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Danning tablet alleviates cholecystitis caused by gallstone through enhancing bile secretion and rinsing

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Abstract: Danning tablets are widely used as a conservative treatment for gallstones in China. However, the underlying pharmacological mechanisms remain to be elucidated. We performed network pharmacology analyses. The intersection between the therapeutic target of Danning tablet and the gallstone target was taken. Adult male C57BL/6J mice were fed with the lithogenic diet only or LD supplemented with Danning tablet for 8 weeks. The gallbladder was stained with HE to assess inflammation, the liver was tested at RNA and protein levels, and bile duct cannulation was used to assess bile secretion function. All these experiments revealed that Danning tablets improved gallbladder inflammation in both humans and mice. Active ingredients such as luteolin in Danning tablet can upregulate the expression of AQP7 by binding to transcription factors such as ESR1, promote the liver to secrete more bile for gallbladder rinsing and relieving cholecystitis. Therefore, we believe that the use of Danning tablets is beneficial for patients with gallstone disease accompanied by inflammation.

Keywords: Gallstone; danning tablets; gallbladder; aquaporin

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INTRODUCTION

Gallstone is a common clinical condition. In Europe, about 20% of the adult population has gallstones, approximately 90% of which are cholesterol stones(Shabanzadeh, 2018). Supersaturation of cholesterol in bile is considered to be a prerequisite for cholesterol gallstone formation. The ratio of cholesterol, bile acids and phospholipids determines the saturation of cholesterol in the bile. These three compositions are secreted into the bile from liver and absorbed in the intestine then back to the liver again, which forms the enterohepatic circulation (Lammert *et al*., 2016). Cholecystitis is common in patients with gallstones. More than 90% acute cholecystitis are due to gallstones or biliary sludge blocking the cystic duct through which bile flows in and out of the gallbladder (Indar and Beckingham, 2002). When the duct is obstructed, bile builds up, causing irritation and pressure in the gallbladder. Sometimes, the bile duct is temporarily blocked. When this happens repeatedly, it can lead to long-term (chronic) cholecystitis. This is sustained swelling and irritation over time. Eventually, the gallbladder becomes thick and hard and loses its ability to store and release bile(Walter, 2022). Some scholars believe that the inflammation is caused by lithogenic bile rather than stone friction or outflow tract obstruction; changes in bile composition or diluted bile may alleviate gallbladder inflammation (Behar *et al*., 2013).

Danning tablet is a traditional Chinese medicine formula, broadly applied in treating hepatobiliary diseases. It consists of seven kinds of herbs, including *Rhei Radix Rhizoma*, *Polygoni Cuspidati Rhizoma ET RADIX*, *Pericarpium Citri Reticulatae, Pericarpium Citri Reticulatae Viride, Radix Curcumae, Fructus Crataegi* and *Rhizoma Imperatae* (Ding *et al*., 2012). The combination of these drugs proved to be effective for NAFLD and cholelithiasis (Ma *et al*., 2021). Traditional Chinese medicine theory, although effective, is too abstract to be understood by people without a Chinese medicine context. With the help of UPLC/MS technology, Zhan *et al*., tried to quantitatively describe the active ingredients of Danning tablets, so as to make the mechanism of Danning tablet more clear (Zhan *et al*., 2016). According to their results, total of 32 constituents in danning tablet were identified based on their physical and chemical characteristics (fig. 1A). *Rhei Radix Rhizoma* and *Polygoni Cuspidati Rhizoma Radix* are thought to be the major drugs responsible for the therapeutic effects of this formula (Zhan *et al*., 2016). The protective effect of Danning tablets against NAFLD is considered by down-regulating SREBP, a key transcription factor in fat synthesis (Ma *et al*., 2021). Danning tablets have also been used in the treatment of cholestasis through signaling pathway of FXR-regulated bile acid and inflammation (Yang *et al*., 2016).

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Rhein, one of the main active ingredients in Danning tablets, is widely used in inflammation treatment in China. Rats pre-treated with rhein attenuated serum TNF-α and IL-6 levels effectively, inhibited the release of inflammatory factors. This effect may be related to the regulation of JAK2/STAT3 signal pathway (Yang *et al*., 2022).

At present, a large number of Chinese cholelithiasis patients choose Danning tablets as conservative therapy or postoperative medication. However, there is a lack of convincing evidence to explain the mechanism by which Danning tablets benefit patients with gallstone. This study combined clinical data with animal experiments and network pharmacological analysis to explore the mechanism of Danning tablets in the treatment of cholecystitis with gallstone, suggesting the clinical value of Danning tablets.

