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Raphanus sativus L. var. *caudatus* Extract Alleviates Impairment of Lipid and Glucose Homeostasis in Liver of High-Fat Diet-Induced Obesity and Insulin Resistance in Mice

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ABSTRACT: The present study investigated the activities of *Raphanus sativus* L. var. *caudatus* extract (RS) on abnormal lipid and glucose homeostasis in a high-fat diet (HFD)-induced obesity and insulin resistance in a mouse model. Institute of Cancer Research mice were rendered obese by 16-week HFD feeding. Obese mice were administered with 100 or 200 mg/kg/d RS orally during the last 8 weeks of diet feeding. Then, the biochemical parameters were determined. The gene and protein expressions regulating lipid and glucose homeostasis in the liver were measured. This study revealed that the state of hyperglycemia, hyperleptinemia, hyperinsulinemia, and hyperlipidemia was reduced after 8 weeks of RS treatment (100 or 200 mg/kg). Administration of RS also improved insulin sensitivity and increased serum adiponectin. The liver total cholesterol and triglyceride concentrations were decreased by both doses of RS. Notably, a decrease in the expression of liver-specific genes, including sterol regulatory element-binding protein 1c, fatty acid synthase, and acetyl-CoA carboxylase, was found in the RS-treated groups. Moreover, administration of RS showed a significant increase in the expression of adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and sirtuin1 (Sirt1) proteins. These findings indicated that RS improved abnormal lipid and glucose homeostasis in the liver of obesity-associated insulin resistance mouse model, possibly through the stimulation of the AMPK/Sirt1 pathway.

Keywords: glucose homeostasis, insulin resistance, lipid homeostasis, obesity, *Raphanus sativus* L. var. *caudatus*

INTRODUCTION

Obesity is commonly linked with insulin resistance, which progresses to more serious conditions, including atherosclerosis, type 2 diabetes mellitus (T2DM), and cardiovascular disorder (Scherer and Hill, 2016). High insulin levels in individuals with insulin resistance can lead to the elevated sterol regulatory element-binding protein 1c (SREBP1c) (Stefan et al., 2008), which belongs to a group of transcription factors involved in the activation of hepatic lipogenic enzymes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Tong et al., 2016). Abnormalities in lipid homeostasis result in liver fat storage and following the progression of non-alcoholic fatty liver disease (NAFLD) (Ipsen et al., 2018).

The obese induction by feeding a high-fat diet (HFD) is a major contributor to metabolic liver disorders. Impairment of lipid and glucose homeostasis, such as abnormal lipid storage, glycogen reduction, and insulin resistance, can also be detected (Fan and Cao, 2013). Adenosine monophosphate-activated protein kinase (AMPK) is a main regulator of energy balance in the liver. AMPK activator can alleviate obesity and obesity-associated metabolic disorders (Hardie, 2008b). Sirtuin 1 (Sirt1) is involved in diverse physiological processes, and Sirt1 deficiency is related to several disorders, such as inflammation, cardiovascular disease, and diabetes (Kitada et al., 2013; Assadiasl et al., 2020). In the progression of insulin resistance, Sirt1 is important for regulating insulin release, adiponectin production, inflammatory process, glu-

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coneogenesis, and oxidative stress (Kitada et al., 2013). AMPK and Sirt1 dysfunction may contribute to the development of T2DM and NAFLD by altering lipid homeostasis, inflammation, and insulin resistance and cause mitochondrial dysfunction (Ruderman et al., 2010).

