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Network pharmacology analysis of "Ling Gui Zhu Gan Decoction" in the treatment of obesity-associated non-alcoholic fatty liver disease

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a prevalent liver condition closely associated with obesity, affecting nearly one billion adults worldwide. "Ling Gui Zhu Gan Decoction," a traditional Chinese medicine composed of Poria, Cinnamon Twig, *Atractylodes macrocephala* and Licorice, has demonstrated clinical efficacy in treating obesity-associated NAFLD. The active components and targets of "Ling Gui Zhu Gan Decoction" were identified using the TCMSP, PubChem and Swiss Target Prediction databases. Disease targets for NAFLD and obesity were retrieved from GeneCards. A protein-protein interaction (PPI) network was constructed, followed by functional enrichment analyses (GO and KEGG). Finally, a drug-disease-pathway network was established. The study identified 108 therapeutic targets for "Ling Gui Zhu Gan Decoction." Key components such as trametenolic acid and cerevisterol, along with targets like PTPN1, ESR1 and EGFR, were implicated in pathways including HIF-1, NF-κB and AMPK signaling. Molecular docking further confirmed that PTPN1 can stably bind with trametenolic acid and cerevisterol. These findings suggest that "Ling Gui Zhu Gan Decoction" exerts its effects through multiple targets and pathways. Network pharmacology elucidates the potential of "Ling Gui Zhu Gan Decoction" in treating obesity-associated NAFLD through multi-target and multi-pathway mechanisms, offering an alternative therapeutic option for this condition.

Keywords: Ling Gui Zhu Gan decoction, obesity, alcoholic liver disease, metabolism

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), or metabolic-associated fatty liver disease, is a leading cause of chronic liver disease and a widespread metabolic disorder globally (Younossi, Z. M *et al.,* 2023, Rong, L *et al.,* 2023). NAFLD ranges from simple fat accumulation to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer (Dufour, J. F *et al.,* 2021. J. F. Dufour *et al.,* 2022, Anstee, Q. M *et al.,* 2022). Obesity, marked by excess body fat, is a major global health issue and a key risk factor for NAFLD (Rinella, M. E *et al*., 2023, Portincasa, P and Baffy, G *et al.*, 2024). Currently, no approved drug treatments exist for NAFLD due to its complexity.

Traditional Chinese Medicine (TCM) is globally acknowledged as a complementary and alternative therapy (Zhou, Z *et al.,* 2022, Zhu, K *et al.,* 2021). Herbal medicines and their extracts have emerged as potential therapeutic agents for preventing and treating NAFLD T. (Yan *et al.,* 2020).Unlike chemical drugs and biologics, it is often challenging to pinpoint the specific bioactive components in TCM preparations guided by traditional theories. TCM prescribes remedies based on ancient experiential philosophy, such as Yin-Yang balance and the hierarchical relationships among herbs. TCM emphasizes a holistic view, treating organs like the viscera as an integrated system. Currently, a formula named "Ling Gui Zhu Gan Decoction" consisting of four herbs: Poria, Cinnamon Twig, Atractylodes Macrocephala and Licorice, has shown significant efficacy in treating obesity-associated NAFLD. However, the comprehensive pharmacological mechanisms underlying this formula's effects on obesity-associated NAFLD remain unclear.

Due to the complexity of the components and the diversity of action targets in TCM formulas, it is challenging to explore their pharmacological mechanisms using traditional methods. With the rapid advancement of computer technology and systems biology, network pharmacology has emerged as an interdisciplinary field with significant advantages in deciphering the multi-component, multi-target and multi-pathway mechanisms of TCM (Gong, W *et al.,*2021). This method aligns with the systematic and holistic characteristics of TCM. Through this approach, our study can provide a systematic and comprehensive view of the therapeutic effects of "Ling Gui Zhu Gan Decoction" on obesity-associated NAFLD. We aim to investigate the potential mechanisms by which "Ling Gui Zhu Gan Decoction" treats obesity-associated NAFLD.

