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# Network Pharmacology-Based Prediction and Verification of Qingluo Tongbi Formula to Reduce Liver Toxicity of *Tripterygium wilfordii* via UGT2B7 in Endoplasmic Reticulum

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Statistical Analysis C  
Data Interpretation D  
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**Background:** The hepatotoxicity of *Tripterygium wilfordii* Hook. f. (TWHF) limits its clinic utilization. Qingluo Tongbi formula (QTF) was formulated based on a basic Chinese medicine theory. Previous studies have confirmed the safety and efficacy of QTF in treating rheumatoid arthritis. Therefore, we considered that TWHF could be detoxified based on its reasonable compatibility with QTF. We investigated the detoxicity mechanism of QTF in reducing the liver toxicity of TWHF.

**Material/Methods:** We used network pharmacology to determine the relevant metabolism targets of TWHF, focusing on the phase II metabolic enzymes uridine diphosphate-glucuronosyltransferase 1A1 (UGT1A1), UGT1A6, and UGT2B7. Based on the molecular mechanisms of these predictions and the results of the network analysis, we designed experiments to verify our hypothesis *in vivo*. We used western blotting, real-time quantitative polymerase chain reaction (RT-qPCR), double immunofluorescence, and laser confocal microscopy to detect the expression of UGTs. Finally, we used transmission electron microscopy to observe the endoplasmic reticulum structure.

**Results:** The results confirmed that QTF reversed the TWHF-induced reduction of UGT content in liver microsomes, upregulated UGT1A1 and UGT1A6 but not UGT2B7 in the liver tissue. UGT2B7 expression in the liver and liver microsomes was inconsistent. QTF upregulated the expression of UGT2B7 in the endoplasmic reticulum, and QTF upregulated UGT2B7 expression levels in the endoplasmic reticulum compared with TWHF, which reduced liver toxicity. Structural changes were observed in the endoplasmic reticulum.

**Conclusions:** The Chinese traditional medicine compound QTF can achieve the effect of detoxification by upregulating the expression of UGT2B7 in the endoplasmic reticulum.

**MeSH Keywords:** **Drug-Induced Liver Injury • Endoplasmic Reticulum • Glucuronosyltransferase • Medicine, Chinese Traditional • Tripterygium**

**Abbreviations:** **RA** – rheumatoid arthritis; **QTF** – Qingluo Tongbi Formula; **TWHF** – *Tripterygium wilfordii* Hook. f.; **PN** – *Panax notoginseng*; **RG** – *Rehmannia glutinosa*; **BB** – *Bombyx batryticatus*; **CS** – *Caulis sinomenii*; **ER** – endoplasmic reticulum; **LM** – liver microsome

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/920376>



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## Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes progressive articular destruction and associated comorbidities in the vascular, metabolic, bone, and psychological domains. RA affects approximately 1% of the population, can present at any age, and is more prevalent in women than in men [1]. At present, effective and cost-effective treatment methods are still urgently required.

Qingluo Tongbi formula (QTF) was formulated based on the basic Chinese medicine theory in which the “kidney (Shen) is in charge of producing marrow” and does so according to the principle of syndrome differentiation [2]. QTF is considered effective in treating RA [3–5]. QTF is composed of the root xylem of *Tripterygium wilfordii* Hook. f. (*Tripterygium wilfordii*, TWHF) 15 g, the root of *Panax notoginseng* (Burkill) F.H. Chen (*Panax notoginseng*, PN) 3 g, the root of *Rehmannia glutinosa* (Gaertn.) DC. (*Rehmannia glutinosa*, RG) 15 g, *Bombyx batryticatus* (BB) 10 g, and the rattan of *Sinomenium acutum* (Thunb.) Rehder & E.H. Wilson (*Caulis sinomenii*, CS) 15 g.

TWHF is a traditional Chinese herb grown in the east and south of China, Japan, and Korea. It has long been used in traditional Chinese medicine, often for RA [6]. But the hepatotoxicity of TWHF has limitations in its clinical utilization [7]. TWHF is the most important herb in QTF, however, these adverse effects have not been observed in QTF. In the past few decades, QTF has been proven to be safe and effective through clinical observations and animal experiments [3,8–11]. QTF shows considerable promise as a new traditional Chinese medicine preparation for the treatment of RA [12–15]. Therefore, we considered that TWHF could be further detoxified by compatibility with QTF since they are reasonably compatibility. Our previous study showed that QTF reduced the toxicity of TWHF by inhibiting the phase II metabolizing enzymes, cytochrome P450 (CYP) 3A and CYP2C19 [16].

Traditional Chinese medicine formulations have multi-target and multi-pathway effects. We used a systems pharmacology approach that combined the prediction of active compounds based on a range of pharmacokinetic parameters and elucidated the multiple drug targets using network analysis based on existing databases and publications. Then, we focused on the ability of QTF to reduce the hepatotoxicity of TWHF via UDP-glucuronosyltransferases (UGTs), which are the most important phase II conjugating enzymes that conjugate various endogenous substances and compounds. These enzymes are responsible for glucuronidation, a major process in phase II metabolism.

## Material and Methods

### Screening chemical compounds in TWHF

The chemical compounds in TWHF were obtained from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (<http://ibts.hkbu.edu.hk/LSP/tcmsp.php>) [17], the Traditional Chinese Medicine Integrated Database (TCMID) (<http://www.megabionet.org/tcmid/>), and the Traditional Chinese Medicine (TCM) database @Taiwan (<http://tcm.cmu.edu.tw/>). The TCM-PTD (<http://pharminfo.zju.edu.cn/ptd>) screening criteria were based on oral bioavailability (OB)  $\geq 30\%$  and drug-likeness (DL)  $\geq 0.18$  according to the absorption, distribution, metabolism, and excretion (ADME) parameters.

### Identifying metabolism related targets of TWHF

We used TCMSP and the Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (BATMANTCM) (<http://bionet.ncpsb.org/batman-tcm/>) [18]; and through literature inquiries, we sought to identify the related targets of TWHF. For compounds that did not have documented protein targets in the TCMSP database, we rechecked BATMANTCM. The BATMANTCM parameters were set as score cutoff of 20 and adjusted *P*-value cutoffs of 0.05. The predicted and known targets with values not smaller than the score cutoff of 20 for each ingredient were presented and further analyzed using bioinformatics. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway/Gene Ontology (GO) terms/trichothiodystrophy (TTD) diseases/Online Mendelian Inheritance in Man (OMIM) diseases with adjusted *P*-values smaller than the adjusted *P*-value cutoff of 0.05 were highlighted in the results. Then, all the related targets were uploaded into String (<https://string-db.org/>) [19] and the KEGG pathways enrichment analysis was used to identify metabolism-related targets (drug metabolism – CYP, metabolism of xenobiotics by CYP; retinol metabolism, drug metabolism – other enzymes, ascorbate and aldarate metabolism, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, metabolic pathways, arachidonic acid metabolism, linoleic acid metabolism). Then, metabolism-related targets were uploaded into the Cytoscapev3.2.1 program to form a network.

