Antidiabetic Activities of Extract from *Malva verticillata* Seed via the Activation of AMP-Activated Protein Kinase

Jeong, Yong-Tae and Chi-Hyun Song*

Department of Biotechnology, Daegu University, Gyeongbuk, Gyeongsan 712-714, Korea

Received: April 14, 2011 / Revised: May 23, 2011 / Accepted: May 28, 2011

Stimulation of AMP-activated protein kinase (AMPK) signaling followed by increase of glucose uptake in L6 myotubes were studied with organic solvent extract of *Malva verticillata* (MV) seeds. Ethanol extract of *M. verticillata* seeds (MVE) significantly increased the phosphorylation level of AMPK, acetyl-CoA carboxylase (ACC), and glucose uptake in L6 myotube cells. The MVE was fractionated with *n*-hexane (MVE-H), chloroform (MVE-C), ethylacetate (MVE-E), *n*-butanol (MVE-B), and water (MVE-W). MVE-H (150 µg/ml) showed the highest phosphorylating activity and increased glucose uptake by 2.3-fold. Oral administration of MVE-H (40 mg/kg) for 4 weeks to type 2 diabetic (*db/db*) mice reduced non-fasting and fasting blood glucose levels by 17.1% and 23.3%, respectively. Phosphorylation levels of AMPK and ACC in the soleus muscle and liver tissue of *db/db* mice were significantly increased by the administration of MVE-H. MVE-H was further fractionated using preparative HPLC to identify the AMPK-activating compounds. The NMR and GC–MS analyses revealed that β-sitosterol was a major effective compound in MVE-H. Phosphorylation levels of AMPK and ACC, and glucose uptake were significantly increased by the treatment of MVE-S (β-sitosterol) isolated from *M. verticillata* to L6 cells, and these effects were attenuated by an AMPK inhibitor (Compound C) pretreatment. These results, taken together, demonstrate that increased glucose uptake in L6 myotubes by MVE-H treatment is mainly accomplished through the activation of AMPK. Our finding suggests that the extract isolated from *M. verticillata* seed would be beneficial for the treatment of metabolic disease including type 2 diabetes and hyperlipidemia.

**Keywords:** *Malva verticillata*, glucose uptake, AMPK, L6 myotube, β-sitosterol, *db/db* mice

The prevalence of diabetes is increasing worldwide. Currently, 150 million people suffer from diabetes and the trend is expected to increase to over 300 million by 2025 [40]. Diabetes mellitus is classified as insulin-dependent diabetes mellitus (IDDM, type 1 diabetes) and non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). About 90% of patients are NIDDM, with insulin resistance playing a key role in the development of the disease [10]. Hyperinsulinemia and insulin resistance (reduced potency of insulin action) are characteristics of NIDDM and usually precede the onset of hyperglycemia. They are also found in the metabolic syndrome, which is defined in the present study as a state of metabolic dysregulation characterized by insulin resistance, hyperinsulinemia, central obesity, and a predisposition to NIDDM, dyslipidemia, hypertension, premature atherosclerosis, and other diseases [32]. Insulin resistance has been increasingly linked to abnormal lipid metabolism, as reflected by an excessive accumulation of triglyceride (TG) in muscle and liver [33].

NIDDM is characterized by insulin resistance in skeletal muscle tissue [39], which accounts for the majority of insulin-stimulated glucose disposal [6]. Whereas the expressed amount of glucose transporter 4 (GLUT4) protein was normal in muscles from subjects with NIDDM, the capacity of insulin to stimulate translocation of GLUT4 to the plasma membrane was impaired [11]. Several defects in the insulin signaling pathway are associated with this phenomenon. Evidence accumulated over the past several years indicates that the AMP-activated protein kinase (AMPK) would be a good target for the pharmacological treatment of type 2 diabetes and obesity, because the AMPK pathway performs a central function in the control of glucose and lipid homeostasis, insulin signaling, and mitochondria biogenesis [5, 16, 17, 36].

Plant and herbal extracts have been used as antidiabetic agents [4]. However, most of them have been shown to exert little or no effect on glycemic control in experimental studies, although some herbs possess hypoglycemic properties.
Malva verticillata seed has been used as diuretic, galactogogue, and laxative treatments [14, 35] and the seeds are known to contain mucilage, polysaccharides, and flavonoids [31]. However, studies have not been conducted regarding whether the seeds are effective on diabetes. In the present research, we obtained several extracts from M. verticillata using various organic solvents, to determine whether these extracts can activate AMPK. A selected extract was further fractionated, identified, and tested for its pharmacological effects on diabetes.

