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Anti-Obesity Property of Lichen *Thamnolia vermicularis* Extract in 3T3-L1 Cells and Diet-Induced Obese Mice

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ABSTRACT: *Thamnolia vermicularis* (TV) is an edible lichen that is prevalent in the alpine zone of East Asia. This study evaluated the feasibility of using TV acetone extracts as a functional food based on experiments using cell line and obese mice. The cellular triglyceride levels and Oil red O staining of 3T3-L1 cells indicated that TV extracts (5 and 10 μg/mL) dose-dependently suppressed adipocyte differentiation and lipid accumulation compared with the control. The TV extract (0.4%, w/w) in a high-fat diet (HFD) was supplemented to C57BL/6N mice for 12 weeks, and TV extract supplement significantly reduced visceral fat mass and body weight compared with HFD feeding alone. The TV extract also induced significant decreases in serum and hepatic lipids, whereas it increased the serum high-density lipoproteins-cholesterol/total cholesterol ratio and fecal lipids levels. Moreover, the TV extract led to significantly lower homeostasis model assessment of insulin resistance in diet-induced obese mice. Taken together, these results suggest that the TV extract may have anti-obesity effects, including lipid-lowering, and it is a natural resource with the potential for use in obesity management.

Keywords: *Thamnolia vermicularis*, lichen, obesity, high-fat diet, 3T3-L1 cells

INTRODUCTION

Obesity is recognized as a major factor of metabolic diseases including insulin resistance, type 2 diabetes, and cardiovascular disease. The management of obesity ultimately begins with modification of diet and exercise (1). However, many people require a quick solution, such as pharmacotherapy. Accordingly, a number of drugs have been developed worldwide, but these often have many side-effects (2). As a result, there have been efforts to develop safer drugs from various natural resources (3).

Lichens are symbiotic organisms composed of a fungal and an algal partner that have been used as natural dyes, foods, traditional drugs, and cosmetics for centuries (4, 5). Even though they are known to have valuable therapeutic effects on a number of diseases and to be a rich repository of natural metabolites useful in folk medicine of many countries, few studies have investigated them in detail. Guo et al. (6) extracted a variety of phenolic compounds from lichen plants, including vermicularin, squamatic acid, barbatinic acid, D-arabitol, mannitol, and baeomycesic acid.

Thamnolia vermicularis (TV) is a lichen that mainly grows in snowy areas at altitudes of 4,000 m above sea level (e.g., Yunnan and Sichuan provinces of China). This lichen is commonly known as "white snow tea" in China (7). TV has recently gained attention because Tibetans and Chinese often drink it as a tea, and those who do are very healthy without abdominal obesity, despite having a diet composed mostly of meat. TV has long been used to counteract inflammation in traditional Chinese medicine (8). Moreover, it is known to be useful for the treatment of chronic constipation and insomnia. However, there is currently no scientific evidence supporting these claims (9). A previous *in vitro* study reported that TV had antioxidant activities including scavenging activity (10), and that these properties imparted it with the potential to prevent or treat obesity (11,12).

Therefore, this study investigated the anti-obesity effects of TV and its potential for use as a functional resource.

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MATERIALS AND METHODS

Preparation of TV extract

TV (KoLRI 002645) was obtained from the Korean Lichen and Allied Bioresource Center (KOLABIC) in the Korean Lichen Research Institute (KoLRI), Sunchon National University, Korea. The air-dried lichen thalli (5 g) were extracted with 200 mL acetone at room temperature for 48 h using sonication. The acetone extract was then filtered and concentrated under vacuum at 40°C using a rotary evaporator. The extract yield from the lichen thalli was 16.03%. The dry extracts were obtained and stored in a freezer at −20°C until further study.

