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Fruiting Body of Niuchangchih (*Antrodia camphorata*) Protects Livers against Chronic Alcohol Consumption Damage

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An alcoholic fatty liver disease was induced by drinking water containing 20% (w/w) alcohol. Therapeutic groups were orally administrated dosages of 0.25 g silymarin/kg body weight (BW) and a low dosage of Niuchangchih (*Antrodia camphorata*) (0.025 g/kg BW) and a high dosage of Niuchangchih (0.1 g/kg BW) per day. Niuchangchih, especially at the high dosage, not only showed a hypercholesterolemic effect ($p < 0.05$) but also reduced ($p < 0.05$) hepatic lipids in alcohol-fed rats. Those beneficial effects could be partially attributed to higher ($p < 0.05$) fecal cholesterol and bile acid outputs, as well as downregulations ($p < 0.05$) of 3-hydroxy-3-methylglutaryl-CoA reductase, sterol regulatory element-binding protein-1c, acetyl-CoA carboxylase, fatty acid synthase, and malic enzyme gene expressions; meanwhile, there was an upregulation of low-density lipoprotein receptor and peroxisome proliferator-activated α gene expression. Besides, Niuchangchih also enhanced ($p < 0.05$) the liver glutathione, Trolox equivalent antioxidant capacity, and activities of superoxide dismutase, catalase, and glutathione peroxidase and decreased the liver malondialdehyde content, which also partially contributed to the lowered ($p < 0.05$) serum aspartate aminotransferase levels and no observed lesion in the histological examination of alcohol-fed rats.

KEYWORDS: *Antrodia camphorata*; alcoholic fatty liver disease; antioxidant capacity; lipid homeostasis; liver damage

INTRODUCTION

Fatty liver is a general term for a liver disease that is a reversible condition, where a large amount of triacylglycerol (TAG) accumulates in hepatocytes via the process of steatosis. Hence, a fatty liver is also named fatty liver disease, steatorrhoeic hepatosis, or steatohepatitis. The pathogenesis of fatty livers is mainly divided into two categories: nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD). NAFLD is mostly associated with obesity, insulin resistance, and metabolic syndrome, while AFLD is due to an excessive consumption of alcohol. An excessive or chronic consumption of alcohol alters the $NADH/NAD^+$ redox potential in the liver, which in turn inhibits mitochondria β-oxidation of fatty acids, thereby reducing the rate of lipid expenditure as well as stimulating lipogenesis, thus increasing lipid accumulation in hepatocytes (*1*). The clinical implication of AFLD is the potential of progression to hepatitis and fibrosis, which may lead to liver cirrhosis (*2*). Hence, a normalization of hepatic lipid homeostasis and antioxidant status in chronic alcohol consumption is a good way to decrease liver damage.

Niuchangchih (*Antrodia camphorata*) is a fungus that only grows on the inner heartwood wall of the endemic evergreen *Cinnamonum kanehirai*, and it can hardly be cultivated (*3*). Currently, the major bioactive compounds of Niuchangchih are identified as polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives (*4*). Hence, the pharmacological properties of Niuchangchih, such as hepatoprotective properties against acute alcohol-, carbon tetrachloride (CCl_4)-, lipolysaccharide-, and hepatitis B virus-induced injuries as well as retardation of liver fibrosis and anti-invasion and antimetastasis of liver cancer cells, have been mentioned and summarized (*4*). On the basis of previous literature, the hepatoprotective effects of Niuchangchih against alcohol and CCl_4-induced injuries were mostly attributed to an enhancement of the antioxidant status in livers (*5*, *6*) and seldom to a normalization of lipid homeostasis. Lipid homeostasis in a biological system is mainly regulated by serum cholesterol clearance (i.e., low-density

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lipoprotein receptor, LDLR), cholesterol biosynthesis (i.e., 3-hydroxy-3-methylglutaryl-CoA reductase, HMG-CoAR) and catabolism (i.e., cholesterol 7-α hydroxylase, CYP7A1), fatty acid biosynthesis (sterol regulatory element-binding protein-1c, SREBP-1c; acetyl-CoA carboxylase, ACC; fatty acid synthase, FAS; and malic enzyme, ME), and energy expenditure (peroxisome proliferator-activated receptor α, PPAR-α) (*7*−*9*). Hence, the regulation of Niuchangchih on lipid homeostasis in chronic alcohol consumption warrants further study.

Because of rareness, the materials used to research the health functionalities of Niuchangchih are its mycelia. Those studies showed that cultivated Niuchangchih mycelia have a good antioxidant capacity (*6*, *10*, *11*). Glutathione (GSH) is the major endogenous antioxidant in hepatocytes and is derived from three amino acids (cysteine, glutamate, and glycine), while superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) could counteract with reactive oxygen species and hydrogen peroxide (H_2O_2) in a biological system (*12*). Therefore, we used a rat model to examine and compare the relative protective effects of wild fruiting bodies of Niuchangchih with silymarin in chronic alcohol consumption. Concurrently, the regulation of wild fruiting bodies of Niuchangchih on lipid homeostasis in livers and its antioxidant capacity were also investigated.

MATERIALS AND METHODS

Fungal Material. Wild fruiting bodies of Niuchangchih (*Antrodia camphorata*) were provided by Antroking Co. Ltd. and were collected from mountain areas in 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 form fine powders (44 μm). Then, it was prepared with sterile distilled water to different dosages of suspension to rats by gastric tubes.

