Therapeutic effects of the traditional medicinal plant Ipomoea stolonifera for the treatment of liver diseases
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Chapter 4

Multifaceted esculetin suppresses hepatic stellate cell activation and CCl4-induced liver fibrosis in mice

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Abstract

**Background:** Chronic liver diseases typically lead to liver fibrosis, which may progress to cirrhosis and liver cancer. Liver fibrosis is caused by overactive hepatic myofibroblasts, but is reversible when the disease-causing insult is abrogated. However, no drugs are available that support resolution of hepatic fibrosis. Esculetin (6,7-dihydroxy-coumarin) is a natural compound that inhibits lipoxygenases and has anti-inflammatory, anti-tumor and anti-arrhythmic properties. Inhibition of 5-lipoxygenase in hepatic stellate cells (HSC) suppresses the transdifferentiation to myofibroblasts. Here, we studied the effect of esculetin on hepatic myofibroblasts and CCl4-induced liver fibrosis in mice.

**Methods:** Culture-activated primary rat HSC and portal myofibroblasts (PMF) were exposed to esculetin. C57BL/6 mice were treated for 4 weeks with CCl4 with esculetin co-treatment (5, 10 or 20 μg/g) during the last 2 weeks.

**Results:** Esculetin inhibited proliferation and expression of activation markers (Col1a1/α-Sma) of *in vitro*-cultured HSC and PMFs. Esculetin dose-dependently reduced CCl4-induced Sirius-red staining (-64%), Masson trichrome staining (-60%) and hydroxyproline levels (-46%) even below levels observed in 2 week CCl4-treated mice. Concomitant significant reductions were observed in mRNA and/or protein levels of TGF-β, collagen type 1, fibroblast-specific protein-1, α-Sma and Smad2/3 phosphorylation. Esculetin did not change Pdgfr-β and Pparγ expression, but did enhance the hepatic ratios of MMP2-9-13/TIMP-1 and GSH/GSSG.

**Conclusion:** Esculetin halts and partly reverses liver fibrosis under persistent CCl4 exposure by suppressing profibrotic TGF-β/Smad signaling and myofibroblast proliferation, while enhancing fibrolysis and the anti-oxidant capacity of the liver. Thus, multifaceted esculetin may be a relevant candidate drug to treat liver fibrosis.
Introduction

Liver fibrosis is the result of an uncontrolled wound healing response to chronic liver injuries, such as viral and autoimmune hepatitis and (non-) alcoholic fatty liver diseases. Liver fibrosis may progress to cirrhosis in which the liver architecture is irreversibly disturbed and predisposes for liver cancer. Hepatic stellate cells (HSC) play a central role in hepatic fibrogenesis. In the healthy liver, HSC contain most of the body supply of vitamin A stored as retinyl esters in large cytosolic lipid droplets. Upon liver injury, HSC transdifferentiate into hepatic myofibroblasts that loose their vitamin A and become highly proliferative, contractile and produce excessive amounts of extracellular matrix proteins (ECM), including collagens and fibronectins. In addition to HSC, ECM-producing myofibroblasts may also originate from portal myofibroblasts (PMF) and bone marrow-derived mesenchymal stem cells [1, 2]. TGF-β plays a pivotal role in the activation of fibrogenic myofibroblasts, mediated through the Smad pathway. Therapeutic resolution of liver fibrosis may be achieved by suppressing the activation and proliferation of myofibroblasts, reducing the synthesis of excess ECM and/or improving the balance of enzymes that modulate the ECM: matrix metalloproteinases (MMPs) versus tissue inhibitor of metalloproteinases (TIMPs). Despite a multitude of studies focusing on molecular targets in these pathways, no efficient and well-tolerated antifibrotic drugs are available yet. Ideally, an antifibrotic drug would affect different aspects of the process of fibrogenesis, e.g. organ inflammation, myofibroblast activation and/or scar tissue formation.

