Effects of Natural Product Extract on the Fatty Liver Induced by Alcohol Diet in Rats

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Liver disease has been increased in proportion to the rise of alcohol and fat consumption. The purpose of this study was to investigate the effects of Natural Product Extract (NPE; *Astragalus membranaceus* + *Salvia miltiorrhiza* + *Pueraria lobata*) on fatty liver induced by alcohol diet. Male Sprague-Dawley rats fed with ethanol-containing diets for 6 weeks showed increase in hepatic lipids, indicating the onset of alcoholic fatty liver. NPE was orally administered (3 g/kg/day, 1 g/kg/day, 0.33 g/kg/day) for 4 weeks following alcohol withdrawal, results revealed reduction in alcohol-induced lipid accumulation and reversed fatty liver. This inhibition of fatty liver by NPE was indirectly accompanied by recovery of hepatic lipase activity. The activity of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased in alcohol fed group, and decreased in NPE treated group. We can conclude from our experiment, NPE can reduce the serum and hepatic contents of triglycerides and cholesterol, which can be used as a remedy for alcoholic fatty liver.

Key words —— fatty liver, *Astragalus membranaceus*, *Salvia miltiorrhiza*, *Pueraria lobata*

INTRODUCTION

Many people who consume large amounts of alcohol encounter a condition known as fatty liver. Fatty liver can lead to liver fibrosis and cirrhosis; therefore, it is important to treat the condition before it can progress into a more serious form. Fatty liver is defined as an excess accumulation of fat in the liver, usually exceeding 5 percent of the total liver weight.[1,2] Potential pathophysiological mechanisms include; decreased mitochondrial fatty acid β-oxidation, increased endogenous fatty acid synthesis or enhanced delivery of fatty acids to the liver, and deficient incorporation or export of triglycerides as very-low density lipoprotein.[3]

We have screened a number of liver protective and anti-hepatic steatosis agents from natural products used for the treatment of liver diseases, and selected *Astragali Radix*, *Salviae Miltiorrhizae Radix* and *Puerariae Radix*. *Astragali Radix*, the root of *Astragalus membranaceus* Bunge, is a perennial leguminous plant native to eastern Asia. *Astragali Radix* is crude drug used widely in oriental medicine used as a preventing liver fibrosis.[4] It is reported to decreased total cholesterol, triglycerides, apoproteins and lipoprotein-a and could also exert an effect of anti-atherosclerosis.[5] And significantly alleviated chemical induced liver injury.[6] *Salviae Miltiorrhizae Radix*, the root of *Salvia miltiorrhiza* Bunge, is a perennial libiatae plant native to eastern Asia. *Salviae Miltiorrhizae Radix* is a traditional medicine for the treatment of liver diseases. It has been shown to reduce carbon tetrachloride (CCl_4)-induced liver fibrosis[7] and to inhibit acute liver injury induced by D-galactosamine in rats.[8] *Puerariae Radix*, the root of *Pueraria lobata* Ohwi, is a perennial leguminous vine of the genus *Pueraria* native to eastern Asia. *Puerariae Radix* is an important Chinese traditional medicine used in the treatment of alcohol related diseases as an anti-intoxication, anti-drinking agent, and in the treatment of various liver diseases caused by alcohol abuse.[9] It has recently demonstrated that has potential anti-diabetic and lipid lowering properties in animals,[10] and also inhibited the elevation of alanine aminotransferase (ALT) activity.[11] It has been reported that *Astragali Radix*, *Salviae Miltiorrhizae Radix* and *Puerariae Radix* effected of liver protection, but the mecha-

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nism responsible for these was unknown. Accordingly, the current study was investigated the effects and mechanism of Natural Product Extract (NPE) on induced fatty liver from male Sprague-Dawley rat by administration of alcohol.

MATERIALS AND METHODS

Preparation of NPE —— *Astragalus membranaceus*, Salviae miltiorrhiza and *Pueraria lobata* (3 : 4 : 3) were purchased from the Korea Pharmaceutical Company. These materials were cut into small pieces. After standing at room temperature for 30 min, the materials extracted in 10 volumes of distilled water at 95–99°C, 0.5–0.6 kgf/cm^2, 3 hr. The fluids were then filtered, and thickened under reduced pressure at 70–80°C. The extracts were spray drying to a dry powder, and humidity maintained below 5%.

