

Full Paper

Protective Effect of a Mixture of *Aloe vera* and *Silybum marianum* Against Carbon Tetrachloride–Induced Acute Hepatotoxicity and Liver FibrosisSung-Hwa Kim¹, Ho Jun Cheon¹, Nari Yun¹, Sun-Tack Oh², Eunju Shin², Kyu Suk Shim², and Sun-Mee Lee^{1,*}¹College of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, Korea²Univera, Inc., Seoul 133-120, Korea

Received July 24, 2008; Accepted November 21, 2008

Abstract. The hepatoprotective effects of ACTIValoe[®]N-931 complex, a mixture of *Aloe vera* and *Silybum marianum*, against acute and chronic carbon tetrachloride (CCl₄)-induced liver injuries were investigated. Acute hepatotoxicity was induced by intraperitoneal injection of CCl₄ (50 µl/kg), and ACTIValoe[®]N-931 complex at 85, 170, and 340 mg/kg was administered orally 48, 24, and 2 h before and 6 h after injection of CCl₄. Hepatotoxicity was assessed 24 h after CCl₄ treatment. Liver fibrosis was induced by intraperitoneal injection of CCl₄ for 8 weeks (0.5 ml/kg, twice per week), and mice were treated with ACTIValoe[®]N-931 complex at 85, 170, or 340 mg/kg once a day (p.o.). In both acute hepatotoxicity and liver fibrosis, serum aminotransferase levels and lipid peroxidation were increased and the hepatic glutathione content was decreased. These changes were prevented by ACTIValoe[®]N-931 complex. The ACTIValoe[®]N-931 complex attenuated the increase in tumor necrosis factor- α (TNF- α), and inducible nitric oxide synthase (*iNOS*), and cyclooxygenase-2 (*COX-2*), mRNA expressions in acute hepatotoxicity. In antifibrotic experiments, tissue inhibitor of metalloprotease-1 (*TIMP-1*) mRNA expression was attenuated by treatment with ACTIValoe[®]N-931 complex. The ACTIValoe[®]N-931 complex decreased the hepatic hydroxyproline content and the transforming growth factor-beta1 levels. Our results suggest that the ACTIValoe[®]N-931 complex has hepatoprotective effects in both acute and chronic liver injuries induced by CCl₄.

Keywords: *Aloe vera*/*Silybum marianum* mixture (ACTIValoe[®]N-931 complex), anti-inflammation, anti-oxidant, carbon tetrachloride, fibrosis

Introduction

Acute and chronic liver diseases constitute a global concern, but medical treatments for these diseases are often difficult to handle and have limited efficacy. Therefore, considerable efforts to obtain useful herbal medicines from documented medicinal plants for a wide variety of clinical conditions are currently underway. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically.

Carbon tetrachloride (CCl₄) is a well-known hepatotoxin that is widely used to induce toxic liver injuries in laboratory animals. The hepatic necrosis caused by CCl₄ is thought to involve bioactivation by cytochrome P450 2E1 (CYP2E1), resulting in the formation of trichloromethyl free radicals and reactive oxygen species (ROS), which initiate lipid peroxidation and protein oxidation and damage the hepatocellular membranes (1). This process is followed by the release of inflammatory mediators from activated hepatic macrophages, which are believed to potentiate the CCl₄-induced hepatic injury. Discrete exposure to CCl₄ results in acute liver injury; continuous exposure to this toxin produces progressive liver injury and fibrosis, eventually causing cirrhosis, portal hypertension, and death (2).

*Corresponding author. sunmee@skku.edu

Published online in J-STAGE

doi: 10.1254/jphs.08189FP

Aloe vera, which is commonly known as Ghee kanwar (Hindi), is a cactus-like plant that grows in hot, dry climates such as the desert. Its survival in such harsh environments leads to the belief that *Aloe vera* has wound-healing and antibiotic effects (3). *Aloe vera* leaves are used in diseases of the eyes and enlargements of spleen and liver (4). Moreover, its liver protective function via anti-oxidative and anti-inflammatory effects is well known (5, 6). *Silybum marianum* is an herbal supplement used to treat liver and biliary disorders (7). It has recently been investigated for use as a cytoprotectant, an anti-carcinogen, and a supportive treatment for liver damage from *Amanita phalloides* poisoning. Silymarin, an active ingredient of *Silybum marianum*, is a strong antioxidant that promotes liver cell regeneration, reduces blood cholesterol, and helps prevent cancer (8). In our previous studies, *Aloe vera* or *Silybum marianum* alone suppressed the increase in serum aminotransferase activities in both CCl₄- and D-galactosamine-treated rats. ACTIVAlloe[®]N-931 complex, a mixture of *Aloe vera* and *Silybum marianum*, appeared to have a synergistic effect in suppressing the increases in serum aminotransferase activities. Furthermore, ACTIVAlloe[®]N-931 complex protected hepatocytes from hepatotoxicity caused by α -naphthylisothiocyanate and ethionine (9).

