Orally administration of *Neolentinus lepideus* extracts attenuated ethanol induced accumulation of hepatic lipid in mice

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Abstract In this study, we examined the effects of the water extract of *Neolentinus lepideus* (WENL), an edible mushroom, on ethanol-induced hepatic lipid accumulation. Ethanol-induced oil red O-positive spots on AML-12 hepatocytes were attenuated by WENL treatment. Furthermore, the oral administration of WENL in acute and chronic ethanol-fed mouse models resulted in the decrease in blood triglyceride and the accumulation of lipid droplets in the liver. Interestingly, the transcriptional expression related to lipid metabolisms, such as sterol regulatory element-binding protein 1, and cytochrome P450 2E1, was decreased by WENL treatment in both ethanol-induced AML-12 hepatocytes and our chronic ethanol-fed mouse models. In addition, WENL effectively attenuated the ethanol induced activation of MAP kinases and NF- κ B in AML-12 hepatocytes. Taken together, our results suggested that WENL can be effective in alleviating alcohol-induced hepatic lipid accumulation and may be used as potential candidate for the prevention of alcoholic fatty liver disease.

Keywords Liver steatosis · *Neolentinus lepideus* · Hepatocytes cells · Fatty liver

Introduction

Alcohol-induced liver disease (ALD) is one of the leading causes of mortality. In the early phase of ALD, fat accumulation usually occurs in the liver and causes hepatic steatosis. Although a fatty liver is considered a benign histological state, if excessive alcohol intake continues, a fatty liver can develop into a severe liver disease, such as fibrosis or cirrhosis. Therefore, preventing the alcohol-induced fat accumulation in the liver may be important in reducing the progression of alcoholic liver diseases. Although the exact pathway for alcoholic fatty liver has not been clearly elucidated, stimulation of lipogenic transcription factors (Chen et al., 2016), mitochondrial dysfunction (Song et al., 2013), and oxidative stress (Leung and Nieto, 2013; Li et al., 2017) seem to be involved in the processing of alcoholic fatty liver. Moreover, chronic alcohol exposure has been reported to induce triglyceride degradation in adipose tissue and increase hepatic fatty acid uptake (Kang et al., 2007; Tan et al., 2012).

As natural compounds have minimal side effects as well as multi-function effects, they have been given much focus for the development of anti-ALD drugs. The aqueous extracts of *Penthorum chinense* Pursh were reported to have a hepatoprotective effect against acute ALD (Cao et al., 2015), and cinnamon extract was also found to have protective effects against alcohol-induced liver steatosis (Kanuri et al., 2009). Cannabidiol, a natural antioxidant, was reported to have anti-ALD effects by attenuating oxidative stress (Yang et al., 2014). Mushrooms are one of the well-investigated natural sources that contain rich dietary fiber, vitamins, and minerals (Mattila et al., 2001). In addition, several important compounds, such as β -glucan, α -tocopherol, and ergosterol, have been identified from mushrooms, and their medicinal functions, such as

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anti-tumor and anti-diabetic effects, have been proposed (Friedman, 2016).

Neolentinus lepideus is an edible mushroom that is widely distributed in East Asia, specifically in China and Korea. *N. lepideus* seems to have a strong anti-hyperlipidemic activity (Yoon et al., 2011b), and fruiting bodies of *Lentinus lepideus* were reported to have several natural antioxidants (Yoon et al., 2011a). The functional components of *N. lepideus* are mainly benzoquinone derivatives, benzofuran, and cinnamic acid derivatives that can be used as inhibitors against nitro oxide (NO) production (Dokocil et al., 2016). However, the suppressive effects of *N. lepideus* on alcohol-induced fatty liver formation have never been studied.

In this study, we investigated whether the administration of the water extract of *N. lepideus* (WENL) could effectively alleviate hepatic lipid accumulation and its action of mechanism.

Materials and methods

Preparation of WENL

Neolentinus lepideus was obtained from Gyeonggido Agriculture Research and Extension Services (Gyeonggi, Korea). Exactly 300 g *N. lepideus* was extracted with 2 L of hot water in 6 h. The supernatant was separated by using whatman filter paper (2 µm pore, Sigma-Aldrich, St. Louis, MO, USA) and concentrated by using a vacuum evaporator (Heidolph Instruments GmbH & Co., Schwabach, Germany) and then lyophilized by using freeze dryer (TFD, ilShinBioBase, Seoul, Korea). The WENL yield was 12.7% (w/w, dry weight 38.1 g).