MATERIALS AND METHODS

Clinical data

We collected the information of patients with gallstone and cholecystitis in the past month, of which 3 patients were not willing to operate and were treated conservatively with Danning tablets. All the three patients were male with an average age of 58 years. The duration of gallstone disease was more than three years. After 7 to 28 days of treatment, pre-and post-treatment ultrasound images were collected, and specialist sonographers were invited to measure gallbladder mucosal thickness.

Animals and diets

SPF eight-week-old male C57BL/6 mice were purchased from Nanjing GemPharmatech Co. Ltd. Danning tablets are gained from manufacturer's donation. The lithogenic diet contained 0.5% cholesterol and 0.25% cholic acid. LD group fed with lithogenic diet, LDNT group fed with lithogenic diet plus 3.6g/kg Danning tablets, and the HDNT group with 10.8g/kg. Animal feed is processed by Trophic Animal Feed High-Tech Co, Ltd, China. Water and food was available ad libitum. The weights of the mice were recorded every week within 8 weeks. Mice were fasted for 12h before sacrificed. Blood, liver and gallbladder were collected, and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Another 18 mice were divided into 3 group feed with LD diet and HDNTdiet respectively. After anesthesia, fresh bile secreted from liver was collected twice for 30 minutes using UT-03 tubes (Unique Medical Co. Ltd, Japan). All experimental protocols were approved by our institutional Animal Care and Use Committee.

Total RNA extraction and determination of gene expression

Total RNA was extracted from liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA concentration was adjusted to 1ug in 20ul system. Single-stranded cDNA was synthesized using PrimeScript™ RT reagent kits with gDNA Eraser (Takara Bio). Real-time PCR preceded using SYBR® Premix Ex Taq™ II (Takara Bio) and a ABI QuantStudio6 Q6 (Thermo Fisher Scientific Inc., USA) with the sequences obtained from Beyotime biotech. Gapdh was used as reference gene, and the amount of target mRNA was calculated by ΔΔCt method.

Western Blot

The frozen proximal small intestine with 500ul RIPA (P0013B, Beyotime Biotechnology, China) and 1mM PMSF was broken up by homogenizer. After centrifuged (13 000g, 15 minutes), took the supernatant and denatured (100 °C, 5 minutes) with SDS-PAGE Sample Loading Buffer (P0015L, Beyotime Biotechnology, China). Samples applied to electrophoresis with 4ug/uL, then proteins were electro-transferred onto PVDF membranes. Subsequent experimental protocol followed the instructions for primary (25131-1-AP, 20536-1-AP, Proteintech, China) or secondary antibodies (YFSA02, YIFEIXUE Biotechnology, China).

Lipids analysis

Commercial purchased kits (E1015, Applygen, China) were used to determined cholesterol levels. The bile was diluted to a suitable concentration and measured according to the commercial kits instructions (bile acids: BI7710, Zhicheng Biological Technology, China; phospholipid: EFR0178, FUJIFILM, Japan). The cholesterol saturation index (CSI) was calculated using the Carey table.

Network pharmacology and molecular docking

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) and HERB database were used to identify the active drug components. Oral bioavailability (OB) of at least 30 % and a drug-likeness (DL) of at least 0.18 are defined as the active ingredient inclusion criteria. The UniProt database is used to translate target names to gene names. Genecard database was used to search for therapeutic targets for gallstone. The PPI network was optimized using Cytoscape 3.9.1. The docking procedure was performed with AutoDock 4.2.6 program. Lamarckian Genetic Algorithm was applied for doing the conformation search. The 3D structure of ESR1 protein was gathered from RCSB Protein Data Bank, and the structures of ligands from PubChem database. Results are visualized by the PyMOL program.

Ethical approval

The use of animals in the study was approved by Tongji University Animal Care and Use Committee with No. TJLAC-0342-025.

The acquisition of clinical data was an observational study, and no ethical approval was involved

STATISTICAL ANALYSIS

All data are expressed as means ± standard error of the mean (SEM). GraphPad Prism 8.0 were used for data output and plotting. Data were compared between groups using Student's t-test. All the statistics were performed using SPSS 23.0. $P < 0.05$ indicated a statistically significant difference.

