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Antifibrotic Effects of Triterpenoids Isolated from The Fruits of *Euscaphis Fukienensis* Hsu. In Mice

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1. Abstract

Liver fibrosis (LF) is the major challenge to current human health. We do this research to document the effects of the triterpenoids from the fruits of *Euscaphisfukienensis* Hsu. (EFT) and the expression of TGF-β_1, PDGF, TNF-α and AngII in a murine model of LF. Sixty mice were equally divided into six study groups. Five groups were subcutaneous injected carbon tetrachloride (0.1 ml/10 g) for six weeks. Two of these five groups were treated with different concentrations of EFT (1.28 and 5.12 mg/10 g), one was treated with the water extracts of the triterpenoids from the fruits of *Euscaphisfukienensis* Hsu. (EFW) 6.39 mg/10 g, one with 0.1 mg/kg of Colchicine (positive control), and one with physiologic saline (negative control). After treating six mouth, mice were executed and then hepatic enzymes/function tests, hyaluronic acid and laminin levels were measured in serum, and hepatic histology, TGF-β_1, PDGF, TNF-α and AngII expression were documented in hepatic tissue. Our results show that both EFT and EFW could significantly attenuate the level of LF. The expression of TGF-β_1, PDGF, TNF-α and AngII mRNA and protein have different degrees of reduction in treated mice. Our researches clarify that triterpenoids are the material basis of the fruits of *Euscaphis fukienensis* Hsu. for the treating LF and preliminarily explain its mechanism.

2. Introduction

Liver fibrosis (LF) occurs as a common pathological consequence of a sustained wound-healing response of liver to toxic, infectious, or metabolic agents. Liver fibrosis is a noteworthy well-being issue that can prompt the progression of liver cirrhosis and hepatocellular carcinoma [1]. At the cellular lever, the main contributors to the fibrotic response are activated hepatic stellate cells (HSCs), orchestrating the deposition of extracellular matrix (ECM) in normal and fibrotic liver [2].Activation of HSCs is considered to be a central event in the development and progression of LF. transforming growth factor-β_1 (TGF-β_1), platelet derived growth factor (PDGF), tumor necrosis factor-α (TNF-α) and angiotensin II (Ang II) play their respective roles in this incident. TGF-β_1 may be the most important pro-fibrogenic mediator which not only promotes the activation of HSCs, but also enhances the synthesis of ECM and inhibits its degradation [3]. In the past decade, the role of the immune response liver disease has gained increasing attention. Immune cells participate in the activation and survival of HSCs and fibrosis regression [4]. The increased expression of TGF-β_1 in LF suggests it may participate in fibrosis development; it also has additive effects regarding ECM production in vivo [5]. TGF-β_1 may be the most important pro-fibrogenic mediator which not only promotes the activation of HSCs, but also enhances the synthesis of ECM and inhibits its degradation [3]. PDGF has strong power to trigger the proliferation and differentiation of HSCs [6,7]. PDGF and TGF-β_1 also form an autocrine cycle of activated HSCs, which is an important mechanism for the continuous activation of HSCs [8,9]. Another cytokine that needs to be mentioned is TNF-α, which induces the effects of liver fibrosis in connection with its promotion of HSCs proliferation and inhibition of activated HSCs apoptosis [10,11]. TNF-α is an inflammatory cytokine involved in liver inflammation and leads to LF. During the progression of LF, TNF-α and its downstream signaling play a pivotal role in a dynamic process of production of ECM and modulation of immune response [12]. The role of Ang II in liver fibrosis should not to be overlooked, because it up-regulates the expression of TGF-β_1 through AT1 receptors [13]. The above-mentioned cytokines promote the development of liver fibrosis to a more severe extent through their respective mechanisms. Thus, down-regulating the express of these bad molecules will be conducive to the outcome of liver fibrosis.*Euscaphis fukienensis* Hsu. is a deciduous shrub that is mainly distributed in the south of the Yangtze River in China. People often use aqueous extracts of its fruits to treat hepatitis and liver fibrosis. In our previous work, we have isolated and identified many triterpene compounds from the fruits of *Euscaphis fukienensis* Hsu [14]. Thus, we speculate that triterpenoids may be the material basis of the pharmacological activity. The present study was designed to prove the anti-liver fibrosis effects of the triterpenoids from the fruits of *Euscaphis fukienensis* Hsu. (EFT). A commonly employed animal model of liver fibrosis, CCl4 induced fibrosis in rats, was used in our work. This work might provide some insights as to the possible mechanisms of action of EFT.