Animal experiments and plasma analysis

Six-week-old male C57BL/6N mice were purchased from Orient Bio (Gyeonggi, Korea). The mice were adapted for 1 week in a pathogen-free environment under a temperature of 23 ± 2 °C, humidity of $50 \pm 10\%$, and a controlled photoperiod (12 h light/dark cycles). The mice were provided with standard mouse food (Orient Bio, Gyeonggi, Korea) and water ad libitum. All experiments were performed in accordance with the standards of the Animal Research Committee of Sungkyunkwan University (SKKUIACUC-17-6-11-2). Acute and chronic alcoholic liver mouse models were constructed according to the protocol of National Institute on Alcohol Abuse and Alcoholism (NIAAA) (Bertola et al., 2013). The mice ($n = 32$) were divided into four groups (control, EtOH, EtOH + WENL 0.5, and EtOH + WENL 1). EtOH was obtained from Sigma-Aldrich (St. Louis, MO, USA). For

the acute alcoholic liver mouse model, the control mouse group was administrated a normal diet (Lieber-DeCarli #710027, Dyets Inc., Bethlehem, PA, USA,) and the others were fed a 5% ethanol diet (Lieber-DeCarli Ethanol diet #710260, Dyets Inc., Bethlehem, PA, USA) with 0.5 or 1 g/kg body weight/day of WENL for 9 days. On day 10, mice in the EtOH, EtOH + WENL 0.5, and EtOH + WENL 1 groups were gavaged a single dose of ethanol (5 g/kg body weight), and the control mouse group was fed an isocaloric amount of dextrin maltose. For the chronic alcoholic liver mouse model, the entire experiment continued for 4 weeks. After 5 days of adaptation to a liquid diet, normal and ethanol diets with 0.5 or 1 g/kg body weight/day of WENL were administrated to each mouse group. Ethanol was introduced by increasing its concentration to 3 (7 days), 4 (7 days), 5 (7 days), and 5 g/kg body weight (7 days). Mice were laparotomized under sevoflurane (Sigma-Aldrich, St. Louis, MO, USA) anesthesia and blood samples were collected by cardiac puncture. Serum was obtained by centrifuging the blood at 3000 g for 20 min at 4 °C and stored in a -80 °C refrigerator until analysis. The concentrations of plasma triglyceride (TG), total cholesterol (TC), serum glutamic-oxaloacetic transaminase (AST), and serum glutamic-pyruvic transaminase (ALT) were estimated by using a commercially available kit (Spotchem SP-4410, Arkray Inc., Kyoto, Japan).

Liver tissue histopathology

Liver tissue was fixed with a paraformaldehyde solution (10%, w/v) and embedded in paraffin. Sections (4 µm thick) were stained with a hematoxylin and eosin solution (Sigma-Aldrich, St. Louis, MO, USA). Images of the liver lipid droplets were taken with an optical microscope (AxioPlan 2 imaging; Zeiss, Jena, Germany).

Cell culture

AML-12 cells were obtained from ATCC and cultured in DME/F-12 media (Hyclone, UT, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin-streptomycin (BioWhittaker Inc., Walkersville, MD, USA), $1 \times$ insulin-transferrin-sodium selenite (ITS) (ITS liquid media supplement premix $100 \times$, Sigma-Aldrich, St. Louis, MO, USA), and 40 ng/mL dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). AML-12 cells of 5×10^5 were seeded on a six-well plate and incubated for 24 h at 37 °C in a CO₂ incubator. WENL (50 and 100 µg/mL) and ethanol (100 mM) were treated and further incubated for 3 days. Media was changed once a day with same media. Cells were stained with an Oil red O solution (Sigma-Aldrich, St. Louis, MO, USA), and Oil red

O-positive spots were counted under an optical microscope (Axioplan 2 imaging; Zeiss, Jena, Germany).

