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ORIGINAL CONTRIBUTION

Antiobesity effect of polyphenolic compounds from molokheiya (*Corchorus olitorius* L.) leaves in LDL receptor-deficient mice

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Abstract
Background Dietary supplementation with polyphenolic compounds is associated with reduced diet-induced obesity and metabolic disorders in humans. The antioxidative properties of polyphenolic compounds contribute to their antiobesity effect in animal experiments and human studies.
Aim The aim of the study was to investigate the antiobesity effect of polyphenolic compounds from molokheiya leaves in LDLR-/- mice fed high-fat diet and to elucidate the mechanism of this effect.
Methods Three groups of LDLR-/- mice were fed with a high-fat diet, supplemented with 0% (control), 1 or 3% molokheiya leaf powder (MLP). Gene expression in the liver associated with lipid and glucose metabolism was analyzed, and physical parameters and blood biochemistry were determined.
Results Compared to controls, mice body weight gain ($P = 0.003$), liver weight ($P = 0.001$) and liver triglyceride levels ($P = 0.005$) were significantly lower in the two MLP groups. Epididymal adipose tissue weight ($P = 0.003$) was reduced in the 3% MLP group. Liver tissue gene expression of gp91phox (NOX2), involved in oxidative stress, was significantly down-regulated ($P = 0.005$), and PPARα and CPT1A, related to the activation of β-oxidation, were significantly up-regulated ($P = 0.025$ and 0.006, respectively) in the 3% MLP group compared to the control group.
Conclusions Our results demonstrate an antiobesity effect of polyphenolic compounds from molokheiya leaves and that this effect is associated with reduction in oxidative stress and enhancement of β-oxidation in the liver. Consumption of molokheiya leaves may be beneficial for preventing diet-induced obesity.

Keywords *Corchorus olitorius* L. · Molokheiya · Obesity · Oxidative stress · β-oxidation · Polyphenolic compounds

Abbreviations

ACOX1	Acyl-coenzyme A oxidase 1
CPT1A	Carnitine palmitoyl transferase 1A
Ehhadh	Enoyl-coenzyme A hydratase/3-hydroxyacyl coenzyme A dehydrogenase
FAS	Fatty acid synthase
GK	Glucokinase
LDLR-/-	Low-density lipoprotein receptor-deficient
MLP	Molokheiya leaf powder
PPARα	Peroxisome proliferator activated receptor alpha

Introduction

The increasing prevalence of obesity has become an urgent worldwide public health problem in the past few decades. Obesity is attributed to an imbalance between energy intake and energy expenditure and is closely associated

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with lifestyle-related diseases (such as diabetes mellitus and hypertension), which are primary risk factors for development of cardiovascular disease. Obesity is known to be related to oxidative stress, through attenuation of antioxidant enzymes levels and increased reactive oxygen species (ROS) production [1, 2].

In recent years, a wide range of polyphenolic compounds that are ubiquitous in edible plants (fruits, vegetables and herbs) and in plant-derived beverages have received increased attention due to their biologic activities. These compounds are believed to possess health-promoting properties including antioxidative, antiatherogenic, antiobesity and anti-inflammatory effects [3, 4]. In previous studies, we reported antioxidative and antiatherogenic activities of a major polyphenolic compound (Q3MG) in mulberry leaves, manifested through increased LDL resistance to oxidative modification [5, 6]. Dulloo et al. [7] were the first to suggest the treatment of obesity with polyphenolic compounds (green tea extract) based on the mechanisms of increased thermogenesis and fat oxidation. Polyphenolic compounds also stimulate lipid catabolism in the liver and suppress dyslipidemia to attenuate diet-induced obesity [8, 9]. In addition, polyphenolic antioxidants may act to attenuate obesity in animals with high-fat diets [10, 11]. These effects of polyphenolic compounds are achieved by changing the activity of certain enzymes related to lipid and glucose metabolisms in the mouse liver [12]. Oxidative stress is increased in high-fat diet–fed animals, and polyphenols can prevent oxidative stress through reducing NADPH oxidase expression, accounting for the antiobesity effect [13, 14]. Research has shown that the hypolipidemic effect of polyphenolic compounds is achieved through enhancement of gene expressions of enzymes related to β-oxidation, such as PPARα, CPT1A and ACOX1 in the liver, increased β-oxidation enhances energy expenditure, helping to prevent obesity [15, 16]. As molokheiya leaves are reported to contain an abundance of caffeoylquinic acid and quercetin glycoside, polyphenolic compounds involved in high antioxidative activity [17], we hypothesized that molokheiya leaves possess similar antiobesity effects through the mechanisms of reducing oxidative stress and enhancing β-oxidation in the liver.