Adipocyte differentiation and lipid accumulation assay in 3T3-L1 cells

The 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and then cultured as previously described (13). The proliferation of 3T3-L1 pre-adipocytes after treatment with different concentrations of TV extract was measured by the sulforhodamine B assay (14). The TV extract had no cytotoxic effects on 3T3-L1 pre-adipocytes at ≤10 μg/mL. Briefly, cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% newborn calf serum (Gibco) until confluent. At 2 days postconfluence (day 0), cell differentiation was induced by a mixture consisting of dexamethasone (0.25 μM), 3-isobutyl-1-methylxanthine (0.5 mM), and insulin (10 μg/mL) in DMEM containing 10% fetal bovine serum (FBS, Gibco). On day 2, the medium was replaced with DMEM containing 10% FBS and insulin. On days 4 and 6, the medium was changed with DMEM containing only 10% FBS. Samples were treated with the TV extract at two different concentrations (5 and 10 μg/mL) on days 0∼8, while the vehicle was treated with dimethyl sulfoxide. To measure the levels of intracellular lipids in differentiated adipocytes, Oil red O staining of the differentiated 3T3-L1 adipocytes was performed on day 8 as previously described (13), then viewed under a Leica microscope Type 090-135.002 (Leica Microsystems GmbH, Wetzlar, Germany) at 200× magnification. The dye retained in the cells was eluted with isopropanol and quantified by measuring the absorbance at 540 nm. To determine the triglyceride (TG) contents, differentiated 3T3-L1 cells were washed in phosphate buffered saline, lysed in cell lysis buffer for 1 h on ice, and then centrifuged at 10,000 rpm for 5 min (13). The supernatant was used to analyze TG levels using an assay kit (Asan Pharmaceutical Co., Ltd., Seoul, Korea).

Animals and diets

Twenty-two male C57BL/6N (four-week-old) mice were purchased from Orient Bio Inc. (Seoul, Korea). The mice were individually housed in polycarbonate cages in a temperature (22±2°C) and humidity (50±5%) controlled room under a 12 h light-dark cycle. After a one-week adaptation period, the mice were randomly divided into three groups and fed a high-fat diet (HFD, n=8, 3% corn oil and 18% lard) or HFD with 0.4% TV extract (n=7) or 0.025% orlistat (n=7, Roche Diagnostics USA, Indianapolis, IN, USA) for 12 weeks. The composition of the experimental diet was based on the AIN-76 semi-synthetic diet (Table 1). All mice received *ad libitum* diet and tap water. Afterwards, mice were fasted for 12-h, then anesthetized with ether, after which their blood samples were taken from the inferior vena cava to determine the serum biomarkers. The liver and visceral white adipose tissue (WAT) were subsequently removed after collecting blood, rinsed with physiological saline solution and immediately stored at −70°C until analysis. The present study was approved by the Sunchon National University Institutional Animal Care and Use Committee (SCNU IACUC-2016-02).

Histological analysis

The liver and epididymal WAT were removed and fixed in a buffer solution containing 10% formalin, after which the fixed tissues were paraffin-embedded and 4 μm sections were prepared and stained with hematoxylin and eosin.

Serum, hepatic, and fecal lipid levels

Serum total cholesterol (TC), high-density lipoproteins-cholesterol (HDL-C), TG (Asan Pharmaceutical Co., Ltd.), and free fatty acid (FFA) (Shinyang Diagnostics, Seoul, Korea) concentrations were determined using commercial kits. The ratio of HDL-C to TC (HTR) was calculated

Table 1. Composition of experimental diets (unit: g/kg)

Ingredient	Experimental group		
	HFD	TV extract	Orlistat
Casein	200	200	200
DL-Methionine	3	3	3
Choline bitartrate	2	2	2
Corn starch	340	336	339.75
Sucrose	150	150	150
Cellulose	50	50	50
Corn oil	30	30	30
Lard	180	180	180
Mineral mixture[1)]	35	35	35
Vitamin mixture[2)]	10	10	10
TV extract	−	4	−
Orlistat	−	−	0.25

[1)]Mineral mixture according to AIN-76.
[2)]Vitamin mixture according to AIN-76.
TV extract, *Thamnolia vermicularis* acetone extract; HFD, high-fat diet.

as follows: HDL-C/TC×100. The feces were collected during the last 7 days to determine the fecal lipid contents. The hepatic and fecal lipids were extracted using the method described by Folch et al. (15), after which the lipids contents were analyzed using the same enzymatic kit that was employed for serum analysis.

Serum hepatotoxic markers

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured based on an enzyme immunoassay (Fuji Dri-Chem 3500, Fujifilm, Tokyo, Japan).

Assessment of insulin resistance

Fasting blood glucose concentration was measured in venous blood drawn from the tail vein using a glucometer (G-doctor, All Medicus Co., Ltd., Anyang, Korea). The serum insulin levels were determined using an ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). A homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: HOMA-IR=[fasting serum insulin level (μIU/mL)×fasting blood glucose concentration (mmol/L)]/22.5.