Western blotting

AML-12 cells of 5×10^5 were seeded on a six-well plate and incubated for 24 h at 37 °C in a CO₂ incubator. After serum starvation for 12 h, WENL (50 and 100 µg/mL) were pre-incubated for 1 h and then ethanol (100 mM) were added, and further incubated for 72 h at 37 °C in CO₂ incubator. Following incubation with ethanol, AML-12 cells were washed with ice-cold PBS and lysis with protein extraction buffer containing 20 mM Tris-HCl (pH 7.4), 70 µM EDTA, 150 mM NaCl, Nonidet P-40 (1%, w/v), and phosphatase inhibitor cocktails (Boston inc., Danvers, MA, USA) on ice for 1 h. After centrifuge at $13,000 \times g$ at 4 °C for 10 min, supernatants were separated, used to determine the concentration of proteins. Protein samples (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE). Gels were transferred to nitrocellulose membranes, blocked with 5% non-fat-milk and then probed with first antibody for 12 h at 4 °C. Rabbit anti-phospho-ERK (c9102S), rabbit anti-ERK (c9101S), rabbit anti-phospho-p38 (c9212S), rabbit anti-p38 (c9211S), rabbit anti-phospho-JNK (c9251S), rabbit anti-JNK (c9252S), rabbit anti-phospho-NFκB (c3031S), and rabbit anti-NFκB (c6956S) antibodies were obtained from cell signaling (Danvers, MA, USA). Following incubation with a rabbit horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK) at room temperature for 1 h, expression of each bands were visualized using an electrochemiluminescence (ECL) detection reagent (Biorad, Hercules, CA, USA) according to the manufacturer's protocols.

RNA isolation and real-time polymerase chain reaction (RT-PCR)

The total RNA of AML-12 cells and mouse liver was isolated by using TRIzol reagent (Invitrogen, Waltham, CA, USA). Oligo (dT) primed total RNA was used for cDNA synthesis, and a quantitative analysis was performed in a CFX connect real-time system (BioRad, Hercules, CA, USA) by using the SYBR Green real time master mix (Toyobo Co., Tokyo, Japan). Relative expression of each gene was determined by using the comparative Ct method and normalized to the expression of GAPDH. The sequences of the sterol regulatory element-binding protein 1 (SREBP1) primers are 5'-TTGTACCTACTGTGGC-TAAATGAGA-3' (forward) and 5'-CTTGTTTGTAA-CATTCTGCTT-3' (reverse); FASN primers, 5'-CCATGCCAGAGGGTGGTTG-3' (forward) and 5'-AACGTCACCTCCAGCTAGAC-3' (reverse); ACC

primers, 5'-CAGGCACACACGATGGAC-3' (forward) and 5'-CGGAGTGAATCTGGGTTGAT-3' (reverse); cytochrome P450 2E1 (CYP2E1) primers, 5'-ACAGGGTTATTGGGCCAA-3' (forward) and 5'-TCTCATGCACTACAGCGT-3' (reverse); GAPDH primers, 5'-CCACCCAGAAGACTGTGGAT-3' (forward) and 5'-CACATTGGGGGTAGGAACAC-3' (reverse).

Estimation of total phenolic contents and total flavonoids

The total phenolic content of WENL was determined by using Folin-Cioaltea method (Abdullah et al., 2012). Initially, 100 µL of WENL solution (5 mg/mL) was mixed with 100 µL of 10% (w/v) Folin-Cioaltea reagent (Sigma-Aldrich, St. Louis, MO, USA). Mixture was incubated at room temperature for 2 min and then 200 µL of 10% (w/v) sodium carbonate solution (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 1 h at room temperature in dark room. Finally, the absorbance of the solution was measured at 750 nm in a 96 well plate by using microplate absorbance reader (Biorad, Hercules, CA, USA). Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) was used to determine standard curve (50–100 µg/mL, $R^2 = 0.999$). The total phenolic contents were expressed as mg of gallic acid equivalents (GAE)/g of WENL. The total flavonoid contents of WENL were determined according to the methods of Moreno et al. (2000) with some modification. Briefly, 110 µL of WENL solution (10 mg/mL), 44 µL of 10% (w/v) aluminium nitrate (Sigma-Aldrich, St. Louis, MO, USA), 44 µL of 1 M potassium acetate (Sigma-Aldrich, St. Louis, MO, USA), 616 µL distilled water, 330 µL of 80% ethanol were mixed and incubated for 40 min at room temperature in the dark room. Finally, the absorbance of the solution was measured at 415 nm by using Optizen POP UV spectrophotometer (MECASYS, Daejeon, Korea). Quercetin (Sigma-Aldrich, St. Louis, MO, USA) was used to determine standard curve (50–400 µg/mL, $R^2 = 0.999$). The total flavonoid contents were expressed as mg of quercetin equivalents (QE)/g of WENL.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and two-tailed unpaired Student's *t* test using SPSS 19.0 for Windows. The data were presented as the mean $\pm$ standard deviation. Statistical significant was considered at $p < 0.05$.