3. Materials and Methods

3.1. Plant Material

The fruits of *Euscaphis fukienensis* Hsu. were collected from Sanming, China, and were identified by Dr. Xiao B.M. of the Pharmacy College of Hunan University of Chinese Medicine. The voucher specimen was deposited in the Chinese Medicine Research Centre of Hunan University of Chinese Medicine for future reference. The air-dried fruits of *Euscaphisfukienensis* Hsu. were smashed and extracted with 95% ethanol under reflux for two hours, three times. After evaporation under reduced pressure, dark-brow fluid extract was obtained, then suspended in water and extracted with chloroform (CHCl3). The CHCl3 portion was subjected to the macroporous resin D-101 and eluted with different concentrations of ethanol. 70% ethanol elution fraction was collected and dried, and then EFT was obtained. The EFT constituent was analyzed by high performance liquid chromatography (HPLC). Another part of the fruits were extracted with water. The aqueous extracts from the fruits of *Euscaphis fukienensis* Hsu. (EFW) were got after we dried the extracts.

3.2. Liver Fibrosis Animals Models

Sixty 4 weeks old Kunming mice (14.0-18.0 g) of both genders (30/30)were purchased from Hunan SJA Laboratory Animal Co., Ltd in Hunan, China License No. SCXK (Xiang) 2011-0005.The mice were bred in a specific pathogen-free facility in the Experimental Animal Center of Hunan University of Chinese Medicine License No.SYXK (Xiang) 2013-0003 and allowed free access to food and water. Carbon Tetrachloride (CCl4), 500 ml per bottle (product lot-No. 20140102) was purchased from Tianjin Hengxing chemical reagent Co., Ltd. (Tianjin, China).Colchicine, 0.5 mg per tablet, was purchased from the Xishuangbanna Pharmaceutical Co., Ltd. (Xishuangbanna, China). Experienced mice were randomly divided into six groups (n=10): group 1, normal group; group 2, model group; group 3, positive group; group 4, EFW treated group; groups 5 and 6: different doses of EFT treated groups (low and high dose, respectively). To groups 2 to 6, 40% $CC1_4$ was injected subcutaneously at a dose of 0.1 ml/10 g with peanut oil every five days for 6 weeks. The dosage was doubled on the first administration. The mice in group 1 were injected with 0.1 ml/10 g Sodium Chloride Physiological Solution in an identical manner. After 6 weeks, groups 3 to 6 received their treatments by intragastric administration of EFW and EFT suspension once a day for 6 weeks. The doses were 6.39, 1.28 and 5.12 mg/10g, respectively. The doses were equivalent to 15, 15 and 60 times of human adultdose. Group 3 was treated with intragastric administration of Colchicine 0.1 mg/kg. The dose of Colchicine was equivalent to dose given to human adult liver disease patients. Group 1 received physiological saline by the same route. The volume of the treatment to each mouse was 0.2 ml/10 g.

3.3. Histopathological Evaluation

Twenty-four hours after the last administration of different intervention factors, mice were anesthetized by inhaling ethylether, and as much as possible peripheral blood was collected from the orbital sinus of each mouse. The mice that were taken blood subsequently sacrificed, potions of liver tissue were washed by PBS, removed in 10% neutralized formaldehyde solution.Paraffinsections (4μm) were developed and stained with Haematoxylinand Eosin. All the sections were examined by light microscopy (×100).