Obesity is a growing health problem globally. Obesity enhances the risk of developing insulin resistance, which can lead to diabetes. Although various medicinal products are available to control obesity, products derived from natural sources are often preferred. *Raphanus sativus* L. var. *caudatus* (Thai rat-tailed radish) is used as a vegetable in Northern Thailand. Several pharmacological effects of *R. sativus* L. var. *caudatus* extract (RS) have been reported, such as antianxiety (Siddiq and Younus, 2018) and antidepression (Younus and Siddiq, 2017). The flower and pod of RS contain two isothiocyanate compounds, namely sulforaphane and sulforaphene, which have anticancer effects in HCT116 cells (Pocasap et al., 2013). The cancer suppressive activity of RS (flower, pod, and dry seed) is associated with the high content of sulforaphene (Pocasap et al., 2017). Unlike sulforaphene, sulforaphane was reported to suppress oxidative stress and inflammation in diabetic rats (Negi et al., 2011). Moreover, sulforaphane reduced obesity in *ob/ob* mice and inhibited adipogenesis in 3T3-L1 cells (Ranaweera et al., 2022). However, how sulforaphane-containing RS pods help regulate lipid and glucose homeostasis in obesity-associated insulin resistance is unclear. We hypothesized that the dichloromethane extract of RS improves abnormal liver lipid and glucose homeostasis by stimulating AMPK/Sirt1 pathway. Thus, RS may be used as a plant-based food for reducing the incidence of obesity-associated insulin resistance and T2DM. To support this hypothesis, we evaluated the activities of RS dichloromethane extract on abnormal lipid and glucose homeostasis by using an HFD-induced obese mouse model associated with insulin resistance. Moreover, we measured the metabolic parameters and the protein expression of AMPK/Sirt1 pathway.

MATERIALS AND METHODS

Plant extraction and phenolic compound screening

Pods of *R. sativus* L. var. *caudatus* were collected from Phrae, Thailand. The identity of plant was given by the Faculty of Pharmaceutical Sciences, Rangsit University, Thailand. Dried pods were soaked in dichloromethane for 30 min. The extract was decanted, filtered, and concentrated. The percentage yield of RS obtained was 10.04% of the starting dry weight of RS pods. RS was then dissolved in 5% gum arabic.

The amount of phenolic compounds was performed by high-performance liquid chromatography with diode array detection and mass spectrometry (Duangjai et al., 2016).

Experimental protocol

All animal experiments were carried out according to the Animal Ethics Committee of Srinakharinwirot University, Bangkok, Thailand (Rec. no. 8/2559). Six-week-old male Institute of Cancer Research mice were purchased from the National Laboratory Animal Center (Nakhon Pathom, Thailand). The animals were maintained in an air condition room (25±2°C) with humidity and a 12-h light-dark cycle. Normal control mice received a standard diet (D12450H, 10 kcal% lard fat, total energy 3.85 kcal/g) and the obese groups received HFD (D12451, 45 kcal% lard fat, total energy 4.73 kcal/g) for 16 weeks. All diets were produced from Research Diets Inc. (New Brunswick, NJ, USA). After 8-week HFD feeding, the state of obese-induced insulin resistance was verified by checking the body weight and intraperitoneal glucose tolerance test (IPGTT). Subsequently, all animals were divided into four groups with 8 mice per treatment group: normal control group received 5% gum arabic, obese control group received 5% gum arabic, and obese group received RS (100 or 200 mg/kg/d). All groups were administered by oral gavage for 8 weeks. The body weight and food intake were evaluated weekly. At the end of treatment, the level of fasting blood glucose (FBG) was determined in 6-h fasted mice. Then, the mice were anesthetized with inhaled isoflurane. Blood samples were collected through cardiac puncture and centrifuged for serum collection to determine the concentrations of lipids, leptin, insulin, and adiponectin. Liver was removed for further examination of biochemical parameters as well as gene and protein expressions.

IPGTT

After 7-week RS treatment, animals were injected intraperitoneally with 2% glucose solution. Blood glucose levels were determined in the fasting state and after glucose injection at 20, 60, and 120 min. The area under the curve of IPGTT was calculated by trapezoidal analysis.

