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Effects of *Antrodia camphorata* on Alcohol Clearance and Antifibrosis in Livers of Rats Continuously Fed Alcohol

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ABSTRACT: Alcoholic fatty liver disease (AFLD) is the result of an excessive or chronic consumption of alcohol. Nine male Wistar rats per group were randomly assigned to one of the following drinking treatments: a 20% (w/w) alcohol solution (ALC); a 20% (w/w) alcohol solution cotreated with 0.25 g silymarin/kg BW/day; or a 20% (w/w) alcohol solution cotreated with 0.025 g Niuchangchih (*Antrodia camphorata*)/kg BW/day for 4 weeks. Rats with cotreatments of silymarin or Niuchangchih had smaller ($p < 0.05$) relative liver size, less ($p < 0.05$) liver lipid accumulation, and lower ($p < 0.05$) liver damage indices [aspartate aminotransferase (AST) and alkaline phosphatase (ALP) values]. In the regulation of alcohol metabolism, the lower serum alcohol level was observed only in alcohol-fed rats supplemented with Niuchangchih. Meanwhile, cotreatment of silymarin or Niuchangchih increased ($p < 0.05$) CAT and ALDH activities but did not ($p > 0.05$) affect ADH and CYP2E1 expressions, which accelerate alcohol metabolism in the body. Additionally, neither silymarin nor Niuchangchih ($p > 0.05$) influenced serum/hepatic MMP-2 activities and NF-κB, AP1, and α-SMA gene expressions, but serum/hepatic MMP-9 activities and TNF-α, KLF-6, and TGF-β1 gene expressions of alcohol-fed rats were down-regulated ($p < 0.05$) by silymarin or Niuchangchih, which also could explain the lower liver damage observed in rats chronically fed alcohol.

KEYWORDS: alcoholic fatty liver, *Antrodia camphorata*, liver lipid accumulation, liver damage index, alcohol metabolism, fibrosis

INTRODUCTION

In 2009 the International Agency for Research on Cancer[1] reported that liver cancer is the third and eighth leading cause of cancer-related deaths in men and women worldwide, respectively. It was reported that chronic liver diseases, such as liver steatohepatitis (fatty liver), hepatitis, fibrosis, and cirrhosis, likely result in liver cancer.[2] Nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) are the two major pathogenic categories of fatty livers. NAFLD is always associated with obesity, insulin resistance, and the metabolic syndrome, whereas AFLD is the result of an excessive or chronic consumption of alcohol.

With regard to AFLD, a change in NADH/NAD$^+$ redox potential by an excessive or chronic consumption of alcohol in the liver results in a decrease of mitochondria β-oxidation of fatty acids in the liver, thereby reducing the lipid expenditure. Furthermore, this stimulates lipogenesis, thus increasing lipid accumulation in hepatocytes.[3] Up-regulations of lipogenic enzyme gene expressions are observed by chronic alcohol consumption, which increases fatty acid biosynthesis and develops hepatic steatosis.[4,5] After all, the liver is the primary organ responsible for the metabolism of alcohol. There are three different enzymes that metabolize alcohol in hepatocytes: (1) alcohol dehydrogenase (ADH) in cytosol; (2) cytochrome P450 in endoplasmic reticulum; (3) catalase (CAT) in peroxisomes.[6] However, liver damage caused by alcohol is most often attributed to cytochrome P450, family 2, subfamily e, polypeptide 1 (CYP2E1) induction because CYP2E1 not only catalyzes alcohol but also generates reactive oxygen radicals ($O_2^{\bullet -}$), which further activates many xenobiotics to hepatotoxic and carcinogenic metabolites.[7] Faster metabolizing alcohol in hepatocytes results in less damage. Hence, an acceleration of alcohol metabolism could be a good way to alleviate liver lipid accumulation and damage during chronic alcohol consumption.

Niuchangchih (*Antrodia camphorata*) is a kind of fungus in Taiwan that grows only on the inner heartwood wall of the endemic evergreen *Cinnamonum kanehirai*, which makes it difficult to cultivate.[8] The major bioactive compounds of Niuchangchih are currently identified as polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives.[9] The pharmacological properties of Niuchangchih include hepatoprotective properties against acute alcohol-, carbon tetrachloride (CCl_4)-, lipolysaccharide-, and hepatitis B virus-induced injuries, as well as retardation of liver fibrosis,

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and anti-invasion and antimetastasis of liver cancer cells.[9] Due to its scarcity, Niuchangchih mycelia are often used to evaluate its hepatoprotective effects; meanwhile, its hepatoprotective effect is attributed to its antioxidant capacity.[10−12] Recently, our previous paper indicated that the wild fruiting body of Niuchangchih has enhanced antioxidant capacity and can normalize lipid metabolism in livers of continuously alcohol-fed rats.[5] That paper also indicated that chronic alcohol consumption caused focal and acute individual necrosis with mild inflammatory cell infiltration, but cotreatments of 0.25 g silymarin/kg BW or 0.025 g Niuchangchih/kg body weight (BW) can reduce lesions in the liver.[5]