**Materials and Methods**

**Preparation of the Extracts**

The seed of *Malva verticillata* were purchased from a Daegu traditional medicinal market. Five types of extracts from the seed of *Malva verticillata* were prepared for the preliminary activity screening. Four flasks each containing 100 g of dried seeds were extracted with hexane (3 × 500 ml), or ethylacetate (3 × 500 ml), or 70% or 100% ethanol (3 × 500 ml) after sonication for 2 h. Another extract was obtained through hot water extraction for 2 h. Each of the combined extracts was evaporated under reduced pressure and these extracts were used for the preliminary screening test.

**Fractionation of the Ethanol Extract**

The combined ethanol extract was partitioned between n-hexane and water, with the more polar layer then partitioned with chloroform, ethylacetate, and n-butanol. After being tested, the most active fraction was selected. The partially purified fraction with high activity was further purified by using preparative high-performance liquid chromatography (LC-9104; Jai Co., Ltd., Tokyo, Japan) equipped with a refractive index detector (UV-310, RI-7) and a 1H column attached. The partially purified fraction with high activity was further purified by using preparative high-performance liquid chromatography (LC-9104; Jai Co., Ltd., Tokyo, Japan) equipped with a refractive index detector (UV-310, RI-7) and a 1H column attached. The partially purified fraction with high activity was further purified by using preparative high-performance liquid chromatography (LC-9104; Jai Co., Ltd., Tokyo, Japan) equipped with a refractive index detector (UV-310, RI-7) and a 1H column attached.

**Bioactivity Test**

**Chemicals and reagents.** L6 myotube cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS), minimum essential medium α (α-MEM), trypsin/EDTA, and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Compound C was purchased from Calbiochem (Schleicher & Shuell), and subsequently subjected to immunoblot experiments.

**Immunoblot analysis.** Immunoblot analysis was carried out as previously described [37]. L6 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin). Cell debris was removed by centrifugation at 14,000 ×g for 15 min at 4°C, and the resulting supernatant was used for immunoblot analysis. The protein concentration of the cell lysate was determined using the Bio-Rad Protein Assay Agent. Proteins in cultured cell homogenates (50–80 µg) were separated on 8% SDS–polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Schleicher & Shuell), and subsequently subjected to immunoblot analysis using specific antibodies. The immunoreactive antigen was then visualized by using the horseradish peroxidase-labeled anti-rabbit IgG from Amersham Biosciences (Piscataway, NJ, USA) and the enhanced chemiluminescence detection kit from Pierce Biotechnology (Rockford, IL, USA).
Animal Experiment
Animal and breeding conditions. Five-week-old C57BLKS/J-db/db mice were purchased from Japan SLC Inc. and were divided into 5 groups in plastic cages. The animal room was maintained at constant temperature (22°C ± 2) and humidity (55% ± 5) with 12 h cycle of light and dark. Mice were fed with commercial pellet diet (FEEDLAB Co., Korea) for 5 weeks. All animal experiments were performed under the guidelines of the Ethics Committee for Animal Experimentation of Korea Food Research Institute.

Experimental design. The highest AMPK-activation of crude extract (MVE-H) isolated from M. verticillata was studied for its antidiabetic potential. The type-2 diabetic mice (db/db mice) were divided into 5 groups. Negative control (olive oil), positive control (rosiglitazone, 5 mg/kg bodyweight), and 3 different concentrations of MVE-H (10, 20, or 40 mg/kg bodyweight) in olive oil were orally administered daily for 4 weeks. The blood sample was collected every week from the tail veins and was measured for non-fasting blood glucose concentration. At the end of oral administration, the animals were fasted for 8 h, blood was collected from the eye, and the organs were dissected.

Separation of plasma and organs. Plasma was separated by centrifugation (1,110 × g for 20 min) of whole blood. Organs were dissected and the weights of the organs were measured after washing with 0.9% NaCl. Livers were perfused with cold saline, excised, and kept frozen at -70°C.

