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Research Article

Lignosus rhinocerus attenuates non-alcoholic fatty liver induced by plant-based high-fat diet in hamster

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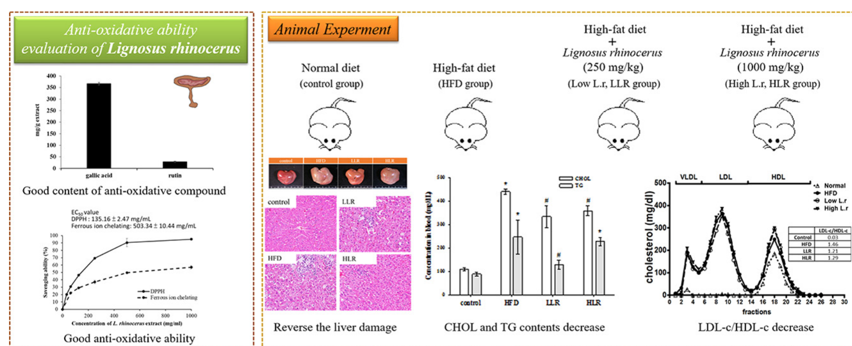
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder globally, and is often caused by abnormal fatty acid metabolism. Plant-based fatty acid may lower the risk of NAFLD, but evidence shows that plant-based fatty acid can still induce fatty liver. *Lignosus rhinoceros* is a mushroom that is widely used as folk medicine in Southeast Asia. Studies indicate that *L. rhinoceros* can act as an antioxidant and immune modulator, might be a potent modulator for NAFLD. In this study, a plant-based high-fat diet (HFD) was used to induce fatty liver in hamsters. Hot water extract of *L. rhinoceros* was applied during the induction period. The antioxidant capacity of *L. rhinoceros* was evaluated.
Results: Data reveal that *L. rhinoceros* extract had promising DPPH scavenging and ferrous ion chelating compacities. The total phenolic and flavonoid contents were 366.23 ± 5.06 mg gallic acid and 28.67 ± 2. 5 mg rutin equivalents per gram of raw *L. rhinocerus* powder, respectively. *Lignosus rhinoceros* extract lowered body weight, and reduced liver damage which was induced by HFD in hamster, possibly by reducing mononuclear cell infiltration in the liver. *Lignosus rhinoceros* treatment also lowered the

Abbreviations: AA, antioxidant activity; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CHOL, cholesterol; CREA, creatinine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DPPH, di(phenyl)-(2,3,6-trinitrophenyl)iminoazanium; HDL, high-density lipoprotein; HLR, *L. rhinocerus* 1000 mg/kg; HFD, high-fat diet; LDL, low-density lipoprotein; LLR, *L. rhinocerus* 250 mg/kg; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TBST, Tris buffer saline tween 20; TG, triglycerides; VLDL, Very low density lipoprotein; GAE/g, gallic acid equivalent per gram.

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Liver disorder
Mushroom
Non-alcoholic fatty liver disease
Plant based oil

cholesterol content and improved the HDL-c/LDL-c level in hamster serum. Finally, *L. rhinoceros* reduced the reactive oxygen species content in the liver which was caused by HFD.
Conclusions: This investigation provides evidence that a plant-based HFD may be a risk factor in fatty liver. It also proves that *L. rhinoceros* promotes liver health, and so can be used in functional food.
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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder worldwide [1], and is characterized by hepatic steatosis in the absence of causes of secondary hepatic fat accumulation. The presence of steatosis and inflammation with liver cell injury (ballooning) defines nonalcoholic steatohepatitis (NASH), which may be accompanied by progressive fibrosis [2]. Untreated and progressive fibrosis can lead to cirrhosis, which is the complete architectural reconstruction of the liver with the deposition of large amounts of connective tissue, resulting in loss of hepatic function. Cirrhosis can also lead to portal hypertension, progressive liver failure and/or hepatocellular carcinoma [3]. The mechanisms of liver fibrogenesis are numerous. In general, an inflammatory response is initiated after tissue injury, and it involves the local vascular system, the immune system, and local systemic mobilization of soluble mediators. This response involves non-parenchymal cells, such as endothelium, stellate and Kupffer cells, and resident immune cells, such as macrophages, dendritic cells, and mast cells. These cells trigger the synthesis and/or release of a large variety of inflammatory and pro-fibrogenic mediators within the injured liver tissue. These soluble mediators lead to the recruitment of more inflammatory immune cells into the liver tissue and lead to the activation of matrix-producing cell populations, driving scar tissue synthesis. Although NAFLD is caused by complex mechanisms, the available therapeutic strategy is promising. Many micronutrients, including vitamin E, vitamin C, vitamin D, and several polyphenols, such as resveratrol, curcumin, caffeine, and quercetin, have been successfully tested in preclinical models and clinical studies [4,5]. Thus, antioxidants and immune-modulation are possible strategies for treating NAFLD.

Lignosus rhinocerus, also called tiger's milk mushroom, is a well-known folk medicine. It belongs to the Polyporaceae family, and can be found in China, Philippines, Sri Lanka, Australia and Malaysia. In Malaysia, *L. rhinoceros* is used to treat fever, asthma, breast cancer, cough, and food poisoning and to promote general wellness. Its health promoting character has been verified recently. The extracts of *L. rhinoceros* provide the neuroprotective effects against glutamate-induced toxicity in HT22 cells [6], and also enhance stress resistance and extend lifespan in *Caenorhabditis elegans* via the DAF-16/FoxO signaling pathway [7]. An open-label prospective study with 50 voluntary participants has proven that the supplementation of *L. rhinocerus* improves respiratory health, immunity and antioxidant status [8]. Like other mushrooms, *L. rhinocerus* is rich in polysaccharide peptide complexes and glucose, which can be extracted from it using hot water. Studies show that *L. rhinocerus* has many health-promoting effects. Lee et al. [9] reported that *L. rhinocerus* extract had an anti-inflammatory effect by reducing carrageenan-induced paw edema in Sprague–Dawley rats, and the aqueous extract of L. *rhinocerus* significantly up-regulates pinocytosis by modulating ROS, NO, and TNF-α production [10]. A previous study found that water and methanol extracts of *L. rhinocerus* exhibit antioxidant properties [11]. These studies indicate that *L. rhinocerus* may be a potent agent for treating NAFLD, but verification is required. This work investigates the potential of *L. rhinocerus* to retard NASH.

In this study, the role of *L. rhinocerus* in protecting against fatty liver is evaluated. First, *L. rhinocerus* extract was made by hot water extraction, and its antioxidant capacity was measured. In a functional test, a plant-based high-fat diet was used to induce fatty liver in hamsters, and the *L. rhinocerus* extract was applied during the induction period. The goal is to evaluate the antioxidant capacity of *L. rhinocerus* and its biological function in modulating NASH.