MATERIALS AND METHODS

Ling Gui Zhu Gan Decoction standard decoction preparation

Take 12g, cassia bark 9g, 6g, 6g licorice in Ling Gui Zhu Gan Decoction formula, add water, fully wet and soak for 30 min, heat and boil for reflux extraction for 30 min, heat filter, then add water to extract for 20 min, filter, combine filtrate twice and concentrate to 500 mL. GC-MS treatment

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conditions: 2 mL of sample solution, added 450 μ L methanol-water (3: 1) and 10 μ L ribool, vortex for 30s, ground at 35 Hz for 4 min, sonicated at 0°C for 5 min and centrifuged at 15 000 r min-1 and 4°C for 15 min. Take 100 μ L of vacuum drying, mix 60 μ L of methoxyamine salt reagent for 30 min; add 80 μL BSTFA (containing 1% TMCS) at 70°C for 1.5 h and cool to room temperature.

Detecting of decoction

GC-MS column condition: DB-5MS (30 mm 250 mm 0.25 μm); carrier gas: high purity helium; flow rate: 1.0 mL min-1; gas inlet temperature: 280°C; column box heating procedure: starting temperature 50°C for 1 min, 10°C min-1 rate to 310°C for 8 min; intake: 1 μL; mass spectrometry interface temperature (transmission line temperature): 280°C; EI ion source temperature: 250°C; ionization voltage: -70 eV; mass scan range: m / z 50-500; scan rate: 12.5 spetra s-1; solvent delay: 6.25 min.

Drug target acquisition

We obtained the active ingredients and pharmacological mechanisms of Poria cocos, cinnamon twig, Atractylodes macrocephala and Licorice by querying the TCM systematology database. For herbal medicine to exert its clinical efficacy, it must be absorbed and metabolized. High OB (≥30%) is essential for determining a substance's drug-likeness (DL) index, used for rapid screening. Components with a DL index ≥0.18 are considered to have high drug sensitivity (Niu, B *et al.,* 2022). The initial screening for active components in "Ling Gui Zhu Gan Decoction" was based on OB ≥30% and DL ≥0.18.

Acquisition of disease targets

Using the keywords "nonalcoholic fatty liver disease" and "obesity," searches were conducted in the GeneCards database (https://www.genecards.org) to collect and organize the disease target results from each database. The top 1000 targets ranked by relevance score were selected as potential targets for the target diseases.

Construction of protein-protein interaction (ppi) network

To clarify the complex relationship between "Ling Gui Zhu Gan Decoction" and obesity-associated NAFLD, this study matched the drug's targets with NAFLD and obesity-related targets, identifying overlapping targets as key therapeutic ones. Drug, NAFLD and obesity targets were input into the InteractiVenn tool for intersection analysis and a Venn diagram was used to highlight key targets for treating both conditions with "Ling Gui Zhu Gan Decoction." These intersecting targets were submitted to the STRING platform (https://cn.string-db.org/), with species set to "Homo Sapiens" and a minimum interaction score of 0.4, to create a PPI network. The data were then analyzed in Cytoscape 3.9.1 to construct a key target PPI network based on degree values.

Functional enrichment analysis

Using the DAVID online analysis tool (https://david.ncifcrf.gov/summary.jsp), the key targets were analyzed for gene ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, with the species set to "Homo sapiens" and $P < 0.01$. The top 20 entries were selected for data visualization.

Construction of the drug-disease-pathway network

To clarify the interactions between "Ling Gui Zhu Gan Decoction" and its therapeutic effects on NAFLD and obesity, we selected the top 20 KEGG pathways most relevant to these conditions from the enrichment analysis. The active components and their associated targets within these pathways were then linked. This data was imported into Cytoscape 3.9.1 to build a "Ling Gui Zhu Gan Decoction Active Components-NAFLD and Obesity Targets-Pathways" interaction network. A topological analysis was conducted to rank the nodes by degree, identifying the main active components and key targets responsible for the therapeutic effects.

