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# Ampelopsin attenuates carbon tetrachloride-induced mouse liver fibrosis and hepatic stellate cell activation associated with the SIRT1/TGF-β1/Smad3 and autophagy pathway

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ABSTRACT

Ampelopsin (Amp), a natural flavonoid found in the vine tea of *Ampelopsis grossedentata*, exhibited anti-cancer, anti-oxidant, anti-inflammatory, anti-apoptosis and hepatoprotective properties. The current study instigates the protective effect of Amp on carbon tetrachloride ($CCl_4$)-induced hepatic fibrosis and explores its underlying mechanisms. The results indicated Amp decreased the levels of liver injury markers. Amp inhibited liver fibrosis, as indicated by decreases in hepatic collagen deposition, extracellular matrix (ECM) deposition and α-smooth muscle actin (α-SMA). Amp blocked the activation of hepaticstellate cells (HSCs) by decreasing the expression of collage I, α-SMA, tissue inhibitor of matrix metalloproteinases (TIMPs) 1, transforming growth factor (TGF)-β1, phosphorylated Smad3 (p-Smad3) and increasing the expression of matrix metalloproteinases (MMPs) 9 and SIRT1 in the model of liver fibrosis and cultured HSCs. The sirtuin 1 (SIRT1) specific inhibitor Sirtinol activated the TGF-β1/Smad3 pathway and enhanced ECM accumulation. Attractively, Amp up-regulates the expression of autophagy-related proteins microtubule-associated protein light chain three II (LC3-II) and Beclin-1 in vivo and in vitro. However, depletion of autophagy by specific inhibitor 3-MA obviously abolished the inhibiting effect of Amp on HSC activation and hepatic fibrosis. Conclusively, these results suggest that Amp could decrease $CCl_4$-induced hepatic fibrosis through regulating the SIRT1/TGF-β1/Smad3 and autophagy pathway.

## 1. Introduction

Liver fibrosis is a compensatory response of wound-healing after repeat injury by a wide range of toxins, which is characterized by the over-deposition of extracellular matrix (ECM) proteins and activation of hepatic stellate cells (HSCs) in the liver [1–3]. Activated HSCs could increase the deposition of collagen type I and α-smooth muscle actin (α-SMA), which further promoted the development and progression of liver fibrosis [1,3,4]. Activated HSCs could break the balance between ECM formation and degradation by increasing tissue inhibitor of matrix metalloproteinases (TIMP) and decreasing collagen-degrading matrix metalloproteinases (MMP) [3]. Several researches had demonstrated carbon tetrachloride ($CCl_4$) cause liver damage and subsequently induced hepatic fibrosis. $CCl_4$ intoxication is a common animal model to study the mechanism of hepatic fibrosis [1,2,5].

Autophagy is a host defense mechanism that degrades the excessive components, protein aggregates, long-lived cytosolic proteins, invading microbes, defective and damaged organelles [6–8]. Microtubule-associated protein light chain 3 (LC3) and Beclin-1 are markers of autophagy [7,8]. Accumulating evidence demonstrated that activated autophagy could prevent hepatic fibrosis and HSC activation [3,6,7]. It is reported that activation of Sirt1-mediated autophagy could inhibit cardiac fibrosis in the diabetic mice [9].

Ampelopsin (Amp, 3,5,7,3′4′5′-hexahydroxyl 2,3 dihydrogen flavonol), also called dihydromyricetin, is a natural flavonoid found in a Rattan tea of *Ampelopsis grossedentata* at concentration 20–30%, which has exhibited anti-cancer, anti-oxidant, anti-inflammatory, anti-apoptosis, anti-diabetes, anti-hypertensive, hepatoprotective, renoprotective and neuroprotective properties [10–12]. Previous study had indicated that Amp could improve ethanol-induced hepatic inflammation by

*Abbreviations:* ALT, alanine aminotransferase; AST, aspartate aminotransferase; Amp, Ampelopsin; TGF-β1, transforming growth factor β1; TIMPs, tissue inhibitor of matrix metalloproteinases; MMPs, matrix metalloproteinases; α-SMA, α-smooth muscle actin

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**Table 1**
Effect of Amp on the activities of ALT and AST in serum.

| Group | ALT activity (U/L) | AST activity (U/L) |
|---|---|---|
| Control | 28.37 ± 6.24 | 41.52 ± 7.64 |
| $CCl_4$ | 148.81 ± 11.03$^{\#\#}$ | 184.46 ± 13.32$^{\#\#}$ |
| $CCl_4$ + Amp(125 mg/kg)) | 112.56 ± 10.84$^{**}$ | 141.29 ± 9.57$^{**}$ |
| $CCl_4$ + Amp(250 mg/kg) | 91.39 ± 8.98$^{**}$ | 78.86 ± 12.05$^{**}$ |

Data are expressed as mean ± S.E.M. (n = 10). One-way ANOVA was used for comparisons of multiple group means followed by post hoc testing ($\#\#P < 0.01$ vs. control;$^{**}P < 0.01$ vs. $CCl_4$ group).

activating autophagy pathway [13]. Amp treatment alleviated the proliferation of cardiac fibroblasts induced by angiotensin II [14]. Several evidences showed that Amp ameliorated behavioral deficits and inhibited apoptosis through activating the silent information regulator 2 homolog 1 (SIRT1) pathways in the brains of the Alzheimer's disease (AD) rat model [15,16]. It is reported that Amp could ameliorate $CCl_4$-induced hepatic fibrosis by regulating the NF-κB pathway and promoting HSC apoptosis[17]. Moreover, Amp could induce apoptosis of Hepal-6 cells and decreased accumulation of ROS by regulating transforming growth factor (TGF-β)/Smads pathway [18]. However, whether Amp against liver fibrosis is associated with the SIRT1/TGF-β1/Smad3 and autophagy pathway has not been fully understood.

In this study, we found that Amp treatment alleviated $CCl_4$-induced hepatic fibrosis in vivo and cultured HSCs in vitro. The evidence demonstrated that Amp attenuated liver fibrosis by regulating the SIRT1/TGF-β1/Smads and autophagy pathway.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ampelopsin (98%), platelet derived growth factor-BB (PDGF-BB), Sirtinol, 3-Methyladenine (3-MA) and $CCl_4$ were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Primary antibodies against p-Smad3, Smad3, Collagen I, Collagen III, α-SMA, SIRT1, LC3, Beclin-1, TIMP1, MMP9, TGF-β1, AKT, p-AKT, p-mTOR and β-actin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA, USA).

