Research Communication

Antidiabetic Activity of *Sedum dendroideum*: Metabolic Enzymes as Putative Targets for the Bioactive Flavonoid Kaempferitrin

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Abstract

The aim of this study was to evaluate the antidiabetic potential of a leaf extract and flavonoids from *Sedum dendroideum* (SD). Additionally, our goals were to establish a possible structure/activity relationship between these flavonoids and to assess the most active flavonoid on the glycolytic enzyme 6-phosphofructo-1-kinase (PFK). SD juice (LJ), a flavonoid-rich fraction (BF), and separately five flavonoids were evaluated intraperitoneally for their acute hypoglycemic activity in normal and streptozotocin-induced diabetic mice. First, the major flavonoids kaempferol 3,7-dirhamnoside or kaempferitrin (1), kaempferol 3-glucoside-7-rhamnoside (2), and kaempferol 3-neohesperidoside-7-rhamnoside (3) were tested. Then, the monoglycosides kaempferol 7-rhamnoside (5) and kaempferol 3-rhamnoside (6) were assayed to establish their structure/activity relationship. The effect of 1 on PFK was evaluated in skeletal muscle, liver, and adipose tissue from treated mice. LJ (400 mg/kg), BF (40 mg/kg), and flavonoid 1 (4 mg/kg) reduced glycemia in diabetic mice (120 min) by 52, 53, and 61%, respectively. Flavonoids 2, 3, 5, and 6 were inactive or showed little activity, suggesting that the two rhamnosyl moieties in kaempferitrin are important requirements. Kaempferitrin enhanced the PFK activity chiefly in hepatic tissue, suggesting that it is able to stimulate tissue glucose utilization. This result is confirmed testing kaempferitrin on C2C12 cell line, where it enhanced glucose consumption, lactate production, and the key regulatory glycolytic enzymes. The hypoglycemic activity of kaempferitrin depends on the presence of both rhamnosyl residues in the flavonoid structure when intraperitoneally administered. Our findings show for the first time that a flavonoid is capable of stimulating PFK in a model of diabetes and that kaempferitrin stimulates glucose-metabolizing enzymes. This study contributes to the knowledge of the mechanisms by which this flavonoid exerts its hypoglycemic activity. © 2014 IUBMB Life, 66(5):361–370, 2014

Keywords: *diabetes; flavonoids; kaempferitrin; PFK;* Sedum dendroideum

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that has important social and economic impacts and has reached epidemic proportions globally (1). The World Health Organization estimates that the number of people with DM worldwide is likely to be more than double between 2000 and 2030 and could amount to more than 300 million (2,3). This disorder is characterized by chronic hyperglycemia caused by a deficiency in insulin production and/or action, majorly characterized as DM type 1 (DM1) and DM type 2 (DM2), respectively, which leads to severe, acute, and chronic complications (4).

The metabolic goal of DM treatment is glycemic control, regardless of the fact that DM is DM1 or DM2. The options available to achieve glycemic control are lifestyle management, such as physical activity and appropriate diet, hypoglycemic drugs, and insulin injections (5,6). Despite the various therapies currently in use, there are some drawbacks that limit their application: impaired effectiveness, failure to significantly change the course of the diabetic complications, and side effects, such as hypoglycemia, weight gain, and renal and hepatic toxicity (5,7). Thus, this scenario justifies the search for new therapies and drugs that present minimal side effects.

Medicinal plants are widely recognized as a potential source of new drugs because of their vast chemical diversity and low side effects rate and toxicity (8). A large number of studies have been carried out on the hypoglycemic potential of plants and bioactive compounds of plant origin (9–11). Among the medicinal species popularly used to treat DM are those belonging to the genus Bauhinia (Fabaceae), which have been extensively studied (12–15). Aqueous and ethanolic extracts from the leaves of *Bauhinia forficata*, a remarkable species of this genus, have shown hypoglycemic activity *in vivo* (12,14,16). The flavonoid kaempferitrin (1), the most abundant compound in these extracts, has been revealed to be the active substance (17,18).

