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Myricetin as the Active Principle of *Abelmoschus moschatus* to Lower Plasma Glucose in Streptozotocin-Induced Diabetic Rats

Abstract

The antihyperglycemic action of myricetin, purified from the aerial part of *Abelmoschus moschatus* (Malvaceae), was investigated in streptozotocin-induced diabetic rats (STZ-diabetic rats). Bolus intravenous injection of myricetin decreased the plasma glucose concentrations in a dose-dependent manner in STZ-diabetic rats. Myricetin at the effective dose (1.0 mg/kg) significantly attenuated the increase of plasma glucose induced by an intravenous glucose challenge test in normal rats. A stimulatory effect of myricetin on glucose uptake of the soleus muscles isolated from STZ-diabetic rats was obtained in a concentration-dependent manner from 0.01 to 10.0 μmol/L. The increase of glucose utilization by myricetin was further characterized using the enhancement of glycogen synthesis in isolated hepatocytes of STZ-diabetic rats. These results suggest that myricetin has an ability to enhance glucose utilization to lower plasma glucose in diabetic rats lacking insulin.

Key words
Myricetin · *Abelmoschus moschatus* · Malvaceae · STZ-diabetic rats · glucose uptake · glycogen synthesis

Introduction

Diabetes, which ranks highly among the top ten causes of mortality around the world, often leads to disability from the vascular complications of coronary artery disease, cerebrovascular disease, renal failure, blindness, and limb amputation in addition to neurological complications and premature death [1]. Clinically, a novel treatment with fewer side effects is desirable for the control of the diabetic disorder, indicating the merit of additional medication for diabetic patients.

Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone; Fig. **1**) is a naturally occurring flavonol that is commonly found in tea, berries, fruits, and medicinal plants including *Abelmoschus moschatus* Medic. (Malvaceae), which has been applied in Chinese traditional medicine for the treatment of depression and anxiety [2]. In addition to possessing an antispasmodic activity in the digestive system, *A. moschatus* has been used to treat diabetes without evidence of adverse effects or toxicity [2]. *A. moschatus* is also applied externally to treat cramp, poor circulation and aching joints [2]. Actually, it has been demonstrated that myricetin possesses both antioxidative properties and a cytoprotective capacity [3], [4]. Considered as a potent anticarcinogen and antimutagen, the therapeutic potential and benefits of myricetin in cardiovascular diseases associated with diabetes mellitus have also been reported [5], [6]. Moreover, insulinomimetic effects of myricetin on lipogenesis and glucose transport in adipocytes of rat with non-insulin-dependent diabetes mellitus have been documented [7]. However, the direct effect of myricetin on glucose homeostasis in type-1 diabetes is still unclear. Thus, we employed streptozo-

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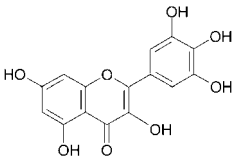

Fig. **1** Chemical structure of myricetin.

tocin induced-diabetic rats (STZ-diabetic rats), a model of type-1 diabetes, to examine the effect of myricetin that was purified from *A. moschatus* on glucose homeostasis under an insulin-deficient state.

Materials and Methods

General procedures

^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AM-500 spectrometer in DMSO-d_6 solution, using the corresponding solvent signal as internal standard. The optical rotation was measured on a Jasco DIP-1020 digital polarimeter. Column chromatography was carried out with Sephadex LH-20 (25 – 100 μ, Pharmacia Fine Chemicals), MCI-gel CHP 20P (75 – 150 μ, Mitsubishi Chemical Industries), silica gel (70 – 230 mesh, Merck). TLC was performed on silica gel plates (60 F-254, Merck), spraying with ferric chloride reagent or 10% sulfuric acid solution followed by heating was used as visualization.

Plant material

The plant, *Abelmoschus moschatus* Medic. (Malvaceae), was purchased from a folk medicinal drug store in Ping-Tung, Taiwan in August 2002. It was identified by Dr. Hsien-Chang Chang (Division of Pharmacognosy, National Laboratories of Food and Drugs, Department of Health, Taiwan). A voucher specimen was deposited at the School of Pharmacy, Taipei Medical University (no. TMU 22 082 002).