Statistical analysis

All data are presented as the means±standard error (SE). Statistically significant differences among the groups were determined by one-way analysis of variance using the SPSS software (SPSS Inc., Chicago, IL, USA), and the differences between means were determined by Duncan's multiple-range test. Values were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Anti-adipogenic effects of the TV extract in 3T3-L1 cells

To determine the anti-obesity activity of the TV extract, we first tested whether it could suppress adipocyte differentiation in 3T3-L1 cells. The 3T3-L1 cell line is considered optimal for differentiation between an immature (pre-adipocyte) and mature state, and it has morphological and biochemical properties similar to the development of obesity in humans (16). The present study demonstrated that the TV extract (≤10 μg/mL) had no effect on the viability of pre-adipocyte 3T3-L1 cells (data not shown). Oil red O staining revealed that the TV extract (10 μg/mL) effectively inhibited adipocyte differentiation by up to 71% when compared with the vehicle (Fig. 1A and 1B). Greater increases in adipocyte levels are known to be associated with higher accumulated lipid contents of adipocytes (17). The cellular TG levels significantly increased in the vehicle when compared with pre-adipocytes, showing an increase in TG accompanied with adi-

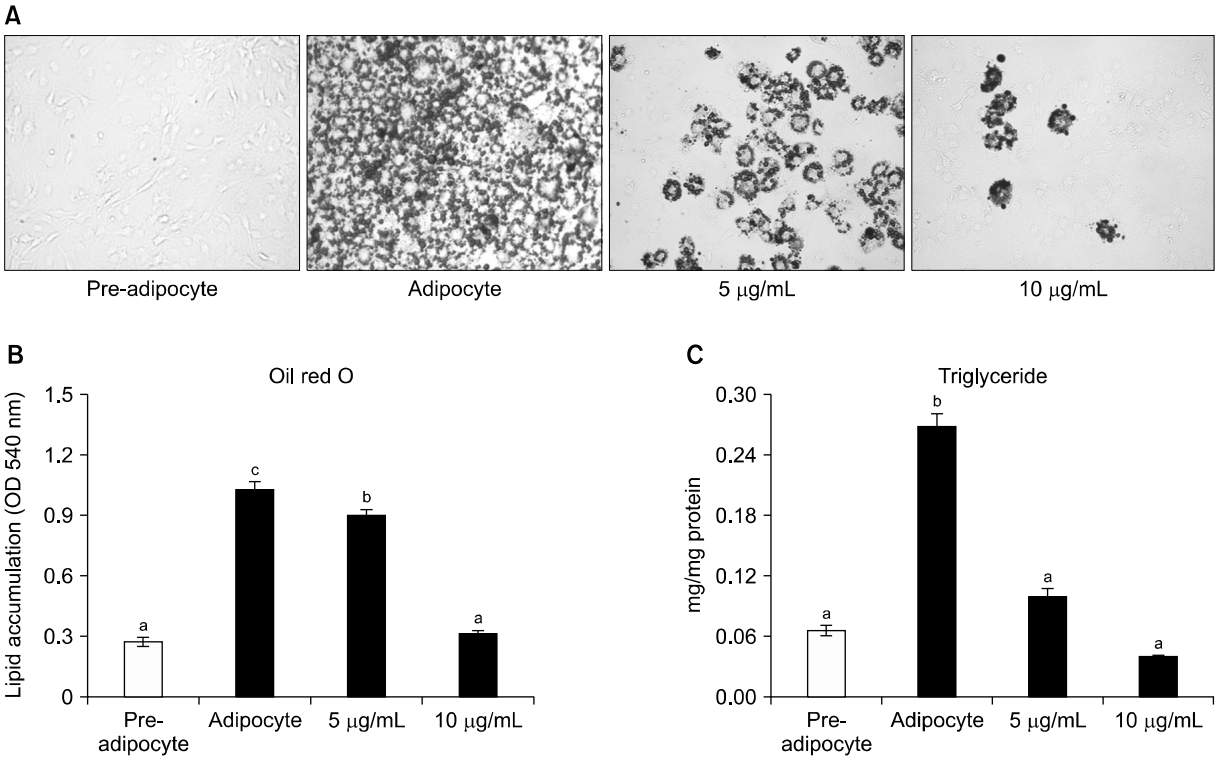

Fig. 1. Effects of *Thamnolia vermicularis* acetone extract on adipocyte differentiation and triglyceride content in 3T3-L1 adipocytes. (A) Representative photographs of lipid droplets stained with Oil red O, (B) corresponding absorbance levels of Oil red O staining, and (C) triglyceride contents. Values shown are means±SE. Values not sharing a common letter (a-c) are significantly different among groups.

