

The “Sweat Soaking Method”-processed *Radix Wikstroemia indica* Extracts Attenuate Liver Toxicity and its Effect on Cytochrome P450 Protein

Wenjing Wang¹, Guo Feng¹ , Qin Xu¹, Wei Li¹, Lailai Li¹, Hongmei Su¹, Guanglin Zhu¹, Xueli Song¹, Ju Zhang¹ and Zhengyan He¹

Abstract

Background: *Radix Wikstroemia indica* (RWI) is one of the most commonly used drugs in Miao medicine. However, RWI is characterized by high toxicity.

Objectives: In this study, we aimed to observe the effect of the “sweat soaking method” processed on reducing the hepatotoxicity of RWI and on cytochrome P450 (CYP) protein expression.

Materials and Methods: The study focused on investigating the impact of both RWI raw products and RWI processed products on the proliferation of L-02 cells. To assess this, a cell counting kit-8 was employed. After the administration of RWI through oral gavage for a duration of 15 days in rats, the corresponding kits were utilized to determine the serum levels of the liver index factor. Furthermore, both hematoxylin and eosin (H&E) staining and western blot analysis were conducted to analyze the liver tissues.

Results: Both the raw RWI and the processed RWI at high doses inhibited the proliferation of L-02 cells, but the cell viability of the concoction group was higher than that of the raw group. The liver tissues of rats in the control and processed product groups were normal, while those in the raw product groups showed different degrees of liver damage. Additionally, compared with the control group, the activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine transaminase (ALT) in the raw product groups was notably increased in a dose-dependent manner; the three enzyme activities of the processed group were lower than those of the raw product group. In addition, the expression levels of CYP3A4, 2C19, 2C9, 1A2, and 2E1 in the liver tissue of rats in the processed group were lower than those in the raw group, while the protein expression trend of CYP2D6 was irregular.

Conclusion: The detoxication mechanism of RWI, after undergoing processing, appears to be associated with reduced protein expression of CYP1A2, CYP2E1, CYP2C9, CYP2C19, and CYP3A4, ultimately leading to decreased liver injury.

Keywords

Radix Wikstroemia indica, sweat soaking method, cytochrome P450, liver toxicity, processing mechanism

Received 05 August 2023; accepted 27 October 2023

Introduction

Drug-induced liver injury (DILI) refers to liver injury induced by different chemical formulations, biological substances, Chinese over-the-counter medicines, healthcare medications, dietary supplements, and their metabolic byproducts, either on an acute or chronic basis. DILI can cause pathophysiological cascade reactions, eventually leading to liver steatosis, hepatitis, liver fibrosis (Miao et al., 2021), liver failure, or even death in more serious

cases (Shen et al., 2019). DILI, despite being infrequently observed, stands as the primary contributor to acute liver failure in both the United States and Europe. Furthermore, it

¹Department of Chinese Materia Medica, Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou, China

Corresponding author:

Guo Feng, Department of Chinese Materia Medica, Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou 550025, China.
E-mail: 453989352@qq.com



represents the leading cause of drug disapproval and market withdrawal (Kullak-Ublick et al., 2017). It has been reported that the interaction between two drugs can alter the enzymatic activity of cytochrome P450 (CYP) and its subtypes, thus increasing hepatotoxic metabolites and hepatotoxicity (Chen et al., 2015).

CYP is considered the largest biotransformation enzymatic system in the liver and is involved in the biotransformation of the majority of clinical drugs. The main mediators of the metabolic CYP system are CYP3A4, CYP2C9, CYP2E1, CYP2C19, CYP1A2, and CYP2D6 (Rendic, 2002; Nelson et al., 2004; Klein et al., 2010; Kanaan et al., 2013). CYP450 enzyme action has shown that it can enhance the efficacy and reduce the toxicity of Traditional Chinese Medicine (TCM); however, some of them may lead to organ damage and even affect the physiological functions of the body (Li et al., 2019; Zhang et al., 2016). The enzymes of the CYP family are implicated in regulating the biosynthesis of several active ingredients in TCM, while changes in their gene expression levels can result in differences in the metabolism of these active ingredients.

Radix Wikstroemia indica (RWI), named “Liao Ge Wang” in Chinese, is one of the most commonly used herbs in Miao medicine. It has been reported that RWI is used to dispel phlegm and blood stasis, while it also exhibits anti-bacterial, anti-tumor, and anti-inflammatory properties (Feng et al., 2018). In Miao medicine, RWI is processed using the “sweat soaking method.” Based on this method, the RWI decoction pieces are tied to the waist of a person and an increase in their efficiency and reduced toxicity can be achieved via their long-term immersion in sweat. In a previous study, artificial sweat was used as an auxiliary material to process RWI, and research was conducted on the quality standards of RWI decoction pieces processed with the “sweat soaking method” (Feng et al., 2017). According to the findings from the aforementioned investigation, no substantial disparity in the effectiveness of RWI was observed among unprocessed commodities, artificially treated products, and perspiration-treated goods. However, the toxicity of the raw products was significantly enhanced compared with the two processed products. Furthermore, acute toxicity experiments revealed that following treatment of RWI with sweat or artificial sweat, its toxicity was reduced.

Hence, this investigation sought to confirm experimentally, both in live animals and in laboratory setups, that the application of the “perspiration immersion technique” had the potential to decrease liver harm in rats. To initially unravel the mechanism through which the “perspiration immersion technique”-treated RWI could mitigate liver toxicity in rats, an initial Western blot analysis was conducted, focusing on the impact on CYP. The results of the current study could provide novel insights into the mechanism underlying the effect of the “sweat soaking method”-processed RWI on reducing liver toxicity, thus providing a reference for the safety and clinical application of RWI.