Extraction and isolation

The aerial parts of *Abelmoschus moschatus* (3 kg) were extracted three times with MeOH (each 20 L, overnight) at room temperature. The combined MeOH extracts were evaporated under vacuum and partitioned to yield *n*-BuOH (63 g) and aqueous extracts (84 g). The *n*-BuOH extract was fractionated on a Sephadex LH-20 column (8 × 100 cm) and eluted with MeOH to yield a saponin (3 g) and a flavonoid-rich fraction (47 g). The flavonoid-rich fraction was subjected to silica gel column chromatography (8 × 80 cm), using $CHCl_3$-MeOH, 9 : 1 – 7 : 3 gradient elution to provide 100 fractions of 12 mL each, which were further combined into 5 fractions according to their TLC patterns. Fraction 5 (8 g) was separated by Sephadex LH-20 column chromatography (7 × 70 cm, H_2O-MeOH gradient, 1 : 0 – 0 : 1). The fractions eluted from MeOH (5.5 g) were purified on an MCI CHP 20P column (5 × 70 cm), eluted with 20%, 30% and 40% MeOH, sequentially, to yield 3 fractions. Finally, the 30 – 40% MeOH elute (3.2 g) was further rechromatographed over silica gel (5 × 65 cm) eluting with $CHCl_3$-MeOH-H_2O (7 : 3 : 0.5) to give 2.5 g of myricetin, and the structure was established by spectral methods [8], [9]. Also, the structurally related compounds present in the flavonoid-rich fractions of *Abelmoschus moschatus* included quercetin, which has been documented to promote normalization of the level of glycemia and increase liver glycogen content in alloxan-induced diabetes [10], and the plasma glucose effect of the major flavonoid myricetin were studied in this paper.

Animal models

Male Wistar rats, aged 8 – 10 weeks (200 – 250 g body weight), were obtained from the Animal Center of National Cheng Kung University Medical College. Diabetic rats were prepared by giving an intravenous injection of STZ (Sigma-Aldrich, Inc., Saint Louis, Missouri, USA) (60 mg/kg), into the fasting rats. Animals were considered to be diabetic if they had plasma glucose concentrations of 20 mmol/L or greater in addition to polyuria and other diabetic features. Plasma insulin levels in STZ-diabetic rats were reduced to 1.18 ± 0.6 pmol/L (n = 8) following STZ injection, a level markedly lower than that of the normal rats (158.7 ± 4.2 pmol/L; n = 8), indicating that insulin deficiency is achieved in these rats. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Effect of myricetin on plasma glucose

After fasting overnight, STZ-diabetic rats received an *i.v.* injection of myricetin at the desired doses using myricetin dissolved in 70% alcohol as stock solution. Actually, the vehicle (70% alcohol:saline = 1 : 19) used to prepare myricetin solution did not modify plasma glucose in the preliminary experiments; the basal plasma glucose was 23.2 ± 1.8 mmol/L and became 23.8 ± 2.4 mmol/L in the vehicle-treated group (n = 8). Thus, the effect of vehicle on plasma glucose of STZ-diabetic rats can be ruled out. Animals were anesthetized with sodium pentobarbital (30.0 mg/kg, *i.p.*) and blood samples (0.1 mL) were collected from the tail vein for measurement of plasma glucose concentrations. In the preliminary experiments, myricetin at 1.0 mg/kg was found to produce the maximal plasma glucose lowering effect in STZ-diabetic rats 30 min after an intravenous injection. Thus, the effect of myricetin on plasma glucose was determined using blood samples collected after 30 min.

Intravenous glucose challenge test (IVGCT)

The basal plasma glucose concentration was obtained from samples from the tail vein of Wistar rats under anesthesia with sodium pentobarbital (30.0 mg/kg, *i.p.*) before the IVGCT. A solution of myricetin at 1.0 mg/kg or the same volume of vehicle was injected into the tail vein of rats. At 30 min later, blood samples (0.1 mL) from the tail vein were drawn and indicated as 0 min. Then, a glucose dose of 60.0 mg/kg was injected through the femoral vein of rats. Rats receiving a similar injection of vehicle at the same volume were taken as control. Blood samples (0.1 mL) from the tail vein were drawn at 5, 10, 20, 30, 60, 90 and 120 min following the glucose injection for the measurement of the plasma glucose concentrations. Rats were maintained under anesthesia by pentobarbital throughout the procedure.

Determination of plasma glucose

Blood samples (0.1 mL) were collected by a chilled syringe containing 10 IU heparin from the tail vein of rats under anesthesia with sodium pentobarbital (30.0 mg/kg, *i.p.*). Blood samples were then centrifuged at 13,000 rpm for 3 min and an aliquot (15 μL) of plasma was added to 1.5 mL of glucose kit reagent (Biosystems S.A., Barcelona, Spain) and incubated at 37 °C in a water

bath (Yamato-BT-25, Tokyo, Japan) for 10 min. The concentration of plasma glucose was then estimated via an analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, Indiana 46515, U.S.A.) with samples run in duplicate.