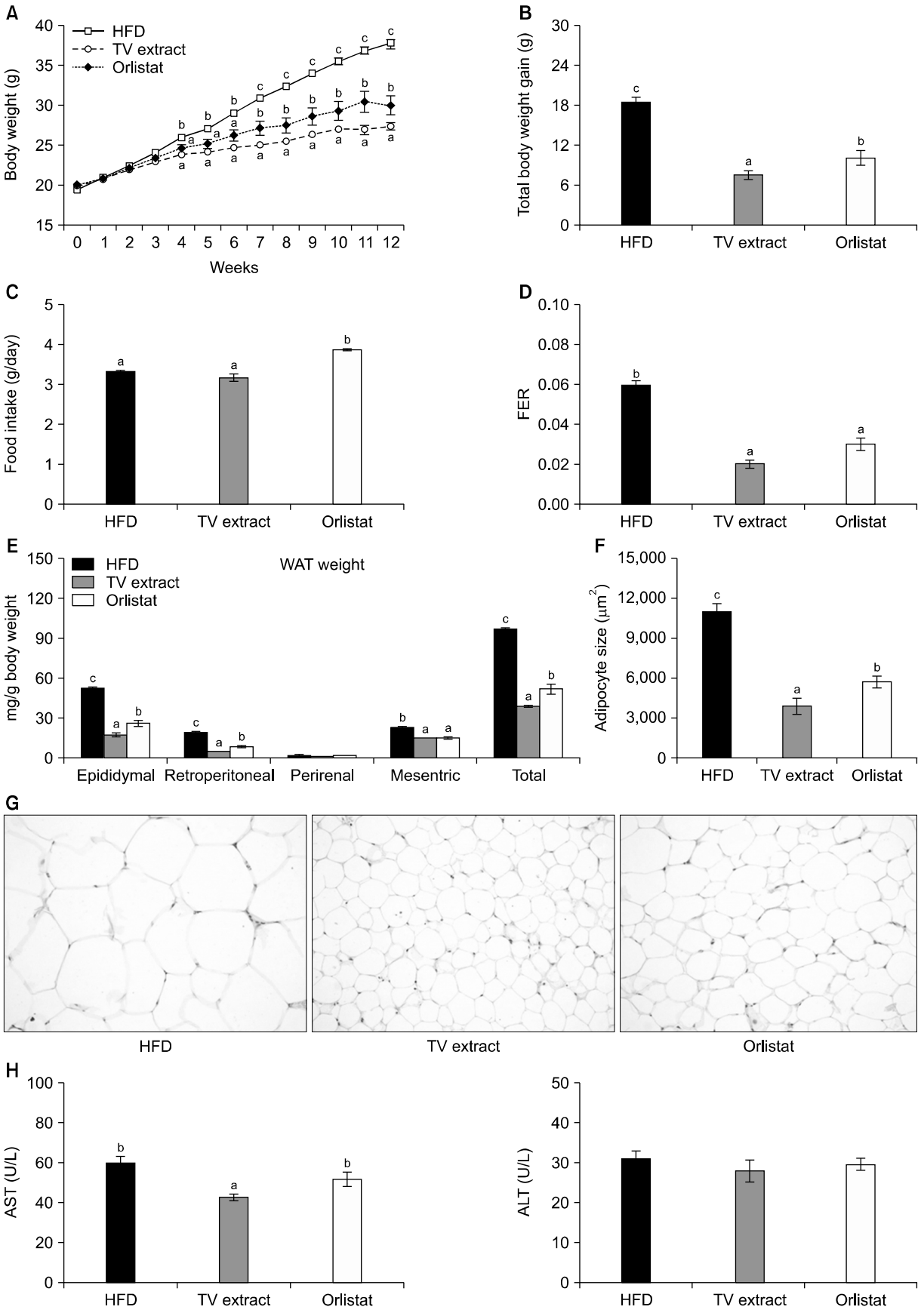

Fig. 2. Effects of *Thamnolia vermicularis* acetone extract (TV extract) supplementation on (A) body weight, (B) total body weight gain, (C) food intake, (D) food efficiency ratio (FER), (E) visceral white adipose tissue (WAT) weight, (F) adipocyte size of epididymal WAT, (G) microscopic images of hematoxylin and eosin stained epididymal WAT, and (H) serum aminotransferase activities in DIO mice. Values shown are means±SE. Values not sharing a common letter (a-c) are significantly different among groups. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