Results and discussion

WENL attenuated the ethanol-mediated lipid accumulation on AML-12 cells

We first determined the cytotoxicity of WENL on AML-12 cells. Administration of WENL up to a concentration of 200 $\mu\text{g/mL}$ did not show cytotoxicity (Fig. 1A). Based on this results, to check the inhibitory effects of WENL on lipid accumulation, WENL (50 and 100 $\mu\text{g/mL}$) was added to the ethanol-induced mouse hepatocytes cell line (AML-12). Lipid accumulation was calculated by counting the Oil Red O-positive spots. As shown in Fig. 1B, the number of Oil Red O-positive spots dramatically increased in ethanol-treated cells compared with the control cells. However, the number of Oil Red O-positive spots dose-dependently decreased in the WENL-treated AML-12 cells (Fig. 1C) ($p < 0.05$). In addition, we checked the transcriptional expression of fatty acid synthesis-related genes, such as sterol regulatory element-binding protein 1 (SREBP1) (Fig. 1E), acetyl-coA carboxylase (ACC) (Fig. 1F), fatty acid synthase (FASN) (Fig. 1G). In addition, we also checked the transcriptional expression of cytochrome P450

2E1 (CYP2E1) (Fig. 1D) which is related with ethanol induced fatty liver. As expected, the administration of ethanol to AML-12 hepatocytes significantly increased the expression of these genes. However, the administration of WENL dose-dependently attenuated the effects of ethanol on the transcriptional expression of these genes (Fig. 1) ($p < 0.05$). These data suggest that WENL has suppressive effects on lipid accumulation induced by ethanol in hepatocytes by attenuating the expression of SREBP1, ACC, FASN and CYP2E1 genes.

Administration of WENL attenuated the concentration of blood TG in ethanol feeding mouse

To further estimate the effects of WENL on ethanol-induced fatty liver formation, WENL was administrated to both acute and chronic ethanol-fed mouse models (Fig. 2). In the acute ethanol-fed mouse liver model, the concentration of blood TG and total cholesterol was significantly ($p < 0.05$) increased in the ethanol-fed mouse groups compared with the control mouse group. In addition, increased levels of AST and ALT were detected in the

Fig. 1 Alcohol-induced lipogenesis on AML-12 hepatocyte was attenuated by administration of WENL. (A) Various concentration of WENL (0–1000 $\mu\text{g/mL}$) was treated to AML-12 cells for 24 h. Cell viability was calculated by using the WST-assay kit. Each data value is expressed as the mean $\pm$ SE of three wells. Asterisks (*) indicate significant ($p < 0.05$) differences compared to the control. (B) AML-12 hepatocytes were treated with ethanol with and without WENL (50–100 $\mu\text{g/mL}$) for 24 h. After fixation, lipid droplets were stained with the Oil red O solution. Scale bar represents 5.0 μm . (C) Oil red

O-positive spots are counted and tabled. The transcriptional expressions of CYP2E1 (D), SREBP1 (E), ACC (F), and FASN (G) were examined by real-time RT-PCR. Each data value is expressed as the mean $\pm$ SE for at least three independent experiments. Different letters indicate significant ($p < 0.05$) differences between the groups. WENL, Water extract of *Neolentinus lepidus*; CYP2E1, cytochrome P450 2E1; SREBP1, sterol regulatory element-binding protein 1; ACC, acetyl-coA carboxylase; FASN, fatty acid synthase

