An Integrative Pharmacology Based Analysis of Refined Liuweiwuling Against Liver Injury: A Novel Component Combination and Hepaprotective Mechanism

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Liver disease is a major cause of illness and death worldwide. In China, liver diseases, primarily alcoholic and nonalcoholic fatty liver disease, and viral hepatitis, affect approximately 300 million people, resulting in a major impact on the global burden of liver diseases. The use of Liuweiwuling (LWWL), a traditional Chinese medicine formula, approved by the Chinese Food and Drug Administration for decreasing aminotransferase levels induced by different liver diseases. Our previous study indicated a part of the material basis and mechanisms of LWWL in the treatment of hepatic fibrosis. However, knowledge of the materials and molecular mechanisms of LWWL in the treatment of liver diseases remains limited. Using pharmacokinetic and network pharmacology methods, this study demonstrated that the active components of LWWL were involved in the treatment mechanism against liver diseases and exerted anti-apoptosis and anti-inflammatory effects. Furthermore, esculetin, luteolin, schisandrin A and schisandrin B may play an important role by exerting anti-inflammatory and hepatoprotective effects in vitro. Esculeti and luteolin dose-dependently inhibited H2O2-induced cell apoptosis, and luteolin also inhibited the NF-κB signaling pathway in bone marrow-derived macrophages. Schisandrin A and B inhibited the release of ROS in acetaminophen (APAP)-induced acute liver injury in vitro. Moreover, LWWL active ingredients protect against APAP-induced acute liver injury in mice. The four active ingredients may inhibit oxidative stress or inflammation to exert hepatoprotective effect. In conclusion, our results showed that the novel component combination of LWWL can protect against APAP-induced acute liver injury by inhibiting cell apoptosis and exerting anti-inflammatory effects.

Abbreviations: APAP, Acetaminophen; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BMDMs, Bone marrow-derived macrophages; CFDA, Chinese Food and Drug Administration; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; HSCs, Hepatic stellate cells; LWWL, Liuweiwuling; LPS, lipopolysaccharide; TCM, traditional Chinese medicine.
INTRODUCTION

Liver diseases lead to severe public health problems owing to their high prevalence worldwide and poor long-term clinical outcomes, including cirrhosis and hepatocellular carcinoma (Wang et al., 2014). Different types of liver diseases, including chronic hepatitis B virus infection, alcoholic liver disease, nonalcoholic fatty liver disease, autoimmune liver disease, and drug-induced liver disease, potentially threaten a large proportion of the global population. In China, approximately 300 million people are affected by liver diseases, which has a major impact on the global burden of liver diseases (Cui and Jia, 2013; Zhang et al., 2016; Sarin et al., 2020).

In China, many patients with liver diseases opt for traditional Chinese medicine (TCM) as an alternative or complementary therapy. In China, the use of Liuweiwuling (LWWL), a TCM compound, has been approved by the Chinese State Food and Drug Administration (CFDA) for decreasing aminotransferase levels induced by different liver diseases (Xin et al., 2009; Du and Jaeschke, 2016; Branch of Hepatobiliary Diseases, CMCA, 2020). LWWL is constituted by the following six traditional Chinese herbs: Schisandrae chinensis fructus, Fructus Ligustri Lucidi, Forsythiae fructus, Curcumae rhizoma, Perennial sow thistle, and Ganoderma spore. In particular, clinical studies have confirmed that LWWL has definite therapeutic effects on liver fibrosis (Wang et al., 2018; Li et al., 2020). The dysregulated inflammatory responses, oxidative stress and cell live/death have been widely documented as primarily involved mechanisms underlying liver diseases (Zhang and Schuppan, 2014; Li et al., 2019). Emerging evidence suggests that TCM directly regulates the production of inflammatory cytokines and chemokines to improve inflammation and liver injury. (Wang C. et al., 2016). Our previous study indicated that LWWL could attenuate hepatic fibrosis via the modulation of TGF-β1 and NF-κB signaling pathways in rat models, based on bile duct ligation (BDL)- and CCl4-induced hepatic fibrosis (Liu et al., 2018a; Liu et al., 2018b). However, it is still unclear whether the underlying mechanisms and core signaling pathways mediate the multi-linked and multi-targeted effects of LWWL against liver diseases.