2. Materials and methods

L. rhinocerus mycelium powder was obtained from the Grape King Biotechnology (Taoyuan, Taiwan). The di(phenyl)-(2,3,6-trinitrophenyl)iminoazanium (DPPH), Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (St. Louis, MO, US). The chow diet was purchased from LabDiet (St. Louis, MO, US). The serum clot activator with gel separator blood collection tube was manufactured by Greiner Bio-One (Monroe, NC, US). The triglycerides and cholesterol assay kits were obtained from the Randox Laboratories (Northern Ireland, UK). Antibody of PPAR-α was obtained from GeneTex (San Antonio, Texas). The anti-actin antibody (A2066) was obtained from Sigma-Aldrich (St. Louis, MO, US). The PVDF membrane was obtained from Millipore Sigma (Burlington, MA, US). All other fine chemicals were obtained from Sigma-Aldrich (St. Louis, MO, US).

2.1. Lignosus rhinocerus extract preparation

Briefly, *L. rhinocerus* extract was prepared from *L. rhinocerus* mycelium powder by 2-fold ddH_2O extraction at 100 °C for 4 h. After extraction, the supernatant was prepared by centrifugal filtering at 10,000 rpm for 15 min, collected, and stored in frozen alternate. One gram of the extract represented 0.5 gram of raw *L. rhinocerus* powder in the following work. The extract was diluted using ddH_2O to obtain the required sample concentration before experiments were conducted.

2.2. DPPH photometric assay

Sample stock solutions were diluted with ddH_2O to concentrations of 100, 75, 50, 25, and 12.5 μg/mL (calculated to the raw *L. rhinocerus* powder). A 1 mL volume of 0.3 mM DPPH ethanol solution was reacted with 2.5 mL of sample solution at room temperature. After 30 min, the absorbance values were measured at 517 nm and the antioxidant activity (AA) was calculated as AA (%) = 100 − {[(sample absorbance − blank absorbance) × 100]/control absorbance}. EC_{50} was calculated using the linear regression of plots, in which the abscissa represents the concentration of *L. rhinocerus* extract and the ordinate represents the percentage of AA.

2.3. Ferrous ion chelating assay

The chelation of ferrous ion by the extracts was evaluated by the method of Decker and Welch [12]. The reaction mixture, containing *L. rhinocerus* extract, $FeCl_2$ (2 mM) and ferrozine (5 mM), was adjusted to 0.8 mL, and incubated for 10 min at room temperature. The absorbance at 562 nm was measured. The ability to chelate ferrous ions was calculated as chelating effect (%) = [1 − (sample absorbance − blank absorbance)] × 100.

2.4. Total phenol content assay

The total amount of phenolic compounds was determined using Folin-Ciocalteu reagent. The sample (1 mL) was mixed with 2% of Na_2CO_3 (1 mL) and incubated for 3 min. Following incubation, Folin-Ciocalteu reagent (0.1 mL) was added and incubated for 60 min. The absorbance was measured at 750 nm. The phenolic content was calculated as gallic acid and rutin equivalents (mg/g of raw *L. rhinocerus*) on the basis a standard curve for gallic acid and rutin.

2.5. Animal care and experimental protocol

The study protocol was approved by the Ethics Committee of National Chung Hsing University, with the approval number IACUC No. 100-86. Twenty-four golden Syrian hamsters were purchased from National Laboratory Animal Center (Tainan, Taiwan) and housed in stainless steel cages in a room at 20–25°C on a 12 hlight–dark cycle with free access to regular rodent chow and water. After 1 week of acclimatization, the hamsters were randomly divided into four experimental treatment groups as follows; group 1 was maintained with a normal diet (control); group 2 was provided a high-fat diet (HFD); groups 3 and 4 were provided an HFD that was supplemented with *L. rhinocerus* 250 mg/kg (LLR) and 1000 mg/kg (HLR), respectively. All *L. rhinocerus* dosages are calculated with respect to raw *L. rhinocerus* powder. For control group basic chow diet was supplied, which was obtained from Labdiet (St. Louis, USA). The high fat diet for the HFD group was basic chow diet (LabDiet 5001, MO, USA) which was supplemented with 11.5% coconut oil (Smith Natural organic virgin coconut oil), 11.5% soybean oil (Fwusow soybean salad oil), and 0.2% cholesterol (Wako Pure Chemical Inc, Osaka, Japan). The HFD was freshly prepared before each administration. The *L. rhinocerus* extract was administered by gavage in groups 3 and 4, whereas water was administered to the control and HFD groups. Food consumption was measured daily, and body weight was recorded every three days. After six weeks of administration, animals were made to fast overnight, before being given mild ether anesthesia. The whole blood was collected by retro orbital sinus puncture in a serum clot activator tube. Plasma was obtained by centrifugation at 2000 rpm at 4°C for 15 min. The liver was excised, rinsed with physiological saline, weighed, flash-frozen in liquid nitrogen, and stored at −80°C until it was assayed.

2.6. Hematoxylin and eosin staining of liver for histopathological examination

Liver tissues were fixed overnight in 10% paraformaldehyde and embedded in paraffin by a routine procedure. Four paraffin sections were obtained consecutively, and processed for histological examination using conventional methods with hematoxylin and eosin stain.

2.7. Serum biomarker measurement

The concentrations of triglycerides (TG), cholesterol (CHOL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (CREA) were measured at the Veterinary Medical Teaching Hospital in National Chung Hsing University. Serum was analyzed using a HITACHI 7050 automatic blood chemical analyzer (Chiyoda, Tokyo, Japan). The lipoprotein (LDL and HDL) from the hamsters' plasma was isolated by the gel-filtration chromatography on a Superose 6HR 10/30 column which was utilized at a flow rate of 0.25 ml/min in phosphate-buffered saline (PBS). The fractions (0.5 mL/tube) were collected and the amount of cholesterol was measured by ELISA kit (Randox, Crumlin, UK).

2.8. Determination of total liver cholesterol and triglyceride concentrations

The liver tissue was homogenized with 19x chloroform/methanol (2:1, v/v). After it had been centrifuged (12,000 rpm for 10 min, 4°C), the clear organic phase solution was transferred into a new glass tube. The concentrations of the liver triglycerides (TG) and cholesterol (CHOL) were measured by enzymatic colorimetric methods. The concentration of the cholesterol was determined following enzymatic hydrolysis and oxidation. Quinoneimine was used as an indicator, and was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The concentrations of triglycerides were determined following enzymatic hydrolysis with lipases. Quinoneimine was used as an indicator, and was formed from hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase.

2.9. Analysis of hepatocyte reactive oxygen species (ROS)

Total ROS was quantified using the DCFH-DA fluorescent probe, as previously described [13]. The liver samples (10 μL liver extract and 275 μL PBS) were placed in the wells of 96-well black plates. After mixing, 1 M DCFH-DA in PBS solution was added to each well and reactions proceeded for 30 min at 37°C. The fluorescence intensity, corresponding to the intracellular ROS level, was measured using a Biotek Microplate Fluorescence Readers FLX800 (Arcugnano Vicenza, Italy) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.10. Western blot was performed to analyze protein expression

Liver protein samples (30 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corporation, USA). Each membrane was blocked with 5% gelatin in Tris-HCl buffer with 0.1% Tween 20 (TBST) and then incubated with the primary antibody overnight at 4°C. Each membrane was washed three times with TBST and incubated with the secondary antibody for 1 h at room temperature. Protein expression was detected using electrochemiluminescence Western blotting detection reagents and exposed in luminescence image systems. The quantity of protein was determined using ImageJ software.