Fig. 2 Administration of WENL alleviated the blood TG levels in acute alcohol liver injury mouse models. Mouse was fed with EtOH with and without WENL (0.5–1 g/kg body weight) for 10 days (acute alcohol liver injury model), and the levels of TG (A), Total cholesterol (B), AST (C), and ALT (D) were compared with those in the control mouse group (n = 8). The increased levels of TG in the EtOH-administrated mouse group (n = 8) were significantly attenuated in the WENL-administrated mouse group (EtOH + WENL 1). To investigate the effect of WENL on chronic alcohol liver injury model, mouse was fed with EtOH with and without WENL (0.5–1 g/

kg body weight) for four weeks, and the levels of TG (E), Total cholesterol (F), AST (G), and ALT (H) were compared with those in the control mouse group (n = 8). The increased levels of TG and Total cholesterol in the EtOH mouse group (n = 8) were significantly attenuated in the WENL administrated mouse groups (EtOH + WENL 0.5 and EtOH + WENL 1). Each data value is expressed as the mean $\pm$ SE, and different letters indicate the significant differences between the groups ($p < 0.05$). WENL, water extracts of *Neolentinus lepidus*; TG, triacylglycerol; AST, glutamic-oxaloacetic transaminase; ALT, glutamic-pyruvic transaminase

acute ethanol-fed mouse group compared with the control mouse group ($p < 0.05$). Although the administration of WENL did not effectively decrease the ethanol-induced AST and ALT levels (Fig. 2C, D), the bloods content of TG significantly ($p < 0.05$) decreased in the WENL-administrated mouse group (EtOH + WENL 1) compared with the control mouse group (Fig. 2A). Interestingly, in our chronic ethanol-fed mouse liver model, the increased concentration of blood TG and total cholesterol in the ethanol-fed mouse groups was significantly ($p < 0.05$) attenuated by WENL administration (Fig. 2E, F). These results suggest that the administration of WENL could effectively attenuate ethanol-induced blood lipids, especially the concentration of TG.

Ethanol-induced hepatic lipid droplets decreased in WENL-administrated mouse liver

To further confirm the effects of WENL on ethanol-induced hepatic lipid accumulation, we compared the number of lipid droplets in the liver between the control and the chronic ethanol-fed mouse groups. As shown in Fig. 3A,

the number of lipid droplets dramatically increased in the ethanol-fed mouse groups compared with the control mouse group. However, the lipid droplets almost disappeared in the WENL-administrated mouse liver (Fig. 3A). Furthermore, the increased transcriptional expression levels of liver CYP2E1 and SREBP1, but not of ACC and FASN, in the ethanol-fed mouse groups were significantly attenuated by WENL administration (Fig. 3B). These results indicate that the administration of WENL could effectively alleviate the ethanol-induced lipid accumulation in the liver by mainly attenuating the transcriptional expression of the CYP2E1 and SREBP1.

Fatty liver is considered a single disease that is associated with other diseases related to fat metabolism (Ferre and Fofelle, 2010). Fatty liver is closely related to excessive intake of alcohol and obesity, and it can develop to severe ALD, such as inflammatory liver (steatosis). Recent studies have suggested that daily alcohol intake (24 g/d) can activate the hepatic lipogenic pathway (Siler et al., 1999), and several molecular and biochemical mechanisms in developing ALD have been suggested (Gramenzi et al., 2006; Han et al., 2015). Given that

Fig. 3 Accumulation of lipid droplets in mouse liver was attenuated by WENL administration. The representative areas of the liver tissue of each mouse group ($n = 8$) of the chronic ethanol-fed mouse liver injury model are shown (A) and transcriptional expressions of CYP2E1, SREBP1, ACC, and FASN were examined by real-time RT-PCR (B). Mouse liver tissues were isolated from each mouse group, and paraffin-embedded sections were stained with a hematoxylin and eosin solution. The numbers of lipid droplets dramatically increased in the EtOH mouse group (EtOH) compared with the control mouse group (Control). However, the lipid droplets were disappeared in the WENL-administrated mouse groups (EtOH + WENL 0.5 and EtOH + WENL 1) compared with the EtOH mouse group (EtOH).

complex mechanisms underlie ALD, developing agents that can completely treat ALD seems a difficult task. However, developing agents than can reduce fat accumulation in the liver is useful in alleviating alcoholic liver injury.

