ORIGINAL ARTICLE

Livistona chinensis Water Extract has the Potential to Repair Thioacetamide-Induced Liver Fibrosis in Mice

San-Ho Hung[1,2] · Tzu-Hsuan Hsu[3] · Chun-Shien Wu[4] · Yi-Ping Lin[3] · Ching-Ting Wei[5,6] · Shyh-Ming Kuo[3]

Abstract

Purpose Liver fibrosis is characterized by the excessive deposition of extracellular matrix, especially collagen, in the liver. Liver fibrosis was initially believed to be irreversible, but currently, various therapeutic strategies or drugs are available for treating liver fibrosis. *Livistona chinensis* is a natural traditional Chinese medicine, and its fruits and seeds contain flavonoids and phenolic compounds with antioxidant, antihyperlipidemia, anti-inflammatory, and antitumor activities. However, high concentrations of the extract may have toxic effects, limiting its use in pharmaceutical applications.

Methods This study evaluated the cell toxicity and anti-inflammatory effect of the water extract of *L. chinensis* (WELC) and its reparative effect on TAA-induced liver fibrosis in mice.

Results The half-maximal inhibitory concentration (IC_{50}) of WELC for hepatic stellate cells was 0.35 mg/mL. The IL-6 assay revealed that WELC exhibited a significantly reduced anti-inflammatory effect at a relatively low concentration of 0.2 mg/mL. Moreover, WELC significantly reduced alanine aminotransferase and aspartate aminotransferase levels and resulted in the recovery of albumin levels to a range similar to that of the normal group after 4-week treatment. Additionally, WELC treatment significantly alleviated liver fibrosis in both the 2-week and 4-week treatment groups. Notably, mice treated with 0.6 mg/mL WELC for 4 weeks exhibited an almost complete restoration of the collagen area to normal levels, indicating the reversal of liver fibrosis. However, the steatosis in TAA-induced fibrotic livers was not significantly alleviated.

Conclusion WELC has the potential to repair TAA-induced liver fibrosis by gradually reducing pericellular fibrosis after the long 4-week treatment period.

Keywords Liver fibrosis · *Livistona chinensis* · Anti-inflammation · Hepatic stellate cell

1 Introduction

Liver fibrosis is characterized by the overproduction and excessive accumulation of extracellular matrix (ECM) produced by fibrotic myofibroblasts. These myofibroblasts, typically rare and transiently activated during wound healing, play a crucial role in ECM synthesis and scar tissue formation. The main precursors of myofibroblasts are hepatic stellate cells (HSCs), which normally remain in a quiescent state. Under injury of or chronic disease in the liver, the quiescent HSCs are activated and transdifferentiate into myofibroblast-like cells. The activated HSCs actively regulate tissue repair processes such as proliferation, contractility, enhanced ECM synthesis, production of inflammatory signals, and release of abundant factors that promote liver repair, including tumor necrosis factor (TNF), transforming growth factor (TGF), and platelet-derived growth factor [1, 2]. These factors play crucial roles in liver inflammation, cell

✉ Shyh-Ming Kuo
  smkuo@isu.edu.tw

1 Department of Physical Therapy, Fooyin University, Pingtung County, Taiwan

2 Department of Radiology, Fooyin University Hospital, Donggang Township, Pingtung County, Taiwan

3 Department of Biomedical Engineering, I-Shou University, No. 8, Yida Rd., Jiaosu Village, Yanchao District, Kaohsiung City, Taiwan

4 Center of General Education, I-Shou University, Kaohsiung City, Taiwan

5 The School of Chinese Medicine for Post Baccalaureate, College of Medicine, I-Shou University, Kaohsiung City, Taiwan

6 Division of General Surgery, Department of Surgery, I-Shou University, E-Da Hospital, Kaohsiung City, Taiwan

necrosis, apoptosis, and ECM production. TGF-β1 has been demonstrated to play a crucial role in liver fibrosis and in promoting the differentiation, migration, and collagen secretion of HSCs. Therefore, TGF-β1 is considered to be one of the main factors causing liver fibrosis [3, 4].

During early stages of liver fibrosis, the fibrous connective tissues formed by dead liver cells can still undergo degradation, digestion, and replacement by healthy surrounding liver cells. Thus, appropriate protective strategies or treatments can be applied to improve or restore liver function. However, chronic liver injury such as fibrosis and cirrhosis, if left untreated, can progress to hepatocellular carcinoma. In the liver, fibrosis serves as a self-protecting mechanism in the wound-healing process. In response to liver injury, HSCs become activated and produce ECM, leading to the formation of scar tissue and ultimately resulting in liver fibrosis [5]. Recently, Chinese medicinal herbs have been used in the treatment and suppression of liver fibrosis as well as in the prevention of liver cirrhosis. Traditional Chinese medicines used for their antihepatic fibrosis properties include Danshen, Bupleurum, and Caulis Spatholobus [6, 7].

Livistona chinensis (LC) is a common ornamental plant that is widely distributed in Taiwan, South China, and Japan. LC and its extracts have been found to contain flavonoids, steroids, and phenols with several biological properties including antioxidant, anti-inflammatory, and anticancer properties. LC seeds are rich in flavonoids, which are notable for their ability to effectively scavenge free radicals, thereby preventing excessive oxidation reactions [8, 9]. LC extracts have been demonstrated to effectively inhibit pro-inflammatory reactions and reduce tumor activity through the inhibition of kinase activity [10]. He et al. isolated and identified 15 phenols from the chloroform soluble fraction of a 70% EtOH extract of LC. Some of these extracts exhibited strong antiproliferative activities against human tumor cell lines [11]. Cheung also indicated that the ethanol extract of LC inhibited the growth of the human promyelocytic mononuclear cell line HL60 [12]. Moreover, the antioxidant properties of the water extract of LC have been demonstrated in cells with LPS-stimulated inflammation, which are mediated by reduction of the levels of interleukin (IL)-1β [13].

Traditionally, LC is used in the form of drinking tea and is stewed with other medicinal materials as a soup for the treatment of nasopharyngeal cancer, leukemia, and hepatitis [14]. However, its clinical applicability remains elusive due to the uncertainties related to its appropriate dosage and potential toxicity in high doses, particularly LC fruit. Considering the marked bioactivity of LC extracts, in the present study, LC fruits were extracted by using an environment friendly method. In brief, LC fruits were extracted through hot water extraction, followed by concentration and lyophilization to obtain a powder form. The toxicity of the LC powder was assessed in HSCs by using the MTT cell viability assay

and flow cytometry apoptosis assay. Additionally, the anti-inflammatory effect of the LC powder on the HSCs exposed to LPS was evaluated. Finally, the protective and reparative effects of the water extract of *L. chinensis* (WELC) were examined in a mouse model of TAA-induced liver fibrosis through histopathology; Western blotting; and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin analyses.