The present study investigated the anti-oxidative and anti-inflammatory properties of ACTIVAlloe[®]N-931 complex against acute CCl₄ liver injury in mice. Its anti-fibrotic effects in the development of fibrosis after repeated CCl₄ injections were also investigated.

Materials and Methods

Chemicals and reagents

ACTIVAlloe[®]N-931 complex was provided by Univera, Inc., Seoul, Korea. Carbon tetrachloride, glutathione, 2,6-di-*tert*-butyl-4-methylphenol, 2-thiobarbituric acid, hydrochloride, *p*-dimethylaminobenzaldehyde, chloramine T hydrate, and *p*-aminophenol were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and reagents used in this study were reagent grade.

Treatment of animals

Male Sprague-Dawley rats (240–260 g) and male ICR mice (25–30 g) were obtained from Daehan Biolink Co., Ltd., Eumseong, Korea. All animals were treated humanely according to Sungkyunkwan University Animal Care Committee guidelines. Acute liver damage was induced in mice by intraperitoneal injection of CCl₄ (50 μ l/kg), and ACTIVAlloe[®]N-931 complex was administered orally 48, 24, and 2 h before and 6 h after CCl₄ injection. Hepatotoxicity was assessed 24 h after

CCl₄ treatment. Chronic liver damage was induced in rats by intraperitoneal injection of CCl₄ for 8 weeks (0.5 ml/kg, twice per week) with ACTIVAlloe[®]N-931 complex treatment once a day (p.o.). The dose and timing of ACTIVAlloe[®]N-931 complex were selected based on previous reports (9–11). The animals were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated CCl₄; iii) ACTIVAlloe[®]N-931 complex at 85 mg/kg (a mixture of 35 mg/kg *Aloe vera* and 50 mg/kg *Silybum marianum*)-treated CCl₄; iv) ACTIVAlloe[®]N-931 complex at 170 mg/kg (a mixture of 70 mg/kg *Aloe vera* and 100 mg/kg *Silybum marianum*)-treated CCl₄; and v) ACTIVAlloe[®]N-931 complex at 340 mg/kg (a mixture of 140 mg/kg *Aloe vera* and 200 mg/kg *Silybum marianum*)-treated CCl₄. The animals were sacrificed by decapitation and blood was collected from the abdominal aorta. The liver was isolated and used immediately to prepare mRNA, which was stored at -75°C for later analysis; part of the left lobe of the liver was used for histological analysis.

Isolation of hepatic microsomal fraction

The excised liver was minced, homogenized with a Teflons pestle homogenizer in 4 volumes of ice-cold 1.15 M KCl for 1 g of the liver, and then centrifuged at $9,000 \times g$ for 60 min. The supernatant was collected and centrifuged at $105,000 \times g$ for 60 min. Microsomal precipitates were resuspended with 4 volumes of 0.1 M phosphate buffer, pH 7.4, for 1 g of the liver microsomes and stored at -75°C until assayed.

Analytical procedures

The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by standard spectrophotometric procedures using the ChemiLab ALT and AST assay kits (IVDLab Co., Ltd., Suwon, Korea), respectively. The total glutathione level was measured spectrophotometrically at a wavelength of 412 nm using yeast glutathione reductase and 5,5'-dithio-bis (2-nitrobenzoic acid) as described by Tietze (12). The oxidized glutathione level was measured using the same method but in the presence of 2-vinylpyridine (13). The level of reduced glutathione (GSH) was determined by the difference between the total glutathione and oxidized glutathione levels. The steady-state level of malondialdehyde (MDA), which is the end-product of lipid peroxidation, in the liver was determined by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at 535 nm using the method described by Buege and Aust (14). CYP2E1 activity was determined by 4-hydroxylation of aniline to *p*-aminophenol (15). The hydroxyproline content in the liver was measured according to the

Table 1. PCR primers used in this study and the amplified product length

Gene (accession number)	Primer sequences (5'-3')	Product length (bp)
<i>TNF-α</i> (M11731)	Sense: AGCCACGTCGTAGCAAACCACCAA	446
	Antisense: AACACCCATTCCTTCACAGAGCAAT	
<i>iNOS</i> (NM_010927)	Sense: AAGCTGCATGTGACATCGACCCGT	598
	Antisense: GCATCTGGTAGCCAGCGTACCGG	
<i>COX-2</i> (NM_011198)	Sense: ACTCACTCAGTTTGTGAGTCATTC	582
	Antisense: TTTGATTAGTACTGTAGGGTTAATG	
<i>TIMP-1</i> (BC099821)	Sense: ACAGCTTTCTGCAACTCG	771
	Antisense: CTATAGGTCTTTACGAAGGCC	
β -Actin (X03672)	Sense: TGGAATCCTGTGGCATCCATGAAA	348
	Antisense: TAAAACGCAGCTCAGTAACAGTCCG	

method of Jamall et al. (16). The serum transforming growth factor- β 1 (TGF- β 1) concentration was quantified using a commercial TGF- β 1 ELISA kit (Biosciences Co., San Diego, CA, USA). Protein concentration was estimated by the dye binding assay of Bradford (17).

