Nutrition Research and Practice 2018;12(2):110-117

http://e-nrp.org

Ethanol extract of *Allium fistulosum* inhibits development of non-alcoholic fatty liver disease

Jin-Taek Hwang[1,2], Eun Ju Shin[1,2], Min-Yu Chung[1], Jae Ho Park[1,2], Sangwon Chung[1] and Hyo-Kyoung Choi[1§]

[1]Korea Food Research Institute, 245 Nongsaengmyeong-ro, Jeonbuk 55365, Korea
[2]Department of Food Biotechnology, Korea University of Science & Technology, Daejeon 34113, Korea

BACKGROUND/OBJECTIVES: Non-alcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease and is closely associated with metabolic syndrome. In the present study, we observed the effect of ethanol extract of *Allium fistulosum* (EAF) on NAFLD and have suggested the possibility of using EAF as a natural product for application in the development of a treatment for NAFLD.
MATERIALS/METHODS: The preventive effect on hepatic lipid accumulation was estimated by using an oleic acid (OA)-induced NAFLD model *in vitro* and a Western diet (high-fat high-sucrose; WD)-induced obese mouse model. Animals were divided into three groups (n = 7): normal diet group (ND), WD group, and WD plus 1% EAF group.
RESULTS: EAF reduced OA-stimulated lipid accumulation in HepG2 cells in the absence of cellular cytotoxicity and significantly blocked transcriptional activation of sterol regulatory element-binding protein 1 and fatty acid synthase genes. Subsequently, we investigated these effects *in vivo* in mice fed either ND or WD in the presence or absence of EAF supplementation. In comparison to the ND controls, the WD-fed mice exhibited increases in body weight, liver weight, epididymal fat weight, and accumulation of fat in hepatocytes, and these effects were significantly attenuated by EAF supplementation.
CONCLUSIONS: *Allium fistulosum* attenuates the development of NAFLD, and EAF elicits anti-lipogenic activity in liver. Therefore, EAF represents a promising candidate for use in the development of novel therapeutic drugs or drug combinations for the prevention and treatment of NAFLD.

Nutrition Research and Practice 2018;12(2):110-117; https://doi.org/10.4162/nrp.2018.12.2.110; pISSN 1976-1457 eISSN 2005-6168

Keywords: Non-alcoholic fatty liver disease (NAFLD), metabolic syndrome, western diet, lipogenesis, HepG2 cells

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the more prevalent metabolic disorders around the world and emerges as a simple form of steatosis before progressing to steatohepatitis and, ultimately, cirrhosis of the liver [1-3]. Chronic lipid accumulation is thought to be the underlying cause of NAFLD, due to its effects on dysregulated lipid metabolism in the liver. The disease is often associated with the most common clinical features of metabolic syndrome including obesity, dyslipidemia, hypertension, insulin resistance (IR), diabetes, and certain types of cancer [4-6]. Although NAFLD is a serious medical problem, its pathogenesis is not yet completely described. It is currently hypothesized that four pathogenic mechanisms are responsible for the accumulation of triglyceride (TG)-based lipid droplets, namely (i) an increased uptake of free fatty acid (FFAs) from high-fat foods by adipocytes in body fat, (ii) an increased synthesis of FFAs in the liver from glucose or acetate by IR, (iii) decreased mitochondrial β-oxidation of FFAs caused by a multitude of drugs, and (iv) decreased secretion of TGs in very low density lipoproteins (VLDL) by the liver [7-9].

Currently, there are no U. S. Food and Drug Administration (FDA)-approved pharmacologic agents or FDA guidelines for the treatment of NAFLD, despite it being the most common liver disease in the USA [10]. Because NAFLD is strongly associated with obesity, IR, and dyslipidemia, pharmacologic therapy directed at weight loss, IR, and/or dyslipidemia have been considered potential therapeutic approaches. However, due to the potentially hazardous side effects of anti-obesity drugs, including orlistat and sibutramine, a number of natural phytochemical compounds to treat NAFLD have been explored. Interestingly, epigenetic and environmental factors such as exercise and diet are known to interact in the definition of the NAFLD phenotype and to determine its progression [11,12]. For this reason, lifestyle modifications, similar to those recommended for obesity, remain the currently recommended therapeutic option [13].

Allium fistulosum, a perennial herb in the genus *Allium* of the

This research was supported by the Main Research Program (E-0150301-02) of the Korea Food Research Institute (KFRI) funded by the Ministry of Science, ICT & Future Planning.
§ Corresponding Author: Hyo-Kyoung Choi, Tel. 82-63-219-9421, Fax. 82-63-219-9876, Email. chkyoung@kfri.re.kr
Received: Octorber 17, 2017, Revised: November 17, 2017, Accepted: February 21, 2018

Alliaceae family, is used widely as an ingredient in Chinese, Japanese, and Korean cuisine [14]. In addition, *A. fistulosum* has been traditionally applied to treat common colds, headache, abdominal pain, and cardiovascular disease [15]. Previously, researchers have reported that *A. fistulosum* also exhibits anti-platelet, anti-oxidative, anti-hypertensive, and anti-hyperlipidemic effects [16-18], and other members of the Allium family have been reported to have inhibitory activity against pathogenic bacteria, fungi, mycotoxins, and putrefactive bacteria [19]. A recent study described the anti-obesity effect of an *A. fistulosum* extract [14].

In the present study, by assessing its effect on various parameters relevant to NAFLD *in vivo* and *in vitro*, we investigated the potential of an ethanol extract of *A. fistulosum* (EAF) as a candidate compound for suppression of NAFLD development.

MATERIALS AND METHODS

Cell culture

Human hepatocellular carcinoma (HepG2) cells were purchased from the American Type Culture Collection (Mannassas, VA, USA) and cultured in a humidified atmosphere of 5% CO_2 at 37°C with high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Welgene, Daegu, Republic of Korea). Cells were incubated with 1% BSA low glucose DMEM (ND), 0.5 mM oleic acid in 1% BSA low glucose DMEM (OA), or with 0.5 mM oleic acid DMEM supplemented with 100 or 200 μg/mL EAF for 24 h.