Materials and Methods

Extraction of RWI

RWI was purchased from Guangxi Yulin Yinfeng International Chinese Medicine Port (Yulin, China; Batch No. 20160115). To obtain the ethanol extracts of RWI, 5.8 kg of crude RWI was stored in a barrel, which was filled with 70% ethanol. After draining the bubbles, the barrel was sealed for 48 h, and ethanol was then extracted by percolation. Following ethanol recovery from the percolate, it was then concentrated and dried, crushed with a pulverizer, and sealed for storage. To isolate ethanol extracts from the “sweat soaking method”-treated RWI, 5.8 kg of crude RWI was first sprayed with 1% artificial sweat, composed of 0.5 g/L L-histidine salt, 5 g/L sodium chloride, and 2.2 g/L sodium dihydrogen phosphate dihydrate. The pH of the artificial sweat was adjusted to a value of 5.5 with 0.05 mol/L sodium hydroxide. Subsequently, the mixture was mixed well, and moisturized, followed by drying in an oven at 37°C for 24 h. The above process was repeated for 2 weeks. For every 100 kg of RWI, 30 kg of 1% artificial sweat were used. Finally, the “sweat soaking method”-processed RWI product was then extracted based on the aforementioned raw product ethanol extraction method (Zheng et al., 2020).

Cell Culture

Hepatocytes L-02, derived from human normal hepatic tissue, were obtained from Feng Hui Biotechnology Co. These cells were maintained in a culture of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% penicillin/streptomycin solution, 10% fetal bovine serum (FBS), and 90% RPMI 1640 medium. The incubation process was carried out under atmospheric conditions of 5% carbon dioxide (CO₂) and 95% air at a temperature of 37°C.

Cell Proliferation Assay

L-02 cells in logarithmic growth were inoculated into 96-well plates at 100 µL per well, containing $4-5 \times 10^4$ cells per well, and incubated in a CO₂ incubator for about 24 h. After the cells were walled and in good condition, the old medium in the culture plate was aspirated, and 100 µL of drugs containing the corresponding concentrations, the raw RWI product, and the raw processed product were added to each well at the concentrations of 300, 150, 75, 37.5, 18.75, 9.38, and 4.69 µg/mL. There were five parallel complexes in each concentration group. After 48 h, 10% cell counting kit-8 reagent was added to each well under protection from light, and the incubation was continued in a constant temperature incubator. After incubation for 2 h, the absorbance value at 450 nm was measured by an enzyme meter. The experiment was repeated three times, the average value was calculated, and the half-maximal inhibitory concentration (IC₅₀) and cell

inhibition rate were calculated according to the formula. IC_{50} values were measured using SPSS 17.0 (SPSS, Inc.).

After 48 h of administration according to the same experimental method, the cells in the different concentration groups were photographed and analyzed for cell morphology using an inverted microscope.

Animals and Experimental Design

TXTMale Sprague–Dawley (SD) rats, free from specific pathogens and raised in a pathogen-free environment, were obtained from Changsha Tianqin Biotechnology Co., Ltd. (Animal License no. SCXK (Xiang) 2019-0013). The rats were given a period of 5 days to acclimate to a new environment with a 12-h light/dark cycle, unrestricted access to standard feed and tap water, and a stable ambient temperature of $25 \pm 1^\circ\text{C}$, along with a relative humidity of $50\% \pm 10\%$. A total of 42 male SD rats, meeting SPF criteria, were randomly divided into seven groups. The groups received different concentrations of raw RWI and processed RWI ethanol extracts (0.1013, 0.2025, 0.4050 g/kg) or a control group (administered an equal volume of 1% CMC-Na via gavage) once daily. The treatment lasted for 15 consecutive days. After the completion of the experiment, the rats were euthanized using an intraperitoneal injection of 2% sodium pentobarbital (0.3 mL/100 g body weight) until complete softening of the entire body was observed. Blood samples were obtained from the abdominal aorta (typically 8–10 mL of blood were collected). The collected blood samples were allowed to stand for 1 h at room temperature before being subjected to centrifugation at 10,000 rpm for 10 min at 4°C , resulting in the separation of serum. In a previous study, the median lethal dose (LD_{50}) of the RWI raw ethanol extraction product in mice was determined to be 4.05 g/kg; therefore, we administered high doses at one-tenth of the LD_{50} value. All animal experiments conducted in this study were reviewed and approved by the Ethics Review Committee for Experimental Animals, following recognized guidelines on animal welfare (Approval No. 20210080).

Liver Index and Biochemical Factor Index Detection

The changes in the serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in rats were measured using the corresponding kits according to the manufacturer's instructions. There were three parallel samples in each group.

We observed and recorded the changes in the body weight of rats and calculated the liver organ index:

Liver index (%) = The weight of the liver/The final weight of the rat $\times 100\%$

Hematoxylin and Eosin (H&E) Staining

In this study, we extracted liver tissues from rats, subjecting them to partial fixation using a 4% formaldehyde solution.

Subsequently, the tissues were embedded in paraffin. To facilitate analysis, we sliced the paraffin-embedded tissues into sections measuring 5 μm in thickness, which were then subjected to H&E staining. The effect of the “sweat soaking method”-processed RWI on liver tissue morphology in rats was evaluated under a microscope.