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissue using TRIzol-reagent (GibcoBRL, Grand Island, NY, USA) according to the manufacturer's protocol. First strand cDNA was synthesized by reverse transcription of total RNA using oligo dT-adaptor primer and AMV Reverse Transcriptase. The PCR reaction was performed with a diluted cDNA sample in a 10 μ l reaction volume. The final reaction concentrations were 10 pmol primers, 2.5 mM MgCl₂, 1 mM dNTP mixture, 1 μ l 10 \times RNA PCR buffer, and 0.5 U Ex Taq-DNA polymerase per reaction. Table 1 lists the gene-specific primers used. All PCRs involved an initial denaturation step at 94°C for 5 min and a final extension at 72°C for 5 min in the GeneAmp 2700 thermocycler (Perkin-Elmer, Inc., Waltham, MA, USA). The cycling conditions for PCR amplification were as follows: 28 cycles of 94°C (30 s), 65°C (30 s), and 72°C (30 s) for tumor necrosis factor- α (*TNF- α*); 35 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s) for inducible nitric oxide synthase (*iNOS*); 40 and 30 cycles of 94°C (30 s), 56°C (30 s), and 72°C (30 s) for cyclooxygenase-2 (*COX-2*) and tissue inhibitor of matrix metalloproteinase-1 (*TIMP-1*), respectively; and 25 cycles of 94°C (30 s), 62°C (30 s), 72°C (60 s), for β -actin. The amplified products were resolved by electrophoresis in 1.5% agarose gel and stained with ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA). The intensity of each PCR product was evaluated semi-quantitatively using a digital camera (DC120; Eastman Kodak, Rochester, NY, USA) and a densitometric scanning analysis program (1D Main; Advanced

American Biotechnology, Fullerton, CA, USA).

Histopathological analysis

Liver slices were prepared from a portion of the left lobe and fixed immediately in 10% formalin-buffered phosphate solution, embedded in paraffin, and sectioned at 5- μ m thickness. Serial sections were stained with hematoxylin and eosin (H&E) to evaluate portal inflammation, inflammatory cell infiltration, centrilobular necrosis, liver cell dysplasia, ballooning degeneration, vascular congestion, fatty change, and fibrosis. The sections were examined in a blind manner under an Olympus CKX 41 microscope (Olympus Optical Co., Ltd., Tokyo).

Statistical analyses

All results are presented as the mean \pm S.E.M. One-way analysis of variance (ANOVA) followed by Student-Neuman-Keuls test was used to determine the significance of the differences between the experimental groups. A *P*-value <0.05 was considered significant.

Results

Effect of ACTIValue[®]N-931 complex on acute liver injury

The serum ALT and AST activities in the control group were 30.8 \pm 0.37 and 109.0 \pm 4.0 U/l, respectively. The serum ALT and AST activities increased markedly 24 h after CCl₄ injection. These increases were attenuated by treatment with ACTIValue[®]N-931 complex at 85, 170, and 340 mg/kg. Similar to the aminotransferase activities, the MDA levels increased significantly in CCl₄-injected mice and were attenuated by ACTIValue[®]N-931 complex at 85 mg/kg. In contrast, the hepatic glutathione content in CCl₄-injected mice decreased to 49% of the control value and this decrease was attenuated by ACTIValue[®]N-931 complex at 85 and

Table 2. Effect of ACTIVAlloe[®]N-931 complex on the serum aminotransferase activities, lipid peroxidation, and hepatic glutathione content in mice with CCl₄-induced acute hepatotoxicity

Groups	ALT (U/l)	AST (U/l)	Malondialdehyde (nmol/mg protein)	Hepatic glutathione content (μmol/g liver)
Control	30.8 ± 0.4	109.0 ± 4.0	0.32 ± 0.01	11.0 ± 0.4
CCl ₄				
Vehicle	2112.7 ± 263.4**	1086.7 ± 165.4**	0.52 ± 0.03**	5.5 ± 0.4**
N-931C85	1142.2 ± 132.0**,#	468.4 ± 74.1**,#	0.49 ± 0.04*	8.3 ± 0.9 [#]
N-931C170	1104.3 ± 154.2**,#	509.1 ± 76.0**,#	0.47 ± 0.05	8.5 ± 0.9 [#]
N-931C340	1266.2 ± 197.3**,#	521.0 ± 95.2**,#	0.48 ± 0.06	6.6 ± 0.7**

Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (* $P < 0.05$, ** $P < 0.01$) from the control group. Significantly different ([#] $P < 0.05$, ^{##} $P < 0.01$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe[®]N-931 complex (85 mg/kg); N-931C170: ACTIVAlloe[®]N-931 complex (170 mg/kg); N-931C340: ACTIVAlloe[®]N-931 complex (340 mg/kg).