Preparation of Allium fistulosum extract

The *A. fistulosum* was purchased from a local market (Sungnam-si, Republic of Korea) and identified by Prof. Sang-In Shim in the Department of Agronomy, Gyeongsang National University, Republic of Korea. A voucher specimen was deposited in the Korea Food Research Institute (KFRI). The *A. fistulosum* samples were cleaned and extracted in a 10-fold volume of 70% ethanol by shaking for 24 h at 25°C, and the precipitate was removed by centrifugation at 8,000 × g for 30 min. The supernatant was lyophilized in a freeze-drier (Il Shin, Dongdochum-Si, Korea).

Cell toxicity

HepG2 cells (5×10^4) were seeded in 24-well plates, and after reaching approximately 70% confluence, cells were treated in the presence or absence of OA, alone or in combination with EAF at 100 or 200 μg/mL. After incubation for 24 h, the cells were treated with 10 μL of WST-1 solution (Enzo Life Sciences, Farmingdale, NY, USA) for 3 h. Subsequently, 100 μL of supernatant was transferred to a 96-well plate, and absorbance was measured at 450 nm (Molecular Devices, Sunnyvale, CA, USA).

Oil red O staining

HepG2 cells (5×10^4) were seeded in 24-well plates and, after reaching approximately 70% confluence, were treated in the presence or absence of OA, alone or in combination with EAF at 100 or 200 μg/mL. After incubation for 24 h, the cells were washed with 200 μL of PBS and fixed with 200 μL of 4% paraformaldehyde for 15 min at room temperature. The cells were then washed three times with PBS and incubated with 200 μL of 60% isopropanol for 5 min, followed by staining with 200 μL of 0.1% oil red O staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After additional washing with water (1 mL), images were captured under a light microscope (Olympus IX51; Olympus, Central Valley, PA, USA). For lipid quantification, isopropanol was added to each well to dissolve the lipid-stained red dye. After 10 min, the absorbance was measured at 510 nm (Molecular Devices).

Quantitative real-time PCR

HepG2 cells (5×10^4) were seeded in 24-well plates and, after reaching approximately 70% confluence, were treated in the presence or absence of OA, alone or in combination with EAF at 100 or 200 μg/mL. After incubation for 18 h, total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed with an I Cycler iQ (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification was carried out with the following primers: for sterol regulatory element-binding protein 1 (*SREBP-1c*), forward 5'-AAACTCAAG CAGGAGAACCTAAGTCT-3', reverse 5'-GTCAGTG TGTCCTCCACC TCAGT-3'; for ATP citrate lyase (*ACLY*), forward 5'-TACCACCTCAG CCATCCAGA-3', reverse 5'-GACCCCAACGAGACCAAGTT-3'; for fatty acid synthase (*FASN*), forward 5'-AACCGGCTCTCCTTCTTCTT CGACTT-3', reverse 5'-TCCGAGCGGCAGTACCCATTC-3'; and for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward 5′-ATGTTCGTCATGGGTGTGAAC-3′, reverse 5′-GCATGGACTGTGG TCATGAGT-3′.

All mRNA expression was normalized by using GAPDH. Reactions were performed in triplicate, and relative expression levels and standard deviation (SD) values were calculated by applying the comparative method.

Animal experiments

Animal experiments were conducted according to the Guide for the Care and Use of Wonkwang University (WKU16-21). Twenty-one male C57/BL6 mice (7 weeks old) were divided into three groups of 7 and housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle. After 1 week of acclimation, the groups of mice fed on a Western diet (high-fat high-sucrose diet; WD, n = 7) or a WD containing 1% EAF (*wt/wt*; WD + 1% EAF, n = 7). Mice fed on a normal chow diet (ND, n = 7) in the absence of EAF were used as controls. The WD was purchased from Research Diets (#D12079B, Research Diets, New Brunswick, NJ, USA). Mouse body weight was measured at the beginning of the experiment and at 1-week intervals for 12 weeks. The extent of food consumption by each group was recorded every week for 12 weeks. At the end of the experiment, the mice were sacrificed to collect serum and tissue samples.

Hematoxylin and eosin staining

Mouse liver specimens were fixed in 4% buffered formalin, embedded in paraffin, and cut into 4-5 μm-thick sections. The sections were stained with hematoxylin and eosin (H&E). Liver morphology was examined and tissue images were captured with a microscope (Nikon ECLIPSE 80i; Nikon Instruments, Melville, NY, USA).

Measurement of aminotransferase enzymes

The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in serum were measured by applying an enzymatic approach using a commercially available kit (Asan Pharm, Seoul, Republic of Korea).

Statistical analysis

The results from the *in vitro* experiment were analyzed by using one-way analysis of variance (one-way ANOVA). Data are expressed as mean ± standard deviation (SD) values. For the *in vivo* data, values are expressed as mean ± standard error (SE) values, and the results were analyzed by using one-way ANOVA with Newman Keul's post-hoc test. The statistical analyses were conducted by using SPSS software (Ver. 20; SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

EAF reduces lipid accumulation in HepG2 cells

During screening of a natural substance library for anti-lipid accumulation, we observed that EAF reduced lipid accumulation in HepG2 cells. To evaluate whether EAF effectively blocks lipid accumulation, its anti-lipogenic properties were examined by Oil Red O staning (Fig.1A, left panel). Lipid accumulation was observed to be significantly increased in the OA-treated cells compared to that in the control group. Importantly, EAF treatment appeared to attenuate the OA-induced lipid accumulation in a dose-dependent manner. For quantitative analysis, Oil Red O dye was dissolved in isopropanol, and absorbance was measured at 510 nm (Fig. 1A, right panel). Colorimetric analysis indicated that OA treatment significantly increased lipid accumulation compared to that in the control group. In OA-treated cells, EAF attenuated lipid accumulation in a dose-dependent manner. We next examined whether the effect of EAF on the suppression of lipid accumulation occurred as a byproduct of cytotoxicity. HepG2 cells were incubated in the presence or absence of OA, alone or in combination with EAF. As shown in Fig. 1B, EAF did not affect the viability of HepG2 cells. Taken together, EFA efficiently blocked OA-induced lipid accumulation in HepG2 cells without inducing cytotoxicity.