Kaempferitrin can also be found in *Sedum dendroideum* (SD) Moc. et Sessé ex DC (Crassulaceae) (19,20). The leaves of this medicinal species are popularly used to treat ulcers and inflammatory conditions (19,21). The antinociceptive and anti-inflammatory activity of the aqueous leaf extract and its flavonoids was confirmed in refs 19 and 20. Like in *B. forficata*, kaempferitrin is the most abundant flavonoid in SD (20). Therefore, the presence of this flavonoid in SD may provide a hypoglycemic activity to this plant extract as has already been observed in *B. forficata*. Interestingly, SD is popularly used in Mexico to treat DM (22), although there are no reports on the antidiabetic activity for this species up to date. Additionally, the chemical composition of SD includes other structurally similar flavonoids that may also possess hypoglycemic activity (20). Thus, the aim of this study was to investigate the *in vivo* hypoglycemic activity of SD aqueous leaf extract and its main flavonoids in normal and streptozotocin (STZ)-induced diabetic mice. In addition, our goals were to establish a possible structure/activity relationship between these flavonoids and to assess the most active flavonoid on the glycolytic enzyme 6-phosphofructo-1-kinase (PFK).

Materials and Methods

Chemical Procedures

Extraction, purification, isolation, and identification procedures were reported in ref. 20. Here, we report a succinct description of the phytochemical approach. Fresh SD leaves were expressed affording a leaf juice (LJ) that was separated by filtration and subjected to precipitation with ethanol (1:1). The supernatant was partitioned successively with ethyl acetate (EtOAc) and *n*-butanol (BuOH), yielding an EtOAc fraction (AF) and a BuOH fraction (BF). BF and AF were chromatographed separately on RP-2 columns (H_2O/methanol gradient), and the flavonoid fractions obtained were then purified on a sequence of RP-2, RP-18, Sephadex G-15, or Sephadex LH-20 column chromatographs. BF afforded the major flavonoids 1 (kaempferol 3-*O*-α-rhamnopyranoside-7-*O*-α-rhamnopyranoside; kaempferitrin), 2 (kaempferol 3-*O*-β-glucopyranoside-7-*O*-α-rhamnopyranoside), 3 (kaempferol 3-*O*-neohesperidoside-7-*O*-α-rhamnopyranoside), and flavonoid 4 (kaempferol 3-*O*-neohesperidoside-7-*O*-β-glucopyranoside). The less polar fraction AF afforded flavonoids 5 (kaempferol 7-*O*-α-rhamnopyranoside; α-rhamnoisorobin), 6 (kaempferol 3-*O*-α-rhamnopyranoside; afzelin), and 7 (kaempferol). In this study, only flavonoids 1–3, 5, and 6 were tested as explained in the "Results" section.

HPLC Analysis

Samples of LJ, BF, and its flavonoids 1–6 were analyzed by HPLC-DAD. The identification of flavonoids in LJ and BF chromatograms was based on the retention time and the UV spectra observed for the corresponding pure compounds.

HPLC-DAD analyses were performed on a Shimadzu liquid chromatograph LC-10AD equipped with an UV SPD-10A wavelength detector, using a Merck reverse-phase column C-18 (5 μm, 250 mm, and 2.5 mm). The mobile phase consisted of water containing phosphoric acid 0.01% (eluent A) and acetonitrile (eluent B). The samples were run for 40 min at 1 mL/min, and the absorbance was monitored between 200 and 500 nm. The gradient used was as follows: 0–10 min (100–80% A), 10–20 min (80–78% A), 20–35 min (78–75% A), and 35–40 min (75–70% A).