3.4. Biochemical Blood Analysis

Serum Alanine Aminotransferase Assay Kit, product lot C052-a, Aspartate Aminotransferase Assay Kit, product lot No. B014, Albumin Assay Kit, product lot No.C008-a and Total Bilirubin Assay Kit, product lot No. C004-g were purchased from ChangChun Huili Biotech CO., LTD, China. A serum Laminin Quantitative Estimation Kit, product lot No. CSB-E04805h, and Hyaluronic Acid Quantitative Estimation Kit, product lot No. CSB-E04644h, were purchased from CUSABIO, USA.The blood was centrifuged10 min (3000 r/min) after coagulating 20 minutes at room temperature,and collected supernatant serum. Serum ALT, AST, Albumin and TBIL levels were detected by commercial kits and a supermatic biochemistry analyzer (Hitachi, Japan)as per the manufacturer's instructions was used to detect the data. Serum HA and LN are detected by ELISA method. In brief, 10 μl sample with 40 μldiluentwas addedto the plate, vibrated for30 seconds, incubated for 30 min at 37°C, washed 5 times, added 100 μl enzyme mark liquid, repeated the operation of incubation and wash, added luminescent substrate (away from light, 37 °C, 15 min), and detected by a supermatic chemiluminescence immune analyzer (Thermo, USA) as per the manufacturer's instructions.

3.5. PCR Measurements

The total RNAs were extracted from liver tissues using trizol reagent according to the manufacturer's instructions. The RNAs were dissolved in RNase-free water and the concentrations were determined spectrophotometrically by ultra-micro spectrophotometer (nanodrop, USA). The cDNAs were reverse transcribed from 1 μg of total RNAs using a reverse transcription system and oligo dT15 as a primer, and the final volume was 20 μl. The reverse transcription product was diluted to 100 μl as a cDNA template. The PCR reaction was carried out on a PCR amplification system (Biometra, USA)with a total volume of 25 μl containing 1 μl of cDNA template, 0.5 μl (10 μm) of primer and 12.5 μl of Go Taq Green Master Mix. The primer sequences were as Table 1.

Table 1: Data of Primer Sequences.

Primer Names	Primer sequences(5'to3')
$TGF\beta_1$-F	ATTCCTGGCGTTACCTTGG
$TGF\beta_1$-R	AGCCCTGTATTCCGTCTCCT
PDGF-F	CAGTGACCTTGGAGGACCAC
PFGF-R	GAATGGTCACCCGAGCTTGA
TNFa-F	TGACCCCCATTACTCTGACC
TNFa-R	TTCAGCGTCTCGTGTGTTTC
AngII-F	CTTCCAGAACACGACGGGAA
AngII-R	TGTATCTGGGCCATCTCCGA
β-actin-F	CCCATCTATGAGGGTTACGC
β-actin-R	TTTAATGTCACGCACGATTTC

3.6.Western Blot Analysis

The obtained liver tissues were placed in 95% saline, rinsed several times and then transferred to a homogenizer containing RIPA buffer lysate. Protein concentrations were measured by the Bradford colorimetric method. The denatured protein was separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in an electrophoresis chamber and transferred to a polyvinylidene fluoride membrane. Blots of TGF-β_1, PDGF, TNF-α and Ang II were blocked with 5% skim milk powder-TBST buffer. Membranes were incubated overnight at 4 °C with antibodies at 1:1000 dilution of TGF-β_1, PDGF, TNF-α and Ang II. The channel load was evaluated using β-actin. The blots were rinsed 4 times with TBST buffer for 10 minutes each. The washed blots were incubated with a 1:10000 diluted horseradish peroxidase-conjugated secondary antibody for 1 hour and washed 4 times with TBST buffer for 10 minutes each. The transferred protein was immobilized with a luminescent liquid and X-rays. The films were scanned and the average optical density of the blot was analyzed using an ImageProPlus 6.0 image processing system.

4. Statistical Analysis

Parametric data were expressed as mean±SD ($x\pm s$) andanalyzed with One-Way ANOVA (analysis of variance) by the Statistical Product and Service Solutions (SPSS) 19.0. The least significant difference (LSD) method was used for individual comparisons. A P value of 0.05 was regarded as statistically significant.

