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*Original Article*

# *Euonymus alatus* and its monomers alleviate liver fibrosis both in mice and LX2 cells by blocking TβR1-Smad2/3 and TNF-α-NF-κB pathways

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**Abstract:** This study aimed to investigate the protective effects, effective constituents and preliminary mechanisms of *Euonymus alatus* on liver fibrosis and screen new high-efficacy drug for fibrosis. 112 male C57BL/6 mice were randomly divided into 14 groups: control group (CG), $CCL_4$ group (CTG), low/medium/high dose of *Euonymus alatus* ethanol extracts (EAE), catechin (CA), dihydroquercetin (DHQ) and kaempferol (KA) groups. The study lasted for 30 days by injecting $CCL_4$ in peritoneal cavity to make fibrosis model, all mice were sacrificed to observe morphological changes and collagenous fiber by HE and Masson staining, to test liver index, ALT, AST, to measure the expression of α-SMA and collagen I by immunohistochemistry and western blotting, to discuss the pathways of TβR1-Smad2/3 and TNF-α-NF-κB by WB and Elisa; after being evaluated the efficacy, anti-fibrosis drug of highest efficacy was chosen to repeat these indexes in human hepatic stellate cells-LX2. Results showed that EAE/CA/DHQ/KA prevented increases in liver index, ALT, AST, α-SMA, collagen I, TβR1, Smad2/3, TNF-α and p-NF-κB caused by CCL4 in dose-dependence, they also improved the liver morphology, decreased inflammatory cell infiltration and collagenous fiber in dose-dependence, CA' efficacy was best in mice; in LX-2, CA also decreased the expression of α-SMA, collagen I, TGF-β, Smad2/3. All findings suggested that *Euonymus alatus* could alleviate liver inflammation and fibrosis by inhibiting TβR1-Smad2/3 and TNF-α-NF-κB pathways, flavonoid were effective constituents and catechin was screened as a new star for its best performance.

**Keywords:** *Euonymus alatus*, catechin, dihydroquercetin, kaempferol, hepatic fibrosis, collagen I

## Introduction

It is known that various agents, including infections, ethanol ingestion, drug intoxication or malnutrition, result in liver damage. When the damage is prolonged, hepatic fibrosis occurs. Fibrosis is the result of excessive accumulation of extracellular matrix (ECM) proteins including collagen that occurs in most types of chronic hepatic diseases. Advanced hepatic fibrosis leads to cirrhosis even liver failure and therapeutic options are limited [1, 2], this process is mainly driven by activation of hepatic stellate cells (HSC) and chronic inflammation to release an altered activity of a multitude of different chemokines and cytokines, resulting in the infiltration by immune cells and increase of matrix-expressing cell types, this process may be reverted, like interfering the activation of HSC or decreasing immune cells infiltration [3].

Traditionally, transforming growth factor (TGF) is the most important factor in profibrogenic pathways, it has been considered to exert profibrogenic actions by binding to its receptor (TβR), which in turn phosphorylates transcription factors called R-Smads (Smad2 and Smad3) [4-6], this is the canonical pathway, because TGF can induce the transdifferentiation of HSC from a non-proliferating cell type into a proliferating activated type. This results in the cell releasing α-smooth-muscle actin (α-SMA) and collagen I, which in turn lead to fibrosis [3, 7].

In recent years, amounts of researches have revealed that hepatic fibrosis is reversible. Oakley F et al reported that the NF-κB signal transduction pathway can promote the activation of HSCs and its inhibitors can inhibit the expression of pivotal proteins and genes, these

inhibitors can induce HSCs apoptosis, so accelerating the regression of hepatic fibrosis [8]. In addition, NF-κB can promote the development of hepatic fibrosis by increasing inflammatory response [9]. Meanwhile some researches also have already showed that TNF-α was associated with hepatic fibrosis, for example: Osawa Y reported that TNF-α promoted hepatic fibrosis in a bile duct ligation model [10, 11] which showed that TNF-α was involved in fibrosis-associated proteins in livers with steatosis. These reports make us can't control our feelings to think whether TNF-α-NF-κB signal pathway exists in hepatic fibrosis and drugs in this paper have inhibitory effect on it, it is unknown but worthy to study, so this signal pathway have been also checked in this paper.

Many factors have taken part in hepatic fibrosis for certain, but no specific medicine have been developed to used in clinical, therapeutics just for fibrosis process is still poor [1]. If effective drugs can be developed, it will be greatly meaningful. *Euonymus alatus* are the wing shape branches or winged appendages of *Euonymus alatus Sieb*. It has the ability of removing blood stasis, dredging meridian, drainage and detoxification and killing insects in Chinese medicine book [12]. Now it is mainly used for diabetes, chronic nephrosis, rheumatoid arthritis and so on, it has been reported that it has the effects of regulating blood sugar level, regulating blood-lipid, oxidation resistance, anti-inflammation and antianaphylaxis, such as: Sun Xuebin [13] reported that total flavonoids which could decrease blood sugar or anti-myocadial ischemia had anti-oxidation effect, Wang Tenghua [14] verified that *Euonymus alatus* ethanol extracts and three different solvent fractions from ethanol extracts all showed anti-inflammation effect, all diseases (diabetes, chronic nephrosis, rheumatoid arthritis et al) which *Euonymus alatus* ethanol extracts had effect on have relationship with inflammation in some degree, so combining hepatic fibrosis has some relationship with inflammation [15], our team considered to choose *Euonymus alatus* ethanol extracts not water extracts as object to study its anti-fibrosis function, our pre-experiment indicated that *Euonymus alatus* ethanol extracts has anti-fibrosis effect indeed [16]. Next goal is to explore active constituents of ethanol extracts. Total flavonoid have been main active ingredients [14] of *Euonymus alatus* ethanol extracts and effective location to play against bacteria and anti-inflammation, this forces us to think flavonoid may be also effective part when playing against fibrosis, some relevant literature about its monomers also gave us this hint: 1. Catechin could attenuate the activation of HSCs by increasing PPARγ and anti-inflammatory cytokine and decreasing collagen I's expression [17-20], 2. Zhou Weichen reported KA-glycosidase can inhibit TGF-β further decrease the activation of HSC [21], 3. Dihydroquercetin has anti-oxidation and anti-inflammatory effect [22], all these substances belong to flavonoid, more importantly, these monomers had been separated and identified in *Euonymus alatus* ethanol extracts, thus, combining literature including our pre-experiemnt and our hypothesis: flavonoid may be effective part to play against fibrosis, catechin, dihydroquercetin and kaempferol will be chosen as representatives to rapidly verify the true effective part of *Euonymus alatus* ethanol extracts and this research design can screen new high-efficacy anti-fibrosis monomer for further clinical study.