Western Blot Analysis

Liver tissue samples weighing 100 mg were stored at -80°C in a refrigerator. The proteins in the liver tissues were extracted through centrifugation, and their concentration was determined using a bicinchoninic acid kit. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane through transfer electrophoresis. Then, the membranes were incubated with the listed antibodies overnight at 4°C , including anti-CYP1A2, anti-CYP3A4, anti-CYP2D6, anti-CYP3C19, anti-CYP2C9, anti-CYP2E1, and glyceraldehyde-3-phosphate dehydrogenase rabbit polyclonal antibody. After washing with phosphate-buffered saline-Tween-20 three times, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody. The protein bands were made visible using an enhanced chemiluminescence solution in a gel imager. The gray value of the protein bands was calculated using ImageJ software from the National Institute of Health.

Statistical Analysis

The data were analyzed using SPSS 17.0 and GraphPad Prism 8 software. Initially, a normality test was conducted, followed by a variance homogeneity test. If the variance was found to be homogeneous, the least significant difference method was utilized to compare group differences. However, if the variance was found to be uneven, Tamhane's method was employed. A significance level of $p < 0.05$ was used to determine statistical significance.

Results

Effect of the Raw RWI on L-02 Cell Morphology

After 48 h of administration, the cell morphology of the raw product low-concentration group was similar to that of the blank group. Cells in the high-concentration group showed larger cell voids, rounder cells, and more floating cells compared to the blank group, indicating a corresponding increase in cytotoxicity with increasing concentrations in the RWI administration group, as shown in Figure 1.

Effect of “Sweat Soaking Method”-processed RWI on L-02 Cell Proliferation

RWI had toxic effects on human normal hepatocytes L-02 before and after the “sweat soaking method,” but its survival

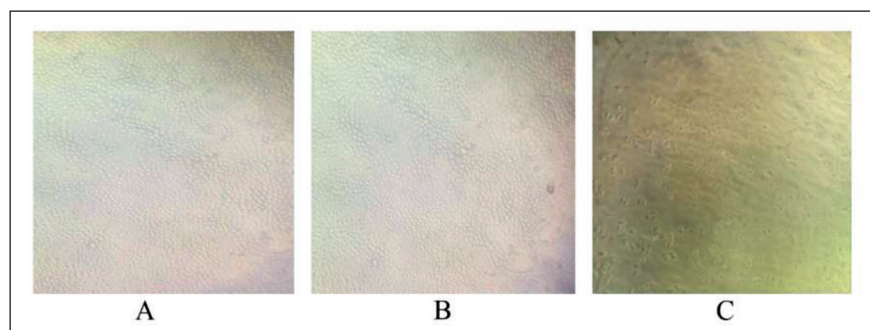


Figure 1. Morphology of Liver Cell L-02 (100×). (A) Blank Control Group, (B) Low-concentration Group, and (C) High-concentration Group.

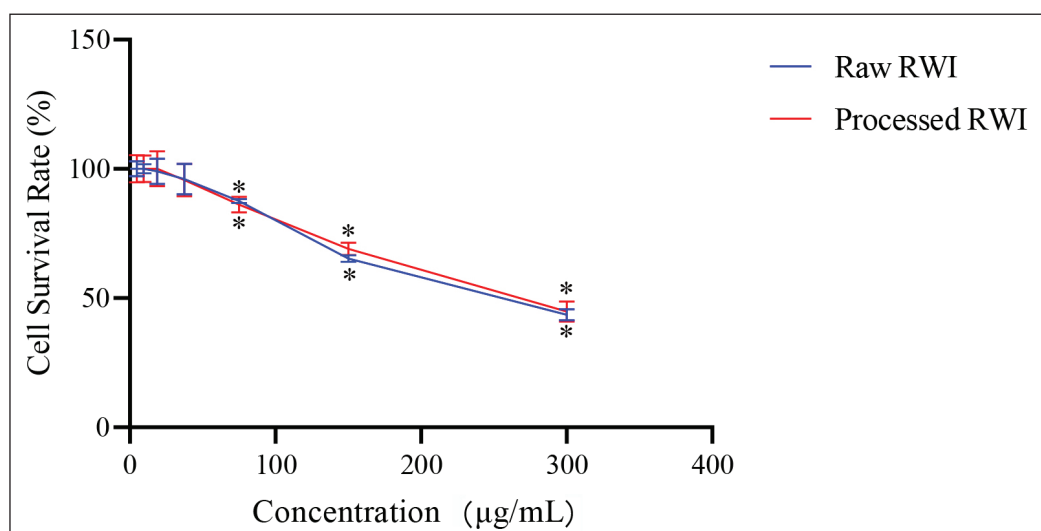


Figure 2. Effect of “sweat soaking method” RWI before and after concoction on the proliferation of L-02 cells.

Note: The outcomes are depicted as the average $\pm$ SD ($n = 5$); * $p < 0.05$ compared to the control group.

Abbreviation: RWI, *Radix Wikstroemia indica*.

rate was 100% at concentrations below 18.75 $\mu\text{g/mL}$. At 75 $\mu\text{g/mL}$ and after, the “sweat soaking method” RWI had an inhibitory effect on the proliferation of L-02 cells compared with the blank group ($p < 0.05$). The IC_{50} values of RWI processed and RWI raw products were 240.3 and 313.9 $\mu\text{g/mL}$, respectively. The results are shown in Figure 2.

Effects of “Sweat Soaking Method”-processed RWI on Body Weight and Liver Organ Index of Rats

The changes in liver index in rats reflected the degree of liver injury (Figure 3). Compared with the control group, the body weight of rats in the administration group increased slowly, and the liver index of rats in the administration group increased to varying degrees. The results indicated that both raw RWI and processed RWI caused liver injury. Compared with the raw RWI group, the liver index of the processed RWI group was lower, but there was no significant difference.