Animals and Diets —— Male Sprague-Dawley rats (Daehan Biolink, Chungbuk, Korea) weighing about 150 g, were purchased. All animals received humane care in compliance with the institution's guidelines criteria for humane care, as outlined in the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.[12] The rats were given free access to standard rodent pellet food (Samtaco, Gyeonggi-do, Korea) and water during the adaptation period. The rats were divided into two groups and then fed with a liquid diet (CD group) or liquid + alcohol diet for 6 weeks. The liquid diet (Table 1) was prepared according to the Lieber-DeCarli liquid diet.[13] The liquid diet provided 1 kcal/ml, containing 28% carbohydrate, 20% protein, 15% fat plus 37% ethanol (ED group) or isocaloric maltose dextrin as CD group. Alcohol supplemented rats were randomly assigned to groups as follows; rats fed with a diet without NPE (ED group) and rats oral administrated with NPE (0.33 g/kg/day; NPE330, 1 g/kg/day; NPE1000, and 3 g/kg/day; NPE3000, respectively) (Fig. 1). CD, ED group included three rats, and NPE group included six rats. The cages were placed in a room with controlled temperature (22 ± 2°C), relative humidity (60 ± 5%), and 12 hr light/dark cycle.

Sampling Procedures —— Rats were anesthetized with ether and sacrificed after 12 hr of fasting. Blood samples were collected from hepatic portal vein and centrifuged (3000 rpm for 15 min at 4°C). Serum was frozen at –70°C for the biochemical analysis. The liver was removed and then weighed after being cleaned with ice-cold saline, and stored at –70°C for the lipid analysis.

Table 1. Composition of Experimental Diets (g/l)

Ingredients	Control Diet	Alcohol Diet
Casein	41.40	41.40
DL-Methionine	0.30	0.30
L-Cystine	0.50	0.50
Cellulose	10.00	10.00
Maltose Dextrin	115.20	25.60
Corn Oil	8.50	8.50
Olive Oil	28.40	28.40
Safflower Oil	2.70	2.70
Mineral Mix[a)]	8.75	8.75
Vitamin Mix[b)]	2.50	2.50
Choline Bitartrate	0.53	0.53
Xanthan Gum	3.00	3.00
Ethanol	—	45.50

a) AIN-76 Mineral mix provided the following g/kg mix: calcium phosphate, dibasic, 500; sodium chloride, 74; potassium citrate·H_2O, 220; potassium sulfate, 52; magnesium oxide, 24; manganous carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium K sulfate.12H_2O, 0.55; sucrose, finely powdered, 118.03; Dyets, Bethlehem, Pennsylvania, U.S.A. *b*) AIN-76A Vitamin mix provided the following g/kg mix: thiamine HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3; calcium pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; Vitamin B12 (0.1%), 1; Vitamin A palmitate (500000 IU/g), 0.8; Vitamin D3 (400000 IU/g), 0.25; Vitamin E acetate (500 IU/g), 10; menadione sodium bisulfite, 0.08; sucrose, finely powdered, 981.15; Dyets, Bethlehem, Pennsylvania, U.S.A.

Biochemical Analysis —— Biochemical analysis was carried out using commercial kits. The analysis of serum triglycerides, total cholesterol, and high density lipoprotein (HDL)-cholesterol concentration were done by automatic analyzer techniques (YD Diagnostics, Seoul, Korea). The concentrations were measured spectrophotometrically at a wavelength of 540 nm, 505 nm, 500 nm respectively (Jasco V-530 UV/VIS, Japan). The low density lipoprotein (LDL)-cholesterol and very low density lipoprotein (VLDL)-cholesterol were estimated using the Friedewald formula as follows; LDL-cholesterol = Total cholesterol – (VLDL-cholesterol + HDL-cholesterol) and VLDL-cholesterol = triglycerides/5.[14]

The activities of the serum aspartate transaminase and alanine transaminase measured using kits (YD Diagnostics).

