typical compound polygalaxanthone (M2).

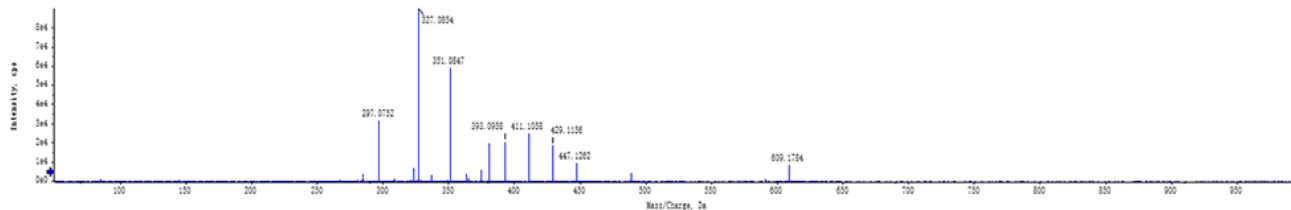


Spectrum from DataSE711.wiff (sample 9) - yuanchisruanzao001, Experiment 2, -TOF MS² (50 - 1000) from 9.921 min Precursor: 675.2 Da, CE: -25.0



Negative mass mode

Spectrum from DataSE711.wiff (sample 11) - yuanchisruanzao001, Experiment 2, -TOF MS² (50 - 1000) from 10.722 min Precursor: 609.2 Da, CE: 35.0



Positive mass mode

Figure 4

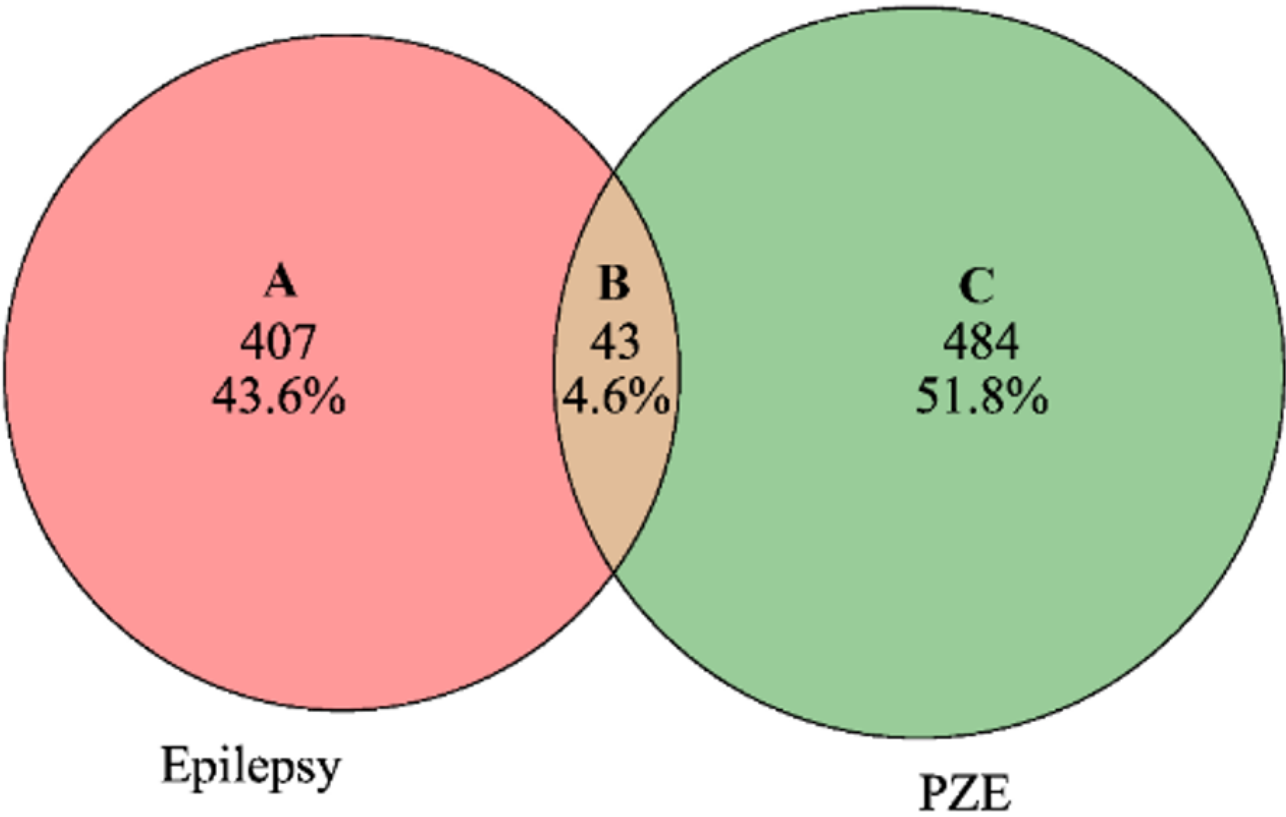


Figure 5

The intersection of active ingredient targets (A+B) and epilepsy targets (B+C) represented in 43 targets (B).