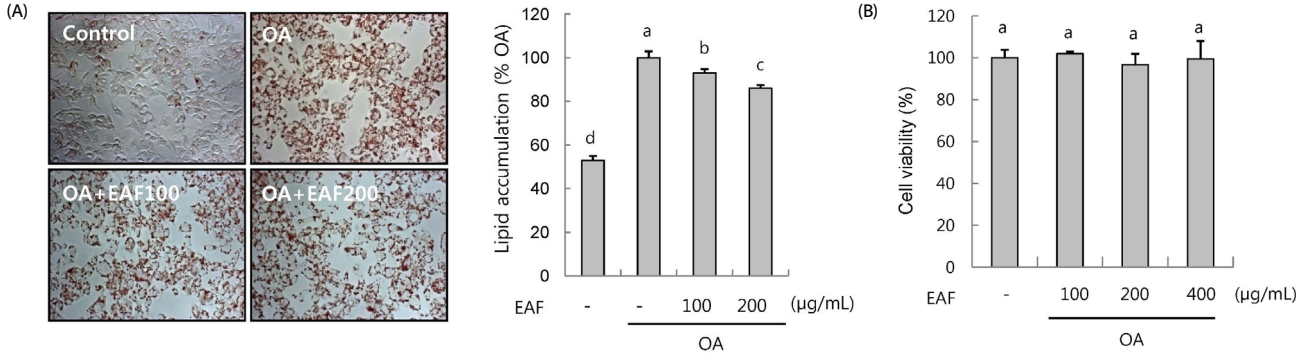

Fig. 1. Ethanol extract of *Allium fistulosum* (EAF) attenuates lipid accumulation in HepG2 cells. (A) Non-cytotoxic concentrations of EAF significantly block oleic acid (OA)-mediated lipid accumulation. HepG2 cells treated in the presence or absence of OA, alone or in combination with EAF, were stained by using an Oil Red O solution. The values presented are mean ± SD for three independent experiments. Means not sharing a common superscript are significantly different, $P < 0.05$. (B) EAF at concentrations up to 400 μg/mL did not produce cytotoxicity in HepG2 cells. Cell viability was measured by using a WST-1 assay in HepG2 cells treated in the presence or absence of OA, alone or in combination with EAF, for 24 h. There was no statistical significance detected.

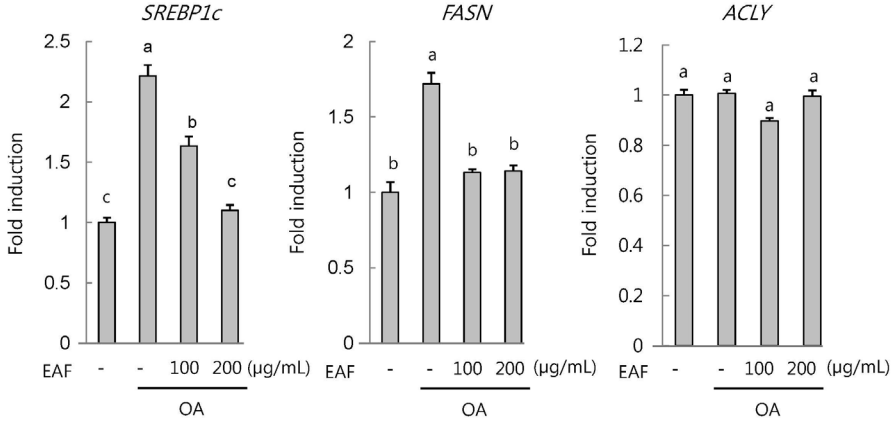

Fig. 2. Ethanol extract of *Allium fistulosum* (EAF) inhibits transcriptional activity of lipogenesis-related genes. The mRNA expressions of genes associated with lipid metabolism were measured by performing qRT-PCR. EAF was treated alone or in combination at either 100 or 200 μg/mL in the presence or absence of oleic acid (OA) in HepG2 cells for 18 h. All experiments were conducted independently three times and data are expressed as mean ± SD. Means not sharing a common superscript are significantly different, $P < 0.05$. SREBP1c, Sterol regulatory element-binding protein 1; FASN, fatty acid synthase; ACLY, ATP citrate lyase.