However, on the basis of our knowledge, the study regarding alcohol clearance and antifibrosis of Niuchangchih against chronic alcohol consumption seems insufficient. Moreover, silymarin extracted from the milk thistle (*Silybum marianum*) is composed of the flavonoids silybin, silydianin, and silychristin and is well-known for its antioxidant and chemoprotective effects on the liver.[13] Therefore, we used a rat model to examine and compare the relative hepatoprotective effects of the wild fruiting body of Niuchangchih and silymarin in continuous alcohol consumption via liver lipid accumulation and liver damage biochemical values. Concurrently, the molecular mechanisms of alcohol metabolism and antifibrosis in rats continuously fed alcohol were investigated as well.

MATERIALS AND METHODS

Fungal Material and Reagents. Wild Niuchangchih (*A. camphorata*) was kindly provided by Antroking Co. Ltd.; it was collected from mountain areas in southern Taiwan. Then wild Niuchangchih was ground by using a grinder (Yu-Chi Machinery Co., Ltd., Chang-Hwa County, Taiwan) and passed through a 325 mesh screen to produce fine powders (44 μm). Finely ground Niuchangchih was prepared with sterile distilled water and fed to rats through a gastric tube.

Ethanol, sulfuric acid, and glacial acetic acid were purchased from E. Merck (Darmstadt, Germany). Phenol, glucose, perchloric acid, Na_2CO_3, gallic acid, Folin−Ciocalteu's phenol reagent, $NaNO_2$, $AlCl_3 \cdot H_2O$, NaOH, and (+)-catechin were all of analytic grade from Sigma-Aldrich, Inc. (St. Loius, MO).

Polysaccharides, Triterpenes, Polyphenols, Flavonoids, and Condensed Tannins of Wild Niuchangchih. Total polysaccharide contents of wild Niuchangchih were extracted according to the method with a modification.[14] Briefly, 50 mg of fine Niuchangchih powders was mixed with 1 mL of ddH_2O in a 90 °C water bath for 2 h. Then, a clear supernatant was collected by centrifuging at 12000*g* for 30 min at 4 °C. Polysaccharides were precipitated with 95% ethanol at 4 °C for 12 h. The concentration of total polysaccharide contents in wild Niuchangchih was measured by the absorbance at 490 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd., Leicestershire, U.K.) against a standard curve (glucose as a standard) by the phenol−sulfuric method.

The total triterpene content of wild Niuchangchih was determined according to a colorimetric method.[15] Briefly, 0.5 g of fine Niuchangchih was extracted with 5 mL of 95% ethanol for 30 min under reflux, then filtered, and centrifuged at 3000*g* for 10 min. After the extraction, 5 mL of the extracted solution was taken out and diluted to 50 mL with ethanol. One milliliter of the diluted solution was dried in a boiled water bath, and then 0.2 mL of 5% (w/v) vanillin−glacial acetic acid was added, followed by an addition of 0.08 mL of perchloric acid. The mixture was shaken and kept in a 60 °C water bath for 20 min and then cooled in an ice−water bath to room temperature. The concentration of total triterpene in wild Niuchangchih was determined by absorbance at 550 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd.) after being added to 3.72 mL of glacial acetic acid against a standard curve (oleanolic acid as standard).

The total phenolic contents of wild Niuchangchih were measured according to a method with Folin−Ciocalteu's phenol reagent with a modification.[16] Briefly, a test solution was prepared with 0.5 g of fine Niuchangchih powder and ddH_2O to 10 mL and then ultrasonicated (model DC150H, Taiwan Delta New Instrument Co., Ltd.) for 60 min followed by a centrifugation at 12000*g* for 10 min. One hundred and twenty-five microliters of the test solution was mixed with 0.5 mL of ddH_2O, followed by 125 μL of Folin−Ciocalteu's phenol reagent, 1.25 mL of 7% (w/v) Na_2CO_3 solution, and 4 mL of ddH_2O. After 20 min, the absorbance was read at 750 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd.) against a standard curve (gallic acid as standard). The concentration of total phenolic contents of wild Niuchangchih was expressed as milligrams of gallic acid equivalent (GAE) per 100 g of Niuchangchih powder.