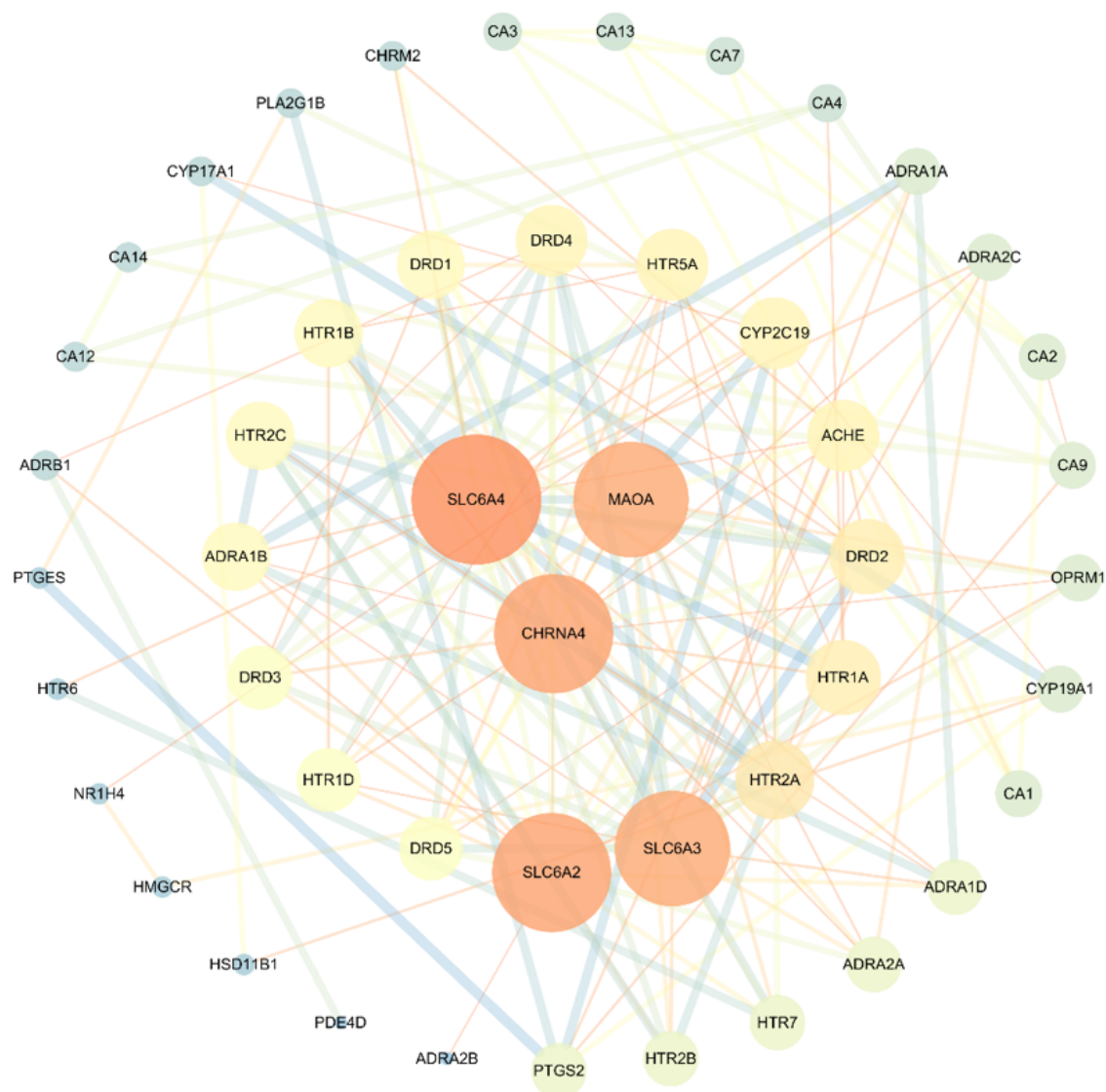


Figure 6

Network diagram of the PPI intersection targets. Size of the node represents the node degree of the target protein in the network. The information about the abbreviation of specific targets are recorded in the **Supporting information**.

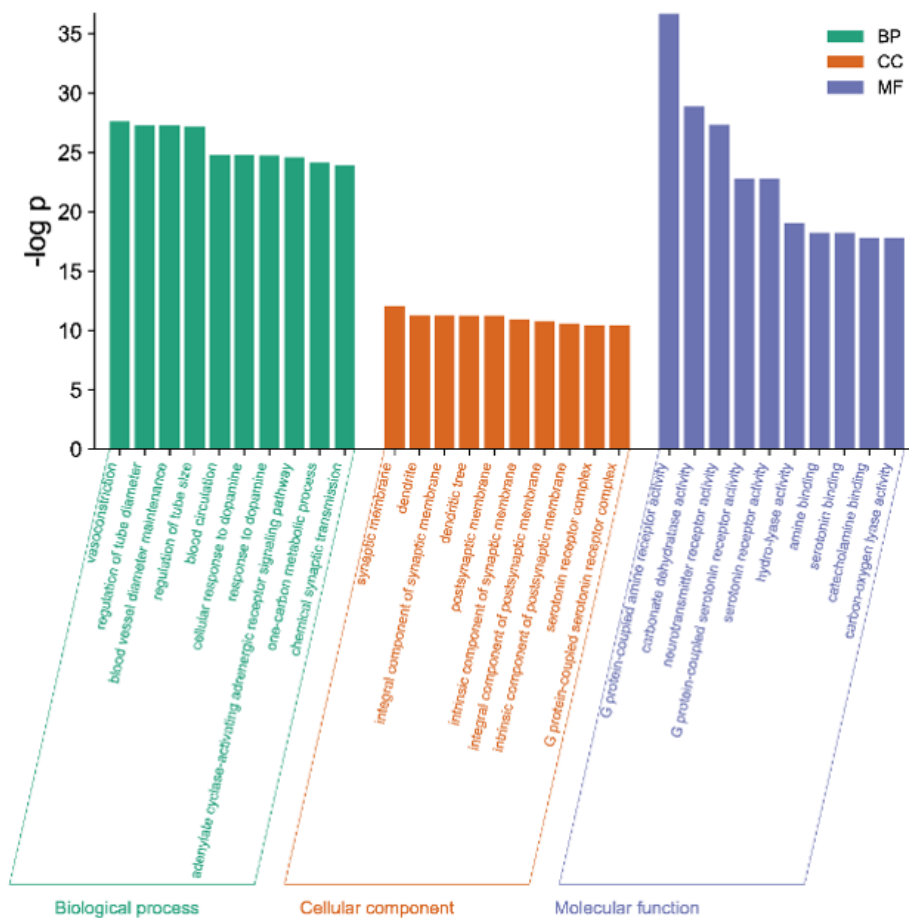


Figure 7

GO function analysis histogram. BP is marked by dark cyan, CC is marked by sienna and MF is marked by steel blue. The bar chart was constructed through the bioinformatics platform.