Biochemical parameters

Serum adiponectin, leptin, and insulin levels were measured by ELISA kits (EMD Millipore, Burlington, MA, USA). Serum triglyceride (TG), total cholesterol (TC), and non-esterified fatty acid (NEFA) levels were determined by colorimetric kits (Wako Pure Chemical Corp., Osaka, Japan). TG and TC in the liver were extracted with isopropanol (Oakes et al., 2001), and the supernatant was collected for measuring TG and TC contents by colorimetric kit (Wako Pure Chemical Corp.).

Liver mRNA expression

Total RNA was extracted by TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City,

CA, USA) was used for cDNA synthesis. The expression of mRNA was quantified with Applied Biosystems TaqMan Gene Expression Kit on a StepOnePlus (Applied Biosystems). TaqMan probes and primer sequences for SREBP1c (Mm00550338_m1), FAS (Mm00662319_m1), ACC (Mm01304257), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) were obtained from Applied Biosystems. The expression of mRNA was calculated by the comparative computed tomography method using the $2^{-\Delta\Delta Ct}$ formula. GAPDH was selected as a reference control.

Liver protein expression

Liver protein (20 μg) was separated by 12% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were transferred from gel to polyvinylidene fluoride membrane and incubated overnight with primary antibodies, including total AMPK (tAMPK) and phospho-AMPK (pAMPK) (EMD Millipore) as well as Sirt1 and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Later, the membranes were incubated with secondary antibody (EMD Millipore) for 2 h. The band intensity was measured using Clarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories). The band intensity were captured with the Odyssey Imager (LI-COR Biosciences, Lincoln, NE, USA). The intensity of the bands was quantitated by the Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA) and normalized with β-actin.

Liver histology

The liver was fixed in 10% formalin, embedded in paraffin, sectioned (3-μm thickness), and stained with hematoxylin and eosin. Liver sections were imaged at 400× magnification (Olympus Corp., Tokyo, Japan).

Statistical analysis

Results were expressed as mean±SEM. One-way analysis of variance followed by Tukey's *post hoc* test using the computer-based software SigmaStat (Systat Software, San Jose, CA, USA). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Metabolic parameters

This study examined the activities of RS in an HFD-induced obese mouse model associated with insulin resistance. After 16-week HFD feeding, the obese control group significantly increased the body weight and energy intake compared with the normal control group fed on a standard diet (Table 1). Interestingly, after 8 weeks of RS treatments, the body weights were significantly reduced compared with the obese control group. However, the levels of food intake and energy intake between obese groups were not significantly different. These results suggest that RS treatment may be beneficial for weight control in obese mice. Furthermore, the normal mice group treated with RS 200 mg/kg did not have significant changes in the amount of food consumed or body weight compared with the normal control group (data not shown).

Findings of this study showed that the obese control group significantly increased the levels of FBG as well as serum insulin and leptin compared with the normal control group (Table 1). RS-treated obese groups significantly decreased the elevation of FBG, insulin, and leptin compared with the untreated obese group. However, a significant increase in serum adiponectin was found in the obese groups treated with RS (Table 1). Among several mediators that regulate energy homeostasis, insulin and leptin are the main mediators of energy balance in

Table 1. Activity of RS on metabolic parameters in high-fat diet-associated insulin resistance mouse model

Parameter	NC	OB	OB + RS (mg/kg)	
			100	200
Body weight (g)	53.0±1.0	59.3±1.0#	54.0±0.7*	54.4±1.1*
Food intake (g/d/mouse)	4.9±0.1	4.4±0.2#	4.3±0.1#	4.3±0.1#
Energy intake (kcal/d/mouse)	19.0±0.4	20.7±0.7#	20.6±0.4#	20.5±0.4#
FBG (mg/dL)	95.6±3.4	164.7±6.5#	100.1±6.1*	99.0±5.5*
Serum insulin (ng/mL)	2.4±0.4	12.5±1.1#	3.3±0.7*	3.1±0.6*
Serum leptin (ng/mL)	8.1±1.2	28.0±1.2#	15.4±0.8*#	13.0±2.4*
Serum adiponectin (μg/mL)	8.5±0.6	6.0±0.3#	8.3±0.4*	8.7±0.5*
Serum TC (mg/dL)	141.3±8.3	205.1±8.7#	146.4±13.5*	126.6±14.4*
Serum TG (mg/dL)	88.1±5.4	127.0±9.9#	91.5±6.2*	85.7±6.3*
Serum NEFA (mEq/L)	2.2±0.1	3.2±0.2#	2.6±0.1*	2.7±0.1*