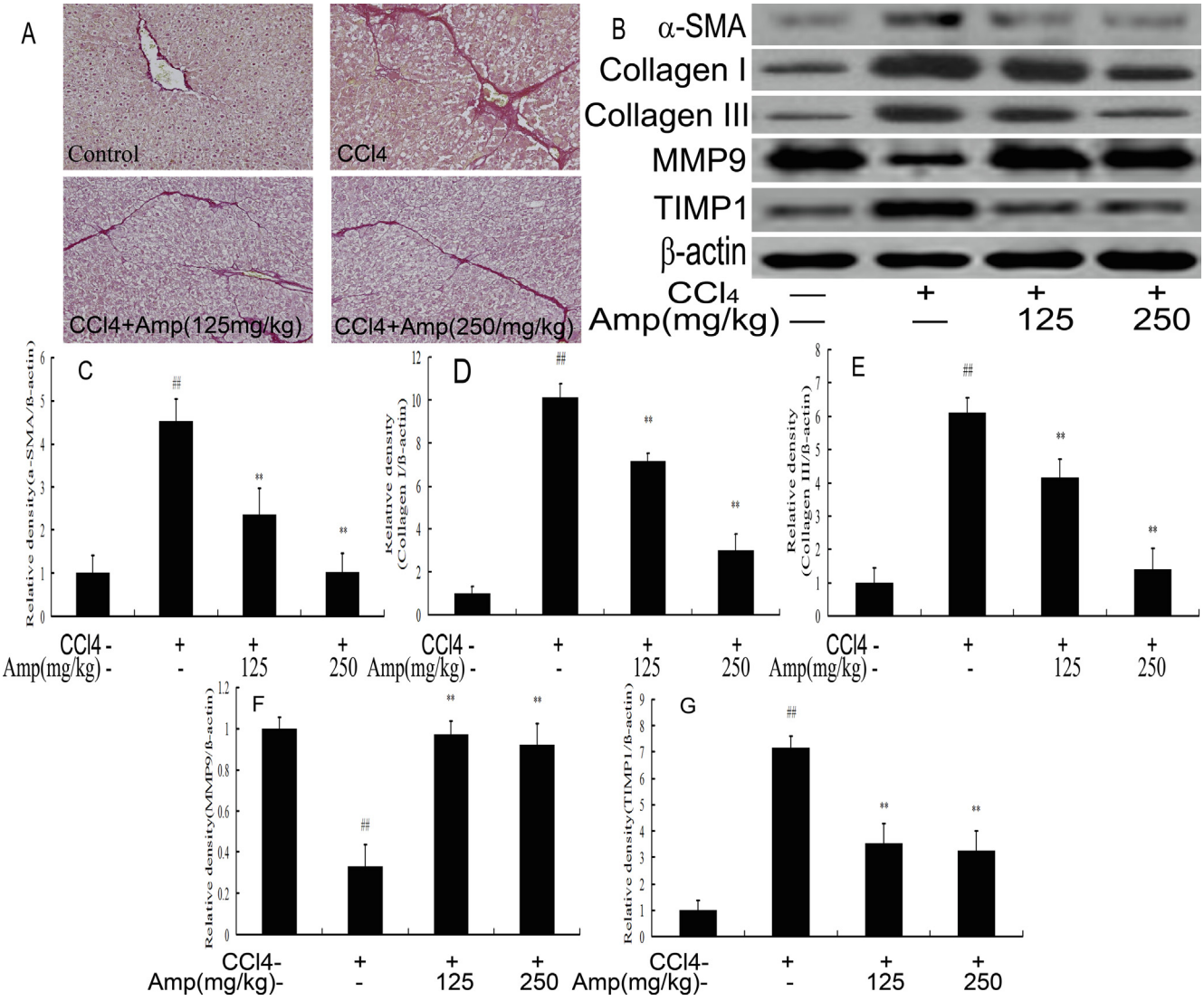


**Fig. 1.** Ampelopsin(Amp) inhibited $CCl_4$-induced hepatic fibrosis and HSC activation in the mice. (A) Sirius red-stained liver sections; (B) Western blot analysis of the markers of hepatic fibrosis; (C) The protein expression of α-SMA; (D) The protein expression of Collagen I; (E) The protein expression of Collagen III; (F) The protein expression of MMP9; (G) The protein expression of TIMP1. β-actin was probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. ##P < 0.01 vs. control; **P < 0.01 vs. $CCl_4$ group.