Effect of “Sweat Soaking Method”-processed RWI on Liver Function Factors in the Serum of Rats

ALT, AST, and ALP play a crucial role in assessing liver function in a clinical setting. In cases of liver disease or injury, the blood levels of ALT and AST increase due to their secretion by liver cells, while ALP fails to be released promptly. Consequently, elevated levels of ALT, AST, and ALP are observed in the bloodstream. The findings are displayed in Figure 4. In comparison with the control group, the enzymatic activity of ALP and AST significantly rose in the raw RWI-H, RWI-M, and RWI-L groups ($p < 0.01$). Likewise, the enzymatic activity of ALT increased significantly ($p < 0.05$ or $p < 0.01$) compared to the control group. Comparing the raw RWI-H group and the processed RWI-H group, significant differences were found in the enzymatic activity of ALP, ALT, and AST. Similarly, significant differences were observed in the enzymatic activity of ALP between the raw RWI-M group and the

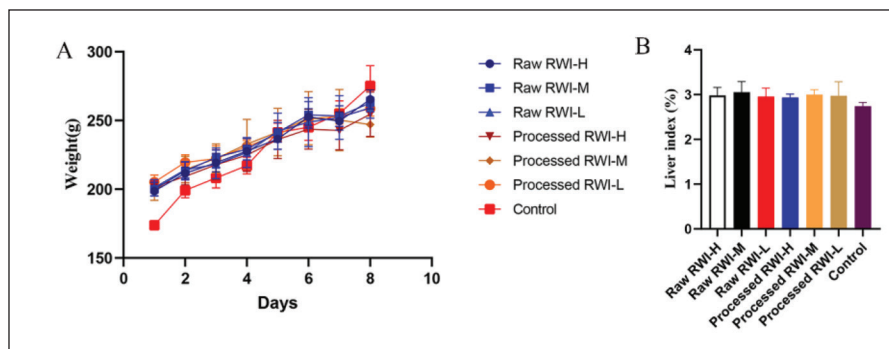


Figure 3. Effects of *Radix Wikstroemia indica* on (A) Body Weight and (B) Liver Index of Rats Before and After Processing.

Note: The results are expressed as the mean $\pm$ SD ($n = 5$).

Abbreviations: RWI, *Radix Wikstroemia indica*; RWI-L, low concentration of RWI (0.1013 g/kg); RWI-M, middle concentration of RWI (0.2025 g/kg); RWI-H, high concentration of RWI (0.4050 g/kg).

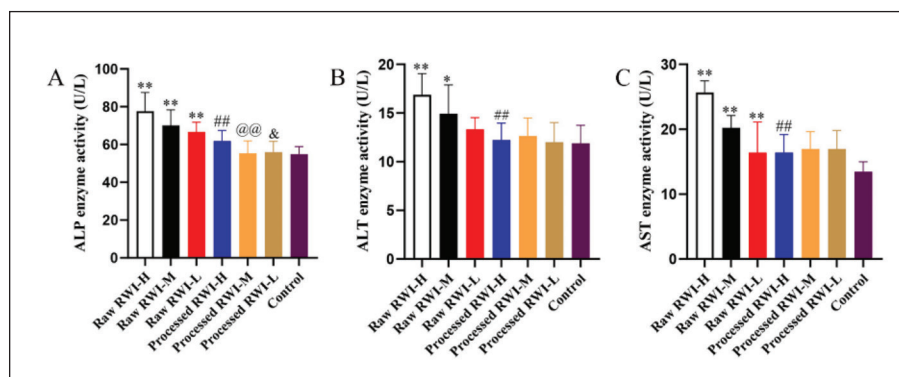


Figure 4. Effect of "Sweat Soaking Method"-processed RWI on the Serum Levels of ALP, AST, ALT in Rats

Note: The results are expressed as the mean $\pm$ SD ($n = 5$). * $p < 0.05$ and ** $p < 0.01$ versus the control group; ### $p < 0.01$ versus the raw RWI-H groups; @@ $p < 0.01$ versus the raw RWI-M groups; * $p < 0.05$ versus the raw RWI-L groups.

Abbreviations: RWI, *Radix Wikstroemia indica*; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine transaminase; RWI-L, low concentration of RWI (0.1013 g/kg); RWI-M, middle concentration of RWI (0.2025 g/kg); RWI-H, high concentration of RWI (0.4050 g/kg).

processed RWI-M group. However, no differences were noted in ALT and AST levels when comparing the raw RWI-M group and the processed RWI-M group. Finally, compared to the raw RWI-L group and processed RWI-L group, the enzymatic activity of ALP was significantly different, and ALT and AST levels had no differences.

Effect of "Sweat Soaking Method"-processed RWI on Rat Liver Tissue Morphology

Histopathological changes in the liver were observed by H&E staining. As shown in Figure 5, the liver cells in the control and processed product groups were arranged neatly, and the intercellular space was clearly visible, while no hepatocyte shrinkage, necrosis, or degeneration and no fibrosis or inflammatory cell infiltration were observed. After 15 days of gavage in the raw RWI group, the hepatocyte gap

was significantly enlarged, the cell gap was unclear, the hepatocytes were shrunken, and necrosis was observed.