Measurement of glucose uptake into soleus muscle

Animals were sacrificed by cervical dislocation and the soleus muscle was quickly excised by a pair of scissors, dissected free of any adjoining connective tissue, blotted, and divided into long longitudinal strips (35 – 25 mg per strip). Muscles were placed in 3 mL of Krebs-Ringer bicarbonate buffer (KRBB) (37 °C, pH 7.4) containing 1 mmol/L glucose, 1% fatty acid-free bovine serum albumin (BSA) under aeration with 5% CO_2 in O_2. After pre-incubation for 30 min, the muscle tissue was incubated with 1.0 nmol/L bovine insulin (Novo Industrias, Bagsvaerd, Denmark) or myricetin at the desired concentrations for 30 min and then with 50 μL KRBB containing 2-[1 – ^{14}C]-deoxy-D-glucose (2-DG) (New England Nuclear, Boston, USA) (1 μCi/mL) for 5 min at 37 °C in the shaking water bath under aeration. Reactions were terminated by quickly blotting the muscles and dissolving them in 0.5 mL of 0.5 N NaOH for 45 min before neutralization with 0.5 mL 0.5 N HCl. After centrifugation, 800 μL of each supernatant was mixed with 1 mL of aqueous counting scintillant and the radioactivity was determined using a β-counter (Beckman LS6000). Non-specific uptake of 2-DG, assessed after an incubation with 20 μmol/L cytochalasin B (Sigma-Aldrich) to block transportation, was subtracted from the total muscle-associated radioactivity. Specific 2-DG uptake was expressed as pmol in 5 min or as the percentage of basal level that was obtained from a sample incubated with KRBB only.

Measurement of glycogen synthesis in hepatocytes

After the 30 min pre-incubation period in KRBB at 37 °C, 2×10^6 hepatocytes were transferred to fresh incubation flasks containing [U-^{14}C]-glucose (0.25 μCi/mL) and then incubated with 1.0 nmol/L bovine insulin or myricetin at the desired concentrations at 37 °C for 1 h, the optimal time obtained from preliminary experiments, under continuous shaking. Glycogen was precipitated with 70% ethanol overnight on ice. Precipitated glycogen was centrifuged at 10,000 *g* for 10 min. Pellets were washed once with 70% ethanol, resuspended in 0.5 mL water, and counted by scintillation counting. The incorporation into glycogen was expressed as pmol per mg of cell protein in 1 h or as the percentage of basal level that was obtained from hepatocytes incubated with KRBB only. The protein content was determined using the BioRad protein dye binding assay.

Statistical analysis

Data are expressed as the mean ± s.e. for the number (n) of animals in the group as indicated in tables and figures. Repeated measures analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. The concentration that produced 50% of the maximum effect (EC_{50}) was obtained from non-linear regression analysis. A P value < 0.05 was considered statistically significant.

Results and Discussion

In the preliminary experiments, we observed that the plasma glucose concentration of STZ-diabetic rats was significantly decreased from 25.1 ± 2.8 mmol/L to 18.2 ± 2.6 mmol/L at 1 h after oral administration of the aqueous crude extract of *Abelmoschus moschatus* (50.0 g/L) in a dose of 100.0 mg/kg. The yield of aqueous extraction of the herb was about 15% (w/w). It seems that oral administration with *A. moschatus* is beneficial for the glucose homeostasis regulation in STZ-diabetic rats. Quercetin has been documented to promote normalization of the level of glycemia and to increase liver glycogen content in alloxan-induced diabetes [10]. As quercetin and myricetin are structurally related flavonoids in *A. moschatus*, it is of special interest to understand whether myricetin possesses the ability to regulate plasma glucose of rats in the absence of insulin.

Giving a drug by mouth is the most common route of administration, but it is also the most variable, and requires the most complicated pathway to the tissues. In order to rule out the pharmacokinetic parameters, myricetin extracted from *A. moschatus* was investigated using intravenous injection into animals. Fig. **2** shows a dose-dependent decrease of plasma glucose in STZ-diabetic rats that received *i.v.* injections of myricetin and the maximal effect (22.6 ± 3.1%) was achieved by myricetin at 1.0 mg/kg. Increasing the myricetin dose to 1.3 mg/kg caused no further decrease of plasma glucose. The effect of myricetin reached a plateau within 30 min and was maintained for 40 min or more. Thus, 1.0 mg/kg of myricetin was employed in subsequent experiments. Actually, thirty minutes after an intravenous injection of short-acting human insulin (1.0 U/kg), the plasma glucose lowering activity produced by insulin was 49.6 ± 3.2% in STZ-diabetic rats. Nevertheless, the role of endogenous insulin is negligible in this STZ-diabetic rat model, an insulin-independent action of myricetin could be considered.