Total flavonoids were determined by a colorimetric assay.[17] Briefly, a test solution was prepared with 0.5 g of fine Niuchangchih and ddH_2O to 10 mL and then ultrasonicated (model DC150H, Taiwan Delta New Instrument Co. Ltd.) for 60 min followed by centrifugation at 12000*g* for 10 min. Test solution (0.1 mL) was mixed with 0.1 mL of ddH_2O and 0.03 mL of 5% (w/v) $NaNO_2$ and kept at a room temperature for 6 min; 0.06 mL of 10% (w/v) $AlCl_3 \cdot H_2O$ was added to the reaction mixture and kept at a room temperature for 5 min. Finally, 0.2 mL of 1 M NaOH and 0.11 mL of ddH_2O were added to the reaction mixture. The absorbance was read at 510 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd.) against a standard curve ((+)-catechin as standard). The concentration of total flavonoids of wild Niuchangchih was expressed as milligrams of catechin equivalent (CE) per 100 mg of Niuchangchih powder.

The total condensed tannins were determined by a vanillin−HCl method.[16] Briefly, a test solution was prepared with 0.5 g of fine Niuchangchih and ddH_2O to 10 mL and then ultrasonicated (model DC150H, Taiwan Delta New Instrument Co. Ltd.) for 60 min followed by centrifugation at 12000*g* for 10 min. Then 0.1 mL of test solution was placed in a tube covered with aluminum foil. One milliliter of 4% vanillin (w/v) in methanol was added, and the tube was shaken vigorously with a mixer. Immediately after that, 0.5 mL of concentrated HCl was pipetted, and the tubes were shaken again. The absorbance was read at 500 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd.) after being allowed to stand for 20 min at room temperature. The results were plotted after a (+)-catechin standard made in the same manner. The interference background of the crude extract was corrected by preparing the test without vanillin. The concentration of condensed tannins of wild Niuchangchih was expressed as milligrams of catechin equivalent (CE) per 100 g of Niuchangchih powder.

Animals and Diets. The animal use protocol was reviewed and approved by the Chung Shan Medical University Animal Care Committee. Twenty-seven male Wistar rats (body weight, 126−150 g) were purchased from BioLASCO Taiwan Co., Ltd. Rats were housed individually in one cage in an animal room at 22 ± 2 °C with a 12/12 h light/dark cycle. Chow diets containing 48.7% (w/w) carbohydrate, 23.9% (w/w) protein, 5.0% (w/w) fat, 5.1% (w/w) fiber, and 7.0% ash (Laboratory Rodent Diet 5001, PMI Nutrition International/Purina Mills LLC, USA) and water were provided for 1 week of acclimation. According to a method for alcoholic fatty liver induction,[5] all rats were given drinking water that contained 20% (w/w) alcohol and then divided into three groups: (1) ALC, 1 mL of sterile distilled water per day; (2) ALC_Sil, 0.25 g silymarin/kg BW (Aldrich Chemical Co., Inc., Milwaukee, WI) in 1 mL of sterile distilled water per day; (3) ALC_Niuchangchih, 0.025 g Niuchangchih/kg BW/day in 1 mL of sterile distilled water per day. The experimental period lasted for 4 weeks. Body weight was measured every week. Feed and drinking solution intake were recorded for obtaining daily feed (g) and water intakes (mL) on a per rat daily basis.

Table 1. Primer Sequences for Semiquantitative RT-PCR

gene	primer sequence	product size (bp)	GenBank accession no.
CYP2E1	F: 5′-CTCCTCGTCATATCCATCTG-3′ R: 5′-GCAGCCAATCACAAATGTGG-3′	474	AF061442.1
NF-κB	F: 5′-CATGAAGCAGCTGACAGAAG-3′ R: 5′-TTCAATAGGTCCTTCCTGCC-3′	496	NM_008689.2
KLF-6	F: 5′-TGTAGCATCTTCCAGGAACTACAGA-3′ R: 5′-TGACACGTAGCAGGGCTCACT-3′	135	AF001417.1
AP1	F: 5′-CCGAGA GCGGTGCCTACGGCTACAG-3′ R: 5′-GACCGGCTGTGCCGCGGAGGTGAC-3′	343	BC078738.1
TGF-β1	F: 5′-CTAATGGTGGACCGCAACAAC-3′ R: 5′- CGGTTCATGTCATGGATGGTG-3′	431	NM_021578.2
α-SMA	F: 5′-GTGCTATGTAGCTCTGGACT-3′ R: 5′-ACATCTGCTGGAAGGTAGAC-3′	419	AK156331.1
TNF-α	F: 5′-ATGAGCACTGAAAGCATGAT-3′ R: 5′-TCACAGGGCAATGATCCCAAAGT-3′	702	NM_000594.2
GAPDH	F: 5′-GACCCCTTCATTGACCTCAAC-3′ R: 5′-GGAGATGATGACCCTTTTGGC-3′	264	DQ403053.1

Collections of Serum, Liver, Heart, Perirenal Fat, and Epididymal Fat, as Well as Determination of Serum Alcohol Value and Liver Damage Indices. At the end of the experiment, the rats were fasted overnight, and drinking solutions were also removed 1 h before sacrifice. Rats were euthanized by CO_2, after which the liver, heart, perirenal fat, and epididymal fat from each rat were removed and weighed. Blood samples were obtained via decapitation. Sera were separated from blood samples by centrifugation at 3000*g* for 10 min and then stored at −80 °C for subsequent analyses. Serum alcohol was detected using a commercial enzymatic kit with automated analyzer (AU2700, Olympus, the First Chemical Ltd., Japan). Serum liver damage indices [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)] were determined using commercial enzymatic kits with the SPOTCHEM EZ SP-4430 biochemistry analyzer (ARKRAY, Inc., Kyoto, Japan).