Molecular docking

Autodock Vina 1.2.2 (Eberhardt, J *et al.,* 2021) was used to evaluate the pharmacological mechanisms of drugs and their targets. The structures of drugs were queried through the PubChem database and the 3D coordinates of proteins were queried through the Protein Data Bank (PDB). The protein and ligand file formats were set to PDBQT, water was removed and polar hydrogen was added. The grid size was set to 30 Å × 30 Å × 30 Å and the spacing between each grid was set to 0.05 nm. Molecular docking and visualization were achieved using Autodock Vina 1.2.2.

RESULTS

Identification of bioactive components in "Ling Gui Zhu Gan Decoction"

The number of chemical components identified for each herb was as follows: Licorice, 280; Poria, 34; Cinnamon Twig, 220; Atractylodes Macrocephala, 55. In total, 589 chemical components were identified. Preliminary screening of blood-active components using ADME parameters (OB ≥ 30%, DL ≥ 0.18) resulted in the identification of 15 active components for Poria, 6 for Cinnamon Twig, 7 for Atractylodes Macrocephala and 92 for Licorice (Supplementary table 1). Next, the SMILE codes of potential active components were collected from the PubChem database. Components without SMILE codes were excluded. The Swiss Target Prediction database was then used to predict the targets of these active components. Components with no predicted targets were also excluded, resulting in 13 active components for Poria, 2 for Cinnamon Twig, 7 for *Atractylodes macrocephala* and 70 for Licorice. Target prediction using the Swiss Target Prediction database, with a "Probability > 0" threshold, identified a total of 793 unique active drug targets in Ling

Gui Zhu Gan Decoction" . Additionally, a keyword search in the GeneCards database yielded 1423 NAFLD targets (Score range: 0.93 to 116.67) and 9965 obesity targets (Score range: 0.10 to 90.49). The top 1000 targets ranked by relevance score were selected as potential targets for these diseases.

Construction of the Key Target PPI Network for "Ling Gui Zhu Gan Decoction" in Treating NAFLD and Obesity The intersection of targets from "Ling Gui Zhu Gan Decoction" components with obesity and NAFLD targets identified 108 key targets for treating these conditions (fig. 1A, Supplementary table 2). Drug-target protein interactions analyzed using the STRING platform, followed by topological analysis with Cytoscape 3.9.1. The nodes were ranked by degree, creating a PPI network with 108 nodes and 3048 edges, where nodes represent proteins and edges represent interactions. In the network, larger and darker purple nodes indicate higher degree values, signifying more interactions and greater importance (fig. 1B). STAT3, PPARG, ESR1, AKT1, IL6, TNF, PPARA and ALB were among the top-ranked genes, suggesting their crucial roles in the therapeutic effects of "Ling Gui Zhu Gan Decoction" on obesity-related NAFLD.

Functional and pathway enrichment analysis of key targets

To explore the functions and pathways of "Ling Gui Zhu Gan Decoction" in treating obesity-associated NAFLD, we performed GO and KEGG enrichment analyses on 108 key targets. GO analysis identified 350 significant terms, including hormone response, lipid metabolism, cell proliferation, hypoxia, aging and inflammation, with the top 20 terms shown in bubble charts (fig. 2A-C, Supplementary tables 3-5). KEGG analysis identified 114 significant pathways, with the top 20 displayed in fig. 2D. These pathways primarily involve HIF-1, NF-κB, insulin resistance, lipid metabolism, AMPK and FoxO signaling, suggesting that "Ling Gui Zhu Gan Decoction" acts through multiple targets and pathways to treat NAFLD.

Construction of the "Ling Gui Zhu Gan Decoction bioactive components-nafld and obesity targets-pathways" network

