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Investigation of the Mechanistic Action of Yinaoan Capsules Hospital Formulation in Epilepsy Therapy Through Multi-Pathway Network Pharmacology Approaches

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ABSTRACT

This study used network pharmacology strategies combined with multi-pathway analysis to investigate the potential bioactive constituents and underlying therapeutic mechanisms of the hospital-prepared Yinaoan capsule (YNA) for epilepsy treatment. The active compounds in YNA were systematically retrieved from multiple resources, including the traditional Chinese medicine system pharmacology database (TCMSP), the traditional Chinese medicines integrated database (TCMID), the encyclopedia of traditional Chinese medicine (ETCM), along with evidence from related literature. The pharmacokinetic properties of these compounds were predicted using the pkCSM platform. The SwissTargetPrediction tool was used to predict the molecular targets of the identified compounds. Meanwhile, known epilepsy-related targets and anti-epileptic drug-related data were extracted from databases such as Genecards and the therapeutic target database (TTD). The intersection between the targets of YNA-derived compounds and epilepsy-related targets was determined for further analysis. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis was conducted via the DAVID database to explore the involved signaling pathways. Network visualization and construction were performed using Gephi software. In addition, molecular docking validation was performed via Autodock Vina to evaluate the binding affinity of the major compounds with core targets. The KEGG analysis identified 27 important signaling pathways, including the calcium signaling pathway and the cAMP signaling pathway, associated with the therapeutic function of YNA. In addition, network analysis revealed 25 core targets, such as MAPK3 and PRKCA, alongside 20 key bioactive compounds, including GC195 and ZNX069. Molecular docking results confirmed strong binding interactions between these pivotal compounds and the identified targets. The findings suggest that YNA may exert anti-epileptic effects by modulating essential targets like MAPK3 and PRKCA, and activating critical pathways such as the calcium signaling pathway and the cAMP signaling pathway, primarily through key compounds such as GC195 and ZNX069.

Keywords: Epilepsy, Network pharmacology, Drug pathway information, Molecular docking, Yinaoan capsules, Disease pathway information

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Introduction

A seizure represents a transient occurrence of clinical manifestations resulting from excessive or synchronous neuronal activity within the brain [1]. Epilepsy is a chronic neurological disorder characterized by a persistent predisposition to generate epileptic seizures [2, 3]. This condition can affect individuals irrespective of their age, ethnicity, socioeconomic status, or geographical location [4, 5]. The Global Burden of Disease study 2016 reported that epilepsy constitutes a significant portion of the global disease burden, with an estimated 46 million individuals affected worldwide [6, 7]. While the underlying cause of epilepsy can often be determined,

approximately 50% of cases remain idiopathic with no identifiable etiology [8]. Presently, epilepsy is recognized as a manageable medical condition, with long-term seizure control achieved in nearly 80% of patients, and around half of these individuals remain seizure-free even after discontinuation of therapy [4, 9]. Pharmacological management with anti-epileptic drugs remains the cornerstone of seizure control, demonstrating effectiveness in approximately 70% of patients, particularly when monotherapy is applied [10]. Nevertheless, around 20-30% of patients exhibit resistance to conventional pharmacological treatments, a condition classified as "drug-resistant epilepsy" [11]. Moreover, the administration of anti-epileptic drugs is frequently associated with a range of adverse effects [12]. Early generations of these medications were primarily linked to neurological side effects. Special consideration is essential when prescribing anti-epileptic drugs during pregnancy to minimize the risk of teratogenicity. Additionally, patients undergoing treatment with these drugs have been shown to face a two- to threefold increased risk of bone fractures [13].

Historical records in China indicate that epilepsy was first mentioned in the classical medical text Huangdi Neijing, where its origin was attributed to congenital deficiencies [14]. Later, Zhu [15] proposed that the occurrence of epilepsy is closely linked to the presence of phlegm. Furthermore, Zhang [16] emphasized that the therapeutic approach to epilepsy should focus on nourishing the kidneys as the root strategy, while the elimination of phlegm should be regarded as addressing the outward manifestation. Traditional Chinese medicine (TCM) has an extensive heritage in managing epilepsy, which can be categorized into four primary therapeutic principles: (1) replenishing qi, promoting vitality, strengthening the spleen, and supporting kidney function; (2) resolving phlegm and improving blood circulation to eliminate stasis; (3) detoxification; and (4) restoring consciousness and revitalizing brain function [17]. TCM interventions for epilepsy are notable for their diversity in treatment techniques, their relatively low incidence of adverse effects, and their capacity to control various seizure types while enhancing patient quality of life [18].