One such product may actually be present in nature. Esculetin (Esc), or 6, 7-dihydroxycoumarin, is present in many plants and medicinal properties have been assigned to stem bark of Fraxinus rhynchophylla [3] and the herbs Cichorium intybus (chicory) [4-6] and Artemisia capillaris (Compositae) [7, 8], which contain high levels of this compound. Esculetin possesses multiple pharmacological activities, including analgesic, anti-inflammatory [8, 9], anti-tumor [10, 11], anti-arrhythmic [12], antisteroidogenic [13, 14] properties. Mechanistically, it has been shown to suppress cell proliferation, particularly of cancer cells [15, 16]. Furthermore, it acts as an anti-oxidant and inhibits apoptosis, e.g. of hepatocytes exposed to CCl₄ [17, 18]. Esculetin is a non-competitive inhibitor of 5-lipoxygenase and 12/15-lipoxygenase, enzymes that produce leukotrienes and lipoxins. Both enzymes have independently been show to promote fibrosis in various organs, including lung [19] and heart [20-22]. Hepatic lipoxygenase expression was assumed to be restricted to Kupffer cells [23, 24], but we [25] and others [26] have recently found that HSC also express 5-LO and that 5-LO is induced upon transactivation of myofibroblasts in vitro. Inhibition of 5-LO, both transcriptionally and pharmacologically, suppressed HSC proliferation and reduced expression of HSC activation markers collagen 1a1 and α-Sma. 5-Alox knockout mice show resistance to hepatic inflammation and develop markedly reduced fibrosis when exposed to hepatotoxic agents like CCl₄ [27]. Esculetin effectively prevents acute liver damage in mice [17] and rats [18, 28, 29] caused by a single dose of CCl₄. In chronic liver injury models, esculetin is typically given
simultaneously with the liver damage-causing agent/condition without or with a short pre-treatment with esculetin. Despite its potent hepatocyte-protective effect, its true value in treatment of liver fibrosis with hepatic myofibroblasts as the main target remains elusive. Here, we studied the direct effect of esculetin on primary rat HSC and its potential as an antifibrotic drug after CCl₄-induced liver injury in mice with established liver fibrosis.

Materials and methods

Animals and CCl₄-induced liver fibrosis

Male C57BL/6 mice (Vital River Laboratories, Beijing, China) were housed in the SPF animal facility of Shantou University Medical College under standard 12hr light/12hr dark cycle and fed standard rodent chow and water ad libitum. After 7 days adjustment, mice were randomly divided into eight treatment groups (CCl₄ groups n=10; control groups n=6). Hepatic fibrosis was induced as described before [30] by twice-weekly injections of CCl₄ (i.p.; 0.4 µl/g BW) diluted 1:3 in olive oil CP (Aladdin Chemistry Co. Ltd, Shanghai, China) for 4 weeks (total nine injections). Esculetin treatment (Alfa Aesar, MA, USA; i.p. 5 μg/g, 10μg/g or 20μg/g BW in PBS) was started at week 3 (together with 5th CCl₄ injection), given once a day for 16 days in total, with continued CCl₄ treatment. Mice injected with CCl₄ for 2 weeks and 4 weeks were included for comparison. The latter group received daily mock injections with PBS as control for esculetin treatment. Control mice received olive oil-injections for 2 or 4 weeks. Additionally, one group received esculetin alone at the highest dose (20μg/g) for 16 days. Mice were sacrificed 48 h after the last CCl₄ injection. Body weight was determined before every CCl₄ injection. Blood samples were collected at sacrifice for biochemical analyses. The left lateral liver lobe was prepared for histological analyses. Remaining liver tissue was snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation.

Isolation and culture of primary rat hepatic stellate cells and portal myofibroblasts

Specified pathogen-free male Wistar rats (Harlan, Zeist, the Netherlands) were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were approved by the local committee for care and use of laboratory animals of the University of Groningen, the Netherlands.

Hepatic stellate cells (HSC) were isolated from 500-600g male rats as described previously [31]. Cell purity was at least 90% after isolation and 100% after 1 day of culturing. HSC were culture-activated for 7 days. HSC were passaged by trypsinization. Cells at passage 1 or 2 were used for experiments.