2.11. Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and *p*-values of <0.05 were considered to indicate significance. Analysis of variance (ANOVA) was used to assess the statistical significance of differences among groups. Statistical analysis was conducted using the SAS, version 9.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Anti-oxidative ability of L. rhinocerus

To characterize *L. rhinoceros*, its antioxidant properties, including DPPH scavenging and ferrous ion chelating activity (Fig. 1a), were measured and total phenol assay was conducted (Fig. 1b). The results indicate that *L. rhinocerus* extract exhibited antioxidant ability, and the EC_{50} values for the scavenging of DPPH and the ferrous ion chelating activity were 135.16 ± 2.47 mg/mL and 503.34 ± 10.44 mg/mL, respectively. From Fig. 1b, total phenolic and contents were estimated to be 366.23 ± 5.06 mg gallic acid and 28.6 7 ± 2.5 mg rutin equivalents per gram of raw *L. rhinocerus* mycelium powder, respectively.

3.2. Effect of L. rhinocerus on body weight and food intake of hamsters

At the end of the experiment, body weights had increased by about 10% in the HFD group over those in the control group (Fig. 2a). This result reveals that co-treatment with *L. rhinocerus* 250 mg (LLR) and 1000 mg (HLR) inhibited the increase in the body weights of hamsters with the HFD for six weeks. Fig. 2b shows that the groups with the high fat diet ate less food than the control group. The food intake of the control group was 8.37 ± 0.49 g/da y, while that of the HFD, LLR and HLR groups was 5.84 ± 1.02 g/d ay, 5.45 ± 0.91 g/day and 5.71 ± 0.87 g/day, respectively.

3.3. Effect of L. rhinocerus on liver in hamsters treated with HFD

Fig. 3a shows the liver morphology after six weeks of treatment. The livers of the HFD group felt oily, and had pale and swollen surfaces. Supplementing with LLR significantly reversed this damage to the liver, but HLR had a weaker reversing effect. Hepatosteatosis of the liver was further examined. Fatty infiltration of the micro- and macro-vesicles and mononuclear infiltration in the hepatocyte were minimal in the control group (Fig. 3b). Histopathological examination revealed no fatty infiltration in the HFD group that was obviously inhibited by *L. rhinocerus*. However, Fig. 3b shows a trend of reduction in mononuclear infiltration in the livers of hamsters that had been treated with HLR, indicating that *L. rhinocerus* might inhibit the fast growth and division of cells such as macrophages.

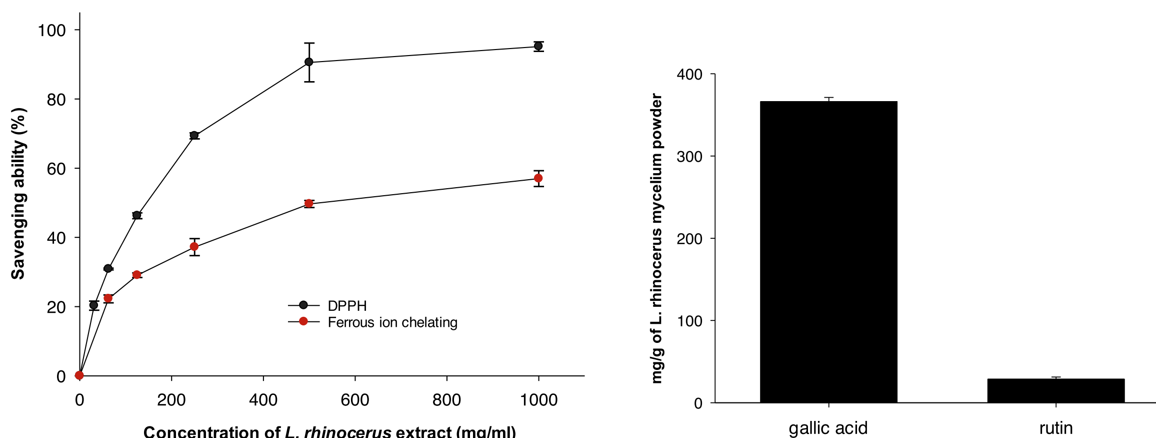


Fig. 1. Antioxidant characteristics of *L. rhinocerus*. The antioxidant capacity of *L. rhinocerus* was evaluated by (a) DPPH scavenging, ferroin chelating activity and (b) total phenol content assay. Both gallic acid and rutin were used as standard phenolic compounds in total phenol content assay. Each value is expressed as mean ± SD (n = 3).