Analysis of blood glucose, plasma glucose, and lipids. Blood glucose level was measured using glucose oxidase strips (Glucocard test strips; Arkray, Inc., Japan). The plasma glucose, total cholesterol, triglyceride, and HDL cholesterol were measured using an enzymatic test kit (Asan Pharm. Co., Ltd., S. Korea). LDL cholesterol [8] and the HDL cholesterol to total cholesterol ratio (HTR) were calculated by the following equation; LDL cholesterol = total cholesterol - HDL cholesterol - (triglyceride/5), HTR = HDL cholesterol / total cholesterol. Statistical analysis. Data were expressed as means ± SE. Group means were compared by one-way analysis of variance and by Duncan’s multiple-range test [7]. The statistical differences were considered significant at p<0.05.

Characterization
The gas chromatography–mass spectrometry (GC–MS) analysis of the isolated compound was performed using a HP 6890 GC coupled to an Agilent 5973 N mass selective detector. A HP 5 MS capillary column (5% PH ME siloxane, 30 m × 0.25 mm, 0.25 μm; USA) was used. Helium served as the carrier gas (flow rate: 0.7 ml/min). The initial oven temperature was 70°C, which was maintained for 1 min and then increased to 300°C at a rate of 7°C/min and held for 30 min. The injector and detector temperatures were 250°C and 200°C, respectively. The injection volume was 1 μl and split ratio was 50:1. The mass spectrometer was operated at 70 eV in the electron impact mode with scan. Identification of compound was tentatively verified using a mass spectral library (Wiley 7nL Mass Spectral Database). NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer (Bruker Co., Billerica, MA, USA) with a 5 mm inverse probe. Proton spectra were run at a probe temperature of 25°C. The sample was dissolve in CDCl3 at a concentration of 5 mg/ml (1H NMR) and 20 mg/ml (13C NMR). 1H chemical shifts were referenced to internal CDCl3 (4.7 ppm at all temperatures).

β-Sitosterol: 1H-NMR (300 MHz, CDCl3): δ: 0.68 (3H, s, CH3-18), 0.81 (3H, d, J = 6.6 Hz, CH3-26), 0.83 (3H, d, J = 6.3 Hz, CH3-27), 0.84 (3H, t, J = 7.2 Hz, CH3-29), 0.92 (3H, d, J = 6.6 Hz, CH3-21), 1.01 (3H, s, CH3-19), 3.52 (1H, m, H-3), 5.35 (1H, d, J = 5.1 Hz, H-6); 13C-NMR (75.5 MHz, CDCl3): δ: 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.8 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.1 (C-17), 11.9 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 34.0 (C-22), 26.1 (C-23), 45.8 (C-24), 29.2 (C-25), 19.0 (C-26), 19.8 (C-27), 23.1 (C-28), 12.0 (C-29).

RESULTS
AMPK-Stimulating Activity Tests of Malva verticillata Seed
M. verticillata (MV) seeds were extracted by hexane, ethylacetate, ethanol, and water. With these MV seeds...
extracts, phosphorylation levels of AMPK and ACC, and glucose uptake were tested in L6 cells. The phosphorylation levels of AMPK and ACC were increased by hexane, ethylacetate and 100% ethanol extracts of MV seeds when treated at 300 µg/ml for 2 h (Fig. 2A). On the contrary, only 100% and 70% ethanol extracts increased glucose uptake by 2.5-fold and 2.4-fold, respectively when treated at 300 µg/ml for 2 h (Fig. 2B). We selected the 100% ethanol extract for further analysis. When 100% ethanol extract was treated to L6 cells, phosphorylation of AMPK and ACC was increased in a dose- and time-dependent manner (Fig. 2C and 2D). The highest phosphorylation of AMPK and ACC was observed at 150 µg/ml for 2 h treatment. At 150 µg/ml, phosphorylation of AMPK and ACC started to increase within 0.5 h and reached the highest at 2 h after the treatment.

To obtain enriched effective compounds stimulating AMPK activity, 100% ethanol extract of *M. verticillata* seed (MVE) was fractionated with *n*-hexane (MVE-H), chloroform (MVE-C), ethylacetate (MVE-E), *n*-butanol (MVE-B), and water (MVE-W). The stimulation of AMPK and ACC phosphorylation in L6 cells was increased by all the subfractions of 100% ethanol extract, except MVE-W, when they were treated at 150 µg/ml for 2 h (Fig. 3A). Among them, only MVE-H could increase glucose uptake by 2.3-fold when it was treated at 150 µg/ml for 2 h (Fig. 3B), and the highest AMPK and ACC phosphorylations were observed when MVE-H was treated at 100 µg/ml for 4 h (Fig. 3C and 3D).