In the present study, we used LDLR-/- mice, which are readily susceptible to obesity and obesity-related metabolic disorders, and fed them a high-fat diet to investigate the antiobesity effects of molokheiya leaf powder (MLP). To further elucidate the mechanism of the antiobesity effect of molokheiya leaves, we conducted quantitative real-time PCR analysis in which we compared gene expression profiles of liver tissue of the 1% and 3% MLP diet and control diet groups.

Materials and methods

Diets

Molokheiya leaves harvested in Shimane Prefecture, Japan, in 2006 were lyophilized and ground to powder using a vibrating sample mill (Heiko Seisakusho, Ltd, Tokyo, Japan). Leaf polyphenolic compounds were extracted by suspending the dried powder in 60% (v/v) ethanol aqueous solution and analyzed by quantitative HPLC system (LaChrom, Hitachi Ltd, Tokyo, Japan) as described previously [5]. The main polyphenolic compounds in MLP included 450 mg hyperoside, 310 mg quercetin 3-glucoside (Q3G) and 1,200 mg quercetin 3-(6-malonylglucoside; Q3MG)/100 g dry weight.

Mouse diets were a high-fat (HF) diet (15 g cocoa butter, 3 g cholesterol and 82 g CE-2/100 g diet), supplemented with 0% (control group), 1% (w/w, 1% MLP group) or 3% (w/w, 3% MLP group) dried molokheiya leaf powder. The CE-2 diet components consisted of 8.6% moisture, 24.9% crude protein, 4.6% crude fat, 51.4% nonfiber carbohydrate, 3.7% crude fiber, and adequate vitamins and minerals necessary for mice.

Animals

A breeding colony was generated from homozygous LDLR-/- mice (aged 7 weeks) with C57BL6/J $\times$ 129 Sv background purchased from Jackson Laboratories (Maine, USA). The mice were housed in the Shimane University School of Medicine Animal Laboratory, under controlled ambient conditions: relative humidity 55 $\pm$ 10%, temperature 23 $\pm$ 2 °C, a 12-h light/dark cycle (07:00–19:00 and 19:00–07:00, respectively), air change 13–15 times/h and consumed food and water ad libitum. At the age of 8 weeks, the mice were randomly divided into three groups: control group, 1% MLP group and 3% MLP group, with 15 mice in each group. The three mice groups were fed their respective experiment diets for 8 weeks. Food intake was recorded once a week, and body weight was measured twice a week. After 8 weeks feeding, the mice were fasted overnight and anesthetized. Blood was collected from the heart. Epididymal adipose tissue and the liver, kidney and soleus muscle were harvested, rinsed and weighed. Liver and blood samples were stored at -80 °C until analysis. This study and all procedures were approved by the Animal Care and Use Committee of Shimane University, Japan.

Plasma biochemical analysis

Blood was placed into tubes containing EDTA2Na, and the plasma was separated immediately by centrifugation at

3,000*g* for 10 min at 4 °C. Concentrations of plasma glucose, triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), total cholesterol (TC), free fatty acid (FFA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyltranspeptidase (γ-GTP) were measured with enzymatic assay kits (Wako Pure Chemical, Osaka, Japan). Hemoglobin A1c (HbA1c) was measured using a DCA 2000 kit (Bayer, Tokyo, Japan). Concentrations of plasma insulin were measured using a Mouse Insulin ELISA Kit (Shibayagi, Gunma, Japan) according to the manufacturer's protocol.