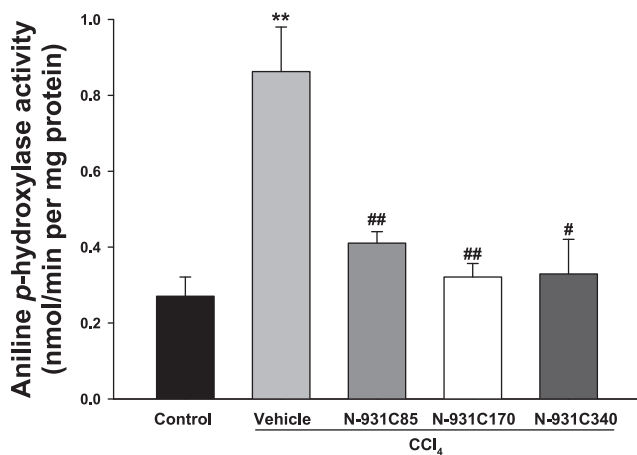


Fig. 1. Effect of ACTIVAlloe[®]N-931 complex on microsomal cytochrome P450 2E1 activity in the liver of mice with CCl₄-induced acute hepatotoxicity. Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (** $P < 0.01$) from the control group. Significantly different ([#] $P < 0.05$, ^{##} $P < 0.01$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe[®]N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe[®]N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe[®]N-931 complex (340 mg/kg).

170 mg/kg (Table 2). No significant effects on serum aminotransferase activities, MDA level, and hepatic glutathione content were observed in mice treated with the ACTIVAlloe[®]N-931 complex at 42.5 mg/kg (data not shown). The CYP2E1 activity markedly increased in the CCl₄-treated group to levels about 318% of those seen in microsomes from the control. This increase was attenuated by ACTIVAlloe[®]N-931 complex at 85, 170, and 340 mg/kg (Fig. 1). The histopathological features of normal liver lobular architecture and cell structure are shown in Fig. 2 for the control livers. However, liver exposed to CCl₄ exhibited multiple, extensive areas of portal inflammation, centrilobular necrosis, and vascular degradation. These histopathological changes were attenuated by the ACTIVAlloe[®]N-931 complex. The *TNF-α*, *iNOS*, and *COX-2* mRNA expressions were significantly higher in CCl₄-treated mice than in the controls. The increases were significantly suppressed by the administration of ACTIVAlloe[®]N-931 complex (Fig. 3).

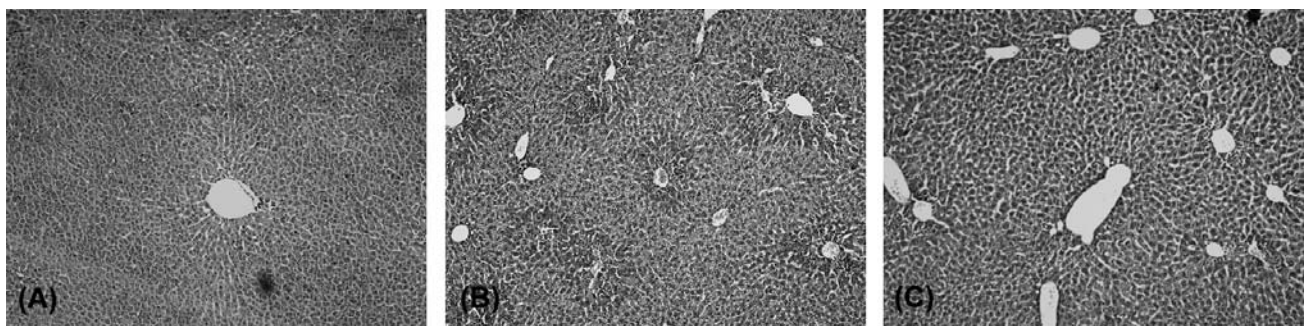


Fig. 2. Effect of ACTIVAlloe[®]N-931 complex on histological changes in CCl₄-induced acute hepatotoxicity (original magnification ×100). A: control group, showing normal lobular architecture and cell structure; B: vehicle-treated CCl₄ group, showing extensive hepatocellular damage with the presence of portal inflammation, centrilobular necrosis, and Kupffer cell hyperplasia; C: ACTIVAlloe[®]N-931 complex (170 mg/kg)-treated CCl₄ group, showing mild portal inflammation, minimal hepatocellular necrosis, and Kupffer cell hyperplasia.