According to the previous view that the intravenous glucose challenge test (IVGCT) is available to characterize the ability of

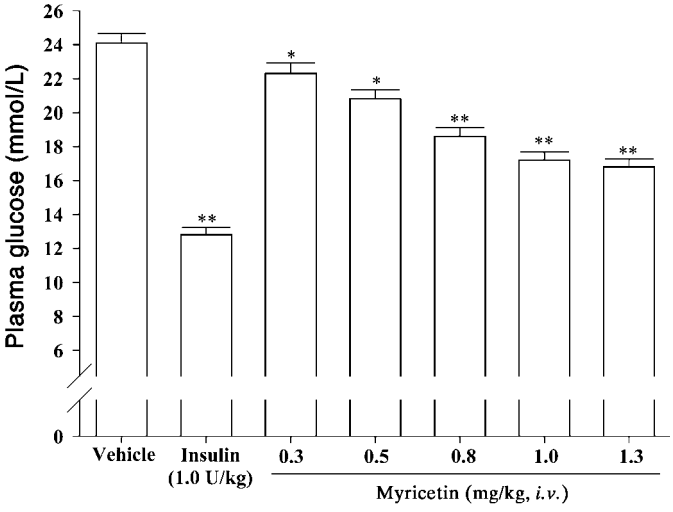

Fig. **2** The plasma glucose lowering action produced by an intravenous injection of myricetin into STZ-diabetic rats. Values (means ± s.e.) were obtained from each group of 8 animals. * P < 0.05 and ** P < 0.01 versus data from animals treated with vehicle given at the same volume.

an animal to clear glucose from the circulation [11], the IVGCT was carried out in the present study. The basal plasma glucose concentration in Wistar rats was 5.6 ± 0.5 mmol/L. As shown in Fig. **3**, thirty minutes after an intravenous injection of myricetin (1.0 mg/kg), the plasma glucose concentration was decreased to 4.3 ± 0.4 mmol/L in rats; however, the plasma glucose concentration was 5.5 ± 0.6 mmol/L in vehicle-treated rats, which was not different from the basal level of plasma glucose. Five minutes after glucose injection, the plasma glucose was elevated to 17. ± 0.6 mmol/L in vehicle-treated rats and was changed to 13.5 ± 0.7 mmol/L in myricetin-treated rats (Fig. **3**). The plasma glucose in rats pretreated with myricetin remained significantly lower than the vehicle-treated group after glucose injection for 20 min (Fig. **3**). No statistical difference ($P > 0.05$) between the myricetin-treated group and vehicle-treated controls was obtained for the plasma glucose level in rats receiving glucose injection 90 min later. The obtained finding shows that the increase of glucose utilization can be considered for the plasma glucose lowering action of myricetin *in vivo*.

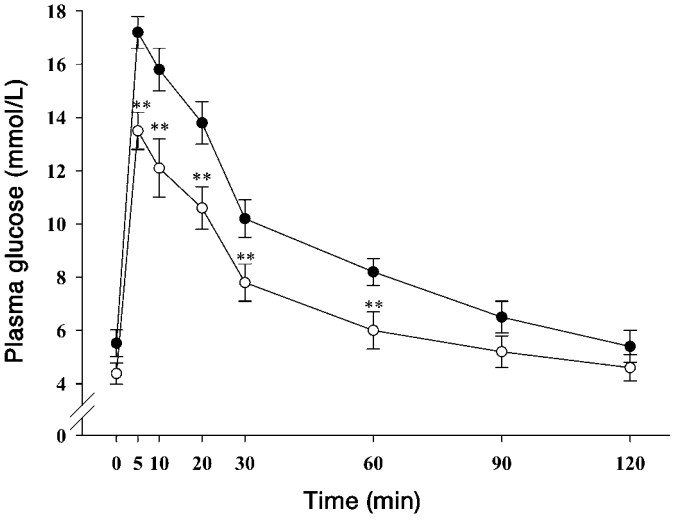

Fig. **3** Effect of myricetin on plasma glucose in normal rats receiving an intravenous glucose challenge test (IVGCT). Myricetin (1.0 mg/kg) was injected into the tail vein (open circles) and compared with the control group of rats receiving a similar injection of vehicle at same volume (closed circles). Then, the IVGCT was performed by an intravenous injection of glucose (60.0 mg/kg) into each group of rats at 30 min later and plasma glucose in samples obtained before injection of glucose was indicated as 0 min. Values (means ± s.e.) were obtained from 8 rats in each group. * $P < 0.05$ and ** $P < 0.01$ versus data from control group.