Diabetes Induction and Treatment

Male Swiss mice (8 weeks old) were housed in a temperature-controlled room, with a 12-h light–dark cycle, with free access to water and food. DM was induced through a single intraperitoneal (i.p.) dose (150 mg/kg) of STZ dissolved in citrate buffer (100 mM, pH 4.5) as described in ref. 23. The control group received only citrate buffer (i.p). After 5 days, mice that developed hyperglycemia (≥300 mg/dL) were considered diabetic. Diabetic mice, as well as normal ones, were divided into control groups ($n = 9$) and treatment groups ($n = 3$). Normal and diabetic mice were treated with an i.p. injection of regular human insulin (Humulin: 2 U/kg), SD LJ (400 mq/kg), BF (40

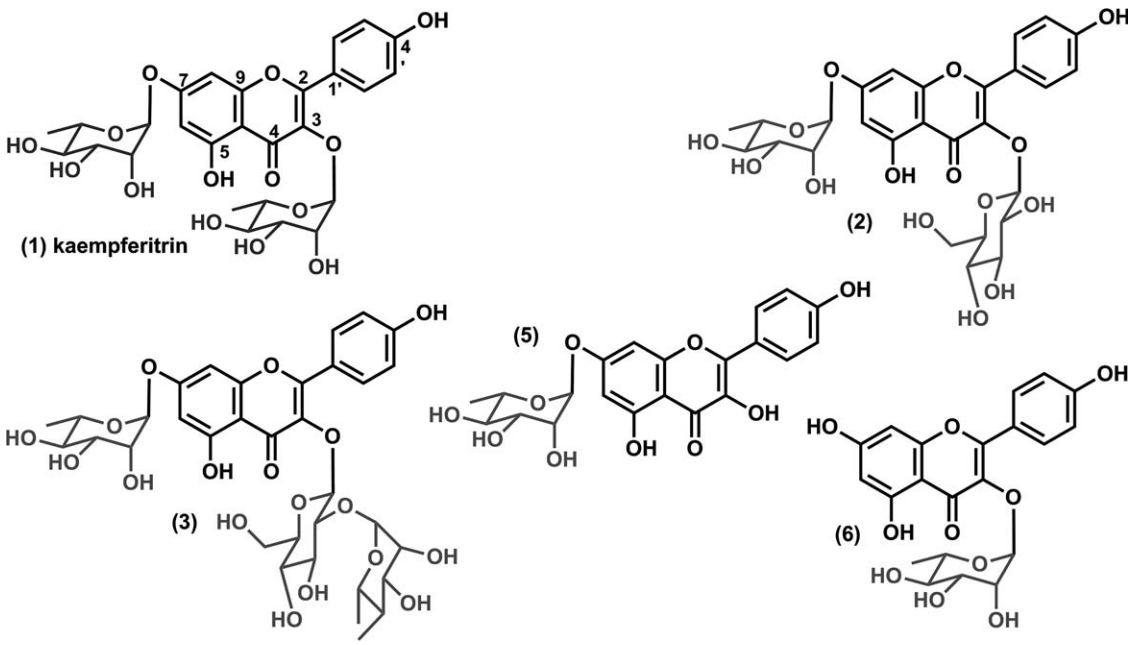

Fig 1 *Chemical structures of kaempferol glycosides from* Sedum dendroideum *tested.*

mg/kg), and the flavonoids 1–3, 5, and 6 separately (4 mg/kg). Doses of fractions and plant compounds were chosen according to their approximate yield from the plant extract. The structures of these flavonoids are shown in Fig. 1. LJ, BF, and its flavonoids were diluted in saline solution (NaCl 0.9%) for 100 μL of injection. Normal and diabetic control groups received only saline solution. Fasting glucose levels were measured from blood mice samples drawn by tail snip before ($t = 0$) and 1 and 2 h after the treatment, using a glucometer (Accu-Chek Active; Roche, Indianapolis, IN, USA). Plasma insulin levels were determined using a commercial Elisa kit (Rat/Mouse Insulin ELISA Kit; Millipore, Billerica, MA, USA) as described previously (23). This protocol (NPPN01) was approved by the internal Animal Ethics Committee (CEUA; CCS/UFRJ).