RESULTS

Danning tablet reduces the thickness of gallbladder mucosa

We collected ultrasound images of patients with gallbladder polyps or gallstones before and after treatment with Danning tablets for 7-28 days. The thickness of gallbladder mucosa was measured by professional sonographers. We found that Danning tablets significantly reduced the thickness of the gallbladder mucosa which indirectly reflected the degree of gallbladder inflammation (fig. 1B). Medical treatment prior to surgery reduced the mean thickness of the gallbladder wall by 1.3mm (fig. 1C). To investigate how Danning tablets alleviate gallbladder inflammation, we conducted animal experiments to replicate this clinical finding. There was no significant difference in body weight among the three groups of mice on the lithogenic diet (LD), lithogenic diet plus low dose of Danning tablets (LDNT), and lithogenic diet plus high dose of Danning tablets (HDNT) during the 8-week period (fig. 1D). This proved that the toxicity of Danning tablets was within the safe range. After 8 weeks of feeding, all the mice in the three groups developed gallstones, and Danning tablets had no obvious preventive effect on gallstones (fig. 1E). The opacity of all the gallbladder walls are poor with macroscopic observation. HE staining showed that the gallbladder thickening was more obvious in the LD group; the mucosal layer proliferated and protruded into the gallbladder cavity. The hyperplastic epithelium is not neatly arranged, suggesting a tendency to form gallbladder Rokitansky-Aschoff sinus and adenomyosis (fig. 1F).

Danning tablets exert effects through core targets such as ESR1

We combined the active ingredients retrieved from the TCMSP database of Danning tablets with the results of HPLC analysis. The obtained names of 7 compounds in both Chinese and English and their corresponding peak serial numbers in fig 1 are shown in Table 1. We obtained 192 target genes for Danning tablet from the TCMSP database and HERB database, and 264 gallstone related genes from Genecard database (fig. 2A). We selected the 12 intersection genes of the two groups as the gene set for subsequent analysis. Cytoscape software was used to draw the network diagram of 7 active compounds and 12 core genes. The network is composed of 19 nodes and 30 edges, indicating that the active ingredients in Daining tablets (inner circle) treat gallstone related diseases through core genes (outer circle). The edges connecting each node represent the interaction between the active component and the gene (fig. 2B). After further analysis of the 12 core gallstone-treatment genes using STRING database, we draw the protein-protein interaction (PPI) network model by Cytoscape. The PPI network will present the more important nodes in the interaction in a darker color and larger circle according to the algorithm of the plug-in centiscape2.2 (fig. 2C). From the fig, we can clearly see that ESR1 (Estrogen Receptor 1), etc. play an important role in the therapeutic effect of Daning tablets.

Four active substances (Aloe-emodin, hesperetin, luteolin, rhein) from Danning tablet that could bind to ESR1 were used for computer simulation molecular docking. The result suggested the interaction between four active substances and ESR1. The maximum predicted binding energy of ESR1 with Aloe-emodin, hesperetin, luteolin and rhein were -3.53, -3.23, -3.54 and -3.31 kcal/mol, respectively. Moreover, the amino acid residues of ESR1 protein can form more than one hydrogen bond with these four substances (fig. 2D).

The relief of gallbladder inflammation is not related to cholesterol excretion

To investigate how the Danning tablet reducing the thickness of gallbladder mucosa, the composition of gallbladder bile in mice treated with Danning tablets was determined. There was no significant difference in the cholesterol and bile acid contents of gallbladder bile among the three groups. Daning tablets slightly increased the phospholipid content, but the difference was not statistically significant (fig. 3A, B, C). The cholesterol saturation index, calculated from the Carey table, was slightly decreased in Danning tablet-treated mice, but there was also no statistical difference (fig. 3D). This suggests that Danning tablet does not reduce the inflammation of the gallbladder by changing CSI. We chose IL-6 as a marker of the degree of inflammation and examined the levels of IL-6 in the homogenized gallbladder and serum. We found that Danning tablets significantly reduced IL-6 levels, reduced inflammation in gallbladder and serum, and exhibited a dose effect (fig. 3E, F). This result prove that Danning tablets indeed decreased the level of inflammation and further reduced the thickness of the gallbladder wall.

The addition of aquaporin by Danning tablets increased the flushing effect of bile

Based on a review of the literature, we examined the mRNA expression levels of liver aquaporins. fig. 4A illustrates the RNA expression of aquaporin isoforms with CT values >30. We found that AQP1 expression was increased successively in LD group, LDNT group and HDNT group, but there was no statistical difference. Danning tablets significantly increased AQP6/7 expression and exhibited a dose effect.