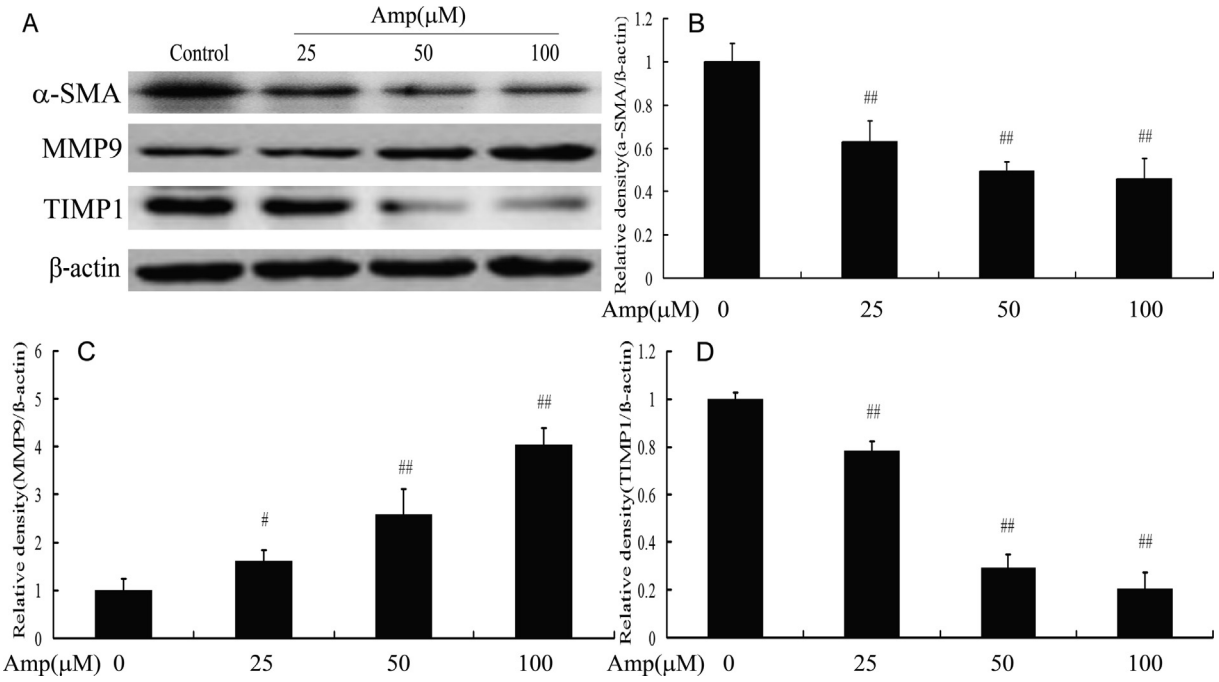


**Fig. 2.** Ampelopsin(Amp) inhibited the activation of HSCs in vitro. HSCs were treated with Amp (25, 50, 100 μM) for 24 h. (A) Western blot analysis of the markers of HSC activation; (B) The protein expression of α-SMA; (C) The protein expression of MMP9; (D) The protein expression of TIMP1. β-actin was probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. # $P < 0.05$, ## $P < 0.01$ vs. control.

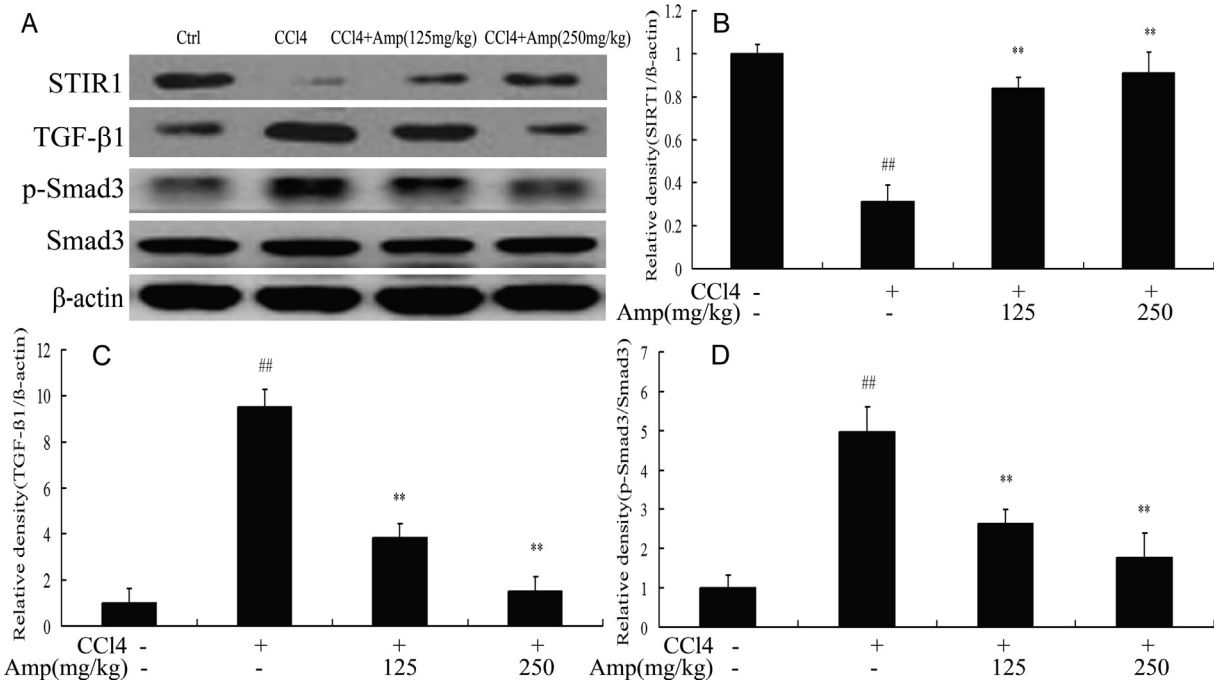


**Fig. 3.** Ampelopsin(Amp) regulated SIRT1/TGF-β1/Smad3 pathway in the hepatic fibrosis of mice. (A) Western blot analysis of the SIRT1, TGF-β1 and Smad3 protein expression in the livers; (B) The protein expression of the SIRT1; (C) The protein expression of the TGF-β1; (D) The protein expression of the p-Smad3. Total Smad3 or β-actin were probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. ##P < 0.01 vs. control;**P < 0.01 vs. $CCl_4$ group.

### 2.2. Animals and ethics

Forty male ICR mice (20 ± 1 g) were provided from Beijing HFK Bioscience CO., LTD. (Beijing, China). The mice were kept under the standard laboratory conditions. Then, the mice were randomly divided into four groups (10 mice/group): I, Control group (saline 0.9% NaCl); II, $CCl_4$ group; III, $CCl_4$ + Amp (125 mg/kg b.w) and IV, $CCl_4$ + Amp (250 mg/kg b.w). In group II, IIII and IV, mice were subjected to intraperitoneal injection of 2 mL of $CCl_4$ in olive oil (1:1, v/v) per kg body weight twice weekly for up to 10 weeks. The mice in III and IV groups were supplied with Amp 125 or 250 mg/kg per body weight, once daily. The doses of Amp and $CCl_4$ were based on previously described