Mushrooms have been used in oriental medicine for the treatment many diseases. Several edible mushrooms have been reported to have nutraceutical effects in the immune system, hyperlipidemia, and tumor progressions (Chang, 1996; Ukawa et al., 2002; Ukawa et al., 2007; Wasser, 2002; Zaidman et al., 2005). Some mushrooms can suppress hepatic fat accumulation. For examples, Mukitake mushroom was found to have suppressive effects on hepatic lipogenesis, and *Pleurotus eryngii* cellulose was also reported to have suppressive effects on high-fat-induced hepatic liver formation in rat. *N. lepideus*, which was used in this study, is an edible mushroom that belongs to the family of Tricholomacetae and order of Agaricales. Although the anti-hyperlipidemic activities of *N. lepideus*

Arrows indicate lipid droplets. Lipid droplets exceed $5\mu\text{m}$ diameter were counted and tabulated. Scale bar represents $100\mu\text{m}$. The expression of CYP2E1 and SREBP1, but not of ACC and FASN, was significantly ($p < 0.05$) increased in the EtOH mouse groups compared with the control group. The administration of WENL significantly ($p < 0.05$) attenuated the ethanol-induced CYP2E1 and SREBP1 expression in the liver. Each data value is expressed as the mean $\pm$ SE, and different letters indicate the significant differences between the groups ($p < 0.05$). WENL, water extracts of *Neolentinus lepideus*; CYP2E1, cytochrome P450 2E1; SREBP1, sterol regulatory element-binding protein 1; ACC, acetyl-coA carboxylase; FASN, fatty acid synthase

was already reported (Yoon et al., 2011b), to our knowledge, this study is the first to investigate the inhibitory activity of *N. lepideus* in alcohol-induced hepatic fat accumulation. Alcohol-induced lipid accumulation in the hepatocyte and mouse liver was dramatically alleviated by the administration of WENL (Figs. 1, 3). Although the daily administration of WENL up to 1 g/kg body weight did not appear to be sufficient for reducing the levels of blood AST and ALT in the acute alcoholic liver mouse model (Fig. 2C, D), the alcohol-induced increase in blood TG concentration was significantly attenuated by the administration of WENL (Fig. 2A). Moreover, the administration of WENL was effective in reducing the blood contents of TG in the chronic alcoholic liver mouse model (Fig. 2E). These results strongly indicate that WENL could be developed as natural agents for preventing alcohol-induced lipid accumulation.

Generally, fatty liver is a result of the accumulation of TG and other fats in liver cells. Although several

mechanisms underlying the process of alcohol-mediated fatty liver have been suggested, the imbalance of alcohol-mediated lipogenesis is one of the critical reasons in the progression of alcoholic fatty liver (Wada et al., 2008). To determine the regulatory role of WENL in alcohol-induced fatty liver formation, we investigated the effects of WENL on the transcriptional expression of lipogenesis-related genes, such as ACC, FASN, and SREBP1. ACC is a well-known lipogenesis-related gene that provides the malonyl-CoA for the biosynthesis of fatty acids (Tong, 2005), and FASN plays an important role in lipogenesis by catalyzing the synthesis of palmitate from acetyl-CoA and malonyl-CoA (Alberts et al., 1975). SREBP1 is a transcription factor that is required for cholesterol and fatty acid biosynthesis (Brown and Goldstein, 1997). Many groups reported that alcohol exposure enhances lipogenesis-related genes, which improve hepatic lipogenesis, thereby resulting in fatty liver. Interestingly, the increased levels of ACC, FASN, SREBP1, and CYP2E1 in ethanol-treated hepatocytes were significantly attenuated by WENL administration (Fig. 1). However, only the transcriptional expression of the SREBP1 and CYP2E1 genes were attenuated by the administration of WENL in our chronic alcoholic liver mouse model (Fig. 3). We thought that the dose of WENL which used in animal study was not sufficient to suppress the expression of ACC and FASN. Considering the fact that SREBP1 is a transcription factor of ACC and FASN genes (Ma et al., 2008; Macfarlane et al., 2008), the use of higher dose of WENL in further our animal study may result in decrease of ethanol induced expression of lipogenesis related genes including ACC and FASN genes.