Determination of Hepatic Triglyceride (TG) and Cholesterol (TC). TG and TC concentrations were measured according to the procedures of Yang et al.[18] Briefly, hepatic lipid was extracted by chloroform and methanol (2:1, v/v). The extract was dried under N_2 and resuspended in isopropanol via an ultrasonic cleaner (model DC150H, Taiwan Delta New Instrument Co. Ltd.). Triglyceride and cholesterol concentrations were measured by using commercial kits (Randox Laboratories Ltd., Antrim, U.K.).

Gene Expressions of Hepatic Alcohol Metabolism and Profibrosis. Total RNA was isolated from the stored frozen liver tissues by using the protocol described by the E.Z.N.A. Tissue RNA kit (Omega Bio-Tek, Inc., Norcross, GA). Reverse transcription was carried out with 2 μg of total RNA, 10 μL of reaction buffer, 1 μL of dNTPs, 2.5 μL of oligo-dT (10 μmol/L), and 0.5 μL of RTase (Improm II, Promega, Madison, WI) with diethyl pyrocarbonate (DEPC) H_2O in a final volume of 50 μL at 42 °C for 1 h. After heat inactivation, 1 μL of cDNA product was used for PCR amplification. The appropriate primers of target genes were designed for rat cytochrome P450 2E1 (CYP2E1), nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), Kruppel-like factor 6 (KLF-6), activating protein-1 (AP1), transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), tumor necrosis factor-α (TNF-α), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as shown in Table 1. The PCR amplification was performed under conditions using a DNA thermal cycler (ASTEC PC-818, ASTEC Co., Ltd., Fukuka, Japan) under the following conditions: CYP2E1, 35 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; NF-κB, KLF-6, AP1, TGF-β1, and TNF-α, 33 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; α-SMA, 33 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; GAPDH, 25 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C. The final products were subjected to electrophoresis on a 2% agarose gel and detected by ethidium bromide staining via a UV light. The relative expression levels of the mRNAs of the target genes were normalized using the GAPDH internal standard.

Preparation of Liver Homogenate and Determination of Liver Homogenate and Serum Protein Levels. First, 0.5 g of liver was homogenized on ice in 4.5 mL of phosphate buffer saline (PBS, pH 7.0, containing 0.25 M sucrose), and filtrate was collected for further analyses. The protein contents in the filtrate and serum sample were measured according to the procedures of a Bio-Rad protein assay kit (catalog no. 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA) and using bovine serine albumin as a standard.

Hepatic Alcohol Dehydrogenase (ADH), Aldehyde Dehydrogenase (ALDH), and Catalase (CAT) Activities. Hepatic ADH and ALDH activities were measured according to the procedure as described by Ward et al.[19] with slight modifications. For the ADH activity measurement, two reaction tubes were prepared. One reaction tube contained 50 μL of liver homogenate, 50 μL of PBS (pH 7.0), and 1 mL of 0.1 M glycine−NaOH buffer, pH 10.8 (containing 10 mM NAD^+). Another reaction tube contained 50 μL of liver homogenate, 50 μL of PBS (pH 7.0), and 1 mL of 0.1 M glycine−NaOH buffer, pH 10.8 (containing 10 mM NAD^+ and 0.016 M ethanol). After a 4 min reaction,

Table 2. Total Polysaccharides, Triterpenes, Polyphenols, Flavonoids, and Condensed Tannins of Niuchangchih

content[a] (mg/100 g Niuchangchih)				
total polysaccharides	total triterpenes	total phenols (GAE)[b]	flavonoids (CE)[c]	condensed tannins (CE)[c]
243.45 ± 0.24	12948.70 ± 299.10	300.02 ± 16.70	50.23 ± 1.92	15.30 ± 0.17

[a] Data are given as mean ± SEM (n = 3). [b] GAE, gallic acid equivalent. [c] CE, catechin equivalent.

the difference of absorbance between the two reaction tubes was measured at 340 nm. Hepatic ADH activity was calculated by taking the extinction coefficient of NADH to be $6.22 \times 10^3\ \mu M^{-1}\ cm^{-1}$. One unit of ADH was expressed as the amount of enzyme that produced 1 mol of NADH per minute at 25 °C.