pocyte differentiation. However, the TV extract significantly lowered the TG content in a dose-dependent manner by up to 63% in response to treatment with 5 μg/mL and up to 85% in response to treatment with 10 μg/mL, respectively, when compared with the vehicle (Fig. 1C). Similarly, Sung et al. (18) found that lichen *Lethariella cladonioides* (25～500 μg/mL) significantly inhibited lipid accumulation in 3T3-L1 adipocytes. Thus, the TV extract appeared to negatively mediate lipid accumulation, even when applied in low doses, thereby leading to greater inhibition of 3T3-L1 adipocytes differentiation than *Lethariella cladonioides*.

Anti-obesity effects of the TV extract in DIO mice

We next investigated whether TV extract supplementation could lead to similar effects *in vivo* by comparison with orlistat, which is well-known as an inhibitor of intestine lipase. In DIO C57BL/6N mice, the TV extract and orlistat both effectively prevented body weight from week 4 of the experiment (Fig. 2A) and inhibited adiposity (Fig. 2E). After seven weeks, body weight in the TV extract was significantly lower than in both HFD and orlistat. At the end of our experiment, the TV extract and orlistat restrained body weight gain by up to 60% and 46%, respectively, when compared with the HFD group (Fig. 2B). Additionally, the TV extract did not lead to a difference in food intake, but the FER decreased (Fig. 2C and 2D), which suggested that TV extract supplementation was less efficient at converting nutrients into body mass in DIO mice (19). On the other hand, the food intake of orlistat increased when compared with HFD or TV extract (Fig. 2C).

Obesity is defined as the increase of adipose tissue mass that results from an increased number of fat cells (hyperplasia) alongside an increased fat cell size (hypertrophy) (20). Particularly, visceral obesity derived from adipocyte hypertrophy is more closely related to various types of metabolic disease and cardiovascular disease (21). The epididymal WAT is a predominant site for fat storage in rodents that is mostly composed of adipocytes within this tissue (22). We found that TV extract-feeding with HFD for 12 weeks dramatically depleted the epididymal WAT weight and changed its morphology, including its size (Fig. 2F and 2G). Both TV extract and orlistat decreased the sizes of the adipocytes when compared with the HFD group. Moreover, when compared with the HFD mice, the weights of epididymal, retroperitoneal, and mesentric WAT of the TV extract decreased significantly as well (Fig. 2E). These results indicate that the TV extract dramatically reduced the body weight and visceral fat accumulation of DIO mice without changing their food intake.

Serum hepatic toxicology markers were analyzed to evaluate the potential toxic effects of the TV extract. The AST activity was significantly lower in the TV extract group than in both the HFD and orlistat groups, whereas the ALT level did not differ among groups (Fig. 2H). Moreover, the TV extract did not induce significant