Morphological and chemical analysis of the liver

Ten liver tissue samples were taken from each of the groups and processed in a standard fashion for light microscopic examination. Briefly, liver biopsy specimens were fixed in 10% (v/v) neutral-buffered formalin for 2–3 days and embedded in paraffin. Sections of 3–5 μm were stained with hematoxylin and eosin (HE). Liver lipids were extracted as per Bligh and Dyer [18], and liver triglyceride concentrations were measured using Triglyceride G test kit (Wako Pure Chemical, Osaka, Japan).

Quantitative real-time PCR

Measurement of gene expression of enzymes related to glucose and lipid metabolism, β-oxidation and oxidative stress in the liver was done by quantitative RT–PCR analysis. Total RNA was extracted from the liver using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the supplier; the quality and quantity were then checked by agarose gel electrophoresis and spectrophotometry. Reverse transcription was performed using a ReverTra Ace -α- cDNA Synthesis kit (Toyobo, Osaka, Japan). Quantitative RT–PCR was performed with Applied Biosystems 7900HT system, using Power SYBR Green PCR Master Mix (Applied Biosystems, California, USA) according to the supplier's manual, with 18S rRNA used for internal control. Gene expression of gp91phox (cytochrome b-245, beta polypeptide), PPARα, CPT1A, ACOX1, Ehhadh, FAS and GK were determined. Primers used in the present study were as follows: 18S rRNA (forward) aagatcgatcgccacatgtatca, (reverse) tgcgtgcttccttggtcttaga; gp91phox (forward) ttgggtcagcactggctctg, (reverse) tggcggt gtgcagtgctatc; PPARα (forward) tcagggtaccactacggagttca, (reverse) ccgaatagttcgccgaaaga; CPT1A (forward) ggatct acaattcccctctgc, (reverse) gcaaaataggtctgccgaca; ACOX1 (forward) agcgagccagagccccag, (reverse) tcaggcagctcactc agg; Ehhadh (forward) gttggcttcccggaagtgat, (reverse) ctcc aacgaccctgggtaga; FAS (forward) ttccaagacgaaaatgatgc, (reverse) aattgtgggatcaggagagc; GK (forward) ccctgagtggc ttacagttc, (reverse) acggatgtgagtgttgaagc.

Statistical analysis

Data were analyzed with SPSS software version 12.0 J (SPSS Inc., Tokyo, Japan). The results are expressed as mean ± SD. Comparisons of the three diet groups were performed by one-way analysis of variance (ANOVA), and post hoc analyses were used with Tukey's test for multiple comparisons. *P*-values of less than 0.05 for ANOVA and 0.025 for post hoc analyses were used to assess significant differences.

Results

Body weight, composition and food intake

Physical and blood parameters within the control and treatment groups are summarized in Table 1. Average body weights within the three mice groups were nearly identical at the onset of the experiment, but there were significant differences in body weight gain between the control and two MLP groups from the 5th week on (Fig. 1). After 8 weeks of feeding, final body weight gains were significantly lower in the 1% MLP (3.8 ± 1.7 g) and 3% MLP (4.3 ± 1.5 g) groups, compared to the control group (5.7 ± 1.2 g, $P = 0.003$). Body weight change was not significantly different between the 1% MLP group and 3% MLP group. Energy intake from diet was not significantly different among the three groups. Epididymal adipose tissue weight was significantly lower in the 3% MLP group (0.30 ± 0.06 g), compared to the control group (0.42 ± 0.10 g, $P = 0.003$). Liver weights were significantly lower in the 1 and 3% MLP groups than in the control group. No significant weight differences were noted for the soleus muscle and kidney samples in all groups.