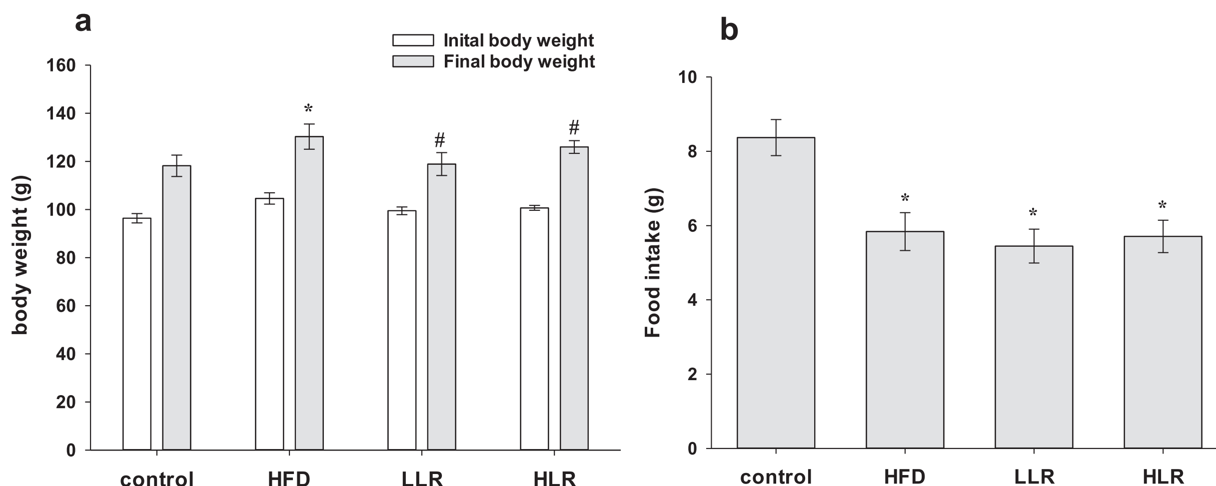
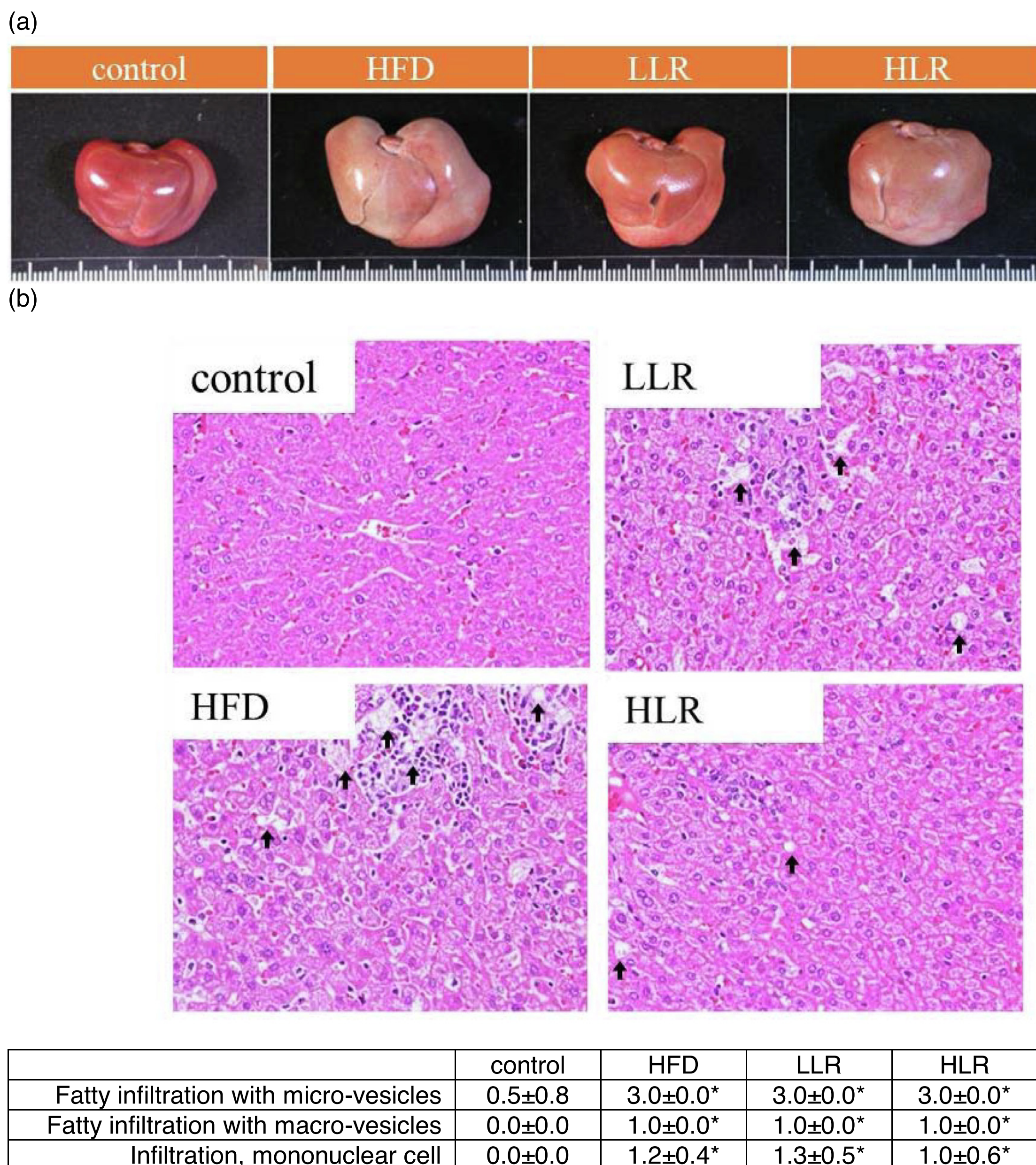


Fig. 2. Effects of *L. rhinocerus on body* weight and food intake of hamster. (a) Changes in body weight of hamsters with HFD and *L. rhinocerus*; (b) average food intake by group. Each value is expressed as mean ± SD (n = 6). *compared to the control group, $p < 0.05$. #compared to the HFD group, $p < 0.05$.



	control	HFD	LLR	HLR
Fatty infiltration with micro-vesicles	0.5±0.8	3.0±0.0*	3.0±0.0*	3.0±0.0*
Fatty infiltration with macro-vesicles	0.0±0.0	1.0±0.0*	1.0±0.0*	1.0±0.0*
Infiltration, mononuclear cell	0.0±0.0	1.2±0.4*	1.3±0.5*	1.0±0.6*

Fig. 3. Effect of *L. rhinocerus* on the liver in hamster treated with HFD. (a) Morphology of liver surfaces of each group; (b) hepatosteatosis in each group. Livers were sectioned and stained with hematoxylin and eosin. The hepatosteatosis in (b) was marked with the arrow. Magnification is 400×. Each value is expressed as mean ± SD (n = 6); *compared to the control group, $p < 0.05$.

3.4. Effect of L. rhinocerus on biochemical parameters of serum in hamster

To estimate the effect of *L. rhinocerus* on the biochemical parameters of serum, AST, ALT, BUN, and CREA, which are related to the functions of the liver and kidneys, were measured. Table 1 shows that HFD treatment significantly elevated AST and ALT (p < 0.05). LLR and HLR had negative-regulatory effects on AST and ALT, which were themselves elevated by HFD treatment. These results reveal that six weeks of the HFD did not affect the BUN or CREA, but HLR treatment reduced the BUN level.

3.5. Effects of L. rhinocerus on TG, CHOL HDL-c and LDL-c contents in hamster blood and liver

The TG and CHOL contents in hamster blood (Fig. 4a) and liver (Fig. 4b) were evaluated. HFD treatment significantly elevated the CHOL and TG contents in blood and liver. In blood, increase in CHOL content that was caused by the HFD was inhibited by both LLR and HLR treatments, but only LLR reduced the TG content for which the HFD was responsible. In liver, the HFD increased CHOL and TG contents (Fig. 4b). Only HLR lowered the TG content in the liver which was elevated by high fat diet, but no treatment reduced the liver CHOL level. The VLDL and HDL contents in the hamsters' serum were evaluated (Fig. 4c). Both LLR and HLR increased HDL in hamsters over the level obtained with the HFD and both improved the LDL-c/HDL-c ratio.