Integrative pharmacology can focus on predicting potential targets, pathways, and consequences and may provide clues for designing subsequent drug studies. This study used an integrative pharmacology approach to understand the systemic, liver disease-related, and molecular effects of LWWL. Our experimental results largely validated the mechanism of action of LWWL, as predicted by the integrative pharmacology analysis.

MATERIALS AND METHODS

Reagents and Antibodies

Dimethyl sulfoxide and ultrapure lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Munich, Germany). Apigenin, esculetin, gomisir N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specuezhenede, schisandrin, luteolin, quinic acid, curcuminol, and acetaminophen (APAP) were obtained from MCE (New Jersey, NJ, United States). Anti-mouse-IL-1β, anti-mouse-NLRP3, and anti-mouse-ASC antibodies were purchased from Santa Cruz Biotechnology (Beijing, China). Anti-mouse-caspase-1 p45, anti-mouse-pro-IL-1β, and anti-GAPDH antibodies were purchased from Proteintech (Chicago, IL, United States). MitoSOX was purchased from Invitrogen (Carlsbad, CA, United States).

Preparation and Analysis of LWWL by LC-MS/MS

The formula of LWWL per dose is listed in Supplementary Table S1. Briefly, the LWWL was accurately measured 1 g in a 25 ml volumetric flask and then dissolved in methanol solution. The sample was kept at 40°C for 1 h after it was sonicated for 5 min. Subsequently, the LWWL extraction was centrifuged at 4°C, 12,000 rpm for 15 min, and then the supernatant was filtered with 0.22 μm filter membrane. Finally, the sample filtrate solution was used to UHPLC analysis. To verify the metabolite components in LWWL, chromatographic analysis was performed using a Triple TOF 5600- quadrupole-LC/MS system (SCIEX Technologies, United States). The SHISEIDO CAPCELL PAK ADME column (2.1 mm*150 mm, 4.6 um) was performed in LC-MS/MS analysis. The flow rate was set at 0.4 ml/min and the sample injection volume was 5 μL. The mobile phase conditions were as follows: mobile phase A was 0.1% formic acid in water, and the mobile phase B was acetonitrile. The multistep linear elution gradient program was as follows: 0 → 0.5 min, 90 → 60% A; 0.5 → 4.0 min, 60 → 10% A; 4 → 9.0 min, 10 → 10% A; 9.0 → 12.01 min, 10 → 90% A; 12.01 → 15 min, 90 → 90% A. Then, A quality control sample was employed to optimize the UHPLC-Q-TOF/MS conditions. MS was performed using Triple TOF 5600-quadrupole-LC/MS with an electrospray ionization source in both positive and negative modes. The electrospray source parameters were fixed as follows: MS data were gathered in the full scan mode from m/z 50–1,250 with a scan rate of 1 spectra/s. The electrospray capillary voltage was 5.5 kV in the negative mode and 5.0 kV in the positive mode. The atomization temperature of the ion source was set to 550°C. The nebulizer pressure was set to 50 psi (G51) and 50 psi (G52). Air curtain gas at 35 psi and cluster voltage DP at 80 V.

Animals

Male Sprague-Dawley rats (weight: 180–220 g) and male C57BL/6 mice (6–8-weeks old) were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). All animals were maintained under 12-h light/dark conditions at 22–24°C with unrestricted access to food and water for the duration of the experiment. All animal
In this study, experiments were conducted according to the guidelines for care and use of laboratory animals, and the study protocol was approved by the Animal Ethics Committee of the Fifth Medical Centre, Chinese People’s Liberation Army (PLA) General Hospital (animal ethics committee approval number: IACUC-2017-003).

**Human Samples and Study Design**

The study protocol was approved by the Medical Ethics Committee of the Fifth Medical Center, General Hospital of PLA (No.2015180D), registered at ClinicalTrials.gov. All volunteers in this study were self-reported as Han Chinese and provided written informed consent. Blood samples were collected from each volunteer who studied or worked at the Fifth Medical Center, General Hospital of PLA (Beijing, China). According to the overall study design, all healthy volunteers took LWWL three times per day according to the specification for 2 consecutive days, and they were prohibited from smoking and consuming alcohol, tea, and coffee drinks.

**Sample Preparation**

Rat plasma, urine, liver tissue homogenate, and human plasma (500 µL) were added to acetonitrile (1,500 µL). The protein was precipitated by shaking for 1 min on a shaker. Centrifugation was performed at 13,000 rpm for 10 min. The supernatant was dried at 45°C under nitrogen. Further, 100 µL methanol:water (V/v = 1:1) was used to redissolve the samples, and 5 µL of the sample was injected for analysis.