Portal myofibroblasts (PMF) were isolated from residues of portal tree of 220-250g male rat as described earlier [32]. Freshly-isolated PMF were passed after 5 days and
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passages 2-4 were used for experiments. HSC and PMF were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1×MEM non-essential amino acids (Invitrogen), 50 µg/mL gentamicin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 µg/ml streptomycin (Lonza), 250 ng/mL fungizone (Lonza) in a humidified atmosphere containing 5% CO₂ at 37°C. Culture-activated HSC and PMF were treated with esculetin (50-100 µmol/L) in 20% serum-containing medium for the indicated time points.

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from liver tissue using RNAiso Plus (Total RNA extraction reagent TaKaRa, Japan) and from HSCs and PMFs using Tri-reagent (Sigma-Aldrich) according to the manufacturer’s instructions. Purity and concentration were analyzed by Nanodrop 2000 (Thermo Scientific). 1 µg of total RNA was reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time TaKaRa, Japan; for liver tissue) or using random nanomers (Sigma-Aldrich; for HSC and PMF). Quantitative reverse-transcription polymerase chain reaction assays were performed on the Eco Real-Time PCR System (Illumina USA) using SYBR Green reagent (TaKaRa, Japan) or ABI PRISM 7700 (PE Applied Biosystems) using taqman probes. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (Ct value) were normalized to the endogenous control (Gapdh for liver tissue; 36B4 for HSC and PMF) and correlated inversely with initial mRNA levels. Relative quantification was performed using the ddCt method. Real time PCR primers and probes are given in Supplementary Table S1.

Biochemical analyses

ALT, AST, and LDH were quantified using the Bio-sinew (Chengdu, China) and Leadman (Beijing, China) kits and the IFCC recommend method on an Automatic Chemistry Analyzer (Accute TBA-40FR, Toshiba medical systems corporation, Japan). Serum levels of mouse TIMP-1 were measured by quantikine ELISA (R&D systems, USA). Tissue hydroxyproline content was determined using the Chloramine-T Hydroxyproline Assay Kit (Nanjing Jiancheng Biotechnology Institute Co., Ltd., Nanjing, China.) according to the manufacturer's protocol. Tissue glutathione levels were determined using the GSH/GSSG assay kit (Beyotime, Jiangsu, China) according to the manufacturer's protocol.

SDS-PAGE and Western blot analysis

Liver samples were homogenized in RIPA lysis buffer (P0013B; Beyotime). Equal amounts of proteins (50 µg) were separated by SDS-PAGE and transferred to nitrocellulose filter membranes. Following blocking, membranes were probed with
primary antibodies: mouse anti α-SMA monoclonal antibody 1:2,000 (Sigma); mouse phospho-Smad2(Ser465/467)/Smad3(Ser 423/425) (D27F4), rabbit monoclonal antibody 1:1,000 (Cell Signaling Technology, USA), anti-TGF-β antibody ab66043 1:1,000 (Abcam, Cambridge, UK), mouse anti-GAPDH monoclonal antibody 1:3,000 (ZSGB-BIO, Beijing), followed by HRP-conjugated secondary mouse antibody 1:60,000 or secondary rabbit antibody 1:80,000 (ZSGB-BIO, Beijing) at room temperature for 1 h. Target proteins were visualized using an enhanced chemiluminescence SuperSignal West Dura detection system (Thermo scientific, IL, USA). Signals were quantified by Quantity One (Bio-Rad).

**Gelatin zymography**

Gelatinase substrate gel electrophoresis was performed using 30% polyacrylamide gels containing 1% gelatin. Samples were prepared in 2x loading buffer consisting of 1 mol/L Tris, pH 6.8, 10% SDS, 20% glycerol, 0.2% bromophenol blue. 50 µg protein was loaded per sample. After electrophoresis, gels were incubated twice in renaturing solution (2.5% Triton X-100) for 30 minutes at room temperature and then for 24 hours at 37°C in a developing buffer containing 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 1% Triton X-100. Gels were stained with Coomassie Brilliant Blue R-250. MMP-2 and MMP-9 gelatinolytic activity was quantified by Quantity One (Bio-Rad).

**Cell Proliferation Assay**

Activated HSC or PMF were seeded in a 96-well plate and grown for 3 days in the absence or presence of esculetin. Proliferation was assessed at day 3 allowing BrdU incorporation for 3 h using cell proliferation ELISA kit (Roche, Almere, the Netherlands) according to the manufacturer’s instructions and measured spectroscopically (The Synergy™HT, BioTek Instruments, Inc.).