We constructed the "Ling Gui Zhu Gan Decoction Bioactive Components-NAFLD and Obesity Targets-Pathways" network using Cytoscape 3.9.1, based on the relationships between the components and targets involved in the top 20 pathways (fig. 3). This network consists of 178 nodes and 1047 edges, where nodes represent targets (green squares for components, blue circles for targets and purple inverted triangles for pathways) and edges represent interactions. Topological parameter analysis of the network was conducted using the Analyze Network tool in Cytoscape 3.9.1. Nodes were ranked by degree, with higher degree values indicated by larger and darker-colored nodes, signifying greater importance in the network. The analysis revealed that among the active components of "Ling Gui Zhu Gan Decoction", FL02 (Trametenolic acid), FL04 (Cerevisterol), FL05 (Stellasterol), FL07 (Eburicoic acid), GC10 (Phaseolinisoflavan), GC9 (1-Methoxyphaseollidin), GC47 (Glyasperin B) and GC52 (Glyasperin F) had high degree, betweenness and closeness centrality values. This suggests these components are likely the core components in Ling Gui Zhu Gan Decoction's treatment of NAFLD and obesity. Among the targets, Protein tyrosine phosphatase non-receptor body type 1 (PTPN1), estrogen receptor 1 (ESR1), epidermal growth factor receptor (EGFR), Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and mammalian target of rapamycin (MTOR) had relatively high degree values, indicating they are key targets through which "Ling Gui Zhu Gan Decoction" exerts its effects. These findings demonstrate that "Ling Gui Zhu Gan Decoction" acts through multiple components, targets and pathways to exert its pharmacological effects in treating NAFLD and obesity.

Molecular docking verification

Since PTPN1 was identified as the top target and FL02 (trametenolic acid) and FL04 (cerevisterol) were the compounds with the highest degree values, the binding affinity of drugs to PTPN1 was analyzed and the binding poses and interactions of FL02 and FL04 with PTPN1 were analyzed. calculating their binding energies (fig. 4A-B). Both compounds successfully occupied the hydrophobic pockets. For KDR, FL02 and FL04 showed low binding energies of -7.1 and -7.8 kcal/mol, respectively, indicating highly stable interactions.

DISCUSSION

NAFLD is the most common liver disease, affecting nearly one billion adults globally *(Pansa, C. C et al., 2023).* Its prevalence in Asia, at around 25%, is similar to that of Western countries *(Teng, M. L et al., 023).* Obesity is a major contributor, with approximately 80% of obese adults developing NAFLD *(Juanola, O et al., 2021, Cotter, T. G and Rinella, M et al., 2020).* Research has increasingly revealed NAFLD's pathogenesis, including liver lipid accumulation, which leads to insulin resistance and inflammation *(Pinter, M et al, 2023, Guo, X et al, 2022).* These processes are key therapeutic targets for NAFLD. However, despite advances in understanding its mechanisms, no approved treatments currently exist for NAFLD (Filali-Mouncef, Y *et al.,* 2022), highlighting the need for effective therapies.

Traditional Chinese Medicine (TCM) has shown promise in treating metabolic diseases like diabetes and NAFLD. "Ling Gui Zhu Gan Decoction," primarily composed of Poria, Cinnamon Twig, Atractylodes Macrocephala and Licorice, has been clinically effective in treating obesity-related NAFLD.