**Network Construction and Analysis**

The components detected in the blood samples from volunteers and the blood, urine, and tissue samples from animals were obtained by their CAS numbers from the open online databases TCM-SP and Chemspider. The putative targets of these compounds were predicted by PharmMapper (http://59.78.96.61/pharmmapper/) with a fit score greater than 3.0 and Z-score greater than 0. Multiple targets associated with liver diseases were collected by keyword-based searches in the Online Mendelian Inheritance in Man database; the disease names and keywords were related to the Medical Subject Headings database from the NIH and the U.S. National Library of Medicine. All collected targets were converted into UniProt IDs, and protein–protein interactions between them were screened from the Database of Interacting Proteins. Thereafter, the components and their putative targets, liver disease-related targets, and interactive proteins were combined to construct a compound–target–disease network, and Cytoscape V3.7.2 was applied to visualize and analyze the network. The topological features of each node in the network were calculated using Network Analyzer in the Cytoscape software, and the most probable disease targets on which LWWL components or their metabolites might act were screened with three topologic parameters (“Degree,” “Betweenness centrality,” and “Closeness centrality”). Only hubnodes (“Degree” greater than 2-fold of the median value) with higher “Betweenness centrality” and “Closeness centrality” (above the median value) values were identified as candidate targets of LWWL. All candidate targets were supplied to the Database for Annotation, Visualization and Integrated Discovery for Gene Ontology (GO) and pathway analysis, and only the results with Bonferroni adjustment p-values less than 0.01 were used for further analysis.

**Cell Culture**

Bone marrow-derived macrophages (BMDMs) were isolated from the femoral bone marrow of 10-week-old female C57BL/6 mice and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 50 ng/ml murine macrophage colony-stimulating factor. L02 and HepaG2 cells were grown in DMEM supplemented with 10% FBS and 1% P/S. All cells were cultured in a humidified 5% (v/v) CO₂ atmosphere at 37°C.

**Cell Viability Assay**

L02 cells were seeded at 8.5 × 10⁴ cells/well onto 96-well plates overnight. The cells were incubated at 37°C, followed by treatment with the active components of LWWL (apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol) for 24 h. Thereafter, the medium was replaced with DMEM containing CCK-8 solution for 30 min. The optical density was determined at a wavelength of 450 nm.

**NF-κB Signaling Pathway Activation**

BMDMs were seeded at 5 × 10⁵ cells/well onto 24-well plates overnight and then treated with apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol for 4 h. BMDMs were then stimulated with LPS (50 ng/ml) for 1 h. The proteins were then analyzed by immunoblotting.

**H₂O₂-Induced Hepatocyte Injury in vitro**

L02 cells were seeded at 8.5 × 10⁴ cells/well onto 24-well plates overnight. The next day, the medium was replaced with DMEM containing apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol; cells were incubated for 1 h and then stimulated with H₂O₂ for 12 h. The cell supernatants were collected, digested, and rinsed with phosphate-buffered saline. An Annexin V-FITC Apoptosis Detection Kit (BD, New York, United States) was used to detect the apoptosis of L02 cells by flow cytometry.

**APAP-Induced Hepatocyte Injury in vitro**

HepaG2 cells were seeded at 0.25 × 10⁵ cells/well onto 24-well plates overnight. The following day, the medium was replaced with DMEM containing apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol; cells were incubated for 1 h.
and then stimulated with APAP (20 mM) for 12 h. The cell supernatants were collected, digested, rinsed with Hank’s balanced salt solution, and stained with 4 μM MitoSOX red mitochondrial superoxide indicator (Invitrogen) at 37°C for 15 min. Thereafter, the cells were washed again with Hanks’ balanced salt solution and assayed by flow cytometry using a BD FACSCanto™ II cell analyzer (Franklin Lakes, NJ, United States) (Liu et al., 2021).

**Western Blotting**

Protein extraction and western blotting assays on cell culture supernatant and whole cell lysis were performed as described previously (Gao et al., 2021).

**Enzyme-Linked Immunosorbent Assay**

ELISA measurements of mouse IL-1β, TNF-α, and IL-6 (Dakewe, Beijing, China) levels were performed in accordance with the manufacturer’s instructions.