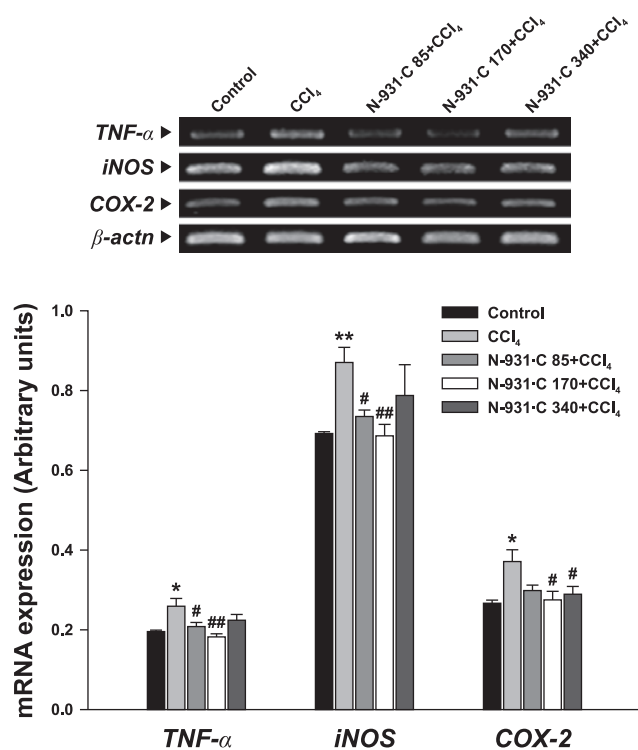


Fig. 3. Effect of ACTIVAlloe[®]N-931 complex on the *TNF-α*, *iNOS*, and *COX-2* mRNA expressions in the liver of mice with CCl₄-induced acute hepatotoxicity. Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (* $P < 0.05$, ** $P < 0.01$) from the control group. Significantly different (# $P < 0.05$, ## $P < 0.01$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe[®]N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe[®]N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe[®]N-931 complex (340 mg/kg).

Effect of ACTIVAlloe[®]N-931 complex on chronic liver injury

The administration of CCl₄ increased the content of hydroxyproline to 2.0 times that of the control (Fig. 4). This increase was significantly attenuated by treatment

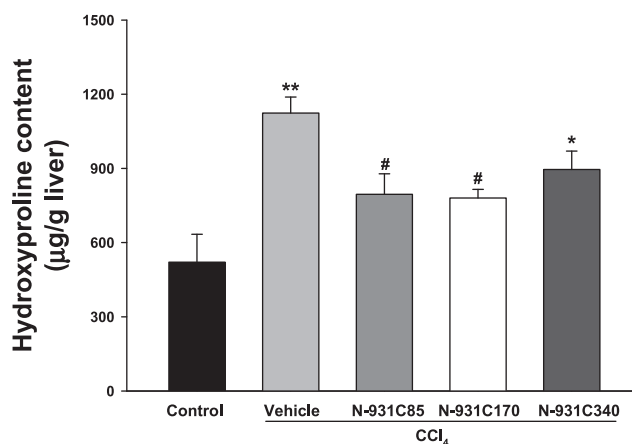


Fig. 4. Effect of ACTIVAlloe[®]N-931 complex on hepatic hydroxyproline content in CCl₄-induced liver fibrosis. Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (* $P < 0.05$, ** $P < 0.01$) from the control group. Significantly different (# $P < 0.05$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe[®]N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe[®]N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe[®]N-931 complex (340 mg/kg).

with ACTIVAlloe[®]N-931 complex at 85 and 170 mg/kg. The histopathological features shown in Fig. 5 indicate a normal liver lobular architecture and cell structure in the control liver. However, livers exposed to CCl₄ exhibited extensive areas of fatty change and gross necrosis, broad infiltration of lymphocytes, and Kupffer cell hyperplasia. These histopathological changes were attenuated by ACTIVAlloe[®]N-931 complex. The serum ALT and AST activities in the control groups were very low. In the vehicle-treated CCl₄ group, the serum ALT and AST levels increased to approximately 48.9 and 19.5 times that of the control group, respectively. Serum ALT activities were slightly attenuated by ACTIVAlloe[®]N-931 complex at 85, 170, and 340 mg/kg, whereas AST activities were significantly attenuated by ACTIVAlloe[®]N-

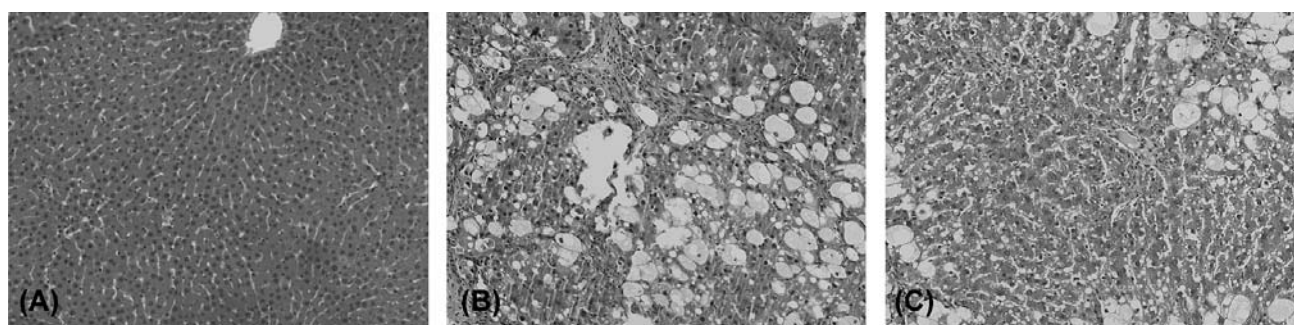


Fig. 5. Effect of ACTIVAlloe[®]N-931 complex on histological changes in CCl₄-induced chronic hepatotoxicity (original magnification ×100). A: control group, showing normal lobular architecture and cell structure; B: vehicle-treated CCl₄ group, showing extensive areas of fatty change and gross necrosis, broad infiltration of lymphocytes, and Kupffer cell hyperplasia; C: ACTIVAlloe[®]N-931 complex (170 mg/kg)-treated CCl₄ group, showing mild fatty changes, minimal hepatocellular necrosis, and Kupffer cell hyperplasia.