The Yinaoan capsules (YNA), investigated in the present study, were formulated by Professor Liu Mao-cai from Guangzhou University of Traditional Chinese Medicine. This preparation integrates modern pharmacological insights with his extensive clinical experience in epilepsy management, and it is based on the classical anti-epileptic herbal prescription ChuXian Powder, developed by Mr. Lin Xia-quan, a well-known veteran TCM physician from Guangdong province. YNA consists of multiple herbal and animal-derived ingredients, including *Ziziphus jujuba* (Suan Zao Ren-SZR), *Paeonia lactiflora* (Bai Shao-BS), *Angelica sinensis* (Dang Gui - DG), *Gastrodia elata* (Tian Ma - TM), *Arisaema erubescens* (Zhi Nan Xing - ZNX), *Buthus martensii* (Quan Xie - QX), *Scolopendra subspinipes mutilans* (Wu Gong - WG), and *Glycyrrhiza uralensis* (Gan Cao - GC). Experimental studies in animal models have demonstrated that YNA is capable of extending the latency period before seizure onset, reducing the intensity of epileptic discharges, and decreasing the duration of seizures. These pharmacodynamic effects were comparable to those observed with phenytoin sodium treatment [19]. Moreover, clinical research indicates that the combined administration of YNA with conventional anti-epileptic drugs yields superior therapeutic outcomes compared to the use of anti-epileptic drugs alone [20].

While previous research has indicated the anti-epileptic potential of YNA, the specific active ingredients and underlying mechanisms have not yet been fully understood. To address this, network pharmacology offers a promising approach for initial investigation [21]. Current network pharmacology studies often explore the therapeutic effects of herbal remedies by examining comprehensive drug data, such as principal component analysis of herbal compounds alongside molecular descriptors of FDA-approved anti-infective and anti-inflammatory drugs. This analysis, grounded in structure-activity relationships, helps predict the anti-infective or anti-inflammatory properties of herbs [22]. Additionally, certain studies apply hierarchical clustering techniques to compare the target profiles of herbal components with those of FDA-approved drugs to better understand the mechanisms of action of herbal medicines [23]. Despite these advances, there remains significant untapped potential in leveraging disease and drug pathway information within network pharmacology research. In the present study, a network pharmacology approach that integrates disease-specific and drug-related pathway data is proposed, aiming to provide valuable insights into the mechanisms underlying YNA's effects.

Materials and Methods

Collection of compounds and targets in YNA

The compounds from the eight medicinal herbs in YNA were gathered from multiple sources, such as the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP), the traditional

Chinese medicine integrated database (TCMID), the encyclopedia of traditional Chinese medicine (ETCM), and relevant scholarly articles. Since glycosylated compounds may undergo hydrolysis by glycosidases in the body, both glycosylated and deglycosylated forms of these compounds were included from the TCMSP database. To assess the pharmacokinetic properties, the pkCSM tool (http://biosig.unimelb.edu.au/pkcsm/) was used, with particular focus on BBB permeability (log BB) and CNS permeability (logPS) to evaluate potential compounds that could affect the brain.

Subsequently, SwissTargetPrediction was employed to predict possible macromolecular targets for these active compounds based on the molecular similarity of their 2D and 3D structures.

Identification of YNA-epilepsy common targets

To identify targets related to epilepsy, several databases were queried, including OMIM (https://omim.org/), Genecards (version 5.13, https://www.genecards.org/), TTD (http://db.idrblab.net/web/), DisGenet (version 7.0, https://www.disgenet.org/), and Drugbank (version 5.1.9, https://go.drugbank.com/). The keyword "epilepsy" was used in the search. The targets of YNA compounds were cross-referenced with epilepsy-related disease targets, identifying potential targets for YNA's anti-epileptic effects.