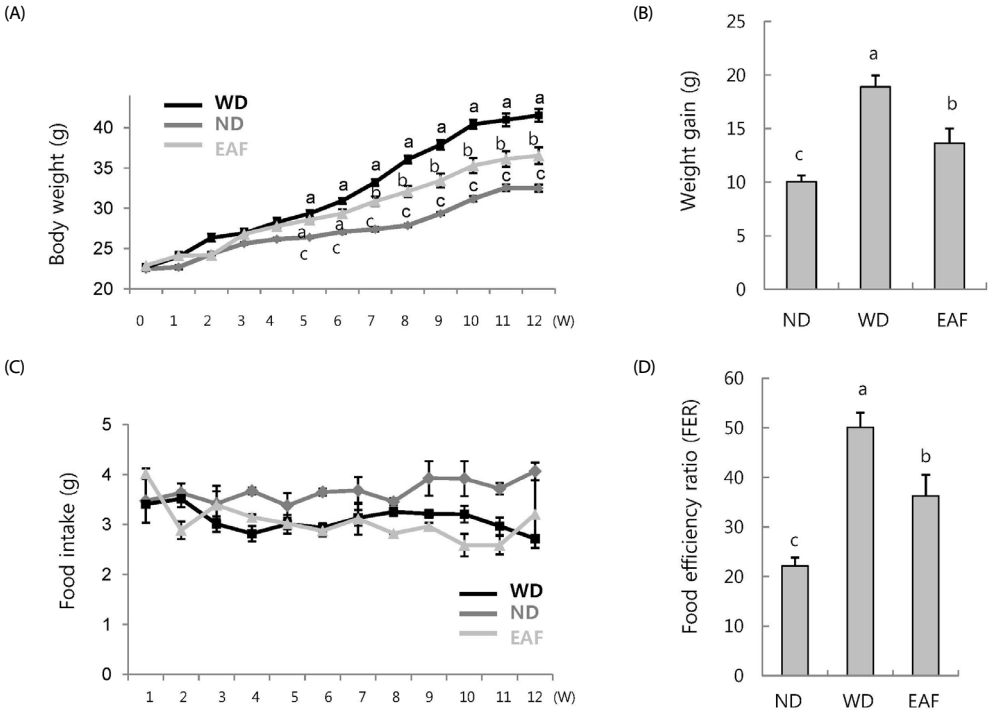

Fig. 3. Body weight, daily food intake, and food efficiency ratio analysis in mice fed on a Western diet (WD) supplemented in the presence or absence of 1% ethanol extract of *Allium fistulosum* (EAF) for 12 weeks. (A) WD-fed mice exhibit increased body weight compared to the controls, while 1% EAF supplementation significantly reduced body weight. Data are expressed as mean ± SE (n = 7/group). (B) The average weight gain after 12-week supplementation is expressed as mean ± SE (n = 7/group). (C) Daily food intake (g) in the WD-fed group is lower than in their control counterparts, but there are no significant differences among the three groups. The data are expressed as mean ± SE (n = 7/group). (D) The increased food efficiency ratio in WD-fed group was reduced by 1% EAF supplementation. Food efficiency ratio (FER) was calculated by applying the equation: FER = (body weight gain (g)/food intake (g)) × 100. FER is expressed as mean ± SE (n = 7/group). Means not sharing a common superscript are significantly different, $P < 0.05$.

EAF inhibits transcriptional activity of SREBP-1c and FASN genes, but not the ACLY gene in HepG2 cells

To examine whether the reduction of lipid accumulation by EAF was related to the transcriptional regulation of lipogenesis-associated genes, we examined changes in the expressions of *SREBP-1c*, *FASN*, and *ACLY* by using qRT-PCR. It was observed that OA treatment significantly increased the mRNA expression levels of both the *SREBP-1c* and *FASN* genes, and both of which were dramatically decreased by EAF treatment in a dose-dependent manner (Fig. 2). Of particular note, the increase in transcriptional activity of *SREBP-1c* and *FASN* by OA was almost normalized to the control group following treatment of 200 μg/mL and 100 μg/mL EAF, respectively. By contrast, there were no differences in *ACLY* expression between the groups.

Dietary supplementation with 1% EAF significantly attenuates body weight in WD-fed mice without influencing food intake

To further examine whether EAF attenuates weight gain and fat accumulation *in vivo*, mice were fed with either ND or WD in the presence or absence of 1% EAF supplementation. At the 5-week mark, the WD group had a significantly higher average body weight than that in the ND group ($P < 0.05$), while 1% EAF supplementation with WD significantly blocked weight gain from 8 weeks after the beginning of supplementation (Fig. 3A). The total weight gain at 12 weeks in the WD group was approximately 20 g, an almost two-fold increase over that in the ND group (≒ 10 g). Relative to the WD group, significantly less weight gain was observed in the 1% EAF supplementation group fed the WD (Fig. 3B). Mice fed only on WD ate less than their ND counterparts, but there were no significant differences in daily food intake between the WD and 1% EAF supplementation groups and the WD group (Fig. 3C). The food efficiency ratio was higher in the WD group than in the ND group, which was significantly decreased after 12 weeks of EAF supplementation (Fig. 3D).

Dietary supplementation with 1% EAF reduces hepatic lipid accumulation, as well as both liver and epididymal fat weight elevated by WD in mice

The liver is the primary site of dietary fat metabolism and regulates fat levels in the blood. To investigate further whether liver weight was responsible for the increases in weight gain, we assessed liver weight and liver to body weight ratio between the groups. In comparison to the ND controls, the WD group had significantly higher liver weight and liver to body weight ratio at 12 weeks ($P < 0.001$), and the increases were relatively lower in the 1% EAF supplementation group (Fig. 4A, left panel). Hepatomegaly was observed in mice fed on WD when compared to ND-fed mice. Liver weights were significantly lower in the 1% EAF supplementation group; although there were no visual differences in liver size between the WD and EAF supplementation groups (Fig. 4A, right panel). To examine whether the reduction in liver weight was attributable to decreased hepatic lipid accumulation, liver samples after 12