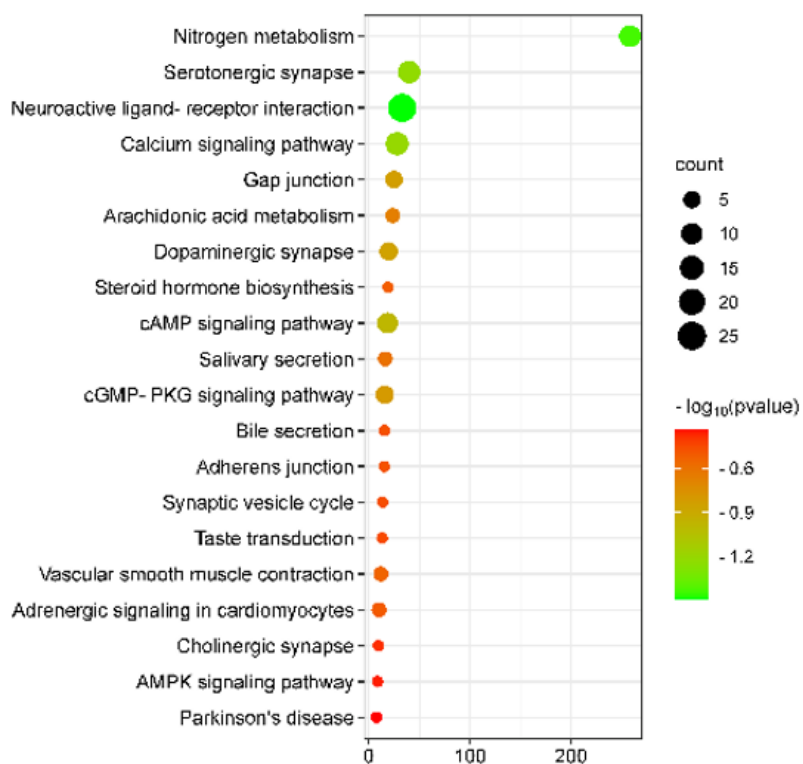


Figure 8

Dot plot of the KEGG pathway enrichment analysis. The horizontal axis represents the enrichment rate of the input genes in the pathway, while the vertical axis represents the pathway name. The color represent different thresholds of the $-\log_{10} P$ value, and the size of the dot indicates the number of genes corresponding to each term. The bubble map was constructed through the bioinformatics platform.

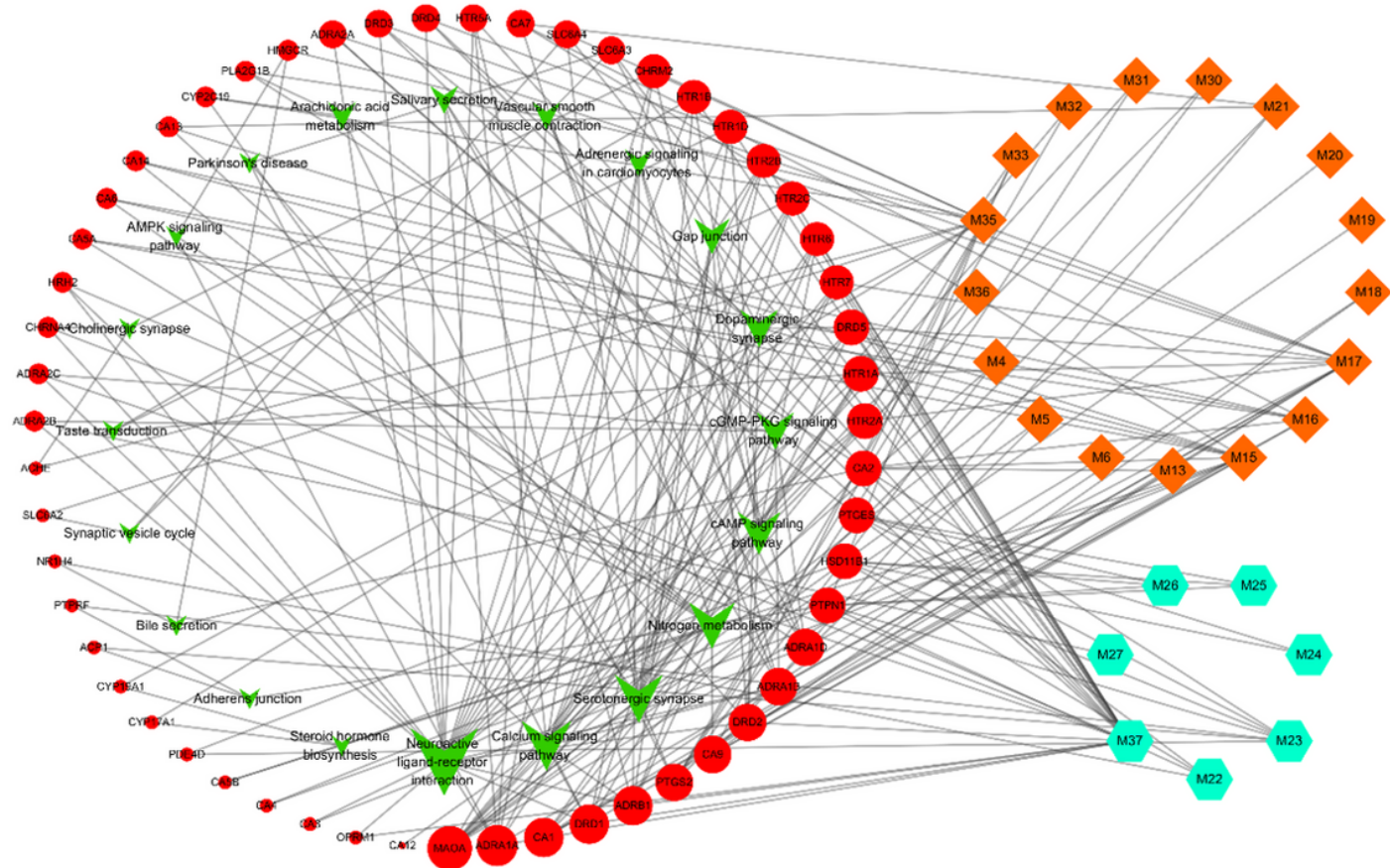


Figure 9

Ingredient-target-pathway network diagram. The active constituents from *P. tenuifolia* displayed as an orange rhombus shape, constituents from *Z. jujuba* is shown as a cyan Hexagon, the overlape target is shown as a red circle, and the signal path is shown as a green V shape.