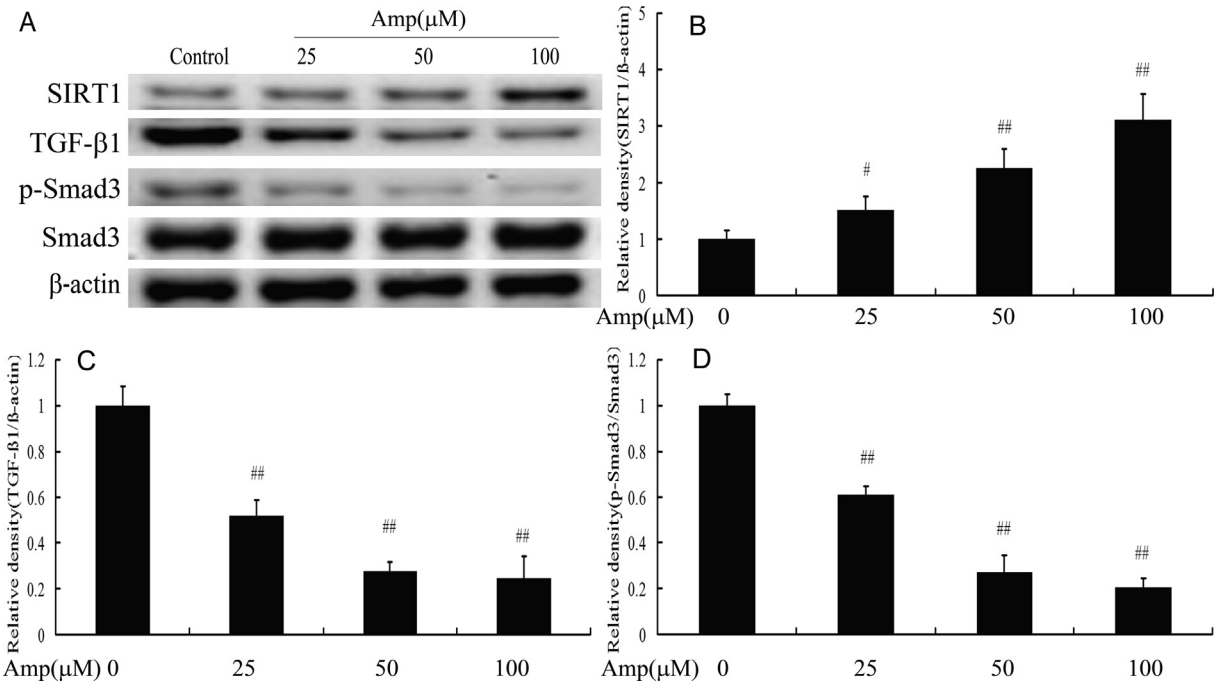


**Fig. 4.** Ampelopsin(Amp) regulated SIRT1/TGF-β1/Smad3 pathway in vitro. HSCs were treated with Amp (25, 50, 100 μM) for 24 h. (A) Western blot analysis of the SIRT1, TGF-β1 and Smad3 proteins in vitro; (B) The protein expression of the SIRT1; (C) The protein expression of the TGF-β1; (D) The protein expression of the p-Smad3. Total Smad3 or β-actin were probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. # $P < 0.05$, ## $P < 0.01$ vs. control.

protocols [2,12].

At the end of 10 weeks, mice were sacrificed by decapitation. The blood samples were collected to analyze the effect of Amp on biochemical parameters. Livers of seven mice were collected immediately for experiments or stored at −80℃for future experiments while liver samples of the other mice were immersed in 4% paraformaldehyde for histology.

All experiments process was approved by Jiangsu Normal University committees (No. IACUC-1.0.15) and performed according to the Chinese Laws on Care of Laboratory Animals and the National Institutes of Health Guidelines for the Care and Use of Animals.

### 2.3. Serum aminotransferase assay

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were analyzed according to the manufacturer's instructions from commercial kits (Jiancheng, Nanjing, China) [2].

### 2.4. Histological evaluations

The changes of hepatic fibrosis were evaluated using standard methods as described by us [2]. In brief, liver slices of 4-μm thick were prepared and stained with Sirius Red according to standard protocols. Microscopy images were acquired using Olympus IX71 microscope.

### 2.5. Cell isolation and cell culture conditions

Primary mouse hepatic stellate cells were isolated from male ICR mice (Jiangsu Normal University, Xuzhou, China) as described [9]. Cells were cultured in DMEM with 10% FBS and 1% streptomycin/penicillin at 37 °C in a 5% $CO_2$, and maintained in sufficiently humid incubator. Hepatic stellate cells were treated with Amp (25 μM, 50 μM, 100 μM) for 24 h [19]

### 2.6. Western blotting analysis

The protein expression of p-Smad3, Smad3, α-SMA, Collagen I, Collagen III, SIRT1, LC3, Beclin-1, TIMP1, MMP9, TGF-β1, AKT, p-AKT, p-mTOR and β-actin were analyzed by western blot as per the manufacturer's guidelines (Bio/Rad, Hercules, CA, USA). Protein content was determined using a protein assay kit (Bio-Rad). Equal amounts of proteins (50 mg) were separated by SDS-PAGE (Sigma-Aldrich) electrophoresis and then transferred to PVDF membranes (Sigma-Aldrich). The protein band intensities were detected by Image-Pro Plus 6.0 software [12].

### 2.7. Statistical analysis

Statistical significant differences between means were evaluated by Student's *t*-test, and one-way ANOVA followed by Turkey's post hoc test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Amp attenuated $CCl_4$-induced liver dysfunction

As shown in Table 1, compared with control group, the activities of ALT and AST in serum of the $CCl_4$ group were increased by 524.5% and 444.3%, respectively. Meanwhile, the daily administration of Amp (125 mg/kg and 250 mg/kg) markedly decreased the activities of ALT (by 24.4%, and 39%, respectively) and AST (by 23.4% and 57.2%, respectively) as compared to the $CCl_4$ group.

### 3.2. Amp suppressed $CCl_4$-induced hepatic fibrosis

To examine the protective effect of Amp on hepatic fibrosis, we determine the α-SMA protein expression and collagen deposition in livers. As is showed in Fig. 1, $CCl_4$ remarkably increased the levels of α-SMA protein, and collagen deposition formed the pseudolobuli bridging