High-Pressure Liquid Chromatography (HPLC) Analysis of Niuchangchih. Wild Niuchangchih powders were extracted with methanol preparation and were performed using a Waters HPLC system equipped with a Waters 996 photodiode array detector (Waters Co., Milford, MA). The sample solution was prepared by dissolving 5 mg of Niuchangchih powder in 1 mL of 100% methanol and then filtering it through a 0.22 μm membrane. The 10 μL sample solution was applied to the analytic column. Separation was performed in an Intertsil ODS-3 C-18 column (4.6 mm × 250 mm, 5 μm), at a flow rate of 1 mL/min, and the absorbance was detected at 275 nm. The mobile phase consisted of A (0.2% formic acid) and B (100% acetonitrile): 0−65 min, 70−53% A and 30−47% B; 65−110 min, 53−53% A and 47−47% B; 110−140 min, 53−0% A and 47−100% B; 140−170 min, 0−0% A and 100−100% B; 170−175 min, 0−70% A and 100−30% B; and 175−200 min, 70−70% A and 30−30% B. Ascorbic acid, 2,3-dimethoxy-5-methyl[1,4]benzoquinone, and 2,4,5-trimethoxybenzaldehyde were purchased from Sigma Co. Ltd. 4,7-Dimethoxy-5-methyl-1,3-benzodioxole was kindly provided by Dr. Horng-Lian Lay (Department of Plant Industry, National Pingtung University of Science and Technology). Zhankuic acid C was kindly provided by Dr. Ya-Ching Shen (School of Pharmacy College of Medicine, National Taiwan University, Taiwan). Dehydrosulfurenic acid, ergostatrien-3β-ol, and dehydroeburicoic acid were kindly provided by Dr. Yueh-Hsiung Kuo (Graduate Institute of Chinese Pharmaceutical Science, China Medical University, Taiwan). Zhankuic acid A was kindly provided by Dr. Tian-Shung Wu (College of Pharmacy, China Medical University, Taiwan). Those compounds were also run on the HPLC as standards to indentify chemical compounds in our wild Niuchangchih.

Animal and Diets. The animal use and protocol was reviewed and approved by Chung Shan Medical University Animal Care Committee. Forty male Wistar rats, 5 weeks of age [body weight (BW), 126−150 g], were purchased from BioLASCO Taiwan Co., Ltd. Two rats were housed 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, United States) and water were provided for 1 week of acclimation. According to a method for alcoholic fatty liver induction (*13*, *14*), all rats were given drinking water that contained 20% (w/w) alcohol, except the control rats drinking the isocalorical glucose solution (30%, w/w), and then, they were divided into four groups: (1) control, 1 mL of sterile distilled water per day; (2) ALC, 1 mL of sterile distilled water per day; (3) ALC_Sil, 0.25 g silymarin/kg BW (Aldrich Chemical Co., Inc., Milwaukee, WI) in 1 mL of sterile distilled water per day; (4) ALC_NiuL, a low dosage of Niuchangchih (0.025 g /kg BW/day) in 1 mL of sterile distilled water per day; and (5) ALC_NiuH, a high dosage of Niuchangchih (0.1 g/kg BW) in 1 mL of sterile distilled water per day. The experimental period lasted for 4 weeks. Feed and drinking solution intakes were recorded for obtaining daily feed (g), and water intakes (mL) were recorded on a per rat daily basis.

Collection of Serum, Liver, Visceral Fat, and Feces. At the end of the experiment, all rats were fasted overnight. All rats were euthanized by CO_2. The liver and visceral adipose tissue in the abdominal cavity from each rat were removed and weighed. Livers were stored at −80 °C for further analyses. Blood samples were also collected via decapitation. Sera were separated from blood samples by centrifugation 3000*g* for 10 min and then stored at −80 °C for further analyses. Feces were collected from each cage 24 h before the end of the experiment and stored at −80 °C for further analyses.

Determination of Serum Cholesterol and TAG and Liver Damage Indices. The serum total cholesterol (TC) and TAG were measured by using commercial kits (Randox Laboratories Ltd., Antrim, United Kingdom). Those methods were based on detection of colored end products at 500 nm. The serum liver damage indices [aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values] were determined by using commercial enzymatic kits with an SPOTCHEMTM EZ SP4430 biochemistry analyzer (ARKRAY, Inc., Kyoto, Japan).

Determination of Hepatic/Fecal Cholesterol, TAG, and Bile Acids. Hepatic and fecal lipid extractions were measured according to the procedures of Yang et al. (*15*). Briefly, hepatic and fecal lipids were 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., TW) for an efficient dissolution. Cholesterol and TAG concentrations were measured using commercial kits (Randox Laboratories Ltd.). The fecal bile acid was also determined by using an enzymatic method (Randox Laboratories Ltd.).

Hepatic mRNA Expressions of Enzymes and Proteins Related to Lipid Homeostasis. Total RNA was isolated from the stored frozen liver tissues by using the protocol described by Rneasy Mini Kits (Qiagen, Valencia, CA). Reverse transcription was carried out with 2 μg of total RNA, 8 μL of reaction buffer, 2 μL of dNTPs, 4.8 μL of $MgCl_2$, 4 μL of Oligo-dT (10 pmol/L), and 200U RTase (Promega, Madison, WI) with diethyl pyrocarbonate (DEPC) H_2O in a final volume of 40 μL at 42 °C for 1 h. After a heat inactivation, 1 μL of cDNA product was used for a PCR amplification. The appropriate primers of target genes were designed for rat's LDLR (GenBank no.: NM_175762.2), HMG-CoAR (GenBank no.: NM_013134.2), SREBP-1c (GenBank no.: AF286470.2), ACC (GenBank no.: EF121987.1), FAS (GenBank no.: X62889.1), ME (GenBank no.: NM_012600), PPAR-α (GenBank no.: NM_013196), and GAPDH (GenBank no.: DQ403053.1) as follows: LDLR sense 5′-GGAGGACTCTGTTCCGAGGAA-3′, antisense 5′-GAGCTAGCTGCTTCTCATCCTC-3′; HMG-CoAR sense 5′-GACACTTACTATCTGTATGATG-3′, antisense 5′-CTTGGAGAGGTAAAACTGCCA-3′; CYP7A1 sense 5′-CTGGCTGAGGGATTGAA-3′, antisense 5′-ATAGCGAGGTGCGTCTT-3′; SREBP-1c sense 5′-GGTGGGCACTGAGGCAAAGC-3′, antisense 5′-CGCACACAGGGCTAGGCGGG-3′; ACC sense 5′-CCTCGGCACATGGAGATGTA-3′, antisense 5′-CCGCTCCTTCAACTTGCTCT-3′; FAS sense 5′-TTGCCCGAGTCAGAGAAC-3′, antisense 5′-CGTCCACAATAGCTTCATAGC-3′; ME sense 5′-CTATTGTGGTGACTGATGGAG-3′, antisense 5′-TCTGACACTTGCTGGGATATG-3′; PPAR-α sense 5′-GGACAAGGCCTCAGGGTACC-3′, antisense 5′-CCACCATCTTGGCCACAAGC-3′; and GAPDH sense 5′-GACCCCTTCATTGACCTCAAC-3′, antisense 5′-GGAGATGATGACCCTTTTGGC-3′. The size of reaction products is as follows: for LDLR, 301 bp; HMG-CoAR, 868 bp; CYP7A1, 134 bp; SREBP-1c, 412 bp; ACC, 210 bp; FAS, 779 bp; ME, 1033 bp; PPAR-α, 461 bp; and GAPDH, 264 bp. GAPDH was used as an internal control in all reactions. The PCR amplification was performed under conditions using a DNA thermal