To analyze hepatic lipid content, the lipids were extracted by homogenizing the liver with 2 : 1 chlo-

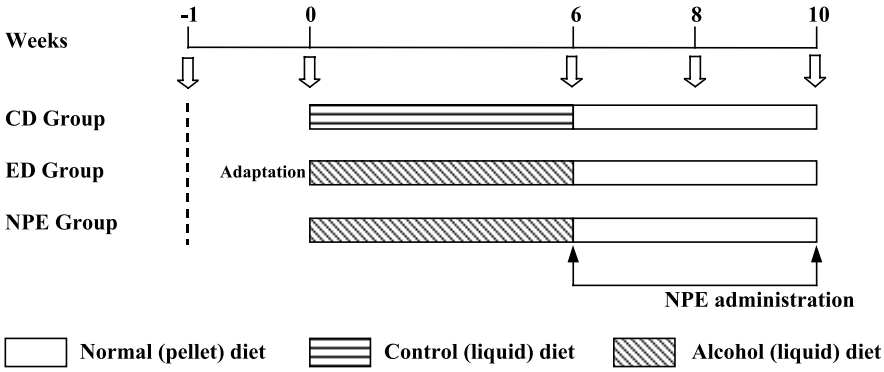

Fig. 1. Schematic Representation of Diet and NPE Treatment in Sprague-Dawley Rats

Group CD rats were treated control diet for 6 weeks and replaced to normal diet. Group ED rats were treated ethanol diet for 6 weeks and replaced to normal diet. Group ED rats were treated ethanol diet for 6 weeks and replaced to normal diet. Group NPE rats were treated ethanol diet for 6 weeks and replaced to normal diet with NPE (0.33 g/kg/day; NPE330, 1 g/kg/day; NPE1000, and 3 g/kg/day; NPE3000, respectively).

Table 2. Body Weight and Liver Weight of Rats Fed Experimental Diets for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

Wks	Group	Body weight	Liver/body weight
		G	g/B.W. 100 g
0		151.8 ± 7.6	2.60 ± 0.10
6	CD	346.9 ± 7.0	2.89 ± 0.44
	ED	303.8 ± 6.3*	3.29 ± 0.29*
8	CD	385.9 ± 9.1$^{a)}$	2.69 ± 0.27$^{b)}$
	ED	346.0 ± 7.3$^{b)}$	3.38 ± 0.55$^{a)}$
	NPE 330	364.0 ± 8.1$^{b)}$	3.22 ± 0.14$^{a)}$
	NPE 1000	365.0 ± 6.8$^{b)}$	3.14 ± 0.26$^{a,b)}$
	NPE 3000	360.8 ± 2.4$^{b)}$	3.07 ± 0.21$^{a,b)}$
10	CD	416.2 ± 6.3$^{a)}$	3.09 ± 0.05$^{a,b)}$
	ED	385.2 ± 10.3$^{c)}$	3.21 ± 0.11$^{a)}$
	NPE 330	397.7 ± 2.1$^{b,c)}$	2.73 ± 0.13$^{b)}$
	NPE 1000	391.1 ± 6.5$^{c)}$	2.86 ± 0.29$^{a,b)}$
	NPE 3000	406.7 ± 14.5$^{a,b)}$	2.84 ± 0.35$^{a,b)}$

Letters (alphabets) different superscripts in a colum are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

roform- methanol (v/v) by the method devised by Folch *et al.*[15] Then, triglycerides and total cholesterol were measured commercial kits (YD Diagnostics).

Lipase activity was determined by enzymatic colorimetric assay with 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methyl-resorufin) ester as substrate. To analyze hepatic lipase activity, the liver was homogenized in 0.2 mol/l Tris buffer (pH 8.8), and centrifuged (13000 rpm, 10 min) and measured by COBAS INTEGRA Lipase colorimetric (Roche company, Switzerland).

Statistical Analysis —— Statistical evaluation of data was performed by Duncan's multiple range tests to make comparisons among the groups. Values were given as the mean ± S.D. Differences of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effects of NPE on Body Weight and Liver Weight

Table 2 shows the body weight and liver weight (g/100 g body weight) in all rats during the 10 weeks.