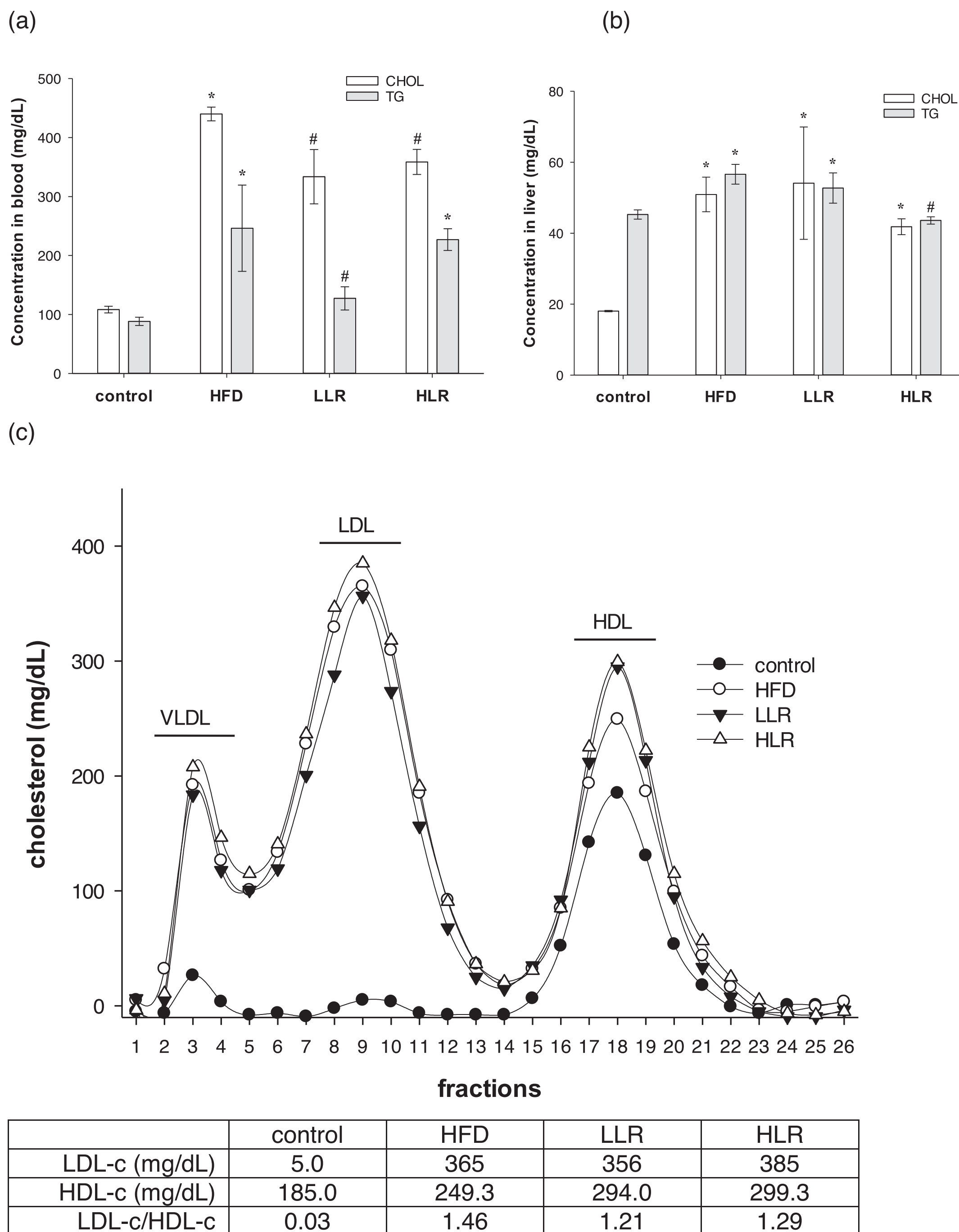
3.6. Effects of L. rhinocerus on ROS content and lipid oxidation in hamster liver

Owing to the antioxidant effect of *L. rhinocerus*, its cytoprotective effects against HFD-induced oxidative damage on the liver were evaluated. The ROS content (Fig. 5a) and ROS-regulated

Table 1
Effect of *L. rhinocerus* on serum biochemical parameters in hamster.

	control	**HFD**	**LLR**	**HLR**
AST (U/L)	44.7 ± 10.3	85.3 ± 24.5*	46.7 ± 8.1#	56.7 ± 14.0#
ALT (U/L)	72.3 ± 0.6	148.0 ± 18.5*	96.0 ± 6.1#	118.0 ± 8.5#
BUN (mg/dL)	20.7 ± 1.5	19.3 ± 0.6	19.3 ± 2.9	16.3 ± 0.6*
CREA (mg/dL)	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0

The serum biochemical parameters, including AST, ALT BUN and CREA were assayed by HITACHI 7050 automatic blood chemical analyzer. Each value is expressed as mean ± SD ($n = 6$). *compared to control group, $p < 0.05$. #compared to HFD group, $p < 0.05$.



	control	HFD	LLR	HLR
LDL-c (mg/dL)	5.0	365	356	385
HDL-c (mg/dL)	185.0	249.3	294.0	299.3
LDL-c/HDL-c	0.03	1.46	1.21	1.29

Fig. 4. Effects of *L. rhinocerus* on TG, CHOL, HDL-c, and LDL-c contents in hamster blood and liver. CHOL and TG contents in blood (a) and liver (b) were measured. (c) Distributions of VLDL, LDL-c, and HDL-c in serum. Each value is expressed as mean ± SD ($n = 6$). *compared to the control group, $p < 0.05$. #compared to the HFD group, $p < 0.05$.

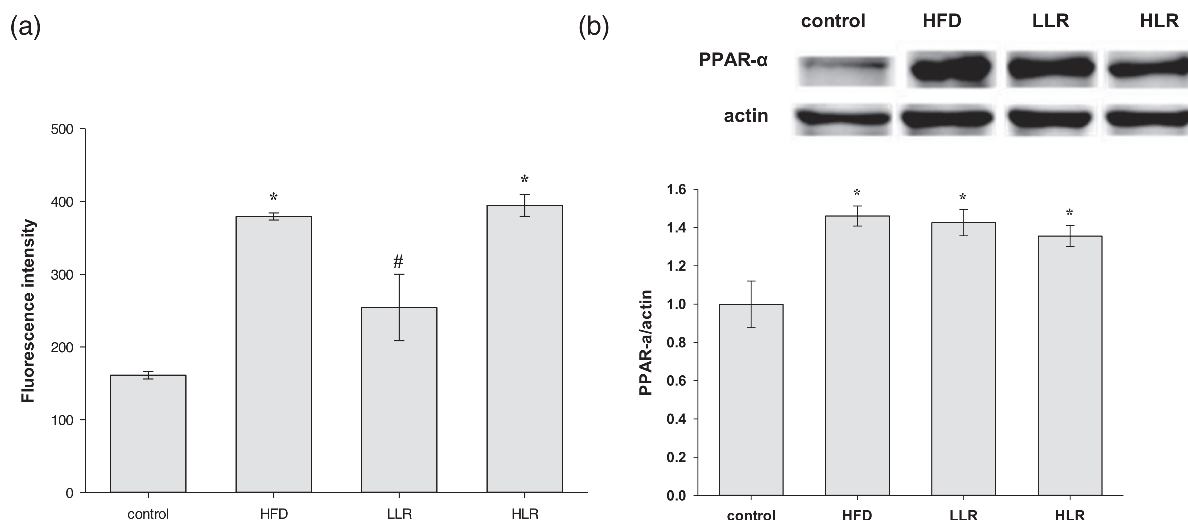


Fig. 5. Effects of *L. rhinocerus* on ROS content and PPAR-α expression in hamster liver. ROS content in liver was quantified using DCFH-DA fluorescent probe (a), and PPAR-α expression was quantified by the Western blot and normalized by actin. Each value is expressed as mean ± SD ($n = 3$). *compared to the control group, $p < 0.05$. #compared to the HFD group, $p < 0.05$.

PPAR-α protein expression (Fig. 5b) in hamster liver were measured. The HFD elevated the ROS content in hamster liver, and LLR treatment reversed this increase. With respect to PPAR-α expression, the HFD elevated the protein level. Neither LLR nor HLR treatment modulated HFD-induced PPAR-α protein expression.