cycler (ASTEC PC-818, ASTEC Co., Ltd., Fukuka, Japan) under the following conditions: LDLR, ME, and CYP7A1: 30 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; HMG-CoAR: 35 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; SREBP-1c and FAS: 35 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; ACC: 35 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; PPAR-α: 33 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; UCP2: 40 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; and 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 by using the GAPDH as an internal control.

Preparation of Liver Homogenate. A 0.5 g amount of liver was homogenized on ice in 4.5 mL of phosphate-buffered saline (PBS, pH 7.0, containing 0.25 M sucrose) and centrifuged at 12000*g* for 30 min. The supernatant was collected for further analyses. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (catalog # 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA) and using bovine serum albumin as a standard.

Determination of Hepatic Malondialdehyde (MDA) and GSH Contents as Well as Trolox Equivalent Antioxidant Capacity (TEAC). The hepatic MDA and GSH contents were according to procedures as described by Yang et al. (*15*). A 0.5 mL amount of liver homogenate was mixed with 0.75 mL of 2-thiobarbituric acid (TBA) solution in a Teflon tube, and then, 4.25 mL of trichloroacetic acid-HCl (TCA-HCl) reagent was added. The tube was flushed with nitrogen and closed. A blank was prepared in the same manner, but PBS (pH 7.0) replaced the liver homogenate. The tubes were boiled for 30 min and then cooled. The colored solution was centrifuged at 4000*g* for 15 min. A clear and colored supernatant was transferred to a cuvette, and the absorbance was measured at 535 nm using an Implen NanoPhotometer (model 1443, Implen GmbH, Munich, Germany). The hepatic MDA content was calculated by taking the extinction coefficient of MDA to be 1.56×10^5 M^{-1} cm^{-1} at 535 nm. Because of the unique thiol compound in GSH, 2,2-dithiobisnitrobenzoic acid (DTNB) is commonly used for the thiol assay. In the analysis of GSH contents, equal volumes of liver homogenate and 10% TCA solution were mixed well and placed in an ice box for 5 min and then centrifuged. The clear layer, Tris base (0.25 M)-ETDA (20 mM) buffer (pH 8.2), and DTNB (10 mM) solution were mixed well. After 5 min, the absorbance was measured at 412 nm using an Implen NanoPhotometer (model 1443, Implen GmbH). The hepatic GSH content was calculated by taking the extinction coefficient of TNB to be 1.36×10^4 M^{-1} cm^{-1}. Hepatic TEAC was analyzed according to a method described previously (*16*). A free radical, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$), can be generated by mixing ABTS (100 μM) with H_2O_2 (50 μM) and peroxidase (4.4 U/mL). The TEAC value was expressed as a scavenging capacity against $ABTS^+$. Briefly, 0.25 mL of a mixture of ABTS, H_2O_2, and peroxidase and 1.5 mL of dd H_2O were mixed well and placed under a dark room. After 30 min, 0.25 mL of diluted liver homogenate (1%, v/v) was then added. The absorbance was measured at 734 nm after the interaction of sample solution for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. A standard curve was plotted for Trolox on scavenging $ABTS^+$ capacity and was calculated as the TEAC. The higher TEAC value of a sample resulted in the stronger antioxidant activity.

Determination of Hepatic SOD, CAT, and GSH-Px Activities. Hepatic SOD was measured by the inhibitory effect of SOD on pyrogallol autoxidation according to the procedure as described by Mueller and colleagues (*17*) with a slight modification. Briefly, 100 μL of liver homogenate was mixed well with 650 μL of PBS (pH 7.0). After a centrifugation at 6000*g* for 10 min (4 °C), 10 μL of supernatant was mixed with 3 mL of Tris-HCl buffer (50 mM, pH 8.2) and 15 μL of pyrogallol (0.2 mM). The absorbance change caused by the formation of the yellow pyrogallol oxidation product, purpurogallin, was recorded at 420 nm in 3 min. One unit of SOD activity was defined as the amount of enzyme that inhibited the autoxidation of pyrogallol by 50%. The hepatic SOD activity was expressed by unit/mg protein.