2 Materials and Methods

2.1 Materials

The dried fruits of LC were obtained from a local store and authenticated by Prof. Dr. Lin Li-wei. A voucher sample, No. ISU-MCMM-200 was preserved in the School of Chinese Medicines for Post-Baccalaureate, I-Shou University for future reference. TAA was purchased from Sigma (St. Louis, MO, U.S.A.). 3–4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), DMEM medium, fetal bovine serum, streptomycin, and penicillin were obtained from Gibco (Waltham, MA, USA). All chemicals used in the present study were of reagent grade. Animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of I-Shou University, Kaohsiung, Taiwan (IACUC-ISU-111–022, Approval Date: 15 June 2022).

2.2 LC Material Extraction

The dried fruits of LC, which consist of a dark shell with a hard seed inside, were ground and pulverized in a blender into a fine powder. The aqueous extract of the LC powder was prepared by boiling the powder in 2000 mL of distilled water. The boiling process was continued until 1000 mL of the water remained. Then, an additional 1000 mL of distilled water was added, and the extraction process was repeated. This procedure was repeated twice. The final 1000-mL solution containing the crude extract was filtered, and the obtained solution was concentrated and frozen at −20 °C for 24 h. Finally, the solution was freeze-dried using a freeze dryer to obtain a powder form for subsequent experiments.

2.3 HPLC–MS/MS Analysis

The WELC samples were analyzed through HPLC–MS/MS by using a Trip-Quant spectrometer from Agilent 6470 with an electrospray ionization (ESI) interface coupled with HPLC Agilent 1290. For gradient separation, an Agilent Zorbax Eclipse Plus C-18 column (3.0 mm × 150 mm × 2.7 μm) was used. A linear gradient system comprising mobile phase A (0.1% formic acid

aqueous solution) and mobile phase B (acetonitrile containing 0.1% formic acid) was used. The gradient elution profile used in the separation process was as follows: 0–1 min, 5% B; 1–35 min, 5–50% B; and 35–40 min, 50% B; the post time was 3 min. The column was maintained at 40 °C, and the flow rate was set to 0.4 mL/min during the gradient separation and column equilibration. The sample injection volume was 5 μL. The MS spectrometer was optimized using the following parameters: dry gas temperature, 325 °C, with a flow rate of 9 L/min; nebulizer pressure, 45 psi; sheath gas temperature, 350 °C, with a flow rate of 10 L/min; and capillary voltage, − 4.0 kV. The characteristic peaks of WELC were identified through Fourier-transform infrared (FTIR) spectroscopy (Jasco 4700, Tokyo, Japan).

2.4 In Vitro Cell Viability Tests of WELC

Mouse LX-2 cells (cell line, purchased from Elabscience, Minneapolis, MN, USA) were seeded at a density of 7×10^3 cells/mL in 96-well plates containing Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. After incubation at 37 °C with 5% CO_2 for 24 h, the medium was replaced with fresh medium containing various concentrations of WELC. LX-2 cell viability was examined using the MTT assay. At 24 h after treatment with WELC, 20 μL of MTT solution was added to each well, followed by incubation for an additional 3 h, with the formation of formazan crystals. The formazan precipitate was dissolved in 200 μL of dimethyl sulfoxide through vortexing, and the absorbance of the solution was measured at 570 nm by using a multiplate reader (Thermo Scientific, Waltham, MA, USA). To further assess cell viability, live/dead cell staining was performed, for which 1 mL of PBS solution containing 4 μM ethidium homodimer-1 (EthD-1) assay solution (2.5 μL/mL) and 2 μM calcein acetoxymethyl solution (1 μL/mL) was prepared. This assay solution (100 μL) was added to the cell culture, and the mixture was incubated at 37 °C with 5% CO_2 for 15 min. The sample was examined under a fluorescence microscope with excitation filters of 494 nm (green, calcein) and 528 nm (red, EthD-1).

Cell viability was examined using a flow cytometry apoptosis assay (BD Biosciences, San Diego, CA, USA). In brief, LX-2 cells were seeded at a density of 6×10^4 cells/well in 24-well plates and incubated for 24 h. The medium in each well was then replaced with fresh medium, and various concentrations of WELC were added to each well, followed by incubation for 24 h. Next, following the manufacturer's instructions, a staining kit with FITC-conjugated annexin V/PI was used for the apoptosis assay. The cells were detached from the culture flask through trypsinization and washed with phosphate buffered saline. The cell pellets were then suspended in $1 \times$ binding buffer and stained with 5 μL of annexin V-FITC and 10 μL of PI for 15 min, after which the cells were subjected to flow cytometry analysis (BD Accuri C6, Franklin, NJ, USA).

2.5 Anti-Inflammatory Tests with H₂O₂-Induced Inflammation in LX-2 Cells

The inflammatory response of LX-2 cells to H_2O_2 exposure was assessed as follows: LX-2 cells were seeded at a density of 7×10^3 cells/well in 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. Subsequently, the cells were treated with 100 μM H_2O_2 for 0.5 h. The cells with H_2O_2-induced inflammation were then treated with various concentrations of WELC and were incubated for 24 h. The culture medium from each well was then collected and centrifuged at $2000 \times g$ for 15 min. The supernatant was assayed for interleukin (IL)-6 and TNF-α by using Elabscience human ELISA kits, and absorbance was measured at 450 nm following the manufacturer's protocol [15].