## Materials and methods

### *Reagents and chemicals*

*Euonymus alatus* produced in Hubei was purchased from Enshi Hongsheng Pharm, identified by associate professor Minying Zhu in Hubei university for nationalities, this specimen is collected in herbarium of Hubei university for nationalities, CA, DHQ and KA (purity ≥98%) were purchased from Shanghai Yuanye Co. LTD.; Bruker Esquire-LC mass spectrometer, Bruker Avance DRX-500 (400) superconducting NMR, X-4 digital display micro melting point tester; macroporous resin column (Nankai university chemical plant, Tianjin), silica gel and silica gel H (Qingdao ocean chemical plant, China), polyamide for column chromatography (Shanghai chemical reagent Co. LTD., China), polyamide-6 film for TLC (Zhejiang Siqing biochemical materials factory, China), Sephadex LH-20 (pharmacia company); Alanine transaminase (ALT) and Aspartate aminotransferase (AST) kits were purchased from Nanjing Jiancheng Co. LTD., China; TNF-α Elisa kit was from BD company, USA; all primary antibodies were from Abcam company, UK; LX-2 was from ATCC, USA; Chemicals were analytically pure.

*Treatment of drugs*

*Extraction of EAE: Euonymus alatus* was smashed, then immersed in 60% ethanol with volume ratio of 10:1 for 2 hours, and completed heating reflux for 2 times. All extracts after filtration were concentrated by vacuum-rotary evaporation and became powder by freeze drying. EAE was stored at room temperature and dissolved in double steaming water when used.

*Isolation and identification of CA, DHQ and KA in EAE*

10 kg of *Euonymus alatus* was extracted by the above method, the extract was concentrated to non-alcoholic condition, filtrated after dilution. Put the supernatant liquid into the macroporous resin column, washed it until it was nearly colorless, then added 70% ethanol to collect the eluent (ethanol was recycled), last got the dry extract. The dry extract was separated by silica gel column chromatography and rinsed off by different gradient chloroform-methanol (30:1, 25:1, 15:1, 10:1, 8:1, 5:1, 2:1), each fraction was identified by thin layer and the same fraction was put together. Different mixed fractions were separated and purified repeatedly by silica gel, Sephadex LH-20 and polyamide chromatographic column, last compound 1-13 were gained. If fraction 315-387 were washed off by Sephadex LH-20 and 5:1 chloroform-methanol, then fraction 118-131 from it were recrystallized, last compound 14 could harvested.

*Animal model and protocol*

Male C57BL/6 mice weighing 15-18 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License number: 2016-0011), the procedures for the care and use of animals were approved by the Ethics Committee of the Hubei University for Nationalities (No: 2017-002), after a week of adjustable feeding, 112 male mice were randomly divided into 14 groups: control group (CG), carbon tetrachloride group (CTG), *Euonymus alatus* ethanol extracts groups of 2/4/8 g/kg (EAE-L/M/H), catechin groups of 100/200/400 mg/kg (CA-L/M/H), dihydroquercetin groups of 10/20/40 mg/kg (DHQ-L/M/H), kaempferol groups of 10/20/40 mg/kg (KA-L/M/H), 8 mice in each group. The study lasted for 30 days. Mice from control group were subjected to intraperitoneal injection of olive oil at the dose of 1.6 ml/kg while mice in other groups were injected with olive oil mixed with 25% CCL4 at the dose of 1.6 ml/kg twice a week to establish hepatic fibrosis model. EAE, CA, DHQ, KA in L/M/H doses were given from the 1$^{st}$ day to the 15$^{th}$ day by gavage. All monomers after identified in *Euonymus alatus* were bought from company directly and dissolved in double steaming water and must be shaken well before use. The diets of all mice was restricted except water on night of the 30$^{th}$ day, 24 hours later, all samples were harvested for analysis. Drug doses and duration of administration were from literature and our preliminary experiment respectively.

*Liver index determination*

The body weight of each mouse was measured before sacrificed and liver weight was measured after sacrificed. Liver index = liver weight (g)/body weight (g) ×100%.

*Serum enzyme activities*

All blood samples were gained by decapitation. After centrifugation, the supernatant was collected and then ALT, AST were measured by assay kits to determine the liver damage.

*Histology and morphology*

Liver samples were fixed in 10% formaldehyde. Tissues were washed with tap water, dehydrated in alcohol and embedded in paraffin. Staining were performed with HE and Masson's trichrome stain. Five sections were selected on glass slide.

*Immunohistochemistry*

After embedded in paraffin, liver tissues were repaired for 2 minutes in high temperature and high pressure, incubated with blocking solution (10% goat serum) at room temperature for 20 min, then incubated with primary antibodies (α-SMA: 1:150, collagen I: 1:600) overnight at 4°C, after that, 50 μl appropriate secondary antibodies for each slide were added and incubated for 30 min at room temperature. After conventional treatment, slides were checked by microscopy.

*Western blot assay*

Liver tissue (100 mg) was homogenized with 1 ml Ripa lysis buffer, then the samples were

sonicated (duration: 3 s, interval: 10 s, times: 10) and centrifuged at 12000 rmp for 20 min, the supernatant was collected and proteins were quantified by BCA method. 40 μg proteins were transferred into 10% polycrylamide and began to electrophoresis (60 V, 30 min; 110 V, 70 min), separated proteins were transferred into a PVDF membrane (110 V, 75 min), then, blots were blocked with 5% skim milk and incubated with specific primary antibodies (α-SMA: 1:3000; collagen I: 1:3000; TβR1: 1:100; Smad2/3: 1:500; NF-κB/p-NF-κB: 1:1000) overnight at 4°C, the next day, membranes were washed with TBST for 3 times and incubated with secondary peroxidase-labeled antibodies in the blocking solution at room temperature for 2 hours. Blots were then washed with TBST again and detected with ECL and digitized and analyzed densitometrically with Image software.

*Cytokine assay*

Enzyme-linked immunosorbent assay (ELISA) kits were used in accordance with the manufacturer's protocol to measure TNF-α in serum.

*Cell culture*

LX-2 was maintained in Dulbecco' Modified Eagle's Medium (DMEM) supplemented with 2% fetal bovine serum (FBS) in a humidified incubator of 5% $CO_2$ in air at 37°C.

*Cell model and protocol*

After cells passage, they were divided into the following groups: control group (CG): use fresh DMEM+2% FBS when making model; model group (MG): use DMEM+20% FBS for 48 h when making model, then collect cells; catechin groups of low, medium, high dose: 0.01/0.02/0.04 mg/ml catechin was given into the medium (DMEM+20% FBS) for 48 h when making model. 48 h later, cells were harvested to test indexes.