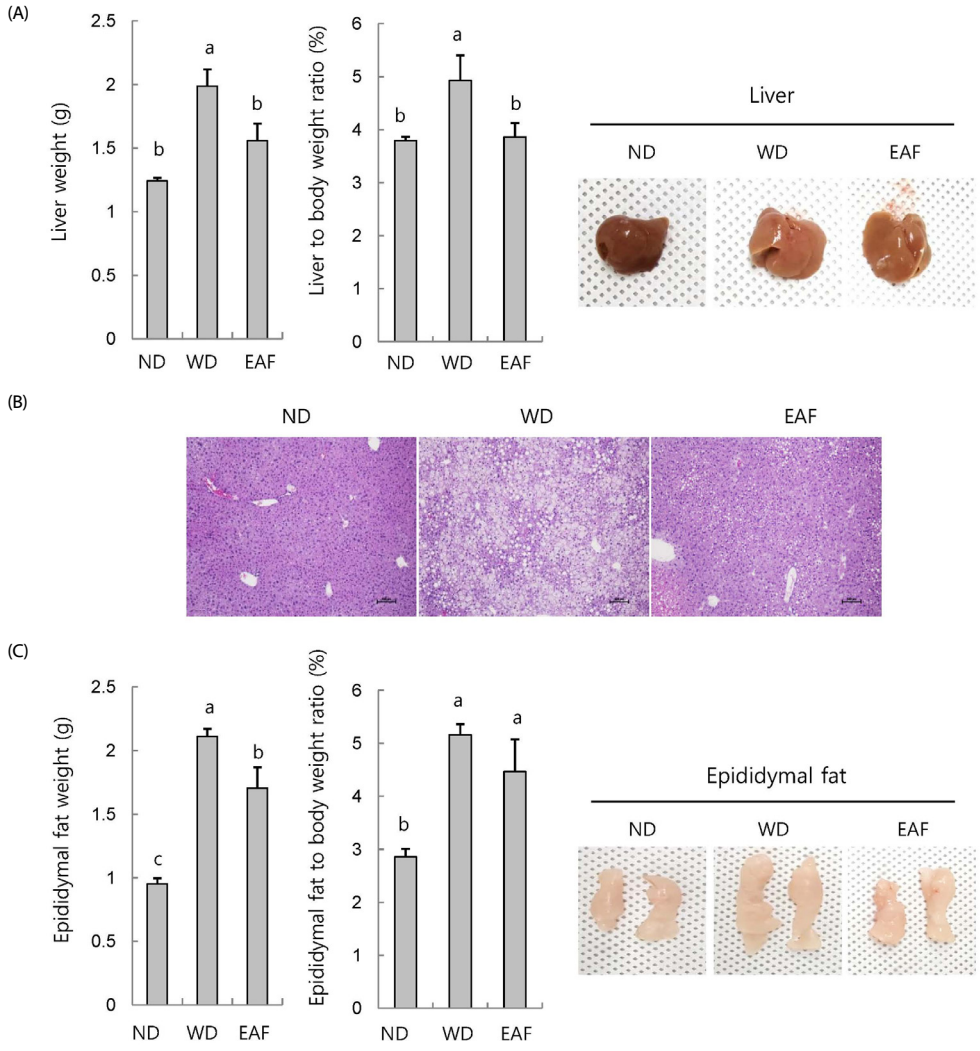

Fig. 4. The 1% ethanol extract of *Allium fistulosum* (EAF) supplement blocked gains in both liver and epididymal fat weights and attenuated hepatic lipid accumulation. (A) Liver weight was significantly increased in Western diet (WD)-fed mice and was rapidly reduced by 1% EAF supplementation with WD. The data are expressed as mean ± SE (n = 7/group). (B) Mice fed with WD exhibit greater lipid accumulation compared to the controls, while EAF supplementation markedly attenuated this effect. Representative images are shown with a 100 μm scale bar. (C) The epididymal fat weight was significantly increased in WD-fed mice and was reduced by 1% EAF supplementation. The data are expressed as mean ± SE (n = 7/group). Means not sharing a common superscript are significantly different, $P < 0.05$.

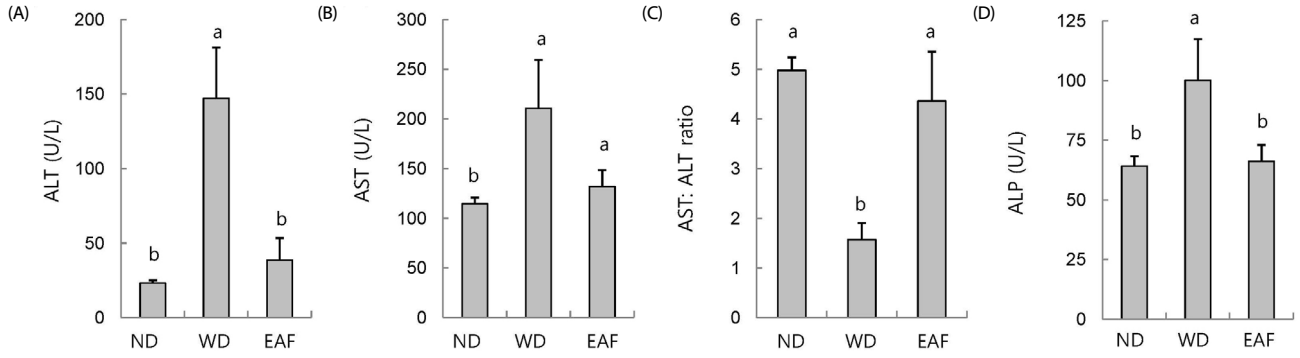

Fig. 5. The 1% ethanol extract of *Allium fistulosum* (EAF) supplement markedly reduced Western diet (WD)-induced liver abnormalities. (A) Plasma levels of alanine aminotransferase (ALT) were dramatically increased in WD-fed mice but were reduced by 1% EAF supplementation. Values are mean ± SE (n = 7/group). (B) Plasma levels of aspartate transaminase (AST) were statistically increased in WD-fed mice, and 1% EAF supplementation reduced that level; however, there was no statistical significance to the changes. Values are mean ± SE (n = 7/group). (C) The reduced AST/ALT ratio in WD-fed group was dramatically recovered by 1% EAF supplementation. The data are expressed as mean ± SE (n = 7/group). (D) Plasma levels of ALP were significantly increased in WD-fed mice and were reduced by 1% EAF supplementation. Values are mean ± SE (n = 7/group). Means not sharing a common superscript are significantly different, $P < 0.05$.

weeks of supplementation were fixed, sectioned, and stained. Histological staining (H&E stain) revealed that the WD group exhibited greater lipid accumulation than that in the ND group, and the WD-induced fat accumulation was markedly attenuated by 1% EAF supplementation (Fig. 4B). Moreover, EAF supplementation significantly reduced the WD-mediated increases in epididymal fat weight (Fig. 4C, left panel) and effectively blocked hypertrophy of epididymal fat; similar to the effects observed in the ND controls (Fig. 4C, right panel). However, there was no statistically significant difference in epididymal fat to body weight ratios between the WD and 1% EAF-fed groups, indicating the increase or decrease of epididymal fat weight induced by either WD or EAF supplementation was associated with the changes in body weight. Altogether, the results indicate that EAF supplementation reduced both liver weight and size, which were increased by WD, thereby preventing hepatic lipid accumulation *in vivo*.