2.6 In Vivo Animal Experiments

2.6.1 Establishment of Thioacetamide-Induced Liver Fibrosis Mouse Model and Treatment with WELC

Animal experiments were performed over 8 weeks. Liver fibrosis was induced using thioacetamide (TAA). A working solution of TAA was prepared using phosphate buffered solution. The liver fibrosis mouse model was established in 12 mice, whereas 2 mice served as the normal controls. The mice were intraperitoneally injected with three doses of 100 μL of TAA solution (80 mg/mL in PBS) per week for a total of 4 weeks. The mice were anesthetized with Zoletil (intraperitoneal administration of 40 mg/kg of tiletamine with 50 mg/kg of zolazepam) and xylazine (10 mg/kg) and were randomly allocated into the following groups (n = 3/group): Group A—liver fibrosis induction with TAA and no treatment (this group served as a negative control group); Group B—untreated for weeks 2 and 4 after liver fibrosis induction with TAA; Group C—oral administration of 100 μL/per day of WECL (0.3 mg/mL); Group D—oral administration of 100 μL/per day of WECL (0.6 mg/mL); and Group E—normal (this group served as positive control group). The surgical procedures on the mice were performed by a qualified surgeon and were closely monitored by experienced veterinarians. During operation, clinical signs of pain, salivation, or abnormal behavior were carefully monitored. If any adverse event occurred as a result of the injection, the affected mice were excluded from the study. Subsequently, randomly selected rats from each group were sacrificed at weeks 2 and 4 after WELC treatments, and liver and serum samples were harvested for subsequent histopathological and biochemical analyses.
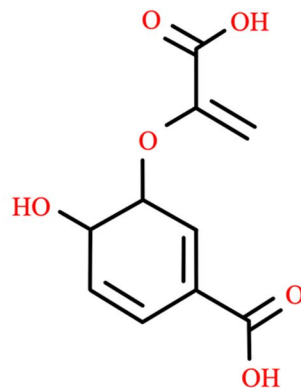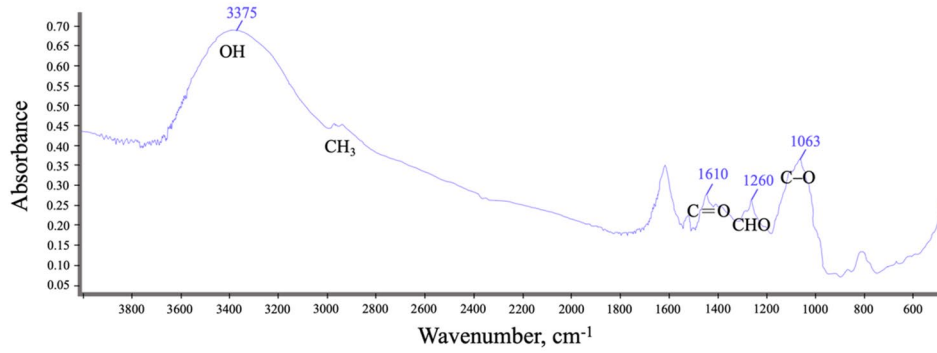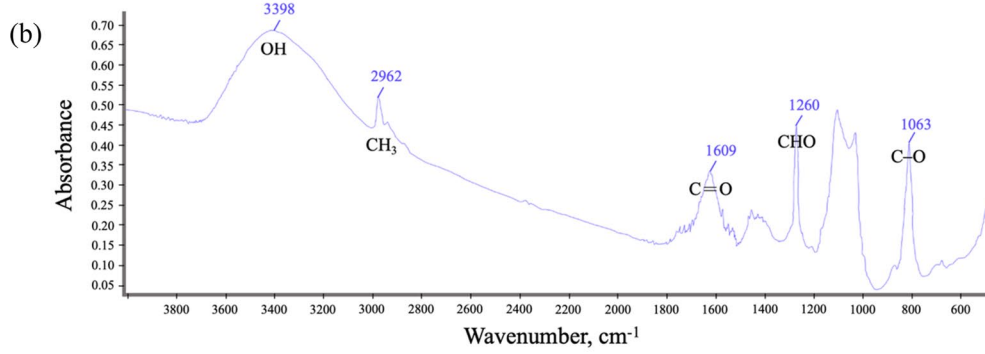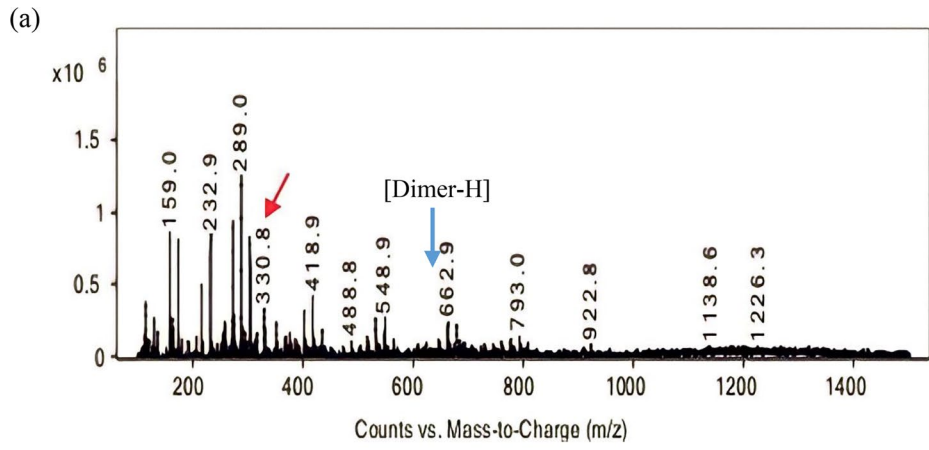
(a)

(b)

2.6.2 Biochemical Assays

To evaluate liver function, blood biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin levels, were measured. Mouse sera were isolated from whole blood samples and subjected to ALT, AST, and albumin biochemical analyses according to the manufacturer's instructions.

2.6.3 Histopathological Analyses

At weeks 2 and 4 after treatment with WELC, the mouse liver tissues from all groups were harvested and fixed in 10% neutral-buffered formalin. The liver tissues were then dehydrated in graded ethanol solutions, cleared in xylene, embedded in paraffin blocks, and cut into 7-µm-thick sections. Hematoxylin and eosin (H&E) staining was performed to examine the general histopathological characteristics of the liver tissues. Masson's trichrome staining was performed to assess changes in the collagen content within the liver tissue. For the analysis of collagen content, three microscopic fields were selected from each liver tissue section and were analyzed under 100× magnification. ImageJ software (Version 1.50; National Institutes of Health, Bethesda, MD, USA) was used to measure the collagen content in each group.

2.6.4 Western Blot Analysis

To assess the reparative effect of WELC treatments, the levels of smooth muscle α-actin (α-SMA) and TGF-β1 were analyzed in mouse liver samples. The proteins from the liver samples were transferred and blotted onto polyvinylidene membranes. The membranes were then incubated in Tris-buffered saline containing 5% skim milk powder and 0.05% Tween 20 for 1 h at room temperature to block nonspecific protein binding. The membranes were then incubated overnight with primary antimouse antibodies for α-SMA, TGF-β1, and GAPDH at 4 °C. The protein levels of α-SMA, TGF-β1, and GAPDH in the experimental groups relative to those in the control group were measured through semiquantitative intensity analysis (normalized by the respective GAPDH and background) by using ImageJ.

2.7 Statistical Analysis

All values are expressed as the mean ± standard error of the mean. Comparisons between multiple groups were conducted using one-way analysis of variance followed by Tukey's multiple comparison. A p value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS, version 17.0.

3 Results and Discussion

3.1 Isolation and Characterization of WELC

In this study, LC fruits were extracted using a hot water extraction method and analyzed through LC–MS and FTIR spectroscopy. Studies have indicated that the phytochemical composition of LC extracted using water is poorly understood, which may be attributed to the lower extraction efficiency of hot water compared with other solvents [12]. Studies have shown that LC fruit primarily contains phenolic compounds, flavonoids, and polyunsaturated fatty acids [16]. Kaur et al. reported the presence of only phenolic compounds in the aqueous extract of LC fruits. Figure 1a presents the results of the LC–MS analysis of the extracted solution (WELC), and the spectrum revealed the presence of phenolic compounds and many flavonoid compounds [17]. Figure 1b presents the FTIR spectrum of WELC extracted from LC fruits and seeds, with characteristic peaks attributed to the functional groups OH–, C=O, C–O, and CHO. These peaks are similar to those of the base molecules.