### Plant materials and reagents

TWHF (batch number: 201403) was purchased from Jiangsu Meitong Pharmaceutical Co., Ltd. PN (batch number: 131202), RG (batch number: 131202), BB (batch number: 131202), and CS (batch number: 131202) were purchased from Anhui Bozhou Herbs Company; the plant material was identified by experts at the School of Pharmacy, Nanjing University of Chinese Medicine, China. All samples met the standard of the Chinese Pharmacopoeia, 2005 Edition. The concentration of the major

toxic compound triptolide (TP) was determined in the TWHF group, the QTF group, the +PN group, the +RG group, the +BB group, and the +CS group samples using high-performance liquid chromatography (HPLC) and compared with standard reference compounds [20]. An endoplasmic reticulum (ER) protein extraction kit BBproExtra™ (BestBio, China) was used to extract the ER protein. The UGT content was determined using an enzyme-linked immunosorbent assay (ELISA) while a rat UGT ELISA kit (Jinyibo, China) was used to determine the liver microsomal total UGT content.

### Oral treatment of animals

We used female Sprague-Dawley (SD) rats (weighing 180 to 220 g) purchased from the Charles River Experimental Animal Company (Beijing, China) for our study. All animal studies were performed with the approval of the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine, and according to the guidelines of the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH publication No. 80-23). The rats were stochastically divided into 7 groups of 10 animals each. The control group rats were administered the vehicle alone while the other 6 groups of rats received TWHF (1.5 g/100 g), QTF (1.93 g/100 g), TWHF+PN (1.5+0.03 g/100 g), TWHF+RG (1.5+0.15 g/100 g), TWHF+BB (1.5+0.10 g/100 g), and TWHF+CS (1.5+0.15 g/100 g) once daily for 28 days. In order to explore the detoxification effect of QTF on TWHF, this study established an animal model of liver injury by using a toxic dose of TWHF. According to the results of the preliminary experiment, the dose of TWHF was set to be 10 times of the clinical dose, and the other drugs used in the study were normal clinical doses. During the experiments, the rats were monitored daily, and their body weights were measured weekly. After the final drug administration, the rats were fasted overnight, anesthetized with 10% chloral hydrate (0.35 mL/100 g) by intraperitoneal injection, and then blood and livers samples were collected. We randomly choose 3 rats from each group for liver perfusion.

### Preparation of liver microsome

Rat liver microsomes were obtained using differential centrifugation. All procedures were performed at 0°C to 4°C. The liver tissue samples were homogenized in 0.3 M sucrose, and the supernatant was obtained by centrifuging the homogenate at 10 000 rpm for 10 minutes and then 90 000 rpm for 20 minutes. Then, the supernatant was further centrifuged at 100 000 rpm for 60 minutes to obtain a microsomal pellet, which was suspended in 0.1 M potassium phosphate buffer (pH 7.4). The preparation was stored at -80°C until used.

### Western blotting

The liver samples were lysed in protein lysis buffer (radioimmunoprecipitation assay (RIPA): phenylmethanesulfonyl fluoride (PMSF, Boster, Wuhan, China); 100: 1). Protein content was measured by bicinchoninic acid (BCA) assay (Thermo Fischer Scientific, Waltham, MA, USA). The proteins were separated using 8% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and wet transferred to a polyvinylidene fluoride (PVDF) membrane for 1 hour at 90 volts, Membranes were blocked with 5% phosphate-buffered saline (PBS) in tris-buffered saline and Tween 20 (TBST) for 2 hours. Then, the membranes were incubated with the following antibodies, UGT1A1 (1: 1000, Abcam), UGT1A6 (1: 1000, Abcam), UGT2B7 (1: 1000, Proteintech), and  $\beta$ -actin (1: 1000, Proteintech), and incubated with goat anti-rabbit secondary antibody (1: 5000, Cell Signaling Technology Inc.). Membranes were exposed by chemiluminescence developing agents and the target protein levels were analyzed by Image Lab Software (Bio-Rad).

### Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA was extracted using the TRIzol reagent (Takara, Dalian, China) and cDNA synthesis was performed using the 5×All-In-One RT Master Mix (Abm, China). The cDNA was then amplified with the EvaGreen 2×RT-qPCR Master Mix (Abm) using the QuantStudio 7 Flex (Applied Biosystems Co., USA). The primers used are listed in Table 1. All operations followed the manufacturer's protocols. The fold changes between groups were analyzed using the comparative CT ( $2^{-\Delta\Delta CT}$ ) method, mRNA expression was normalized to  $\beta$ -actin.

### Double immunofluorescence and laser confocal microscopy

The perfused livers were subjected to gradient dehydration in sucrose solution, embedded in optimal cutting temperature compound (OCT, Sakura, USA) before freezing, and then the frozen tissue was sectioned using a Leica CM1950 cryostat (Leica, Germany). Permeabilization was performed sequentially with 0.1% Triton X-100 in PBS (0.1%) plus Tween-20 (PBS-T) for 30 minutes at room temperature (25°C). Then, tissue slices were rinsed 3 times with PBS-T, incubated with 5% goat serum in PBS-T for 1 hour at room temperature, and then incubated overnight with UGT2B7 antibody diluted in PBS-T at 4°C. Then, the sections were incubated with the fluorescent secondary antibody, washed, further incubated with 5% goat serum in PBS-T for 1 hour at room temperature, and then incubated overnight with Calnexin (Proteintech) antibody diluted in PBS-T overnight at 4°C. This was followed by incubation with the fluorescent secondary antibody, washing, and then the sections were cover slipped, followed by scanning

**Table 1.** Sequence of primers.

Target gene		Sequence of primers
UGT1A1	Forward	5'AACGATCTGCTTGGTCATCC3'
	Reverse	5'CAGGTCCAGAGGCTCGATAG3'
UGT1A6	Forward	5'AACGATCTGCTTGGTCATCC3'
	Reverse	5'CAGGTCCAGAGGCTCGATAG3'
UGT2B7	Forward	5'TGTAACCTCATGGTGGAGCCAAT3'
	Reverse	5'GATGACTGCCTTCAGTGCCT3'
β-actin	Forward	5'TCACCACACTGTGCCATCTATGA3'
	Reverse	5'CATCGGAACCGCTCATTGCCGATAG3'

and image acquisition using an Olympus BX-60 microscope (Olympus, Japan).

**Transmission electron microscope (TEM)**

For the TEM analysis, we selected the right lobe of each liver near the fossae transversalis hepatis; the tissue was cut into 1 mm<sup>3</sup> pieces, quickly rinsed with PBS, and then immediately placed in glutaraldehyde. Furthermore, to routinely process the samples for TEM, they were rinsed, fixed, dehydrated, soaked,

and embedded in Spon812. Subsequently, the tissue was cut into ultra-thin sections after semi-thin sectioning under a light microscope and then stained with lead citrate and uranium acetate. The ultra-structures of the sections were observed under a JEM-1200Ex electron microscope (Hitachi, Japan, provided by the Nanjing Medical University Electron Microscopy Center).