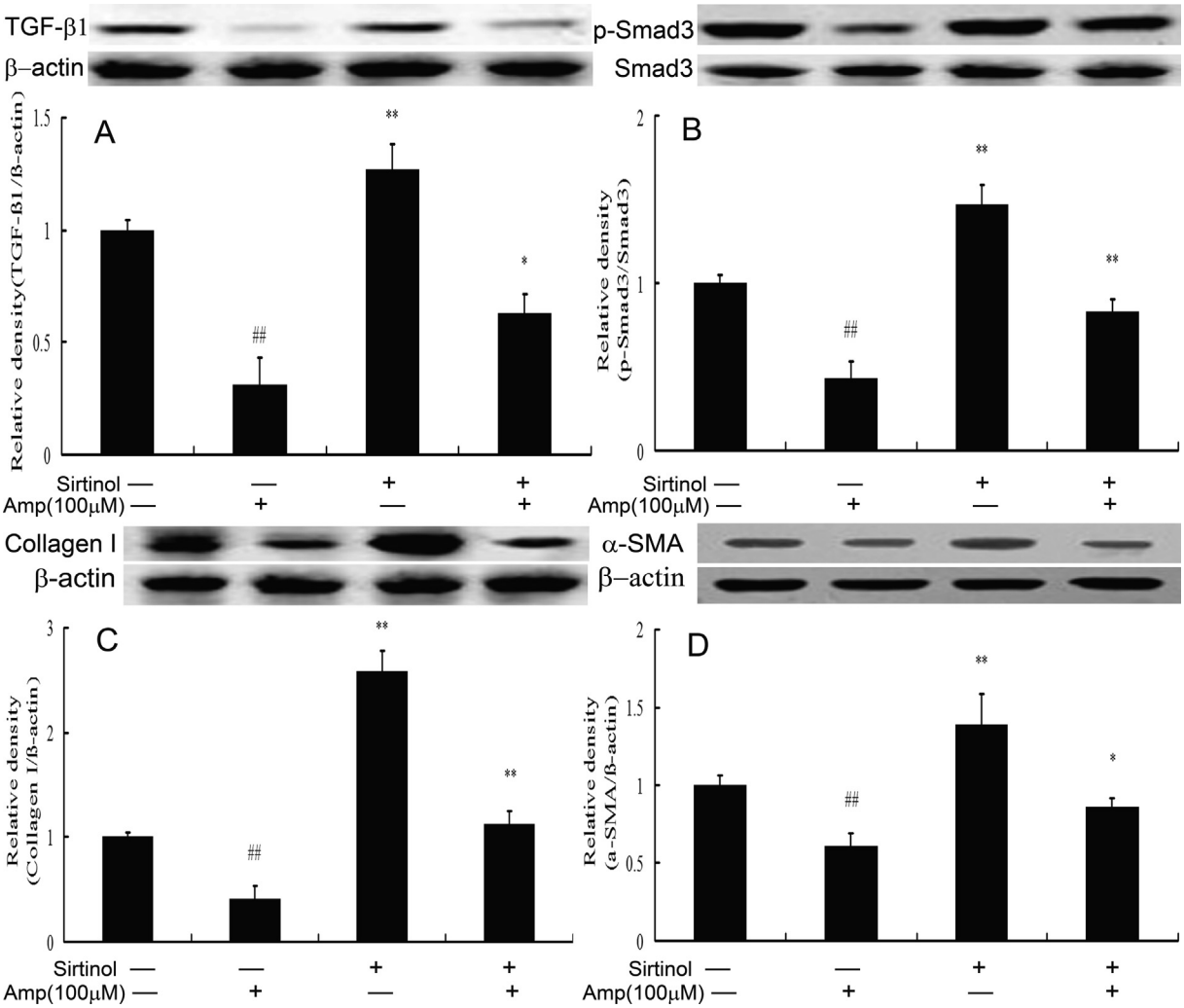


**Fig. 5.** Inhibition of SIRT1 expression abolished the inhibiting effect of Ampelopsin(Amp) on TGF-β1/Smad3 signaling pathway and hepatic fibrosis in vitro. Activated HSCs were exposed to 3-MA, followed by 100 μM Amp treatment for 24 h. (A) The protein expression of the TGF-β1; (B) The protein expression of the Smad3. (C) The protein expression of Collagen I; (D) The protein expression of α-SMA. Total Smad3 and β-actin were probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. ##$P < 0.01$ vs. control; *$P < 0.01$,**$P < 0.01$ vs. $CCl_4$ group.

fibrosis. However, Amp supplement remarkably attenuated the accumulations of collagen fibers and the productions of α-SMA in liver tissues of mice ($P < 0.01$).

### 3.3. Amp inhibited the HSC activation in liver fibrosis

Activation of HSCs can antagonize the liver damage and the development of hepatic fibrosis [3,6]. We further measured the α-SMA protein expression, one indicator of activated HSCs. As shown in the Fig. 1, compared to control group, $CCl_4$ increased the α-SMA protein expression. However, the oral administration of Amp significantly suppressed $CCl_4$-induced activation of HSCs in liver fibrosis. Simultaneously, the administration with Amp (25 μM, 50 μM, 100 μM) remarkably decreased the α-SMA protein expression in cultured HSCs (by 37%, 51%, 54%, respectively), as compared to the control (Fig. 2). Moreover, $CCl_4$ decreased the protein expression of MMP9 by 67% and increased the protein expression of TIMP1 (the major inhibitor of MMPs) by 615% in livers of mice compared to control group. The oral administration of Amp significantly suppressed $CCl_4$-induced ECM deposition in the livers (Fig. 1). Additionally, these results were further confirmed in vitro experiments (Fig. 2).

### 3.4. Amp regulated SIRT1/TGF-β1/Smad3 signaling pathway in liver fibrosis

SIRT1/TGF-β1/Smads signaling pathway is tightly linked to hepatic fibrosis. Here, we measured the expression of SIRT1, TGF-β1 and Smad3 in the livers of mice. As showed in the Fig. 3, the protein expression of SIRT1 was significantly down-regulated by 67%, the protein expression of TGF-β1 and p-Smad3 were remarkably up-regulated by 851% and 397% in the livers of $CCl_4$ group in comparison to control. Interestingly, treatment with Amp (125 mg/kg and 250 mg/kg) increased the protein expression of SIRT1 (by 168% and 192%, respectively) and reduced the levels of TGF-β1 (by 60% and 84%, respectively) and p-Smad3 (by 47% and 64%, respectively) in liver tissues of $CCl_4$ group ($P < 0.01$).

### 3.5. Amp regulated SIRT1/TGF-β1/Smad3 signaling pathway in cultured HSCs

We further analyzed SIRT1/TGF-β1/Smads signaling pathway in cultured HSCs. The results indicated that the administration with Amp (25 μM, 50 μM, 100 μM) remarkably increased the SIRT1 protein expression (by 50%, 126%, 210%, respectively) and decreases the protein