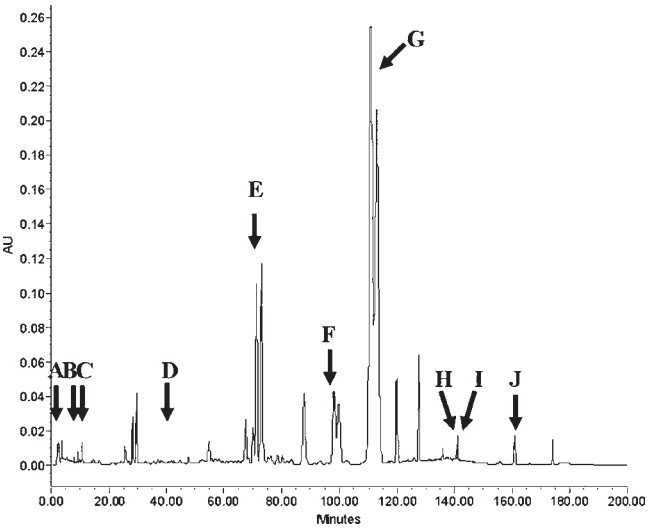

Figure 1. HPLC chromatogram of Niuchangchih (*A. camphorata*). Identification of peaks: A, ascorbic acid; B, 2,3-dimethoxy-5-methyl[1,4]benzoquinone; C, 2,4,5-trimethoxybenzaldehyde; D, 4,7-dimethoxy-5-methyl-1,3-benzodioxole; E, *R*,*S*-zhankuic acid C; F, dehydrosulphurenic acid; G, *R*,*S*-zhankuic acid A; H, zhankuic acid B; I, dehydroeburicoic acid; and J, ergostatrien-3β-ol.

The hepatic CAT activity was performed according to the procedure as described by Hong and Lee (*18*) with a slight modification. Briefly, 450 μL of liver homogenate was mixed well with 50 μL of triton X-100 (10% v/v). After a centrifugation at 6000*g* 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. The 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 decomposes 1 mol H_2O_2 per min at 25 °C. The hepatic CAT activity was expressed by unit/mg protein.

The hepatic GSH-Px activity was measured by using a commercial kit (Randox Laboratories Ltd.). The hepatic GSH-Px activity was expressed by unit/mg protein.

Histopathological Analysis. For histopathological study, the liver tissues were placed in formalin for no more than 24 h and were fixed in neutral-buffered formalin solution, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. These blockers were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section, and stained with hematoxylin and eosin (H&E).

Statistical Analysis. The experiment was conducted using a completely random design. Data were analyzed using analysis of variance. A significant difference was used at the 0.05 probability level, and differences between treatments were tested using the least significant difference test. All statistical analyses of data were performed using SAS (SAS Institute, Inc., 2002).

RESULTS

Chemical and Bioactive Compounds of Wild Niuchangchih. The analysis of chemical and bioactive compounds in our wild Niuchangchih was performed by using an HPLC and was demonstrated in **Figure 1**. The HPLC chromatogram revealed retention times of five major compounds of our wild Niuchangchih: A, ascorbic acid, 2.42 min; B, 2,3-dimethoxy-5-methyl[1,4]-benzoquinone, 11.69 min; C, 2,4,5-trimethoxybenzaldehyde, 13.09 min; D, 4,7-dimethoxy-5-methyl-1,3-benzodioxole, 41.86 min; E, *R*,*S*-zhankuic acid C, 71.38 and 72.99 min; F, dehydrosulphurenic acid, 101.92 min; G, *R*,*S*-zhankuic acid A, 110.83 and 112.94 min; H, zhankuic acid B, 141.4 min; I, dehydroeburicoic acid, 142.43 min; and J, ergostatrien-3β-ol, 164.3 min locations.

Table 1. BW Gain, Feed and Water Intake, and Relative Sizes of Liver, Heart, and Visceral Fat in the Experimental Rats

				g/100 g BW		
	BW gain[a](g)	feed intake[a](g)	water intake[a](mL)	liver[a]	heart[a]	visceral fat[a]
control[b]	135.74 ± 3.80 a	17.36 ± 0.37 a	21.88 ± 0.38 a	2.91 ± 0.06 b	0.33 ± 0.01 a	1.30 ± 0.77 bc
ALC[b]	107.49 ± 9.19 b	11.37 ± 0.45 c	19.17 ± 1.08 b	3.27 ± 0.04 a	0.33 ± 0.01 a	1.79 ± 0.13 a
ALC_Sil[b]	122.68 ± 7.12 ab	13.40 ± 0.58 b	18.72 ± 0.44 b	3.16 ± 0.09 a	0.32 ± 0.01 a	1.66 ± 0.12 ab
ALC_NiuL[b]	135.11 ± 1.61 a	14.19 ± 0.30 b	20.78 ± 0.70 ab	3.16 ± 0.12 a	0.34 ± 0.00 a	1.73 ± 0.05 ab
ALC_NiuH[b]	134.03 ± 6.32 a	13.44 ± 0.33 b	19.46 ± 1.48 b	3.13 ± 0.06 ab	0.34 ± 0.01 a	1.34 ± 0.06 c

[a] Data are given as means ± SEMs ($n = 10$, except feed and water intakes, $n = 5$). Mean values with different letters in each testing parameter were significantly different ($p < 0.05$). [b] Control, 30% glucose + sterile distilled water; ALC, 20% alcohol + sterile distilled water; ALC_Sil, 20% alcohol + 0.25 g silymarin/kg BW/day; ALC_NiuL, 20% alcohol + 0.025 g Niuchangchih/kg BW/day; and ALC_NiuH, 20% alcohol + 0.1 g Niuchangchih/kg BW/day.