For measurement of ALDH activity, two reaction tubes were prepared. One reaction tube contained 100 μL of liver homogenate and 1 mL of 50 mM sodium pyrophosphate buffer, pH 8.8 (containing 1 mM NAD^+, 0.2 mM 4-methylparazole, 1 mM $MgCl_2$, 2 μM rotenone, 1% Triton X-100). Another reaction tube contained 100 μL of liver homogenate and 1 mL of 50 mM sodium pyrophosphate buffer, pH 8.8 (containing 1 mM NAD^+, 0.2 mM 4-methylparazole, 1 mM $MgCl_2$, 2 μM rotenone, 1% Triton X-100, 5 mM acetaldehyde). After a 30 min reaction, the difference of absorbance between the two reaction tubes was measured at 340 nm. Hepatic ALDH activity was calculated by taking the extinction coefficient of NADH to be $6.22 \times 10^3\ \mu M^{-1}\ cm^{-1}$. One unit of ALDH was expressed as the amount of enzyme that produced 1 mol of NADH per minute at 25 °C.

Hepatic catalase (CAT) activity was measured according to the procedure described by Chang et al.[20] Briefly, 450 μL of liver homogenate was mixed well with 50 μL of triton X-100 (10% v/v). After centrifugation at 6000g for 10 min (4 °C), a mixture of 10 μL of supernatant and 9990 μL of PBS, pH 7.0, was reacted with 0.5 mL of H_2O_2 (30 mM). The optical density decrease caused by the disappearance of H_2O_2 was measured at the end of 1 min against the blank at 240 nm. The difference of absorbance between 0 and 3 min was measured at 340 nm. Hepatic CAT activity was calculated by taking the extinction coefficient of H_2O_2 to be $39.5\ M^{-1}\ cm^{-1}$. One unit of CAT was expressed as the amount of enzyme that decomposed 1 mol of H_2O_2 per minute at 25 °C. Hepatic CAT activity was expressed by microunits per milligram of protein.

Serum and Hepatic of Matrix Metalloproteinase-2 (MMP-2) and Matrix Metalloproteinase (MMP-9) Activities. The activities of MMP-2 and MMP-9 in serum samples and liver tissues were measured by gelatin zymography protease assays as described by Yang et al.[21] Briefly, serum samples and liver homogenate were prepared with a SDS sample buffer without boiling or reduction and subjected to 0.1% gelatin−8% SDS-PAGE. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$; and 0.01% NaN_3) at 37 °C for 12 h. Then the gel was stained with Coomassie brilliant blue R-250. The gelatinolytic activities were detected as clear bands against the blue background.

Statistical Analysis. The experiment was conducted using a completely random design (CRD). Data were analyzed using analysis of variance (ANOVA). When a significant difference in the interaction effect was obtained, the least significant difference (LSD) test at 0.05 probability level was used to test differences between combination treatments. All statistical analyses of data were performed using SAS (SAS Institute, Inc., 2002).