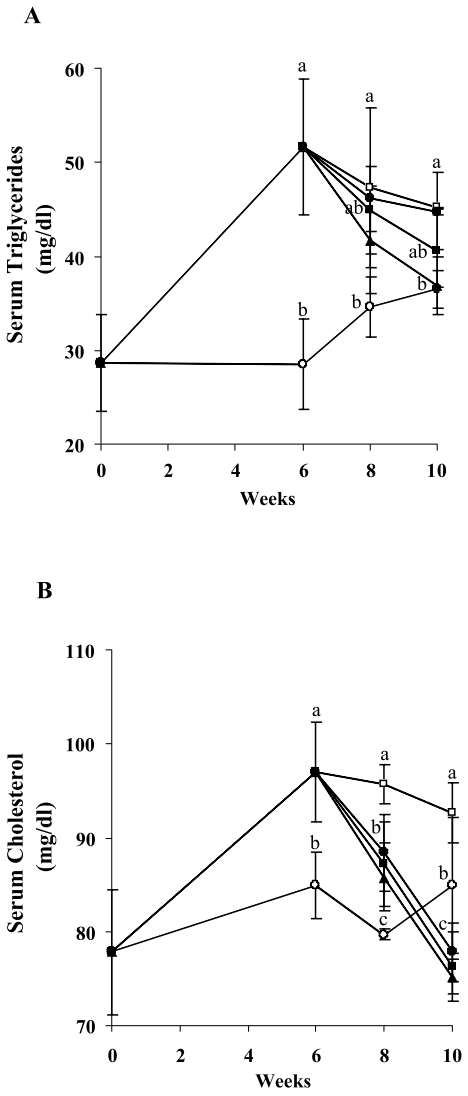

Fig. 2. Concentration of Serum Triglycerides (A) and Total Cholesterol (B) of Rats Fed Experimental Diet for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

○; CD, □; ED, ▲; NPE3000, ■; NPE1000, ●; NPE330. All values are mean ± S.D. Letters with different superscripts in the same raw are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

At 6 week, the body weight was significantly lower in alcohol fed rats than in the CD group. It could be attributed to the reduction of intake and absorption of nutrients.[16,17] However, rats administrated NPE was trended to greater in body weight compared to ED group. The liver weight was trended to higher in alcohol fed rats than in the CD group. However, after administration of NPE for 4 weeks, the NPE groups significantly decreased liver weight. It is considered that there is inhibition of hepatic lipids accumulation in rats treated with NPE.

Effects of NPE on Serum Lipid Profiles

Serum triglycerides and total cholesterol were significantly elevated by about 1.8- and 1.3-fold by alcohol feeding compared to CD group (Fig. 2). In rat administration of NPE, the triglycerides and total cholesterol decreased about 30% and 23%, respectively (Fig. 2). It is considered that there is inhibition of hepatic lipids accumulation in rats treated with NPE.

VLDL-cholesterol was triglycerides-rich lipoproteins and transfer triglycerides from the liver to peripheral tissues. Previous studies indicate that impaired ability of hepatocyte to secrete lipid components as VLDL-cholesterol is likely to be one

Table 3. Serum HDL-Cholesterol, LDL-Cholesterol and VLDL-Cholesterol Concentration of Rats Fed Experimental Diet for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

Wks	Group	LDL-cholesterol	VLDL-cholesterol	HDL-cholesterol
			mg/dl	
0		12.14 ± 3.50	12.78 ± 0.68	53.09 ± 3.84
6	CD	13.28 ± 2.87	9.90 ± 0.28	76.65 ± 4.98
	ED	30.56 ± 16.50	3.67 ± 0.31*	52.96 ± 13.70**
8	CD	18.19 ± 13.00$^{b)}$	9.20 ± 0.46$^{b)}$	59.32 ± 5.32
	ED	25.41 ± 16.10$^{a)}$	4.64 ± 0.34$^{a)}$	53.46 ± 15.14
	NPE 330	17.00 ± 5.60$^{b)}$	6.59 ± 0.34$^{a,b)}$	60.31 ± 8.95
	NPE 1000	19.82 ± 11.86$^{b)}$	6.63 ± 0.24$^{a)}$	61.74 ± 8.58
	NPE 3000	14.98 ± 2.13$^{b)}$	7.24 ± 0.15$^{a)}$	61.05 ± 20.12
10	CD	13.67 ± 7.80$^{b,c)}$	9.14 ± 0.42$^{b)}$	62.26 ± 13.37
	ED	22.90 ± 11.47$^{a)}$	6.56 ± 0.37$^{a)}$	60.98 ± 3.55
	NPE 330	14.50 ± 6.27$^{a,b)}$	9.02 ± 0.18$^{b)}$	72.99 ± 9.93
	NPE 1000	16.54 ± 4.28$^{c)}$	8.65 ± 0.39$^{a)}$	69.09 ± 10.98
	NPE 3000	14.10 ± 3.85$^{b,c)}$	8.00 ± 0.34$^{a)}$	67.90 ± 18.79