PFK Activity Assay in Mice Tissues

Normal and STZ-induced diabetic mice were treated with flavonoid **1** (F1) as described above. After 2 h, animals were euthanized by cervical displacement, and skeletal muscle from the back limbs, liver, and epididymal adipose tissue were removed and frozen in liquid nitrogen. To measure the PFK activity, tissues were afterward homogenized in a polytron (Brinkmann Instruments, Westbury, NY, USA) with 100 mM Tris-HCl, pH 7.4, 100 mM sucrose, 10 mM EDTA, 46 mM KCl, 20 mM β-mercaptoethanol, and 1 mM sodium pyrophosphate. After homogenization, they were centrifuged for 10 min at 1,000*g* at 4 °C. The supernatant obtained was used to evaluate PFK activity using the radiometric method described in ref. 24, with modifications introduced in refs. 25 and 26. The enzyme activity assay was carried out in a reaction media containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM [1-^{32}P] ATP (4 μCi/μmol), 5 mM $(NH_4)_2SO_4$, and 1 mM fructose 6-phosphate (F6P). The reaction was started by the addition of the supernatant and interrupted by the addition of an activated charcoal suspension with 0.1 M HCl and 0.5 M mannitol. The suspension obtained was centrifuged for 10 min at 1,500*g* at 4 °C, and the radioactivity present in 400 μL of the resultant supernatant was measured in a liquid-scintillation counter (Tri-Carb; Perkin Elmer, Whaltam, MA, USA). Blanks for this assay were submitted to the same experimental conditions without F6P. PFK activity was determined through the formation of [1-^{32}P] fructose 1,6-bisphosphate in function of the reaction time. Protein concentration was determined according to method proposed in ref. 27.

C2C12 Cell Culture

C2C12 myoblast cell lineage was obtained from the Cell Bank of Hospital Universitário Clementino Fraga Filho, UFRJ, Brazil. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, São Paulo, SP, Brazil) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, São Paulo, SP, Brazil) and L-glutamine at 37 °C in 5% CO_2 atmosphere.

Spectrophotometric Assay for Enzyme Activity

All enzyme activities were measured as described previously (28). Cells were seeded in 24-well plates and grown to confluence. Then, the medium was removed, fresh medium was added, and the cells were returned to the incubator in the presence of different concentrations of kaempferitrin for 24 h. After this incubation, cells were removed from the plates by trypsinization and counted using a hemocytometer. Protein concentrations of cell lysates were measured (29), and the glycolytic enzyme activities were evaluated.