Table 3. Effect of ACTIVAlloe®N-931 complex on the serum aminotransferase activities, lipid peroxidation, and hepatic glutathione content in the CCl₄-induced fibrosis

Groups	ALT (U/l)	AST (U/l)	Malondialdehyde (nmol/mg protein)	Hepatic glutathione content (μmol/g liver)
Control	29.8 ± 0.4	112.0 ± 4.6	0.32 ± 0.04	7.3 ± 0.8
CCl ₄				
Vehicle	1469.5 ± 289.3**	2188.1 ± 270.2**	0.61 ± 0.09**	5.2 ± 0.2*
N-931C85	895.8 ± 169.8**	1269.9 ± 256.5** [#]	0.44 ± 0.02**	5.2 ± 0.2*
N-931C170	830.2 ± 150.3**	1275.5 ± 201.5** [#]	0.39 ± 0.01 ^{###}	5.8 ± 0.5
N-931C340	1037.7 ± 103.6**	1422.8 ± 199.9** [#]	0.40 ± 0.03 ^{###}	5.2 ± 0.4*

Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (* $P < 0.05$, ** $P < 0.01$) from the control group. Significantly different ([#] $P < 0.05$, ^{###} $P < 0.01$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe®N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe®N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe®N-931 complex (340 mg/kg).

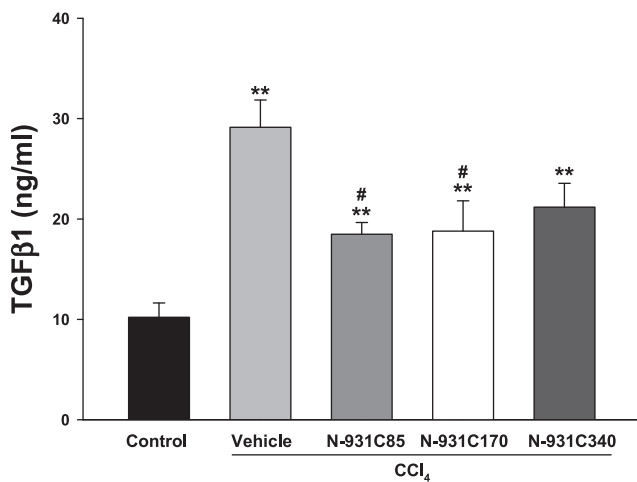


Fig. 6. Effect of ACTIVAlloe®N-931 complex on serum TGF-β1 levels in CCl₄-induced liver fibrosis. Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (** $P < 0.01$) from the control group. Significantly different ([#] $P < 0.05$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe®N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe®N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe®N-931 complex (340 mg/kg).

931 complex 85, 170, and 340 mg/kg. The administration of CCl₄ increased the hepatic level of MDA to approximately 2 times that of the control group. This elevation was attenuated by ACTIVAlloe®N-931 complex at 170 and 340 mg/kg. The GSH content in the control group was $7.3 \pm 0.8 \mu\text{mol/g}$ liver. The GSH content decreased significantly after CCl₄ administration, but was slightly attenuated by ACTIVAlloe®N-931 complex at 170 mg/kg (Table 3). As shown in Fig. 6, the level of TGF-β1 was markedly elevated to 2.8 times that of the control, and this increase was significantly attenuated by ACTIVAlloe®N-931 complex at 85 and 170 mg/kg. The *TIMP-1* mRNA expression in the CCl₄-injected group was 1200-fold higher than that in the control group. The increase in *TIMP-1* mRNA level was significantly