3.2 Effects of WECL on Cell Viability and Inflammation Through In Vitro Cell Viability and Anti-Inflammatory Tests on LX-2 Cells

The results of the MTT assay revealed that WELC exerted moderate cytotoxicity in LX-2 cells. As shown in Fig. 2, treatment with increasing concentrations (0.1–0.4 mg/mL) of WELC for 24 h reduced the cell viability in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) was approximately 0.35 mg/mL. The cell pseudopodia gradually shrank, and the cells became round and detached from the culture, indicating that higher WELC concentrations resulted in cell death (Fig. 2b). Fluorescence staining of live cells corroborated the MTT assay results; the number of dead cells (red color) increased with increasing concentrations of WELC (Fig. 2c). To further examine the cytotoxicity of WELC in LX-2 cells, annexin V-FITC/PI staining and flow cytometry analysis were performed. The control cells displayed a low background of staining with either annexin V-FITC or PI (Fig. 2d). However, treatment

Fig. 2 a MTT assay. **b** Cell morphology (magnification, 100×). **c** Live/dead staining (green fluorescence, live cells; red fluorescence, dead cells) and semiquantitative analysis of stained cells. **d** Flow cytometry assay for apoptosis in LX-2 cells treated with different concentrations of WELC. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with the control group. Solvent control: cells treated with DMSO solution used to dissolve WELC (The final concentration of DMSO in the medium for all cell experiments was $< 0.5\%$)
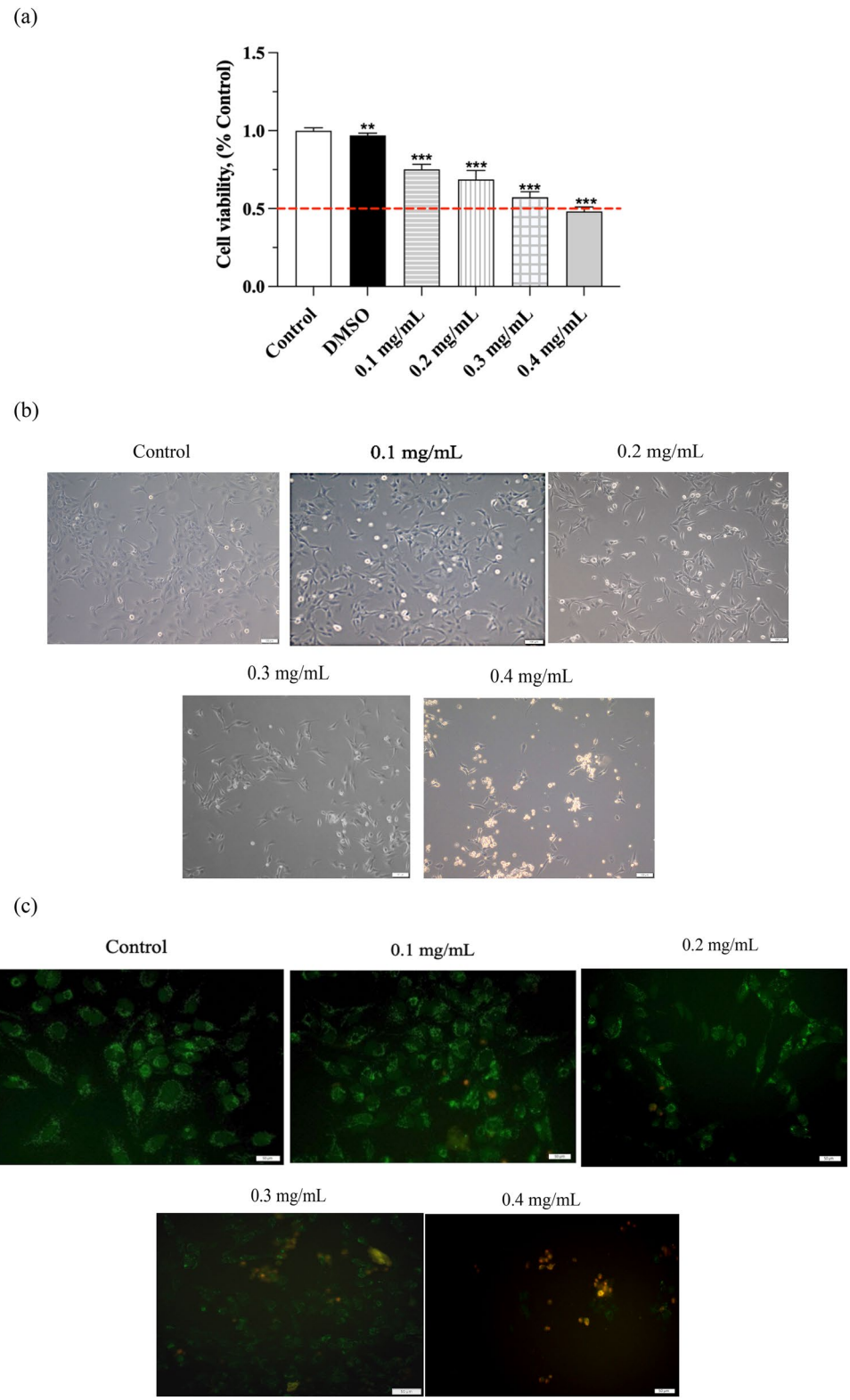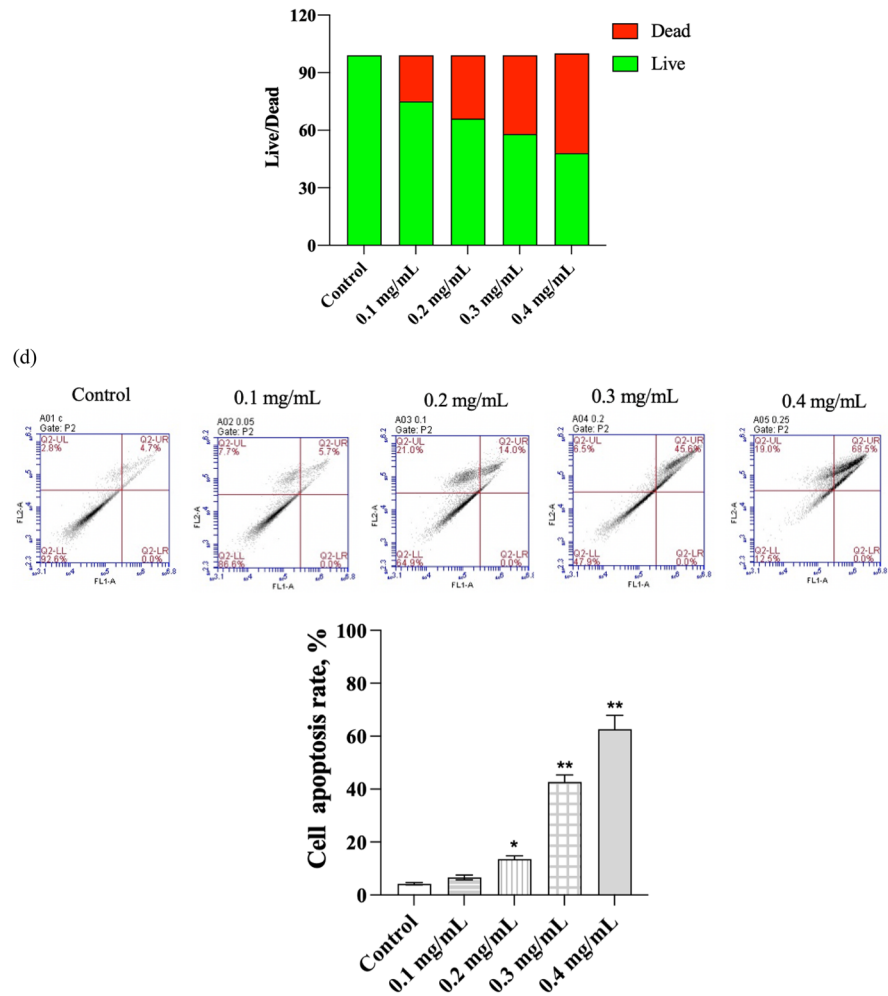
(d)

3.3 Histopathological Analysis of Liver Fibrosis in Mice Treated with WELC

with increasing concentrations of WELC (0.1, 0.2, 0.3, and 0.4 mg/mL) resulted in a gradually increase in the number of annexin V-FITC- and PI-positive cells, representing late apoptosis. The semiquantitative analysis of apoptosis revealed similar cytotoxic effects of WELC in LX-2 cells.