Effect of "Sweat Soaking Method"-processed RWI on CYP450 Protein Expression

The expression levels of CYP protein in rat liver tissues were assessed using Western blot analysis, as depicted in Figure 6. Following RWI administration, the expressions of various proteins exhibited varying degrees of increase in comparison to the control group. Notably, the processed RWI-H group displayed significant reductions in the expressions of CYP3A4, CYP1A2, and CYP2C19 when compared to the raw RWI-H group ($p < 0.05$, $p < 0.01$). Similarly, the processed RWI-M group exhibited significant decreases in the expression of CYP2E1, CYP1A2, and CYP2C19 as compared to the raw RWI-M group ($p < 0.05$, $p < 0.01$). Furthermore, the processed RWI-L group demonstrated significant reductions in the

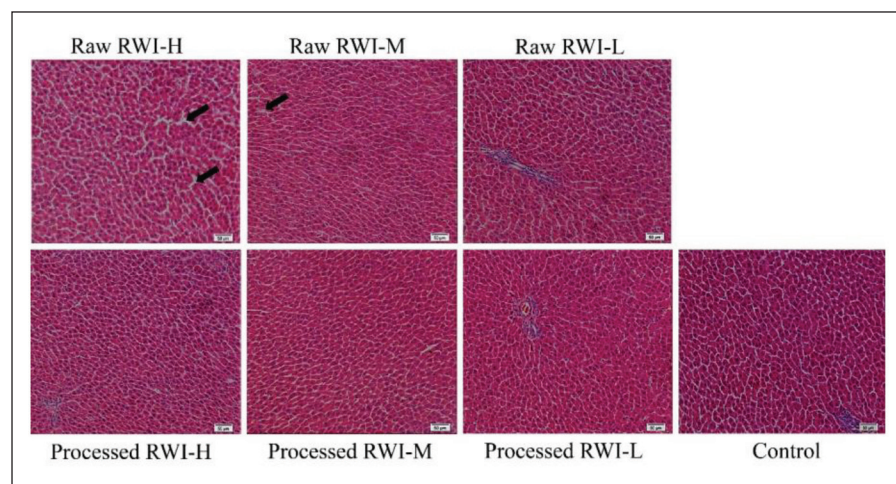


Figure 5. Effect of “Sweat Soaking Method”-processed *Radix Wikstroemia indica* on the Morphology of Liver Tissue in Rats.

Note: Representative microscopy images of hematoxylin and eosin-stained liver tissues are shown (magnification, 200×).

Abbreviations: RWI, *Radix Wikstroemia indica*; RWI-L, low concentration of RWI (0.1013 g/kg); RWI-M, middle concentration of RWI (0.2025 g/kg); RWI-H, high concentration of RWI (0.4050 g/kg).

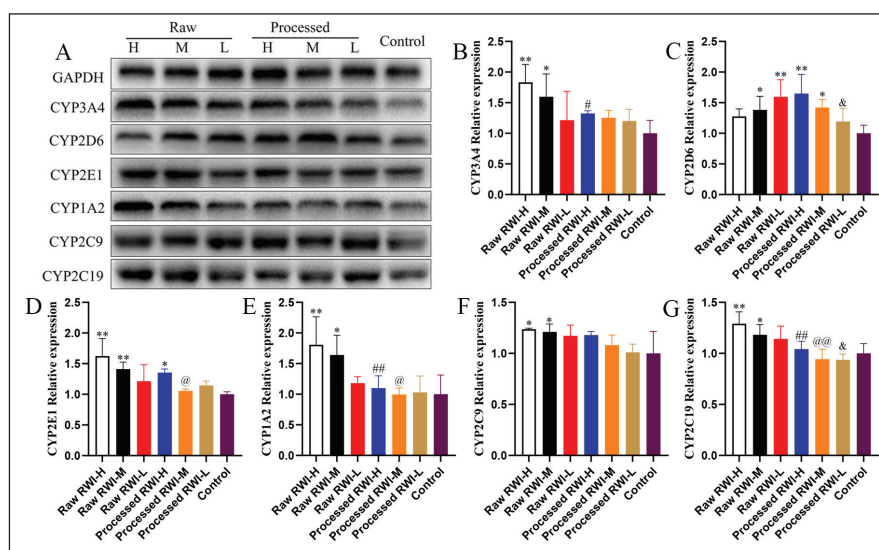


Figure 6. Effect of “Sweat Soaking Method”-processed RWI on the Protein Expression Levels of CYP in the Liver Tissues of Rats.

The Protein Expression Levels of (A) CYP, (B) CYP3A4, (C) CYP2D6, (D) CYP2E1, (E) CYP1A2, (F) CYP2C9, and (G) CYP2C19 Are Shown.

Note: * $p < 0.05$ and ** $p < 0.01$ versus the control group; # $p < 0.05$ and ## $p < 0.01$ versus the raw RWI-H groups; @ $p < 0.05$ and @@ $p < 0.05$ versus the raw RWI-M groups; & $p < 0.05$ versus the raw RWI-L groups.

Abbreviations: CYP, cytochrome P450; RWI, *Radix Wikstroemia indica*; RWI-L, low concentration of RWI (0.1013 g/kg); RWI-M, middle concentration of RWI (0.2025 g/kg); RWI-H, high concentration of RWI (0.4050 g/kg).

expressions of CYP2D6 and CYP2C19 when compared to the raw RWI-L group ($p < 0.05$).

Discussion

The liver, being the most crucial metabolic organ in the human body, remains susceptible to harm caused by numerous internal and external elements. In recent years, the studies on DILI have increased year by year. However, the application

of several drugs against DILI is restricted (Zhao & Xie, 2016; Garrido & Djoudar, 2021). Therefore, the frequent events of liver injury highlight the need for in-depth study and clarification of the mechanisms underlying liver injury and to avoid or reduce the incidence of liver injury in order to improve drug development (Vega et al., 2017).