Effects of molokheiya leaf powder on plasma biochemistry

The effects of MLP on plasma parameters are summarized in Table 1. Fasting plasma glucose and plasma triglyceride levels were significantly lower in the 3% MLP group compared to the control group ($P < 0.001$ and $= 0.009$, respectively). Plasma HDL-C levels showed significantly higher values in the 1 and 3% MLP groups ($P = 0.006$ and <0.001, respectively), while plasma FFA concentrations were significantly lower ($P = 0.013$ and 0.001, respectively) than in the control group. Plasma

Table 1 Effects of molokheiya leaf powder on physical and blood parameters after feeding 8 weeks

	Diet			ANOVA
	Control ($n = 15$)	1% MLP ($n = 15$)	3% MLP ($n = 15$)	P-value
Food intake				
Flavonoids intake (mg/mouse/day)	0	0.66 ± 0.03	2.03 ± 0.08	
Energy intake (kcal/mouse/day)	14.7 ± 0.4	14.8 ± 0.7	14.9 ± 0.6	0.865
Body weight and organ weight (g)				
Initial body weight	22.6 ± 0.9	22.9 ± 1.5	22.9 ± 1.3	0.753
Final body weight	28.3 ± 1.4	26.7 ± 1.4*	27.2 ± 2.1	0.032
Body weight gain	5.7 ± 1.2	3.8 ± 1.7*	4.3 ± 1.5*	0.003
Epididymal adipose tissue	0.42 ± 0.10	0.35 ± 0.12	0.30 ± 0.06*	0.003
Liver	1.74 ± 0.13	1.5 ± 0.20*	1.43 ± 0.29*	0.001
Soleus muscle	0.32 ± 0.03	0.31 ± 0.03	0.32 ± 0.03	0.751
Kidney	0.37 ± 0.03	0.35 ± 0.03	0.39 ± 0.04	0.52
Plasma parameters				
Triglyceride (mg/dl)	262 ± 45	204 ± 71	192 ± 70*	0.009
HDL-cholesterol (mg/dl)	25 ± 5	33 ± 7*	35 ± 7*	<0.001
Free fatty acid (mEq/l)	1.7 ± 0.2	1.4 ± 0.2*	1.3 ± 0.3*	0.001
Total cholesterol (mg/dl)	333 ± 23	314 ± 28	256 ± 13	0.054
Glucose (mg/dl)	167 ± 16	156 ± 28	137 ± 14*	0.001
Insulin (ng/ml)	0.32 ± 0.23	0.24 ± 0.07	0.31 ± 0.34	0.588
HbA1c (%)	3.4 ± 0.3	3.2 ± 0.4	3.2 ± 0.3	0.179
AST (IU/l)	17 ± 7	20 ± 8	18 ± 5	0.635
ALT (IU/l)	12 ± 3	13 ± 9	13 ± 7	0.801
γ-GTP (IU/l)	22 ± 8	20 ± 18	25 ± 12	0.556
Liver triglyceride levels				
Relative value (mg/g liver)	27.6 ± 8.0	18.1 ± 7.0*	18.9 ± 7.0*	0.006
Absolute value (mg)	42.7 ± 13.0	27.8 ± 10.0*	28.8 ± 11.0*	0.005

Values are mean ± SD. ANOVA, post hoc pairwise comparison with Tukey's test. $P < 0.025$ means significant differences