4. Discussion

Fatty liver is a global growing health problem. Traditionally, fatty liver is associated with alcohol and an animal-based diet. A plant-based diet is known to lower the probability of having a fatty liver [14,15]. However, an unhealthy plant diet may increase the risk of NAFLD [16]. Considering that the vegan population is growing, plant diet-induced fatty liver may become the next important cause of NAFLD. In this research, a plant-based diet was used to induce fatty liver and *L. rhinocerus* was used to reverse the damage to the liver and the syndrome that it induces.

Monosaccharides and disaccharides are reportedly the major constituents of *L. rhinocerus* sclerotia at a fat content of <1%. In the aqueous extract, β-glucans was the dominant glucans, representing 82–93% of total glucan (w/w) [17]. Polysaccharides are known to be the major bioactive compounds in mushrooms. Many studies have revealed the immunomodulatory effects of *L. rhinocerus*, which, for example, ameliorates airway inflammatory hypersecretion and airway hyperresponsiveness in a murine model of asthma [18]. Polysaccharide is a well-known agent of immune modulation. In fatty liver, ROS is a critical factor that drives hepatic inflammation and fibrosis [4]. In this research, the antioxidant capacity of *L. rhinocerus* is assumed to have a role in fatty liver. Yap et al. [11] found total phenolic contents of water extracts of *L. rhinocerus* were between 2.82 and4.67 mg GAE/g (gallic acid equivalent). For wild-type *L. rhinocerus*, the total phenolic content is 0.12–0.23 mg GAE/g [11]. In the *L. rhinocerus* sample herein, it was 366.23 mg GAE/g, which is much higher than those obtained in previous investigations. The GAE of the *L. rhinocerus* which was used in this research is much higher than other edible macro-fungi which were measured by Guo et al. [19]. The cause of this difference may lie in the cultivation and extraction methods. Interestingly, Yap et al. [11] also found that methanol extract has a lower total phenolic content. The aqueous extract might be the proper method to obtain the antioxidant character of *L. rhinocerus*.

A Mediterranean diet, which is rich in antioxidants, helps to prevent NAFLD [20]. Unsaturated fatty acids, such as olive oil, are partially responsible for this protection [21]. Cholesterol, a well-known risk factor for NAFLD, is abundant in animal-based diets, but it presents in much smaller quantities in plant foods [22]. This work may be very first in which a plant-based diet is used induce fatty liver. In this study, 23% of oil content is plant-based fatty acid and 0.2% is cholesterol. After six weeks of administration, body weight (Fig. 2a), liver morphology and histopathology (Fig. 3), AST, ALT (Table 1) TG and CHOL contents in the blood and liver (Fig. 4), the HDL-c/VLDL-c ratio in blood (Fig. 4c) and the ROS content in the liver (Fig. 5) had all changed, indicating that a plant-based HFD with cholesterol can induce fatty liver.

The results in Fig. 2 show that the HFD increased the weight of hamsters, even though their food intake declined (Fig. 2b). The control group had a higher food intake than other groups perhaps because the HFD had more food calories than the normal diet. Therefore, the hamsters with the HFD might have required less food to obtain a similar amount of calories. A comparison of body weights and food intakes among the groups reveals that the body weight reduction was not associated directly with appetite or energy intake. Hence, *L. rhinocerus* may be responsible for metabolic regulation on an HFD, and so may reduce body weight, mitigating the development of obesity.

In this work, fatty liver was induced using plant-based oil, and liver morphology may provide preliminary evidence of fatty liver. Fig. 3 presents liver morphology after six weeks of the HFD and *L. rhinocerus* treatment. Based on the liver morphology, treatment with *L. rhinocerus* prevented the morphological change that would otherwise have caused the HFD, maintaining normal morphology. Fig. 3b shows that HFD treatment also caused micro/macro-vesicular steatosis and mononuclear cell infiltration. Macro-vesicular steatosis refers to a site of fatty degeneration, and micro-vesicular steatosis is characterized by small intracytoplasmic fat vacuoles, like liposomes, that accumulate within hepatocytes [23]. Mononuclear cell infiltration is caused by a change in the immune microenvironment, to which the excessive deposition of fat in the hepatocytes may contribute [24]. These are two indicators of fatty liver. No sign of reversal of vesicular steatosis was observed in the LLR and HLR treatment groups. *L. rhinocerus* treatment only had a minor dose-dependent effect on mononuclear cell infiltration in liver (Fig. 3b). Since studies have found that

polysaccharides from *L. rhinocerus* can contribute to immune modulation, *L. rhinocerus* may modulate liver inflammation that is induced by an HFD, but not significantly improve vesicular steatosis that is associated with a plant-based HFD.

Table 1 presents assayed serum AST, ALT, BUN, and CREA values. Treatment with *L. rhinocerus* strongly supported the recovery of AST and ALT levels. Aspartate transaminase (AST/GOT) and alanine transaminase (ALT/GPT) are biomarkers that can be clinically used to monitor liver health, especially in cases of NAFLD [25], possibly indicating that *L. rhinocerus* can provide a benefit to liver cells, preventing their death. BUN and CREA are two indicators of kidney function, and are especially useful in acute kidney injuries [26]. Table 1 shows that the HFD did not affect BUN and CREA levels after six weeks of treatment. Notably, HLR treatment (equivalents 1000 mg/kg of raw *L. rhinocerus*) down-regulated the BUN level. BUN is a marker of kidney function but it is also affected by non-renal factors. For example, protein intake, catabolic state, upper gastrointestinal bleeding, intravascular volume status, and therapy with high-dose steroids can affect BUN level [20]. Since the ratios of liver and kidney weights to body weight did not differ (Table S1), *L. rhinocerus* was determined to be a novel agent for kidney protection, but further study is required.

CHOL and TG levels are two indicators of fatty liver and NAFLD [5]. The HFD increased CHOL and TG levels in both blood and liver, and *L. rhinocerus* treatment reversed these increases in blood (Fig. 4a). *L. rhinocerus* treatment had limited effects on CHOL and TG contents in the liver (Fig. 4b). *L. rhinocerus* treatment elevated HDL-c levels and improved the ratio of HDL-c to LDL-c in blood (Fig. 4c). Polysaccharides are known to modulate CHOL, TG, HDL and LDL levels in blood [27,28]. Since the *L. rhinocerus* extract was prepared by hot water extraction, which can yield a large amount of water-soluble polysaccharides, the data herein may demonstrate that *L. rhinocerus* has potential to improve hyperlipidemia that is induced by an HFD. The mechanisms by the changes in CHOL and TG levels in liver are hard to determine. The soybean oil in the HFD herein were assumed to provide unsaturated fatty acids which can lower the TG content in the liver [29], potentially interfering with fatty acid metabolism. We believe that six weeks of a plant-based HFD may not suffice significantly to damage fatty acid metabolism in the liver. The protective role of *L. rhinocerus* is limited.