5. Results

5.1. Standardization of EFT

EFT was identified by oleanolic acid and pomolic acid using HPLC. 10.00 mg EFT was mixed with 100.0 mL acetonitrile, and filtered through a 0.2 μm filter membrane after sonicating. HPLC was performed by a SHIMADZU Essential LC-16 series instrument and chromatographic separation was achieved on a Speak Technologies C18 column (250 mm × 4.6 mm, 5 μm). Gradient elution was carried out with A: B (water: acetonitrile)= 40: 60. The flow rate was 1.0 mL/min and the detection wavelength was 210 nm. The column temperature was maintained at 30°C. EFT was characterized based on the contents of oleanolic acid and pomolic acid (Figure 1).

EFT improves the morphological structure of liver fibrosis $CC1_4$-induced model of liver fibrosis was used to determine if EFT could attenuate this injury. Serum ALT, AST, Alb, TBIL, HA, and LN were detected, and hepatic histology were observed at the end of this study. Colchicine was used as the positive control (group 3). As shown in Figure 2, the levels of serum ALT in all treated groups

(groups 3 to 6) were significantly lower than the data of model group (group 2), while the level in model group is much higher than those of normal group (group 1). Likewise, the levels of AST and TBIL in these four treated groups are obviously lower than the same indicators of group 2. It is worth mentioning that the levels of ALT, AST, and TBIL in EFT high dose group (group 6) are similar with group 3 and lower than EFW group (group 4).

The trends of HA and LN are consistent with liver injury indicators. The levels of serum HA and LN in four treated groups were lower than those model group with statistical significance ($P<0.05$). Moreover, compare with EFW group, the data in EFT high dose group was lower or similar ($P<0.05$) (Figure 3). In terms of histology, slices in model group showed the serious tissue fibrous lesions and hepatocytes necrosis, while in no CCl4 exposed group, cells staining was uniform. The deterioration of tissues was less prominent in our treated groups (Figure 3).The results of biochemical and histologic analysis forcefully suggest that the fruits of *Euscaphisfukienensis* Hsu. could attenuate liver fibrosis in CCl4 induced model, and triterpenoid constituents may be the material basis of the pharmacological activity.

Effects of EFT on hepatic TGF-β_1, PDGF, TNF-α and AngIImRNA expression

In terms of mRNA expression, the results of RT-PCR were shown in Fig. 4. Both TGF-$\beta_1$and TNF-α mRNA in four treated groups were significantly lower than in model group ($P<0.05$). But there were no statistical significance difference between other two indicators, meaning that the fruits of *Euscaphis fukienensis* Hsu. had no effects on these makers.

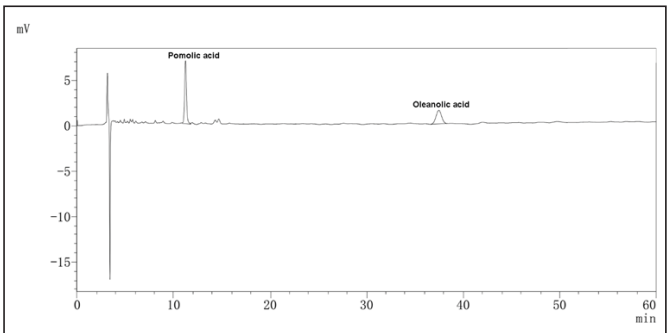

Figure 1: HPLC chromatogram of the EFT by HPLC analysis. The contents of oleanolic acid and pomolic acid in the EFT extracts were 80.30±1.16% and 16.73±0.25%, respectively.