*Real time PCR*

Expression of collagen I, α-SMA, TGF-β1, Smad-2, Smad-3 and 18 s were measured after extraction of RNA, total RNA was reversely transcribed using PrimeScriptR RT Master (Takara, Japan), quantitative PCR was carried out with SYBRR Premix Ex TaqTM II in ABI 7200 system in accordance with kit instruction, data analysis was performed using the $2^{-\Delta\Delta CT}$ method for relative quantification, all of the gene expression levels were calculated relative to the 18 s gene. The sense and anti-sense primers were as follows: Collagen I: forward: 5'-TCCTGGTCCTGCTGGCAAAGAA-3'/reverse: 5'-CACGCTGTCCAGCAATACCTTGA-3', α-SMA: forward: 5'-GACAATGGCTCTGGGCTCTGTAA-3'/reverse: 5'-CTGTGCTTCGTCACCCACGTA-3', TGF-β1: forward: 5'-ACCTGAACCCGTGTTGCTCT-3'/reverse: 5'-CTAAGGCGAAAGCCCTCAAT-3', Smad2: forward: 5'-CGTCCATCTTGCCATTCACG-3'/reverse: 5'-CTCAAGCTCATCTAATCGTCCTG-3', Smad3: forward: 5'-GGTCAAGAGCCTGGTCAAGA-3'/reverse: 5'-TTGAAGGCGAACTCACACAG-3', 18 s: forward: 5'-CGGCTACCACATCCAAGGAA-3'/reverse: 5'-GCTGGAATTACCGCGGCT-3'.

*Statistical analysis*

The data were shown as mean ± standard deviation ($\bar{X}$ ± S) and analyzed with SPSS 24.0, click "analyze" button to test homogeneity of variance, click "independent-samples T Test" button to do Dunnett T Test for intra-group comparison. *P* value less than 0.05 was considered statistically significant.

## Results

*Structural identification results of EAE*

After extraction and separation, the compounds 1-14 were naringenin (1), aromadendrin (2), dihydroquercetin (3), hesperidin (4), kaempferol (5), quercetin (6), kaempfero-7-O-α-L-rhamnoside (7), kaempfero-7-O-β-D-glycosidase (8), quercetin-7-O-α-L-rhamnoside (9), hyperin (10), kaempferol-3,7-2-O-α-L-rhamnoside (11), quercetin-3,7-2-O-α-L-rhamnoside (12), dehydrodicatechin A (13), catechins (14) respectively. To show that the drugs mentioned really existed in EAE, here identification data for these three drugs mentioned in this article were provided. Dihydroquercetin (3): white powder, soluble in ethanol and methanol, HCl-Mg reaction: positive, EI-MS m/z: 304 [M]+. 1H-NMR (400 MHz, DMSO-d6) δ: 6.90 (1H, d, J = 2.0 Hz, H-2'), 6.78 (1H, dd, J = 8.4, 2.0 Hz, H-6'), 6.74 (1H, d, J = 8.4 Hz, H-5'), 5.86 (1H, d, J = 2.0 Hz, H-8), 5.82 (1H, d, J = 1.6 Hz, H-6), 4.85 (1H, d, J = 11.2 Hz, H-2), 4.43 (1H, d, J = 11.2 Hz, H-3); 13C-NMR (100 MHz, CD3OCD3) δ: 85.1 (C-2), 73.6 (C-3),

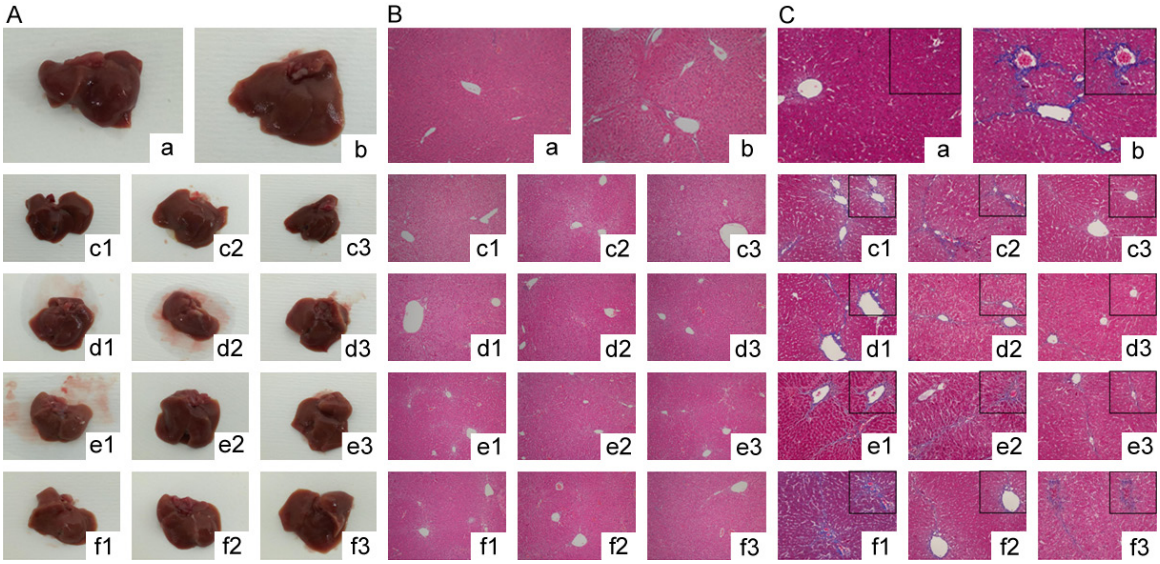


**Figure 1.** EAE, CA, DHQ and KA attenuated edema, inflammatory infiltration, pseudolobule, and collagennous fiber in liver in dose-dependence. At the termination of the study, liver was removed for morphological evaluation, fixed and cut into sections for histopathological evaluation, same magnification times for appearance (A), 100× augmentation for HE staining (B) and 200× augmentation for Masson staining: blue section was collagennous fiber (C) (a: CG, b: CTG, c1-c3: low/medium/high dose of EAE, d1-d3: low/medium/high dose of CA, e1-e3: low/medium/high dose of DHQ, f1-f3: low/medium/high dose of KA).

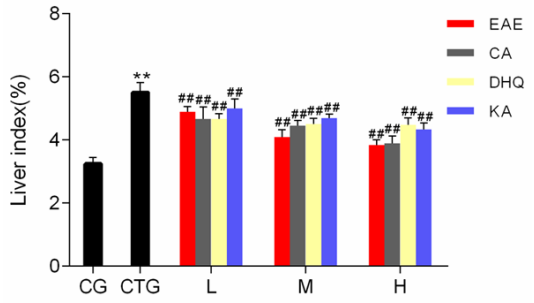


**Figure 2.** EAE, CA, DHQ and KA decreased mice liver index caused by $CCL_4$ in dose-dependence. Live index reflected liver swell in some degree. Data were presented as mean ± SD. N = 8. (L: low dose; M: medium dose; H: high dose). ***P*<0.01 *vs* CG; ##*P*<0.01 *vs* CTG.