Collection of targets for anti-epileptic drugs

The databases Drugbank and TTD were used to gather data on anti-epileptic drugs and their corresponding targets. Information on whether these drugs remain approved and marketed in major regions was confirmed by cross-referencing data from the US FDA, the National Medical Products Administration (NMPA), the Pharmaceuticals and Medical Devices Agency (PMDA), and the European Medicines Agency (EMA).

KEGG pathway enrichment analysis

Gene symbols for the YNA-epilepsy targets, epilepsy-related targets, and anti-epileptic drug targets were submitted to the DAVID platform (version 2021, https://david.ncifcrf.gov/), using OFFICIAL_GENE_SYMBOL, Homo sapiens, and KEGG_PATHWAY for identifiers, background, and pathways, respectively. This generated pathway enrichment data for the YNA-epilepsy, epilepsy, and anti-epileptic drug targets based on KEGG.

Construction of networks

Based on the results from the KEGG pathway enrichment analysis, Gephi (Version 0.9.6) was used to construct two key networks: one depicting the relationships between targets and key pathways, and the other highlighting the connections between compounds and their core targets in YNA. These networks were analyzed to identify the central targets and most influential compounds.

Molecular docking procedures

The 3D molecular structures of the compounds were sourced from TCMSP and Pubchem. For compounds lacking 3D structural data, ChemDraw (Version 16.0, Cambridge Soft, USA) was used to generate the necessary structures. To facilitate docking, SDF files were batch-converted to mol2 format using Open Babel GUI (Version 2.4.1). Docking simulations were performed using Autodock Vina (Version 1.1.2, Scripps Research, USA). Target protein structures were retrieved from the PDB database (http://www.rcsb.org/) and saved in pdbqt format. The docking box was placed at the binding site for targets with known ligands, while for others, the docking box was adjusted to encompass the target protein as much as possible. The docking configuration was set with parameters: exhaustiveness = 8, energy_range = 3, and num_modes = 9, aiming for optimal binding affinity with lower docking scores.

Results and Discussion

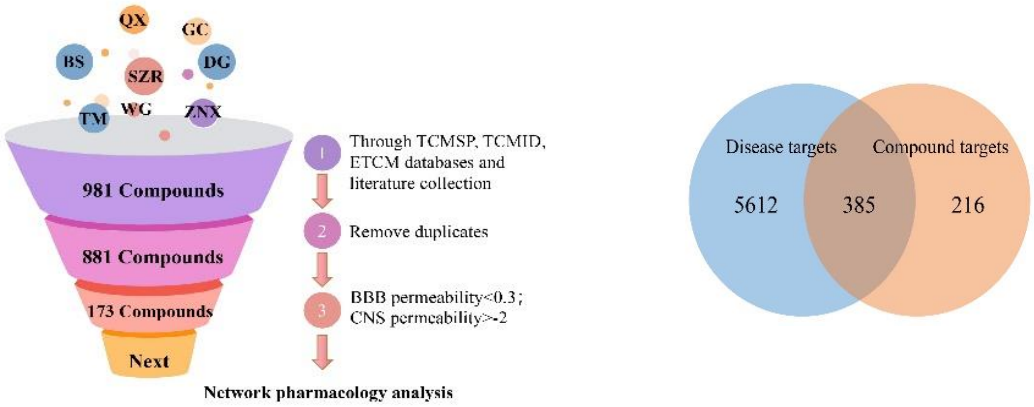
Compounds in YNA and target collection outcomes

A total of 981 components from the herbal ingredients used in YNA were compiled from various databases and scientific publications. After reviewing these compounds, 881 remained, with 100 of them being recurrent across different herbal formulations. Out of these, 173 compounds were selected based on their log BB (> 0.3) and logPS (> -2) values. These compounds are more likely to cross the blood-brain barrier and have potential effects on the central nervous system [24], which could contribute to their anti-epileptic activity. The process of compound

screening is illustrated in **Figure 1a**. Following this, SwissTargetPrediction was used to predict 601 potential targets for these compounds.