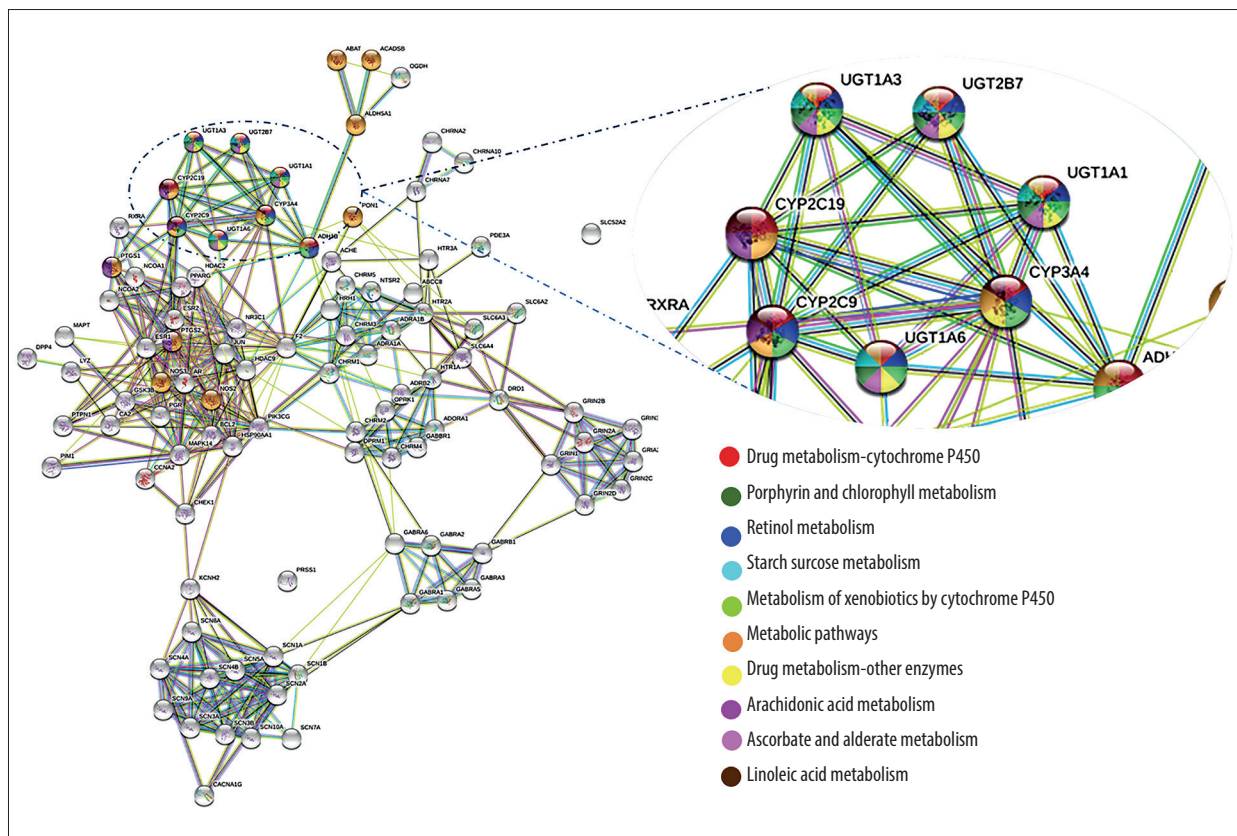
**Statistical analysis**

Data were expressed as the mean±standard deviation. For all quantitative data, statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Comparison among the groups were carried out by one-way ANOVA with Tukey's post hoc test. P<0.05 or P<0.01 was considered significant.

**Results**

**Identification of metabolism-related targets of TWHF**

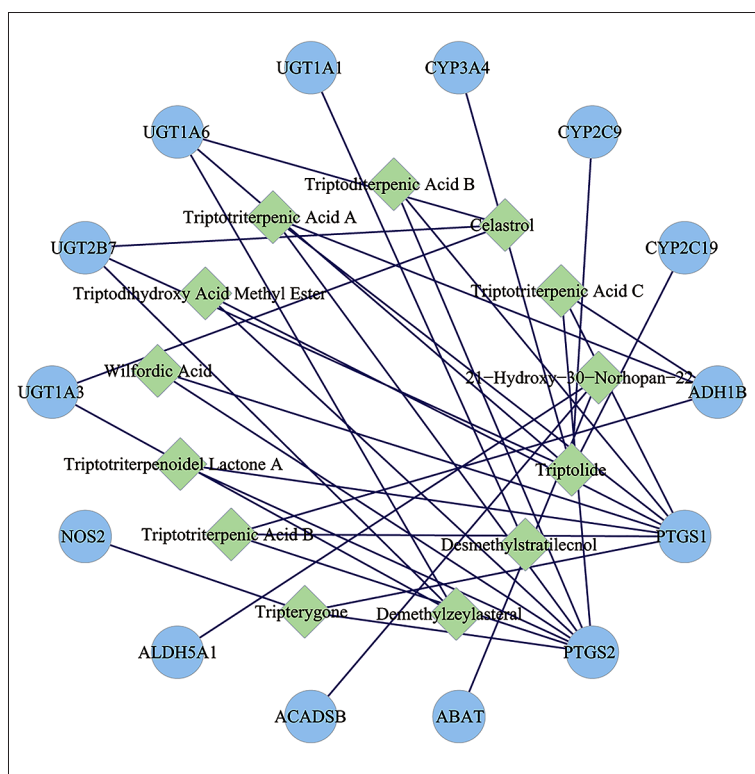
In this study, we first identified 114 chemical constituents of TWHF from the TCMSP and BATMANTCM databases and screened out 40 candidates based on OB ≥30% and DL ≥0.18 according to the ADME parameters. Then, these 40 compounds