Data are presented as mean±SEM (n=8).
#*P*<0.05 compared with the normal control group.
**P*<0.05 compared with the obese control group.
NC, normal control mice; OB, obese control mice; RS, *Raphanus sativus* L. var. *caudatus* extract; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; NEFA, non-esterified fatty acid.

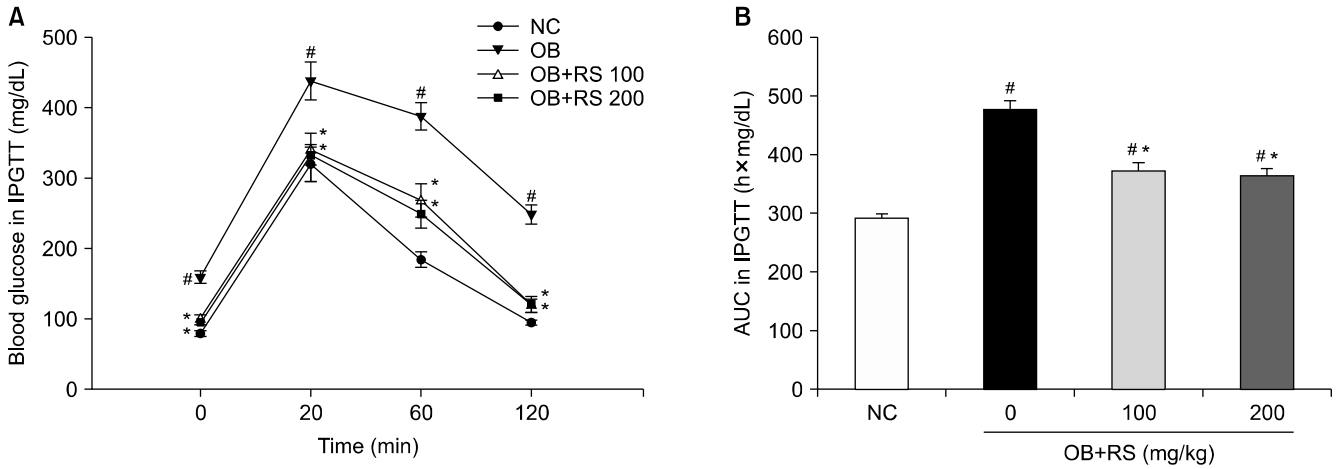

Fig. 1. Activity of *Raphanus sativus* L. var. *caudatus* extract (RS) on intraperitoneal glucose tolerance test (IPGTT) (A) and area under the curve (AUC) (B) in high-fat diet-associated insulin resistance mouse model. Results are presented as mean±SEM (n=8). $^{\#}P<0.05$ compared with the normal control (NC) group. $^{*}P<0.05$ compared with the obese control (OB) group.

maintaining body weight (Enriori et al., 2006; Sohn et al., 2013). The leptin production is primarily controlled by insulin (Obradovic et al., 2021). Long-term hyperinsulinemia in obese conditions is associated with an elevation of plasma leptin levels (Obradovic et al., 2021); consequently, the appetite cannot be suppressed, which causes weight gain (de Assis and Murawska-Ciałowicz, 2021). Our findings demonstrated that obese mice treated with both doses of RS had decreased leptin levels. Thus, body weight reduction in the RS-treated obese groups may be associated with the improvement of leptin function. Moreover, the 8-week RS administration did not present any undesired effects, such as diarrhea, that altered body weight. It is thus reasonable to consider RS as an alternative for controlling body weight.