Dietary supplementation with 1% EAF blocks increases in plasma indicators for liver abnormalities

Plasma ALT levels rise dramatically with acute liver-specific damage, and the plasma level of AST is used as an indicator of hepatic and extrahepatic tissue damage. The mice in the WD group had significantly higher plasma levels of both ALT ($P < 0.000$) and AST ($P < 0.016$) than those in the ND group (Fig. 5A, B). The group fed 1% EAF with WD had a lower ALT level ($P < 0.002$) than that in the WD group (Fig. 5A). The plasma levels of AST were also lower in the mice fed the 1% EAF supplement, but there was no statistical significance to the difference ($P < 0.066$, Fig. 5B). However, as shown in Fig. 5C, we observed that 1% EAF supplementation normalized the AST:ALT ratio ($P < 0.000$), which was dramatically decreased in the WD-fed group ($P < 0.004$). An increased ALP level is another indication of liver abnormalities, including obesity. The increased plasma levels of ALP induced by WD were also significantly reduced by 1% EAF supplementation (Fig. 5D).

DISCUSSION

NAFLD is the most common liver disease and is a hepatic manifestation of obesity and metabolic syndrome [20]. Due to serious side effects associated with drugs used to treat the symptoms of NAFLD, dietary intervention using natural phytochemicals has gained interest as a potentially important concept in therapeutic intervention and public health. During ongoing library screening to identify natural phytochemicals for the treatment of NAFLD, we observed that EAF attenuates OA-induced lipid accumulation without cytotoxicity (Fig. 1). To validate whether EAF could reduce the development of NAFLD, we investigated OA-induced NAFLD in HepG2 cells *in vitro* [21,22] and an WD-induced NAFLD model *in vivo*. Anti-lipogenic mechanisms in the liver are generally associated with the expression of lipogenic enzymes, cholesterol biosynthesis, TG biosynthesis, and fatty acid β-oxidation in HepG2 cells [23]. In the present study, we first demonstrated that EAF significantly prevents NAFLD by reducing the transcriptional activities of both *SREBP-1c* and *FASN* genes, which are representative lipogenesis-related genes in the liver (Fig. 2). Lipid accumulation in the liver is known to be the result of enhanced *de novo* lipogenesis, the activation of lipid uptake, and the lowering of its catabolism. These mechanisms are associated with the expression of *SREBP-1c*, which is the transcription factor responsible for fatty acid and triglyceride synthesis in the liver [24-27]. *FASN* encodes a key enzyme critical for *de novo* fatty acid and TG synthesis and catalyzes the final step in fatty acid biosynthesis; thus, *FASN* is believed to be a major determinant of the maximal hepatic capacity to generate fatty acids by *de novo* lipogenesis. The effect of *A. fistulosum* on the transcriptional activity of lipogenesis-related genes has yet to be investigated. However, there is a study showing the anti-inflammatory effect of *A. fistulosum* through inhibition of *tumor necrosis factor-a* and attenuation of excessive nitric oxide and prostaglandin generation [28-30], which are considered as pro-inflammatory mediators, induces inflammation [31,32]. It has also been reported that hyper inflammation is a major factor contributing to the development of NAFLD [33,34]. Lipogenic targets such as *PPAR-γ*, *SREBP-1c*, and *FASN* were overexpressed in the liver of the patients with abnormally increased inflammation from hepatitis C virus infection with hepatic steatosis [35]. Based on the results in previous studies, the anti-inflammatory property of *A. fistulosum* is probably a strong predictable mechanism that mediates prevention of NAFLD by reducing the transcriptional activity of lipogenesis-related genes in the liver. However, further studies are required to elucidate the exact molecular mechanisms involved.

Classic Western diets are high in both saturated fat and sugar, and the WD was originally developed as a model for NAFLD progression in mice [36-38]. It also appears to model obese humans with mild non-alcoholic steatohepatitis, as recently reported in a thorough analysis of liver pathophysiology phenotypes [39]. On that basis, the WD consumption model has been used in studies related to chronic conditions associated with obesity. In this study, we adopted the WD-fed mouse model to examine whether EAF could prevent the development of NAFLD. The EAF supplementation group showed a significant decrease in body weight gain, liver to body weight ratio, and epididymal white fat to body weight ratio compared to the WD group, and the EAF had no cytotoxic effect (Fig. 5); moreover, there was no difference in daily food intake among the groups (Fig. 3, 4). Hepatic lipid accumulation in the WD group was higher than that in the ND group (Fig. 4B). The data for the WD-fed mice are consistent with the previously reported results for a phenotype associated with HFD-induced obesity [40,41]. EAF significantly suppressed increases in various risk factors, including gain in body weight, liver to body weight ratio, and the degree of hepatic lipid accumulation, all of which are known to be involved in the progression of NAFLD (Fig. 3, 4).

There have been many reports on the hypolipidemic effects of *Allium* species. Yamamoto (2010) reported that welsh onion attenuates hyperlipidemia in rats fed on a high-fat high-sucrose diet [18]. Also, extracts of garlic, which is another *Allium* crop, have also been reported to lower plasma lipids in rats fed on a diet with or without cholesterol [42-44]. However, there were no significant changes in lipid profiles in our data (data not shown). These results are consistent with those reported by Aoyama *et al*. [32], thus supporting our observation that the

anti-lipidemic effect of *A. fistulosum* in liver was caused by attenuating the transcriptional activity of lipogenic-related genes, not through its hypolipidemic property.