Targets related to YNA and epilepsy

A search for epilepsy-related targets using the Genecards, OMIM, DisGenet, TTD, and Drugbank databases yielded 5997 results with the keyword "Epilepsy." After intersecting the targets of the YNA compounds with those associated with epilepsy, 385 potential targets were identified, as shown in **Figure 1b**. These targets are believed to be involved in the therapeutic effects of YNA on epilepsy.



a) b)

Figure 1. a) The compounds screening process, and b) Venn map of YNA target for treating epilepsy.

Collection of anti-epileptic drugs and their targets

From the Drugbank and TTD databases, 64 anti-epileptic drugs were compiled. Out of these, 45 drugs were linked to corresponding targets and are confirmed to be available in major global markets, as validated by the FDA, NMPA, EMA, and PMDA. However, 11 drugs have been discontinued or are not authorized in the aforementioned regions, while 8 others lack specific target data. In total, 108 targets were identified as relevant to the anti-epileptic actions of these drugs.

KEGG pathway enrichment analysis outcomes

The intersection of the YNA-epilepsy targets (385), epilepsy disease-related targets (5997), and anti-epileptic drug targets (108) was processed using DAVID, revealing 92, 117, and 21 significant pathways, respectively, with a P-value of < 0.05. These pathways were ranked according to their P-values. To determine the main pathways relevant to YNA's effect on epilepsy, three strategies were applied: 1) mapping the top 10% of epilepsy-related pathways to the YNA-epilepsy intersection targets, 2) identifying the top 10% of pathways for YNA-epilepsy targets, and 3) incorporating all anti-epileptic drug pathways into the YNA-epilepsy network. The first strategy identified 9 important pathways, including the calcium signaling pathway, cAMP signaling pathway, and serotonergic synapse. The second strategy revealed 8 additional pathways, such as retrograde endocannabinoid signaling, FoxO signaling, and neutrophil extracellular trap formation. Notably, while the lysosome and thermogenesis pathways were ranked 8th and 9th in the epilepsy disease pathways, they were not part of the YNA-epilepsy network, suggesting their irrelevance to YNA's therapeutic effects on epilepsy. The third strategy identified another 10 relevant pathways, such as the GABAergic synapse, glutamatergic synapse, and taste transduction. Notably, pathways like cardiac muscle contraction, Cortisol synthesis, Aldosterone synthesis, and Renin secretion ranked highly in the anti-epileptic drug pathways but were not present in the YNA-epilepsy network, and thus were excluded. Ultimately, 27 key pathways were identified, with detailed relationships between these pathways and epilepsy summarized in **Table 1**.

From the analysis, it is evident that the first 9 YNA-epilepsy pathways align with the epilepsy disease pathways, and both the cAMP signaling pathway and Apoptosis rank significantly in both datasets. Furthermore, all of the top 10 disease-related pathways were found in the YNA-epilepsy pathway, providing evidence for YNA's potential anti-epileptic effects. Interestingly, while 4 out of the top 9 YNA-epilepsy pathways are not linked to

current anti-epileptic drugs, 17 out of the 21 pathways for anti-epileptic drugs overlap with those of YNA-epilepsy. This suggests that YNA influences multiple pathways to treat epilepsy, highlighting the distinct advantages of Traditional Chinese Medicine (TCM) in comparison to conventional pharmaceutical treatments. New research is continually shedding light on the mechanisms of epilepsy, with neuroinflammation now recognized as a significant factor in the onset and progression of the disease. Consequently, targeting neuroinflammation has become an important strategy in treating epilepsy [25]. Based on the KEGG pathway data, YNA's involvement in the PI3K-Akt signaling pathway also suggests its potential role in managing neuroinflammation.

Table 1. Information on key pathways.