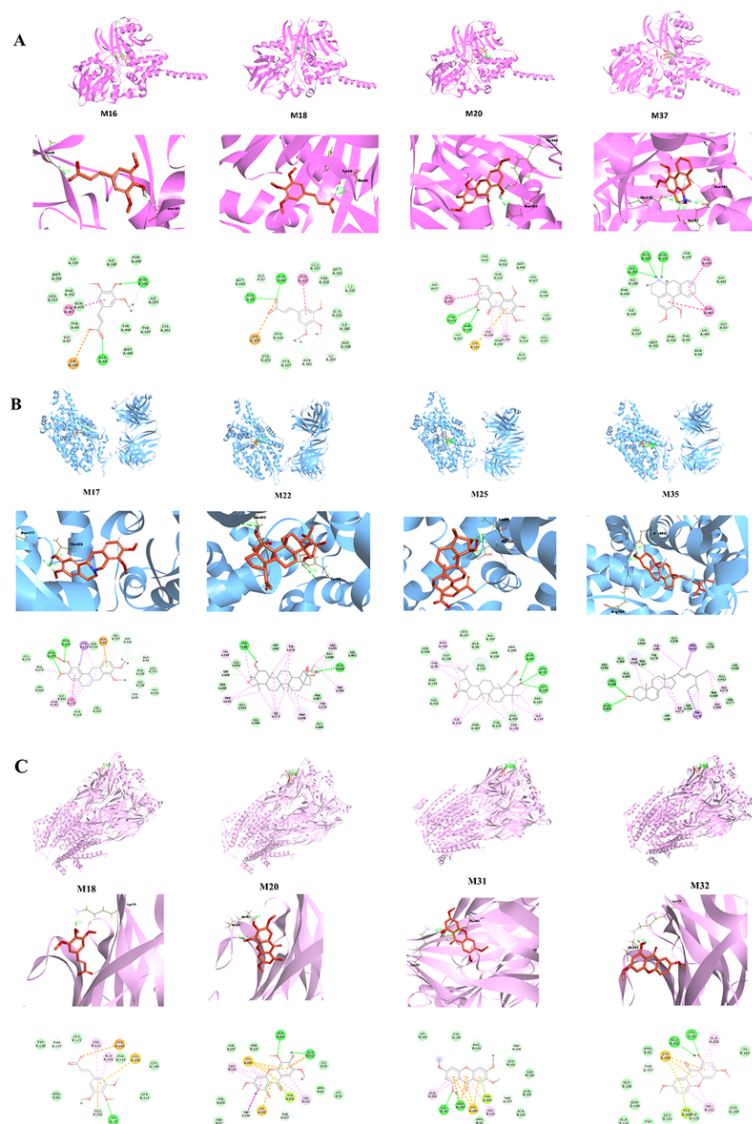


Figure 10

The pictures show molecular docking of the 3D interaction patterns. The legend inset means the type of interaction between ligands and the amino acid residues of the protein, and the important H-bonds. The middle row of pictures shows the important interactions between ligands and the amino acid residues of the protein. The bottom of the figure shows the 2D docking of the compound in the binding pocket. The (A) show protein Ligand-protein interactions of compounds **M16**, **M18**, **M20** and **M37** with the active site of (2Z5Y); The (B) show protein Ligand-protein interactions of compounds **M17**, **M22**, **M25** and **M35** with the active site of (6AWN); The (C) show protein Ligand-protein interactions of compounds **M18**, **M20**, **M31** and **M32** with the active site of (5KXI).

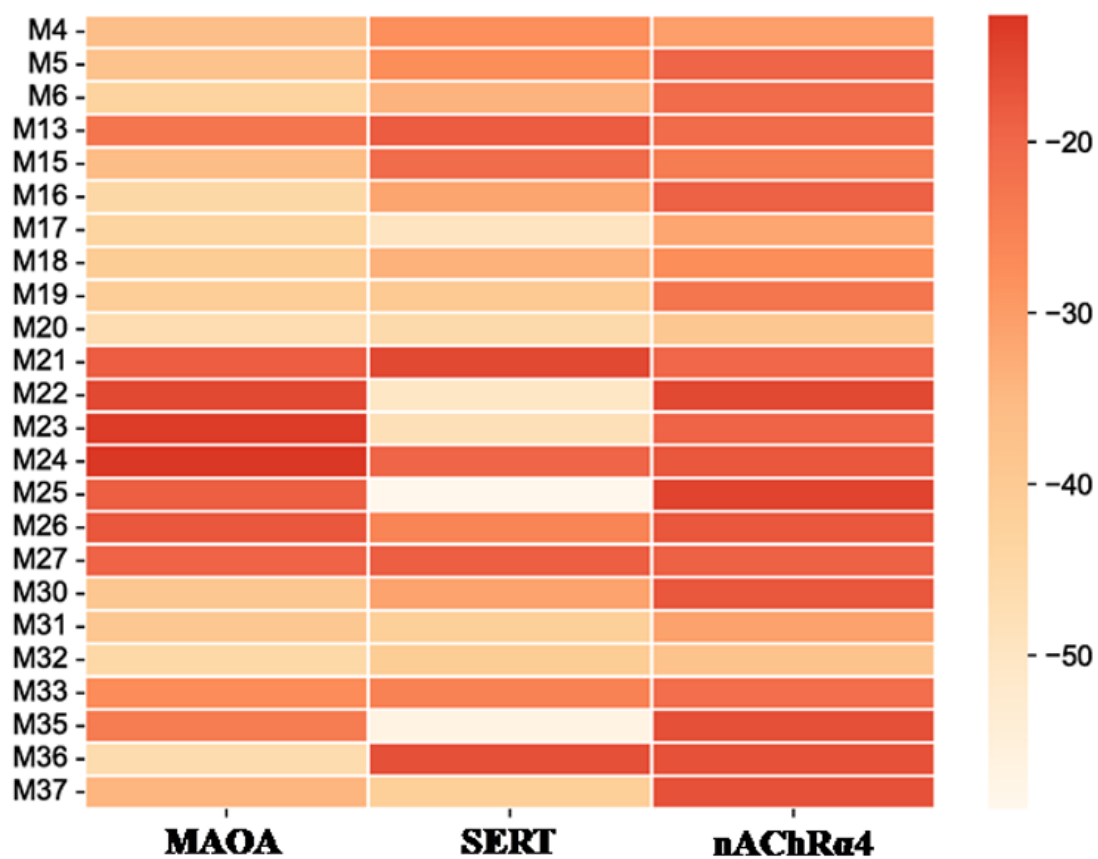


Figure 11

A heat map ranking the docking scores of components in PZE and potential antiepileptic targets.