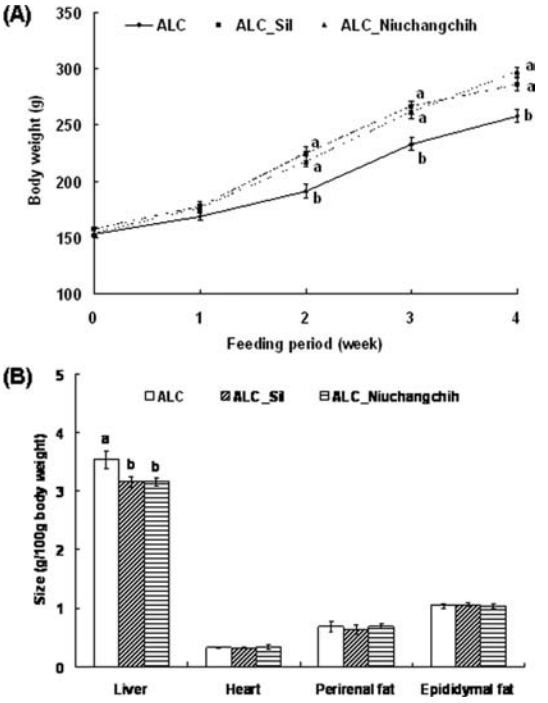

Figure 1. Body weight change (A) as well as sizes of liver, heart, perirenal fat, and epididymal fat (B) of alcohol-fed rats as affected by silymarin or Niuchangchih supplementation. The data are given as the mean ± SEM (n = 9). Different letters on data points and bars in each feeding period and each tested parameter, respectively, indicate significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Compositions of Wild Niuchangchih. The major bioactive compounds of wild Niuchangchih are identified as polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives.[9] Hence, the bioactive compound analysis is demonstrated in Table 2. The major components in wild Niuchangchih are triterpenes, followed by phenols, polysaccharides, flavonoids, and condensed tannins, which are 12948.70 mg/100 g Niuchangchi, 300.02 mg GAE/100 g Niuchangchih, 243.45 mg/100 g Niuchangchih, 50 mg CE/100 g Niuchangchih, and 15.30 mg CE/100 g Niuchangchih, respectively. The hepatoprotective effect of Niuchangchih mycelia was most often attributed to their antioxidant capacity.[10−12] Furthermore, the antioxidant activities of the filtrate and mycelia extracts of Niuchangchih result from their total polyphenols, triterpenoids, and polysaccharide.[10] The hepatoprotective effect of wild Niuchangchih against alcohol was also attributed to not only enhancement of antioxidant capacity but also normalization of the lipid metabolism in rats continuously fed alcohol.[5] On the basis of our study of the literature, information on the mechanism related to alcohol metabolism and antifibrosis through the supplementation with Niuchangchih is rather limited. Hence, in vivo effects of Niuchangchih on alcohol metabolism and antifibrosis in the liver of rats continuously fed alcohol were investigated in this study.

Effects of Wild Niuchangchih on Body Weight and Relative Liver, Heart, Perirenal Fat, and Epididymal Fat Sizes in Rats Continuously Fed Alcohol. During a continuous alcohol consumption over 4 weeks, higher ($p < 0.05$) body weights of alcohol-fed rats supplemented with either silymarin or Niuchangchih were observed after 2 weeks when compared to those fed only

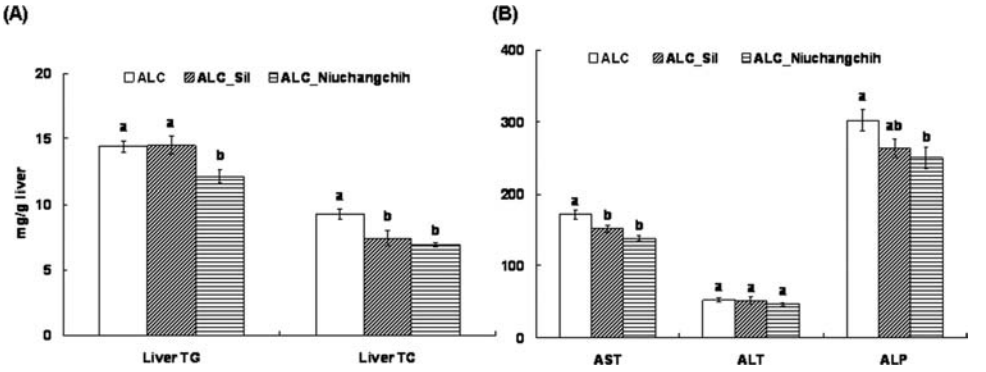

Figure 2. Liver triglyceride and cholesterol levels (A) as well as serum liver damage indices (AST, ALT, and ALP) (B) of alcohol-fed rats as affected by silymarin or Niuchangchih supplementation. The data are given as the mean ± SEM (n = 9). Different letters on data bars in each tested parameter indicate significant differences (p < 0.05).

Table 3. Serum Alcohol Level, as Well as Hepatic Alcohol Dehydrogenase (ADH), Catalase (CAT), and Aldehyde Dehydrogenase (ALDH) Activities of Alcohol-Fed Rats As Affected by Silymarin or Niuchangchih Supplementation

	ALC[a]	ALC_Sil[a]	ALC_Niuchangchih[a]
serum alcohol (mg/dL)	9.00 ± 0.30a	8.30 ± 0.33a	6.70 ± 0.33b
CAT (μunit/mg protein)	169.67 ± 8.70b	263.79 ± 10.59a	273.39 ± 5.96a
ADH (μunit/mg protein)	11.85 ± 0.85ab	9.13 ± 0.75b	13.57 ± 1.50a
ALDH (μunit/mg protein)	22.23 ± 0.47b	22.54 ± 0.79b	26.35 ± 1.09a

[a] Data are given as the mean ± SEM (n = 9). Mean values with different letters in each testing parameter were significantly different (p < 0.05).

alcohol (Figure 1A). Silymarin or Niuchangchih decreased (p < 0.05) the relative liver size of alcohol-fed rats at the end of the experiment (Figure 1B), whereas no differences (p > 0.05) on relative sizes of heart, perirenal fat, and epididymal fat were observed (Figure 1B).

Alcohol-induced steatohepatitis due to up-regulation of lipogenesis and down-regulations of fatty acid transport ability and β-oxidation in the liver by continuous alcohol consumption was shown.[3] Silymarin, as a hepatoprotective agent, can decrease the relative liver size of rodents induced by continuous alcohol consumption.[5] On the basis of our current data, wild Niuchangchih also showed effects similar to those of silymarin on lowering relative liver size in rats continuously fed alcohol.