The anti-inflammatory effect of WELC on LX-2 cells with H_2O_2-induced inflammation is depicted in Fig. 3. Treatment with WELC at concentrations of 0.1–0.3 mg/mL significantly suppressed the inflammatory factor IL-6. The inflammatory factor TNF-α was also slightly suppressed after treatment with the same concentration range of WELC.

Considering the cell cytotoxicity and anti-inflammatory effects observed in LX-2 cells, the WELC concentrations of 0.3 and 0.6 mg/mL were selected for in vivo animal experiments involving the treatment of TAA-induced liver fibrosis.

Figure 4 illustrates the timeline and assays used in the in vivo animal experiments. The TA induction period for liver fibrosis was 4 weeks. Following the induction period, the mice received oral administration of WELC at either 0.3 or 0.6 mg/mL per day for the next 2 or 4 weeks. WELC concentrations were set at 0.3 mg/mL near the IC_{50} of WELC in LX-2 cells and approximately twofold of IC_{50} (i.e., 0.6 mg/mL).

The reparative effect of WELC on TAA-induced liver fibrosis in vivo was evaluated through histopathological analysis. Figure 5a presents the H&E and Masson's trichrome–stained images of the normal mouse liver. Figure 5b presents the H&E and Masson's trichrome–stained images of the liver of mice that underwent 4 weeks of TAA induction of liver fibrosis and subsequent oral administration of

Fig. 3 Anti-inflammatory effects of H_2O_2-induced LX-2 cells treated with various concentrations of WELC. $*p < 0.05$ and $**p < 0.01$, compared with the control group. $\#p < 0.05$, compared with the untreated group
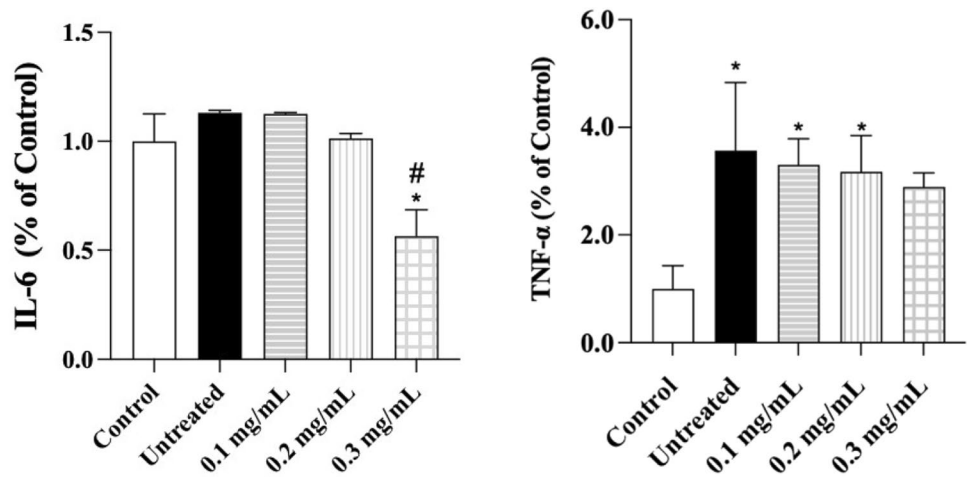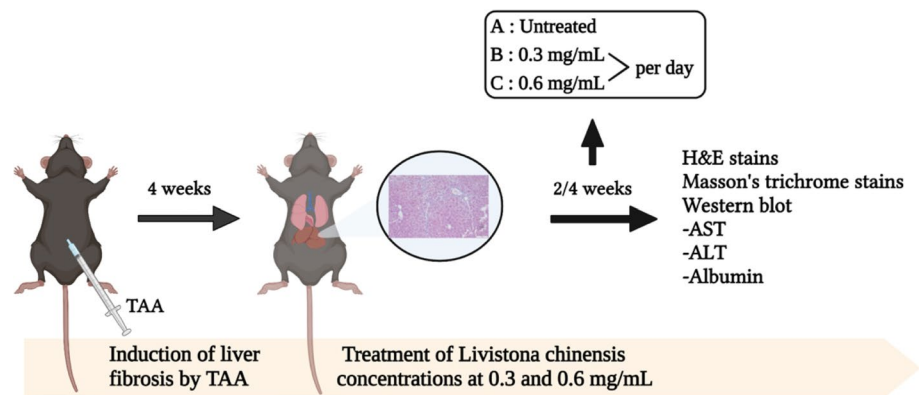

Fig. 4 Schematic of the TAA-induced liver fibrosis mouse model and treatment procedures

0.3 or 0.6 mg/mL WELC for the following 2 weeks. In the untreated group, after 4 weeks of TAA induction, moderate steatosis and diffuse pericellular fibrosis were observed, indicating a mild inflammatory response in the liver. After treatment with 0.3 mg/mL WELC, a slight decrease in pericellular fibrosis but no obvious alleviation of steatosis was observed. The inflamed livers treated with 0.6 mg/mL WELC exhibited more alleviation of inflammation (Fig. 5b and Table 1). After 4 weeks of treatment, the reparative efficacy of WELC was noted compared with the untreated livers. The livers exhibited significantly less pericellular fibrosis and steatosis (Fig. 5c). Mild steatosis was still observed in the inflamed livers, which may be attributed to TAA induction. The histopathological staining results revealed that the inflamed liver gradually healed after the 4-week period in the untreated group compared with the 2-week untreated group. The inflamed livers treated with 0.3 or 0.6 mg/mL WELC exhibited marked alleviation in steatosis and pericellular fibrosis and recovery to a normal appearance compared with the normal group. However, mild steatosis was still present in these livers. Tables 1 and 2 present the histological findings obtained from histopathological staining analysis. Oral administration of WELC repaired the TAA-induced inflamed livers by alleviating pericellular fibrosis and steatosis, particularly after the long 4-week treatment period.