198.4 (C-4), 164.5 (C-5), 97.3 (C-6), 168.7 (C-7), 96.3 (C-8), 165.3 (C-9), 101.8 (C-10), 129.9 (C-1'), 120.9 (C-2'), 146.5 (C-3'), 147.2 (C-4'), 115.9 (C-5'), 116.1 (C-6'), the above spectral data were consistent with the literature report [23]. Kaempferol (5): yellow powder, mp 279~282°C, soluble in ethanol and methanol, HCl-Mg reaction: positive, $FeCl_3$ reaction: positive, UV λMeOH (nm): 265, 364. ESI-MS m/z: 285 [M-H]-. 1H-NMR (400 MHz, CD3OD) δ: 8.02 (2H, d, J = 8.0 Hz, H-2', 6'), 6.84 (2H, d, J = 8.0 Hz, H-3', 5'), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.12 (1H, d, J = 2.0 Hz, H-6), the above spectral data were consistent with the literature report [24]. Catechin (14): mp 240~242°C, soluble in ethanol and methanol, HCl-Mg reaction: negative, $FeCl_3$ reaction: positive, UV λ (nm): 279, 1HNMR (CD3OD-d4, 400 MHz) δ: 6.78 (1H, d, J = 1.6 Hz, H-2'), 6.70 (1H, d, J = 8.4 Hz, H-5'), 6.66 (1H, dd, J = 8.4, 1.6 Hz, H-6'), 5.87 (1H, d, J = 2.0 Hz, H-8), 5.79 (1H, d, J = 8.0 Hz, H-6), 4.50 (1H, d, J = 7.2 Hz, H-2), 3.91 (1H, m, H-3), 2.79 (1H, dd, J = 16.0, 5.6 Hz, H-4a), 2.45 (1H, dd, J = 16.0, 8.4 Hz, H-4b). 13 CNMR (CDOD-d4, 100 MHz) δ: 82.9 (C-2), 68.8 (C-3), 28.5 (C-4), 157.8 (C-5), 96.3 (C-6), 157.6 (C-7), 95.5 (C-8), 156.9 (C-9), 100.8 (C-10), 132.2 (C-1'), 116.1 (C-2'), 146.2 (C-3'), 146.2 (C-4'), 115.2 (C-5'), 120.0 (C-6'), the above spectral data were consistent with the literature report [25]. These data showed flavonoid in EAE really contained KA, DHQ and CA.

*Anti-fibrosis effect of EAE, CA, DHQ and KA in mice*

*EAE, CA, DHQ and KA attenuated $CCL_4$-induced hepatic injury and fibrosis:* To evaluate the effect of these four drugs in mice fibrosis model, the efficacy of EAE, CA, DHQ and KA to relieve the liver injury and fibrosis indexes was

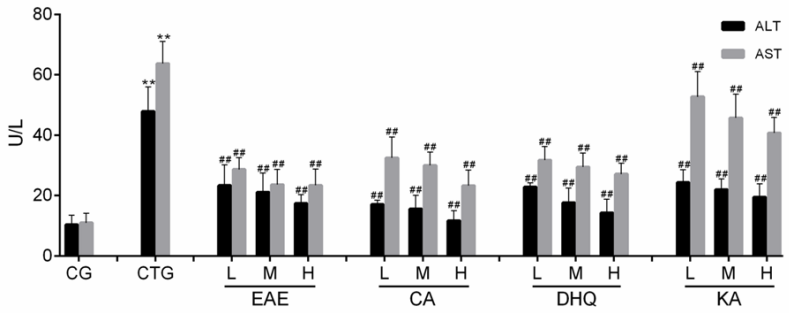


**Figure 3.** EAE, CA, DHQ and KA decreased transaminase elevation in dose-dependence. ALT and AST were determined by kits. Data were presented as mean ± SD. N = 8. ***P*<0.01 *vs* CG; ##*P*<0.01 *vs* CTG.

tested. Appearance and HE staining can show morphological change, such as: edema, inflammatory infiltration and pseudolobule, Masson staining shows the amount of collagennous fiber. Liver index can reflect liver swelling in some degree, serum ALT and AST levels, as the golden biomarker for liver damage, are indicators to evaluate liver injury. Corrugation in appearance, edema, inflammation, pseudolobule and collagennous fiber could be seen more clearly in CTG than those in CG by HE staining and Masson staining (**Figure 1**), liver index in CTG was higher than that in CG (5.51 ± 0.31 *vs* 3.24 ± 0.21, **Figure 2**), ALT and AST in CTG were also higher than those in CG (ALT: 47.89 ± 8.09 *vs* 10.35 ± 3.12; AST: 63.71 ± 7.33 *vs* 10.98 ± 3.17, *P*<0.01, **Figure 3**), these data demonstrated that 30 days administration of $CCL_4$ successfully induced liver fibrosis. However, this injury was reversed after using different dose of EAE, CA, DHQ and KA, corrugation in appearance, edema, inflammation, pseudolobule and collagennous fiber were reduced in drug groups in different degree respectively (**Figure 1**), liver index, ALT, AST in drug groups were also lower than those in CTG (**Figures 2**, **3**), the data together revealed that four drugs inhibited the increases of liver index, liver edema, inflammatory infiltration, pseudolobule, the amount of collagennous fiber and the level of serum ALT and AST caused by $CCL_4$ in dose-dependence (*P*<0.05), but their efficacy against hepatic injury and fibrosis are different.

*EAE, CA, DHQ and KA inhibited HSC activation in $CCL_4$-induced hepatic fibrosis*

The activation of HSC is the key process to lead to hepatic fibrosis. α-SMA represents the unique marker for activated HSC [26], while collagen I which can lead to fibrosis is the main matrix released by activated HSC [27-29], in order to discuss the effect of EAE, CA, DHQ and KA on the suppression of HSC activation, the expression of α-SMA and collagen I by western blotting and immunohistochemical staining were needed. α-SMA expressed higher in CTG than that in CG both in immunohistochemical staining and western blotting (**Figure 4A**, **4B**), its protein gray ratio against CG was 2.43 ± 0.18 *vs* 0.23 ± 0.03, EAE, CA, DHQ and KA downregulated α-SMA expression significantly (1.66 ± 0.24 *vs* 2.43 ± 0.18, 0.94 ± 0.11 *vs* 2.43 ± 0.18, 2.04 ± 0.25 *vs* 2.43 ± 0.18, 1.59 ± 0.12 *vs* 2.43 ± 0.18, *P*<0.01, **Figure 4C**); on the other hand, collagen I also expressed higher in CTG than that in CG both in immunohistochemical staining and western blotting (**Figure 5A**, **5B**), its protein gray ratio against CG was 3.23 ± 0.23 *vs* 0.30 ± 0.05, EAE, CA, DHQ and KA also downregulated collagen I expression significantly (2.29 ± 0.31 *vs* 3.23 ± 0.23, 0.52 ± 0.09 *vs* 3.23 ± 0.23, 0.90 ± 0.14 *vs* 3.23 ± 0.23, 1.36 ± 0.14 *vs* 3.23 ± 0.23, *P*<0.01, **Figure 5C**). All these results suggested that these four drugs inhibited the activation of HSC, further prevented the progress of hepatic fibrosis.