**Serum Biochemistry**

Serum ALT, AST, DBIL, and TBA levels were determined using a commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

**Animal Experiments**

After 7 days of adaptive breeding, mice were randomly divided into the following seven groups (n = 6): control group, APAP group (300 mg/kg), APAP + SA (schisandrin A [159.78 mg/kg]), APAP + SB (schisandrin B [162.43 mg/kg]), APAP + E (esculetin [0.93 mg/kg]), APAP + L (luteolin [8.56 mg/kg]) and APAP + SSEL group (schisandrin A [159.78 mg/kg] + schisandrin B [162.43 mg/kg] + esculetin [0.93 mg/kg] + luteolin [8.56 mg/kg]). Schisandrin A, schisandrin B, esculetin, and luteolin were injected into the mice for 6 consecutive days. The normal and APAP groups were treated with the vehicle in the same manner. One hour after the final administration of schisandrin A, schisandrin B, esculetin, and luteolin were injected into the mice for 6 consecutive days. The blood was collected from the orbital vein at 1, 2, 4, 8, 12, and 24 h. Thereafter, the serum was fully mixed after centrifugation (Supplementary Figure S2A). We also detected active components in the liver of the rats (Supplementary Figure S2B). During the animal gavage, the urine of rats was collected in a metabolic cage (Supplementary Figure S3). Subsequently, we used UPLC/Q-TOF-MS to analyze the active components of LWWL in the human body. Volunteers took LWWL for 2 consecutive days (three tablets at a time, three times per day). After the last dose, one of the volunteer’s blood was collected from the vein at 1, 2 h, the other one of the volunteers’ blood was collected from the vein at 4, 8 h, and the serum was then completely mixed after centrifugation (Supplementary Figure S4). Overall, as a result, apigenin, esculetin, gomisin N, schisandanhol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol were identified according to the described chromatographic conditions.

**RESULTS**

**Identification of the Active Components of LWWL in Rats and Humans**

Firstly, we analysis the active components of LWWL by LC-MS/MS. As shown in Supplementary Figure S1 and Supplementary Table S1, a total of 20 active compounds were confirmed in LWWL. To examine the effective components of LWWL in vivo, we used UPLC/Q-TOF-MS for qualitatively analyzing the active components of LWWL in rats and humans. Rats were gavaged with LWWL (6.4 g/kg) for 2 consecutive days. After the last administration, the blood was collected from the orbital vein at 1, 2, 4, 8, 12, and 24 h. Thereafter, the serum was fully mixed after centrifugation (Supplementary Figure S2A). We also detected active components in the liver of the rats (Supplementary Figure S2B). During the animal gavage, the urine of rats was collected in a metabolic cage (Supplementary Figure S3). Subsequently, we used UPLC/Q-TOF-MS to analyze the active components of LWWL in the human body. Volunteers took LWWL for 2 consecutive days (three tablets at a time, three times per day). After the last dose, one of the volunteer’s blood was collected from the vein at 1, 2 h, the other one of the volunteers’ blood was collected from the vein at 4, 8 h, and the serum was then completely mixed after centrifugation (Supplementary Figure S4). Overall, as a result, apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol were identified according to the described chromatographic conditions.

**Prediction of the Effective Targets of LWWL With Network Pharmacology**

In total, 18 compounds of LWWL, 289 drug targets, 358 liver disease-related targets, and 785 interactive proteins were
Table 1: The targets related to liver diseases.