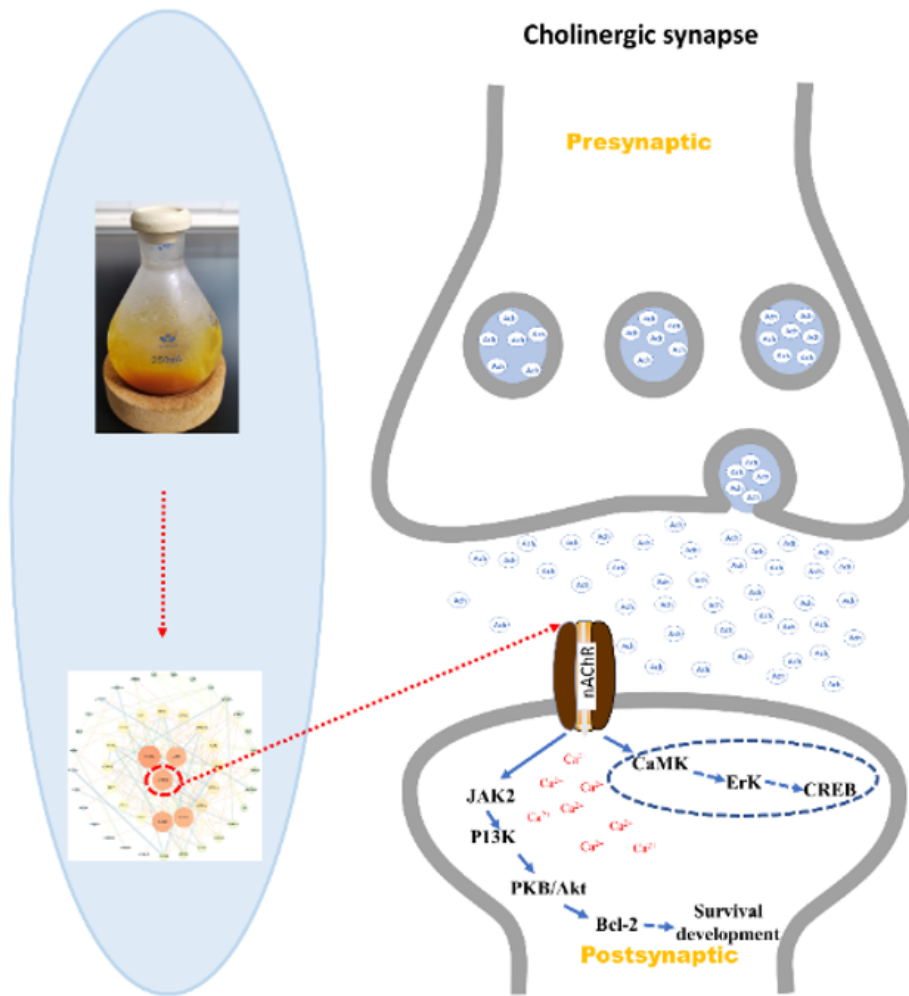


Figure 12

Schematic diagram illustrating the anti-epilepsy cascade activated by $\alpha 4$ nAChRs. Activation of the $\alpha 4$ nAChR, which regulates the flow of Ca^{2+} plasma inside and outside the cell. activates CaMK, and modulate ERK and CREB downstream of the $\alpha 4$ nAChR signal transduction pathway.

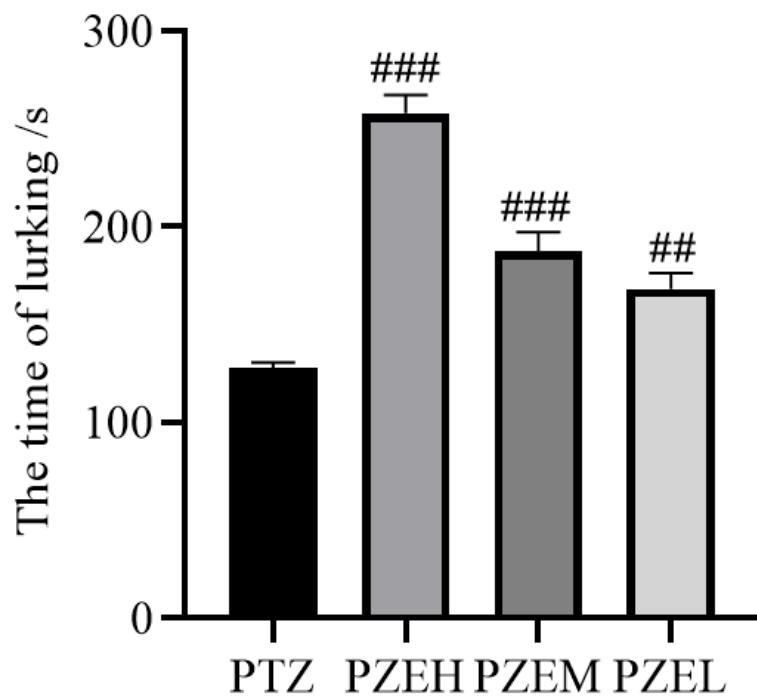


Figure 13

After PTZ modeling, the time of lurking, PTZ vs. PZEH, $###p < 0.001$, PTZ vs. PZEM, $###p < 0.001$, PTZ vs. PZEL $##p < 0.01$; Data are expressed as means $\pm$ SEM.