During the entire IPGTT analysis, the levels of blood glucose in the obese control group were significantly higher than the normal control group (Fig. 1A). However, RS administration significantly decreased the high blood glucose levels at 20, 60, and 120 min after glucose loading in treated obese mice compared with obese control mice. RS treatments significantly reduced the area under the curve of blood glucose (Fig. 1B). Thus, RS administration can regulate obesity as well as insulin and leptin sensitivity.

Dyslipidemia and abnormal fat storage, especially in the liver, often occur in metabolic disorders, such as obesity, insulin resistance, diabetes, and NAFLD (Godoy-Matos et al., 2020). The liver TG storage is an important indicator of hepatic insulin resistance and NAFLD (Mu et al., 2019). The glucose-lipid connection was observed in obese mice, which had high concentrations of serum glucose, serum and liver lipids, and serum insulin. In this study, all lipid parameters, including serum TC, TG, and NEFA, were significantly increased in the obese control group compared to the normal control group (Table 1). The concentrations of serum TC, TG, and NEFA were significantly decreased in the RS-treated obese groups compared with the untreated obese group (Table 1). Moreover, the treated mice displayed a significant decrease in liver weight as well as TG and TC deposition (Fig. 2A～2C). Analysis of liver histology revealed that the RS-treated obese groups had lesser lipid accumulation than the untreated obese group (Fig. 2D). These findings clearly demonstrated that RS administration decreased lipid contents in the circulation and liver tissue. Moreover, the blood glucose and insulin resistance (as seen in the results of improved glucose tolerance) were significantly reduced in RS-treated obese mice. Therefore, improvement of hyperglycemia, hyperlipidemia, glucose tolerance, and liver TG storage by RS may ameliorate insulin resistance.

Expression of lipogenic genes and AMPK/Sirt1 protein in the liver

The elevated SREBP1c, FAS, and ACC gene expressions were significantly observed in the obese control group compared with the normal control group (Fig. 3A～3C, respectively). However, RS treatments significantly reduced the increase in gene expression in treated obese mice compared with untreated obese mice. This expression was even lower than that in normal control mice. Next, we determined the protein expression involved in the AMPK/Sirt1 pathway. The RS-treated obese groups had increased expression of phosphorylated AMPK (Fig. 4A) and Sirt1 (Fig. 4B) proteins compared with the untreated obese group.

Because activated AMPK plays a main role in decreased liver fat deposition (Hardie, 2008a), the modulation of energy balance in the liver is associated with AMPK via several mechanisms, such as increase in fatty acid oxidation, inhibition of fat synthesis, and suppression of glu-