RWI is a type of toxic TCM. Preliminary studies demonstrated that extracts from different polar parts of RWI could cause different degrees of liver injury in rats. Chinese

folk medicine habits and methods suggested that 1% artificial sweat could be used to prepare RWI, while pharmacodynamics and acute toxicity tests, as evaluation indicators, could be considered the best processing technology to screen out the effects of RWI on liver injury (Feng et al., 2017).

The liver serves as the primary site for substance metabolism and is highly vulnerable to harm caused by drug toxicity. In this study, we focused on investigating the impact of Miao RWI on human normal hepatocytes L-02. Specifically, we aimed to compare the extent of hepatocyte damage before and after employing the “sweat soaking method” for processing RWI. Additionally, we analyzed the IC_{50} values of both raw and processed RWI products to determine any changes in toxicity levels. The findings indicated a decrease in toxicity after the processing of RWI.

ALP, ALT, and AST are significant indicators for the clinical evaluation of liver function (Kamoun et al., 2017). When the liver is injured, increasing amounts of ALT and AST are secreted into the blood circulation by hepatocytes, while ALP cannot be discharged in time. The above events eventually lead to increased ALT, AST, and ALP levels in the blood (Kazemi et al., 2017; Cengiz et al., 2019). Ma et al. (2018) investigated the improving effect of lentinan on ethanol-induced L-02 cells. The findings indicated that anthocyanins had a significant impact on reducing ALT and AST activities induced by ethanol, thereby demonstrating a protective effect. Herein, following gavage administration of RWI in rats for 15 days, the serum levels of ALT, AST, and ALP were measured. The results indicated that raw RWI can cause liver damage. With the increase in dose, there was increased liver damage. The effect of processing RWI by the “sweat soaking method” was more obvious in alleviating liver injury when the dose was higher. This indicates that the “sweat soaking method” for processing RWI can reduce the liver damage caused by raw RWI.

Preliminary pathological evaluation of liver tissues revealed that in the raw RWI-H group, the hepatocyte gap was significantly enlarged, the cell gap was not clear, hepatocytes were shrunken, and the majority of them were necrotic. In addition, liver cell necrosis was observed in the local area of the liver tissue of rats in the raw RWI-M group. No obvious hepatocyte necrosis was observed in the raw RWI-L group, accompanied by reduced infiltration of inflammatory cells. Interestingly, the morphology of the liver tissue was normal in both the control and processed RWI groups. Furthermore, the changes in liver toxicity in rats treated with processed RWI were evaluated using comprehensive liver tissue slices and liver function indexes. The results showed that in rats treated with the “sweat soaking method”-processed RWI, liver injury was significantly reduced compared with those treated with raw RWI.

CYPs are significant enzymes involved in phase I drug metabolism (Fukami et al., 2022), which are mainly expressed in the liver. Their enzymatic activity is closely associated with the occurrence of DILI and serves a key role in the

evaluation of drug-induced hepatotoxicity (Yu et al., 2014). CYPs are involved in the biotransformation of exogenous substances such as drugs, thereby affecting their efficiency, toxicity, and metabolism (Pan et al., 2020; Rendic & Guengerich, 2021; Bao et al., 2020). CYP3A4 (Nelson et al., 2004) is the most abundant CYP isoform enzyme, with levels reaching 60% of the total hepatic CYP. The expression of CYP2D6 in the liver is relatively low, but it is involved in the conversion of 15%–25% of clinical drugs. CYP1A2 (Klein et al., 2010) accounts for about 13% of the total amount of liver drug enzymes. It is an aromatic hydrocarbon-mediated induction effect and plays a very important role in the activation of some pre-toxic substances and pre-carcinogens in the body, such as the transformation of aflatoxin, aromatic amines, halohydrocarbons, and heterocyclic amines. CYP2E1 plays a crucial role in the body, aiding in the metabolism of a wide range of low-molecular weight chemicals. It not only contributes to the breakdown of numerous endogenous and exogenous medications but also facilitates the conversion of various pre-carcinogens into carcinogens and toxic compounds (Kenaan et al., 2013). CYP2C19 and CYP2C9 have high homology, and their DNA and protein sequences are highly correlated, but each has a different role in drug metabolism. The prevalence of DILI has gradually increased in recent years, and CYP450 plays a very important role in drug metabolism. To delve deeper into understanding the mechanism underlying the impact of the “sweat soaking method” on hepatic CYP activity in rats, Western blot analysis was employed at the molecular level to assess the protein expression levels of CYP3A4, CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP2E1 in rat liver tissues detected by Western blot at the molecular level. After the administration of RWI, CYP protein expression changed in all groups of rats. Compared with the RWI raw product group, CYP3A4, CYP2E1, CYP1A2, CYP2C9, and CYP2C19 protein expression decreased, and CYP2D6 protein expression level showed no significant trend.

Due to the complex chemical composition of RWI, the chemical components that cause changes in P450 protein expression levels by the “sweat soaking method” of the RWI concoction need to be studied in depth. The results provide a basis for the mechanism of hepatotoxicity reduction of RWI by the “sweat soaking method” and provide a reference for the safety and clinical application of RWI. When combined with drugs metabolized by CYP enzymes, attention should be paid to the dose and drug–drug interactions to avoid more serious liver injury.

Conclusion

The processed RWI is less toxic than the raw RWI product. The expression levels of six CYP proteins were changed after the “sweat soaking method” of RWI processing, which may be related to the reduced toxicity of RWI processed by the

“sweat soaking method.” This provides some experimental basis for exploring the “sweat soaking method”-processed RWI to reduce hepatotoxicity; however, the regulation of CYP450 enzymes by TCM is extremely complex, and this experiment is not yet sufficient to explain the attenuation of hepatotoxicity by RWI through regulation of CYPs, and further experiments are needed to support our experimental conclusions.