Hepatic fibrosis is a progressive wound-healing response of the liver to repeated injuries. If the hepatic injury persists, then hepatocytes are substituted with high amounts of ECM, particularly fibrillar collagen [18]. Determining the collagen content in the liver is considered a reliable marker for assessing the presence and severity of liver fibrosis. Masson's trichrome staining is a commonly used histological staining technique for the visualization of collagen fibers in tissues. This staining method stains collagen fibers blue, and ImageJ software can be used to analyze the stained images and calculate the percentage of blue color staining, which corresponds to collagen content in the liver tissue. Figure 5d presents the results of the semiquantitative analysis of collagen content through Masson's trichrome staining in this study. The TAA-induced liver fibrosis group without WELC treatment exhibited significantly higher collagen content compared with the groups treated with WELC. Similarly, the liver exhibited auto-healing capability after 4-week treatment with WELC (2-week untreated and 4-week untreated group). Liver fibrosis was significantly alleviated after

Fig. 5 Results of H&E staining and Masson's trichrome staining of the sections of **a** the normal liver and TAA-induced fibrotic liver treated with WELC after **b** 2 and **c** 4 weeks of treatment. **d** Semiquantitative analysis of collagen levels between groups through Masson's trichrome staining (magnification, 100×). *p < 0.05, **p < 0.01, ***p < 0.001, compared with the normal group. #p < 0.05, compared with the untreated group after a 2-week treatment. ++p < 0.01, compared with the untreated group after 4-week treatment
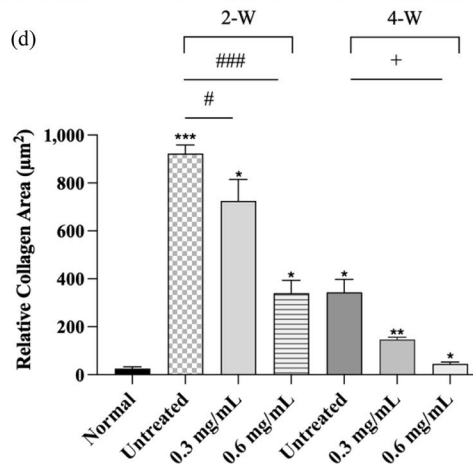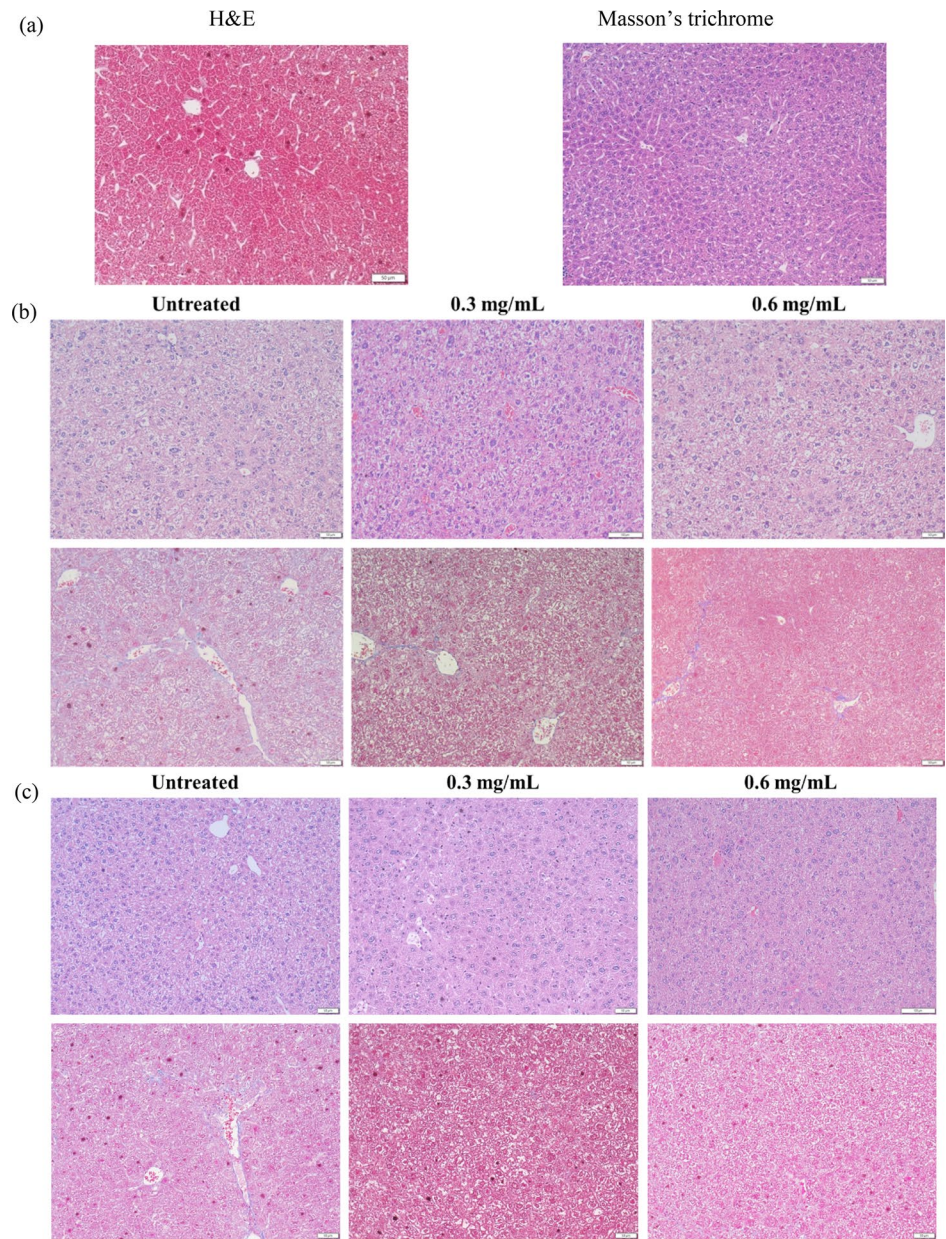
Table 1 Histological findings in TAA-induced fibrotic livers after a 2-week treatment

	Normal	Untreated	0.3 mg/mL	0.6 mg/mL
Steatosis	+	+~++	++	++
Pericellular fibrosis	+	++~+++	++	+~++

Table 2 Histological findings in TAA-induced fibrotic livers after 4-week treatment

	Normal	Untreated	0.3 mg/mL	0.6 mg/mL
Steatosis	+	++	+~++	+~++
Pericellular fibrosis	+	++~+++	+	−/+

Percentage of steatosis (steatosis area/total liver area in the slide): +++ (> 66%), ++ (33%–66%), + (0%–33%), Degree of pericellular fibrosis: +++ (diffuse), ++ (patchy), + (focal), −/+ (very focal)

2-week and 4-week WELC treatments. Notably, in the mice treated with 0.6 mg/mL WELC for 4 weeks, the collagen levels almost returned to normal, indicating that liver fibrosis was resolved. The histopathological results revealed that the oral administration of WELC in the mice with TAA-induced liver fibrosis promoted healing and exerted protective effects on the injured liver. Moreover, longer treatment durations and higher WELC concentrations had a more pronounced reparative effect on liver fibrosis.

