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• *BASIC RESEARCH* •

# Effect of *Astragalus complanatus* flavonoid on anti-liver fibrosis in rats

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## Abstract

**AIM:** To observe the anti-liver fibrosis effect of *Astragalus complanatus* flavonoids (ACF) in rats.

**METHODS:** The liver fibrosis model in rats was established by injecting interperitoneally 0.2 mL/100 g 0.5% dimethylnitrosamine, thrice a week. Meanwhile, the rats were administered ACF (30, 60, 120 mg/kg) or colchicine (0.1 mg/kg) once a day for 1 mo. Serum *N*-propeptide of type I procollagen (PINP) and type III procollagen (PIIINP) was measured using ELISA. Malondialdehyde (MDA) and superoxide dismutase (SOD) in hepatic tissue were evaluated. Matrix metal protease-1 (MMP-1) mRNA expression was assayed by RT-PCR and the protein expression of tissue inhibitor of metal protease-1 (TIMP-1) was analyzed by immunohistochemistry.

**RESULTS:** In the ACF groups, SOD activity increased and MDA content decreased in comparison to the liver fibrosis model group. The serum PINP and PIIINP contents in ACF-2 and -3 group decreased compared to those in model group. In ACF-2 and -3 group, the expression of MMP-1 mRNA increased significantly and the protein expression of TIMP-1 decreased compared to that in model group.

**CONCLUSION:** The antifibrotic mechanisms of ACF are associated with its influence on lipid peroxidation and collagen synthesis and degradation.



**Key words:** *Astragalus complanatus*; Liver fibrosis; *N*-propeptide of type I procollagen; *N*-propeptide of type III procollagen; Malondialdehyde; Superoxide dismutase; Matrix metal protease-1; Tissue inhibitor of metal protease-1

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## INTRODUCTON

Fibrosis is a common manifestation in most chronic liver diseases. The progression of hepatic fibrosis often leads to cirrhosis and is associated with liver cancer. Therefore, interrupting or reversing hepatic fibrosis is very important in preventing cirrhosis. Semen *Astragali* (SA) is a tonic traditional Chinese medicine for liver and kidney. We previously reported that SA has a protective effect on liver[1]. Our further investigation has revealed that the flavonoids extracted from SA are useful in preventing rat liver fibrosis induced by dimethylnitrosamine (DMN)[2]. However, how *Astragalus complanatus* flavonoids (ACF) interrupt the formation of liver fibrosis remains unknown. This study was designed to observe the anti-liver fibrosis effect and mechanisms of DMN in rats.

## MATERIALS AND METHODS

### *Materials*

Astragalus complanatus (AC) was obtained from Leiyunshang Pharmaceutical Corporation (Suzhou, China) and identified by a chief pharmacist (Suzhou Drug Administration Bureau). Colchicine was obtained from Kunming Pharmaceutical Corporation (Kunming, China). DMN was purchased from Shanghai Chemical Company (Shanghai, China). All other chemicals were of analytical grade.

### *Preparation of ACF*

After being extracted with ligarine, the seed powder of AC was extracted with 80% ethyl acetate (EtOH). The 80% EtOH extract was further subjected to ZTC-5 adsorptive resin (Nankai University) column and eluented with water, 50% and 95% EtOH to afford three fractions. The 50% EtOH fraction was dried under vacuum, recrystallized with EtOH and ACF was obtained as a yellow powder containing complanatuside, neocomplanatuside, myricomplanoside, rhomnocitrin-3-O-β-D-glucoside and astragalin, etc. The complanatuside content in ACF was about 20% by RP-HPLC and determined on a Shim-pack VP-ODS analytical column (4.6 mm×150 mm, 5 μm) with the mobile phase consisting acetonitrile-1% acetic acid (18:82) as eluent at the flow rate of 1 mL/min. The UV detection was set at 266 nm. The total ACF was 80%, determined at 266 nm by UV spectroscopy with complanatuside as a standard substance.