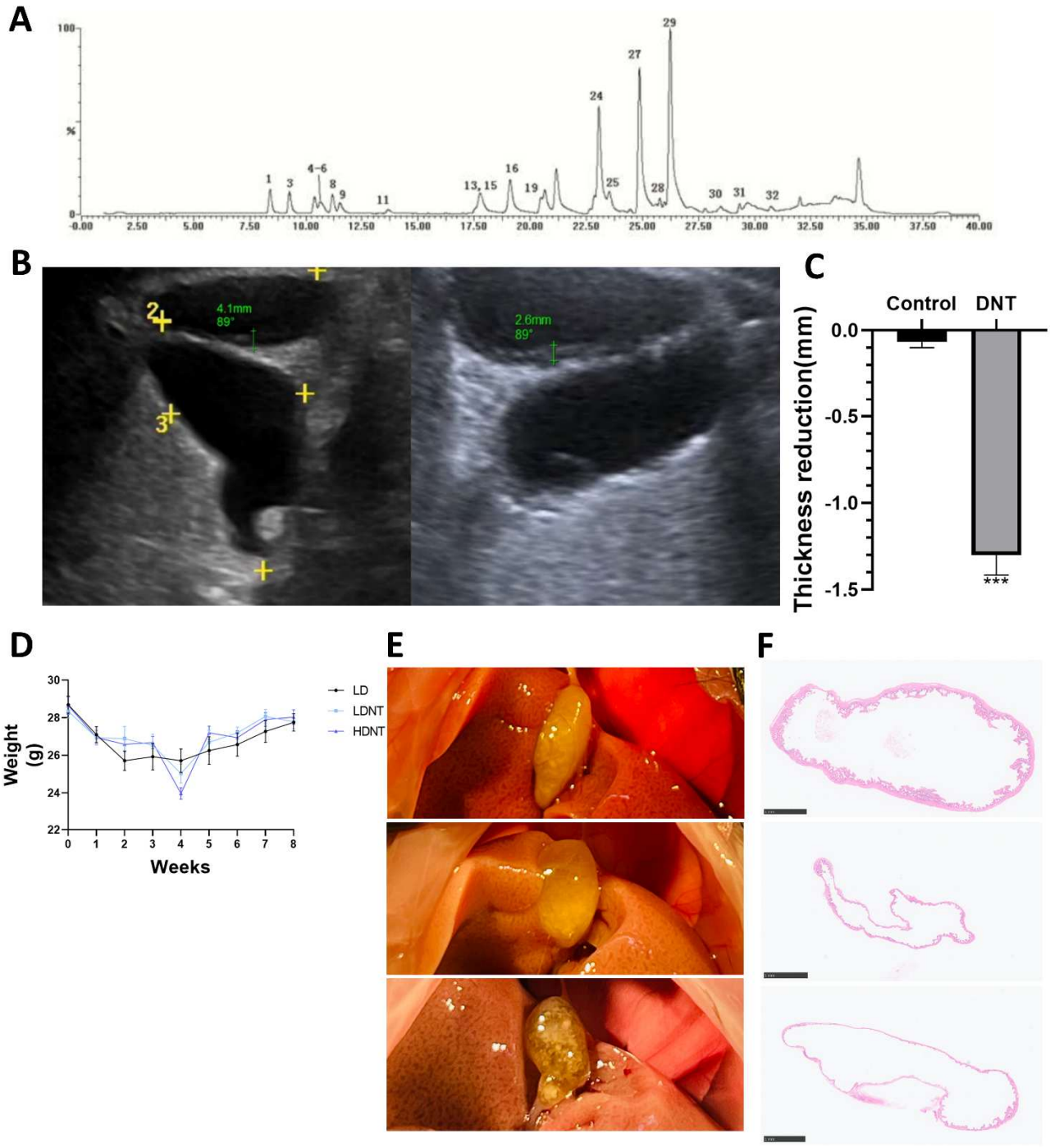

Fig. 1: Danning tablets reduced the thickness of gallbladder mucosa in both human and mice, but had no effect on gallstone formation. (A) UPLC-MS chromatograms of Danning Tablets sample at positive ion mode, quoted from PMID: 27187345. (B) Ultrasound image of the gallbladder of the patient before (left) and after (right) taking Danning tablets. (C) The reduction value of gallbladder mucosal thickness in patients taking Danning tablets for 7-28 days versus patients without medication. (D) Changes in the body weights of the mice. (E) Gallbladder appearance of LD, LDNT, HDNT (From up to down). (F) Three groups gallbladders with H&E staining (up: LD, middle: LDT, down: HDNP). Data are expressed as mean ± SEM (n =10 each group in animal experiment and n=3 in clinical data).