3.4 Biochemical Analysis of Serum AST, ALT, and Albumin Levels

Altered serum AST and ALT levels have been widely used as markers to indicate liver function and for the prognosis of liver diseases [19]. In this study, we therefore measured the serum levels of AST and ALT to evaluate liver function in the mice with TAA-induced liver fibrosis that received the oral administration of WELC. The mice with TAA-induced liver fibrosis that did not receive WELC treatment exhibited high serum levels of AST and ALT, indicating that the liver function had deteriorated after the 4-week TAA induction (Fig. 6a and b). Significant reductions in the serum levels of AST and ALT were observed in the mice that received WELC treatment for 2 and 4 weeks. A slight increase in serum levels of AST and ALT was observed after treatment with a higher concentration of WELC (i.e., 0.6 mg/mL) for 2 weeks. However, after 4-week treatment with the same concentration (0.6 mg/mL), the AST and ALT levels significantly decreased. These changes may be attributed to the cytotoxicity associated with the higher dosages of WELC and the initial rejection response of the liver to the WELC treatment. In summary, WELC treatment, particularly at a higher concentration of 0.6 mg/mL and for a longer period of 4 weeks, significantly reduced the serum levels of AST

and ALT, indicating an improvement in liver function in the TAA-induced liver injury model.

Physiologically, serum albumin is an important functional marker used to assess liver function. Serum albumin has considerable antioxidant potential and acts as a free radical scavenger in the liver [20]. Figure 6c shows that the serum albumin levels returned to the normal range after treatment with WELC at a higher concentration of 0.6 mg/mL for 2 or 4 weeks. However, a lower dosage of 0.3 mg/mL WELC resulted in a minor reparative efficacy for liver fibrosis.

3.5 Western Blot Analysis

The presence of the inflammatory factors α-SMA and TGF-β1 in the mouse liver was assessed through Western blotting. As shown in Fig. 7, the 2-week untreated group had the highest levels of α-SMA and TGF-β1. As time progressed to 4 weeks, the fibrotic liver gradually healed, leading to lower levels of α-SMA and TGF-β1. However, the oral administration of 0.6 mg/mL WELC for 4 weeks resulted in an obvious reparative effect on the fibrotic liver, which exhibited the lowest levels of α-SMA and TGF-β1.

Liver fibrosis is characterized by the excessive deposition of ECM in response to chronic injury and inflammation. α-SMA, a marker of activated myofibroblasts, is often overexpressed in fibrotic disorders such as liver fibrosis. Myofibroblasts are activated in fibrotic or inflammatory tissues with increased levels of ECM components, such as type I collagen, and the cells exhibit neo-expression of α-SMA in liver fibrosis induced by CCl_4 [21]. Moreover, activated myofibroblasts contribute to the activation of integrin-bound latent TGF-β1, a potent fibrogenic growth factor [21]. In the present study, liver fibrosis was induced using TAA for 4 weeks. Subsequently, the fibrotic livers were treated with 0.3 or 0.6 mg/mL WELC for an additional 2 and 4 weeks. The results revealed that WELC treatment had beneficial effects on liver fibrosis and promoted the repair of fibrotic livers.

Many studies have reported the anticancer and antiproliferative properties of the ethanol extracts of LC and the use of these extracts as a traditional or folk remedy to treat various types of cancer. However, these extracts are not formal candidates in clinical trials due to their cytotoxicity at high concentrations. To realize the potential of the components of LC extracts and explore their applications in other medicinal fields, an environmentally friendly and simplified extraction method is required.

Chronic and persistent liver inflammation can lead to the development of liver fibrosis and liver cirrhosis [22]. Effective treatment strategies for liver fibrosis involve targeting the underlying causes, such as alcohol or drug-induced liver injury. Silymarin, known for its antioxidant, anti-inflammatory, and antifibrotic properties, is widely
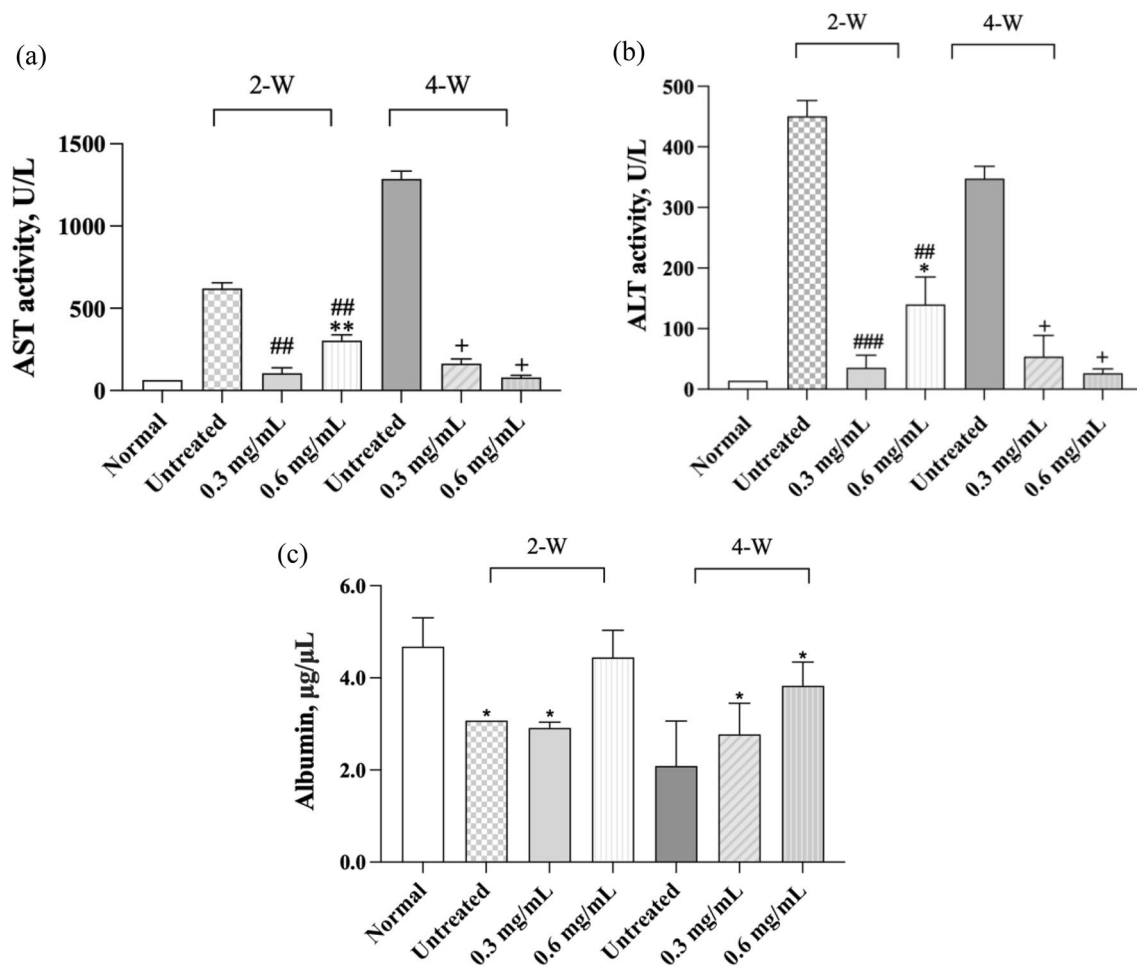
(a)

(b)

(c)