**Histology and immunohistochemical studies**

Liver samples were fixed in 4% paraformaldehyde and embedded in paraffin. 4-µm paraffin sections were processed following routine methods. Masson trichrome and Sirius-red staining (Beijing Leagene Biotechnology Co.,Ltd.) were performed according to manufacturer’s instructions. Deparaffinized liver sections were immunostained for α-SMA (Sigma; dilution 1:200) using a two-step immunohistochemistry kit (Super Vision, Boster, Wuhan, China). Staining was determined in at least 10 separate fields and quantified using a computer-aided image analysis system IPP6.0.

**Immunofluorescence microscopy**

HSC and PMF were cultured on glass coverslips and fixed in 4% paraformaldehyde/PBS. Cells were permeabilized by 1% Triton X-100 for 5 min.
Nonspecific antibody binding sites were blocked in 0.5% BSA/PBS for 30 min. Next, cells were incubated with primary antibodies α-SMA (Sigma Aldrich, 1:500) and collagen 1α1 (Southern Biotech, 1:30) in 0.5% BSA/PBS for 1-2 h at room temperature. Secondary antibodies were Alexa fluorophores (Molecular Probes, 1:500). Coverslips were mounted in fluorescence mounting medium S3023 containing DAPI for nuclear staining (DAKO). Staining was visualized using a Zeiss 410 inverted laser scan microscope.

Statistical analysis

All data are presented as mean ± standard deviation. Significance of differences between groups was tested by one-way ANOVA and t-test. Calculations were made using the software of GraphPad Prism 5. Results were considered statistically different when the p value <0.05.

Results

Esculetin inhibits proliferation of primary rat HSCs and PMFs

First, we analyzed whether esculetin directly affects proliferation and/or activation of the 2 main sources of hepatic myofibroblasts, portal myofibroblasts (PMF) and hepatic stellate cells (HSC) (Fig. 1). Culture-activated primary rat PMF and HSC were treated for 3 days with or without esculetin (50 or 100 µmol/L). Both BrdU incorporation (Fig. 1A) and PCNA protein levels (Fig. 1B) were sharply decreased in PMF and HSC after esculetin treatment, suggestive of a potent suppression of cell proliferation. Messenger RNA (Fig. 1C) and protein levels (Fig. 1D) of collagen1a1 and α-Sma were slightly reduced after treatment with 50 µmol/L esculetin, but a clear reduction of these myofibroblast activation markers was observed at higher concentrations (100 µmol/L) of esculetin (Fig. 1D). No significant induction of PMF or HSC cell death was observed at these conditions as indicated by the absence of cellular LDH release during the course of the treatment (Supplementary Fig. S1).
Esculetin inhibits proliferation and activation of primary rat HSC and PMF. Primary HSC and PMF were cultured for 3 days in the absence or presence of esculetin. BrdU incorporation was quantified on day 3 (A). PCNA protein levels (B) and Col1a1 and Acta2 mRNA levels (C) were determined by Western blotting and Q-PCR, respectively. (D) Immunofluorescent microscopy of intracellular collagen (green) and α-Sma (red) in PMF (left panels) and HSC (right panels) in the absence (top panels) and presence of 50 or 100 µmol/L esculetin (Esc) (original magnification 200×). *p <0.05, **p <0.01 compared to control. Experiments were performed from at least 3 different HSCs and PMFs isolations.