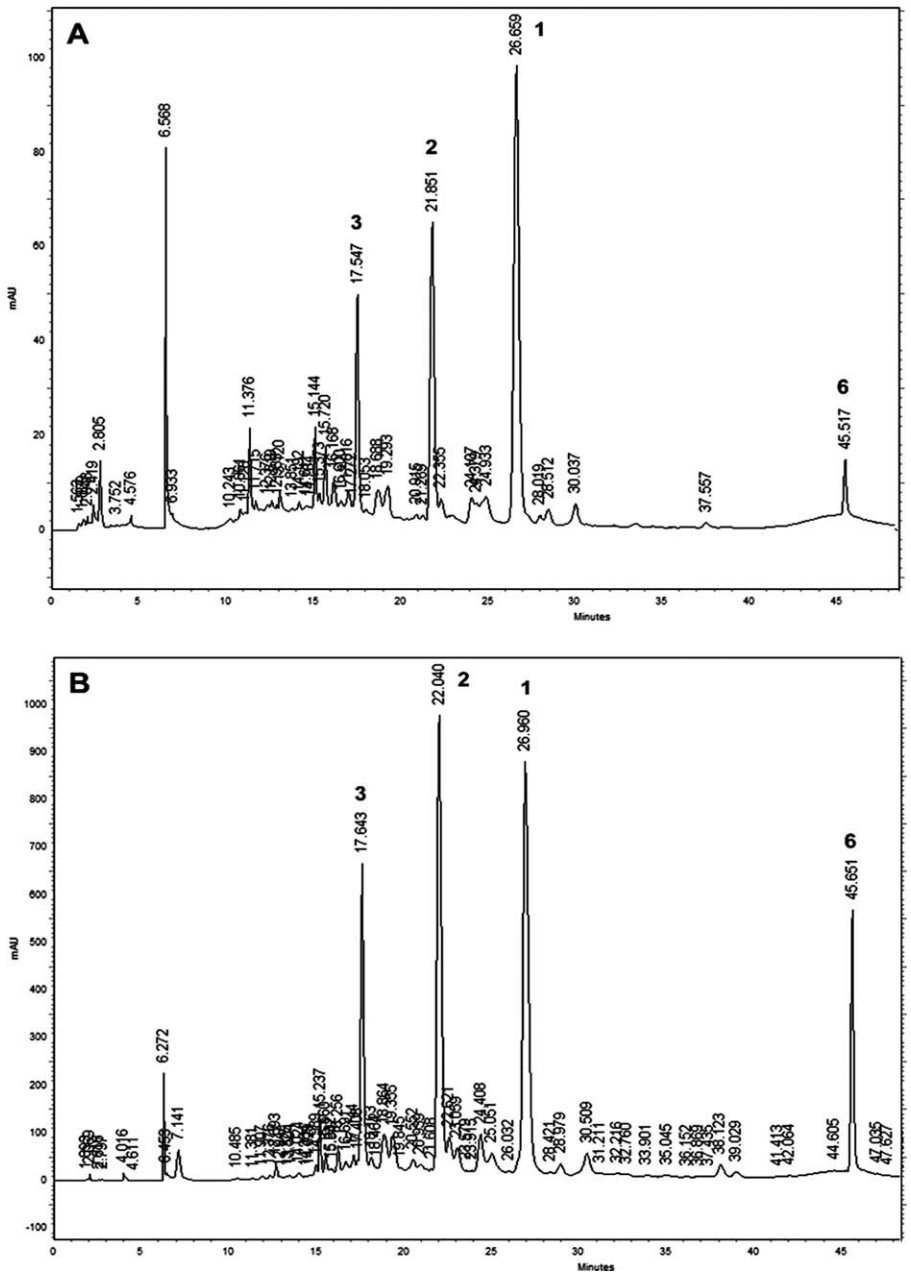

Fig 2 *HPLC chromatograms of LJ (A) and BF (B). Concentration: 10 mg/mL; UV detection: 254 nm.*

PFK activity was assayed using an enzyme-coupled method, where the oxidation of NADH was monitored spectrophotometrically (30). The basic medium contains 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM F6P, 1 mM ATP, 0.2 mM NADH, 2 U/mL aldolase, 4 U/mL triosephosphate isomerase, and 2 U/mL α-glycerophosphate dehydrogenase.

Hexokinase (HK) activity was assessed in a basic medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM glucose, 1 mM ATP, 0.2 mM NAD^+, and 1 U/mL glucose 6-phosphate dehydrogenase as described previously (31).

Pyruvate kinase (PK) activity was measured in a medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 120 mM KCl, 1 mM phosphoenolpyruvate, 1 mM ADP, 0.2 mM NADH, and 20 U/mL lactate dehydrogenase (LDH) as described previously (31).

Glucose 6-phosphate dehydrogenase (G6PDH) activity was assayed as previously described (32). The basic medium contains 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM glucose 6-phosphate, and 0.2 mM $NADP^+$.

For all enzymatic analyses, NADH oxidation or $NAD(P)^+$ reduction was determined by measuring the absorbance at 340 nm in a microplate reader (VICTOR 3; Perkin Elmer, Whaltam, MA, USA). The reactions were initiated by the addition of an aliquot of cellular homogenate and followed online during the first-order kinetics period. Enzyme rates were

determined calculating the first derivative of these curves. Blanks containing none of the coupled enzymes (or without glucose 6-phosphate in the case of G6PDH) were performed to control for nonspecific oxidation/reduction. Each curve was performed in quadruplicate.