Abbreviations

RWI: Radix *Wikstroemia indica*; CYP: Cytochrome P450; TCM: Traditional Chinese Medicine; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkalinephosphatase; HE: Hematoxylin and eosin (H&E) staining; RWI-L: Low concentration of RWI (0.1013 g/kg); RWI-M: Middle concentration of RWI (0.2025 g/kg); RWI-H: High concentration of RWI (0.4050 g/kg).

Acknowledgments

The experimental equipment, provided by Guizhou University of Traditional Chinese Medicine, is sincerely acknowledged by the authors. Appreciation is expressed to the editors and reviewers for their valuable suggestions, which have significantly enhanced the quality of this manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

Funding for this research was provided by the National Natural Sciences Foundation of China (Grant nos. 81760766 and 82060767), the Guizhou Province Science and Technology Foundation Project (Grant no. Guizhou Scientific Basis ZK [2022] General 461), the Guiyang College of TCM Doctor Startup Fund Project (Grant no. Guizhongyi Doctor Fund [2017] 1), the National training program for innovative backbone talents for TCM (Grant no. Zjjh [2019] 128), the “Thousand” level Innovative Talents Project in Guizhou Province (Grant no. Qrlf [2020] 4), and the Fund project of Guizhou administration of Traditional Chinese Medicine (Grant no. QZYY-2020-083).

Statement of Informed Consent and Ethical Approval

All animal studies were performed as per the procedures of the Ethics Review Committee for Animal Experimentation (Approval No. 20210080).

ORCID iD

Guo Feng  <https://orcid.org/0000-0002-2236-1275>

References

Bao, Y. F., Wang, P., Shao, X. Y., Zhu, J. J., Xiao, J. C., Shi, J., Zhang, L. R., Zhu, H. J., Ma, X. C., Manautou, J. E., & Zhong, X. B. (2020). Acetaminophen-induced liver injury alters expression

and activities of cytochrome P450 enzymes in an age-dependent manner in mouse liver. *Drug Metabolism and Disposition*, 48(5), 326–336. <https://doi.org/10.1124/dmd.119.089557>

Cengiz, M., Yildiz, S. C., Demir, C., Sahin, L. K., Teksoy, O., & Ayhanci, A. (2019). Hepato-preventive and anti-apoptotic role of boric acid against liver injury induced by cyclophosphamide. *Journal of Trace Elements in Medicine and Biology*, 53, 1–7. <https://doi.org/10.1016/j.jtemb.2019.01.013>

Chen, R., Wang, J., Zhang, Y., Tang, S. W., & Zhan, S. Y. (2015). Key factors of susceptibility to anti-tuberculosis drug-induced hepatotoxicity. *Archives of Toxicology*, 89(6), 883–897. <https://doi.org/10.1007/s00204-015-1473-1>

Feng, G., Chen, Y. L., Li, W., Li, L. L., Wu, Z. G., Wu, Z. J., Hai, Y., Zhang, S. C., Zheng, C. Q., Liu, C. X., & He, X. (2018). Exploring the Q-marker of “sweat soaking method” processed Radix *Wikstroemia indica*: Based on the “effect-toxicity-chemicals” study. *Phytomedicine*, 45, 49–58. <https://doi.org/10.1016/j.phymed.2018.03.063>

Feng, G., Li, W., He, X., Zheng, C. Q., Leng, A. B., & Tian, X. F. (2017). Comparison of acute toxicity effects of ethanol extract from different processed products of miao medicine *Wikstroemia indica* on mice. *China Pharmacy*, 28(25), 3536–3540. <https://doi.org/10.6039/j.issn.1001-0408.2017.25.22>

Fukami, T., Yokoi, T., & Nakajima, M. (2022). Non-P450 drug-metabolizing enzymes: Contribution to drug disposition, toxicity, and development. *Annual Review of Pharmacology and Toxicology*, 62, 405–425. <https://doi.org/10.1146/annurev-pharmtox-052220-105907>

Garrido, A., & Djouder, N. (2021). Cirrhosis: A questioned risk factor for hepatocellular carcinoma. *Trends in Cancer*, 7(1), 29–36. <https://doi.org/10.1016/j.trecan.2020.08.005>

Kamoun, Z., Kamoun, A. S., Bougatef, A., Kharrat, R. M., Youssfi, H., Boudawara, T., Chakroun, M., Nasri, M., & Zeghal, N. (2017). Hepatoprotective and nephroprotective effects of sardinella (*Sardinella aurita*) protein hydrolysate against ethanol-induced oxidative stress in rats. *Environmental Science and Pollution Research International*, 24(2), 1432–1441. <https://doi.org/10.1007/s11356-016-7424-4>

Kazemi, S., Kani, S. N. M., Rezazaden, L., Pouramir, M., Kasman, M. G., & Moghadamnia, A. A. (2017). Low dose administration of Bisphenol A induces liver toxicity in adult rats. *Biochemical and Biophysical Research Communications*, 494(1–2), 107–112. <https://doi.org/10.1016/j.bbrc.2017.10.074>

Kanaan, C., Shea, E. V., Lin, H. L., Zhang, H. L., Pratt-Hyatt, M. J., & Hollenberg, P. F. (2013). Interactions between CYP2E1 and CYP2B4: Effects on affinity for NADPH-cytochrome P450 reductase and substrate metabolism. *Drug Metabolism and Disposition*, 41(1), 101–110. <https://doi.org/10.1124/dmd.112.046094>

Klein, K., Winter, S., Turpeinen, M., Schwab, M., & Zanger, U. M. (2010). Pathway-targeted pharmacogenomics of CYP1A2 in human liver. *Frontiers in Pharmacology*, 1, 129. <https://doi.org/10.3389/fphar.2010.00129>