Table 2. Serum, Liver Lipids, and Fecal Lipids and Fecal Bile Acid and Liver Damage Indices of the Experimental Rats

groups	control[b]	ALC[b]	ALC_Sil[b]	ALC_NiuL[b]	ALC_NiuH[b]
			serum[a]		
TC (mg/dL)	48.75 ± 1.17 bc	54.36 ± 2.39 a	51.98 ± 1.88 ab	45.78 ± 1.25 c	48.68 ± 1.84 bc
TAG (mg/dL)	135.81 ± 3.15 b	162.38 ± 10.63 a	161.72 ± 6.49 a	158.77 ± 6.80 a	159.51 ± 2.20 a
			liver[a]		
TC (mg/g tissue)	4.69 ± 0.31 c	7.54 ± 0.47 a	6.25 ± 0.53 b	5.90 ± 0.17 b	5.84 ± 0.51 bc
TAG (mg/g tissue)	7.25 ± 0.30 c	14.10 ± 0.62 a	14.14 ± 0.83 a	12.49 ± 0.66 a	10.57 ± 0.70 b
			feces[a]		
TC (mg/g dried feces)	4.03 ± 0.16 a	1.98 ± 0.25 c	2.92 ± 0.19 b	3.28 ± 0.19 b	2.96 ± 0.11 b
TAG (mg/g dried feces)	3.74 ± 0.14 a	3.12 ± 0.33 a	3.34 ± 0.30 a	3.08 ± 0.09 a	3.39 ± 0.16 a
bile acid (nmol/g dried feces)	331.06 ± 16.30 a	253.32 ± 15.98 b	330.53 ± 7.73 a	347.27 ± 8.93 a	339.821 ± 7.27 a
			liver damage index[a]		
AST (U/L)	102.78 ± 3.24 d	153.00 ± 8.49 a	137.75 ± 6.30 ab	125.14 ± 4.18 bc	117.43 ± 4.48 cd
ALT (U/L)	34.89 ± 2.46 b	52.33 ± 3.26 a	51.50 ± 4.98 a	44.57 ± 2.83 ab	42.86 ± 4.39 ab

[a] Data are given as means ± SEMs ($n = 10$, except fecal TA, TAG, and bile acid, $n = 5$). Mean values with different letters in each testing parameter were significantly different ($p < 0.05$). [b] Control, 30% glucose + sterile distilled water; ALC, 20% alcohol + sterile distilled water; ALC_Sil, 20% alcohol + 0.25 g silymarin/kg BW/day; ALC_1X, 20% alcohol + 0.025 g Niuchangchih/kg BW/day; and ALC_4X, 20% alcohol + 0.1 g Niuchangchih/kg BW/day.

The highest amounts of those major compounds in wild Niuchangchih are *R*,*S*-zhankuic acid A (31.12 and 27.96%), followed by *R*,*S*-zhankuic acid C (5.59 and 6.06%), dehydrosulphurenic acid (3.55%), ergostatrien-3β-ol (0.7%), ascorbic acid (0.1%), 4,7-dimethoxy-5-methyl-1,3-benzodioxole (0.05%), dehydroburicoic acid (0.03%), 4,5-trimethoxybenzaldehyde (0.03%), zhankuic acid B (0.02%), and 2,3-dimethoxy-5-methyl[1,4]benzoquinone (0.01%).

Effects of Wild Niuchangchih on Growth Performances, Serum, Liver, and Fecal Lipids, and Fecal Bile Acids. During the experimental period, growth performances of rats, that is, BW gain, feed and water intakes, and sizes of liver, heart, kidney, spleen, and visceral fat were calculated and are illustrated in **Table 1**. Lower ($p < 0.05$) BW gain and lower ($p < 0.05$) feed and water intakes of rats were obtained in the ALC group as compared to those in the control group. However, in chronic alcohol consumption, therapeutic groups (ALC_Sil, ALC_NiuL, and ALC_NiuH) increased ($p < 0.05$) BW gain and feed intake as compared to the disease group (ALC). Although the relative sizes of the heart were not ($p > 0.05$) different among groups, the relative sizes of liver only in the ALC_NiuH and visceral fat in all therapeutic groups, respectively, were similar ($p > 0.05$) to those in the ALC group. Serum and liver lipids, as well as fecal lipids and bile acids after 4 weeks of feeding, are demonstrated in **Table 2**. The serum TAG was higher in rats drinking alcohol as compared to those drinking glucose solution (control group), while it was not ($p > 0.05$) altered among rats drinking alcohol. Although serum TC and liver TC and TAG were increased in alcohol-fed rats, supplementing Niuchangchih decreased ($p < 0.05$) those values. Conversely, higher ($p < 0.05$) fecal cholesterol and bile acid outputs were measured in alcohol-fed rats with therapeutic agents (silymarin and Niuchangchih) as compared to those without therapeutic agents.

Effects of Wild Niuchangchih on Regulations of Enzymes and Proteins Related to Lipid Homeostasis. Gene expressions related to lipid homeostasis, that is, LDLR, HMG-CoAR, CYP7A1, SREBP-1c, ACC, FAS, ME, and PPAR-α, were analyzed in the present study (**Figure 2**). Dysregulation of lipid homeostasis was observed in alcohol-fed rats, but therapeutic agents could normalize lipid homeostasis of alcohol-fed rats. HMG-CoAR gene expression was downregulated ($p < 0.05$) by supplementing therapeutic agents in chronic alcohol consumption. Meanwhile, LDLR and CYP7A1 mRNA expressions were upregulated ($p < 0.05$) only by supplementing Niuchangchih. In TAG biosynthesis and energy expenditure, gene expressions of SREBP-1c, ACC, FAS, and ME in alcohol-fed rats were downregulated ($p < 0.05$) by supplementing therapeutic agents, while only Niuchangchih could upregulate ($p < 0.05$) PPAR-α mRNA expression in alcohol-fed rats.