Cell Proliferation and Viability Assay, Glucose Uptake, and ATP Quantitative Evaluation

The metabolic rates were measured as described previously (28). Cells were seeded in 96-well plates in the appropriate medium and grown to confluence. Then, the medium was removed, fresh medium was added, and the cells were returned to the incubator in the presence of different concentrations of kaempferitrin. After 24 h, the medium was removed, and the cell viability was determined by measuring the amount of leaked LDH, whereas the mitochondrial activity (proliferation assay) was determined in the remaining cells using an MTT assay (33,34).

Glucose uptake was determined by incubating cells with fresh medium containing 5 mM 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG; Molecular Probes; Invitrogen; Life Technologies, Carlsbad, CA, USA), a fluorescent glucose analog. After 15-, 30-, and 45-min incubation, the medium was removed, the cells were washed with 6-NBDG-free medium, and 6-NBDG taken up by the cells was evaluated by fluorescence emission, according to the manufacturer's instructions. The glucose uptake rate was calculated by the linear regression of the increase in fluorescence incorporated by the cells.

Statistical Analysis of Data

The results were expressed as mean ± SEM. The differences between two values were determined using Student's *t*-test, and the data were considered different at a significance level of $P < 0.05$. The differences among groups were determined using ANOVA test with Bonferroni's post-test, and the data were considered different at a significance level of $P < 0.05$. The analyses were performed using SigmaPlot 11.0 software.

Results

Flavonoid Composition of LJ and BJ

The HPLC-DAD chromatogram (Fig. 2A) of LJ shows that compounds 1, 2, and 3 are the major flavonoids obtained by aqueous extraction, and for this reason, they were chosen to be evaluated in the hypoglycemic activity assays. These