**Figure 1.** Identifying metabolism related targets of TWHF (*Tripterygium wilfordii* Hook. f.).

**Table 2.** KEGG pathway enrichment analysis of metabolism related targets.

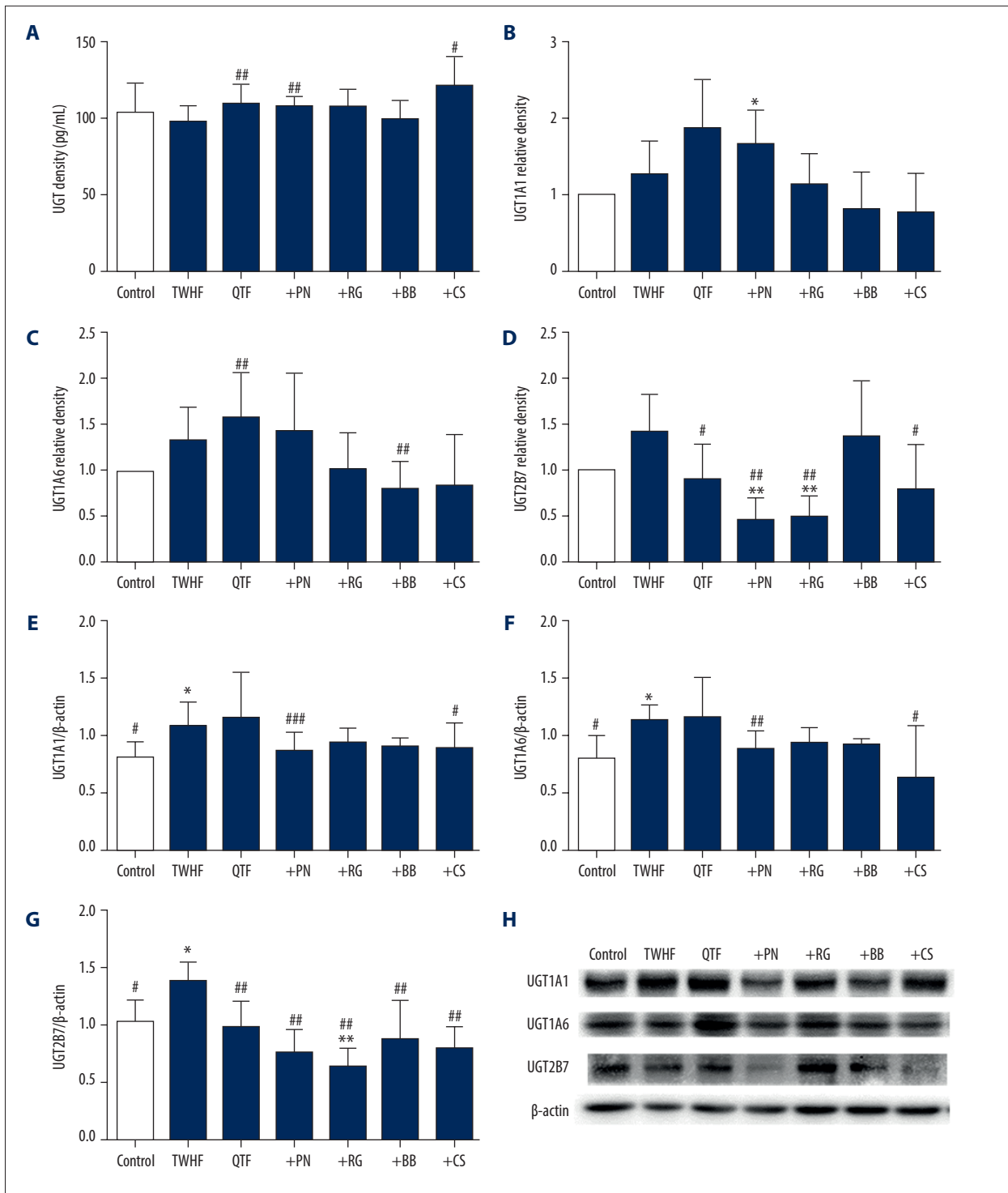
KEGG Pathway (false discovery rate)	Gene targets
Drug metabolism – cytochrome P450 (2.24e-08)	UGT1A1 UGT1A3 UGT2B7 CYP3A4 UGT1A6 CYP2C9 CYP2C19 ADH1B
Retinol metabolism (2.36e-07)	UGT1A1 UGT1A3 UGT1A6 UGT2B7 CYP3A4 CYP2C9 ADH1B
Metabolism of xenobiotics by cytochrome P450 (5.48e-07)	UGT1A1 UGT1A3 UGT1A6 UGT2B7 CYP3A4 CYP2C9 ADH1B
Drug metabolism – other enzymes (2.29e-05)	UGT1A1 UGT1A3 UGT1A6 UGT2B7 CYP3A4
Ascorbate and aldarate metabolism (6.19e-05)	UGT1A1 UGT1A3 UGT1A6 UGT2B7
Porphyrin and chlorophyll metabolism (0.000384)	UGT1A1 UGT1A3 UGT1A6 UGT2B7
Starch and sucrose metabolism (0.000737)	UGT1A1 UGT1A3 UGT1A6 UGT2B7
Metabolic pathways (0.000984)	UGT1A1 UGT1A3 UGT1A6 UGT2B7 CYP2C9 CYP2C19 CYP3A4 ABAT ACADSB ADH1B ALDH5A1 PON1 PTGS1 PTGS2 NOS2 NOS3
Arachidonic acid metabolism (0.00145)	CYP2C9 CYP2C19 PTGS1 PTGS2
Linoleic acid metabolism (0.00206)	CYP2C9 CYP2C19 CYP3A4



**Figure 2.** Metabolism related targets network of TWHF (*Tripterygium wilfordii* Hook. f.).

were input into TCMS and BATMANTCM, which generated 100 corresponding molecular targets. In addition, the project team also included 8 related targets from previous studies and publications. Next, these 108 protein targets were transformed into gene targets and entered into String to obtain the target network. The KEGG pathway in the string analysis was used for all metabolic pathways, and the results are shown in Figure 1

and Table 2. In addition, the metabolism-related target network of TWHF is shown in Figure 2. Therefore, it can be speculated that the inhibitory effects of active constituents of TWHF on CYP450, UGT1A1, UGT1A3, UGT1A6, and UGT2B7 are one of the mechanisms of toxicity. After the compatibility of QTF, the drug metabolism of TWHF can be regulated by regulating drug metabolism enzymes, thereby reducing the liver toxicity



**Figure 3.** Qingluo Tongbi formula (QTF) can reverse the reduction of UGT caused by TWHF (*Tripterygium wilfordii* Hook. f.), and QTF can upregulate UGT1A1 and UGT1A6, but cannot upregulate UGT2B7 in liver. (A) Enzyme-linked immunosorbent assay measured the content of UGTs in liver microsomes. (B–D) The mRNA expression of UGT1A1, UGT1A6, UGT2B7 was analyzed by real-time quantitative polymerase chain reaction. (E–H) Western blot measured the protein expression of UGT1A1, UGT1A6, UGT2B7 in liver. mRNA and protein expression were normalized to β-actin. Data are means ± standard error of the mean (SEM), each bar represents the mean of 3 independent experiments carried out in triplicate. \*/\*\*/\*\*\* P<0.05/0.01/0.001 compared with control group, and #/###/#### P<0.05/0.01/0.001 compared with TWHF group.

of TWHF. Based on the molecular mechanisms of these predictions and the results of the network analysis, we designed experiments to verify our hypothesis *in vivo* of SD rats. CYP450 is an important representative of the phase I metabolic enzymes. Our research team has conducted in-depth research on its mechanism of detoxification in QTF [12], so this study focused on the phase II metabolic enzymes UGT.