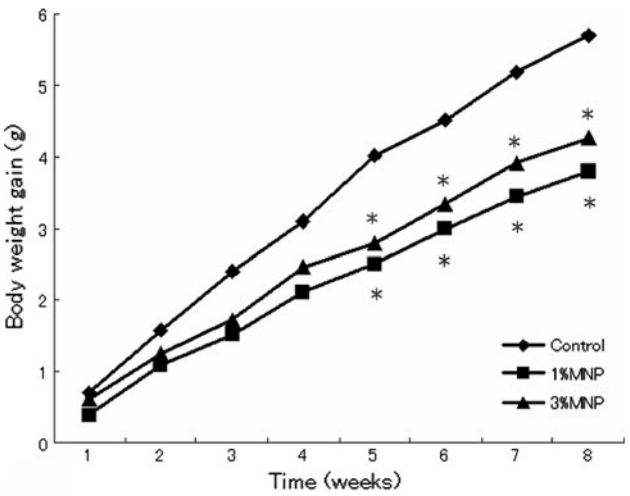

Fig. 1 Effects of molokheiya leaf powder on body weight gains of mice. *Filled circle* Control group, *filled square* 1% MLP group, *filled triangle* 3% MLP group, each point represents mean ± SD ($n = 15$). * $P < 0.025$ (Tukey's test) compared with those of the control group

insulin, total cholesterol, HbA1c, AST, ALT and γ-GTP levels were not significantly different among the control and MLP groups.

Effect of molokheiya leaf powder on liver fat accumulation and TG levels

Since liver weight and plasma TG levels were significantly lower in the MLP groups, we evaluated the effect of MLP on fat accumulation and TG levels in the liver. As shown in Fig. 2, lipid droplets were abundant around the center vein in the liver in the control group, but less so in the MLP groups. The relative and absolute values of liver TG in the 1% and 3% MLP groups are shown in Table 1. Liver TG levels were significantly lower in the 1% and 3% MLP groups, compared with those in the control group ($P < 0.025$).

Effects of molokheiya leaf powder on gene expression in the liver

For greater understanding of the mechanism of the anti-obesity effect of MLP, we measured the effects of the three experimental diets on gene expressions in the liver. The genes were selected according to metabolic function in terms of β-oxidation, oxidative stress and glucose metabolism, as summarized in Table 2. The gene expression of