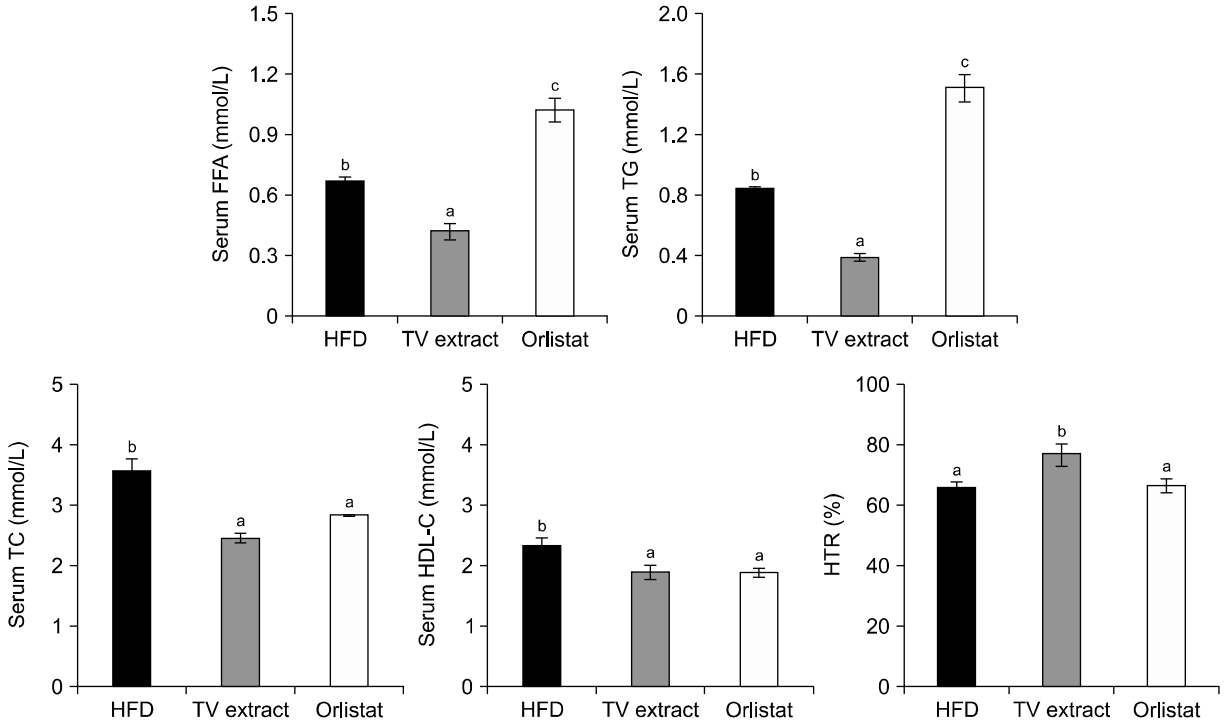

Fig. 3. Effects of *Thamnolia vermicularis* acetone extract (TV extract) supplementation on serum lipid profiles in DIO mice. Values shown are means±SE. Values not sharing a common letter (a-c) are significantly different among groups. FFA, free fatty acid; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; HTR, ratio of HDL-C to TC.

changes in liver weight (data not shown). Thus, long-term TV extract (500 mg/kg/d) supplementation had no harmful effects in DIO mice. Çolak et al. (23) also reported that *Cetraria islandica* and *Pseudevernia furfuracae* lichen species could be safely used at 500 mg/kg body weight in normal and diabetic rats. These data showed that the decreased body weight and fat mass in TV extract-supplemented mice did not result from the toxicity of the TV extract.

Anti-hyperlipidemic effects of the TV extract in DIO mice

Hyperlipidemia is a risk factor for cardiovascular disease (24-26). We previously reported that hyperlipidemia in DIO mice resulted in significantly greater body weight (27,28). Therefore, the present study evaluated the hypolipidemic effects of the TV extract in DIO mice. Supplementation with the TV extract significantly decreased serum FFA and TG levels by up to 37% and 54%, respectively, when compared to HFD feeding alone, whereas the orlistat-supplementation elevated these markers (Fig. 3). It is well known that serum HDL-C levels serve as a major indictor to evaluate the potential HDL for readily translocating of cholesterol from peripheral tissue to the liver for catabolism (29). In the present study, the HTR was only increased by TV extract supplementation even though both the TV extract and orlistat significantly decreased TC contents (Fig. 3) when compared with HFD. Hyperlipidemia results from increased serum TG, TC, low-density lipoprotein-cholesterol, very-low-density lipoprotein-cholesterol, and decreased HDL-C (30). Thus, it is assumed that the TV extract had a potent lipid-lowering effect in DIO mice.

Orlistat is a commercially successful diet drug that is well-known to inhibit lipase in the intestine; therefore, we measured the fecal lipid contents and observed increased lipid (TG and cholesterol) levels in the orlistat as well as the TV extract groups when compared with the HFD group (Table 2). As expected, orlistat supplementation led to dramatic increases in fecal TG levels up to 23-fold compared to that of the HFD group. These results implied that the TV extract increases fecal lipid excretion in a manner similar to orlistat, which may partly contribute to the lower visceral fat mass and serum lipid levels that were observed.