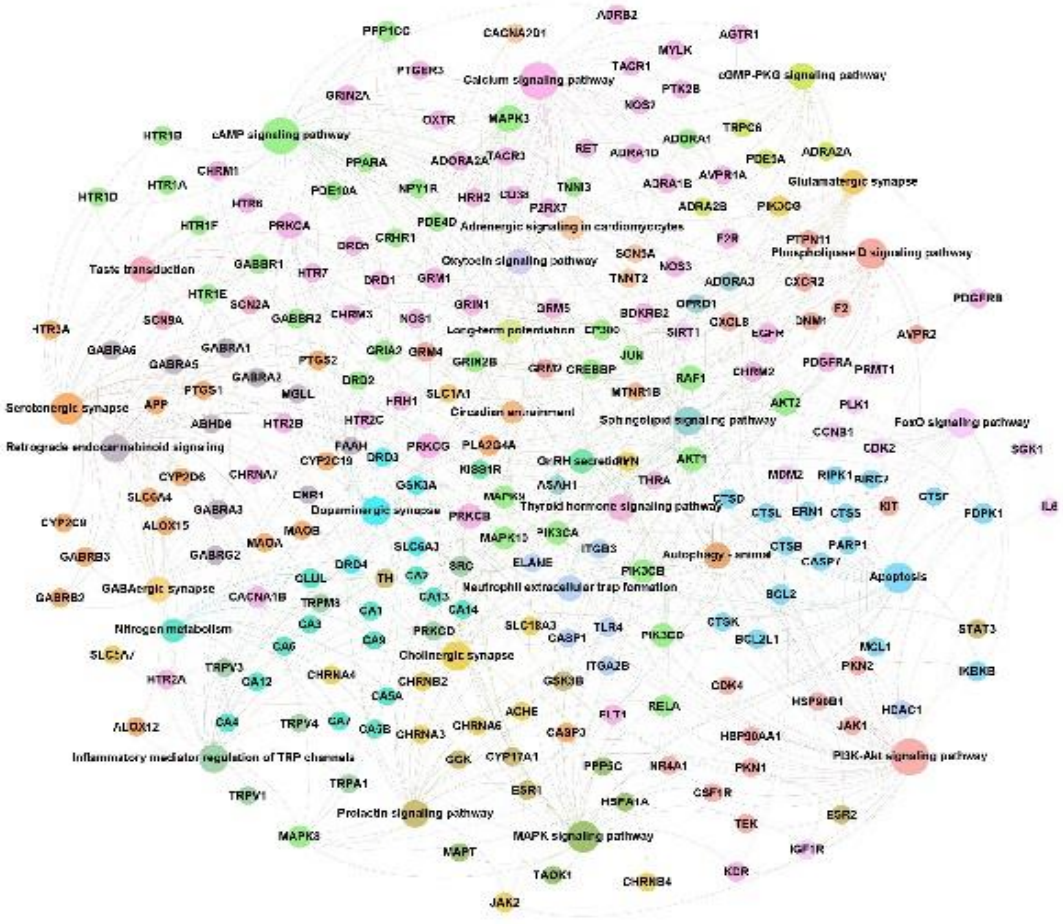
No.	Pathway	Function	Ranking in the YNA-epilepsy pathway	Rank in the disease pathway	Rank in the anti-epileptic drug pathway
1	Calcium signaling pathway	This pathway plays a significant role in epilepsy [26].	1	35	4
2	cAMP signaling pathway	It is involved in both the onset and management of epilepsy [27].	2	4	9
3	Serotonergic synapse	It is associated with epilepsy treatment [28].	3	29	6
4	Nitrogen metabolism	This pathway is crucial in regulating ammonia balance and preventing epilepsy [29].	4	40	-
5	Apoptosis	Epilepsy contributes to neuronal apoptosis [30].	5	5	-
6	Cholinergic synapse	It plays a key role in modulating epilepsy [31].	6	28	10
7	Inflammatory mediator regulation of TRP channels	It could serve as a potential target for treating seizure disorders [32].	7	69	14
8	Phospholipase D signaling pathway	This pathway has a distinct pathophysiological role in epileptic seizures [33].	8	77	-
9	Prolactin signaling pathway	Prolactin is known for its neuroprotective properties [34].	9	19	-
10	Retrograde endocannabinoid signaling	It regulates GABA release [35].	21	1	2
11	FoxO signaling pathway	Seizure-induced neuronal death is influenced by this pathway [36].	10	2	-
12	Neutrophil extracellular trap formation	Neutrophils act as mediators of neuronal hyperexcitability [37].	69	3	-
13	PI3K-Akt signaling pathway	This pathway is linked to both the development and treatment of epilepsy [38].	11	6	-
14	GnRH secretion	Epilepsy can disrupt the regulation of this pathway [39].	35	7	17
15	Sphingolipid signaling pathway	This pathway is implicated in a range of neurological conditions [40].	13	10	-
16	Autophagy-animal	It plays a role in epileptogenesis [41].	47	11	-
17	Thyroid hormone signaling pathway	It contributes to a form of juvenile myoclonic epilepsy [42].	28	12	-
18	GABAergic synapse	It is the mechanism of action for several anti-epileptic medications [43].	37	14	1
19	Glutamatergic synapse	It represents a key therapeutic pathway in epilepsy [44].	58	43	3
20	Taste transduction	Impaired taste transduction results from altered calcium and ion influx through this pathway [45].	24	-	5
21	Circadian entrainment	Sleep disturbances are commonly observed in individuals with epilepsy [46].	82	59	7
22	Adrenergic signaling in cardiomyocytes	In chronic epilepsy, this pathway becomes activated, leading to myocardial damage [47].	71	49	8