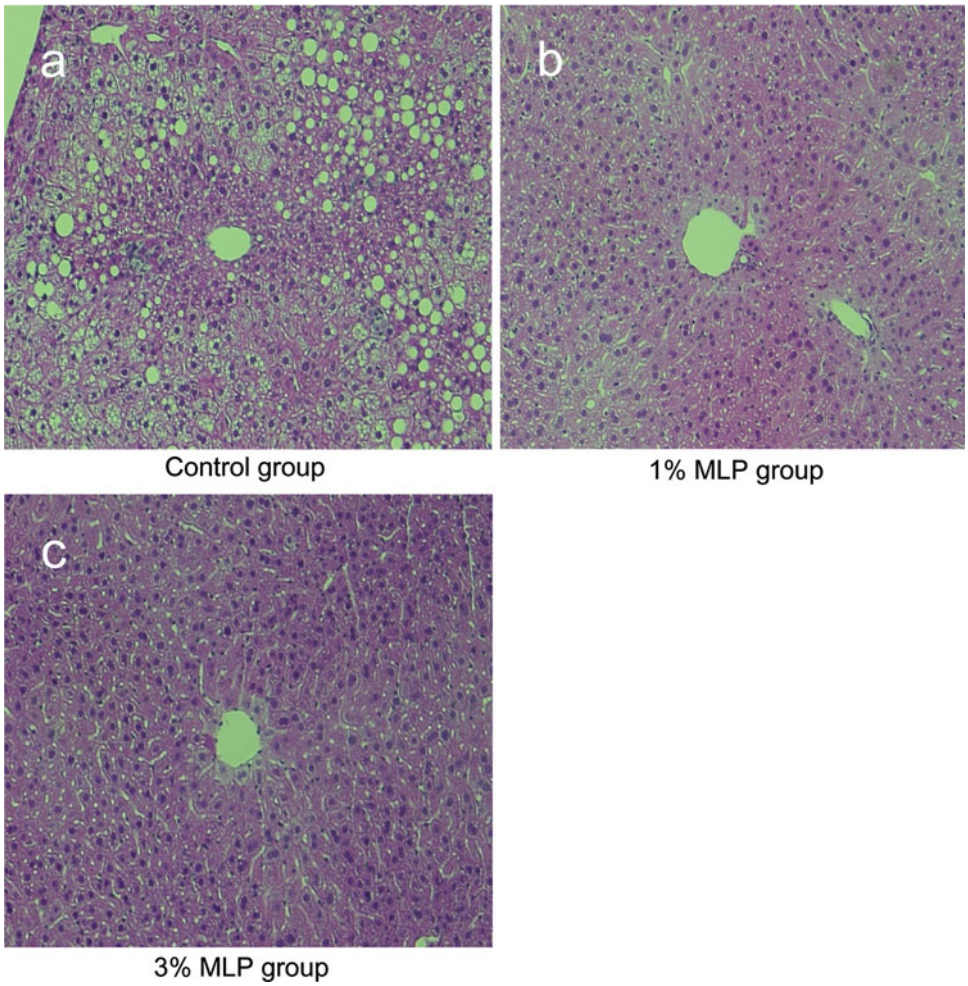

Fig. 2 Histological staining of liver sections of control group (**a**), 1% MLP group (**b**) and 3% MLP group (**c**)

PPARα, a regulatory molecule in fatty acid β-oxidation, was significantly enhanced in the 3% MLP group (1.56 fold, $P = 0.025$) compared to the control group. The expression of CPT1A, related to β-oxidation, was also significantly up-regulated (1.61 fold, $P = 0.006$) in the 3% MLP group, while that of gp91phox, a key subunit of NADPH oxidase and involved in oxidative stress, was significantly down-regulated in the 3% MLP group (−1.61-fold, $P = 0.005$) compared to the control group. Expressions of GK, ACOX1, Ehhadh and FAS were not significantly different among the three groups.

Discussion

In the present study, we investigated the antiobesity effect of polyphenolic compounds from molokheiya leaves in LDLR-/- mice fed high-fat diets. Our results clearly showed that supplementation with MLP rich in polyphenolic compounds is effective in reducing diet-induced obesity, attenuating oxidative stress and enhancing β-oxidation.

Our study showed that body weight gain was significantly lower in the MLP groups as early as the 5th week, and ultimate body weight gains were significantly lower in both MLP groups in comparison with the control group. During the experiment period, average energy intake was not significantly different among the three groups, contrasting with the results of a previous study [19]. Our results indicate that the antiobesity effect of molokheiya leaves was unrelated to levels of energy intake, so we evaluated other mechanisms for the antiobesity effect.

Phenolic antioxidants from plants may act as regulators of obesity in mice or rats fed with high-fat diets [10, 20]. In previous studies, we have also reported the remarkable antioxidative activities of Q3MG, the predominant flavonol glycoside in mulberry leaves [5, 6]. Molokheiya leaf powder contains an abundance of Q3MG, Q3G and hyperoside, which led us to hypothesize that it may have been the polyphenolic compounds in molokheiya leaves

Table 2 Gene expression changes in the liver induced by molokheiya leaf powder

Gene name	Control ($n = 14$)	1% MLP ($n = 14$)	3% MLP ($n = 12$)	ANOVA *P*-value
gp91phox	1.00 ± 0.33	0.78 ± 0.55	0.62 ± 0.52*	0.005
PPARα	1.00 ± 0.22	1.16 ± 0.22	1.56 ± 0.83*	0.025
CPT1A	1.00 ± 0.28	1.36 ± 0.31	1.61 ± 0.65*	0.006
Acox1	1.00 ± 0.38	1.15 ± 0.47	1.35 ± 0.79	0.171
Ehhadh	1.00 ± 2.09	1.42 ± 3.08	1.49 ± 3.85	0.236
FAS	1.00 ± 0.41	0.9 ± 0.51	0.88 ± 0.47	0.631
GK	1.00 ± 0.64	1.39 ± 1.06	1.81 ± 0.95	0.21

Values are means ± SD. ANOVA, *post hoc* pairwise comparison with Tukey's test. * $P < 0.025$ denotes significant differences compared with those of the control group

that were responsible for antioxidative activities stimulating lipid metabolism in the liver, suppressing fat accumulation in adipose tissue and the liver and reducing plasma and hepatic lipid levels, leading to the antiobesity effect.