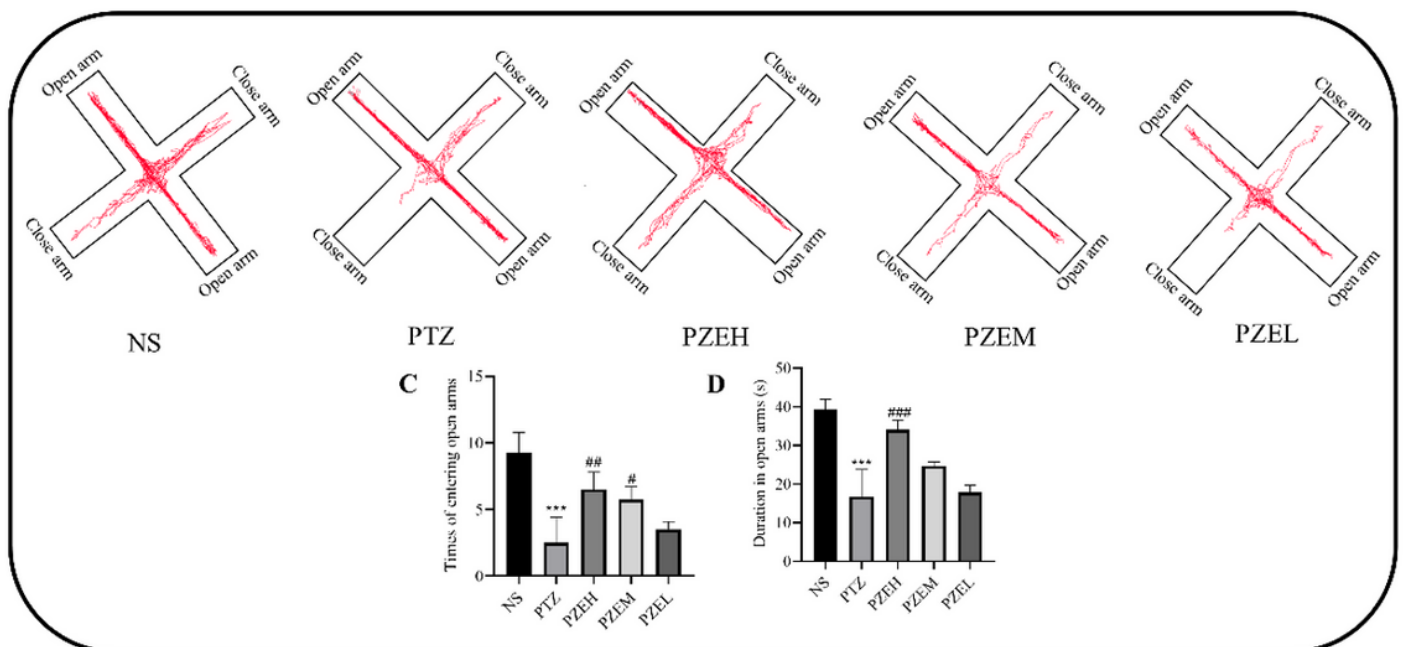


Figure 14

Effect of PZE on Elevated plus maze of epileptic model mice, showing times of entering open arms (C) and the time spent on entries into the open arms (D). N = 4/group. *p < 0.05, ***p < 0.001, ****p < 0.0001. Data are expressed as means± SEM.

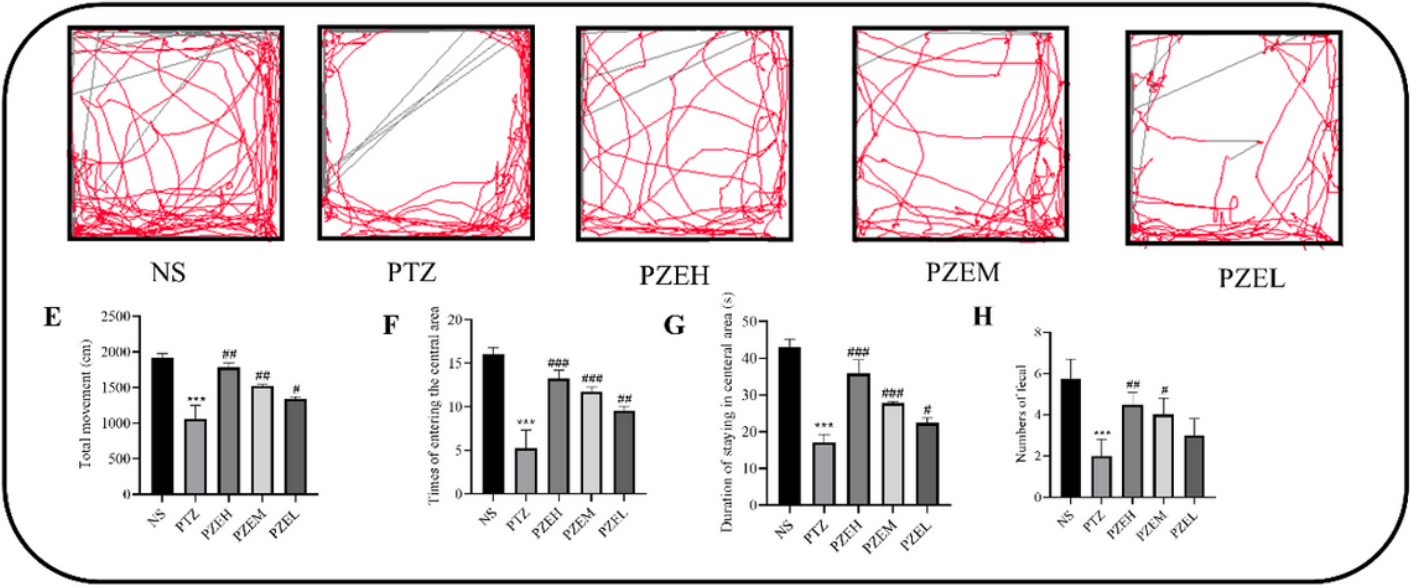


Figure 15

Effect of PZE on Open Field Test of epileptic model mice, total movement(E), times of entering the central area(F), duration of staying in central area(G), numbers of fecal(H).

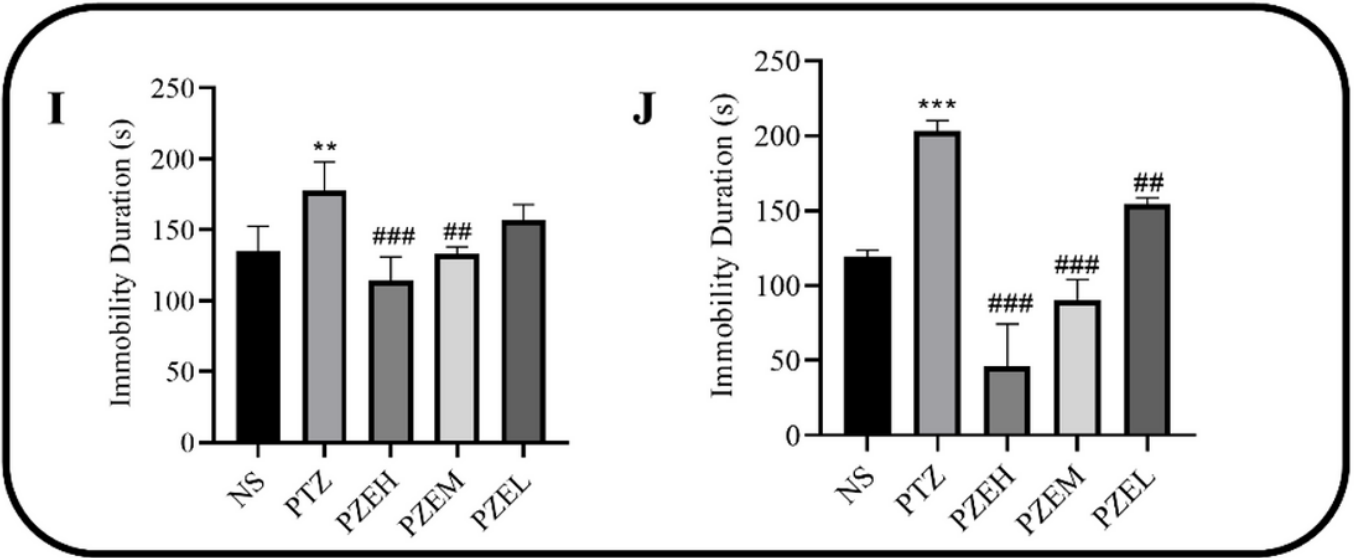


Figure 16

Effect of PZE on swimming test (I) and tail suspension test (J) of epileptic model mice, immobility duration.