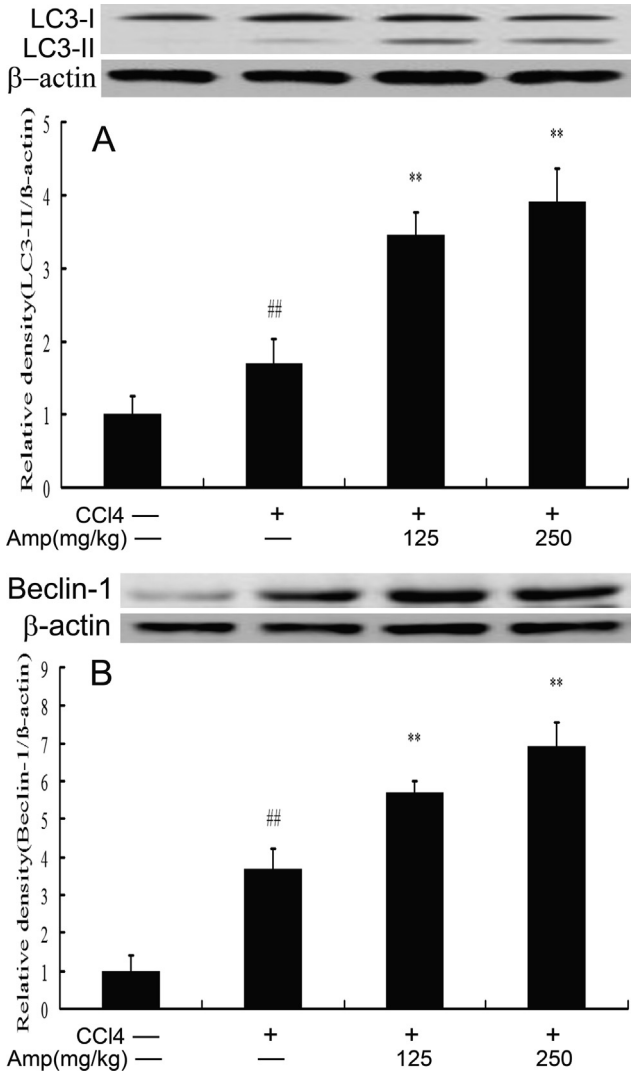


**Fig. 6.** Ampelopsin(Amp) promoted $CCl_4$-induced autophagy in the livers of mice. (A) The protein expression of the LC3-II; (B) The protein expression of the Beclin-1. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. $\#\#P < 0.01$ vs. control;$^{**}P < 0.01$ vs. $CCl_4$ group.

expression TGF-β1 (by 48%, 72%, 75%, respectively) and p-Smad3 (by 39%, 73%, 80%, respectively) in cultured HSCs, as compared to the control. However, the administration with Amp not affected the total level of Smad3 in cultured HSCs (Fig. 4).

### 3.6. Inhibition of SIRT1 expression abolished the inhibiting effect of Amp on the TGF-β1/Smad3 signaling pathway and hepatic fibrosis in vitro

To investigate whether SIRT1 is involved in the anti-fibrotic effect of Amp, we further examined the expression of TGF-β1, Smad3, collagen I and α-SMA in cultured HSCs. As shown in Fig. 5, Amp treatment remarkably decreased the protein levels of TGF-β1 (by 68%), p-Smad3 (by 57%), collagen I (by 59%) and α-SMA (by 39%) in cultured HSCs. However, SIRT1 specific inhibitor sirtinol significantly abolished the inhibiting effect of Amp on the TGF-β1/Smad3 signaling pathway and hepatic fibrosis in vitro (Fig. 5).

### 3.7. Amp promoted $CCl_4$-induced autophagy in the livers of mice

Autophagy is a dynamic process that involved in liver fibrosis [3,6]. To examine the effect of Amp on autophagy, we measured protein levels of the autophagy-related markers. As Fig. 6 show, $CCl_4$ increased the expression of LC3-II by 70% and Beclin-1 by 271%. Intriguingly, Amp supplement further increased these protein expression and promoted the $CCl_4$-induced autophagy in livers ($P < 0.01$).

### 3.8. Inhibition of autophagy impaired Amp-induced anti-fibrosis effects in vitro

To further investigate whether autophagy is involved in the anti-fibrotic effect of Amp, Chemical autophagy specific inhibitor 3-MA was used to inhibit autophagy in activated HSCs. As expected, Amp treatment remarkably increased the protein levels of the autophagy-related markers LC3-II (by 157%) and Beclin-1 (by 436%) in cultured HSCs. However, 3-MA treatment significantly inhibited Amp-stimulated autophagy (Fig. 7A and B). Moreover, Amp treatment remarkably decreased the protein levels of HSC activation markers α-SMA (by 49%) and collagen I (68%) in cultured HSCs, but autophagy specific inhibitor 3-MA treatment obviously abolished the inhibiting effect of Amp on HSC activation and hepatic fibrosis (Fig. 7C and D).

### 3.9. The AKT/mTOR pathway involved in the Amp-induced anti-fibrosis effects

The AKT/mTOR pathway is a classic negative regulatory pathway for autophagy, which plays a crucial role in cell autophagy [3,5]. To further investigate whether the PI3K/AKT/mTOR pathway is involved in the anti-fibrotic effect of Amp, we measured the phosphorylation levels of AKT and mTOR. As Fig. 8 show, Amp treatment significantly decreased the phosphorylation of AKT (by 37%) and mTOR (by 53%) in cultured HSCs. However, 3-MA treatment impaired the down-regulated p-AKT and p-mTOR expression induced by Amp treatment (Fig. 8).

## 4. Discussion

Amp, a kind of flavonoid purified from the vine tea of *Ampelopsis grossedentata*, elicited protective functions in many disorders [10–12]. Currently, we indicate that Amp displayed the hepatoprotective effects against $CCl_4$-induced hepatic fibrosis and activation of HSCs. Amp promoted $CCl_4$-induced autophagy and regulated SIRT1/TGF-β1/SmadSIRT1 signaling pathway in livers of mice.

$CCl_4$ can cause tissue injuries in multiple organs and primarily impairs livers [1,3,4]. It is reported that Amp exhibited the protective effects on ischemia/reperfusion induced hepatic injury and apoptosis [20]. Amp could improve ethanol-induced liver steatosis and damage [13]. Amp prevented $CCl_4$-induced acute liver damage by JNK pathway in mice [10]. Consistent with previous study, Amp treatment can dramatically decrease the activities of liver injury markers, ALT and AST in serum of $CCl_4$ group (Table 1), which displayed the hepatoprotective effects of Amp on $CCl_4$-induced liver dysfunction.

Several studies had demonstrated that $CCl_4$ could induce the deposition of ECM proteins and activation of HSCs, and subsequently induced hepatic fibrosis [1–3]. It is reported that Amp could prevent the HFD-induced fatty liver and decrease the expression of collagen I protein [21]. Amp treatment alleviated the proliferation of cardiac fibroblasts induced by angiotensin II [14]. Amp could prevent hepatoma cell metastasis by regulating MMP9 expression [1]. In our study, Sirius red staining result showed that Amp treatment remarkably decreased the collagen deposition and the formation of fibrous nodules in livers of $CCl_4$ group (Fig. 1A). Furthermore, Western blot analyses revealed that Amp treatment dramatically down-regulated the protein expression of α-SMA, collagen I, collagen III and TIMPs, while up-regulated the protein expression of MMP9 in livers of mice (Fig. 1). These results were further confirmed in cultured HSCs in vitro (Fig. 2). Our study supported that Amp exerts its hepatoprotective property on $CCl_4$-induced ECM formation during fibrogenesis partly via inhibiting HSC activation and maintaining balance of MMP9/TIMP1.