### *Animals*

Sixty male Wistar rats weighing 130-160 g were housed in conventional cages with free access to water and rodent chow at 20-22 ℃ with a 12-h light-dark cycle. All procedures involving the use of laboratory animals were in accordance with National Institutes of Health Guidelines. Rat liver fibrosis was produced according to the established protocol[3]. Except for the normal group, rats in other groups were injected interperitoneally 5 g/L DMN (0.2 mL/100 g, thrice a week). Meanwhile, the rats were administered 30 mg/kg ACF-1, 60 mg/kg ACF-2, 120 mg/kg ACF-3 or 0.1 mg/kg colchicine, respectively, once a day for 1 mo. The doses of ACF and its course of treatment were based on a pilot experiment. The rats in the normal control group and DMN model group received saline by gavage instead of ACF. After 4 wk of ACF treatment, all rats were anesthetized, sera were collected and liver tissues were removed rapidly. Liver tissue samples were immediately rinsed in sterile saline, snap-frozen in liquid nitrogen, and stored at -70 ℃ until analysis.

### *MDA and SOD determination*

The livers were homogenized at 100 g/L in cold saline at 0-4 ℃ using a glass-potter-type homogenizer at 500-800 r/min in ice. The homogenates were centrifuged at 100 000 *g* for 20 min at 4 ℃ to obtain supernatants of tissue homogenates. The resultant supernatants were immediately pipetted for measuring the content of malondialdehyde (MDA) and the enzymatic activity of superoxide dismutase (SOD) by the test kits. Data were expressed as nanomolar MDA per gram wet liver tissue and units SOD per milligram wet liver tissue.

### *PINP and PIIINP determination*

Serum *N*-propeptide of type I procollagen (PINP) and *N*-propeptide of type III procollagen (PIIINP) were measured by ELISA (LIFEKEY BioMeditech Corporation).

### *Immunohistochemistry*

Five-micrometer-thick sections of formalin-fixed and paraffin-embedded livers were processed routinely. Tissue inhibitor of metal protease-1 (TIMP-1) was stained with polyclonal antibody (Santa Cruz Biotechnology, Inc.). Briefly, sections were deparaffinized in xylene and hydrated in a series of graded alcohol, and endogenous peroxidase was blocked with 5% hydrogen peroxide. The sections were then incubated with primary antibodies (1:50) against TIMP-1 at 37 ℃ for 1 h, followed by incubation with goat anti-rabbit IgG conjugated-horseradish peroxidase (1:200). The signals were visualized by ABC immunostaining system. The images were captured and digitized with an image analysis system (Jieda 501, version 3.10). The TIMP-1 positive cell ratios were automatically evaluated. For each specimen at least five fields were analyzed at 20× magnification.

### *RT-PCR reaction*

Semiquantitative RT-PCR with β-actin as internal control was performed to examine the expression of matrix metal protease-1 (MMP-1) mRNA. Total RNA was extracted from liver by TRIzol method (GIBCO specification). The first-strand cDNA was synthesized from the total RNA using 40 μL reverse transcription mixture containing 2 μg total RNA, 1.0 μg M-MLV reverse transcriptase (Promega) and random primers (Sangon, Shanghai), 10 mmol/L dNTP. Reverse transcription reaction was preformed at 37 ℃ for 1 h. At the end of reverse transcription, the mixture was heated at 95 ℃ for 5 min and immediately cooled in ice for 5 min.

PCR amplification was carried out with 2 μL cDNA product in a 40 μL reaction volume containing 10 pmoL of each specific oligonucleotide primer (Table 1), 10 mmol/L dNTP, and 2.5 IU Taq DNA polymerase. Amplification was performed for 30 cycles of denaturation (at 94 ℃ for 30 s), annealing (at 58 ℃ for 30 s), and elongation (at 72 ℃ for 50 s). PCR products were electrophoresed on 20 g/L agarose gel and visualized by ethidium bromide staining under UV light. This semiquantitative measure was expressed as ratios compared to β-actin.

**Table 1** Primers for PCR amplification of MMP-1 and β-actin mRNA

| Primer name | Primer sequence | Product Size/bp | GenBank acc. no. |
|---|---|---|---|
| MMP-1 | Sense: 5′-tactagccaccgcttcttcc-3′ (192–211) | 311 | U53605 |
| | Anti-sense: 5′-ggccagaaatagctgaatgc-3′ (483–502) | | |
| β-actin | Sense: 5′-ctgtgcccatctatgagggt-3′ (485–504) | 199 | NM-031144 |
| | Anti-sense: 5′-gccatctcttgctcgaagtc-3′ (664–683) | | |

### *Statistical analysis*

All numerical data were expressed as mean±SD. Statistical analyses were done using the SPSS for Window release 11.0 (SPSS Inc., USA). The parameters were evaluated with one-way ANOVA and Dunnett's *t* test. $P<0.05$ was considered statistically significant.