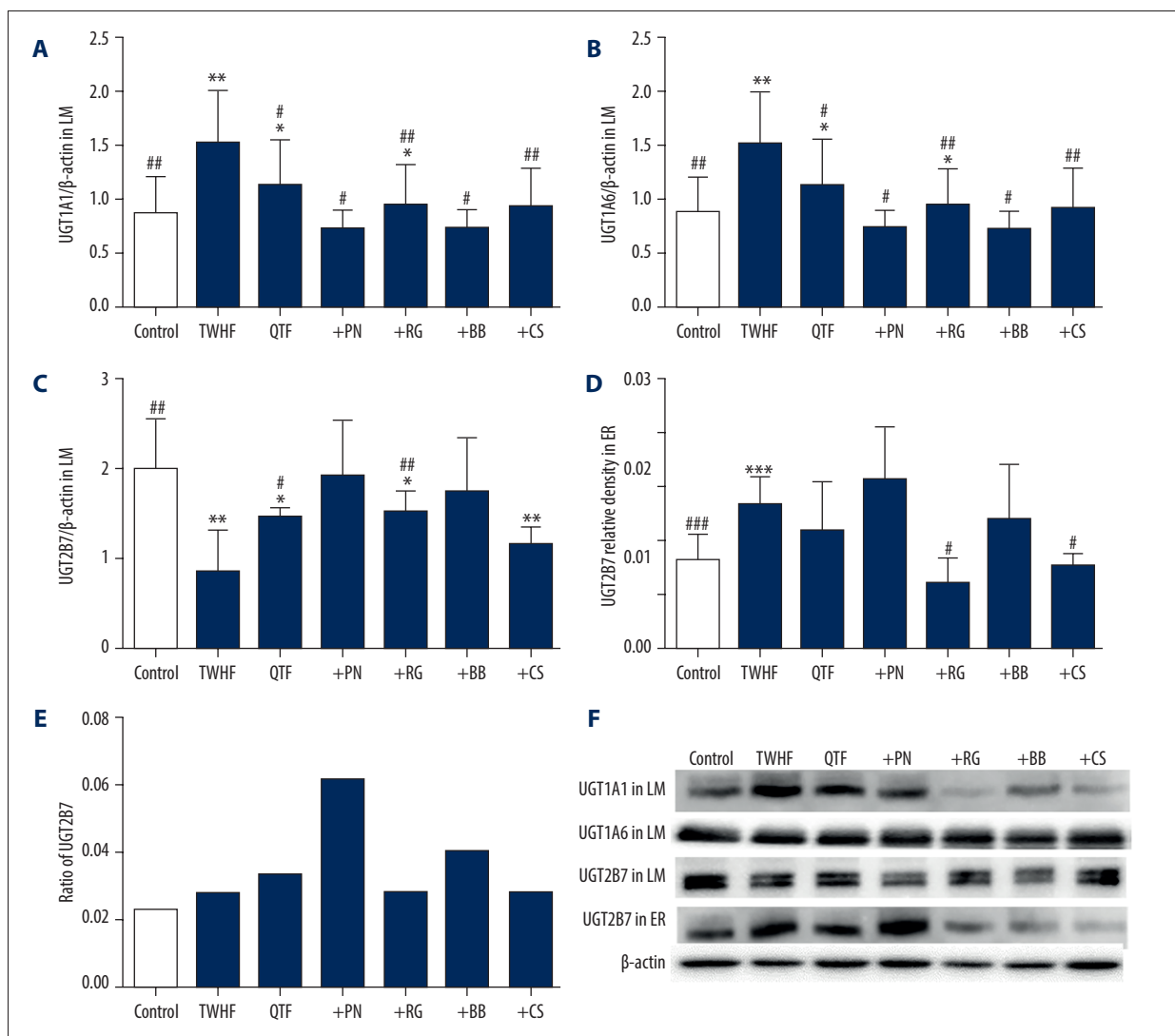
### QTF reversed reduction of UGT caused by TWHF

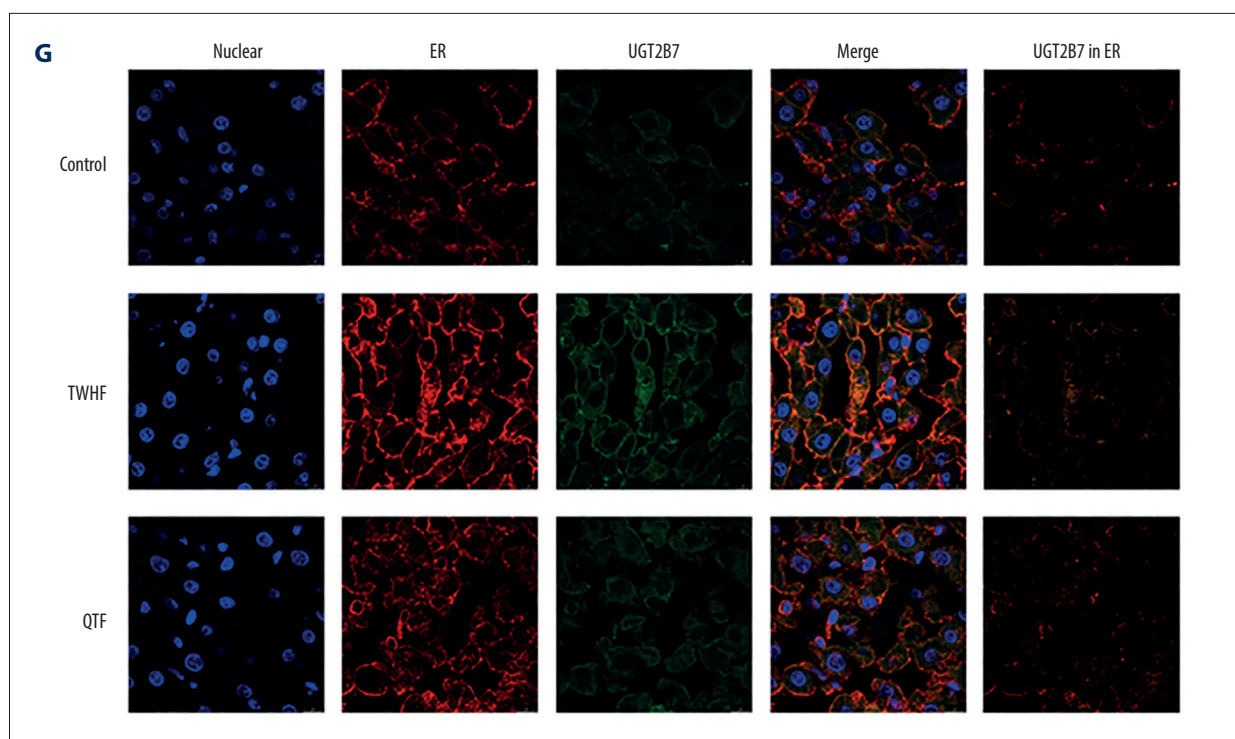
To confirm the role of UGT in the attenuating effects of the compounds in the QTF prescription, the content of UGT in liver microsome was determined using an ELISA. The UGT content of the TWHF and BB group was lower than that of the control group. Furthermore, the UGT content of the QTF, +PN, and +CS groups were higher than TWHF group ( $P < 0.05$ ). These results indicated that the toxicity of TWHF was related to its inhibitory

effects on the UGT enzyme. The compatibility of TWHF as QTF increased the UGT content in the liver microsome, indicating the detoxification of TWHF (Figure 3A).