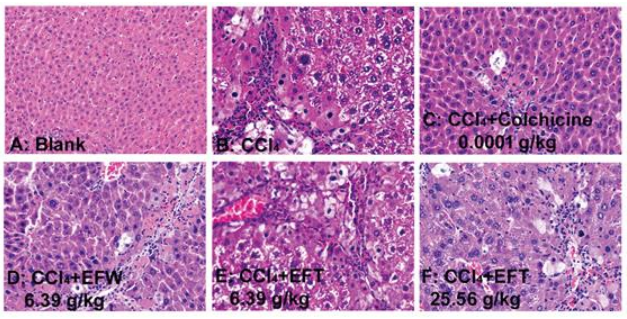

Figure 2: Diagnostic results of liver fibrosis tissue sections (magnification: ×100). (A) normal group: hepatocyte nucleus round, uniform staining, consistent cytoplasmic staining, normal hepatic sinusoids, no vacuoles, and few intrahepatic macrophages. (B) Model group: fibroid lesions were severe, and hepatocytes were massively edematous and necrotic. (C) Positive drug group: tissue had inflammatory cell infiltration, and some hepatocytes showed edema and necrosis. (D) EFW group: The tissue has certain fibroid lesions, inflammatory cell infiltration, macrophage increase, vacuolar-like changes, hepatocyte edema and necrosis, and hepatocyte size varies. (E) EFT low-dose group: mild fibrosis, inflammatory cell infiltration, vacuolar-like changes, and partial hepatocyte edema and necrosis. (F) EFT high-dose group: tissue with mild fibrosis, inflammatory cell infiltration, and vacuolar-like changes.

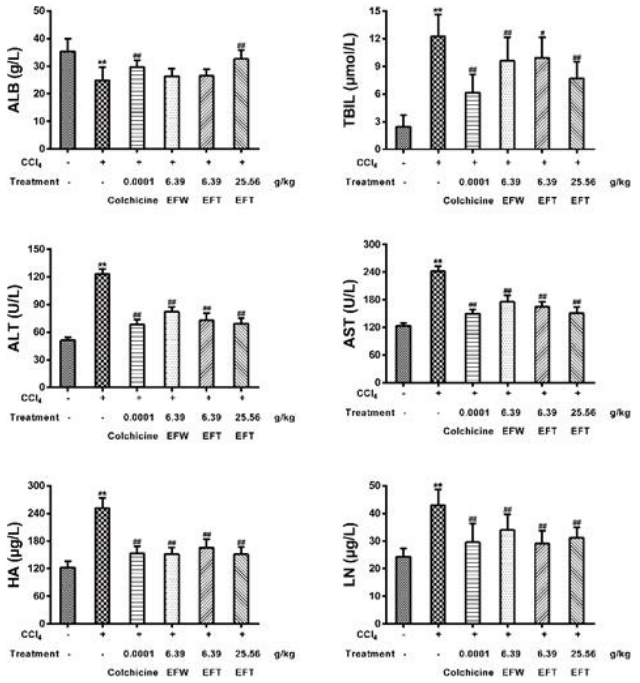

Figure 3: Effects of EFT and EFW on liver function damage and fibrosis-related factors in a CCl_4-induced liver injury model. $^{**}P<0.01$, compared with the blank group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the model group (n=10).

Effects of EFT on hepatic TGF-β_1, PDGF, TNF-α and AngIIprotein expression

To determine whether EFT altering the levels of TGF-β_1, PDGF, TNF-α and AngII cytokines, western blot analyses were performed using liver tissue extracts. The results revealed that treatment with all four concentrations of TGF-β_1, PDGF, TNF-α and AngII protein were depressed when compared to untreated group ($P<0.05$). And data in EFT high dose group was much lower than in EFW treated group (Fig. 5). Together with the results of mRNA expression, EFT could reduce the secretion of TGF-β_1, PDGF, TNF-α and AngII, which indicated that EFT could alleviate the level of LF by changing the immune environment.