## RESULTS

### *Effect of ACF on MDA and SOD in liver tissue of rats*

As shown in Figure 1A, the SOD activity decreased and the MDA content increased significantly in model group in comparison to those in normal group ($P<0.01$, $P<0.01$). However, the SOD activity in ACF groups increased and MDA contents decreased in comparison to those in model group ($P<0.01$).

### *Effects of ACF on PINP and PIIINP contents in rat serum*

The serum PINP and PIIINP contents in ACF-2 and -3 group decreased ($P<0.05$, $P<0.01$) compared to those in model group (Figure 1B).

### *Expression of MMP-1 mRNA in rat hepatic tissue*

There was no significant difference in expression of MMP-1 mRNA between normal group and model group. But the expression of MMP-1 mRNA in ACF groups tended to increase. In ACF-2 and -3 group, the expression of MMP-1 mRNA increased significantly as compared to that in model group (Figure 2).

### *TIMP-1 protein expression in rat hepatic tissue*

The positive expressions of TIMP-1 protein were observed in all groups. In normal group, the mild expression was

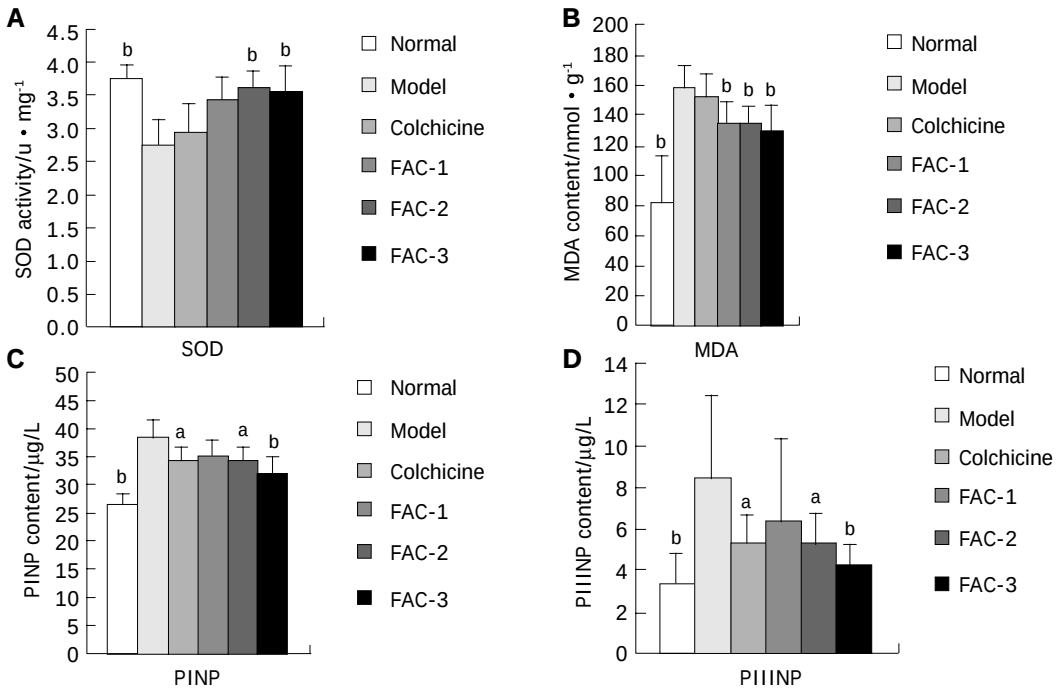


Figure 1 Effects of ACF on SOD activity and MDA content in rat liver tissue (A) and on PINP and PIIINP contents in rats serum (B). [a]$P<0.05$, [b]$P<0.01$ *vs* model group.