### QTF upregulated liver UGT1A1 and UGT1A6 but not UGT2B7

To confirm whether QTF changes the expression of UGT, the mRNA and protein expressions of UGT1A1, UGT1A6, and UGT2B7 in tissues were detected using RT-qPCR and western blot, respectively. The gene and protein expression levels of UGT1A1, UGT1A3, and UGT2B7 were comparable (Figure 3B–3H). The mRNA transcription levels and protein expression levels of UGT1A1 and UGT1A6 in both groups were significantly higher than those in the blank group ( $P < 0.05$ ) and QTF showed a more significant increase compared to TWHF ( $P < 0.05$ , Figure 3B, 3C, 3E, 3F, 3H)). This finding suggested that





**Figure 4.** Qingluo Tongbi formula (QTF) can upregulate UGT2B7 expression in endoplasmic reticulum (ER). (A–C) UGT1A1, UGT1A6, and UGT2B7 in liver microsomal (LM) expression in different groups. (D) Western blotting results of UGT2B7 in ER. mRNA and protein expression were normalized to  $\beta$ -actin. Data are means  $\pm$  standard error of the mean (SEM), each bar represents the mean of 3 independent experiments carried out in triplicate. \*\*\*\*/\*\*\*\*  $P < 0.05/0.01/0.001$  compared with control group, and #####/####  $P < 0.055/0.01/0.001$  compared with TWHF (*Tripterygium wilfordii* Hook. f.) group. (E) Ratio of ER UGT2B7 in tissues = mean of relative expression of UGT2B7 in ER protein/mean of relative expression of UGT2B7 in liver tissue. (G) Double immunofluorescence and laser confocal microscopy.

the stress of UGT1A1 and UGT1A6 increased after test substance administration, and the compatibility of QTF increased more significantly than that of TWHF.

However, compared with TWHF, QTF, +PN, +RG, and +CS downregulated the expression of UGT2B7 mRNA in the liver ( $P < 0.05$ , Figure 3D). Compared with the control group treatment, TWHF increased the UGT2B7 protein expression while the QTF and its compatibility downregulated UGT2B7 protein expression ( $P < 0.05$ , Figure 3G, 3H). These results indicated that QTF did not increase the expression of UGT2B7 in the liver.

#### UGT2B7 expression was not consistent in liver microsomal protein and tissue

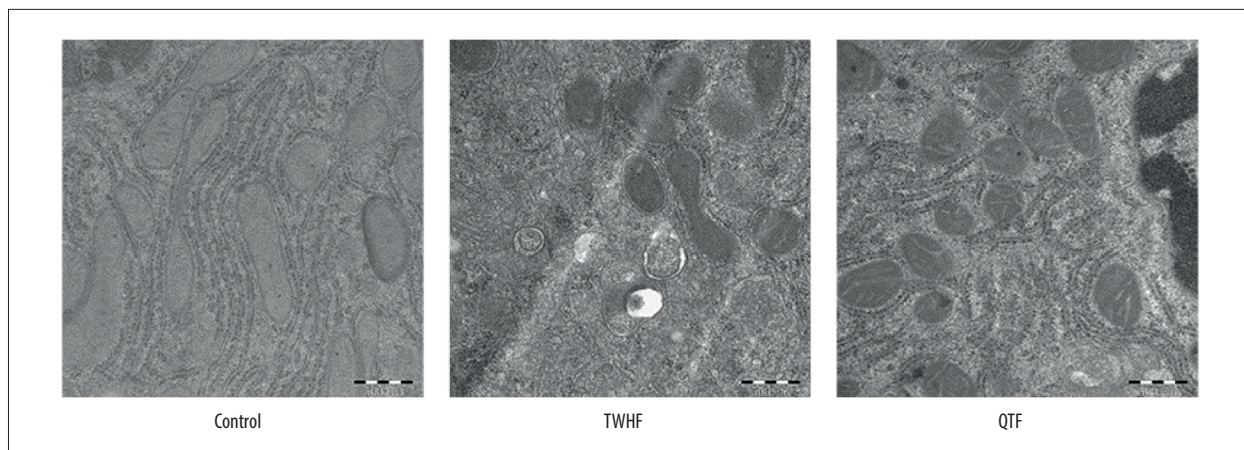
UGT2B7 attracted our interest contrary to expectations. Liver microsomes are the nearly spherical vesicle-like structures formed by self-fusion of broken ER produced during cell homogenization and differential centrifugation. They consist of 2 basic components of the ER: membrane and ribosomes. Therefore, we detected the liver microsomal proteins using western blot, to identify possible discoveries. In the liver microsomes, UGT1A1

and UGT1A6 protein expression induction by QTF and compatibility were higher and lower than that in the control and TWHF groups, respectively ( $P < 0.05$ , Figure 4A, 4B). However, the expression of the UGT2B7 protein was significantly lower in the TWHF, QTF, +RG, and +CS groups than it was in the control ( $P < 0.05$ ). The control, QTF, and +RG groups showed higher levels than that of the TWHF group ( $P < 0.05$ , Figure 4C). This finding indicated that QTF and its compatibility upregulated UGT2B7 in liver microsomes, which was in contrast to the results of the liver tissue total protein analysis. Liver microsomal and ER proteins are similar, and thus, we hypothesized that QTF and its combinations would reduce the toxicity of the components by increasing the UGT2B7 content in the ER. Therefore, we conducted verification experiments and the results are described.

#### QTF upregulated UGT2B7 expression in ER

To verify the previous hypothesis, the ER protein was extracted to detect the expression of UGT2B7 in the ER using western blotting. The relative expression of UGT2B7 in the ER was analyzed based on the ratio of the ER UGT2B7 in the liver tissue.





**Figure 5.** Endoplasmic reticulum (ER) structure changes were detected by transmission electron micrographs. Control: ER rich, no rupture, expansion and other changes. TWHF (*Tripterygium wilfordii* Hook. f.): ER reduced, visible fracture. Qingluo Tongbi formula (QTF): ER rich, no rupture, expansion and other changes.

The formula used was as follows: ratio of ER UGT2B7 in tissues=mean relative expression of UGT2B7 in ER protein/mean relative expression of UGT2B7 in liver tissue. The control as well as +RG and +CS groups showed lower UGT2B7 expression in the ER than the TWHF group ( $P<0.05$ , Figure 4D). The ratio of UGT2B7 in the ER to the tissues of the various groups was in the following increasing order: control  $<$ +CS  $<$ TWHF  $<$ +RG  $<$ QTF  $<$ +PN (Figure 4E). These results indicated that the expression of UGT2B7 in the ER increased after administration, and the effect of QTF was more significant than that of TWHF, which could be detoxified by phase II metabolism.