**Esculetin halts progression and partly reverses liver fibrosis in CCl4 treated mice**

Esculetin has been shown to protect hepatocytes against acute toxicity by a single high dose of CCl4. To determine its efficacy as an antifibrotic drug, we subjected mice to the chronic model of CCl4-induced fibrosis, where mice were given CCl4 injections twice a week for 4 weeks and esculetin treatment was started from week 3 on a daily
basis until sacrifice at the end of week 4. Body weight characteristics were comparable for all CCl₄-treated groups, showing an initial drop after the first CCl₄ injection after which body weight gain is observed during the consecutive CCl₄ treatments (Fig. 2A). Liver weight at sacrifice was similar for all experimental groups and comparable to the untreated controls (Fig. 2B). Serum transaminases (ALT, AST) were strongly elevated after 2 weeks CCl₄ treatment (Fig. 2C). After 4 weeks CCl₄, AST and LDH were not significantly elevated anymore, while ALT levels were 40% reduced compared to 2 week CCl₄-treated mice, but still significantly enhanced above normal levels. Albumin and total bilirubin levels were comparable in all groups and not different from untreated controls (Supplementary Fig. S2). Sirius-red (Fig. 2D) and Masson trichrome (Fig. 2E) staining progressively increased in livers after 2 and 4 week CCl₄ treatment, with evident perilobular fibrosis and marked bridging fibrosis after 4 week CCl₄ treatment. Hepatic hydroxyproline levels were significantly enhanced in 2-week and 4-week CCl₄ treated mice compared to controls (Fig. 2F).

Esculetin dose-dependently reduced serum ALT levels in CCl₄ treated mice, but levels remained increased compared to untreated controls (Fig. 2C). AST and LDH levels were not elevated after 4-week CCl₄ treatment and esculetin did not change this. Sirius-red and Masson trichrome staining revealed that esculetin dose-dependently reduced hepatic collagen deposition in CCl₄-treated mice (Fig. 2D and E). The Sirius red-positive area dropped from 3.99% in 4 week CCl₄-treated mice to 1.44% in animals receiving the highest dose of esculetin, which was similar to the level after 2 weeks CCl₄ treatment (Fig. 2D). Even more pronounced, esculetin reduced the Masson trichrome-positive area from 8.45% to 3.39% after 4 weeks of CCl₄ treatment, which is significantly lower compared to mice treated for 2 weeks with CCl₄ (6.17%) (Fig. 2E). Similarly, esculetin dose-dependently reduced hepatic hydroxyproline below levels in livers from mice treated for 2 weeks CCl₄, marking the start of esculetin treatment (Fig. 2F). In line with the histological observations, hepatic mRNA levels of collagen 1a1, Acta2 and fibroblast-specific protein (Fsp1) were dose-dependently reduced by esculetin (Fig. 3A) and accompanied with a reduction in α-Sma protein (Fig. 3B). Strong periportal staining was observed for α-Sma in livers of 4 week CCl₄-treated mice, which was almost completely absent when co-treated with the highest dose of esculetin (Fig. 3C). Moreover, parenchymal staining of α-Sma was observed in the initial stages of fibrosis after 2 week CCl₄-treatment, which was not detected in mice subsequently treated for 2 weeks with CCl₄ together with 20 µg esculetin/g body weight (Fig. 3D).
Figure 2. Esculetin halts progression and partly reverses liver fibrosis under persistent CCl₄ administration in mice. (A) Body weight development during CCl₄ treatment ("Time" indicates CCl₄ injections). (B) Liver mass as percentage of body weight at sacrifice. (C) Serum liver damage makers (ALT, AST and LDH) at sacrifice. Collagen deposition as determined by Sirius-red staining (D) and Masson trichrome staining (E) (original magnification 200×) including quantification of staining by densitometry. (F) Hepatic hydroxyproline content (µg/g). *p <0.05, **p <0.01, compared to CCl₄ 4w. ###p <0.01, ####p <0.001 compared to control 4w.
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**Figure 3.** Esculetin suppresses CCl₄-induced mRNA and protein expression of hepatic myofibroblast activation markers. Liver homogenates analyzed for (A) mRNA levels of *Collagen 1a1*, *Acta2* and *Fsp1* by Q-PCR and (B) α-SMA protein expression by Western blotting. GAPDH was used as loading control. Protein bands were quantified by densitometry and corrected for GAPDH. (C) Immunohistochemical staining for α-SMA (original magnification 200×). (D) shows a zoom of (C) providing details of the cellular distribution of α-Sma staining in the parenchyma. *p < 0.05, **p < 0.01 compared to CCl₄ 4w. #p < 0.05, ##p < 0.01, ###p < 0.001 compared to control 4w.