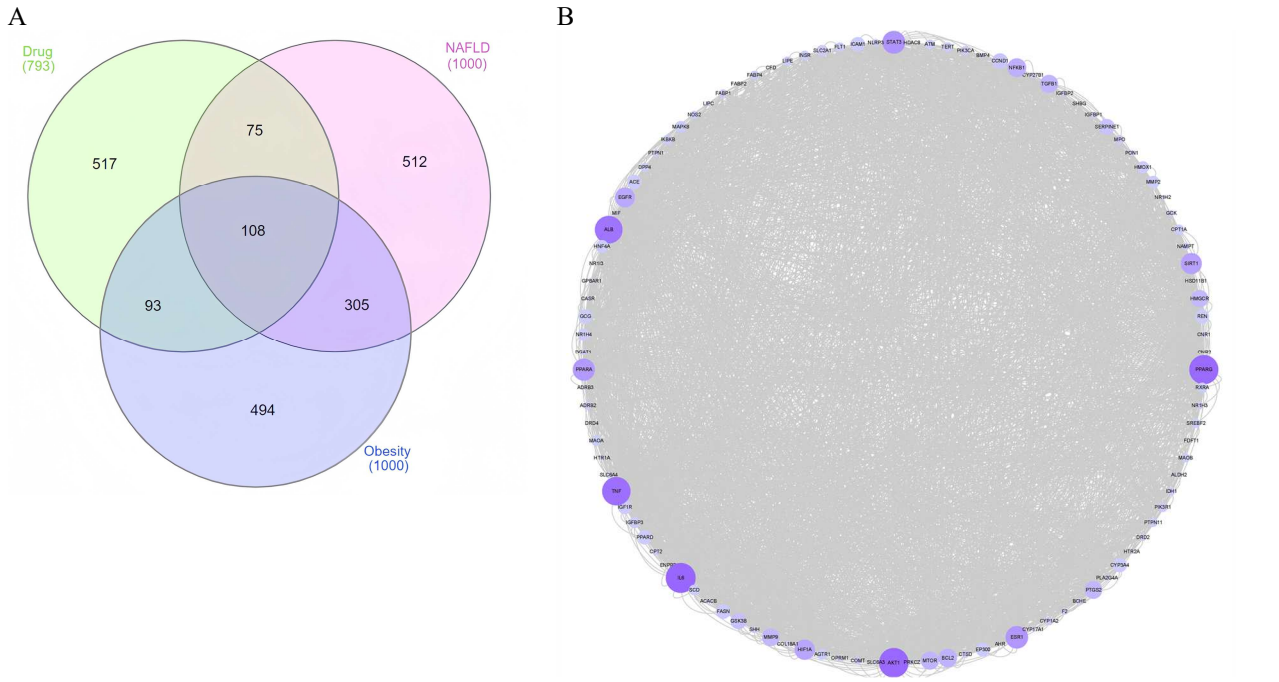

Fig. 1: Targets (A) and PPI Network (B) of "Ling Gui Zhu Gan Decoction" in Treating Obesity-Associated NAFLD.