A double-labeled immunofluorescence confocal technique with the ER marker calnexin was used to further validate our findings, and the results were consistent with those of western blot analysis of the ER protein. The expression of the UGT2B7 protein in the ER induced by QTF treatment was higher than that induced by TWHF (Figure 4G). Similarly, these results were verified, after QTF compatibility increased the expression of UGT2B7 in the ER, which reduced liver toxicity.

#### **QTF improved the structure of the ER more than TWHF did**

To investigate the different expression of UGT2B7 in the ER, the ER structure changes were detected by TEM. Figure 5 shows ER of the control group was rich, no ruptures, expansions and other changes; the TWHF group was reduced, visible fracture; and the QTF group was rich, no ruptures, expansions and other changes. After, QTF compatibility improved the structure of the ER more than TWHF did.

## **Discussion**

We used network pharmacology to determine the relevant metabolism targets of TWHF, focusing on the phase II metabolic enzymes, UGTs. The results confirmed that QTF reversed the reduction of the UGT content caused by TWHF in liver microsomes, and upregulated UGT1A1 and UGT1A6 but not UGT2B7 in the liver. UGT2B7 expression in the liver and liver microsomes was used as an index to confirm that QTF upregulated the expression of UGT2B7 in the ER, which reduced liver toxicity.

The mechanism underlying the QTF-mediated alleviation of the liver toxicity of TWHF has been studied from many perspectives. Sun et al. [8] measured the dissolution contents of 3 toxic components of QTF (triptolide, tripteryne, and wilfordide) and its individual ingredients. The results revealed that the dissolution of these 3 toxic components was significantly reduced in QTF. Liu et al. [20] investigated the dissolution changes of triptolide a liver toxic component of TWHF after QTF and its compatibility from chemical compound aspect to determined that the compatibility of QTF can reduce triptolide dissolution to detoxicity. Zhang et al. [12] used metabolomics technology to verify the QTF can decrease the liver toxicity of TWHF. Xie et al. [10] also used metabolomics to confirm the QTF can decrease the liver toxicity of TWHF by reversing amino acid metabolism.

Inhibition of phase II metabolic enzymes is the key factor in drug toxicity and drug interactions. In the process of evolution, the body has developed a complex system for the metabolic transformation of exogenous toxins called biotransformation. Drugs, poisons, carcinogens, and other exogenous substances in the body generally undergo 2-phase metabolism consisting of phase I and II metabolic processes. Phase II metabolic processes are mainly catalyzed by enzymes and some endogenous

polar small molecules with covalent bonds. The activation of the resultant reaction products or foreign substances enhance their polarity and promote excretion. Phase II metabolism deactivates toxic substances and therefore, phase II metabolic enzymes are also known as phase II metabolism detoxification enzymes, such as UGT, which is one of the most important microsomal enzymes in phase II metabolism. UGTs are mainly located in the liver, particularly in the cell membrane of the ER cavity edge. UGTs catalyze the glucuronic acid group of uridine diphosphate glucuronic acid (UDPGA) in the ER to transfer it to a variety of polar-containing groups of endogenous or exogenous compound molecules. This generates glucuronides with the loss of the original activity, and conversion to a hydrophilic material, which facilitates its excretion in the urine and bile. Therefore, UGTs need to be combined with UDPGA in the ER for detoxification.

Studies on TWHF toxicity have focused mainly on phase II metabolic enzymes. Zhao et al. [21] demonstrated that demethylzeylasteral, an active ingredient of TWHF, strongly inhibits UGT1A6 and UGT2B7 in inhibitory studies on UGT1A6, UGT1A9, and UGT2B7. This observation indicates the need to monitor the potential clinical interactions between demethylzeylasteral-containing herbs and clinical drugs that mainly undergo UGT1A6- and UGT2B7-catalyzed metabolism. Zhang et al. [22] reported the strong inhibition of UGT1A6 and UGT2B7 by celastrol, which indicated the possibility of celastrol-drug interactions or celastrol-containing herb-drug interactions. Yu et al. [23] confirmed the inhibitory effect of demethylstratilenol on UGT1A1 using the recombinant UGT1A1. This observation indicated that the potential clinical interactions between the combination of drugs and these preparations need to be considered, mainly with substances that are metabolized by UGT1A1. Wang et al. [24] confirmed the inhibitory effect of demethylzeylasteral on UGT1A3 using recombinant UGT1A3 and suggested that attention should be paid to the interaction between traditional Chinese medicine formulations based on their inhibition of UGT1A3. Huang et al. [25] showed that the high risk of drug-drug interactions between celastrol and drugs was mainly due to their elimination by UGT1A3-mediated metabolism.

Thus, it can be speculated that the hepatotoxic effects of components of TWHF are closely related to their metabolism by UGT1A1, UGT1A3, UGT1A6, UGT1A9, and UGT2B7, which is in agreement with the results of our network pharmacology analysis. Most of these research studies were conducted using recombinase administration. Such *in vitro* experiments are associated with unavoidable problems that could affect endogenous and exogenous substances after metabolism of TWHF, and the involvement of phase II metabolic enzymes cannot be investigated. Therefore, we used an *in vivo* animal model to

detect the expression of UGTs and found that the liver toxicity of TWHF was related to the expression of UGT2B7 in the ER.

Mammalian UGTs are located in the ER of cells and catalyze the covalent addition of glucuronic acid from UDP glucuronic acid to a variety of aglycone substrates, rendering them more polar and thus more readily excreted in the bile or urine [26–28]. Among them, UGT2B7 is the most important enzyme in phase II metabolism [29,30].

Based on the differential UGT2B7 expression in ER, their substrates and metabolites need to be transported through the ER membrane, we considered the UGT membrane association and retention signal, or other reasons that could cause structural changes in the ER. Meech et al. [28] examined UGT2B1 ER subcellular localization and membrane association, and the construction and analysis of an active truncated form of UGT2B1 indicated that the cytosolically located dilysine motif, which is a putative ER membrane targeting signal, might be redundant in the residency of UGT in the ER. The TEM results showed that QTF improved the structure of the ER more than TWHF did. However, the underlying cause of ER structural damage is unclear, and the repair mechanisms mediated by QTF are not entirely understood. This could be considered a limitation of this study and a direction for our continuing future research.

## Conclusions

In summary, our results indicated that QTF attenuates TWHF-induced hepatotoxicity by affecting phase II metabolic enzymes (UGT1A1, UGT1A6, and UGT2B7). We also found that QTF can upregulate the relative expression of UGT2B7 in ER, thereby better reducing liver toxicity, and we found that PN and BB play an important role in it. We initially believe that this change may be related to structural changes of ER, but further research and supporting evidence are necessary.

## Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

## Ethical approval

The procedures for care and use of animals were approved by the Ethics Committee of the Nanjing University of Chinese Medicine.

## Conflicts of interest

None.