*EAE, CA, DHQ and KA inhibited TβR1-Smad2/3 signaling pathway in $CCL_4$-induced fibrosis*

TGF-β is recognized as a profibrogenic cytokine due to its role in HSC activation and extracellular matrix production. TGF-β signals its effect on liver cells by binding to single transmembrane type I and type II receptors (i.e. TβR I and TβR II) that are endowed with serine threonine kinase activity [4-6], later the intracellular signaling is initiated by the phosphorylation of specific proteins, among which the receptor-regulated (R) Smads, i.e. Smad2 and Smad3, have a prominent role. In order to discuss whether the anti-fibrosis effects of EAE, CA, DHQ and KA were related to this classical pathway, the expressions of TβR I and Smad2/3 after drug intervene by WB were examined. Results showed that TβR I and Smad2/3 were

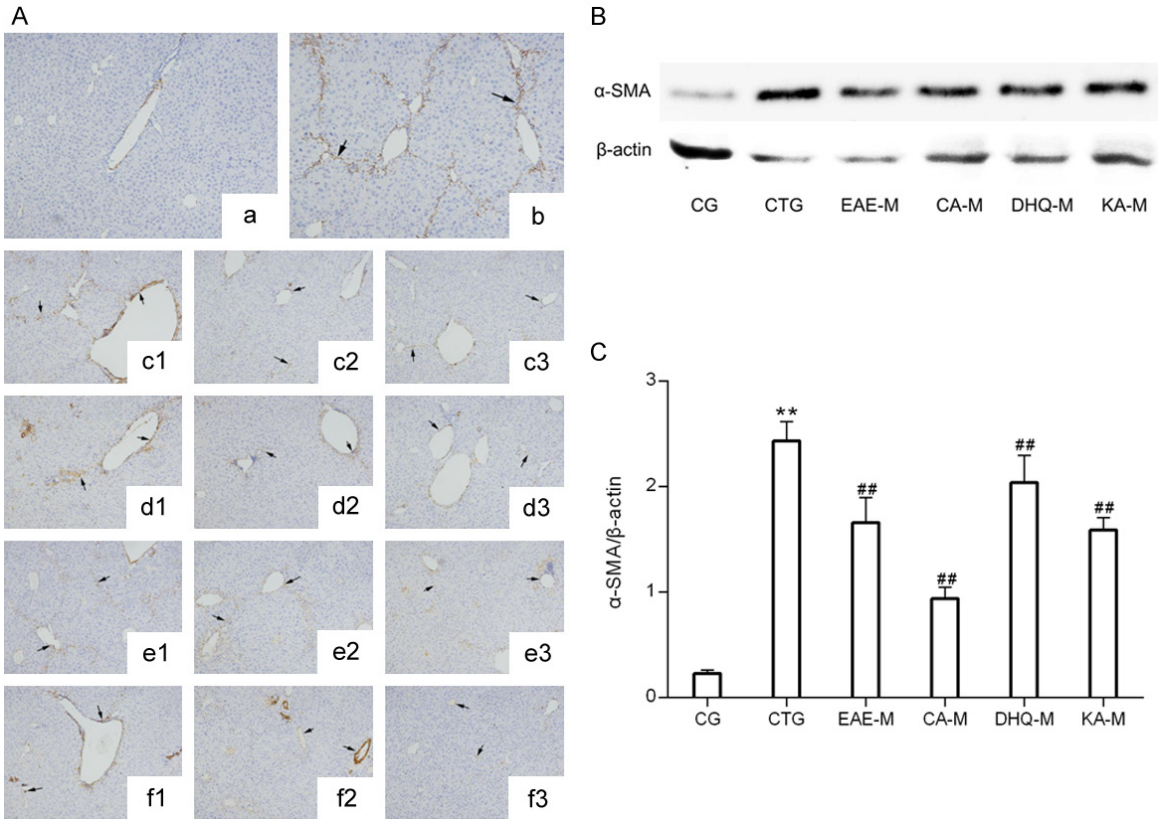


**Figure 4.** EAE, CA, DHQ and KA acted through decreasing α-SMA level. α-SMA was the marker of liver fibrosis. A: α-SMA level was tested by immunohistochemistry (100×, a: CG, b: CTG, c1-c3: low/medium/high dose of EAE, d1-d3: low/medium/high dose of CA, e1-e3: low/medium/high dose of DHQ, f1-f3: low/medium/high dose of KA), B: α-SMA level was tested by western blotting, EAE, CA, DHQ and KA were in medium dose, C: Gray ratio of α-SMA protein to β-actin protein. Data were presented as mean ± SD. N = 8. $^{**}P<0.01$ *vs* CG; $^{\#\#}P<0.01$ *vs* CTG.

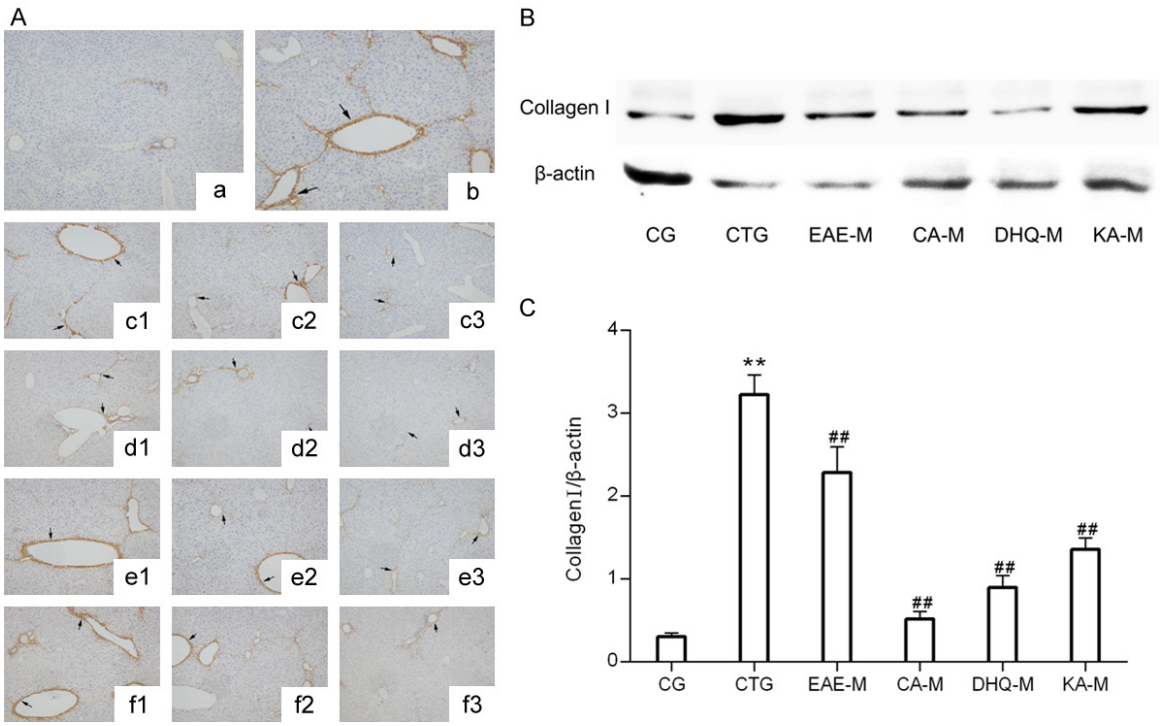

**Figure 5.** EAE, CA, DHQ and KA acted through decreasing collagen I level. Collagen I was the main factor to induce liver fibrosis. A: Collagen I level was tested by immunohistochemistry (100×, a: CG, b: CTG, c1-c3: low/medium/high dose of EAE, d1-d3: low/medium/high dose of CA, e1-e3: low/medium/high dose of DHQ, f1-f3: low/medium/high dose of KA), B: Collagen I level was tested by western blotting, EAE, CA, DHQ and KA were in medium dose, C: Gray ratio of collagen I protein to β-actin protein. Data were presented as mean ± SD. N = 8. ***P*<0.01 *vs* CG; ##*P*<0.01 *vs* CTG.