| No | Uniprot IDs | Degree | Closeness Centrality | Betweenness Centrality | Direct target |
|----|-------------|--------|----------------------|------------------------|---------------|
| 1  | P10275      | 43     | 0.276                | 0.108                  | Direct        |
| 2  | P06213      | 24     | 0.293                | 0.059                  | Direct        |
| 3  | P00533      | 23     | 0.269                | 0.049                  | Direct        |
| 4  | P03372      | 23     | 0.263                | 0.033                  | Direct        |
| 5  | P04150      | 21     | 0.267                | 0.025                  | Direct        |
| 6  | P15086      | 19     | 0.262                | 0.025                  | Direct        |
| 7  | P19793      | 19     | 0.250                | 0.019                  | Direct        |
| 8  | P13926      | 18     | 0.252                | 0.024                  | Direct        |
| 9  | P35685      | 15     | 0.262                | 0.016                  | Direct        |
| 10 | Q02750      | 15     | 0.257                | 0.009                  | Direct        |
| 11 | P12931      | 14     | 0.263                | 0.026                  | Direct        |
| 12 | P31749      | 14     | 0.263                | 0.022                  | Direct        |
| 13 | P01112      | 14     | 0.243                | 0.017                  | Direct        |
| 14 | P00519      | 12     | 0.269                | 0.017                  | Direct        |
| 15 | P02879      | 12     | 0.253                | 0.002                  | Direct        |
| 16 | Q48641      | 10     | 0.278                | 0.043                  | Direct        |
| 17 | P13501      | 10     | 0.234                | 0.037                  | Direct        |
| 18 | P08069      | 10     | 0.226                | 0.014                  | Direct        |
| 19 | P35221      | 10     | 0.249                | 0.011                  | Direct        |
| 20 | P20339      | 10     | 0.255                | 0.009                  | Direct        |
| 21 | P55055      | 10     | 0.241                | 0.008                  | Direct        |
| 22 | P22830      | 10     | 0.268                | 0.003                  | Direct        |
| 23 | Q06187      | 10     | 0.236                | 0.002                  | Direct        |
| 24 | P08581      | 9      | 0.252                | 0.006                  | Direct        |
| 25 | P11766      | 9      | 0.266                | 0.002                  | Direct        |
| 26 | P04637      | 60     | 0.285                | 0.147                  | Indirect      |
| 27 | P09403      | 33     | 0.236                | 0.053                  | Indirect      |
| 28 | P38398      | 30     | 0.250                | 0.049                  | Indirect      |
| 29 | Q06653      | 28     | 0.220                | 0.017                  | Indirect      |
| 30 | Q09619      | 22     | 0.244                | 0.029                  | Indirect      |
| 31 | P35222      | 22     | 0.226                | 0.024                  | Indirect      |
| 32 | P19838      | 21     | 0.221                | 0.013                  | Indirect      |
| 33 | Q04206      | 19     | 0.235                | 0.034                  | Indirect      |
| 34 | P01106      | 19     | 0.255                | 0.028                  | Indirect      |
| 35 | Q07812      | 15     | 0.247                | 0.015                  | Indirect      |
| 36 | Q14200      | 15     | 0.240                | 0.012                  | Indirect      |
| 37 | P05412      | 13     | 0.248                | 0.019                  | Indirect      |
| 38 | Q06216      | 12     | 0.225                | 0.016                  | Indirect      |
| 39 | Q16665      | 12     | 0.207                | 0.014                  | Indirect      |
| 40 | P15692      | 12     | 0.211                | 0.007                  | Indirect      |
| 41 | P09874      | 11     | 0.236                | 0.030                  | Indirect      |
| 42 | P25445      | 11     | 0.229                | 0.017                  | Indirect      |
| 43 | Q00255      | 11     | 0.215                | 0.006                  | Indirect      |
| 44 | Q09472      | 10     | 0.247                | 0.018                  | Indirect      |
| 45 | Q55816      | 10     | 0.224                | 0.011                  | Indirect      |
| 46 | Q13315      | 9      | 0.237                | 0.014                  | Indirect      |
| 47 | P27986      | 9      | 0.235                | 0.007                  | Indirect      |
| 48 | Q07820      | 9      | 0.226                | 0.006                  | Indirect      |
ELISA assay kit. Taken together, these results demonstrate that luteolin inhibits the NF-κB signaling pathway in BMDMs in vitro.

Luteolin and Esculetin Suppress H₂O₂-Induced Apoptosis

Furthermore, the protective effects of the active constituents of LWWL on hepatocytes were examined using the Annexin V-FITC Apoptosis Detection Kit. We selected a typical hydrogen peroxide (H₂O₂)-induced cell apoptosis model. The results demonstrated that luteolin and esculetin treatment significantly inhibited H₂O₂-induced apoptosis of L02 cells. Thereafter, we treated H₂O₂-induced apoptosis of L02 cells with different concentrations of esculetin or luteolin.

The results showed that esculetin and luteolin could dose-dependently inhibit H₂O₂-induced cell apoptosis at 5, 10, and 20 μmol/L, and esculetin and luteolin could also inhibit the total apoptotic cells, early apoptotic cells, and late apoptotic cells (Figure 5). These results indicated that esculetin and luteolin could protect against hepatocyte injury.