**Esculetin differentially affects lipoxygenase mRNA expression**

Esculetin is a non-competitive inhibitor of 5-lipoxygenase (5-LO) and 12/15-lipoxygenase (12/15-LO). We did not observe compensatory expression of either of the enzymes in the esculetin-treated mice. Both *Alox5* (16-fold) and *Alox15*
Figure 4. Differential effect of esculetin on CCl₄-induced lipoxygenase expression. *Alox5* and *Alox15* mRNA levels were strongly increased after 2 week CCl₄ treatment after which both decreased at 4 week CCl₄ treatment, but remain elevated compared to untreated control. Esculetin did not affect *Alox5* mRNA levels, but fully suppressed *Alox15* expression. ***p <0.001 compared to CCl₄ 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.

(35-fold) mRNA levels were strongly increased after 2 weeks CCl₄ treatment (Fig. 4), after which they decreased at 4 weeks CCl₄ treatment to 4-fold and 23-fold enhanced levels compared to untreated controls, respectively. Esculetin treatment during continued CCl₄ administration did not change *Alox5*, mRNA levels (Fig. 4). In sharp contrast, esculetin fully suppressed *Alox15* mRNA levels in some conditions (10 µg Esc/g body weight) even below levels observed in untreated animals (Fig. 4).

**Esculetin suppresses Tgf-β/Smad-signaling**

As expected, Tgf-β expression was progressively induced after 2- and 4- week CCl₄ treatment (Fig. 5A). Esculetin (at doses of 10 and 20 µg/g body weight) inhibited Tgf-β expression both at mRNA and protein level (Fig. 5A,B), which was accompanied by reduced phosphorylation of the downstream effector proteins Smad2/3 (Fig. 5B). In contrast, expression of key factors of other pathways that may contribute to CCl₄-induced liver fibrosis, including *Pdgfr-β* and *Ppary*, were not changed by esculetin (Fig. 5A).
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**Figure 5.** Esculetin suppresses CCl4-induced TGF-β/Smad signaling. CCl4 induced mRNA levels of Tgf-β and Pdgfr-β, while reducing Pparγ (A) the typical signature of the development of fibrosis. Esculetin only affected Tgf-β transcript levels, which was accompanied by reduced hepatic TGF-β protein levels and Smad2/3 phosphorylation, as determined by Western blotting (B). **p <0.01 compared to CCl4 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.

**Esculetin affects Timp-1 and Mmp expression towards fibrosis resolution.**

CCl4 progressively induced mRNA levels of Mmp2 and Mmp9, while Mmp13 expression peaked at 2 weeks (29-fold), but remained significantly enhanced (14-fold) compared to controls after 4 weeks CCl4 treatment (Fig. 6A). Esculetin only reduced Mmp9 mRNA expression (-52%), which was accompanied by reduced MMP9 activity (-25%; Fig. 6B). Most pronounced was the effect of esculetin on CCl4-induced Timp1 expression (+3.2-fold; Fig. 6C). Timp1 mRNA levels were fully suppressed to control levels, which was accompanied by strongly reduced TIMP-1 protein levels in serum.

**Esculetin improves the hepatic GSH/GSSG balance in CCl4 treated mice**

Reduced glutathione (GSH) levels in the liver were decreased after 4-week CCl4 treatment, which was accompanied by enhanced oxidized glutathione (GSSG) levels (Fig. 7). This leads to a strongly decreased GSH/GSSG ratio, which was significantly enhanced in all esculetin-treated groups (Fig. 7), implying that esculetin significantly improves the hepatic anti-oxidant status under hepatotoxic CCl4 treatment.
Figure 6. Esculetin promotes fibrolysis by increasing the MMP2-9-13/TIMP-1 ratio. (A) CCl₄ strongly enhanced mRNA levels of Mmp2, Mmp9 and Mmp13. Esculetin did not change (Mmp2 and Mmp13) or only moderately reduced (Mmp9) metalloproteinase expression. MMP2 and MMP9 activity were confirmed by zymography and quantified by densitometry (B). (C) CCl₄ strongly induced hepatic Timp-1 mRNA levels (left panel), which was fully suppressed by esculetin. Serum TIMP-1 levels (right panel) mirrored hepatic Timp-1 transcript levels. *p <0.05, **p <0.01 compared to CCl₄ 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.
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Discussion