Effects of Wild Niuchangchih on Liver Antioxidant Capacity and Liver Damage. The liver MDA content was to represent the liver peroxidation status, while liver GSH and TEAC were to evaluate the liver antioxidant capacity in the present study. Lower ($p < 0.05$) liver MDA and higher ($p < 0.05$) GSH and TEAC were observed in the control rats as compared to those in alcohol-fed rats (**Figure 3A−C**). Lower ($p < 0.05$) liver MDA contents in alcohol-fed rats were observed when supplementing Niuchangchih (**Figure 3A**). Besides, alcohol-fed rats supplemented with

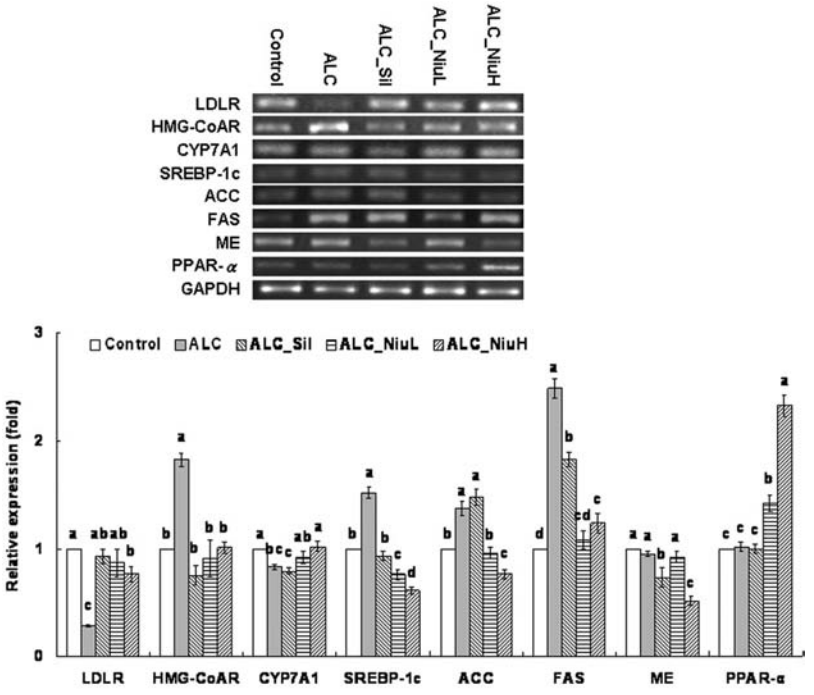

Figure 2. LDLR, HMG-CoAR, CYP7A1, SREBP-1c, ACC, FAS, ME, and PPAR-α mRNA expressions of the experimental rats. The data are given as means ± SEMs ($n = 10$). Mean values in each target gene with different letters indicate a significant difference ($p < 0.05$). Control, 30% glucose + sterile distilled water; ALC, 20% alcohol + sterile distilled water; ALC_Sil, 20% alcohol + 0.25 g silymarin/kg BW/day; ALC_NiuL, 20% alcohol + 0.025 g Niuchangchih/kg BW/day; and ALC_NiuH, 20% alcohol + 0.1 g Niuchangchih/kg BW/day. The values of LDLR, HMG-CoAR, CYP7A1, SREBP-1c, ACC, FAS, ME, and PPAR-α mRNA were normalized to the value of GAPDH, and values for the rats in control, ALC, ALC_Sil, ALC_NiuL, and ALC_NiuH groups are expressed relatively to the average values for rats in the control group, which was set to 100.

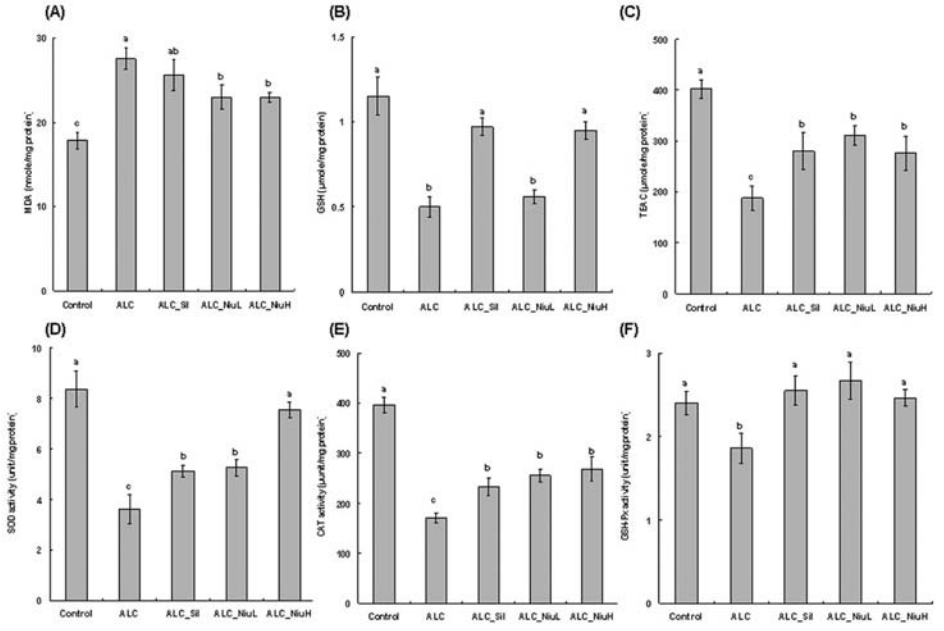

Figure 3. Liver peroxidation and antioxidant capacity and enzyme activities of the experimental rats. The data are given as means ± SEMs ($n = 10$). Different letters on data bars indicate significant differences ($p < 0.05$). Control, 30% glucose + sterile distilled water; ALC, 20% alcohol + sterile distilled water; ALC_Sil, 20% alcohol + 0.25 g silymarin/kg BW/day; ALC_NiuL, 20% alcohol + 0.025 g Niuchangchih/kg BW/day; and ALC_NiuH, 20% alcohol + 0.1 g Niuchangchih/kg BW/day.

silymarin and Niuchangchih also increased ($p < 0.05$) liver TEAC and GSH in livers (**Figure 3B**,**C**). **Figure 3** also showed liver antioxidant enzyme activities (SOD, CAT, and GSH-Px). Apparently, chronic alcohol consumption decreased ($p < 0.05$) antioxidant enzyme activities. However, silymarin and Niuchangchih increased ($p < 0.05$) SOD, CAT, and GSH-Px activities in alcohol-fed rats, but the ALC_NiuH group had the highest ($p < 0.05$) SOD activities as compared to ALC_Sil and ALC_NiuL groups (**Figure 3D**−**F**). Serum AST and ALT values were applied to evaluate liver damage in the present study. Although alcohol-fed rats had higher ($p < 0.05$) serum AST and ALT values as compared to the control rats, lower