Letters (alphabets) different superscripts in a colum are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

major mechanism linked to alcoholic fatty liver.[2,18–20] One important mechanism involved in the NPE-induced recovery from an alcoholic fatty liver is decrease in serum LDL-cholesterol and VLDL-cholesterol concentrations. The current study, LDL-cholesterol and VLDL-cholesterol concentration were significantly higher in alcohol fed rats than in the CD group (Table 3). However, a trend toward decreased LDL-cholesterol was seen after NPE administration. While, HDL-cholesterol concentration was not significantly different among the groups.

Effects of NPE on Hepatic Triglycerides and Total Cholesterol

In alcohol fed rats, hepatic lipid content raised significantly compared to rats fed with an isocaloric diet without alcohol (Fig. 3). Hepatic triglycerides and total cholesterol concentration, respectively, showed 2.9-, and 3.3-fold increase ($p < 0.05$) in rats fed with an alcohol compared to those fed a control diet. Therefore, ingestion of alcohol containing diets for 6 weeks causes the onset of fatty liver. Administration of NPE reduced accumulation of lipids in the liver. In rats administrated NPE, triglycerides and total cholesterol decreased to 20% and 30% (Fig. 3).

The current study demonstrated that the ability of the NPE administration to inhibit the hepatic lipids accumulation may have been mainly due to the recovery of hepatic lipase activity.

Effects of NPE on Serum Aspartate Aminotransferase (AST)/ALT

The administration of alcohol to rats significantly raised the activities of aspartate aminotransferase (AST) and ALT (Table 4). Supplement with NPE reduced the activity of serum AST and ALT in rats with fatty liver.

Effects of NPE on Hepatic Lipase Activity

Hepatic lipase was known to be important in the final steps in the lipolytic conversion of VLDL to LDL by hydrosis of the LDL triglyceride. To put it more concretely, Apo B is incorporated into VLDL by hepatocytes, preparing this lipoprotein for transport of triglycerides and cholesterol from liver to other tissues. When VLDL encounters lipoprotein lipase (LPL) in tissue capillaries the Apo C2 on the VLDL activates the enzyme, which hydrolyzed much of the triglycerides of the VLDL to produce intermediate denisty lipoprotein (IDL). About 1/2 of the resulting IDL, which is poorer than VLDL in triglycerides and relatively richer in cholesterol and its esters, is taken up by the liver by a receptor that recognizes the Apo B of the IDL. The triglycerides of the other 50% of the IDL is hydrolyzed by another enzyme hepatic lipase producing LDL, a lipoprotein that is richer than IDL in cholesterol and its es-

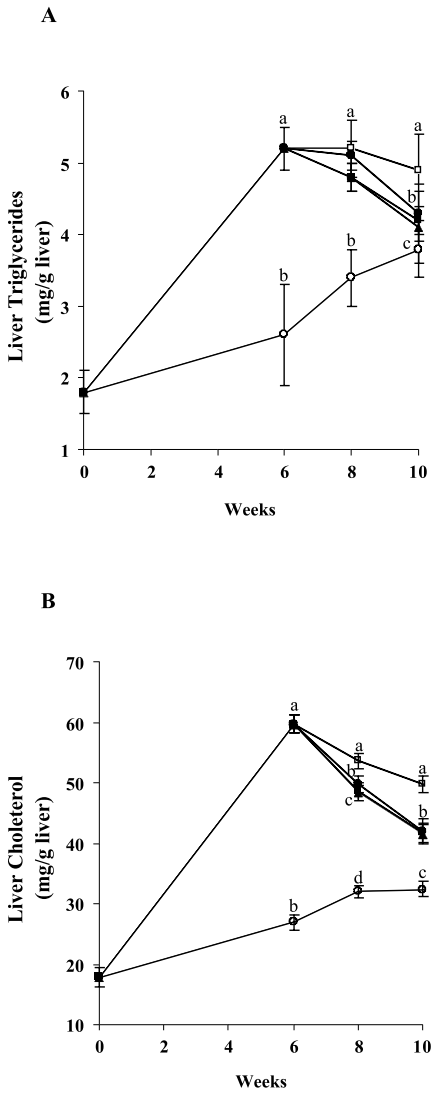

Fig. 3. Triglycerides and Total Cholesterol Concentrations in Liver of Rats Fed Experimental Diet for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