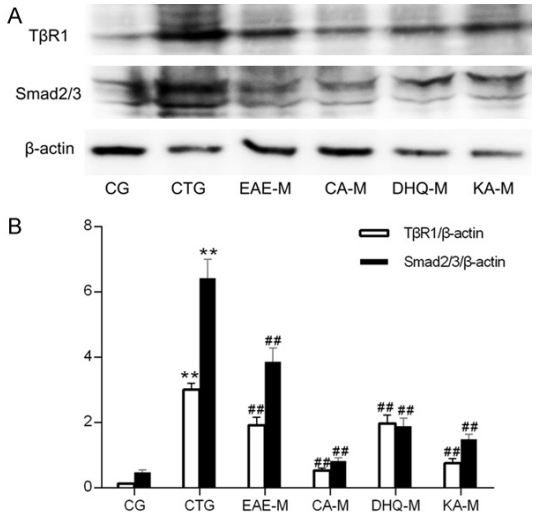


**Figure 6.** EAE, CA, DHQ and KA in medium dose blocked the function of profibrogenic pathway: TβR1-Smad. Western blotting indicated EAE, CA, DHQ and KA decreased TβR1 and Smad2/3 protein expression. A: Livers were homogenated for western blotting, B: Gray ratio of TβR1 and Smad2/3 protein to β-actin protein respectively. Data were presented as mean ± SD. N = 8. ***P*<0.01 *vs* CG; ##*P*<0.01 *vs* CTG.

high-expression when liver fibrosis (**Figure 6A**), their protein gray ratio in CTG and CG were 3.01 ± 0.20 *vs* 0.14 ± 0.02 and 6.43 ± 0.59 *vs* 0.48 ± 0.07 respectively (*P*<0.01, **Figure 6B**), EAE, CA, DHQ and KA decreased the expression of TβR I and Smad2/3 significantly (**Figure 6A**), their gray ratio against CTG in TβR I were 1.92 ± 0.24 *vs* 3.01 ± 0.20, 0.54 ± 0.07 *vs* 3.01 ± 0.20, 1.98 ± 0.26 *vs* 3.01 ± 0.20, 0.75 ± 0.14 *vs* 3.01 ± 0.20; while gray ratio against CTG in Smad2/3 were 3.87 ± 0.42 *vs* 6.43 ± 0.59, 0.83 ± 0.09 *vs* 6.43 ± 0.59, 1.90 ± 0.25 *vs* 6.43 ± 0.59, 1.49 ± 0.15 *vs* 6.43 ± 0.59 (*P*<0.01, **Figure 6B**). All these data showed that EAE, CA, DHQ and KA inhibited TβR1-Smad2/3 classical signaling pathway.

*EAE, CA, DHQ and KA suppressed TNF-α-NF-κB-mediated inflammatory response in $CCL_4$-induced fibrosis*

Inflammatory mediators, like TNF-α, are involved in $CCL_4$-induced liver damage [11]. More-

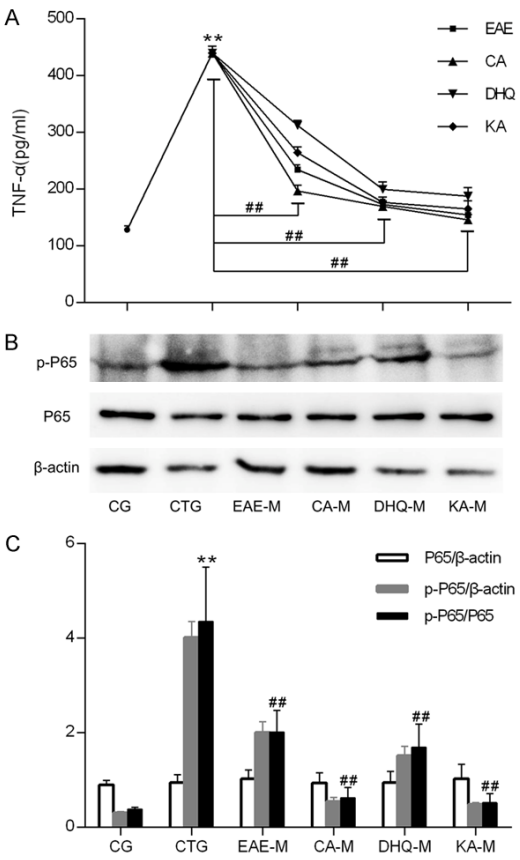


**Figure 7.** EAE, CA, DHQ and KA in medium dose blocked the function of inflammatory pathway: TNF-α-NF-κB. Western blotting indicated EAE, CA, DHQ and KA decreased TNF-α and p-P65 protein expression. A: TNF-α in serum was determined by Elisa, B: Livers were homogenated for p-P65 and P65 by western blotting, C: Gray ratio of p-P65 and P65 protein to β-actin protein respectively. Data were presented as mean ± SD. N = 8. ***P*<0.01 *vs* CG; ##*P*<0.01 *vs* CTG.

over NF-κB which promoted inflammatory factors amount was activated by $CCL_4$ stimulation [9], besides our preliminary work revealed that EAE decreased THF-α, so whether this inflammatory signal (TNF-α-NF-κB) participated liver fibrosis and drugs had inhibitory effect on this pathway was also discussed. Results showed that $CCL_4$ increased the amount of TNF-α (439.84 ± 12.09 *vs* 128.55 ± 6.61, **Figure 7A**) and p-NF-κB (4.02 ± 0.34 *vs* 0.31 ± 0.02, **Figure 7B**, **7C**), EAE and its monomers of flavo-

**Table 1.** Parameters of EAE, CA, DHQ and KA in its dose-effect curve of reducing α-SMA

| Parameters | EAE | CA | DHQ | KA |
|---|---|---|---|---|
| Bottom asymptote | 12.37801 | 43.91424 | 12.53497 | 16.61064 |
| Top asymptote | 33.38129 | 79.91388 | 18.76958 | 69.04304 |
| EC50 (mk/kg) | 2895.94678 | 202.565 | 19.01272 | 23.56528 |

Efficacy of reducing α-SMA was judged by its top asymptote.