23	Long-term potentiation	This pathway has therapeutic potential for epilepsy treatment [48].	36	56	12
24	MAPK signaling pathway	This pathway is important in both the onset and treatment of epilepsy [27].	41	32	13
25	Dopaminergic synapse	The primary function of GABA neurons and receptors is to regulate this pathway [49].	15	21	15
26	Oxytocin signaling pathway	Oxytocin exerts anticonvulsant and neuroprotective effects [50].	63	46	16
27	cGMP-PKG signaling pathway	It influences synaptic transmission and membrane excitability [51].	51	22	21

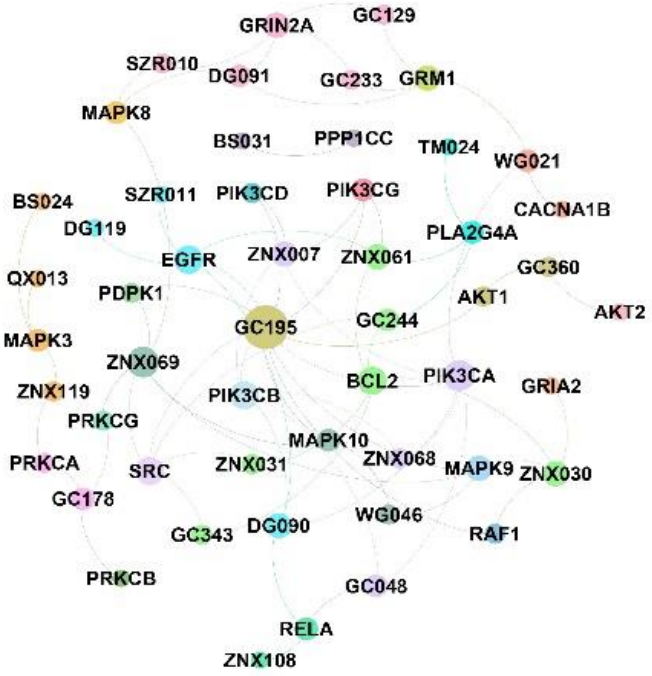
-: Does not exist in the corresponding pathways.

KEGG pathway enrichment analysis results

In this investigation, 27 essential pathways and their associated targets were compiled to establish a pathway-target network that identifies the critical targets of YNA for epilepsy treatment. **Figure 2a** illustrates a network composed of 242 nodes, representing 27 pathways and 215 targets, interconnected by 632 edges. **Table 2** and **Figure 2** highlight 25 core targets with a degree value surpassing 5, emphasizing their relevance in treating epilepsy. This suggests that YNA may act through various mechanisms to address epilepsy. A secondary network, linking the core targets to their respective compounds, was constructed to pinpoint the most relevant compounds, as shown in **Figure 2b**. **Table 3** presents 20 compounds with a degree value greater than 1, marking them as key candidates for treating epilepsy. Notably, Anethole has demonstrated anti-epileptic effects [52], while cis-isoeugenol [53] and methyleugenol [54] are known for their antioxidant properties, which contribute to neuroprotective actions in epilepsy treatment [55]. Methyleugenol is additionally recognized for its anti-epileptic and neuroprotective benefits [56], warranting further exploration into its therapeutic potential.



a)



b)

Figure 2. Network analysis: a) the key pathway-target network, and b) the core target-compound network.

Table 2. The core targets retrieved from the key pathway-target network.