6. Discussion

Liver fibrosis is an over-repair reaction to liver damage. Although the pathogenesis of the formation of fibrosis is complicated and still not completely cleared, the activated HSCs and over-synthesized ECM are considered to be the central roles in the development of liver fibrosis. The synthesis of ECM mainly comes from activated HSCs. However, after the drug enters the liver, it is hardly to absorbed by HSCs, for HSCs are in a small proportion in the intrahepatic cell population and are in a matrix-wrapped microenvironment15. This reason leads to the difficulty of the drug acting directly on HSCs in vivo to treat LF. However, The proliferation and activation of HSCs are not only their own changes, but also are regulated by cytokines in the liver microenvironment. HSCs are located in the space of "Disse", and many other cells, such as kupffer cells and dendritic cells, connect with them in this space. These cells, including HSCs, and the cytokines they release are important components of liver's innate immunity [16,17].In the inflammatory response phase of LF initiation, plentiful cytokines are secreted by immune cells, and trigger multiple signaling pathways involved in inflammation, proliferation, and apoptosis, as cascade of signals following liver injury. The secretion of TGFβ1 in particular leads to transdifferentiation of HSCs

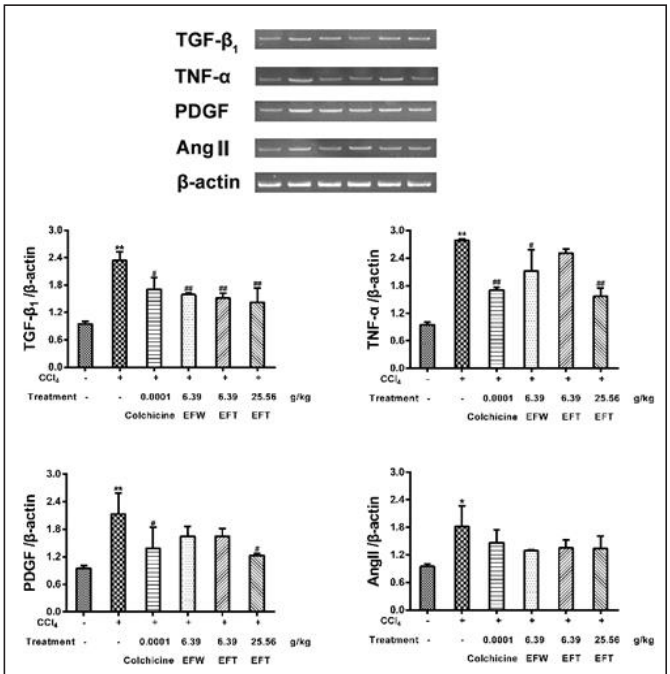

Figure 4: Effects of EFT and EFW on hepatic inflammatory factor gene in CCl_4-induced liver injury model were examined by PCR. $^{***}P<0.01$, $^{*}P<0.05$, compared with the blank group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the model group (n=6). 1: blank group, 2: model group, 3: positive drug group, 4: EFW group, 5: EFT low dose group, 6: EFT high dose group.

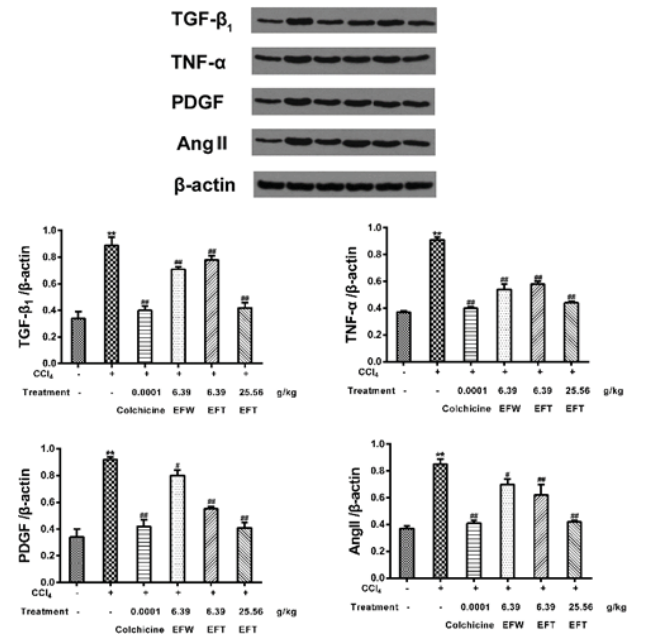

Figure 5: Western blot was used to detect the effects of EFT and EFW on hepatic inflammatory factors in CCl4-induced liver injury model. $^{***}P<0.01$, compared with the blank group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.01$, compared with the model group (n=6). 1: blank group, 2: model group, 3: positive drug group, 4: EFW group, 5: EFT low dose group, 6: EFT high dose group.