Effect of Wild Niuchangchih on Liver Lipids and Damage Indices in Rats Continuously Fed Alcohol. In measurements of liver lipids, wild Niuchangchih decreased (p < 0.05) hepatic TG and TC accumulations in alcohol-fed rats but silymarin decreased (p < 0.05) only hepatic TC accumulation (Figure 2A). Furthermore, wild Niuchangchih lowered (p < 0.05) AST and ALP values but silymarin lowered (p < 0.05) only AST values, although ALT values were not (p > 0.05) influenced by supplementing alcohol-fed rats with either silymarin or wild Niuchangchih (Figure 2B).

Alcohol consumption up-regulates lipogenesis in the liver,[5,8] which can cause increased TG and TC accumulations in livers. Reductions of plasma and liver TC of Wistar rats fed a high-fat diet are mainly due to decreased cholesterol absorption by supplementing silymarin.[22] Niuchangchih also showed down-regulations of gene expressions in cholesterol (HMG-CoA reductase) and fatty acid (SREBP-1c, ACC, and FAS) biosyntheses.[5] Therefore, these reductions in liver lipids of alcohol-fed rats with either silymarin or wild Niuchangchih could be closely related to the lower relative liver size observed as well (Figures 1B and 2A). In the liver

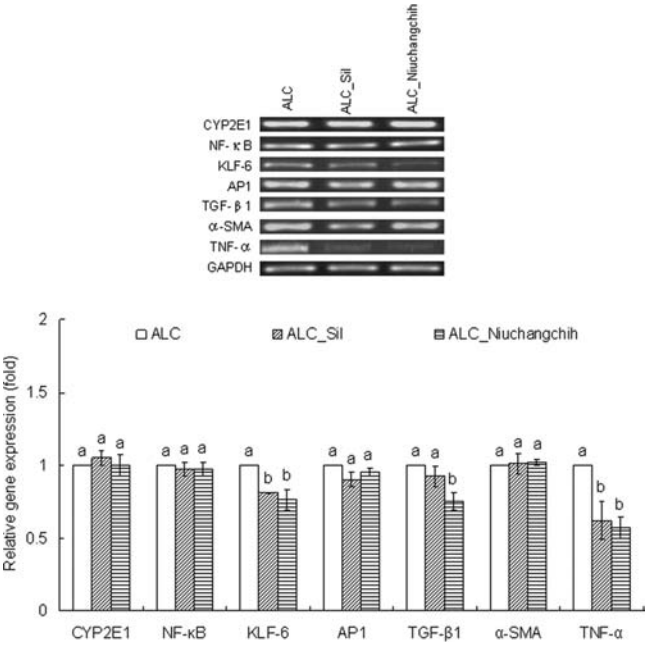

Figure 3. Gene expressions of CYP2E1, NF-κB, KLF-6, AP1, TGF-β1, α-SMA, and TNF-α in alcohol-fed rats as affected by silymarin or Niuchangchih supplementation. Data are expressed as the mean ± SEM (n = 9). Different letters on data bars in each target gene indicate significantly different values (p < 0.05). Gene expression was normalized to GAPDH and expressed relative to ALC group.

damage indices, lower liver lipid accumulation is always coupled with lower liver damage indices.[5,18,20,21] Our data (Figure 2) also concur with the previous studies. Hence, wild

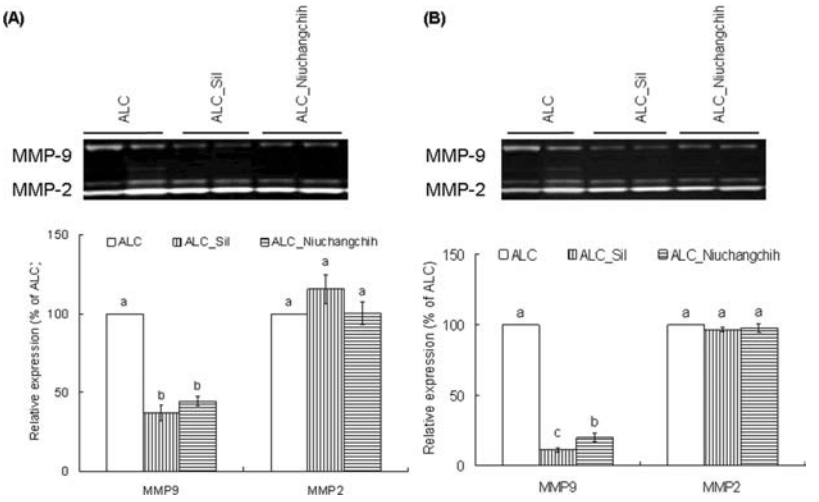

Figure 4. Serum (A) and hepatic (B) MMP- 9 and MMP-2 activities of alcohol-fed rats as affected by silymarin or Niuchangchih supplementation. The data are given as the mean ± SEM ($n = 9$). Different letters on data bars in each tested parameter indicate significant differences ($p < 0.05$). MMP-9 and MMP-2 activities were determined by a densitometric analysis with those of ALC group as 100%, respectively.