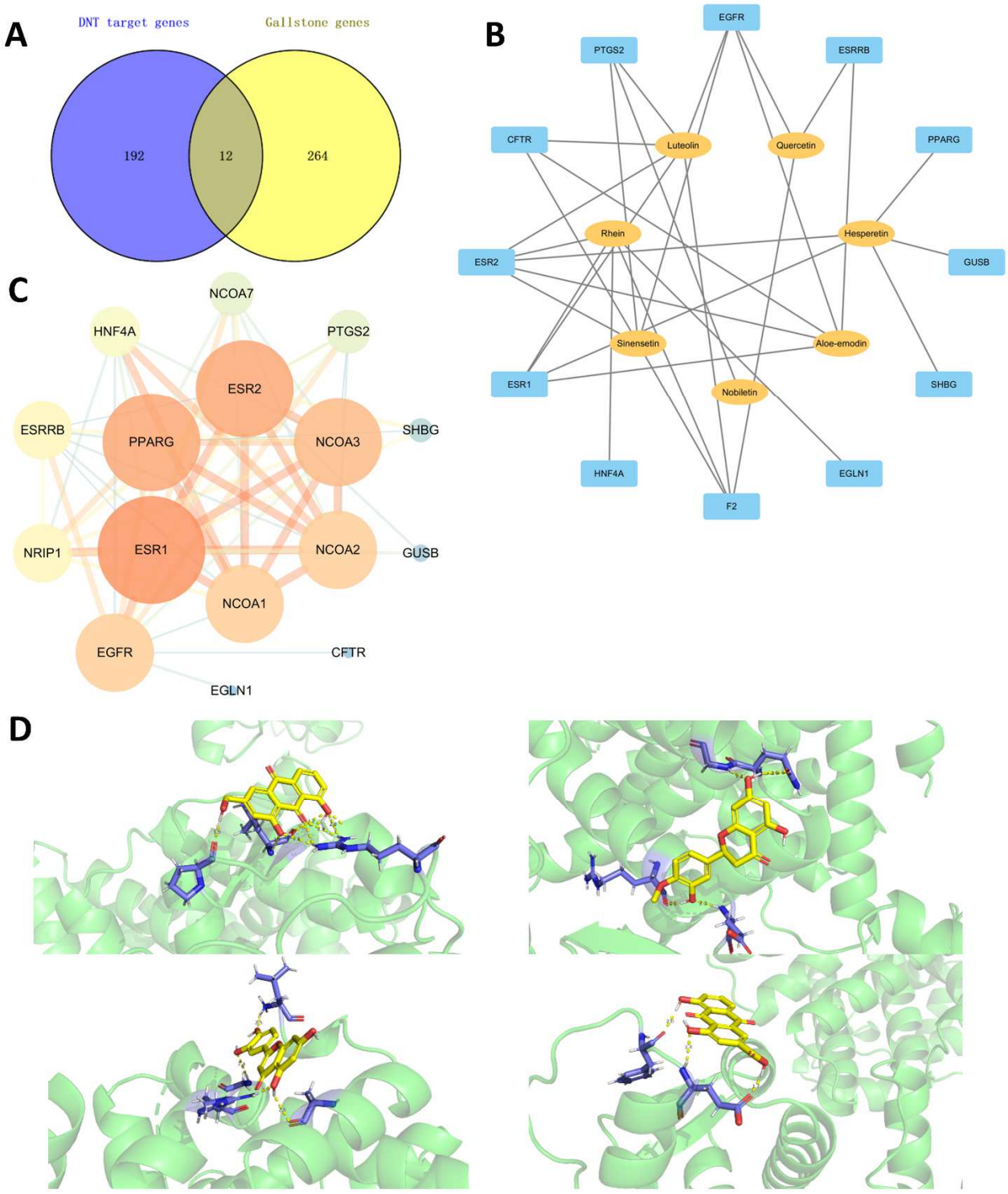

Fig. 2: Network pharmacology and molecular docking revealed the underlying mechanism of Danning tablets in the treatment of cholecystitis. (A) Venn diagram of the intersection of target genes of Danning tablets and cholelithiasis related genes. (B) PPI network of predicted targets of Danning tablet against gallstone. (C) The interaction of key protein in PPI network, node size and color represent the importance of the protein. (D) Predicted 3D structures of ESR1 protein in interaction with Aloe-emodin (left up), hesperetin (right up), luteolin (left down) and rhein (right down).