into myofibroblasts. PDGF stimulates myofibroblast proliferation [18]. The inflammatory phase is perpetuated because of TNF-α production, which leads to the activation of silent HSCs into fibrogenic myofibroblasts [12]. Previous research has showed that Ang II increased TGF-β_1 expression [19]. The role of them in promoting the development and progression of liver fibrosis is relatively clear. And they are able to interact and ultimately promote the proliferation and activation of HSCs, promote the formation of ECM, and lead to LF.

Traditional Chinese medicine (TCM) has a long history and exact curative effect on LF. A number of previous papers have described the being effective in preventing or decreasing liver fibrosis in animal models. For example, Xi et al. described lower HA, LN and less histologic evidence of fibrosis with the TCM Tao-Hong-Si-Wu which was intervened the mice with CCl4 induced LF [20]. According to the theory of TCM, Tao-Hong-Si-Wu treats LF by regulating "blood" in the bodies. Another substance maintaining liver health is "gas". TCM believes that the abnormal of "gas" is one of the causes of liver fibrosis, especially at the early stage. As a result, regulating "gas" is an important method for treating liver fibrosis by TCM. Lim et al. reported "gas" medicine Citrus aurantium could efficiently regulate bile duct ligation-induced liver injury [21]. Thus, our findings were consistent with this report and extend the benefits of the "gas" medicine in TCM to include the more commonly employed, $CC1_4$-induced liver fibrosis model. People in southern China often use the decoction of the fruits of *Euscaphisfukienensis* Hsu.to treat liver fibrosis. Chemical investigations have demonstrated that the fruits of *Euscaphisfukienensis* Hsu.contain many types of compounds, such as triterpenoids, flavonoids and aromatic acids. Triterpenoids are a kind of compounds with a wide range of physiological activities, and there have been numerous reports on their studies in liver diseases. Wei et al. reported asiatic acid, a triterpenoid isolated from Centella asiatica, exhibits efficient activity to attenuate CCl4-induced LF by regulating the PI3K/AKT/mTOR and Bcl-2/Bax signaling pathway [22]. Li et al. reported antcin K and antcin B, two triterpenoid compounds from Antrodia camphorata showed hepatoprotective effects against CCl4-induced mice liver injury [23]. The present data demonstrate that EFT, the triterpenoids composition isolated from traditional Chinese medication*Euscaphis fukienensis* Hsu., decreased serum biochemical and histologic evidence of $CC1_4$-induced hepatic inflammation, and that liver fibrosis was also attenuated in this model. And its therapeutic effect was comparable to EFW.To clarify the mechanisms underlying the therapeutic effects of EFT on liver fibrosis, we learned from the theory of "gas" in TCM. According to some modernization researches of TCM, "gas" is closely related to some immune factors in human bodies. There are also many reports of these factors on liver fibrosis. In LF rats, CCl4 induces liver release of a large number of cytokines including TGF-β_1, PDGF, TNF-α and AngII. After the intervention of EFT and EFW, the expression of these proteins was significantly downregulated. From the perspective of the compounds, oleanolic acid and ursolic acid are antagonists to TGF-β_1 signal [24]. oleanolic acid could also inhibits AngII expressing and the TGF-β_1 increasing followed by AngII stimulating [25].

7. Conclusion

In conclusion, our researches showed that EFT was the active ingredient of the fruits of *Euscaphis fukienensis* Hsu. for the treatment of liver fibrosis. These actions might be associated with the downregulation of TGF-β_1, PDGF, TNF-α and AngII.

8. Conflicts of Interest

All authors declare no presents or potential conflicts of interest. All authors are responsible for the content and writing of the paper and approved of its publication.

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