Table 2. Effects of TV extract supplementation on hepatic and fecal lipid levels in DIO mice

	HFD	TV extract	Orlistat
Liver			
FFA (μmol/g)	16.90±0.46	16.01±0.82	16.20±0.40
TG (mmol/g)	0.35±0.01^{b}	0.16±0.01^{a}	0.44±0.01^{c}
Cholesterol (mmol/g)	0.08±0.003^{b}	0.06±0.003^{a}	0.07±0.001^{ab}
Feces			
TG (mmol/g)	0.82±0.07^{a}	1.67±0.24^{b}	18.61±0.26^{c}
Cholesterol (mmol/g)	1.78±0.13^{a}	4.17±0.36^{b}	4.41±0.35^{b}

Values shown are means±SE.
Values not sharing a common letter in the same row (a-c) are significantly different among groups.
HFD, high-fat diet; TV extract, *Thamnolia vermicularis* acetone extract; FFA, free fatty acid; TG, triglyceride.

The recent rise in obesity has been found to be closely associated with increases in hepatic steatosis. In the present study, morphological analysis demonstrated that TV extract supplementation led to a significant reduction in hepatic lipid droplets (Fig. 4) as well as lipid (TG and cholesterol) contents (Table 2) against HFD feeding alone. Taken together, these results suggest that the TV extract significantly decreased hepatic lipid accumulation.

Anti-insulin resistance effects of the TV extract in DIO mice

Increases in visceral fat are associated with insulin resistance, metabolic syndrome and cardiovascular disease (31). The effects of the TV extract on obesity-induced insulin resistance are shown in Table 3. Although the TV extract and orlistat did not decrease blood glucose levels in DIO mice, serum insulin levels were markedly reduced in the TV extract and orlistat groups by 50% and 35%, respectively, which leads to improvement of the insulin resistance marker, HOMA-IR, when compared with

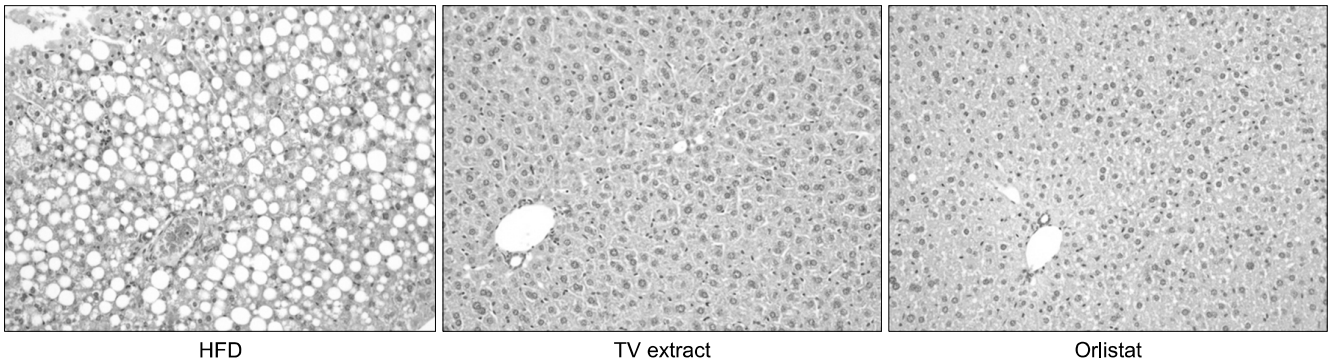

Fig. 4. Effects of *Thamnolia vermicularis* acetone extract (TV extract) supplementation on hepatic histology in DIO mice. Hematoxylin and eosin staining (200× magnification).

Table 3. Effects of TV extract supplementation on fasting blood glucose, serum insulin level and HOMA-IR in DIO mice

	HFD	TV extract	Orlistat
Blood glucose (mmol/L)	7.94±0.19	8.32±0.59	7.50±0.51
Serum insulin (μIU/mL)	26.78±1.61[b]	13.29±2.83[a]	17.40±1.80[a]
HOMA-IR	9.48±0.68[b]	4.80±0.90[a]	5.85±0.77[a]

Values shown are means±SE.
Values not sharing a common letter in the same row (a,b) are significantly different among groups.
HFD, high-fat diet; TV extract, *Thamnolia vermicularis* acetone extract; HOMA-IR, homeostasis model assessment of insulin resistance.

HFD feeding alone (Table 3). These data indicate that the TV extract could effectively prevent and treat obesity-related diseases such as diabetes.

In conclusion, these results suggest that the TV extract may have an anti-obesity effect based on the suppression of adipocyte differentiation and adiposity both *in vitro* and *in vivo*, and indicate that it has the potential for use as a natural resource capable for obesity management. However, future studies should be conducted to investigate the bioactive components in the TV extract and its molecular mechanism for anti-obesity effects.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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