Schisandrin A And Schisandrin B Inhibit The Release Of Reactive Oxygen Species

Oxidative stress and mitochondrial dysfunction play important roles in the pathogenesis of APAP-induced acute liver injury (Miele et al., 2007). Therefore, we examined the effect of active components of LWWL on the production of ROS induced by...
FIGURE 3 | Effects of the active components of LWWL on cell viability. The viability of L02 cells treated with the active components of LWWL for 24 h was determined. Data are presented as mean ± SD using biological samples.
FIGURE 4 | Luteolin inhibits the NF-κB signaling pathway. (A) Western blotting of pro IL-1β, NLRP3, Casp-1 p45, ASC, and GAPDH in BMDMs treated with apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisandrin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol (40 μM) for 4 h and then stimulated with LPS (50 ng/ml) for 1 h. (B,C) ELISA of TNF-α (B) and IL-6 (C) in SN from samples described in A. (D) Western blotting of pro IL-1β, NLRP3, Casp-1 p45, ASC, and GAPDH in BMDMs treated with luteolin for 4 h and then stimulated with LPS (50 ng/ml) for 1 h. (E,F) ELISA of TNF-α (E) and IL-6 (F) in SN from samples described in D. GAPDH served as a loading control. Data are represented as the mean ± SD using biological samples.
FIGURE 5 | Luteolin and esculetin suppress \( H_2O_2 \)-induced apoptosis. (A) Apoptosis of L02 cells treated with apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumenol (40 \( \mu \)M) and then exposed to APAP, as detected by flow cytometry. (B) The percentage of early apoptotic cells from samples described in A. (C) The percentage of total apoptotic cells from samples described in A. (D) Apoptosis of L02 cells treated with esculetin or luteolin (5, 10, and 20 \( \mu \)M) and then exposed to APAP, as detected by flow cytometry. (E–G) The percentage of early apoptotic cells (E), late apoptotic cells (F), and total apoptotic cells (G) treated with luteolin (5, 10, and 20 \( \mu \)M). (H–J) The percentage of early apoptotic cells (H), late apoptotic cells (I), and total apoptotic cells (J) treated with esculetin (5, 10, and 20 \( \mu \)M). Data are represented as the mean ± SD using biological samples. The significance of the differences was analyzed using unpaired Student’s t-test: 
\* \( p < 0.05 \), \*\* \( p < 0.01 \), \*\*\* \( p < 0.001 \) vs. the control, NS, not significant.
APAP \textit{in vitro}. Our results showed that several active components, especially schisandrin A and schisandrin B, could inhibit the release of ROS after APAP treatment (Figures 6A,B). As shown in Figures 6C–E, schisandrin A and schisandrin B dose-dependently inhibited the production of mitochondrial ROS. Therefore, schisandrin A and schisandrin B exert protective effects against liver injury by inhibiting the release of ROS \textit{in vitro}.

FIGURE 6 | Schisandrin A and schisandrin B inhibit the release of ROS. (A) HepaG2 cells were treated with apigenin, esculetin, gomisin N, schisandrin, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisantherin B, specnuezhenide, luteolin, quinic acid, and curcumenol (40 μM) before being stimulated with APAP. HepaG2 were loaded with MitoSOX red mitochondrial superoxide indicator (Ex/Em: 510/580 nm). After staining and washing, flow cytometry was conducted to test mtROS production. (B) Percentage of ROS-positive cells in HepaG2 cells from samples described in A. (C) The production of mtROS was detected by flow cytometry in HepaG2 cells treated with schisandrin A or schisandrin B (10, 20, and 40 μM). (D,E) Percentage of ROS-positive cells in HepaG2 cells pretreated with schisandrin A (D) or schisandrin B (E) (10, 20, and 40 μM) and then stimulated with APAP, followed by staining with MitoSox. Data are represented as the mean ± SD using biological samples. The significance of the differences was analyzed using unpaired Student’s t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control, NS, not significant.
Combination of LWWL active ingredients protect against APAP-induced acute liver injury in vivo. (A–G) Eight-week-old C57BL/6 male mice were administered with a vehicle, schisandrin A, schisandrin B, esculetin, and luteolin every day by gavage for 7 days. Further, 1 h after the final gavage of schisandrin A, schisandrin B, esculetin and luteolin, mice in all groups (except the control group) were administered with APAP (300 mg/kg) by a single intraperitoneal injection. (A–D) Serum levels of ALT (A), AST (B), DBIL (C) and TBA (D) ELISA of IL-1β (E) and TNF-α (F). (G) H&E staining. The significance of the differences was analyzed using unpaired Student’s t-test: #p < 0.05, ##p < 0.01, ###p < 0.001 vs. control group; *p < 0.05, **p < 0.01, ***p < 0.001 vs. the APAP group, NS, not significant.
Combination of LWWL Active Ingredients Protect APAP-Induced Acute Liver Injury in vivo