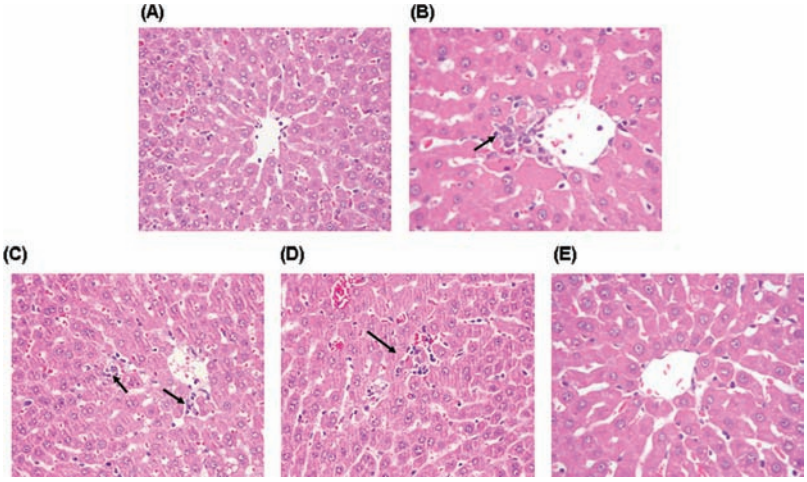

Figure 4. H&E stain under microscope (400×) of liver tissues in the experimental rats. (**A**) Control group, (**B**) ALC group, (**C**) ALC_Sil group, (**D**) ALC_NiuL group, and (**E**) ALC_NiuH group. Arrows points out hepatic necrosis with inflammatory cell in livers. Control, 30% glucose + sterile distilled water; ALC, 20% alcohol + sterile distilled water; ALC_Sil, 20% alcohol + 0.25 g silymarin/kg BW/day; ALC_NiuL, 20% alcohol + 0.025 g Niuchangchih/kg BW/day; and ALC_NiuH, 20% alcohol + 0.1 g Niuchangchih/kg BW/day.

($p < 0.05$) serum AST was only observed in alcohol-fed rats with Niuchangchih as compared to those without therapeutic agents (**Table 2**). Via a histological examination of rat liver, chronic alcohol consumption caused focal and acute individual necrosis (arrow) with mild inflammatory cell infiltration (**Figure 4B−D**). It is inspiring that the high dosage of Niuchangchih (0.1 g/kg BW) showed no significant lesion in the livers of alcohol-fed rats (**Figure 4B−D**) similar to the control group (**Figure 4A**).

DISCUSSION

The major bioactive compounds of Niuchangchih are identified as polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives, while it is summarized that Niuchangchih possesses several health functions, such as anticancer, hepatoprotection, etc. (*4*). The HPLC analysis reveals that our wild Niuchangchih can be identified as belonging to *A. camphorata* in accordance with previous literature (*4*, *19*). 2,4,5-Trimethoxybenzaldehyden has been recognized as a COX-2 inhibitor, which possesses many functions such as anti-inflammation, pain reduction, and chemoprotective effects against colon and breast cancers (*19*). Zhankuic acid C and dehydroeburicoic acid also showed an in vitro cytotoxic effect and anti-inflammatory activity, respectively (*4*). In addition, zhankuic acid A and dehydroeburicoic acid were also mentioned to have an anti-inflammatory activity in vitro (*19*). So far, less information is available regarding to the bioactivity of individual compounds in Niuchangchih. Hence, the hepatoprotective mechanism of Niuchangchih against chronic alcohol consumption was investigated in this study.

Decreased BW gain and feed intakes in chronic alcohol consumption were shown in the current study and also reported previously (*20*). The causes are complex, but most contributed to the morphological changes in hepatic mitochondria and the reduction of ATP syntheses (*21*), or NADPH was wasted to produce heat in the oxidation of alcohol via a microsomal ethanol-oxidizing system (MEOS) (*22*). Although a higher ($p < 0.05$) relative liver size was measured in alcohol-fed rats, there was a tendency toward lower liver sizes in alcohol-fed rats with therapeutic agents (**Table 1**). Sozio and Crabb (*23*) also indicated that alcohol consumption could increase lipogenesis but decrease fuel metabolism and fatty acid transport ability in livers, which may cause fatty liver development. Hence, reduced sizes of liver and visceral fat due to the high dosage of Niuchangchih therapies may be accounted for normalizing lipid metabolism of rats in chronic alcohol consumption. A report indicates that the reduced liver TC content was accounted for higher cholesterol secretion into bile, thus leading to a depletion of the intrahepatic pool of cholesterol (*24*). However, little or no study pointed out the mechanism of reduced serum and liver lipids. Moreover, higher fecal lipid and bile acid outputs are highly associated to lower serum lipid levels, thus alleviating the hepatic lipid accumulation (*25*). Hence, it is speculative that the cholesterol-lowering effects of Niuchangchih on serum and liver are highly associated with higher cholesterol and fecal bile acid outputs, which also partially accounted for a lower tendency toward liver sizes in alcohol-fed rats with Niuchangchih. Besides, the lipid (TC and TAG) homeostasis in body was also investigated in this study.