○; CD, □; ED, ▲; NPE3000, ■; NPE1000, ●; NPE330. Values are mean ± S.D. Letters with different superscripts in the same raw are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

ter.[21] Therefore, with hindering hepatic lipase activity, it can be reduced to remodel lipoprotein into LDL. In current study, we investigated the NPE's role in hepatic lipase. It is already well known that *Astragali Radix*, *Salviae Miltiorrhizae Radix* and *Puerariae Radix* is lowered the blood lipid, we demonstrated that the administration of NPE was lowered the hepatic lipase activity in a dose-dependent manner (Table 5). It was found from this result that NPE was not only lowered liver and blood lipid concentration but also engaged generally lipid metabolism by regulated the balance of VLDL, LDL and HDL.

In conclusion, administration of NPE recovered the hepatic lipase activity and it diminished the accumulation of lipids in liver. These results suggested that intake of NPE (*Astragalus membranaceus*, *Salviae miltiorrhiza* and *Pueraria lobata*) may be useful in preventing and improving fatty liver induced by alcohol. However, It is not enough mechanism from the current observation; therefore, follow-up investigations identifying mechanism related to the reduced the lipids in liver.

Table 4. AST and ALT Activities of Rats Fed Experimental Diet for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

Wks	Group	AST	ALT
		Unit/ml	
0		113.53 ± 9.32	27.39 ± 4.94
6	Control	105.10 ± 8.11	32.27 ± 5.19
	Ethanol	137.82 ± 9.58	65.09 ± 8.55*
8	CD	113.75 ± 5.59$^{d)}$	26.54 ± 3.81$^{c,d)}$
	ED	148.15 ± 4.64$^{a)}$	45.97 ± 0.28$^{a)}$
	NPE 330	128.01 ± 8.74$^{b)}$	32.85 ± 2.18$^{b)}$
	NPE 1000	124.92 ± 7.69$^{b,c)}$	30.74 ± 4.48$^{b,c)}$
	NPE 3000	113.44 ± 5.05$^{d)}$	27.40 ± 3.22$^{c,d)}$
10	CD	105.06 ± 8.30$^{a,b)}$	35.07 ± 7.84$^{a,b)}$
	ED	119.30 ± 5.98$^{a)}$	41.30 ± 2.85$^{a)}$
	NPE 330	114.59 ± 11.66$^{a)}$	29.50 ± 5.27$^{b)}$
	NPE 1000	98.46 ± 6.59$^{b)}$	29.27 ± 7.16$^{b)}$
	NPE 3000	93.81 ± 8.69$^{b)}$	26.31 ± 5.51$^{b)}$

Letters (alphabets) different superscripts in a colum are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

Table 5. Hepatic Lipase Activity of Rats Fed Experimental Diet for 6 weeks and Replaced to Normal Diet or NPE for the Next 4 weeks

Wks	Group	Hepatic lipase activity
		U/l
0		51.37 ± 4.55
6	Control	65.00 ± 7.92
	Ethanol	426.10 ± 72.43*
8	CD	51.20 ± 8.91$^{c)}$
	ED	363.43 ± 123.97$^{a)}$
	NPE 330	90.97 ± 13.94$^{c)}$
	NPE 1000	148.58 ± 69.84$^{b,c)}$
	NPE 3000	246.95 ± 17.89$^{b)}$
10	CD	55.20 ± 1.41$^{b)}$
	ED	254.75 ± 22.27$^{a)}$
	NPE 330	48.55 ± 2.14$^{b)}$
	NPE 1000	56.38 ± 6.90$^{b)}$
	NPE 3000	117.95 ± 68.14$^{b)}$

Letters (alphabets) different superscripts in a colum are significantly different ($p < 0.05$) among the groups by Duncan's multiple range test.

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