CYP2E1 is a member of the cytochrome P450 and is responsible for the breakdown of a large number of toxic chemicals in human body (Rendic and Di Carlo, 1997). CYP2E1 have been reported to be a major regulator to ethanol mediated oxidative stress (Leung and Nieto, 2013; Lu and Cederbaum, 2008). In addition, in the chronic ethanol induced fatty liver, CYP2E1 seems to have critical roles in pathogenic regulation. For examples, the expression of CYP2E1 is closely related with the degree of steatosis in rat (Jarvelainen et al., 2000). Inhibiting the expression of CYP2E1 by using specific inhibitor of CYP2E1, chlormethiazole, attenuated the ethanol induced liver injury (Gouillon et al., 2000). Ethanol-induced steatosis was not occurred in CYP2E1 deficient mice (Lu et al., 2008). Therefore, isolation of natural agents which can attenuate the expression of CYP2E1 may be effective to develop anti-steatosis drug. In our study, administration of WENL significantly attenuated the ethanol expression of CYP2E1 in hepatocyte and chronic alcoholic liver mouse model. So, if we determined the active single compound

from WENL, WENL could be developed as anti-steatosis agents.

WENL attenuated alcohol induced intracellular signaling pathway

To further investigate the underlying mechanisms of WENL mediated hepatic changes, we investigated the expressional changes of mitogen activated protein kinases (MAPKs) such as ERK, p38 and JNK. Administration of ethanol to AML-12 cells dramatically increased the phosphorylation of ERK, p38 and JNK. Such elevation was abolished by the treatment of WENL (Fig. 4A). Furthermore, we found that WENL can be effective in attenuating the alcohol induced phosphorylation of NF- κ B (Fig. 4B). Since it has been suggested that MAPKs/NF- κ B pathway are involved in ethanol induced hepatic injury (Xiao et al., 2014), the inhibitive effects of WENL on ethanol induced MAPKs/NF- κ B activation could be a mechanism on WENL mediated inhibitive effects on alcohol induced hepatic lipids accumulations.

Total phenolic acids and total flavonoids of WENL

Finally, to determine the active component of WENL, we first measured the total amount of phenolic acids and flavonoids of WENL. Gallic acid and quercetin were used for preparing standard curve for total phenolic acids and total flavonoids respectively. The total amount of phenolic acids and flavonoids of WENL was 20.9 ± 0.55 mg GAE/g and 6.5 ± 1.62 QE/g respectively. Only a few active components of *Neolentinus lepideus* such as lepidopyrone, a gamma-pyrone derivative, and novel tyrosinase inhibitors have been reported as active components of *Neolentinus lepideus* (Hosoe et al., 2007; Ishihara et al., 2018). However, there were no reports about functional study of active phenolic acids and flavonoids from *Neolentinus lepideus*. Since relatively high amount of phenolic acids was detected in WENL (20.9 ± 0.55 mg GAE/g) when compared with other mushrooms (Abdullah et al., 2012), we think that one of the phenolic acids could be an active component for WENL mediated inhibitive effects on alcohol induced hepatic lipids accumulation.

In the present study, we provided the first evidence that the administration of WENL effectively attenuates the alcohol-induced lipid accumulation in the hepatocyte and mouse liver. Although the active components of WENL was not determined in this study, it clearly showed the effects on lowering blood TG and hepatic fat accumulation of alcohol-administrated hepatocyte and mouse liver. In addition MAPKs/NF- κ B pathway has been proposed as a possible mechanism for inhibitive effects of WENL on alcohol induced hepatic fat accumulation. Further studies

Fig. 4 Administration of WENL attenuated the alcohol-induced intracellular signaling pathway. AML-12 cells were pre-treated with WENL (50–100 μg/mL) and then further incubated with and without 100 mM ethanol. AML-12 cells were harvested, and the expression of p38, phosphor p38 (p-p38), total JNK (JNK), phosphor JNK (p-JNK), total ERK (t-ERK) and phosphor ERK (p-ERK) was examined by

western blotting (A). Ethanol-induced expression of NF-κB and phosphor-NF-κB (p-NF-κB) were measured by western blotting (B). Each band was quantified by using image J program. Each data value is expressed as the mean ± SE (n = 3), and different letters indicate the significant differences between the groups ($p < 0.05$)

will be performed to determine the chemical identity of WENL that regulates alcohol-mediated fat accumulation in the liver.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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