No.	Gene Nane	Degree
1	MAPK3	21
2	PRKCA	19
3	PRKCG	16
4	PRKCB	16
5	AKT2	16
6	AKT1	16
7	RAF1	16
8	PIK3CD	13
9	PIK3CB	13
10	PIK3CA	13
11	MAPK9	10
12	MAPK8	10
13	MAPK10	10
14	CACNA1B	7
15	GRM1	7
16	RELA	7
17	PPP1CC	7
18	EGFR	6
19	GRIN2A	6

20	GRIA2	6
21	PLA2G4A	6
22	PDPK1	6
23	BCL2	6
24	PIK3CG	6
25	SRC	6

Table 3. The core targets attained from the key pathway-target network.

No.	Mol ID	Degree	Compound
1	GC195	11	2-Tetradecanone
2	ZNX069	6	1-Acetyl-beta-carboline
3	ZNX030	4	7,10-Octadecadienoic acid, methyl ester
4	ZNX061	4	Methyl pentadecanoate
5	DG090	4	cis-Isoeugenol
6	ZNX007	4	Trioxsalen
7	GC244	3	Methyl 12-methyltetradecanoate
8	WG021	3	2-Decanone
9	DG091	3	2-Methyl-5-decanone
10	GC048	3	Anethole
11	GC178	3	1-Pentadecanol
12	GC360	2	Tetrahydroharmine
13	GC343	2	Methyl linoleate
14	ZNX031	2	8,11,14-Docosatrienoic acid methyl ester
15	GC129	2	5,6,7,8-Tetrahydro-2,4-dimethylquinoline
16	GC233	2	5,6,7,8-Tetrahydro-4-methylquinoline
17	SZR010	2	O-Nornuciferine
18	WG046	2	4-Methylbenzoic acid anhydride
19	ZNX119	2	Methyl cis-11-eicosenoate
20	ZNX068	2	Methyleugenol

The 20 compounds were individually docked with the 25 core targets. A majority of the binding scores were below -6 kcal/mol, indicating strong interactions between the compounds and their corresponding core targets, suggesting they are promising candidates for further investigation. **Figure 3** presents the potential binding mechanisms of the two compounds, 2-tetradecanone and 1-acetyl-beta-carboline, with their top-scoring targets. Both compounds exhibited degree values above 4. 2-tetradecanone established hydrogen bonds with the amino acids Cys424 of RAF1, Gly219 of GRIA2, and Gly250 of GRIN2A, while 1-acetyl-beta-carboline formed hydrogen bonds with Gly250 and Ser242 of GRIN2A and GRIA2. Hydrophobic interactions accounted for the remaining binding forces. These results suggest that hydrophobic interactions play a dominant role in the binding of these active compounds to their core targets. The docking results are not only valuable for future experimental validation but also provide compelling evidence of the significant connections between the key compounds and the main targets.