**In Vivo Antidiabetes Effect of MVE-H**

To test the *in vivo* effect of crude extract (MVE-H), the antidiabetic effect of MVE-H was analyzed using type 2 diabetic *db/db* mice. For this purpose, different doses of MVE-H (10, 20, and 40 mg/kg bodyweight/day) were orally administered to diabetic mice for four weeks and the blood glucose levels of the mice were analyzed every week. Non-fasting blood glucose levels were decreased in the 20 mg/kg and 40 mg/kg treatment groups (Fig. 4A). In mice treated with MVE-H (40 mg/kg), the non-fasting blood glucose level was 17.1% lower than that of the control group after 4-week treatment. The effect of MVE-H on lowering blood glucose level was a dose-dependent manner (Fig. 4B). Fasting blood glucose level was also significantly decreased (23.3%) with administration of MVE-H (40 mg/kg) compared with the control group.

The effects of MVE-H on levels of plasma total cholesterol, triglycerides, and HDL cholesterol are summarized in Table 1. In MVE-H treated mice (40 mg/kg), reductions of 15.3% of total plasma cholesterol and 20.3% of plasma triglycerides levels were observed, but the HDL cholesterol levels of MVE-H-treated mice (114.9 ± 2.8 mg/dl) were not significantly different from that of the control group (109.7 ± 20.3 mg/dl).

In accordance with these beneficial effects of MVE-H on lowering blood glucose, total cholesterol, and triglycerides levels, phosphorylations of AMPK and ACC in the soleus muscle and in the liver of *db/db* mice were all increased by administration of MVE-H (Fig. 4C and 4D).

**Purification of AMPK-Stimulating Active Compounds and Analysis of *M. verticillata* Ethanol Extract**

The bioactive subfraction MVE-H was further fractionated by recycling preparative HPLC. Among the 10 fractions obtained from recycling preparative HPLC, fractions 3 and 4 showed the highest activity for increasing the phosphorylation of AMPK and ACC (data not shown). When these two fractions were pooled and further separated into 7 fractions,
fraction 2 showed the highest activity for increasing the phosphorylation of AMPK and ACC. This fraction 2 was further separated into 4 fractions. Among them, fraction 4 (MVE-S) showed the highest activity for increasing the phosphorylation of AMPK and ACC (data not shown). The compound isolated from MVE-S was characterized to

Table 1. Plasma lipid and blood glucose levels in db/db mice after administration of MVE-H obtained from Malva verticillata.

<table>
<thead>
<tr>
<th>Group</th>
<th>Triglyceride (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>HDL cholesterol (mg/dl)</th>
<th>HTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>126.6±6.9b</td>
<td>251.2±28.6b</td>
<td>109.7±20.3 NS</td>
<td>0.52±0.03b</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>72.7±6.0ab</td>
<td>286.7±34.7b</td>
<td>85.2±9.8</td>
<td>0.33±0.04a</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>90.6±6.4a</td>
<td>228.8±12.6b</td>
<td>116.6±2.8</td>
<td>0.57±0.02b</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>105.9±7.2ab</td>
<td>210.7±9.9f</td>
<td>113.8±5.5</td>
<td>0.58±0.04b</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>100.8±6.9b</td>
<td>207.1±12.1b</td>
<td>114.9±2.8</td>
<td>0.58±0.04b</td>
</tr>
</tbody>
</table>

The animals (db/db mice) of each experimental group were administered orally with either olive oil (control) or different doses of MVE-H (10–40 mg/kg body weight/day) for 4 weeks. HTR=HDL-cholesterol/total cholesterol. Each value represents the mean±SE for 8 mice.

a, b Values with different letters are significantly different among the groups at p<0.05.
β-sitosterol (molecular mass was 414 g/mol) as shown in Fig. 5A by NMR and GC–MS [12, 13].

Stimulation of AMPK and ACC Phosphorylation, and Glucose Uptake by MVE-S in L6 Myotubes

Treatment of L6 myotube cells with 100 µM β-sitosterol from *Malva verticillata* (MVE-S) increased the phosphorylation of the Thr<sup>172</sup> residue of the AMPKα subunit and the Ser<sup>79</sup> subunit of ACC in a dose- and time-dependent manner. Treatment with MVE-S at a concentration of 25 µM for 2 h increased AMPK phosphorylation, whereas the increasing effect on AMPK phosphorylation was not detected with lower than 25 µM (Fig. 5B and 5C).