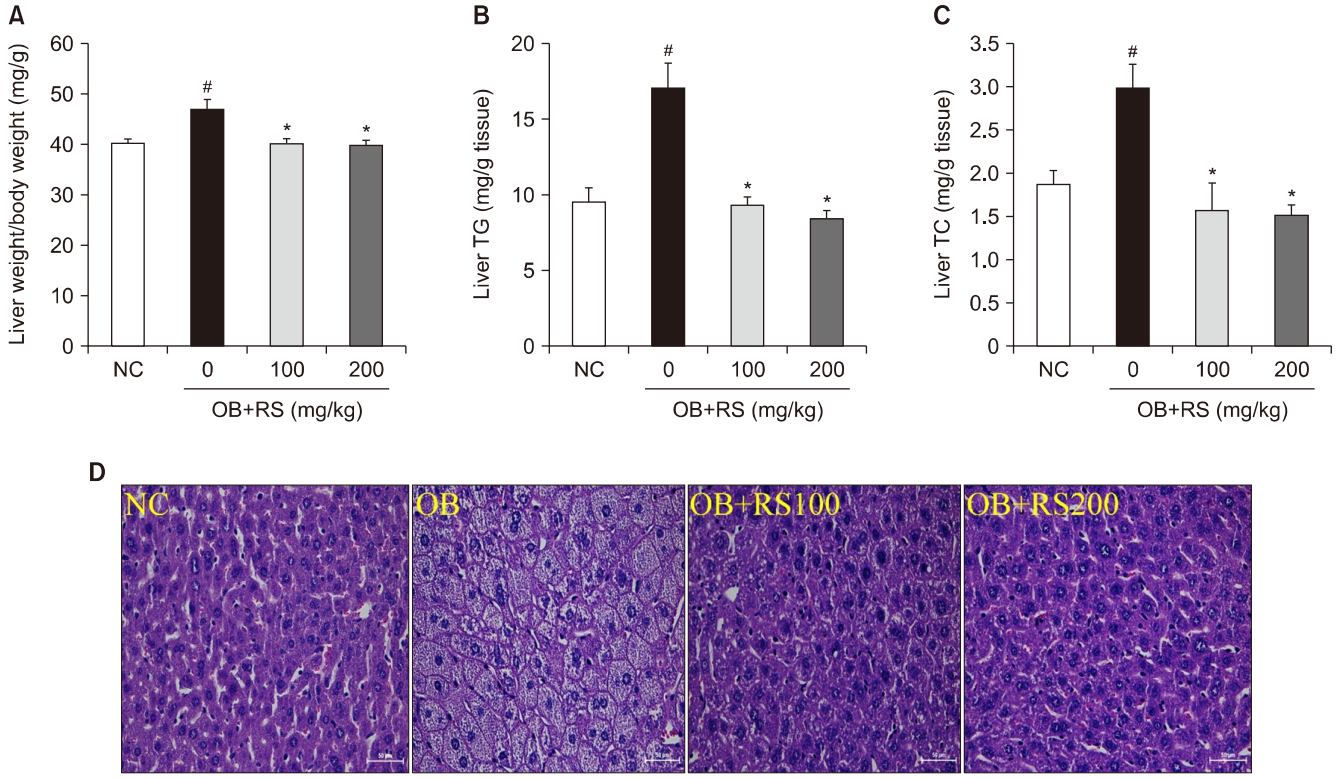

Fig. 2. Activity of *Raphanus sativus* L. var. *caudatus* extract (RS) on liver weight (A), liver triglyceride (TG) (B), liver total cholesterol (TC) (C), and liver histology (H&E, 400×, scale bar 50 μm) (D) in high-fat diet-associated insulin resistance mouse model. Results are presented as mean±SEM (n=8). $^{\#}P<0.05$ compared with the normal control (NC) group. $^{*}P<0.05$ compared with the obese control (OB) group.