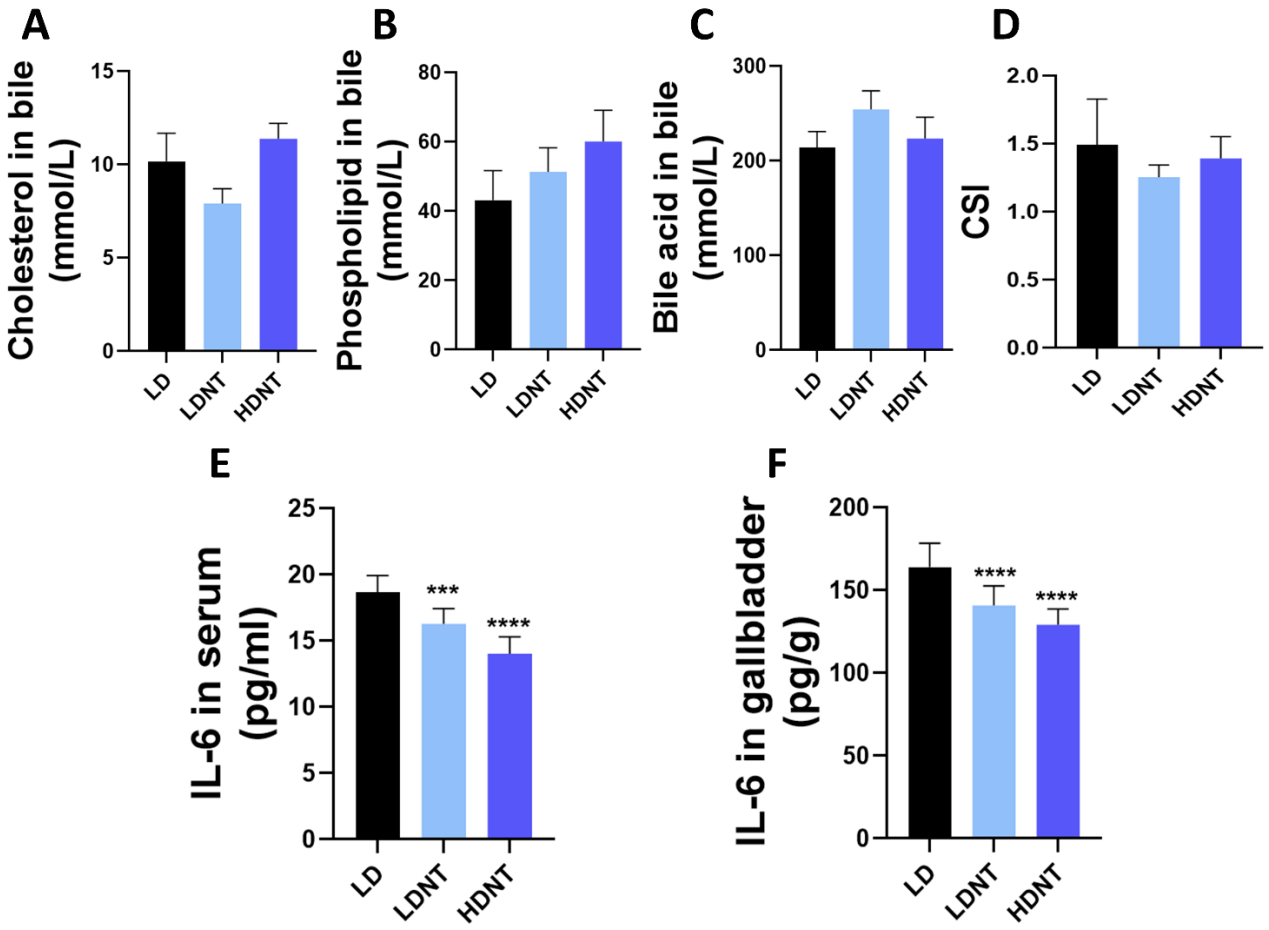

Fig. 3: Effects of Danning tablets on bile composition and gallbladder inflammatory factors (A)–(C) Biliary cholesterol, phospholipid and bile acid levels, respectively. (D) Cholesterol Saturation Index (CSI) of each group. (E), (F) Serum and gallbladder IL6 level of three group mice. Data are expressed as mean ± SEM (n =10 each group)

The expression of AQP8/9 was disordered. Low dose of Danning tablet seemed to decrease the expression of AQP8/9, but the expression increased after high dose of Danning tablet treating. Based on literature reports, AQP7 was selected for the determination of protein level. Consistent with the mRNA expression, the expression of AQP7 protein was higher in the HDNT group (fig. 4B). Subsequently, we measured the amount of bile produced from liver as functional level detection. There was no significant difference in bile secretion in mice treated with Danning tablets for one week. However, after 4 weeks of feeding with Danning tablets, the amount of hepatic bile secretion of DNT group was almost triple higher than that LD group. This choleretic effect was more pronounced at 8 weeks of feeding (fig. 4C).

DISCUSSION

This study is the first to investigate the mechanism by which Danning tablets alleviate cholecystitis induced by gallstones. Notably, our findings reveal that aquaporins play a significant role in the therapeutic effects of Danning tablets. The administration of Danning tablets stimulates the liver to produce a greater volume of dilute bile, which facilitates the flushing of the biliary system and the removal of proinflammatory substances, such as supersaturated cholesterol and bile acids.(Behar *et al*., 2013). With the reduction of inflammatory substances, the edema and congestion of gallbladder mucosa were relieved to a certain extent, and the pain of patients was relieved. On the other hand, the reduced thickness of the gallbladder wall greatly reduces both the difficulty of cholecystectomy and the probability of conversion from laparoscopy to open cholecystectomy (Balbaloglu and Tasdoven, 2023, Kokoroskos *et al*., 2020). Thanks to advancements in ultra-high performance liquid chromatography technology, we were able to comprehensively characterize the active components of Danning tablets (Wang *et al*., 2025). Our results suggested Danning tablet to be promising drug on non-obstructive gallstones and cholecystitis.