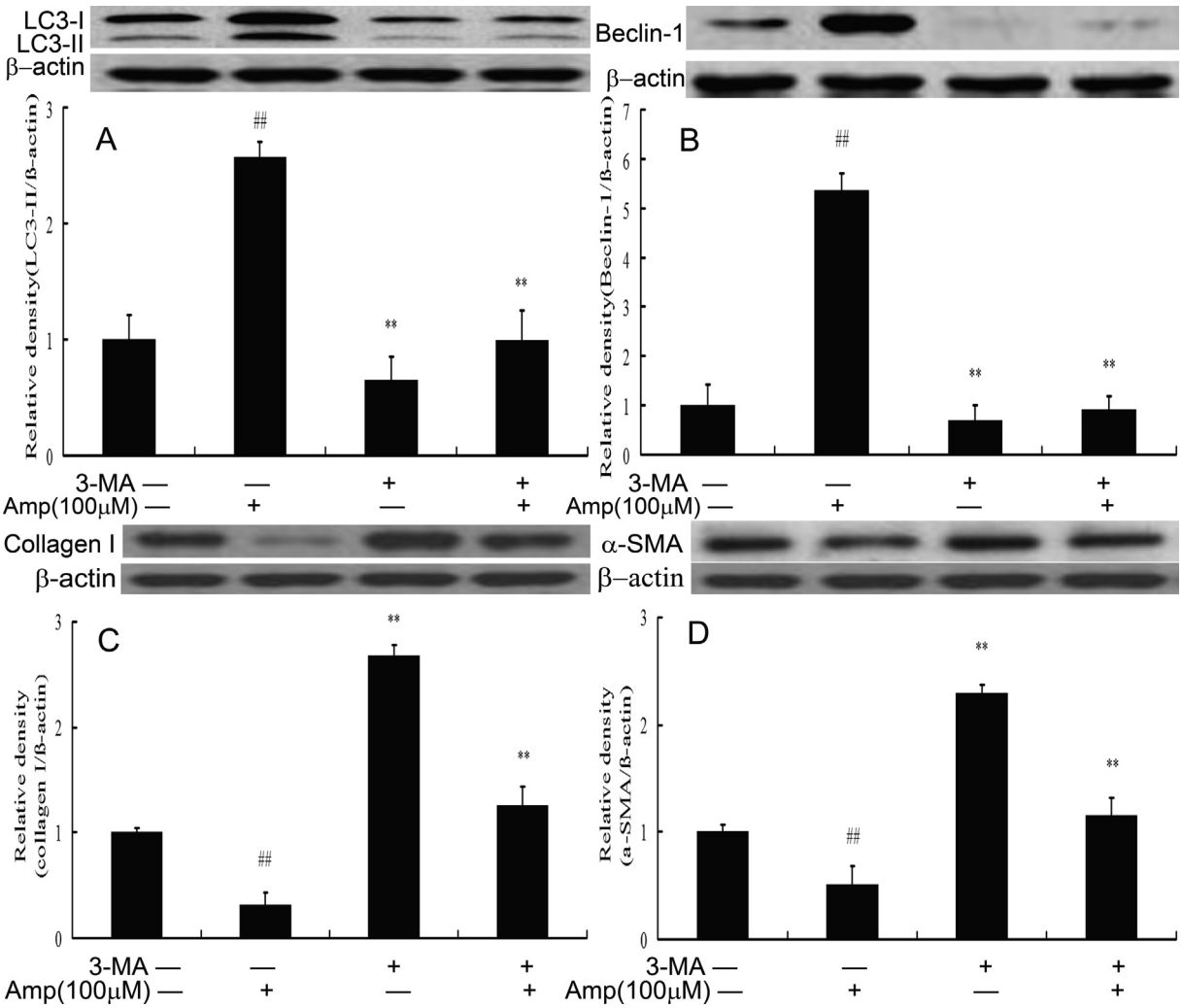


**Fig. 7.** Inhibition of autophagy impaired ampelopsin(Amp)-induced anti-fibrosis effects in vitro. Activated HSCs were exposed to 3-MA, followed by 100 μM Amp treatment for 24 h. (A) The protein expression of the LC3-II; (B) The protein expression of the Beclin-1. (C) The protein expression of Collagen I; (D) The protein expression of α-SMA. β-actin was probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. ##$P < 0.01$ vs. control;**$P < 0.01$ vs. $CCl_4$ group.

SIRT1 is considered as a pivotal regulator in many diseases, including oxidative stress, inflammation, apoptosis, autophagy, diabetes, aging, proliferation, cardiovascular disease, hypertension, neurological disease, liver disease and kidney disease [22–24]. Grape seed oil alleviated $CCl_4$-induced liver damage by up-regulation of SIRT1 gene expression [22]. Previous studies had demonstrated that activated SIRT1 could inhibit $CCl_4$-induced liver fibrosis and HSC activation [23,24]. Studies also revealed that Amp could improve brain aging, apoptosis and autophagy by SIRT1 pathways [15,16]. Here, our results indicated $CCl_4$ treatment decreased the expression of SIRT1. However, administration of Amp remarkably increased the expression of SIRT1 protein in vivo and in vitro (Figs. 3 and 4). The results revealed that SIRT1 was involved in the protective effects of Amp against $CCl_4$-induced hepatic fibrosis.