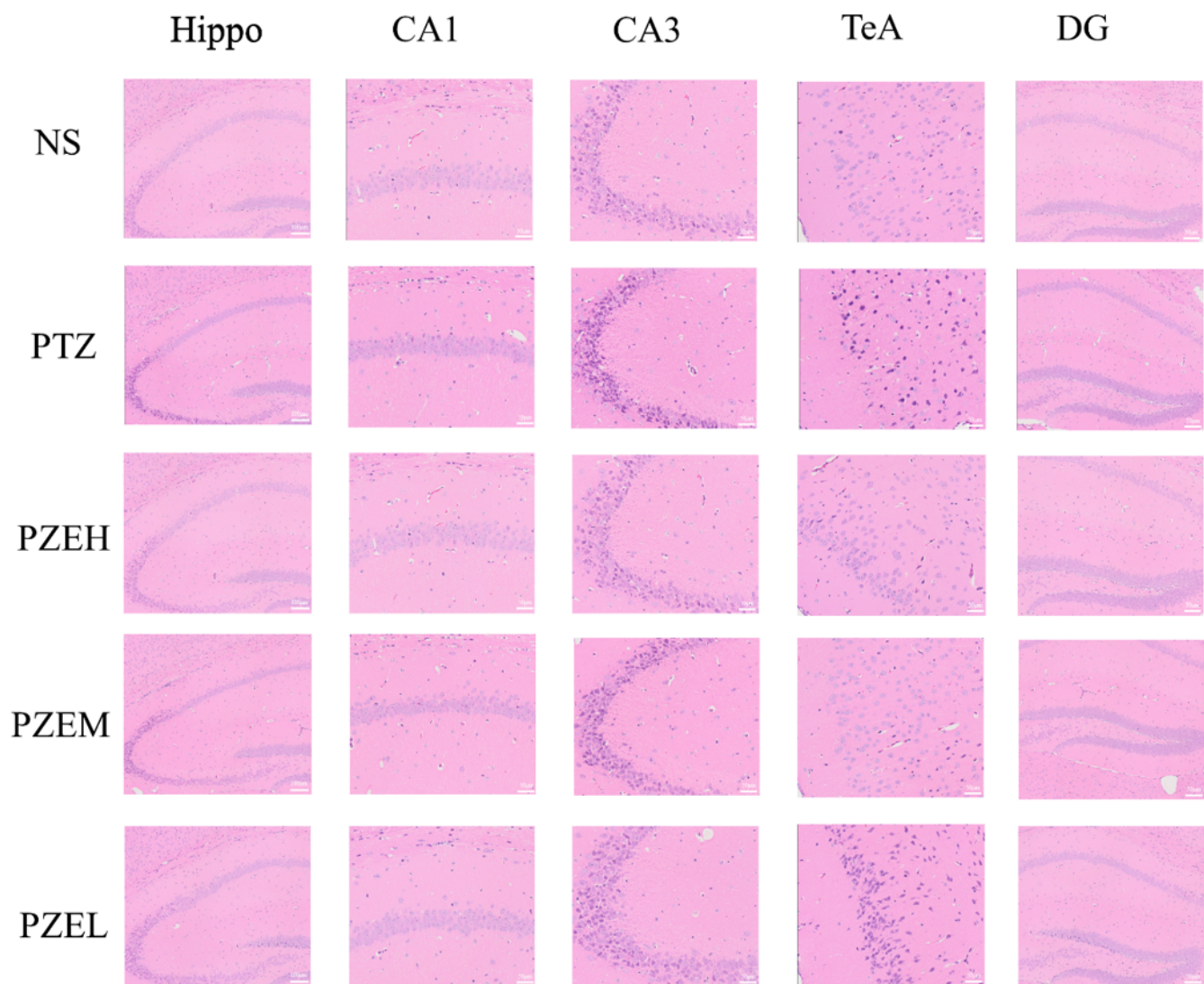


Figure 17

PZE can improve the morphological changes of hippocampal in epileptic mice. Hematoxylin and eosin (H&E) staining, entire hippocampus (× 100). CA1, CA3, TeA and DG regions of the hippocampus (× 50).