Safe and effective extract dosing is necessary, regardless of the purpose of the supplementation. In animal experiments using extract administration, conversion methods based on the common perception of scaling of dose based on the body weight (mg/kg) have been used [45]. However, the animal experiments in our study adopted the method of food supplementation through diet; thus, it was not reasonable to apply dose scaling based on body weight. Therefore, we simply used a method that calculated the amount of food intake. The EAF supplemented diet contained 1% EAF, and the average of daily food intake of the study mice was 2.9 g over the 12-week experimental period, indicating that the average mouse ate 29 mg of EAF through the diet. Based on 24-hour dietary recall data obtained from 7,042 subjects in the 2016 Korea National Health and Nutrition Examination Survey, on average, Korean subjects ate 9,828 mg of *A. fistulosum* (data not shown). Considering the body weights of both mouse and human, we used a relatively high dosage of EAF in this study. Fortunately, there was no hepatic toxicity detected (Fig. 5).

Taken together, our results demonstrate that an ethanol extract of *A. fistulosum* attenuates the development of NAFLD, with EAF eliciting anti-lipogenic activity in liver. Therefore, EAF is a promising candidate for the development of novel therapeutic drugs or drug combinations for the prevention and treatment of NAFLD. Although functioning of the representative phytochemicals within *A. fistulosum* has been shown in hepatic lipogenesis at the phenotype level, there are no previous reports showing its anti-lipogenic effect via attenuation of lipogenic-related genes in the liver, even if it was limited *in vitro*. Regardless, further studies should be conducted to elucidate the exact molecular mechanism involved in the capacity of *A. fistulosum* to regulate lipogenesis in liver. In addition, to accurately determine the amount of *Allium fistulosum* required to protect against the development of NAFLD, an in-depth study is needed.

ABBREVIATIONS

NAFLD: Non-alcoholic fatty liver disease, EAF: Ethanol extract of *Allium fistulosum*, IR: Insulin resistance, TG: Triglyceride, FFAs: free fatty acids, VLDL: very low density lipoproteins, SREBP1c: Sterol regulatory element-binding protein 1, *FASN*: fatty acid synthase, ACLY: ATP citrate lyase, WD: Western diet, FER: Food efficiency ratio, AST: Aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase

AUTHORS' CONTRIBUTIONS

HKC conceived and designed the experiments and also wrote the manuscript. JTH performed the experiments and wrote a draft of the manuscript. MYJ, EJS, and SWJ supported the experiments. JHP critically reviewed the manuscript. HKC supervised the work and critically reviewed the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare no potential conflicts of interest.

ORCID

Jin-Taek Hwang: http://orcid.org/0000-0002-6650-7934
Min-Yu Chung: http://orcid.org/0000-0001-8981-215X
Eun Ju Shin: http://orcid.org/0000-0002-1727-6367
Jae Ho Park: http://orcid.org/0000-0003-4428-436X
Sang-Won Chung: http://orcid.org/0000-0001-7773-2195
Hyo-Kyoung Choi: http://orcid.org/0000-0001-9424-0432

REFERENCES

1. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129: 113-21.
2. Cohen JC, Hortaon JD, Horbbes HH. Human fatty liver disease: old questions and new insights. Science 2011;332:1519-23.
3. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. J Clin Invest 2004;114:147-52.
4. Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. Nat Med 2004;10:355-61.
5. Lei F, Zhang ZN, Wang W, Xing DM, Xie WD, Su N, Du LJ. Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced ovese mice. Int J Obes (Lond) 2007;31:1023-9.
6. Watanabe T, Hata K, Hiwatashi K, Hori K, Suzuki N, Itoh H. Suppression of murine peradipocyte differentiation and reduction of visceral fat accumulation by a Petasites Japonicus ethanol extract in mice fed a high-fat diet. Biosci Biotechnol Biochem 2010;74: 499-503.
7. Willebrords J, Pereira I, Maes M, Yanguas S, Colle I, Bossche B, Silva T, Oliveira C, Andraus W, Alves V, Cogliati B, Vinken M. Strategies, models and biomarkers in experimental non-alcoholic fatty liver disease research. Prog Lipid Res 2015;59:106-25.
8. Tannapfel A, Denk H, Dienes HP, Langner C, Schirmacher P, Trauner M, Flott-Rahmel B. Histopathological diagnosis of non-alcoholic and alcoholic fatty liver disease. Virchows Arch 2011;458:511-23.
9. Burt AD, Mutton A, Day CP. Diagnosis and interpretation of steatosis and steatohepatitis. Semin Diagn Pathol 1998;15:246-58.
10. Tolman KG, Dalpiaz AS. Treatment of non-alcoholic fatty liver disease. Ther Clin Risk Manag 2007;3:1153-63.
11. Chalasani N, Guo X, Loomba R, Goodarzi MO, Haritunians T, Kwon S, Cui J, Taylor KD, Wilson L, Cummings OW, Chen YD, Rotter JI; Nonalcoholic Steatohepatitis Clinical Research Network. Genome-wide association study identifies variants associated with histologic features of nonalcoholic fatty liver disease. Gastroenterology 2010; 139:1567-76, 1576.e1-6.
12. Anstee QM, Day CP. The genetics NAFLD. Nat Rev Gastroenterol Hepatol 2013;10:645-55.
13. Ferré P, Foufelle F. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. Diabetes Obes Metab 2010;12 Suppl 2:83-92.
14. Sung YY, Yoon Y, Kim SJ, Yang WK, Kim HK. Anti-obesity activity of Allium fistulosum L. extract by downregulation of the expression of lipogenic genes in high-fat diet-induced obese mice. Mol Med