Kullak-Ublick, G. A., Andrade, R. J., Merz, M., End, P., Benesic, A., Gerbes, A. L., & Aithal, G. P. (2017). Drug-induced liver injury: Recent advances in diagnosis and risk assessment. *Gut*, 66(6), 1154–1164. <https://doi.org/10.1136/gutjnl-2016-313369>

Li, N., Zhou, T., Wu, F., Wang, R., Zhao, Q., Zhang, J. Q., Yang, B. C., & Ma, B. L. (2019). Pharmacokinetic mechanisms underlying the detoxification effect of Glycyrrhizae Radix et Rhizoma

- (Gancao): Drug metabolizing enzymes, transporters, and beyond. *Expert Opinion on Drug Metabolism and Toxicology*, 15(2), 167–177. <https://doi.org/10.1080/17425255.2019.1563595>
- Ma, L., Huo, C. Y., Zhang, X. Y., Qin, C. Q., Ren, D. F., & Lu, J. (2018). Protective effect of Letinous edodes foot peptides against ethanol-induced liver injury in L02 cells. *Molecular Medicine Reports*, 18(2), 1858–1866. <https://doi.org/10.3892/mmr.2018.9093>
- Miao, Z., Lai, Y., Zhao, Y. Y., Chen, L. M., Zhou, J. N., Li, C. Y., & Wang, Y. (2021). Protective property of scutellarin against liver injury induced by carbon tetrachloride in mice. *Frontiers in Pharmacology*, 12, 710692. <https://doi.org/10.3389/fphar.2021.710692>
- Nelson, D. R., Zeldin, D. C., Hoffman, S. M. G., Maltais, L. J., Wain, H. M., & Nebert, D. W. (2004). Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*, 14(1), 1–18. <https://doi.org/10.1097/00008571-200401000-00001>
- Pan, X. Q., Zhou, J., Chen, Y., Xie, X. F., Rao, C. L., Liang, J., Zhang, Y., & Peng, C. (2020). Classification, hepatotoxic mechanisms, and targets of the risk ingredients in traditional Chinese medicine-induced liver injury. *Toxicology Letters*, 323, 48–56. <https://doi.org/10.1016/j.toxlet.2020.01.026>
- Rendic, S. (2002). Summary of information on human CYP enzymes: Human P450 metabolism data. *Drug Metabolism Reviews*, 34(1–2), 83–448. <https://doi.org/10.1081/dmr-120001392>
- Rendic, S. P., & Guengerich, F. P. (2021). Human family 1-4 cytochrome P450 enzymes involved in the metabolic activation of xenobiotic and physiological chemicals: An update. *Archives of Toxicology*, 95(2), 395–472. <https://doi.org/10.1007/s00204-020-02971-4>
- Shen, T., Liu, Y. X., Shang, J., Xie, Q., Li, J., Yan, M., Xu, J. M., Niu, J. Q., Liu, J. J., Watkins, P. B., Aithal, G. P., Andrade, R. J., Dou, X. G., Yao, L. F., Lv, F. F., Wang, Q., Li, Y. G., Zhou, X. M., Zhang, Y. X., Zong, P. L., Wan, B., Zou, Z. S., Yang, D. L., Nie, Y. Q., Li, D. L., Wang, Y. Y., Han, X. A., Zhuang, H., Miao, Y. M., & Chen, C. W. (2019). Incidence and etiology of drug-induced liver injury in Mainland China. *Gastroenterology*, 156(8), 2230–2241. <https://doi.org/10.1053/j.gastro.2019.02.002>
- Vega, M., Verma, M., Beswick, D., Bey, S., Hossack, J., Merriman, N., Shah, A., & Navarro, V. (2017). Drug Induced Liver Injury Network (DILIN), the incidence of drug- and herbal and dietary supplement-induced liver injury: Preliminary findings from gastroenterologist-based surveillance in the population of the state of Delaware. *Drug Safety*, 9, 783–787. <https://doi.org/10.1007/s40264-017-0547-9>
- Yu, K., Geng, M. J., Zhang, J., Wang, B. S., Llic, K., & Tong, W. (2014). High daily dose and being a substrate of cytochrome P450 enzymes are two important predictors of drug-induced liver injury. *Drug Metabolism and Disposition*, 42(4), 744–750. <https://doi.org/10.1124/dmd.113.056267>
- Zhang, N. Y., Qi, M., Zhao, L., Zhu, M. K., Guo, J., Liu, J., Gu, C. Q., Rajput, S. A., Krumm, C. S., Qi, D.S., & Sun, L.H. (2016). Curcumin prevents aflatoxin B₁ hepatotoxicity by inhibition of cytochrome P450 isozymes in chick liver. *Toxins*, 8(11), 327. <https://doi.org/10.3390/toxins8110327>
- Zhao, H., & Xie, W. (2016). Current status of the treatment of drug-induced liver injury. *Zhonghua Gan Zang Bing Za Zhi*, 24(11), 804–806. <https://doi.org/10.3760/cma.j.issn.1007-3418.2016.11.002>
- Zheng, C. Q., Feng, G., Li, W., Zhou, Z. R., Xu, Q., Li, Z. P., Yi, D.B., & Li, J.H. (2020). Correlation study of “Dose-effect-toxicity” of miao medicine *Wikstroemia indica* on anti-immune inflammation of mice before and after processed by “Sweat Soaking Method.” *China Pharmacy*, 31(06), 661–665. <https://doi.org/10.6039/j.issn.1001-0408.2020.06.05>