Finally, the ROS content and ROS-mediated PPAR-α expression in hamster liver after HFD and *L. rhinocerus* treatments were measured. Peroxisome proliferator-activated receptor α (PPAR-α) is a ligand-activated transcription factor, and PPARs mainly exist in three subtypes – α, β/δ, and γ. All of them belong to the NR1C nuclear receptor subfamily [30]. The PPAR-α, also called NR1C1, is highly expressed in the liver, and was initially identified as the molecular target of xenobiotics inducing peroxisome proliferation in rodents [31]. The ROS content in the HFD-treated liver group was about double that in the untreated group (Fig. 5a). The relevant data reveal that ROS may be a cofactor that contributes to fatty liver. Data also interestingly demonstrate that an HFD and *L. rhinocerus* can elevate the protein PPAR-α expression (Fig. 5b). Recently, Lee et al. [32] showed that an HFD (lard-induced) reduced PPAR-α expression in liver. However, the HFD in this research differs entirely from that in other studies. Herein work, unsaturated plant-based oil provided most of the fat on the HFD whereas most studies use lard (saturated animal oil). The effect of lard on hepatic steatosis is clear but the role effective of poly or mono-unsaturated fatty acids in hepatic steatosis is not fully understood. Studies show that coconut oil can increase PPAR-α mRNA expression [33] and medium-chain fatty acids can act as pan-PPAR partial agonists [34]. It is known that PPAR-α can be modulated by many factors, including ROS [35], saturated fatty acids, unsaturated fatty acids and other naturally occurring and synthetic ligands [36]. The plant oil that is used in this research is assumed to provide some naturally occurring fatty acids that up-regulated PPAR-α ligands; however, excess fatty acid also changes the cellular redox status of the liver and favors the accumulation of oxidative damage [37]. In Fig. 5, LLR reduced the ROS in liver but HLR had no effect on liver ROS. Both LLR and HLR had minor effects on PPAR-α expression in the liver. The mechanisms by which *L. rhinocerus* modulates liver ROS and PPAR-α are unclear. It is known that PPARα expression is enriched in the tissues with high fatty acid oxidation rates, such as liver, heart, skeletal muscle, and brown adipose tissue [38]. Polysaccharides and antioxidant capacity are assumed to be involved in the protective ability of *L. rhinocerus* which requires further study.

The protection that is provided by *L. rhinocerus* against HFD-induced fatty liver in this study is weaker than in similar studies, perhaps because of the method by which fatty liver was induced by plant-based fatty acids, coconut oil and soybean oil in the HFD herein. Soybean oil has been proven to have no effect on HDL and LDL levels [39]. The major cause of fatty liver in this investigation is assumed to be cholesterol, and plant-based oil might have provided excess free fatty acid that contributed to this phenomenon. Whereas another study used 12% fat (lard) and 0.2% cholesterol in the HFD [40], a higher fat content (23%) was used herein, and the resulting syndrome was less severe. Unsaturated oils, including monounsaturated oil and polyunsaturated oil, are known to reduce the risk of NAFLD [41]. This study provides new information about the effects of vegetable oil on NAFLD, and on the uses of *L. rhinocerus* in health foods and clinical applications to protect against fatty liver.

5. Conclusions

This study suggested that a plant-based diet, especially one high in oil and cholesterol, can induce NAFLD in hamster. This model motivates speculations that an unhealthy plant-oil diet is a possible factor in human NAFLD. *L. rhinocerus* extract can reverse the damage that is caused by NAFLD. This investigation may support the claim that a plant-based diet with a high oil content is a powerful risk factor for fatty liver, and it demonstrates that *L. rhinocerus* may reduce the damage that is associated with fatty liver.

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Conflicts of interest

The authors have indicated no financial conflicts of interest.