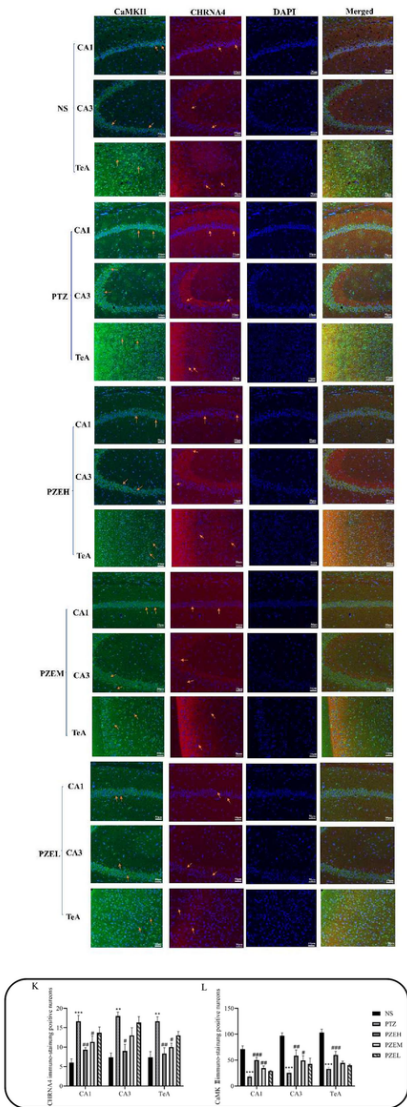


Figure 18

The expression in the hippocampus was evaluated by Immunofluorescence staining. Statistical results are as follows: CHRNA4: CA1: CHRNA4: $F(4,10) = 66.60, p < 0.0001$, NS vs. PTZ, $***p < 0.001$, PTZ vs. PZEH, $###p < 0.001$, PTZ vs. PZEM, $##p < 0.01$; CA3: $F(4,10) = 28.46, p < 0.0001$, NS vs. PTZ, $***p < 0.001$, PTZ vs. PZEH, $##p < 0.01$, PTZ vs. PZEM, $#p < 0.05$; TeA: $F(4,10) = 98.62, p < 0.0001, n = 3$; NS vs. PTZ, $***p < 0.001$; PTZ vs. PZEH, $##p < 0.001$; $##p < 0.001$; CaMK II CA1: $F(4,10) = 66.60, p < 0.0001$, NS vs. PTZ, $***p < 0.001$, PTZ vs. PZEH, $###p < 0.001$, PTZ vs. PZEM, $##p < 0.01$; CA3: $F(4,10) = 28.46, p < 0.0001$, NS vs. PTZ, $***p < 0.001$, PTZ vs. PZEH, $##p < 0.01$, PTZ vs. PZEM, $#p < 0.05$; TeA: $F(4,25) = 246.6, p < 0.0001, n = 3$; NS vs. PTZ, $***p < 0.001$; PTZ vs. PZEH, $###p < 0.001$; $###p < 0.001$.

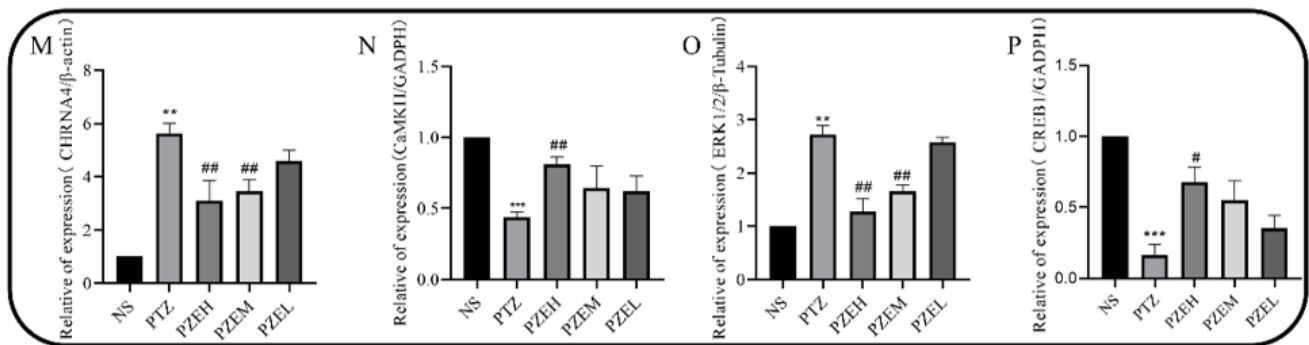
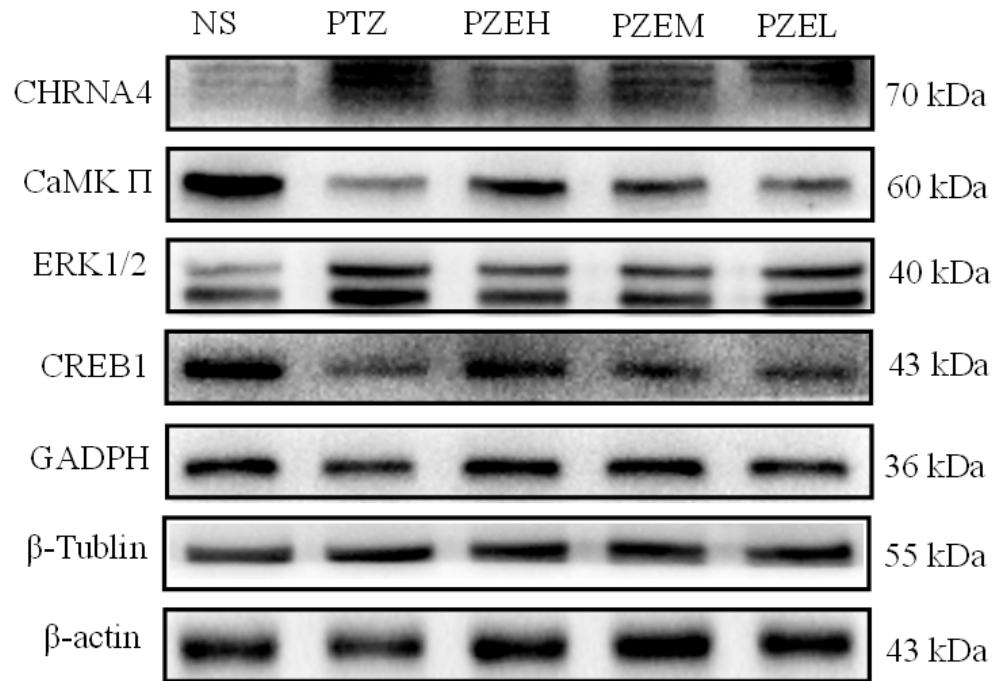


Figure 19

The expression in the hippocampus was investigated by Western blotting. Statistical results are as follows: CHRNA4: $F(4,10) = 41.91$, $p < 0.0001$, NS vs. PTZ, $**p < 0.01$, PTZ vs. PZEH, $##p < 0.01$, PTZ vs. PZEM, $##p < 0.01$; CaMKII: $F(4,10) = 16.84$, $p < 0.0002$, $n = 3$; NS vs. PTZ, $*p < 0.001$; PTZ vs. PZEH, $##p < 0.01$; ERK1/2: $F(4,10) = 82.09$, $p < 0.0001$, NS vs. PTZ, $**p < 0.01$, PTZ vs. PZEH, $##p < 0.01$, PTZ vs. PZEM, $##p < 0.01$; CREB1: $F(4,10) = 35.54$, $p < 0.0001$, NS vs. PTZ, $***p < 0.001$, PTZ vs. PZEH, $#p < 0.05$, PTZ vs. PZEM, $##p < 0.01$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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