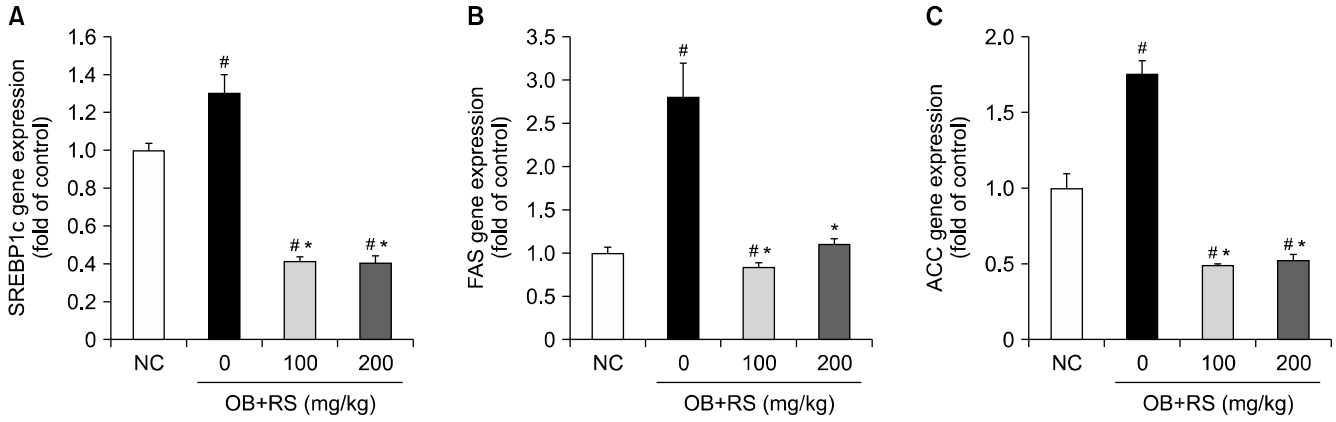

Fig. 3. Activity of *Raphanus sativus* L. var. *caudatus* extract (RS) on SREBP1c (A), FAS (B), and ACC (C) gene expression in the liver of high-fat diet-associated insulin resistance mouse model. Results are presented as mean±SEM (n=8). $^{\#}P<0.05$ compared with the normal control (NC) group. $^{*}P<0.05$ compared with the obese control (OB) group.

coneogenesis (Viollet et al., 2009). Sirt1 is another regulatory key in controlling lipid and glucose homeostasis in the liver (Li, 2013). Phosphorylated AMPKα was shown to directly activate Sirt1 (Baskaran et al., 2016). Overexpression of Sirt1 suppressed hepatic steatosis in HFD-fed mice (Pfluger et al., 2008) and reduced symptoms of fatty liver via suppressing the lipogenic genes in monosodium glutamate-treated mice (Yamazaki et al., 2009). Elevation of Sirt1 reduced HFD-induced obesity (Lee et al., 2019), but depletion of Sirt1 increased fat mass, repressed glucose tolerance, and reduced insulin sensitivity (Li et al., 2019). Resveratrol, a phytochemical Sirt1 activator, decreased SREBP1c expression (Ponugoti et al., 2010), reduced hepatic fat content, and improved insulin resistance in obese humans (Timmers et al., 2011). In this study, we demonstrated that RS treatment could upregulate the liver protein expression of AMPK phosphorylation and Sirt1. These data suggest that stimulation of the AMPK/Sirt1 has a relationship with decreased lipogenic genes (SREBP1c, FAS, and ACC), in RS-treated obese