We also analyzed the effect of MVE-S on glucose uptake in L6 myotube cells. For this purpose, L6 myotube cells were exposed to 75, 150, and 300 µM of MVE-S. All the treatments of β-sitosterol increased glucose uptake in L6 cells by around 1.7-fold compared with untreated control (Fig. 5D).

To verify the specificity of MVE-S for AMPK signaling, compound C, an inhibitor of AMPK [38], was pretreated to L6 cells prior to β-sitosterol treatment and changes of AMPK and ACC phosphorylations were tested. The increasing effect of phosphorylation of AMPK and ACC by MVE-S treatment was almost completely abrogated by the pretreatment of 10 µM Compound C (Fig. 6A). Similarly, the increase of glucose uptake by β-sitosterol treatment was not induced in L6 cells when Compound C was pretreated (Fig. 6B).

**DISCUSSION**

AMPK is a highly conserved fuel sensor of cellular energy status and is activated by a variety of cellular stresses that deplete ATP, including exercise, muscle contraction, changes in fuel availability, and adipocyte-derived hormones, adiponectin and leptin, as well as by hypoxia, oxidative...
A subfractionated sample (MVE-H) of MVE showed remarkably increasing phosphorylating activity on the AMPK and ACC, and increased glucose uptake in L6 cells. Further purification of MVE-H-S was done with MVE-H by recycling preparative HPLC. A major AMPK-activating activity in MVE-S was characterized to β-sitosterol by NMR and GC–MS analyses. This phytosterol compound is already known to possess a cholesterol-lowering effect [4]. Similarly, another phytosterol, stigmasta, isolated from various plants have beneficial effects on metabolic diseases such as antilipidemic and antidiabetic effects [15, 20]. However, the mechanisms underlying the beneficial effects of these phytosterols have not been elucidated.

In L6 myotube cells, MVE-S not only increased phosphorylation of AMPK and ACC but also stimulated glucose uptake in a dose- and time-dependent manner. In addition, MVE-S also exerted profound antidiabetic and antihyperlipidemic effects with increasing AMPK phosphorylation in the soleus muscle and liver tissue of type 2 diabetic mice. These antidiabetic effects of MVE-S may be driven by increased translocation of GLUT4 into the plasma membrane since activation of AMPK can increase GLUT4 translocation independent to insulin signaling [16]. It was also demonstrated that the expression of the constitutively active form of AMPK stimulated glucose uptake into the cells, in association with the enhanced translocation of GLUT4 to the plasma membrane [9]. The increased phosphorylations of AMPK and ACC were remarkably inhibited by Compound C [38]. These beneficial effects of MVE-S may be specifically achieved by activation of AMPK signaling, since pretreatment of Compound C abrogated MVE-S-mediated increase of AMPK and ACC phosphorylation and glucose uptake in L6 cells. However, an upstream kinase for MVE-S-mediated activation of AMPK signaling remains to be elucidated, although possible involvement of LKB1 or calcium/calmodulin-dependent protein kinase kinase (CaMKK) has been reported to be the upstream kinase of AMPK [19].

In type 2 diabetic db/db mice, oral administration of MVE-H at a dose of 40 mg/kg significantly decreased blood glucose concentration, triglycerides, and total cholesterol levels, whereas HDL cholesterol and HTR levels were marginally decreased after 4-week treatment. This result is in good agreement with other reports that administration of some plant extracts containing an AMPK activator or synthetic small molecule AMPK activator could reduced the blood glucose level as well as blood lipid level in db/db mice [5, 21, 23, 27]. However, administration of MVE-S did not reduce the gain of body weight, although activation of AMPK can exert an anti-obesity effect [26, 28]. This inability of MVE-H for anti-obesity activity might be caused by insufficient dose or duration of treatment.
In conclusion, our results provide evidence that a phytosterol, MVE-S (β-sitosterol), isolated from *M. verticillata* seeds effectively reduced blood glucose and lipid levels in diabetic *db/db* mice by increasing AMPK activity in the skeletal muscle and liver tissue, implicating that it can be a potential drug candidate for the metabolic syndrome including type 2 diabetes and hyperlipidemia.

**Acknowledgment**

This work was supported by a Daegu University research grant, 2008.

**References**