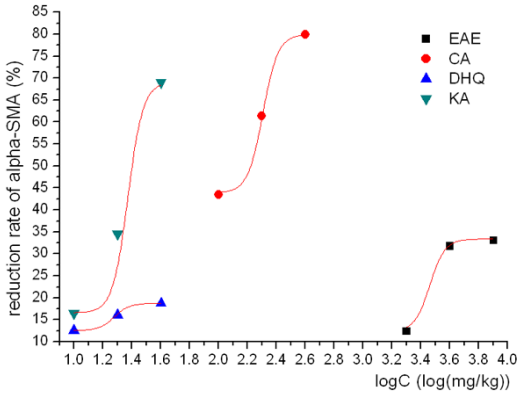


**Figure 8.** Dose-effect curve of EAE, CA, DHQ and KA on reducing α-SMA. Parameters of reducing α-SMA was tested from this curve.

noid decreased the expression of TNF-α and p-NF-κB, had no effect on NF-κB (**Figure 7**). These data suggested that EAE, CA, DHQ and KA affected TNF-α-NF-κB-mediated inflammatory response.

*Efficacy comparison of EAE, CA, DHQ and KA*

In animal study, $CCL_4$ successfully induced liver fibrosis, EAE and its flavonoid monomers: CA, DHQ and KA inhibited liver fibrosis in dose dependence but in different efficacy. Till now, the first aim of this study had been done, but from the perspective of new drug development, it was necessary to choose a drug with best effect. Usually two indices could be used to describe effect of a drug: potency (or median effective dose: EC50) and efficacy (maximum effect). Therefore, the dose-effect curves were simulated between α-SMA and each drug through software OriginPro 8, then the efficacy and potency were evaluated on this fibrogenic factor, usually the smaller the EC50 was, the less the drug dose would be used, the bigger the top asymptote was, the higher the drug effect was, results (**Table 1**; **Figure 8**) showed that DHQ's EC50 was the lowest: 19.01272 mg/kg, but efficacy was also lower than other drugs (top asymptote: 18.76958), on the contrary, CA's efficacy was the highest (top asymptote: 79.91388), the effect of reducing α-SMA by CA was the most obvious although its EC50 seemed like a bit higher, but efficacy of a new drug will be more concerned about when there is little adverse reaction within the selected dose range especially for severe and acute diseases. Based on new drug development consideration, last CA was selected for cell experiment further.

*Anti-fibrosis effect of CA in LX-2*

*CA inhibited LX-2 activation in FBS-induced human hepatic stellate cell fibrosis: α-SMA represents* the unique marker for activated HSC, while collagen I which can lead to fibrosis is the main matrix released by activated HSC, in order to observe whether LX-2 was activated and released matrix, the expression of α-SMA and collagen I were detected by real time PCR in LX-2. Results showed that 20% FBS increased α-SMA (MG *vs* CG: 6.07 ± 0.25 *vs* 1) and collagen I mRNA (MG *vs* CG: 5.37 ± 0.25 *vs* 1) successfully, compared to MG, catechin decreased their expression in dose-dependence (**Figure 9A**), these data suggested that catechin could inhibit activation of human hepatic stellate cells.

*CA inhibited TGF-β1-Smad2/3 signaling pathway in FBS-induced human hepatic stellate cell fibrosis*

The classical pathway (TGF-β1-Smad2/3) was also tested in LX-2, effect of catechin against this signal pathway was firstly verified in LX-2. TGF-β1, Smad2, Smad3 mRNA were examined by real time PCR, 18 s was reference gene. Results showed that 20% FBS increased TGF-β1, Smad2, Smad3 mRNA obviously (MG *vs* CG: 3.27 ± 0.21 *vs* 1, 6.03 ± 0.35 *vs* 1, 4.30 ± 0.26 *vs* 1, $P<0.01$), catechin reduced their expression in dose-dependence in some degree respectively (**Figure 9B**). These data implied that catechin interfered with this fibrosis-related pathway.

## Discussion

The *Euonymus alatus Sieb* has long been used as a crude drug. Now in many countries, crude

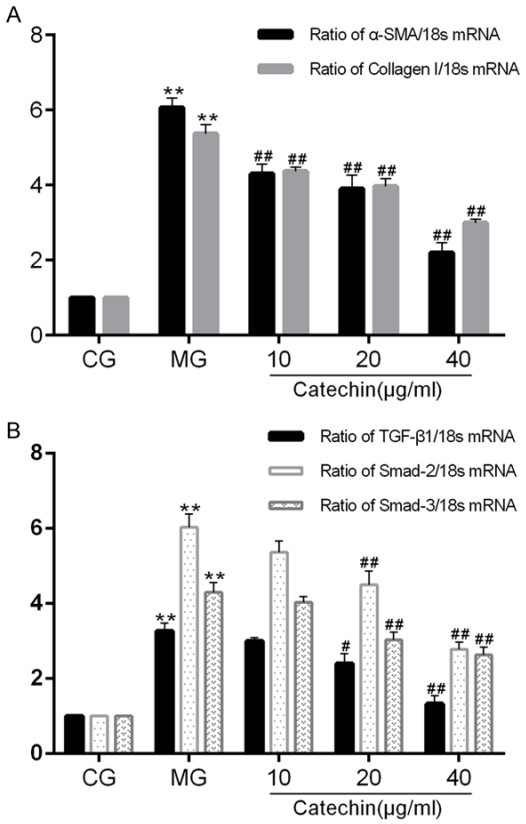


**Figure 9.** CA inhibited gene expression and signal pathway function related to liver fibrosis in LX-2 cells in dose dependence. α-SMA and collagen I as unique gene were tested by real time PCR (A), TGF-β1, Smad2, Smad3 as canonical pathway were tested by Real time PCR in LX-2 cells (B). Data were presented as mean ± SD. N = 3. ***P*<0.01 *vs* CG; #*P*<0.05 *vs* MG; ##*P*<0.01 *vs* MG.

drugs prepared from this plant are traditionally used and their pharmacological effects have been extensively explored from various viewpoints, especially for regulating blood circulation, relieving pain, eliminating stagnant blood, treating dysmenorrhea and anti-cancer in Asian countries. Reported bioactive constituents of *E.alatus* include sesquiterpenes, triterpenes, flavonoid and phenolic compounds [30-36].

Based on reported literature of our group by Huang De-bin [37] and the research by other teachers in our university [38]: *E.alatus* ethanol extracts and flavan in it have obvious anti-inflammation effect, besides, other scholars also reported flavonoid have best anti-oxidation effect [13], and even Kim [39] reported flavonoid have anti-fibrotic activity over HSC, this is the first time for us to discuss whether *E.alatus* has effect on liver fibrosis, so *E.alatus* ethanol extracts (flavonoid are in this part) were chosen as research object, in order to confirm whether the flavonoid were effective sites, three different flavonoid structure monomers were also selected for rapid experiments to verify our ideas, therefore, CA, DHQ and KA were also chosen as research objects.