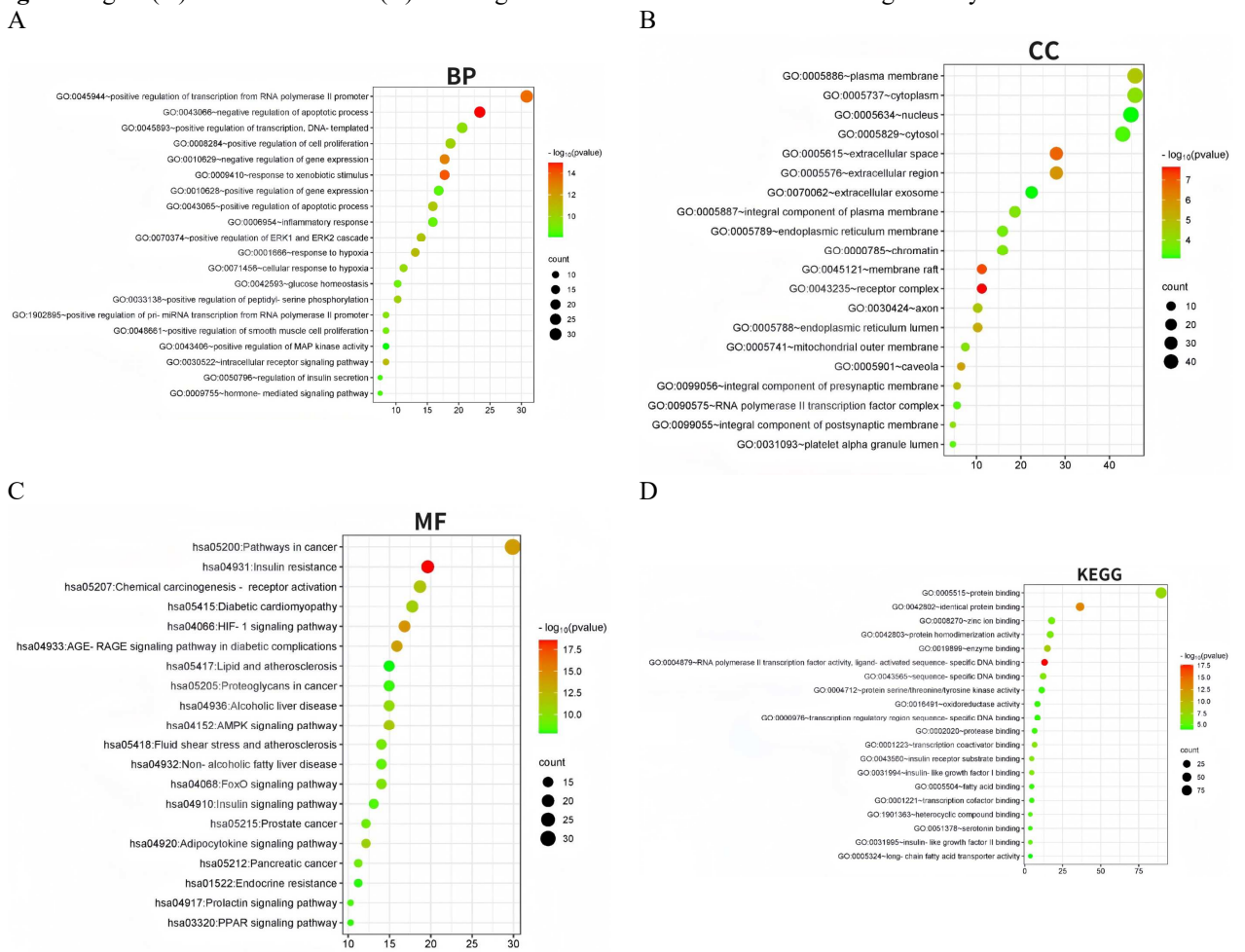

Fig. 2: GO (A-C) and KEGG (B) Enrichment Analysis of Key Targets for "Ling Gui Zhu Gan Decoction" in Treating Obesity-Associated NAFLD.