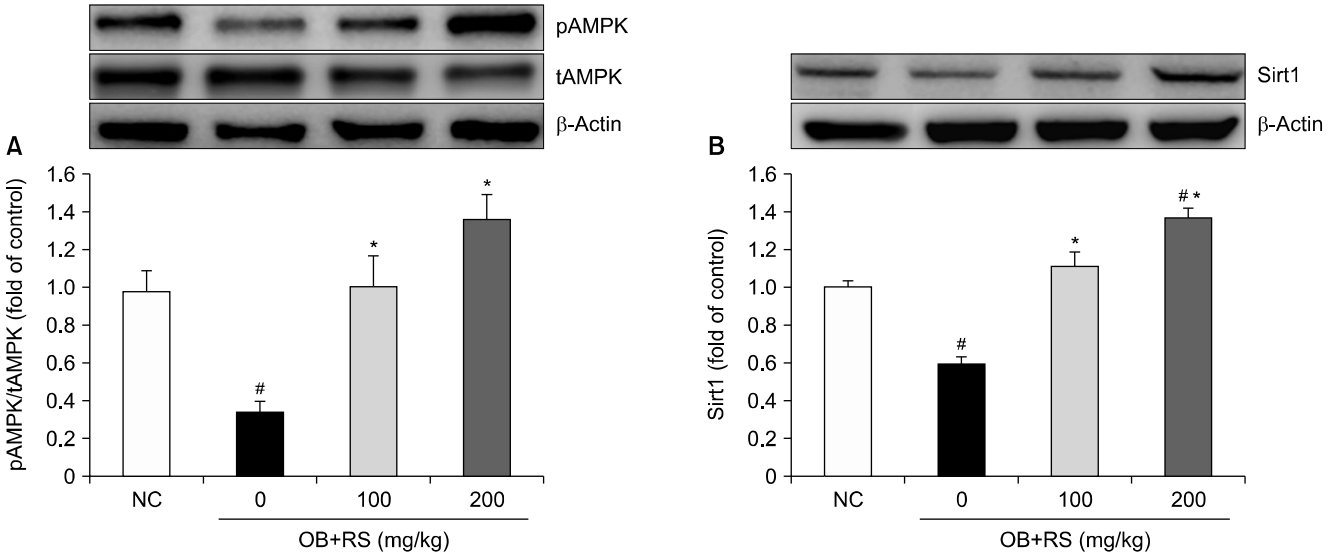

Fig. 4. Effect of *Raphanus sativus* L. var. *caudatus* extract (RS) on pAMPK/tAMPK ratio (A) and sirtuin1 (Sirt1) (B) protein expression in the liver of high-fat diet-induced obese mice. Data are presented as mean±SEM (n=8). $^{\#}P<0.05$ compared with the normal control (NC) group. $^{*}P<0.05$ compared with the obese control (OB) group.

mice. Our findings reveal the benefits of RS in alleviating the symptoms of impaired lipid and glucose homeostasis in obesity-associated insulin resistance.

Phytochemical contents of RS

The presence of several phenolic compounds in RS, such as quercetin, sinapic acid, and caffeic acid, is shown in Table 2. Caffeic acid was a major compound in RS, with a high concentration of 179.27 μg/g. Caffeic acid exerts antidiabetic effects (Oršolić et al., 2021) and neuroprotective effects through the amyloid-tau-neuroinflammation axis and AMPK/Sirt1 pathway (Hao et al., 2020). Caffeic acid was reported to strongly induce AMPK activation in various cell types (Vasileva et al., 2020). However, the contents of isothiocyanates, which are commonly found in RS, were not measured in our study. Numerous studies have shown that sulforaphane, a natural isothiocyanate mainly found in RS (Pocasap et al., 2013), displayed pharmacological activities, such as suppression of inflammatory process and oxidative stress in diabetic rats (Negi et al., 2011). Sulforaphane stimulated the AMPK pathway in *ob/ob* mice and suppressed adipogenesis in 3T3-L1 cells (Ranaweera et al., 2022). Thus, sulforaphane effectively regulates impaired liver glucose and lipid metabolism and the phenolic compounds detected in this study in HFD-induced obese mice. Nevertheless, increasing RS concentrations to alleviate insulin resistance should be performed with caution because ingredients in RS that were not assessed in this study may have deleterious effects on regulation of lipid and glucose homeostasis.

Table 2. Phytochemical contents of *Raphanus sativus* L. var. *caudatus* extract in μg/g

Phytochemicals	
Gallic acid	−
Caffeic acid	179.27±3.96
Coumaric acid	−
Ferulic acid	−
Sinapic acid	47.92±1.85
Catechin	24.77±0.96
Rutin	17.43±1.11
Quercetin	47.68±0.96

Data are presented as mean±SEM (n=3).
−, not detected.

In conclusion, our findings indicated that RS administration improved abnormal lipid and glucose homeostasis in HFD-induced obese mice. This improvement was related to a reduction in serum lipids, liver TG and TC accumulation, and liver lipogenic gene expressions (SREBP1c, FAS, and ACC). RS treatment also improved insulin resistance, which resulted in the reduction of hyperglycemia, hyperleptinemia, and hyperinsulinemia. Impaired lipid and glucose homeostasis in the liver may be improved by stimulating the AMPK/Sirt1 pathway. Hence, RS can be used as a regulator to treat metabolic disorders, such as obesity-associated insulin resistance.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: LC, SW, JN. Analysis and interpretation: LC, JN. Data collection: LC, UN, JN. Writing the article: JN, PT, PH. Critical revision of the article: JN, PH. Final approval of the article: all authors. Statistical analysis: LC, CJ, JN. Obtained funding: LC. Overall responsibility: LC, JN.

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