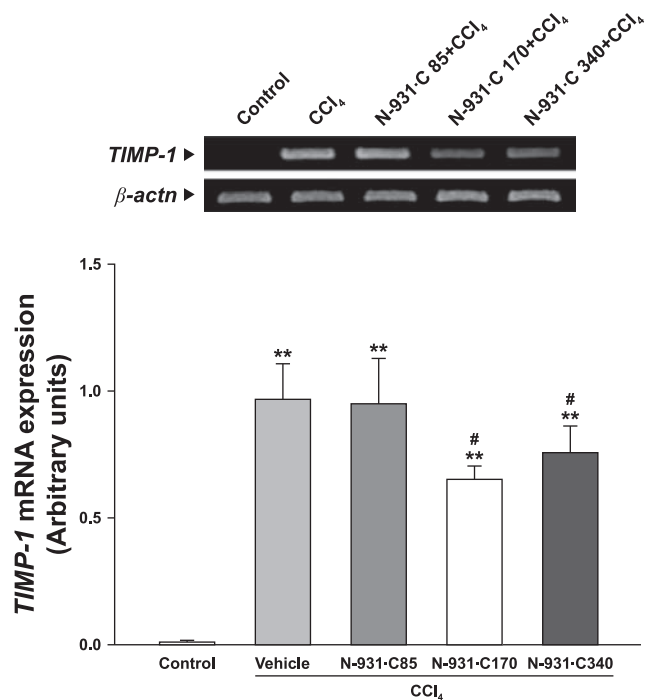


Fig. 7. Effect of ACTIVAlloe®N-931 complex on the *TIMP-1* mRNA expression in the liver of the CCl₄-induced liver fibrosis. Each value is the mean ± S.E.M. of 8–10 animals per group. Significantly different (** $P < 0.01$) from the control group. Significantly different ([#] $P < 0.05$) from the vehicle-treated CCl₄ group. N-931C85: ACTIVAlloe®N-931 complex (85 mg/kg), N-931C170: ACTIVAlloe®N-931 complex (170 mg/kg), N-931C340: ACTIVAlloe®N-931 complex (340 mg/kg).

suppressed by ACTIVAlloe®N-931 complex at 170 and 340 mg/kg (Fig. 7).

Discussion

In the present study, the hepatoprotective effects of ACTIVAlloe®N-931 complex in acute and chronic liver injury models were investigated. We first examined the

effect of ACTIValue[®]N-931 complex on acute hepatotoxicity induced by single injection of CCl₄. In the vehicle-treated CCl₄ group, ALT and AST levels increased dramatically compared with those in the control group, indicating severe hepatocellular damage. In contrast, treatment with ACTIValue[®]N-931 complex markedly attenuated the release of ALT and AST. Histological observations of liver samples strongly supported the release of aminotransferases by damaged hepatocytes as well as the protective effect of ACTIValue[®]N-931 complex. CCl₄ caused various histological changes in the liver, including cell necrosis, fatty metamorphosis in the adjacent hepatocytes, ballooning degeneration, cell inflammation, and infiltration of lymphocytes and Kupffer cells. These alterations were significantly attenuated by the ACTIValue[®]N-931 complex so that treated livers showed only minor hepatocellular necrosis and inflammatory cell infiltration and mild portal inflammation. These results suggest that the ACTIValue[®]N-931 complex has potential clinical applications for treating liver disorders.

Many hepatotoxicants including CCl₄, nitrosamines, and polycyclic aromatic hydrocarbons require metabolic activation, particularly by liver CYP enzymes to form reactive toxic metabolites, which in turn cause liver injury in experimental animals and humans. Alterations in the activity of CYP2E1 can affect the susceptibility to hepatic injury from CCl₄. The trichloromethyl radical formed during the metabolism of CCl₄ is capable of binding to lipids, and this binding initiates lipid peroxidation and liver damage (18). GSH constitutes the first line of defense against free radicals and is a critical determinant of tissue susceptibility to oxidative damage. In this study, the ACTIValue[®]N-931 complex exhibited protective effects by impairing CCl₄-mediated oxidative stress through decreased production of free radical derivatives, as evidenced by the decreased CYP2E1 activity and MDA level. Furthermore, the ACTIValue[®]N-931 complex attenuated hepatic glutathione depletion 24 h after CCl₄ injection. The increase in the hepatic glutathione level in ACTIValue[®]N-931 complex-treated mice could be due either to its effect on the *de novo* synthesis of glutathione, its regeneration, or both. These results suggest that the antioxidant properties may be one mechanism by which the ACTIValue[®]N-931 complex protects against CCl₄-mediated liver damage.

The liver is a major inflammatory organ and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release pro-inflammatory mediators either in response to necrosis or in direct response to an activated hepatotoxin; these pro-inflammatory mediators are believed to aggravate CCl₄-induced hepatic injury (19).

TNF- α is a major endogenous mediator of hepatotoxicity in several experimental liver injuries (20) through its direct cytotoxicity (21), nitric oxide production (22), and the triggering of an inflammatory cascade. It has been well established that both oxidative stress and abnormal production of cytokines, especially TNF- α , play important etiological roles in the pathogenesis of alcoholic liver disease. Acute ethanol administration to mice has been found to induce hepatic TNF- α production and mRNA expression and down-regulated TNF- α production by antioxidants was paralleled by attenuated hepatic lipid peroxidation, steatosis, and liver injury (23). Nitric oxide, which is produced by iNOS, is a highly reactive oxidant that plays a role in a number of physiological processes such as vasodilation, neurotransmission, and nonspecific host defense (24). As nitric oxide has a range of effects on a variety of biological processes, it is unclear if it is beneficial or detrimental in the liver injury induced by hepatotoxins. Previous studies reported that the induction of COX in the inflammatory response is a secondary effect of CCl₄-induced hepatotoxicity (25). COX-2, the mitogen-inducible isoform of COX, is induced in macrophages by several pro-inflammatory stimuli such as cytokines and growth factors, leading to COX-2 expression and the subsequent release of prostaglandins (26). This study confirmed a significant increase in the TNF- α , iNOS, and COX-2 mRNA expressions in the liver after CCl₄ administration; these increases were attenuated by treatment with the ACTIValue[®]N-931 complex. These results suggest that the ACTIValue[®]N-931 complex modulates the expressions of TNF- α , iNOS, and COX-2 mRNA. Several microarray studies have been reported describing gene expression changes caused by acute CCl₄ toxicity (27), although the significance of these changes has not been fully understood. Our results provide evidence for the pharmacological effect of ACTIValue[®]N-931 complex in CCl₄-induced hepatotoxicity. Overall, the ACTIValue[®]N-931 complex not only provides conjugation with injurious free radicals and diminishes their toxic properties, but also suppresses the proinflammatory response of a CCl₄-induced liver injury. Further studies will be needed to fully understand the association between oxidative stress and the inflammatory responses in the hepatoprotective effect of ACTIValue[®]N-931 against CCl₄-induced hepatotoxicity.