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Supplementary material

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References

[1] Ayonrinde OT. Historical narrative from fatty liver in the nineteenth century to contemporary NAFLD – Reconciling the present with the past. JHEP Rep 2021;3(3):. https://doi.org/10.1016/j.jhepr.2021.100261 PMid: 34036255100261.
[2] Van Herck MA, Vonghia L, Francque SM. Animal models of nonalcoholic fatty liver disease-a starter's guide. Nutrients 2017;9(10):1072. https://doi.org/10.3390/nu9101072 PMid: 28953222.
[3] Tacke F, Weiskirchen R. Non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)-related liver fibrosis: mechanisms, treatment and prevention. Ann Transl Med 2021;9(8):729. https://doi.org/10.21037/atm-20-4354 PMid: 33987427.
[4] Weiskirchen R. Hepatoprotective and anti-fibrotic agents: it's time to take the next step. Front Pharmacol 2016;6:303. https://doi.org/10.3389/fphar.2015.00303 PMid: 26779021.
[5] Ullah R, Rauf N, Nabi G, et al. Role of nutrition in the pathogenesis and prevention of non-alcoholic fatty liver disease: recent updates. Int J Biol Sci 2019;15(2):265–76. https://doi.org/10.7150/ijbs.30121 PMid: 30745819.
[6] Kittimongkolsuk P, Pattarachotanant N, Chuchawankul S, et al. Neuroprotective effects of extracts from tiger milk mushroom *Lignosus rhinocerus* against glutamate-induced toxicity in HT22 hippocampal neuronal cells and neurodegenerative diseases in *Caenorhabditis elegans*. Biology 2021;10(1):30. https://doi.org/10.3390/biology10010030 PMid: 33466350.
[7] Kittimongkolsuk P, Roxo M, Li H, et al. Extracts of the tiger milk mushroom (*Lignosus rhinocerus*) enhance stress resistance and extend lifespan in *Caenorhabditis elegans* via the DAF-16/FoxO signaling pathway. Pharmaceuticals 2021;14(2):93. https://doi.org/10.3390/ph14020093 PMid: 33513674.
[8] Tan ESS, Leo TK, Tan CK. Effect of tiger milk mushroom (*Lignosus rhinocerus*) supplementation on respiratory health, immunity and antioxidant status: an open-label prospective study. Sci Rep 2021;11:11781. https://doi.org/10.1038/s41598-021-91256-6 PMid: 34083710.
[9] Lee SS, Tan NH, Fung SY, et al. Anti-inflammatory effect of the sclerotium of Lignosus rhinocerotis (Cooke) Ryvarden, the tiger milk mushroom. BMC Complement Altern Med 2014;14:359. https://doi.org/10.1186/1472-6882-14-359 PMid: 25256382.
[10] Guo C, Wong K-H, Cheung PCK. Hot water extract of the sclerotium of *Polyporus rhinocerus* Cooke enhances the immune functions of murine macrophages. Int J Med Mushrooms 2011;13:237–44. https://doi.org/10.1615/IntJMedMushr.v13.i3.30 PMid: 22135875.
[11] Yap YHY, Tan N, Fung S, et al. Nutrient composition, antioxidant properties, and anti-proliferative activity of *Lignosus rhinocerus* Cooke sclerotium. J Sci Food Agric 2013;93(12):2945–52. https://doi.org/10.1002/jsfa.6121 PMid: 23460242.
[12] Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem 1990;38(3):674–7. https://doi.org/10.1021/jf00093a019.
[13] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27(5–6):612–6. https://doi.org/10.1016/S0891-5849(99)00107-0.
[14] Chiu TH, Lin M-N, Pan W-H, et al. Vegetarian diet, food substitution, and nonalcoholic fatty liver. Tzu-Chi Med J 2018;30(2):102–9. https://doi.org/10.4103/tcmj.tcmj_109_17 PMid: 29875591.
[15] Li H-Y, Gan R-Y, Shang A, et al. Plant-based foods and their bioactive compounds on fatty liver disease: effects, mechanisms, and clinical application. Oxid Med Cell Longev 2021;2021:e6621644. https://doi.org/10.1155/2021/6621644 PMid: 33728021.
[16] Mazidi M, Kengne AP. Higher adherence to plant-based diets are associated with lower likelihood of fatty liver. Clin Nutr Edinb Scotl 2019;38(4):1672–7. https://doi.org/10.1016/j.clnu.2018.08.010 PMid: 30578029.
[17] Lau BF, Abdullah N, Aminudin N. Chemical composition of the tiger's milk mushroom, *Lignosus rhinocerotis* (Cooke) Ryvarden, from different developmental stages. J Agric Food Chem 2013;61(20):4890–7. https://doi.org/10.1021/jf4002507 PMid: 23597270.
[18] Johnathan M, Muhamad SA, Gan SH, et al. *Lignosus rhinocerotis* Cooke Ryvarden ameliorates airway inflammation, mucus hypersecretion and airway hyperresponsiveness in a murine model of asthma. PLoS ONE 2021;16(3):e0249091. https://doi.org/10.1371/journal.pone.0249091 PMid: 33784348.
[19] Guo Y-J, Deng G-F, Xu X-R, et al. Antioxidant capacities, phenolic compounds and polysaccharide contents of 49 edible macro-fungi. Food Funct 2012;3(11):1195–205. https://doi.org/10.1039/c2fo30110e PMid: 22868715.
[20] Anania C, Perla FM, Olivero F, et al. Mediterranean diet and nonalcoholic fatty liver disease. World J Gastroenterol 2018;24(19):2083–94. https://doi.org/10.3748/wjg.v24.i19.2083 PMid: 29785077.
[21] Linseisen J, Welch AA, Ocké M, et al. Dietary fat intake in the European Prospective Investigation into Cancer and Nutrition: results from the 24-h dietary recalls. Eur J Clin Nutr 2009;63:S61–80. https://doi.org/10.1038/ejcn.2009.75 PMid: 19888281.
[22] Sonawane PD, Pollier J, Panda S, et al. Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. Nat Plants 2016;3:16205. https://doi.org/10.1038/nplants.2016.205 PMid: 28005066.
[23] Tandra S, Yeh MM, Brunt EM, et al. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. J Hepatol 2011;55(3):654–9. https://doi.org/10.1016/j.jhep.2010.11.021 PMid: 21172393.
[24] Feng D. The alteration of immune cells in the pathogenesis of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Liver Res 2020;4(1):23–7. https://doi.org/10.1016/j.livres.2020.02.003.
[25] Lala V, Goyal A, Bansal P, et al. Liver function tests. StatPearls, Treasure Island (FL): StatPearls Publishing; 2021. PMid: 29494096.
[26] Griffin BR, Faubel S, Edelstein CL. Biomarkers of drug-induced kidney toxicity. Ther Drug Monit 2019;41(2):213–26. https://doi.org/10.1097/FTD.0000000000000589 PMid: 30883514.
[27] Chen G, Luo YC, Ji BP, et al. Effect of polysaccharide from *Auricularia auricula* on blood lipid metabolism and lipoprotein lipase activity of ICR mice fed a cholesterol-enriched diet. J Food Sci 2008;73(6):H103–8. https://doi.org/10.1111/j.1750-3841.2008.00821.x PMid: 19241585.
[28] Seedevi P, Ramu Ganesan A, Moovendhan M, et al. Anti-diabetic activity of crude polysaccharide and rhamnose-enriched polysaccharide from *G. lithophila* on Streptozotocin (STZ)-induced in Wistar rats. Sci Rep 2020;10:556. https://doi.org/10.1038/s41598-020-57486-w PMid: 31953455.
[29] Lopez-Alvarenga JC, Ebbesson SOE, Ebbesson LOE, et al. Polyunsaturated fatty acids effect on serum triglycerides concentration in presence of metabolic syndrome components. The Alaska-Siberia Project. Metabolism 2010;59(1):86–92. https://doi.org/10.1016/j.metabol.2009.07.010 PMid: 19766268.
[30] Pawlak M, Lefebvre P, Staels B. Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. J Hepatol 2015;62(3):720–33. https://doi.org/10.1016/j.jhep.2014.10.039 PMid: 25450203.
[31] Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 1990;347:645–50. https://doi.org/10.1038/347645a0 PMid: 2129546.
[32] Lee C-Y, Lee C-L. Comparison of the improvement effect of deep ocean water with different mineral composition on the high fat diet-induced blood lipid and nonalcoholic fatty liver disease in a mouse model. Nutrients 2021;13(5):1732. https://doi.org/10.3390/nu13051732 PMid: 34065270.
[33] Manio MC, Matsumura S, Inoue K. Low-fat diet, and medium-fat diets containing coconut oil and soybean oil exert different metabolic effects in untrained and treadmill-trained mice. J Int Soc Sports Nutr 2018;15(1):29. https://doi.org/10.1186/s12970-018-0234-y PMid: 29914522.
[34] Liberato MV, Nascimento AS, Ayers SD, et al. Medium chain fatty acids are selective peroxisome proliferator activated receptor (PPAR) γ activators and pan-PPAR partial agonists. PLoS ONE 2012;7(5):e36297. https://doi.org/10.1371/journal.pone.0036297 PMid: 22649490.
[35] Ku T, Zhou M, Hou Y, et al. Tebuconazole induces liver injury coupled with ROS-mediated hepatic metabolism disorder. Ecotoxicol Environ Saf 2021;220:. https://doi.org/10.1016/j.ecoenv.2021.112309 PMid: 34015629112309.
[36] Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta 2011;1812(8):1007–22. https://doi.org/10.1016/j.bbadis.2011.02.014 PMid: 21382489.
[37] Chen Z, Tian R, She Z, et al. Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. Free Radic Biol Med 2020;152:116–41. https://doi.org/10.1016/j.freeradbiomed.2020.02.025 PMid: 32156524.
[38] Lefebvre P, Chinetti G, Fruchart J-C, et al. Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. J Clin Invest 2006;116(3):571–80. https://doi.org/10.1172/JCI27989 PMid: 16511589.
[39] Unhapipatpong C, Shantavasinkul PC, Kasemsup V, et al. Tropical oil consumption and cardiovascular disease: an umbrella review of systematic reviews and meta analyses. Nutrients 2021;13(5):1579. https://doi.org/10.3390/nu13051549 PMid: 34064496.
[40] Chen J-W, Lin Y-L, Samuel Wu Y-H, et al. Ameliorative effects of functional crude-chalaza hydrolysates on the hepatosteatosis development induced by a high-fat diet. Poult Sci 2021;100(4):. https://doi.org/10.1016/j.psj.2021.01.031 PMid: 33647717101009.
[41] Ravaut G, Légiot A, Bergeron K-F, et al. Monounsaturated fatty acids in obesity-related inflammation. Int J Mol Sci 2021;22(1):330. https://doi.org/10.3390/ijms22010330 PMid: 33396940.