In this study, we show that esculetin directly suppresses proliferation and activation of hepatic myofibroblasts in vitro. Esculetin halts and in part reverses liver fibrosis (e.g. hydroxyproline content, Masson trichrome staining) during persistent CCl₄-induced liver injury. The TGF-β/Smad signaling pathway was suppressed by esculetin, while PPARγ and PDGFR-β expression was not affected. Expression of metalloproteases (MMP2, 9 and 13) were reduced by esculetin, but remained elevated compared to untreated controls. In contrast, CCl₄-induced TIMP-1 expression was fully suppressed by esculetin. In addition, esculetin rescued the CCl₄-associated reduction in hepatic GSH/GSSG ratio. Thus, esculetin suppresses profibrotic signaling, improves fibrolysis and maintains an anti-oxidant status during CCl₄-induced liver toxicity.

Esculetin is a natural product and is a main constituent of medicinal plant extracts. A plethora of therapeutic properties have been assigned to esculetin, including analgesic, anti-inflammatory [8, 9], anti-tumor [10, 11], anti-arrhythmic [12], antisteroidogenic [13, 14] properties. With respect to hepatoprotective activities, esculetin has so far only been studied in models of acute liver toxicity, such as t-butyl hydroperoxide [33] and high single-dose CCl₄ [29]. Esculetin is a non-competitive inhibitor of 5-lipoxygenase and 12/15-lipoxygenase. These enzymes are essential for leukotriene and lipoxin synthesis that are potent inflammatory signaling molecules. We and others have shown that rat HSC express Alox5 [26] and is under inhibitory control of the retinoid-related orphan receptor-alpha (RORα). Both RORα agonists (melatonin, SR1078) and a 5-LO antagonist (AA861) suppress HSC proliferation and activation [25]. In line, several recent studies have shown that melatonin potently suppresses liver fibrosis in the chronic CCl₄-model in mice and rats [34-37]. Part of these effects may be exerted by the suppression of 5-LO. Long-term drug treatment with melatonin to treat liver fibrosis may, however, not be an ideal approach as this
pineal gland hormone controls circadian rhythm and induces sleep [38-40]. Thus, we reasoned that esculetin, as a direct inhibitor of 5-LO and a component of save medicinal plant extracts may be an attractive alternative.

Hepatic expression of \( \text{Alox5} \) and \( \text{Alox15} \) was strongly induce by \( \text{CCl}_4 \) treatment with highest levels after 2 weeks, at which point the esculetin treatment started. Esculetin treatment did not change hepatic \( \text{Alox5} \) levels, reminiscent of its effect on 5-LO enzyme activity and not on transcriptional regulation, while \( \text{Alox15} \) expression returned to levels observed in untreated animals. Remarkably, we were unable to detect \( \text{Alox15} \) expression in freshly-isolated (quiescent) or culture-activated rat HSC (data not shown), suggesting that these effects occur in non-HSC liver cells, most likely including Kupffer cells [41]. It remains to be determined whether esculetin directly affects transcription of the \( \text{Alox15} \) gene or whether this is a result of feedback mechanisms associated with reduced inflammation and/or fibrosis.

Esculetin has been shown to induce expression PPAR\( \gamma \) and suppress expression of PDGF [42], TGF-\( \beta \) [43, 44], various MMPs and TIMP-1 [45, 46], all factors that are involved in fibrogenesis and/or fibrolysis.

In \( \text{CCl}_4 \) treated mice, esculetin did not change \( \text{Ppar}\gamma \) and \( \text{Pdgfr}-\beta \) expression. Instead, significant suppression of TGF-\( \beta \) expression and downstream Smad2/3 phosphorylation was observed, suggesting that this profibrotic pathway was most sensitive to esculetin. Most pronounced, however, was the strong suppression of \( \text{CCl}_4 \)-induced TIMP-1. TIMP-1 is a generic inhibitor of MMPs and thereby prevents fibrosis resolution. \( \text{CCl}_4 \)-induced expression of \( \text{Mmp2} \) and \( \text{Mmp13} \) were not changed by esculetin, while \( \text{Mmp9} \) was only moderately reduced. Thus, the esculetin-increased MMP/TIMP-1 balance favors fibrolysis.