Rep 2011;4:431-5.
15. Fenwick GR, Hanley AB. The genius Allium-part 1. Crit Rev Food Sci Nutr 1985;22:199-271.
16. Chen JH, Chen HI, Wang JS, Tsai SJ, Jen CJ. Effects of Welsh onion extracts on human platelet function in vitro. Life Sci 2000;66:1571-9.
17. Yamamoto Y, Aoyama S, Hamaguchi N, Rhi GS. Antioxidative and antihypertensive effects of Welsh onion on rats fed with a high-fat high-sucrose diet. Biosci Biotechnol Biochem 2005;69:1311-7.
18. Yamamoto Y, Yasuoka A. Welsh onion attenuates hyperlipidemia in rats fed on high-fat high-sucrose diet. Biosci Biotechnol Biochem 2010;74:402-4.
19. Beuchat LR. Control of foodborne pathogens and spoilage microorganisms by naturally occurring antimicrobials. In: Wilson CL, Droby S, editors. Microbial Food Contamination. Boca Raton (FL): CRC Press; 2001. p.149-70.
20. Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, Cassader M, David E, Cavallo-Perin P, Rizzetto M. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: further evidence for an etiologic. Hepatology 2002;35:367-72.
21. Cui W, Chen SL, Hu KQ. Quantification and mechanisms of oleic acid-induced steatosis in HepG2 cells. Am J Transl Res 2010;2: 95-104.
22. Zhu C, Xie P, Zhao F, Zhang L, An W, Zhan Y. Mechanism of the promotion of steatotic HepG2 cell apoptosis by cholesterol. Int J Clin Exp Pathol 2014;7:6807-13.
23. Kang OH, Kin SB, Seo YS, Joung DK, Mun SH, Choi JG, Lee YM, Kang DG, Lee HS, Kwon DY. Curcumin decreases oleic acid-induced lipid accumulation via AMPK phosphorylation in hepatocarcinoma cells. Eur Rev Med Pharmacol Sci 2013;17:2578-86.
24. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 2002;109:1125-31.
25. Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation o sterol regylatiory element binding proteins in livers of fasted and refed mice. Proc Natl Acad Sci U S A 1998;95:5987-92.
26. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci U S A 2003;100:12027-32.
27. Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. J Clin Invest 1997;99:838-45.
28. Wang BS, Huang GJ, Lu YH, Chang LW. Anti-inflammatory effects of an aqueous extract of Welsh onion green leaves in mice. Food Chem 2013;138:751-6.
29. Wang BS, Chen JH, Liang YC, Duh PD. Effects of welsh onion on oxidation of low-density lipoprotein and nitric oxide production in macrophage cell line RAW 264.7. Food Chem 2005;91:147-55.
30. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. Inflammopharmacology 2007;15:252-9.
31. Sala A, Folco G. Actual role of prostaglandins in inflammation. Drug Investig 1991;3:4-9.
32. Aoyama S, Hiraike T, Yamamoto Y. Antioxidant, lipid-lowering and antihypertensive effects of red welsh onion (Allium fistulosum) in spontaneously hypertensive rats. Food Sci Technol Res 2008;14: 99-103.
33. Asrih M, Jornayvaz FR. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. J Endocrinol 2013;218:R25-36.
34. Tilg H, Moschen AR. Evolution on inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 2010;52:1836-46.
35. Lima-Cabello E, Garcia-Mediavilla MV, Miguilena-Colina ME, Vargas-Castrillon J, Lozano-Rodriguez T, Femandez-Bermejo M, Olcoz JL, Conzalez-Gallego J, Garcia-Monzon C, Sanchez-Campos S. Enhanced expression on pro-inflammatory mediators and liver X-receptor-regulated lipogenic genes in non-alcoholic fatty liver disease and hepatitis C. Clin Sci 2011;120:239-50.
36. Charlton M, Krishnan A, Viker K, Sanderson S, Cazanave S. MeComico A, Masuoko H, Gores G. Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. Am J Physiol Gastrointest Liver Physiol 2011;301:G825-34.
37. Kohli R, Kirby M, Xanthakos SA, Softic S, Feldstein AE, Saxena V, Tang PH, Miles L, Miles MV, Balistrere WF, Woods SC, Seeley RJ. High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis. Hepatology 2010;52:934-44.
38. Tetri LH, Basaranoglu M, Brunt EM, Yerian LM, Neuschwander-Tetri BA. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. Am J Physiol Gastrointest Liver Physiol 2008;295:G987-95.
39. Machado MV, Michelotti GA, Xie G, Almeida Pereira T, Boursier J, Bohnic B, Guy CD, Diehl AM. Mouse models of diet-induced nonalcoholic steatohepatitis reproduce the heterogeneity of the human disease. PLoS One 2015;10:e0127991.
40. Almind K, Kahn CR. Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice. Diabetes 2004;53:3274-85.
41. Hoevenaars EP, Keijer J, Swarts HJ, Snaas-Alders S, Bekkenkamp-Grovenstein M, van Schothorst EM. Effects of dietary history on energy metabolism and physiological parameters in C57BL/6J mice. Exp Physiol 2013;98:1053-62.
42. Bordia A, Verma SK, Vyas AE, Khabya BL, Rathore AS, Bhu N, Dedi HK. Effect of essential oil and garlic on experimental atherosclerosis in rabbits. Atherosclerosis 1977;26:379-86.
43. Bordia A, Verma SK, Srivastava KC. Effect of garlic (Allium sativum) on blood lipids, blood sugar, fibrinogen and fibrinolytic activity in patients with coronary artery disease. Prostaglandins Leukot Essent Fatty Acids 1998;58:257-63.
44. Thomson M, Al-Qattan KK, Bordia T, Ali M. Including garlic in the diet may help lower blood glucose, cholesterol, and triglycerides. J Nutr 2006;136:800S-802S.
45. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7:27-31.