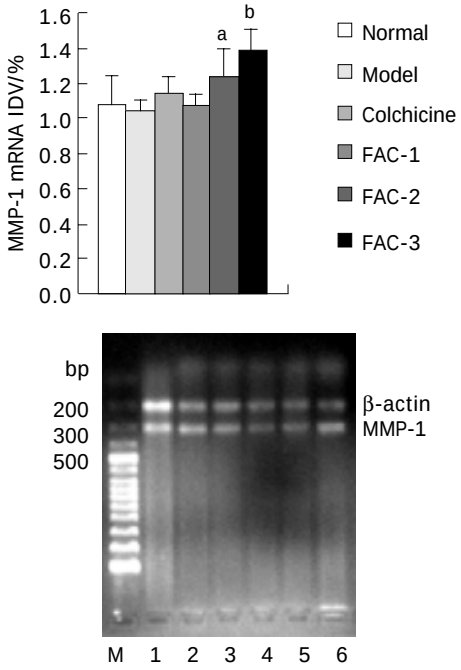


Figure 2 Analysis of MMP-1 mRNA expression in rat hepatic tissue. M: molecular weight markers (bp); lane 1: normal group; lane 2: model group; lane 3: colchicine group; lane 4: ACF-1 group; lane 5: ACF-2 group; lane 6: ACF-3 group; $n = 4$. [a]$P<0.05$, [b]$P<0.01$ *vs* model group.

presented in vascular endothelial cells and sinusoidal space. However, in model group the strong expression was noted around lobule median vein and in portal areas with a positive ratio of 8.84%. After the rats were treated with ACF, TIMP-1 protein expression decreased significantly ($P<0.01$, Figure 3).

## DISCUSSION

Following chronic liver injury of any etiology, there is progressive fibrosis. The fibrotic process recognizes the involvement of various cells and different factors, leading to excessive fibrogenesis with disruption of intercellular contacts and interactions and of extracellular matrix composition[4-6]. However, hepatic stellate cells (HSCs), together with recruited mononuclear cells and Kupffer cells are by far the key players in liver fibrosis[7-10]. Their cross-talk is triggered and favored by a series of chemical mediators, with a prominent role played by the transforming growth factor beta. Both expression and synthesis of this inflammatory and pro-fibrogenic cytokine are mainly modulated through redox-sensitive reactions. Furthermore, involvement of reactive oxygen species and lipid peroxidation products can be clearly demonstrated in other fundamental events of hepatic fibrogenesis, like activation and effects of stellate cells, expression of metalloproteinases and their specific inhibitors[11-14]. Therefore, anti-oxidative stress can inhibit the process of fibrosis.

Liver fibrosis is characterized by the deposition of excess extracellular matrix (particularly collagen types I and III) in the liver. The aminoterminal procollagen type-I peptide and type-III peptide, which release during collagen types I and III deposition, have been suggested as serum markers of fibrogenesis[15-17]. Their concentrations in serum reflect the ongoing collagen formation in the liver. Extracellular degradation of matrix protein is regulated by MMPs produced by HSCs, which in turn are regulated by several mechanisms including regulation at level of the gene (transcription and proenzyme synthesis), cleavage of the proenzyme to an active form and specific inhibition of activated forms by TIMPs[18].

Extracellular degradation of matrix proteins is regulated by a family of enzymes known as MMPs[19-21]-types I, II,