Esculetin strongly suppressed proliferation, as well as collagen1a1 and \( \alpha \text{Sma} \) expression, of \textit{in vitro} cultured HSC and PMF. These results are highly similar as effects observed with melatonin, SR1078 (ROR\( \alpha \) antagonist) and AA861 (5-LO antagonist), and suggest that these all act (in part) through inhibition of 5-LO. The anti-proliferative properties of esculetin are well-documented, especially in relation to cancer cells. Though this has not been specifically studied for the various forms of liver cancer (cells) yet, this feature may also be beneficial for preventing progression of cirrhosis to liver cancer.

Finally, we also found that esculetin prevents the \( \text{CCl}_4 \)-induced reduction of the GSH/GSSG balance, thereby preserving the anti-oxidant capacity in the liver. Most of the glutathione in the liver resides in hepatocytes, suggesting that esculetin also has a cytoprotective effect on the major liver cell type, as has been documented before. It is important to note that the esculetin did not fully prevent \( \text{CCl}_4 \)-induced hepatocyte damage as indicated by elevated transaminases in the absence and presence of esculetin. Thus, the antifibrotic effect of esculetin cannot be explained solely by protection of the hepatocytes.
Recent reports revealed very similar effects of melatonin and *Fraxinus rhynchophylla* extracts on CCl₄-induced liver fibrosis. Remarkably, the concentrations of esculetin present in the *Fraxinus rhynchophylla* extracts (given at 0.1, 0.5 and 1.0 mg/g body weight with 33.54 mg esculetin/mg *Fraxinus rhynchophylla* extract) were in the same range as our study (5, 10 and 20 µg esculetin/g body weight). This suggests that esculetin may be the most prominent antifibrotic factor in this plant extract.

Taken together, this study reveals multiple antifibrotic properties of esculetin, including suppression of hepatic stellate cell activation, profibrotic TGF-β/Smad signaling and induction of fibrolysis by enhancing the MMP/TIMP-1 balance. Moreover, the anti-oxidant capacity of the liver is improved by increasing the GSH/GSSG ratio.

These results warrant follow-up studies with the long term application of esculetin before and at various stages of established fibrosis to further establish it preventive and therapeutic capacity in the treatment of liver fibrosis. Moreover, experiments to test the anti-fibrotic potential on human liver, e.g. using precision-cut human liver slices [47], are urgently needed.
References


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Multifaceted esculetin suppresses hepatic stellate cell activation and CCl₄-induced liver fibrosis


## Supplementary Material

### Supplementary Table S1. Real time PCR primers used in this study

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**Supplementary Figure S1.** Esculetin does not induce necrosis in PMF or HSC. Primary rat PMF (white bars) and HSC (black bars) were cultured for 3 days in the absence or presence of esculetin (see also Fig. 1) and the LDH activity present in the medium was determined.

**Methods:**

**Cell necrosis measurement**

The lactate dehydrogenase (LDH) release assay was used to determine necrotic cell death. Primary HSC and PMF were cultured for 3 days in the absence or presence of esculetin. Medium was harvested and LDH activity was determined by monitoring the oxidation of NADH to NAD parallel to the conversion of pyruvate to lactate [19]. The oxidation of NADH was measured at 340 nm for 30 min with 1 min interval at 37°C using a Bio-Tek EL808 Thermo microplate reader (Bio-Tek). The linear portion of the kinetic curve was used to calculate LDH activity when compared to a standard curve.
Supplementary Figure S2. CCl₄ and/or esculetin treatment do not change serum albumin and total bilirubin levels in mice. Mice were treated with CCl₄ with or without esculetin co-treatment as indicated (see also Fig. 2). Serum albumin and total bilirubin levels were determined at sacrifice. No significant differences were detected between treatment groups and control mice.

Methods:

Biochemical analysis

Serum albumin and total bilirubin were quantified by Bio-sinew kits (Chengdu, China) according to the suppliers’ protocol using an automatic chemistry analyzer (Accute TBA-40FR, Toshiba Medical Systems Corporation, Japan).