In the first part, the identification data indicated that flavonoid were separated successfully and *E.alatus* ethanol extracts contained CA, DHQ and KA indeed. The pathogenesis of liver fibrosis is complicated, in which the activation of HSC via transformation to myofibroblast-like cells that express α-SMA is a key issue [40]. It was reported that in normal rat liver, α-SMA was observed only in vascular smooth muscle cells. With the progression of fibrosis induced by $CCL_4$ injection, α-SMA-positive cells appeared in the perisinusoidal space and fibrous septa, and ultimately surrounded regenerative nodules [41]. Therefore, α-SMA is a good biomarker of liver fibrosis, in the second part in our study, we observed the expression of α-SMA in CTG, which could be reduced after EAE, CA, DHQ and KA treatment, the reduction ability of these four drugs were different. The events subsequent to HSC activation, including the augmented production and disposition of ECM, are crucial for the hepatic fibrogenesis cascade [42]. In advanced stages, the liver contains 6 times more ECM than normal, including collagen [1], so in second part in our study, accumulated collagen I in the liver tissue was also observed by IHC and WB, which also could be attenuated by EAE, CA, DHQ and KA treatment in different efficacy, these data were also consistent with that of Masson staining of liver tissue. TGF-β1 may stimulate and activate HSC to proliferate, and the activated HSC may synthesize and secrete ECM constantly by autocrine and paracrine mechanisms, which subsequently activate adjacent HSC, leading to a sustained fibrosis, TGFβ/Smad pathway is the most extensively investigated molecular mechanism in hepatic fibrogenesis [43], cell type-specific intervention of TGFβ/Smad signaling suppresses collagen gene expression and hepatic fibrosis in mice [44], thus, in second part in this study, an inhibitory effect of EAE, CA, DHQ and KA on this pathway was also observed by WB, EAE, CA, DHQ and KA decreased the expression of TGFβ receptor and Smad2/3, but how these four drugs effected on these two transcription factors were still unknown, and need-

ed to investigate in the following studies, besides TGFβ, many inflammatory cytokines, such as TNF-α were also secreted by the damaged hepatocytes and activated Kupffer cells to activate HSC, therefore, in the second part in this study, a new signaling: TNF-α-NF-κB was explored and result showed that EAE, CA, DHQ and KA also decreased the expression of TNF-α, NF-κB to interfered this pathway with their own abilities. Once fibrosis was alleviated, liver damage would be reduced and liver would be protected, so in the second part in this study, the most common indicators of liver injury: ALT, AST, were examined too, high expression of these two serum factors were decreased by EAE, CA, DHQ and KA in dose dependence significantly, all results suggested that *E.alatus* ethanol extracts, CA, DHQ and KA had effects on fibrosis and two signal pathways in animal experiment, but CA in the dose used in this paper performed best in many indexes, such as: HE, Masson, college I and TβR-1/Smad2/3 pathway, this apparent difference in efficacy were exciting, therefore, we were curious whether new specific drug for fibrosis would emerge, thus, in the third part in this study, four dose-effect curves were mapped to analyze the anti-fibrotic capacity of EAE, CA, DHQ and KA, according to the results and a comprehensive analysis of Emax and EC50, it was found that CA was indeed a potential high-effect drug, in order to further determine the role and mechanism of CA, experiments were also designed in human HSC cell: LX-2 in the fourth part in this study, and results showed that CA also had inhibitory effects on α-SMA, collagen and TGFβ/Smad mRNA. Although effects of EAE, CA, DHQ and KA were determined, although it was found that flavonoid were active sites and CA had high efficacy against fibrosis, some results, for example, KA had better effect on NF-κB expression and EAE were showed advantages in liver damage indexes, like: liver index, ALT and AST, even including why ethanol extract and monomers all have anti-fibrosis effect but the effect of ethanol extract is lower than that of monomers, were still worth our attention, thus, we tried to make some inferences based on the current literature and the nature of these drugs.

Catechin is a flavan-3-ol monomer, existing in a variety of food and plant, based on this structure, most reports focused on its antioxidant and anti-inflammatory properties [45, 46]. Only a few articles [17-20] directly reported that it could attenuate the activation of HSC by increasing PPARγ and anti-inflammatory cytokine and decrease collagen I's expression, so according to its chemical structure and these few literature, we inferred that antioxidation of Catechin was the basis to play effect, it could also influence the expression of some inflammatory cytokine, but these two causes were not the leading ways because it also has significant effects on some fibrosis factors, so performing well in HE, Masson, college I and TβR-1/Smad2/3 pathway was understandable in this study, however, gene chip screening hasn't been done, now it was hard for us to infer out the drug target and full signal pathway it acts on. This required further study in the future.

Kaempferol was reported to have anti-inflammatory, anti-oxidative, anti-cancer and anti-proliferation actions [47-50], even Zhou Wei-chen reported KA-glycosidase could inhibit TGF-β further decrease the activation of HSC, last decreased the secretion of collagen I and collagen III [21], so it was chosen as research object in this paper, from our results, it did work and had better effect on NF-κB, so we inferred that its anti-inflammatory effect like the inflammatory effect of CA was also the basis, whether this though was right or not, it was needed to make more research about KA on other inflammatory cytokines, like: interleukin.

Dihydroquercetin was mostly reported to have anti-oxidation and anti-inflammatory effects [22]. No English literature had reported the relationship between this substance and fibrosis. From our results, it also worked when playing against liver fibrosis, and had effect on TNF-α-NF-κB and TβR1-Smad2/3 pathways, we guessed that both its anti-oxidation and anti-inflammatory effects contributed to its anti-fibrosis effect, specific target and full pathway against were also needed to be done further.

As for the role of *Euonymus alatus* ethanol extracts, now we made sure it had effect on liver fibrosis and its efficacy was middle among these flavonoid monomers, we made the following guess: 1. flavonoid make contribution to *Euonymus alatus* ethanol extracts in inhibiting liver fibrosis, its mechanism may be related to their anti-oxidation effect, anti-inflammatory

effect and anti-fibrotic factors, but it was not clear whether other ingredients in ethanol extract would work; 2. even if flavonoid were the only effective parts, monomers in primitive compatibility maybe an "error" because they weaken the efficacy of ethanol extract, so it is imperative to choose effective monomers in it to rematch to get a highest compound preparation.

## Conclusions

In summary, *Euonymus alatus* ethanol extracts have significant effect on liver fibrosis, three flavonoid monomers in it show anti-fibrosis effect at same time, so flavonoid is the effective constituent, among which CA performed best, the mechanism of these flavone may be related to inhibiting TNF-α-NF-κB and TβR1-Smad2/3 pathways. This study screened out a new hopeful drug: catechin, for clinical use further and confirmed that inflammation is a pathogenesis of fibrosis indeed.

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## Disclosure of conflict of interest

None.

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