To test whether the combination of LWWL active ingredients (schisandrin A, schisandrin B, esculetin and luteolin) protects against acute liver injury in vivo, we chose APAP to induce acute liver injury. Figures 7A–D showed that the serum levels of ALT, AST, DBIL, and TBA in the APAP-treated group were higher than those in the control group. Schisandrin A, schisandrin B, esculetin and luteolin prevented the increase in serum ALT and AST levels compared with those in the APAP group. Consistent with the results of ALT and AST, treatment with the combination of schisandrin A, schisandrin B, esculetin and luteolin attenuated the expression of DBIL and TBA compared with that in the APAP group. We also detected that the combination of schisandrin A, schisandrin B, esculetin and luteolin influenced the production of IL-1β and TNF-α (Figures 7E,F). As expected, the combination of schisandrin A, schisandrin B, esculetin and luteolin treatment significantly decreased the production of IL-1β and TNF-α in vivo. Moreover, histopathologic studies showed that the combination of LWWL active ingredients treatment definitely alleviated the liver failure with reduced hepatocyte necrosis and liver cell degeneration (Figure 7G). Collectively, these results suggest that the four LWWL active ingredients play an important role in the regulation of hepatoprotective activity.

DISCUSSION

At present, TCM has been confirmed to have significant therapeutic efficacy for complex diseases by exerting pharmacological effects in a multi-component and multi-target manner (Wang et al., 2018). Some Chinese patent medicines, such as LWWL and San-Cao granule, have been used clinically to treat liver diseases for many years (Wei et al., 2016). However, unclear active medicinal ingredients and mechanisms of Chinese patent medicine restrict the widespread use of TCM.

In China, LWWL, a Chinese medicine formula, is widely used to treat liver injury induced by chronic hepatitis B (Lei et al., 2015). Previous clinical and experimental studies showed that LWWL inhibits liver injury and reverses the progression of hepatic fibrosis (Liu et al., 2018a; Liu et al., 2018b). Hepatocyte injury, a primary inducer of hepatic fibrosis, can increase inflammation and activation of hepatic stellate cells (HSCs) (Ai et al., 2021). Thus, reversal of hepatocyte injury is an important way to prevent and treat hepatic fibrosis (Lee et al., 2015). Our previous studies indicated that LWWL could significantly suppress HSC activation and reverse histological fibrosis and liver injury (Liu et al., 2018b). In addition, LWWL regulates the expression of inflammatory cytokines by inhibiting the activation of NF-κB p65 and phosphorylation of IκBα (Liu et al., 2018a).

In this study, we designed an integrated strategy to explore and identify the components of LWWL by integrating network pharmacology with systems biology. We successfully employed this strategy to demonstrate the preventive effect and elementary mechanisms of LWWL against inflammatory diseases of the liver.

We used UPLC/Q-TOF-MS to analyze the active components of LWWL in vivo. As a result, apigenin, esculetin, gomisin N, schisanhenol, schisandrin A, schisandrin B, anwulignan, schisantherin A, schisandrin B, specnuezhenide, schisandrin, luteolin, quinic acid, and curcumin were identified. Thereafter, we predicted the effective targets of LWWL with network pharmacology. According to network pharmacology, LWWL can be used to treat liver diseases owing to its anti-inflammatory and anti-apoptotic functions.

NF-κB is one of the most important transcription factors and plays a role in the expression of pro-inflammatory genes, such as cytokines, chemokines, and adhesion molecules (Oeckinghaus and Ghosh, 2009; Aziz et al., 2018). NF-κB and upstream kinase cascades are known to have promotional roles in inflammation (Reuter et al., 2010). Inhibition of cellular inflammation has been considered a promising approach to lower the risk of inflammation-driven diseases (Rakariyatham et al., 2018). Luteolin is an abundant flavone found in LWWL, and in vitro and in vivo experiments have revealed its anti-inflammatory activity. Luteolin exerts its anti-inflammatory effects by altering the NF-κB signaling pathway (Aziz et al., 2018). In addition, cytokine regulation is crucial because cytokines are key modulators of both acute and chronic inflammation (Turner et al., 2014; Aziz et al., 2018). Moreover, luteolin significantly attenuates TNF-α-induced intracellular ROS generation (Xia et al., 2014). As expected, luteolin is an anti-inflammatory active component of LWWL and exerts its anti-inflammatory effects by inhibiting NF-κB signaling and regulating inflammatory mediators such as IL-6 and TNF-α.