## References:

1. Mcinnes IB, Schett G: Pathogenetic insights from the treatment of rheumatoid arthritis. *Lancet*, 2017; 389(10086): 2328–37
2. Zhou T, Zhou L, Zhou C et al: Inhibition mechanism of Qingluo Tongbi granule (QTG) on osteoclast differentiation induced by synovial fibroblast and monocytes co-culture in adjuvant-induced arthritic rats. *Chin J Integr Med*, 2015; (04): 291–98
3. Huang Y, Li M, Zhou L et al: Effects of Qingluo Tongbi Decoction on gut flora of rats with adjuvant-induced arthritis and the underlying mechanism. *Evid Based Complement Alternat Med*, 2019; 2019: 6308021
4. Zhou X, Zhou Z, Jin M et al: Clinical study of Qingluo Tongbi granules in treating 63 patients with rheumatoid arthritis of the type of yin-deficiency and heat in collaterals. *J Trad Chin Med*, 2004; 24(2): 83–87
5. Liu TY, Zhou LL, Zhou C et al: Inhibition mechanism of Qingluo Tongbi Granule on osteoclast differentiation induced by synovial fibroblast and monocytes co-culture in adjuvant-induced arthritic rats. *Chin J Integr Med*, 2015; 21(4): 291–98
6. Wang X, Xue M, Gu J et al: Transdermal microemulsion drug delivery system for impairing male reproductive toxicity and enhancing efficacy of *Tripterygium wilfordii* Hook f. *Fitoterapia*, 2012; 83(4): 690–98
7. Liu J, Lee J, Salazar Hernandez MA et al: Treatment of obesity with celastrol. *Cell*, 2015; 161(5): 999–1011
8. Sun L, Zhang B, Zhu H et al: [Chemical basis of *Tripterygium wilfordii* compatibility for detoxicity in Qingluo Tongbi Decoction based on 'diversity restriction'.] *Chinese Journal of Experimental Traditional Medical Formulae*, 2017; 23(11): 50–54 [in Chinese]
9. Xie T, Li H, Lu Y et al: [Effects of Qingluo Tongbi Decoction combined with *Radix Rehmanniae Recens* and *Radix Notoginseng* on hepatotoxicity of *Tripterygium wilfordii* based on metabonomics.] *China Journal of Traditional Chinese Medicine and Pharmacy*, 2017; 32(8): 3462–67 [in Chinese]
10. Xie T, Zhou X, Lin L et al: [Metabolomics analysis of *Tripterygium wilfordii* formulation based on theory of detoxicity compatibility.] *Zhongguo Zhong Yao Za Zhi*, 2016; 41(6): 1124–29
11. Correction for Yan et al. Imbalanced signal transduction in regulatory T cells expressing the transcription factor FoxP3. *Proc Natl Acad Sci USA*, 2016; 113(2): E256
12. Zhang B, Zhang Q, Liu M et al: Increased involvement of *Panax notoginseng* in the mechanism of decreased hepatotoxicity induced by *Tripterygium wilfordii* in rats. *J Ethnopharmacol*, 2016; 185: 243–54
13. Zhou X, Zhou Z, Jin M et al: Clinical study on Qingluo Tongbi granule in treating 63 cases of rheumatoid arthritis with yin deficiency syndrome. *J Tradit Chin Med*, 2003; (3): 191–93
14. Liu Z, Zhou X: Clinical Study of Qingluo Tongbi Decoction in treating rheumatoid arthritis Rebi syndrome. *Guo Yi Forum*, 2004; (2): 23–25
15. Zhou X, Zhou Z, Jin M et al: Clinical study of Qingluo Tongbi Granules in treating 63 patients with rheumatoid arthritis of the type of yin-deficiency and heat in collaterals. *J Tradit Chin Med*, 2004; (2): 83–87
16. Lu Y, Xie T, Zhang Y et al: [Influence of compound prescription of *Tripterygium wilfordii* on CYP450 enzyme family based on theory of 'heterogeneous restriction'.] *China Journal of Traditional Chinese Medicine and Pharmacy*, 2017; (3): 1050–56 [in Chinese]
17. Ru J, Li P, Wang J et al: TCMSp: A database of systems pharmacology for drug discovery from herbal medicines. *J Cheminform*, 2014; 6(1): 13
18. Xiao H, Jiao J, Wang L et al: HDAC5 controls the functions of Foxp3(+) T-regulatory and CD8(+) T cells. *Int J Cancer*, 2016; 138(10): 2477–86
19. Franceschini A, Szklarczyk D, Frankild S et al: STRING v9.1: Protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res*, 2013; 41(Database issue): D808
20. Liu M, Zhang X, Pan L et al: [Chemical basic research of reducing toxicity of *Tripterygium wilfordii* in Qingluo Tongbi.] *Chinese Journal of Experimental Prescriptions*, 2014; (7): 107–12 [in Chinese]
21. Zhao JW, Wang GH, Chen M et al: Demethylzeylasteral exhibits strong inhibition towards UDP-glucuronosyltransferase (UGT) 1A6 and 2B7. *Molecules*, 2012; 17(8): 9469–75
22. Zhang YS, Tu YY, Gao XC et al: Strong inhibition of celastrol towards UDP-glucuronosyl transferase (UGT) 1A6 and 2B7 indicating potential risk of UGT-based herb-drug interaction. *Molecules*, 2012; 17(6): 6832–39
23. Yu ML, Lin JJ, Yang Y et al: Evaluation of inhibition of UDP-glucuronosyltransferase (UGT) 1A1 by demethylzeylasteral. *Latin American Journal of Pharmacy*, 2012; 31(7): 1067–70
24. Wang Y, Gong HY, Wan Y et al: *Tripterygium wilfordii* Hook F. bioactive component shows significant inhibition towards metabolic clearance of 4-methylumbelliferone (4-MU). *Latin American Journal of Pharmacy*, 2012; 31(9): 1351–54
25. Huang T, Jiang F, Mou XD et al: Drug metabolizing enzymes inhibition-based prediction of celastrol-drugs interaction. *Latin American Journal of Pharmacy*, 2012; 31(9): 1363–66
26. Aitio A: Conjugation reactions in drug biotransformation: Elsevier/North-Holland Biomedical Press, 1978; 122–23
27. Frederick CKPD: Conjugation-deconjugation reactions in drug metabolism and toxicity. Springer Berlin Heidelberg, 1994; 2434–45
28. Meech R, Mackenzie PI: Determinants of UDP glucuronosyltransferase membrane association and residency in the endoplasmic reticulum. *Arch Biochem Biophys*, 1998; 356(1): 77–85
29. Abdullah NH, Ismail S: Inhibition of UGT2B7 enzyme activity in human and rat liver microsomes by herbal constituents. *Molecules*, 2018; 23(10): pii: E2696
30. Hu DG, Meech R, McKinnon RA, Mackenzie PI: Transcriptional regulation of human UDP-glucuronosyltransferase genes. *Drug Metabol Rev*, 2014; 46(4): 421–58