Previous therapeutic compounds for cholelithiasis generally focused on decreasing intestinal cholesterol absorption such as ezetimibe, phytosterols, diosgenin, etc

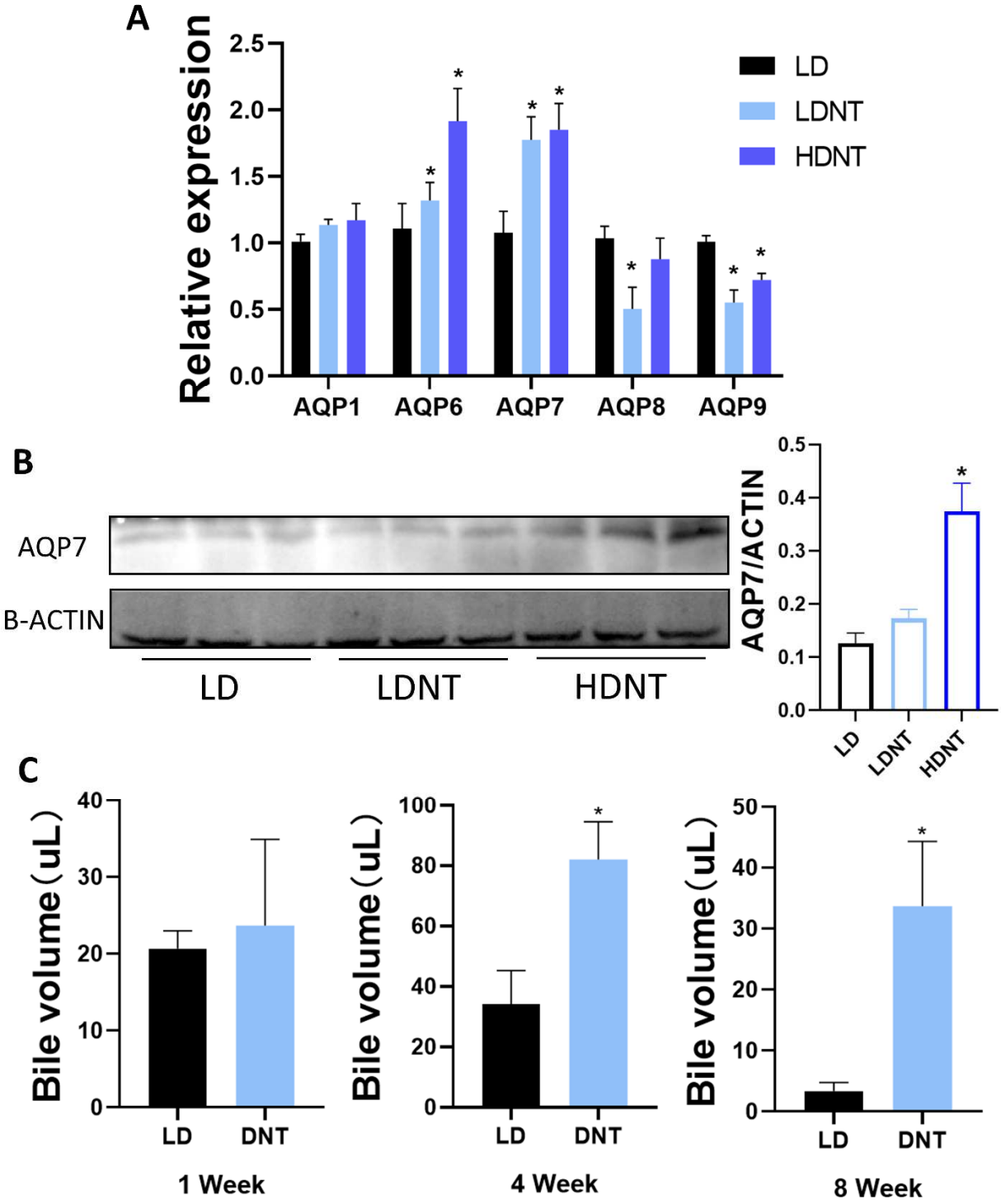

Fig. 4: Danning tablet can enhance expression of liver AQP7 and promote bile secretion and rinsing. (A) mRNA expression of genes involved in hepatic aquaporins. (B) Protein level of AQP7 in the mice liver. The histogram shows grayscale values ratio of AQP7/ACTIN. (C) Hepatic bile secretion at 30 min in LD and LDNT groups. Data are expressed as mean ± SEM (n =10 each group in qPCR experiment and n=3 in WB and function experiment).