Apoptosis is a biochemical process strictly controlled by an organism to scavenge dead cells through natural physiological methods (Chen et al., 2017). ROS are normal metabolites of various redox reactions in cells (Kalyanaraman et al., 2018; Gao et al., 2019). Hydrogen peroxide, the major ROS contributor in cells, is an intermediate product of oxidative metabolism in the body, affecting the structure and function of nucleic acids, membrane phospholipids, or proteins, resulting in cell damage and death (Pessayre et al., 2002). It is commonly used to evaluate antioxidant capacity, particularly for evaluating ROS scavenging capacity in cells (Wang L. et al., 2016). This study investigated whether the 14 active constituents of LWWL exert protective effects against H2O2-induced oxidative damages; we found that esculetin and luteolin could dose-dependently inhibit H2O2-induced cell apoptosis. Therefore, esculetin and luteolin are both anti-apoptosis active components of LWWL.

Some studies indicated that mitochondrial dysfunction and altered mitochondrial ROS levels affect signaling pathways, contributing to liver fibrogenesis, inflammation, and innate immune responses to viral infections (Pessayre et al., 2002; Gao and Bataller, 2011; Tell et al., 2013; Koliaki et al., 2015; Vacca et al., 2015). *Schisandra chinensis* is a commonly used traditional herbal medicine and nutritive food in many countries (Sőza et al., 2017) and has pharmacological effects in stimulating immune response and anti-inflammatory effects (Chen et al., 2019; Zhu et al., 2019). Several studies identified that the whole extract and bioactive lignans of *Schisandra chinensis* protect...
Clinical efficacy using an integrated approach. The strategy proposed in this study provides a better understanding of the pharmacological effects of LWWL from the perspective of its bioactive ingredients. The development of TCM formulae as a complementary and alternative therapy for liver inflammation is extremely urgent. Therefore, further development and incorporation of many disciplines (such as biochemistry, molecular biology, and bioinformatics) are necessary to elucidate the curative and biological mechanisms of TCM. The strategy proposed in this study provides a new method to identify modern indications for TCM with notable mechanistic studies and analyzed the data. RL and YA performed most of the experiments. HY performed the animal study was reviewed and approved by the All animal ethics committee approval number: IACUC-2017-003.

CONCLUSIONS

In this study, we show that luteolin could inhibit the NF-κB signaling pathway in BMDMs, Esculetin and luteolin also dose-dependently suppressed H2O2-induced cell apoptosis, schisandrin A and schisandrin B decreased the serum levels of ALT, AST, DBIL, and TBA, and the combination of active ingredients significantly decreased the production of IL-1β and TNF-α. These data suggest that the combination of LWWL active ingredients could effectively protect against APAP-induced liver injury. This study provides a better understanding of the pharmacological effects of LWWL from the perspective of its bioactive ingredients. The development of TCM formulae as a complementary and alternative therapy for liver inflammation is extremely urgent. Therefore, further development and incorporation of many disciplines (such as biochemistry, molecular biology, and bioinformatics) are necessary to elucidate the curative and biological mechanisms of TCM. The strategy proposed in this study provides a new method to identify modern indications for TCM with notable clinical efficacy using an integrated approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The study protocol was approved by the Medical Ethics Committee of the Fifth Medical Center, General Hospital of PLA (NO.2017056D), registered at ClinicalTrials.gov. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Ethics Committee of the Fifth Medical Centre, Chinese People’s Liberation Army (PLA) General Hospital (animal ethics committee approval number: IACUC-2017-003).

AUTHOR CONTRIBUTIONS

ZB and MN supervised the project. ZB acquired the funding for this study. MN and JZ designed the experiments. YG and WS performed most of the experiments. HY performed the mechanistic studies and analyzed the data. RL and YA analyzed the immunohistochemistry data. ZW, TL, and WD analyzed the data of mice experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.747010/full#supplementary-material
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