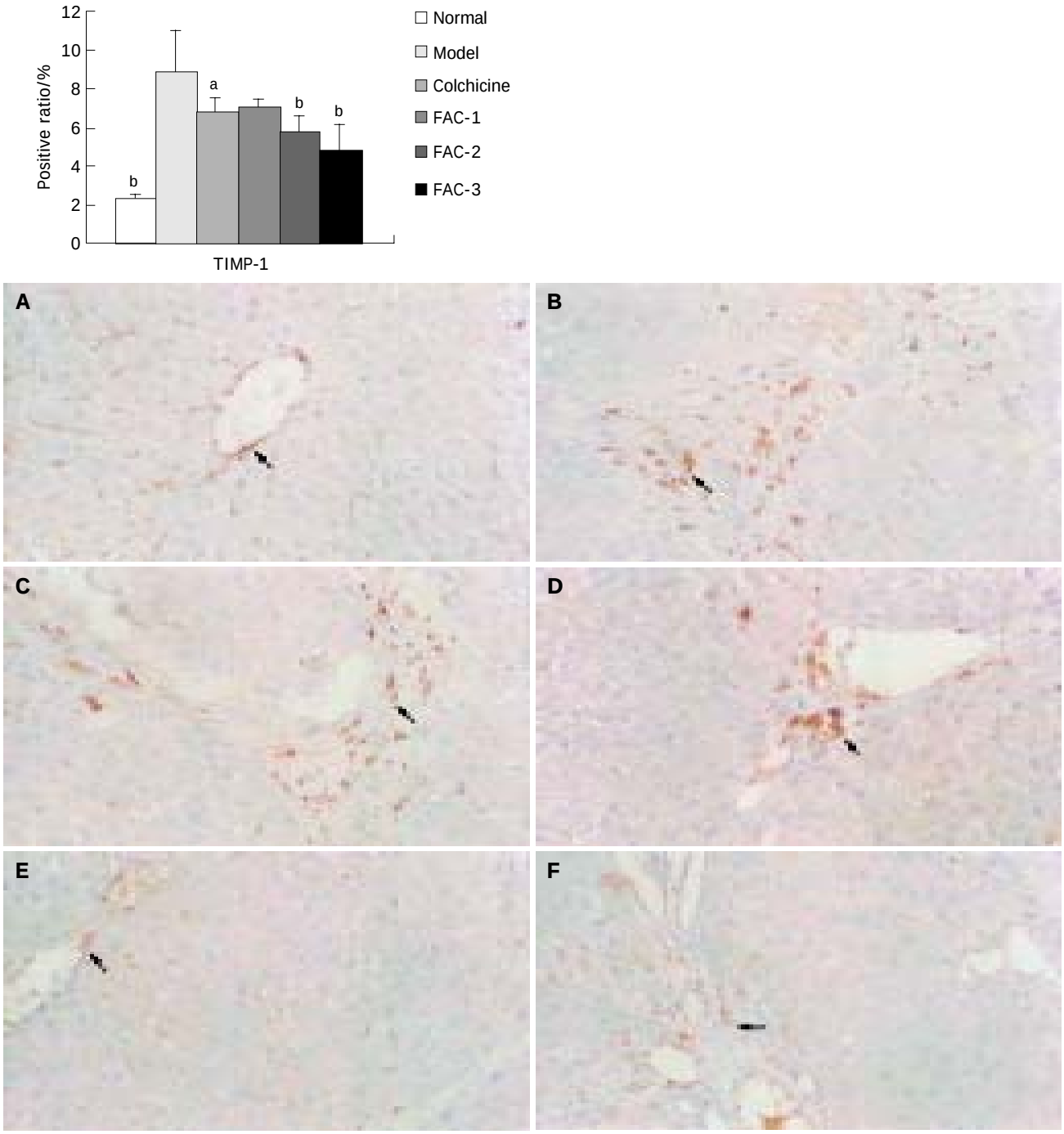


Figure 3 Immunohistochemical expression of TIMP-1 protein in rat hepatic tissue. (1) The positive ratio detected by Jieda 501 image-analysis system was used as the total protein expression. (2) TIMP-1 is shown as yellow grains in hepatic tissue (immunohistochemistry, ×200). A: normal group; B: model group; C: colchicine group; D: ACF-1 group; E: ACF-2 group; F: ACF-1 group; $n$ = 5. $^{a}P<0.05$, $^{b}P<0.01$ *vs* model group.

and III collagenases degrade interstitial collagens. Type IV collagenases/gelatinases degrade basement membrane collagen and gelatins and stromelysins which degrade a broad range of substrates including proteoglycans, laminin, gelatins, and fibronectin. However, the activated forms of MMPs in turn are inhibited specifically by TIMPs, such as TIMP-1 and -2[22-26].

Based on this pathophysiological mechanism, therapeutic methods are developed to diminish collagen accumulation, stimulate the collagenolytic process, and/or inhibit stellate cell proliferation[27,28]. However, current therapies targeting at arresting or reversing liver fibrosis are largely ineffective and some have unacceptable side effects in long-term therapy. Recent clinical and experimental observations have demonstrated that Chinese medicines might be of some preventive and therapeutic values against fibrosis[29,30]. SA is a traditional Chinese medicine containing flavonoids, organic acids and triterpenoids, etc.[31]. It can protect animal livers against injury by $CCl_4$ and cirrhosis caused by DMN[1,2].

The present results indicate that ACF decreases the serum content of *N*-propeptide of types I and III procollagen,

elevates the activity of SOD and reduces the MDA content in DMN-treated rat hepatic tissue. In addition, the protein expression of TIMP-1 reduces and the MMP-1 mRNA expression increases in liver fibrosis rats with induced by DMN. The antifibrotic effect of ACF is associated with its influence on lipid peroxidation and collagen synthesis and degradation.

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