Glucose transport, which depends on insulin-stimulated translocation of glucose carriers to the cell membrane, is the rate-limiting step in the carbohydrate metabolism of skeletal muscle, a major site for glucose disposal [12]. Reduction in insulin-mediated glucose uptake in diabetes has been reported [13]. Thus, we prepared soleus muscle samples from STZ-diabetic rats to evaluate the effect of myricetin on glucose uptake. In the present study, stimulation of 2-DG uptake into soleus muscle by a 30-min exposure to 1.0 nmol/L bovine insulin was about 236.5 ± 4.8% of the basal 2-DG uptake that was taken as 100% from samples incubated with KRBB only (Table **1**). Incubation with myricetin for 30 min increased the 2-DG uptake into isolated soleus muscle in a concentration-dependent manner (Table **1**). Maximal 2-DG uptake obtained in samples treated with myricetin at 1.0 μmol/L was about 182.2 ± 4.9% of the basal uptake, the 2-DG uptake by myricetin at 10.0 μmol/L was similar with the value obtained from 1.0 μmol/L of myricetin (Table **1**). The EC_{50} of myricetin to increase 2-DG uptake into soleus muscle was about 0.1 μmol/L. Therefore, myricetin could increase the uptake of glucose into muscle and this can be considered as one of the mechanism(s) to lower plasma glucose without the presence of insulin.

Otherwise, the liver is mainly responsible for the maintenance of regular blood glucose through its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or gluconeogenic precursors [14]. In diabetes, an elevation of blood glucose is a consequence of increased hepatic glucose output together with reduced peripheral glucose utilization [15]. Thus, we used liver samples to investigate the effect of myricetin on incorporation of glucose into glycogen that can be related to the decrease of plasma glucose. In isolated hepatocytes of STZ-diabetic rats, a 30-min exposure of 1.0 nmol/L bovine insulin increased the level of glucose incorporation into glycogen to about 2.4-fold of the basal glycogen synthesis that was taken as 100% from samples treated with the same volume of KRBB (n = 6). Myricetin at 1.0 μmol/L increased the glycogen synthesis in isolated hepatocytes of STZ-diabetic rats to about 1.8-fold of the basal level (Table **1**). Even at 10 μmol/L, myricetin did not further increase glucose incorporation into glycogen (Table **1**). The EC_{50}

Table **1** Effects of myricetin on the glucose uptake into isolated soleus muscle and the glycogen synthesis into hepatocytes isolated from STZ-diabetic rats[a,b]

	Glucose uptake (pmol/5 min)	*Glycogen synthesis (pmol/mg protein/h)*
Vehicle	702.2 ± 27.6	1352.3 ± 26.3
Insulin (1.0 nmol/L)	1685.3 ± 35.2**	3264.5 ± 36.7**
Myricetin (μmol/L)		
0.001	706.5 ± 28.9	1485.2 ± 31.2
0.01	848.8 ± 30.2*	1642.7 ± 25.3*
0.1	996.2 ± 26.2*	2027.9 ± 24.1*
1.0	1289.4 ± 33.3**	2485.5 ± 28.6**
10.0	1315.4 ± 29.4**	2507.6 ± 30.4**

[a] Values (mean ± s.e.) were obtained from each group of 8 animals. The glucose uptake and the glycogen synthesis were determined as described in Materials and Methods.
[b] *$P < 0.05$ and ** $P < 0.01$ versus data from samples incubated only with vehicle, respectively.

of myricetin on glycogen synthesis was similar to that on glucose uptake (about 0.1 μmol/L). This result can be used to implicate the increase of glucose utilization by myricetin in peripheral tissue for lowering of plasma glucose under an insulin deficient state. Although myricetin was not so effective as bovine insulin to lower the plasma glucose in STZ-diabetic rats, our data showed that myricetin seems to be valuable as a plasma glucose lowering agent in type-1 diabetes by increasing glucose utilization. However, the mechanism of myricetin to lower plasma glucose needs more studies.

In summary, the present study has demonstrated that intravenous injection of myricetin, the active component of *Abelmoschus moschatus*, could lower the plasma glucose in STZ-diabetic rats through an increase of glucose utilization. These data show that myricetin can be used as a therapeutic intervention or an attractive adjuvant for type 1-diabetic patients.

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