TGF-β1 is a pleiotropic cytokine (the pro-fibrogenic and inflammatory factor) that participate in immune response and the development of hepatic fibrosis [1]. TGF-β1 resulted in activation and proliferation of HSCs and fibrotic process. Activated HSCs further secrete TGF-β1, which increases collagen production and promotes the progression of liver fibrosis [1,5]. TGF-β1 could promote the progression of liver injury and fibrosis genesis though phosphorylation of Smad2/3 [3]. TGF-β1/Smads pathway could regulate autophagy by affecting transcription of Beclin-1 gene [25]. It reported that nicotinamide riboside could prevent $CCl_4$-induced liver fibrosis and activation of HSCs by increasing the activity of SIRT1 and inhibiting TGF-β1/Smads pathway [4]. Many studies revealed that Amp induced apoptosis in HepG2 cells and mouse hepatoma Hepal-6 cell via TGF-β1/Smads pathway [18,26]. In our study, we observed that Amp down-regulates the expression of TGF-β1 and p-Smad3 in livers of $CCl_4$ group and in cultured HSCs (Figs. 3 and 4). Inhibition of SIRT1 protein expression by specific inhibitors sirtinol partly abolished the inhibiting effect of Amp on the TGF-β1/Smad3 pathway and hepatic fibrosis (Fig. 5). This result indicated that Amp displayed its hepatoprotective function on $CCl_4$-induced hepatic fibrosis by modulating TGF-β1/Smads singling pathway.

Autophagy is a critical intracellular process in which the excessive components, defective and damaged organelles are delivered to lysosomes for degradation [1,3,7]. Many studies had demonstrated that $CCl_4$ could induce autophagy in the model of liver fibrosis [1,5,6]. Activated SIRT1 could enhance autophagy and inhibit apoptosis [27]. It is reported that natural compounds treatment could alleviate liver fibrosis by activating mTOR-dependent autophay pathway [28]. Caffeic acid phenethyl ester could alleviate $CCl_4$-induced liver fibrosis via induction of autophagy and inhibition of TGF-β expression [3]. Oroxylin A could ameliorate $CCl_4$-induced hepatic fibrosis and HSC activation via induction of autophagy pathway [6]. Amp could ameliorate liver

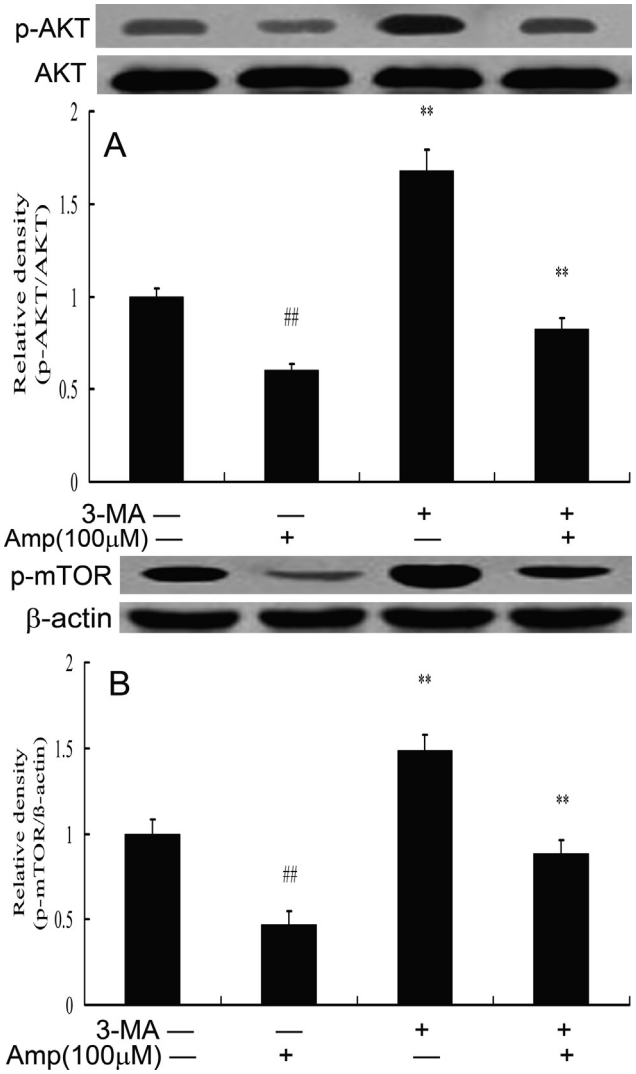


**Fig. 8.** The AKT/mTOR pathway involved in the ampelopsin(Amp)-induced anti-fibrosis effects in vitro. Activated HSCs were exposed to 3-MA, followed by 100 μM Amp treatment for 24 h. (A) The protein expression of the p-AKT; (B) The protein expression of the p-mTOR. Total AKT and β-actin were probed as an internal control in relative density analysis. Data are expressed as mean ± S.E.M. and representative of at least three independent experiments. $##P < 0.01$ vs. control;$**P < 0.01$ vs. $CCl_4$ group.

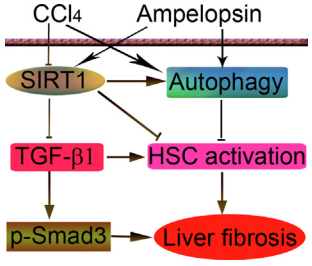


**Fig. 9.** Schematic diagram showed the possible protective effects of ampelopsin (Amp) in $CCl_4$-induced hepatic fibrosis. The → indicates activation or induction, and ⊣ indicates inhibition or blockade.

damage by activating autophagy pathway [13,20]. Amp also caused autophagy and suppressed the proliferation of HepG2 cells [29]. Our results are consistent with previous reports that $CCl_4$ treatment markedly increased the expression of Beclin-1 and LC3-II. Interesting, Amp supplement obviously increased these markers of autophagy in the livers (Fig. 6). Several studies had revealed that the AKT/mTOR signal pathway plays a crucial role in cell autophagy and inhibited phosphorylation of AKT and mTOR could alleviate autophagy and hepatic fibrosis [3,30,31]. In this study, we observed that Amp treatment promoted autophagy in cultured HSCs. Inhibition or depletion of autophagy by specific inhibitors 3-MA partly abolished the inhibiting effect of Amp on HSC activation and hepatic fibrosis (Fig. 7). Moreover, Amp treatment reduced the expression levels of p-mTOR and p-AKT in cultured HSCs. However, 3-MA treatment impaired the down-regulated p-mTOR and p-AKT expression induced by Amp treatment (Fig. 8). There results suggested that Amp could ameliorate the hepatic fibrosis via promoting autophagy.

Taken together, this is the first study showing that Amp possesses protective property against $CCl_4$-induced hepatic fibrosis in mice. Our findings suggest that Amp could decrease $CCl_4$-induced hepatic fibrosis by regulating the SIRT1/TGF-β1/Smad3 and autophagy pathway (Fig. 9). The results suggested Amp may be developed as a potential nutritional target for the prevention of $CCl_4$-induced liver injury. Although our results suggested the underlying mechanisms for the anti-fibrosis effect of Amp, the question warrants further investigation.

## Declaration of Competing Interest

None.

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