It was reported that alcohol consumption causes upregulations of lipogenic enzyme gene expressions, thereby increasing fatty acid biosynthesis and the development of hepatic steatosis (*23*). Our data also confirmed that previous report (**Figure 2**). In the cholesterol homeostasis, LDLR transports serum cholesterol to the liver; hence, upregulating its gene expression helps normalized serum TC, while HMG-CoAR is the control point for cholesterol biosynthesis and CYP7A1 is the rate-limiting enzyme for cholesterol catabolism/output. On the basis of our knowledge, the net effect of dietary cholesterol absorption, cholesterol biosynthesis, and biliary cholesterol excretion regulates body cholesterol balance. Skottová and Krecman (*26*) reported that silybin, one of the silymarin constituents, can decrease cholesterol biosynthesis, since it showed an inhibition of HMG-CoAR. Inspiringly, Niuchangchih also showed the same inhibition of HMG-CoAR as silymarin, which may explain that Niuchangchih has cholesterol-lowering effects on serum and liver in alcohol-fed rats. Moreover, LDLR and CYP7A1 in alcohol-fed rats were also upregulated by supplementing Niuchangchih. Hence, it could be explained that cholesterol-lowering effects in serum and livers of alcohol-fed rats with Niuchangchih are due to both decreasing hepatic TC biosynthesis and increasing serum cholesterol clearance and cholesterol catabolism abilities, thus increasing fecal bile acid outputs (**Figure 2** and **Table 2**). In the TAG biosynthesis, SREBP-1c is a structurally related protein that controls lipid homeostasis and also increases the lipogenic

enzyme transcription, such as ACC and FAS (*7*). Segawa et al. (*27*) indicated that SREBP-1c is overexpressed by alcohol intake. A previous report indicated that the suppressions of SREBP-1c and FAS gene expressions can decrease the deposition of visceral fat and serum and liver TAG levels of rats (*28*). Donohue (*1*) pointed out that excessive or chronic alcohol consumption alters $NADH/NAD^+$ redox potential in the liver, thus simulating TAG biosynthesis, while a chronic alcohol consumption downregulates fatty acid oxidation and transport (*23*). ME is a major enzyme that produces NADH in the TAG biosynthesis (*7*), and increasing PPAR-α gene expressions results in higher β-oxidation in livers, thus increasing energy expenditure and alleviating hepatic steatosis (*8*, *9*). The present study indicated that the Niuchangchih treatment could suppress SREBP-1c, ACC, FAS, and ME but upregulate PPAR-α (**Figure 2**). Therefore, the hepatoprotection of Niuchangchih against alcohol consumption could be partially explained as follows: The hepatic TAG biosynthesis was normalized via downregulating lipogenic enzymes and upregulating energy expenditure (**Table 2** and **Figure 2**).

The liver is the main organ in charge of oxidizing alcohol. There are three different enzymes to oxidize alcohol in hepatocyte: (1) alcohol dehydrogenase (ADH) in cytosol, (2) cytochrome P450 (CYP2E1) in endoplasmic reticulum, and (3) CAT in peroxisomes (*29*). Alcoholic liver damage most accounts for CYP2E1 induction because CYP2E1 not only catalyzes alcohol but also generates reactive oxygen radicals ($O_2^{-\bullet}$) and activates many xenobiotics to hepatotoxic and carcinogenic metabolites (*30*). MDA is often used as an indicator of lipid peroxidation in the biological system (*15*, *16*, *22*); meanwhile, GSH is a major antioxidant component that defends free radical damage in the body (*31*). Besides, SOD, CAT, and GSH-Px are capable of counteracting reactive oxygen species and H_2O_2 in the biological system (*12*). Overproduction of reactive oxygen species ($O_2^{-\bullet}$) due to lipid peroxidation and alcohol oxidation in the body can be converted into H_2O_2 and oxygen (O_2) by SOD, and furthermore, H_2O_2 can be detoxified by either CAT or GSH-Px to form water and O_2. Song and Yen (*10*) analyzed the antioxidant properties of different extracts of cultivated Niuchangchih. They summarized that dry matter of filtrated and water extractd of mycelia of cultivated Niuchangchih show a marked free radical scavenging, which is correlated with the presence of total polyphenols, the crude triterpenoids, and the protein/polysaccharide ratio of the crude polysaccharides. They even suggested that the dry matter of filtrates of Niuchangchih could possibly be a chemopreventive agent with respect to free radical-related diseases. Our data indicated that liver MDA contents in alcohol-fed rats were decreased by supplementing Niuchangchih, although only higher liver GSH contents in therapeutic groups (ALC_Sil and ALC_NiuH) were detected. This lower lipid peroxidation may be attributed to higher antioxidant enzyme activities, which could explain that silymarin and Niuchangchih counteract $O_2^{-\bullet}$ produced by CYP2E1 and further detoxify H_2O_2 to form H_2O and O_2. Besides, it was reported that PPAR-α can enhance CAT activity and reduce the extracellular H_2O_2 (*32*), which also confirms higher PPAR-α expression and CAT activity in alcohol-fed rat with Nuichangchih (**Figures 2** and **3E**). Chronic alcohol consumption also results in higher serum AST and ALT (*20*, *22*). Higher hepatic MDA contents always couple with higher serum AST and ALT values, thus inducing liver damage; however, higher antioxidant contents, that is, GSH and TEAC in tissues, result in lower oxidative stress, thus lowering MDA contents (*15*, *16*). Similar results were demonstrated in our study (**Table 2** and **Figures 3** and **4**). Hence, Niuchangchih demonstrates a good free radical scavenging in alcohol-fed rats via an elevated antioxidant capability, thus decreasing liver damage by alcohol consumption (**Figure 4**).

In conclusion, Niuchangchih, especially at a high dosage (0.1 g/kg BW/day), not only shows a hypercholesterolemic effect but also reduces hepatic lipids in alcohol-fed rats. Those beneficial effects could be attributed to increased fecal TC and bile acids outputs, as well as downregulations of HMG-CoAR, SREBP-1c, ACC, FAS, and ME gene expressions, thus alleviating lipid biosynthesis; meanwhile, there was an upregulation of LDLR and PPAR-α gene expression, thus increasing the serum cholesterol clearance ability and energy expenditure, respectively. Besides, Niuchangchih enhanced the liver antioxidant capacity (GSH, TEAC, SOD, CAT, and GSH-Px) and decreased liver lipid peroxidation (MDA content), which attenuated liver damage in alcohol-fed rats. On the basis of those benefits from our study, the hepatoprotective effect of Niuchangchih (0.1 g/kg BW/day) against chronic alcohol damage seems better than silymarin; hence, Niuchangchih is worth applying to the therapy of AFLD.

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