It is known that the liver plays a vital role in lipid metabolism, prevention of oxidative stress and regulation of β-oxidation, which in turn may contribute to the prevention of both abdominal fat accumulation and increase in lipid levels in plasma and the liver [9, 21]. PPARα is highly expressed in the liver and plays a crucial role in the induction of hepatic fatty acid oxidation and regulation of lipid metabolism [15, 22]. The gene expression of many lipid-metabolizing enzymes, including ACOX1 and CPT1A, is transcriptionally regulated by PPARα in the liver [23, 24]. Polyphenolic compounds have been reported to improve hyperlipidemia by enhancing hepatic PPARα and other mRNA expression related to β-oxidation in HF-diet-fed animals [9, 16]. Therefore, in the present study, we investigated the effect of MLP on the gene expression of enzymes related to oxidative stress and β-oxidation in the liver for purposes of clarifying the antiobesity mechanism.

We found that the expression of PPARα and CPT1A were significantly up-regulated in the 3% MLP group. We suggest that molokheiya leaf polyphenolic compounds promote fatty acid oxidation and increase energy expenditure by enhancing the expression of these enzymes related to β-oxidation. Oxidative stress results from excess ROS production and/or deficient antioxidant capacity and is observed in diet-induced obesity [1]. The NADPH oxidase system is an important source of ROS generation, and a significant up-regulation of gp91phox can induce ROS overproduction, contributing to the pathogenesis of oxidative stress observed with diet-induced obesity [1, 25]. Polyphenolic compounds have abundant antioxidants and are considered to play an important role in the decrease of NADPH oxidase expression and contribution to partially preventing oxidative stress [13, 14]. In the present study, gp91phox expression in the liver was significantly down-regulated in the 3% MLP group, indicating oxidative stress may be attenuated. We believe that molokheiya leaf polyphenolic compounds prevent or attenuate oxidative stress, contributing to the antiobesity effect.

The liver also plays an important role in regulating blood glucose levels, and polyphenolic compounds have been reported to decrease blood glucose through their influence on glucose metabolism enzymes in the liver [26]. In the present study, we found that plasma glucose concentrations were significantly lower in the MLP groups. Furthermore, the gene expression of GK related to glycolysis was up-regulated in the MLP ingestion groups. These findings may indicate that polyphenolic compounds in MLP regulate gene expression involved in glucose metabolism in the liver, thus attenuating the increase in blood glucose levels.

There are a few limitations in the present study. MLP contains small amounts of fiber, which may contribute to the antiobesity effect. However, to our knowledge, dietary fiber does not have antioxidative functions or bioactivities which affect gene expression related to oxidative stress and β-oxidation in the liver. We therefore believe that it is the abundant polyphenolic compounds in molokheiya leaves, which provide the main contribution to the antiobesity effect. Body weight gains in the 1 and 3% MLP groups were nearly the same (no statistical difference), although flavonoids intake of the 3% MLP group was much higher. It is possible that the ingestion of 1% MLP maximally affects body weight change, with higher concentrations of MLP producing no additional antiobesity effects. The present study was a preliminary animal experiment, and the MLP-supplemented diet was provided for 8 weeks. Future studies are required to investigate long-term and possible toxic effects of MLP and to extract polyphenolic compounds of molokheiya leaves to confirm the components responsible for the antiobesity effect.

In conclusion, the present study shows that the polyphenolic compounds from molokheiya leaves prevent obesity in high-fat-fed mice, and these effects may be related to the decrease of oxidative stress and enhancement of β-oxidation in the liver. The present study raises the possibility that polyphenolic compounds from molokheiya

leaves may have potential value as a health supplement to help reduce obesity.

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