Fig. 6 Effects of WELC on **a** AST, **b** ALT, and **c** albumin levels of mice with TAA-induced liver fibrosis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with the normal group. $\#p < 0.05$, compared with the untreated group after a 2-week treatment. $++p < 0.01$, compared with the untreated group after 4-week treatment

used in the treatment of chronic liver diseases [23]. In our previous study, we discovered that oleanolic acid exhibits similar therapeutic efficacy to that of silymarin, indicating its potential as a liver-protective and reparative agent against inflammation [23]. In the present study, aqueous extracts of LC were obtained using the hot water extraction method. Hot water extraction yields lower amounts of extracted components compared with ethanol extraction. Reports have indicated that water extracts of LC mainly contain phenolic compounds, flavonoids, and polyunsaturated fatty acids. Figure 1a shows the presence of phenolic compounds and many flavonoid compounds in the LC–MS spectrum of the extracts of both the LC fruit and seed. These compounds exhibited characteristic peaks attributed to the functional groups of OH–, C=O, C–O, and CHO. These peaks are similar to those of the base molecule (Fig. 1b). These compounds possess antioxidant and anti-inflammatory properties and have been

used to as liver-protective agents. Therefore, the objective of this study was to extract the beneficial components of LC by using a simple hot water extraction method and to evaluate the reparative effects of the extracted solution on TAA-induced liver fibrosis. First, we examined the cytotoxicity of WELC and obtained an IC_{50} of approximately 0.35 mg/mL. In our in vivo animal studies, histopathological examination, biochemical analysis, and Western blotting revealed the obvious reparative effects of WELC, particularly from the oral administration of 0.3 or 0.6 mg/mL WELC over a longer 4-week period (Figs. 5, 6, 7). A shorter 2-week treatment period exerted less improvement in TAA-induced liver fibrosis. However, it is worth noting that mild liver fibrosis was observed during the 4-week TAA induction period. To evaluate the full reparative or protective efficacy of WELC, a longer induction period may be necessary to induce more severe and obvious liver fibrosis and injury.
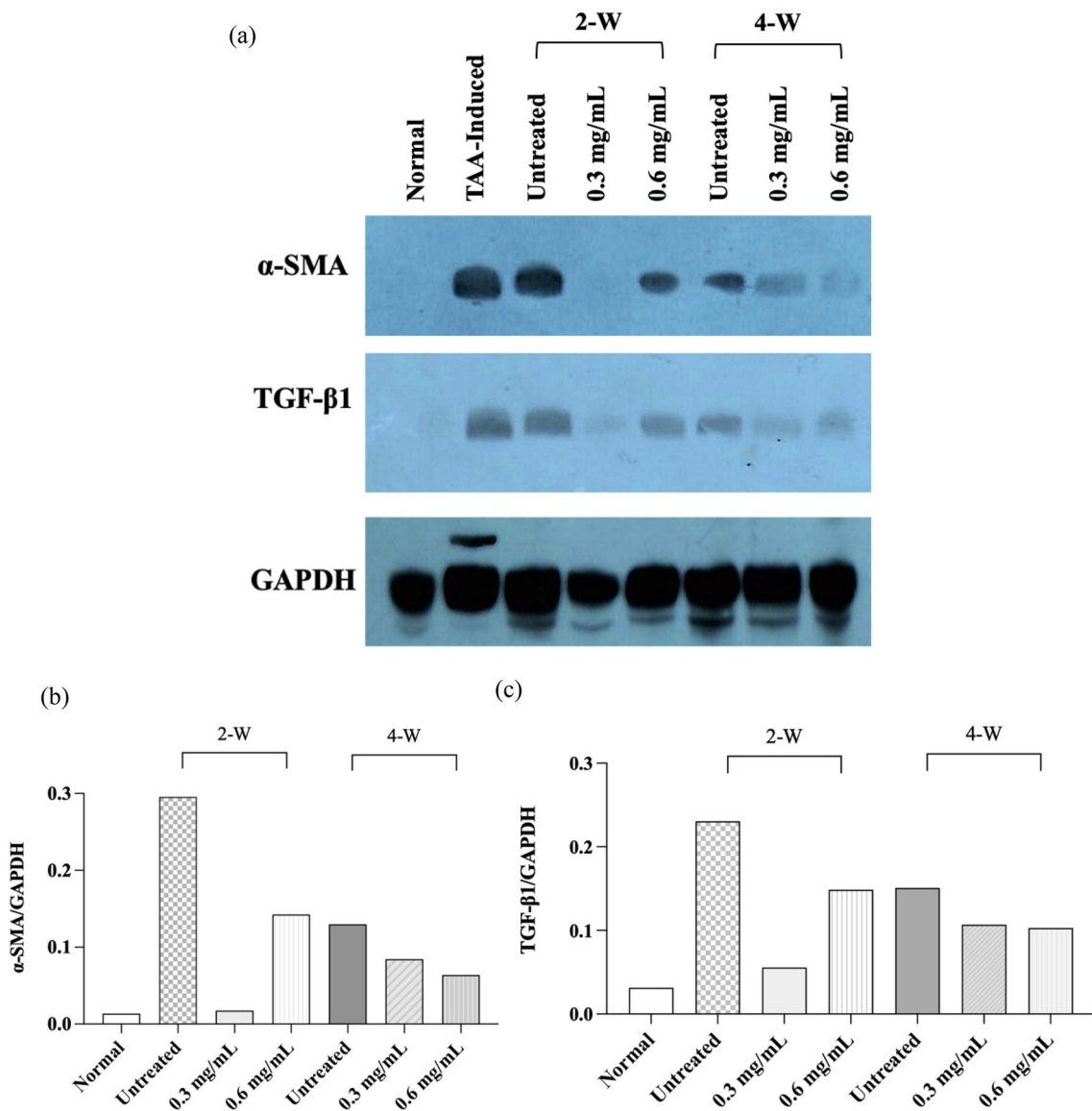
Fig. 7 Western blot analysis of α-SMA and TGF-β levels in TAA-induced liver fibrosis samples after treatment with WELC. The semiquantitative levels of **b** α-SMA vs GAPDH and **c** TGF-β1 vs GAPDH in the fibrous liver ($n=2$)

4 Conclusion

Our study demonstrated that the oral administration of the aqueous extract of LC can effectively repair TAA-induced liver fibrosis. The aqueous extract contains phenolic and flavonoid compounds, which possess various health benefits, suggesting that this extract can be used in the formulation of dietary drinks as a potentially alternative protective strategy for liver fibrosis.

Author Contributions All authors contributed to the study conception and design. Materials preparation, data collection and analysis were performed by SHH, THH, CSW, YPL. The histopathological data was evaluated by CTW. The draft of the manuscript was written by SMK. All authors read and approved the final manuscript.

Data Availability The data that support the findings of this study are available within the article.

Declarations

Conflict of interest The authors have no conflicts to disclose.

Ethical Approval Animal experiments conducted in this study were approved by the by the Institutional Animal Care and Use Commit-

tee of I-Shou University, Kaohsiung, Taiwan (IACUC-ISU-111–022, Approval Date: 15 June 2022).

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