(Shen *et al*., 2023, Shen *et al*., 2021, Wang *et al*., 2008). Other drugs target bile acid metabolism in the gut and liver such as ursodeoxycholic acid(Guarino *et al*., 2013). Researchers are also developing more convenient and natural drugs for gallstone treatment, such as vinegar-soaked Ficus carica fruit (Bukhari *et al*., 2024). The latest research found that hepatic water channel plays a key role in bile dilution and gallstone formation, modulation of hepatic water transport may provide a universal therapeutic strategy for all types of gallstone diseases (Huo *et al*., 2024). The present study also confirmed the above views, Danning tablets promoted the liver to secrete more bile to wash the gallbladder, and reduced gallbladder inflammation and gallbladder wall thickness. The liver expresses a variety of aquaporin isoforms，AQP1, AQP3, AQP7, AQP8, and AQP9 mRNA and protein expressions were detected in human liver. On primary culture of murine hepatocytes, AQP1 and AQP7 mRNAs were identified, while the presence of AQP3, AQP8, AQP9, and AQP11 mRNAs was confirmed (Gregoire *et al*., 2015). Many studies revealed that AQP8 plays an important role in gallstone formation (Portincasa *et al*., 2008, Huo *et al*., 2024). However, the effect of AQP8 was not evident in the present study, this may be due to we focus on aquaporins on the liver and differences in different mouse strains. In this study, upregulation of liver AQP7 was found to reduce gallstone induced inflammation. Multiple components in Danning tablets could bind to ESR, which is a transcriptional regulator of AQP7 (Xing *et al*., 2019). In addition, estrogen is also a ligand of ESR, suggesting that Danning tablets may have different effects on male or female. The effect of Danning tablets on patients with different genders needs to be further studied.

Danning tablet as a traditional Chinese medicine formula, has a rather complex composition. Aloe-emodin, hesperetin, luteolin, rhein, nobiletin, sinensetin, quercetin are seven of the key compounds screened in this study. There are many studies on these plant derived compounds, especially quercetin and luteolin. Quercetin has a strong anti-inflammatory effect, this is mainly through inhibition of NF-kB activity and reduction of inflammatory cytokines (Hosseini *et al*., 2021).This effect of anti-inflammatory could be magnified by combination with other drugs. Quercetin combined with catechin (a flavonoid commonly found in the diet) significantly attenuate the LPS-stimulated increase of some inflammatory mediators and cytokines in RAW 264.7 macrophages (Li *et al*., 2019). Quercetin in danning tablet is mainly from *Polygoni Cuspidati Rhizoma ET RADIX* which is a common medicinal herb in China. This herb itself can be used to treat cholelithiasis and fatty liver by promoting autophagy (Li *et al*., 2024). The synergistic effect of quercetin and several flavonoids in Danning tablet may be another mechanism of anti-inflammatory effect of Danning tablet. Luteolin is also known to alleviate tissue inflammation (Xue *et al*., 2023), and has agonist potential for estrogen receptor (Puranik *et al*., 2019). Loren Kline believed that luteolin may also affect the electrophysiology and contractile function of the gallbladder(Kline, 2019). Some unpublished studies from our group have suggested that luteolin could act on intestinal cholesterol absorption and reduce the occurrence of gallstones. In China, several herbal prescriptions containing ingredients similar to those in Danning tablets have demonstrated therapeutic efficacy in the treatment of cholelithiasis. Research has identified quercetin and other bioactive compounds as the key active ingredients responsible for their pharmacological effects (Wang *et al*., 2024), which is consistent with the present study.

The role of liver-derived bile flush in the prevention and treatment of gallstones and inflammation has been gradually recognized. Mice lack of hepatic AQP8 are more susceptible to gallstones (Huo *et al*., 2024), highlight the importance of hepatocyte AQP-mediated water secretion as a critical mechanism for normal bile flow. Interestingly, the expression of AQP8 was also regulated by estradiol, which is similar to the results of the present research, but the underlying mechanisms remain to be studied (de Oliveira *et al*., 2020). It is generally believed that Danning tablets have a strong effect on promoting bile excretion, which was confirmed by the animal experiment in this study. Unfortunately, this rinsing effect does not seem to be of much help in stone prevention. This suggests that clinicians should pay more attention to the direction of Danning tablet in the treatment of gallbladder inflammation and pain.

CONCLUSION

Danning tablet promotes bile secretion through upregulation of hepatic AQP7 expression mediated by transcription factors including ESR1, thereby facilitating gallbladder irrigation and alleviating cholecystitis symptoms. The anti-inflammatory effects of Danning tablet contributed to the thinning of gallbladder wall thickness, which may potentially reduce surgical complexity in clinical settings.

Author's contribution

§ These authors contributed equally to this work and should be considered as co-first authors

Conflict of interest

This study does not involve any conflicts of interest.

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