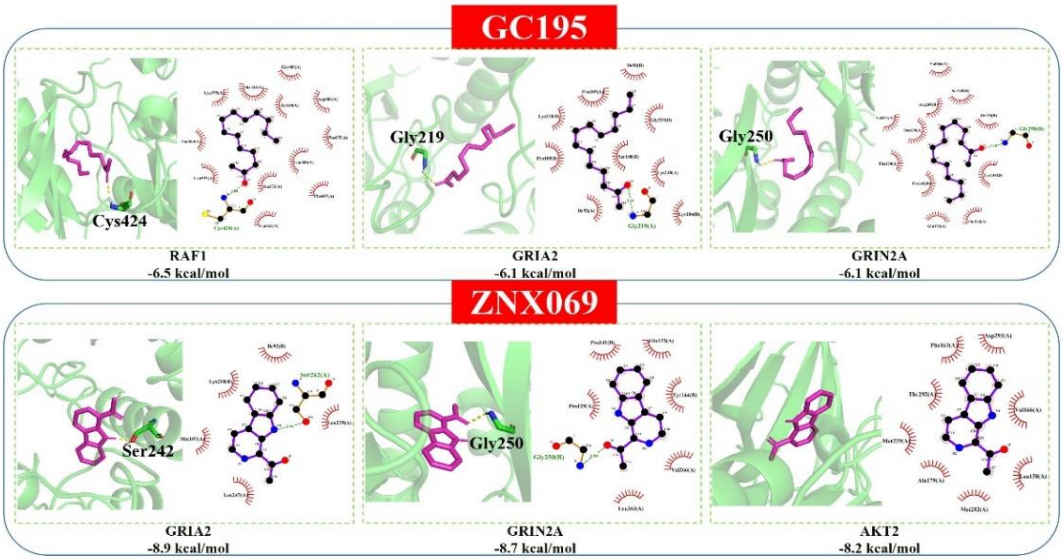


Figure 3. The predicted binding mode of GC195 and ZNX069 to their respective top three-scoring targets.

The KEGG pathway and network analysis have provided valuable insights into how YNA might exert its anti-epileptic effects. As shown in **Figure 4**, compounds like 2-Tetradecanone could target core proteins such as MAPK3 and PRKCA, subsequently activating important pathways, including the Calcium signaling pathway, cAMP signaling pathway, Serotonergic synapse, and GABAergic synapse. According to the KEGG pathway database [57], these effects may include the regulation of synaptic transmission, modulation of neuronal excitability, neuroprotection, and reduced excitability through hyperpolarization. The study employed network pharmacology, a method frequently utilized in traditional Chinese medicine research [58], to predict the active components of YNA and its mechanisms for treating epilepsy. Nonetheless, as the conclusions are based on computational predictions, experimental confirmation is necessary, which is a limitation of this work. In the future, we plan to conduct pharmacological experiments to further investigate the anti-epileptic actions of YNA, ultimately leading to more refined clinical applications.

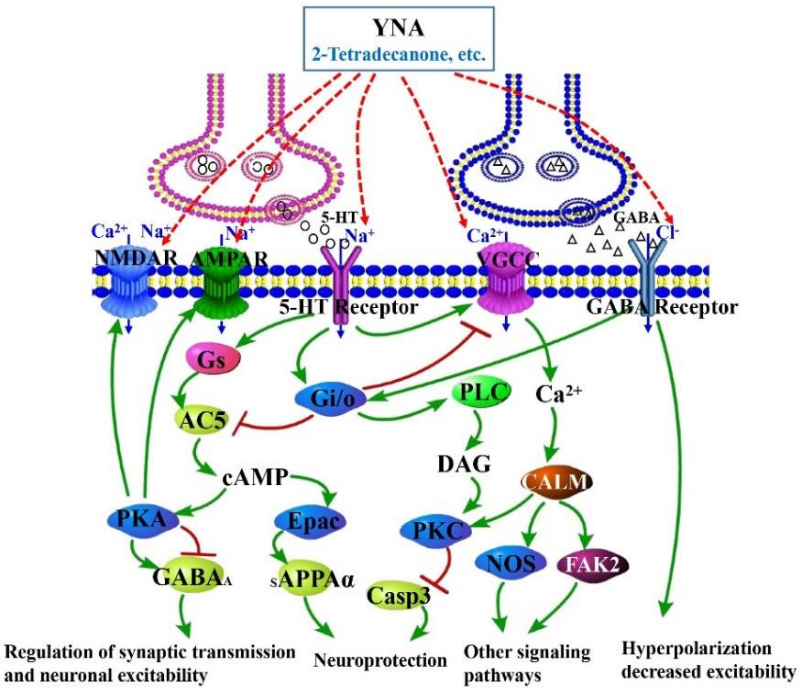


Figure 4. The potential mechanism of YNA in treating epilepsy.

Conclusion

This study utilized network pharmacology, incorporating both disease and anti-epileptic drug pathway data, to investigate the potential bioactive components and mechanisms of YNA for epilepsy treatment. Through molecular docking, significant pathways, targets, and compounds linked to YNA's therapeutic effects were identified. The analysis suggests that YNA may exert its anti-epileptic activity by modulating key pathways, including calcium signaling and cAMP signaling, through interaction with central targets like MAPK3 and PRKCA, and compounds such as GC195 and ZNX069. The findings provide a basis for future clinical studies and support the therapeutic potential of YNA. Moreover, the approach of integrating multiple pathway data offers new avenues for network pharmacology research.

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Conflict of Interest: None

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