Niuchangchih shows a hepatoprotective effect similar to or even better than that of silymarin.

Effects of Wild Niuchangchih on Alcohol Clearance and Antifibrosis of Livers in Rats Continuously Fed Alcohol. After 4 weeks of continuous alcohol consumption, wild Niuchangchih decreased ($p < 0.05$) serum alcohol concentration compared to rats fed only alcohol, but silymarin did not ($p > 0.05$) (Table 3). With regard to the effects of alcohol clearance, wild Niuchangchih apparently increased ($p < 0.05$) hepatic CAT and ALDH activity (Table 3), but did not ($p > 0.05$) influence hepatic CYP2E1 and ADH gene expression (Figure 3). Although hepatic gene expressions of NF-κB, AP1, and α-SMA of alcohol-fed rats were not ($p > 0.05$) affected by supplementing either silymarin or Niuchangchih in the regulation of liver fibrosis, hepatic gene expressions of KLF-6 and TNF-α (Figure 3), as well as serum and hepatic MMP-9 activities (Figure 4), were down-regulated ($p < 0.05$) in the alcohol-fed rats supplemented with either silymarin or Niuchangchih. However, a down-regulation ($p < 0.05$) of TGF-β1 gene expression was observed only in alcohol-fed rats supplemented with wild Niuchangchih (Figure 3).

The liver is the primary organ in charge of alcohol metabolism. Alcohol dehydrogenase (ADH) in cytosol, cytochrome P450 in endoplasmic reticulum, and catalase (CAT) in peroxisomes are the three main enzyme systems that metabolize alcohol into acetaldehyde, which is further metabolized into acetic acid by an aldehyde dehydrogenase (ALDH).[6] CYP2E1 induction during alcohol metabolism is most responsible for the alcohol-related liver damage because CYP2E1 not only catalyzes alcohol but also generates reactive oxygen radicals ($O_2^{\bullet -}$).[7] Hence, faster alcohol metabolism in hepatocytes results in less damage. According to the data from the present study, accelerated serum alcohol clearance by Niuchangchih could be related to increased CAT and ALDH activities (Table 3); meanwhile, our previous study[5] indicated that wild Niuchangchih also elevated other antioxidant enzyme activities, that is, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), which also could explain the less extensive oxidative damage due to CYP2E1 induction in alcohol-fed rats.

MMPs are zinc- and calcium-dependent proteases that participate in the degradation of the extracellular matrix (ECM) molecules. Major components of ECM in liver fibrosis include collagen types I, III, and IV. MMP-1, -2, -8, -9, and -13 cause fibrinolysis and tissue remodeling, which result in the progression of liver fibrosis.[23,24] Previous studies reported that hepatic MMP-2 and -9 activities are regarded as one of the major causes of liver damage.[21,25] Moreover, Fernandes et al. reported that MMPs are induced by TNF-α as a consequence of response to inflammation.[26] Our data are also in accordance with the previous studies. Rats fed only alcohol had higher serum and hepatic MMP-9 activity and TNF-α gene expression, as well as AST and ALP values, but oral supplementation of silymarin or Niuchangchih indeed lowered those results (Figures 2B, 3, and 4). Liver fibrosis is regarded as an activation of hepatic stellate cells (HSCs). Several transcriptional factors, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), and Kruppel-like factor 6 (KLF-6), are associated with HSC activation. HSC activation generates the α-smooth muscle actin (α-SMA)-positive myofibroblast-like cells that are responsible for scar tissue formation in the fibrotic liver.[27] Additionally, KLF-6 also activates transforming growth factor-β1 (TGF-β1) expression in activated HSCs.[28] On the basis of our data, the hepatoprotection of Niuchangchih in rats continuously fed alcohol could partially account for down-regulations of KLF-6 and TGF-β1 expressions (Figures 2 and 3).

In summary, the major components in wild Niuchangchih are triterpenes, polysaccharides, and phenols. An acceleration of alcohol clearance in rats continuously fed alcohol and cotreated with Niuchangchih is primarily the result of increasing CAT and ALDH enzyme systems but not ADH and CYP2E1. With regard to the liver fibrogenic mechanism, Niuchangchih decreases both serum and hepatic MMP-9 activity and down-regulates TNF-α, KLF-6, and TGF-β1 gene expressions. Therefore, the protective mechanism of Niuchangchih could additionally account for acceleration of alcohol clearance and suppression of the alcohol-mediated inductions of MMP-9, TNF-α, KLF-6, and TGF-β1.

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NOTE ADDED AFTER ASAP PUBLICATION

There was an error involving the forward primer sequence of TNF-α in Table 1 of the version of this paper published March 14, 2011. The correct version published March 16, 2011.