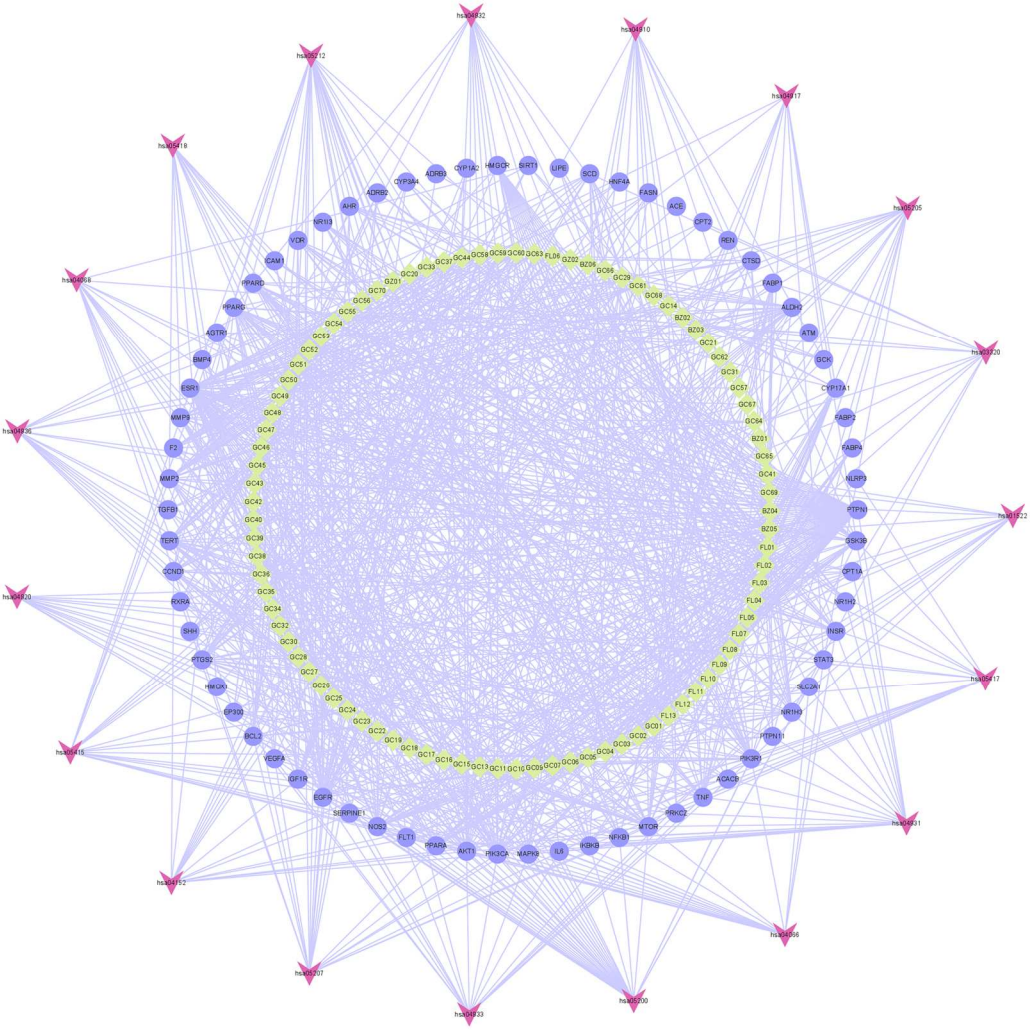

Fig. 3: Construction of the "Ling Gui Zhu Gan Decoction Bioactive Components-NAFLD and Obesity Targets-Pathways" Network. This network consists of 178 nodes and 1047 edges, where nodes represent targets (green squares for components, blue circles for targets, and purple inverted triangles for pathways), and edges represent interactions.

A B

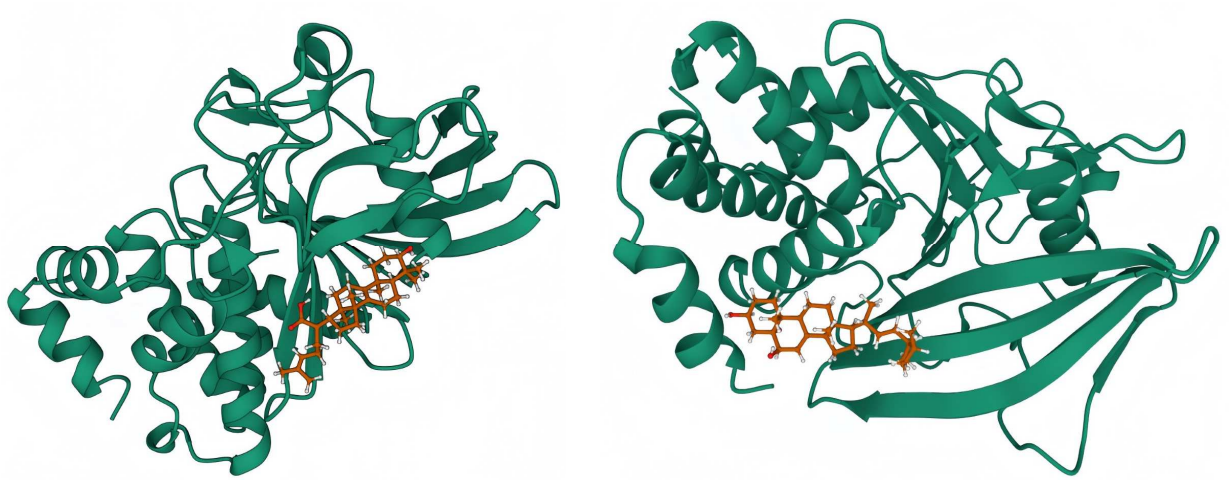

Fig. 4: Molecular docking analysis of candidate drugs FL02 (trametenolic acid) (A) and FL04 (cerevisterol) (B) with protein target PTPN1.