Hepatic fibrosis is a consequence of various chronic liver diseases caused by hepatitis viruses, drugs, alcohol, parasites, and autoimmune mechanisms. Studies in animal models of hepatic fibrosis show that extracellular matrix components accumulate in interstitial regions of the liver around central veins or in the portal tract. There are now several animal models of liver fibrosis, among

which the CCl₄-induced rat liver fibrosis model has been extensively investigated and applied. Normally, hepatic stellate cells are quiescent, but they become activated following liver injury. These activated hepatic stellate cells are primarily responsible for the excess production of extracellular matrix (28). Thus, reduction of extracellular matrix production by activated hepatic stellate cells is crucial for the prevention of fibrogenesis. In this study, liver fibrosis was observed as significant histological changes and significant increases in hydroxyproline content in an assessment of liver collagen content. Administration of CCl₄ to rats twice a week for 8 weeks caused a variety of histological changes in the liver including extensive areas of fatty change and gross necrosis, broad infiltration of lymphocytes, and Kupffer cell hyperplasia. These changes were significantly attenuated by the ACTIVAlone[®]N-931 complex. Repeated injections of CCl₄ also markedly increased the ALT and AST levels, and treatment with ACTIVAlone[®]N-931 complex slightly attenuated the release of ALT and markedly prevented the release of AST.

A large body of evidence suggests that oxidative stress is a major mechanism in the etiology of fibrosis (29). Excess ROS play a major mediatory role in inducing the synthesis of fibrillar extracellular matrix (30). The anti-fibrotic effect of ACTIVAlone[®]N-931 complex can also be ascribed to the suppression of lipid peroxidation as well as its propagation in the liver because ACTIVAlone[®]N-931 complex attenuates the CCl₄-induced increase in hepatic MDA content. Severe liver damage is followed by a phase of liver repair wherein surrounding hepatocytes proliferate and extracellular matrix synthesis increases. During liver repair, TGF- β 1 limits the proliferative response of hepatocytes and increases the production of collagen and other extracellular matrix proteins (31). Consistent with these findings, the present study demonstrated that, during the development of liver fibrosis, TGF- β 1 levels were markedly elevated and these increases were significantly attenuated by ACTIVAlone[®]N-931 complex.

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent enzymes that specifically degrade extracellular matrix components (32). Among them, the key enzymes in the degradation of fibrillar collagens are MMP-1 in humans and MMP-13 in rodents (33). However, during fibrogenesis, the expression of MMP-1 or MMP-13 is very limited, whereas that of MMP-2 increases (34). The activity of MMPs is regulated by TIMP. TIMP-1 is produced by Kupffer cells, hepatic stellate cells, and myofibroblasts in liver, but it is mainly produced by activated hepatic stellate cells. Injured liver tissue expresses more TIMP-1, which in turn causes interstitial fibrils to accumulate (35). In our model, there

were no significant changes in the expression of *MMP-2* mRNA in any of the experimental groups (data not shown). However, there was increased expression of *TIMP-1* mRNA in liver following repeated injection of CCl₄. We observed that increased *TIMP-1* mRNA levels were significantly attenuated by ACTIVAlone[®]N-931 complex. These results suggest that the anti-fibrotic effect of ACTIVAlone[®]N-931 complex is related to increased removal of deposited collagen, that is, enhanced collagenolytic activity due to decreased TIMP-1 levels.

Several studies have been reported that TGF- β 1 stimulates the production of *TIMP-1* mRNA (36). It also acts synergistically with other cytokines such as epidermal growth factor, basic fibroblast growth factor, and embryonal carcinoma-derived growth factor to increase the level of expression of *TIMP-1* mRNA (37). In our results, ACTIVAlone[®]N-931 complex at 85 mg/kg did not suppress the *TIMP-1* mRNA levels, whereas it attenuated the TGF- β 1 levels in CCl₄-induced chronic hepatotoxicity. This result may involve other factors such as epidermal growth factor, basic fibroblast growth factor, and embryonal carcinoma-derived growth factor.

In conclusion, the hepatoprotective and anti-fibrotic activity of ACTIVAlone[®]N-931 complex could be attributed to its mechanistic intervention in several cellular events, providing beneficial effects, most importantly, by 1) attenuating oxidative stress, 2) preventing the inflammatory response, and 3) enhancing collagenolytic activity. This study provides evidence that ACTIVAlone[®]N-931 complex could be used to prevent hepatocellular damage and liver fibrosis.

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