This study found that "Ling Gui Zhu Gan Decoction" may exert therapeutic effects on obesity-associated NAFLD through various components, such as FL02 (Trametenolic acid), FL04 (Cerevisterol), FL05 (Stellasterol), FL07 (Eburicoic acid), GC10 (Phaseolinisoflavan), GC9 (1-Methoxyphaseollidin), GC47 (Glyasperin B) and GC52 (Glyasperin F). These components target key proteins like PTPN1, ESR1, EGFR, PIK3CA and MTOR, influencing the HIF-1, NF-κB, AMPK and FoxO pathways, reflecting a multi-component, multi-target and multi-pathway treatment approach. GO enrichment analysis revealed that the therapeutic effects are closely related to hormone response and inflammation. NAFLD is more prevalent in men than women, potentially due to sex hormone levels and receptor expression (Balakrishnan, M *et al.,* 2021). Research shows that higher testosterone reduces NAFLD risk in men but increases it in women, while higher sex hormone-binding globulin levels reduce risk in both genders (Pafili, K and Roden, M *et al.,* 2021, Zhang*, X et al.,* 2022). The PPI results highlight ESR1 as a key estrogen-related target for treating NAFLD, suggesting that hormone response plays a critical role in "Ling Gui Zhu Gan Decoction's" effectiveness. NAFLD, being a chronic metabolic liver disease, is linked to chronic inflammation. Pathways like JNK-AP-1 and IKK-NF-κB are involved in NAFLD progression (Shi, S *et al.,* 2023). KEGG analysis also showed that key targets are enriched in the HIF-1 (Zhang, Y *et al.,* 2020). *NF-κB* (Xu, M *et al.,* 2023) and AMPK (Johanns, M *et al*., 2022) signaling pathways, indicating that the decoction may also work by regulating inflammatory responses.

The bioactive components-targets-pathways network indicates that trametenolic acid (FL02), cerevisterol (FL04) and ergosta-7 (FL05) are key components in the network. Among them. Studies have shown that TA can improve the progression of diabetic nephropathy in mice (Chen, J *et al.*, 2022, Duan, Q *et al.,* 2022). Additionally, TA has demonstrated effective anti-cancer and anti-inflammatory activities in human prostate and breast cancers . Cerevisterol and ergosta-7 are found in various traditional Chinese medicine components and exhibit anti-inflammatory and antioxidant physiological effects (Alam, M. B *et al.,* 2020, Huang, J *et al.,* 2023, Huang, Y. P *et al.,* 2021, Hsieh, WT *et al.,* 2021). Furthermore, PTPN1, EGFR, PIK3CA and MTOR are core targets in the network. Research has shown that pharmacological inhibition of EGFR can suppress non-alcoholic fatty liver disease (Hardesty, JE *et al.,* 2021). Additionally, a reduction in lncRNA NEAT1 alleviates NAFLD through the mTOR/S6K1 signaling pathway (Fan, L *et al.,* 2022). Although there is no direct report on the effect of PTPN1 on NAFLD, it is considered a potential target for diabetes and obesity treatment. PTPN1 dephosphorylates activated STAT3 and JAK2 (Chen, CX *et al.,* 2023, Ito, Y *et al.,* 2022). A study on a rat NAFLD model found that IL-22 significantly reduces hepatic lipid droplets and triglyceride accumulation by activating the JAK1/STAT3 signaling pathway and inhibiting the apoptosis factor BAX (Song, Q *et al.,* 2023), suggesting that PTPN1 and STAT3 are potential targets for alleviating NAFLD.

This study also has some limitations. First, network pharmacology approaches have their inherent limitation of the inability to predict up-or down regulation of targets, which hinders an accurate understanding of the mechanisms by which chemical components act on disease targets. Secondly, the traditional decoction can cause the evaporation of the volatile components at high temperatures, which is an inherent defect of the thermal extraction method. However, the thermal extraction method has higher extraction efficiency for non-volatile components such as alkaloids, flavonoids and polysaccharides, which may be the main material basis of compound efficacy. In the future, the volatile components can be extracted separately by supplementing other methods to fully cover the active substances in the compound. Third, due to the selection criteria, we only analyzed the main compounds in "Linggui chocolate soup", which may have limited the results to some extent. Finally, although network pharmacology can identify numerous targets and pathways, these findings still need to be validated through basic research and clinical trials, which will be the focus of our future research.

CONCLUSION

Our results suggest that "Ling Gui Zhu Gan Decoction" acts through multiple targets and pathways. Network pharmacology has clarified the potential of treating obesity-related NAFLD through multi-target and multipathway mechanisms, providing an alternative treatment option.

Conflict of interest

This study does not involve any conflicts of interest.

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