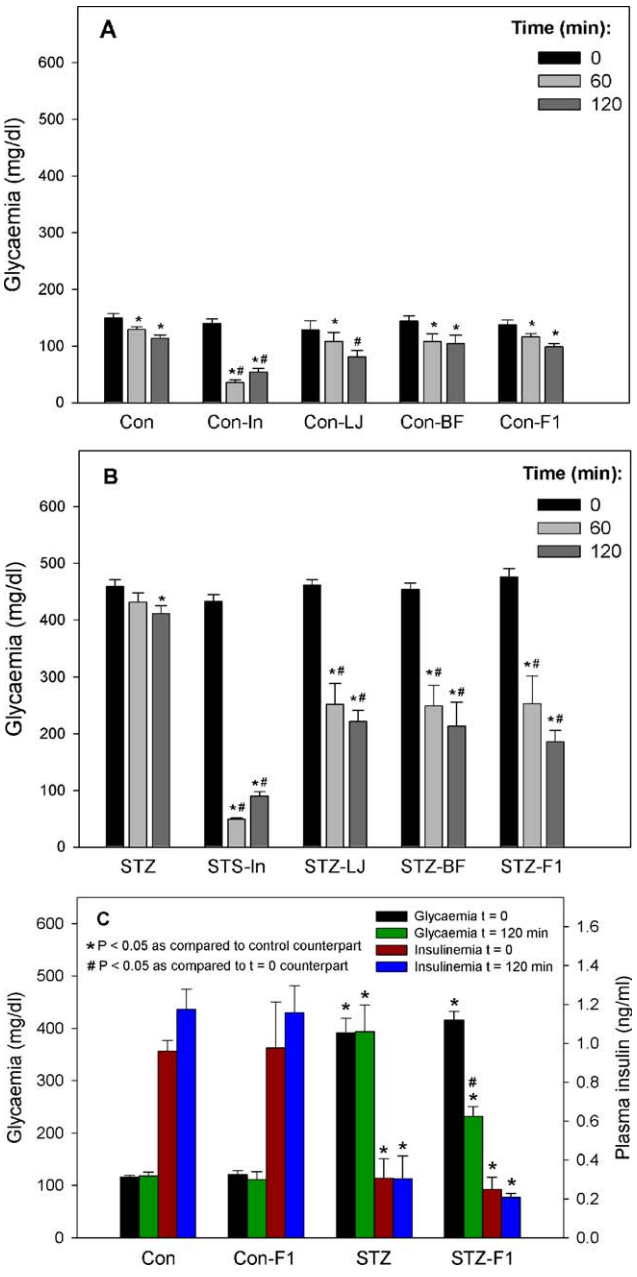

Fig 3

*Hypoglycemic activity of LJ, BF, and flavonoid **1** (F1). A: Con: normal mice treated with saline (n = 9); Con-In: normal mice treated with insulin (2 U/kg; n = 3); Con-LJ: normal mice treated with LJ (400 mg/kg; n = 3); Con-BF: normal mice treated with BF (40 mg/kg; n = 3); and Con-F1: normal mice treated with F1 (4 mg/kg; n = 3). B: STZ: diabetic mice treated with saline (n = 9); STZ-In: diabetic mice treated with insulin (2 U/kg; n = 3); STZ-LJ: diabetic mice treated with LJ (400 mg/kg; n = 3); STZ-BF: diabetic mice treated with BF (40 mg/kg; n = 3); and STZ-F1: diabetic mice treated with F1 (4 mg/kg; n = 3). C: Con: normal mice treated with saline (n = 4); Con-F1: normal mice treated with F1 (4 mg/kg; n = 4); STZ: diabetic mice treated with saline (n = 4); and STZ-F1: diabetic mice treated with F1 (4 mg/kg; n = 4). Glycemia (black and green bars) and insulinemia (red and blue bars) were measured at time 0 (black and red bars) and 120 min (green and blue bars) after treatment with saline or F1. Data represent the mean ± SEM. *P < 0.05 compared with t = 0 (Student's t-test). #P < 0.05 compared with control mice (Student's t-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

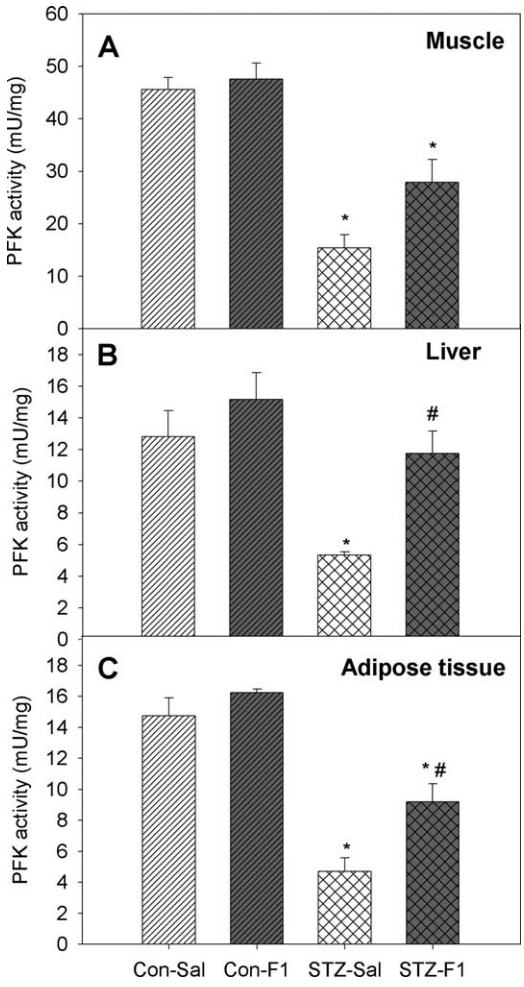

Fig 4

*PFK activity of skeletal muscle (A), liver (B), and epididymal adipose tissue (C). Data represent the mean ± SEM (n = 3). *P < 0.05 compared with untreated control mice (Student's t-test). #P < 0.05 compared with untreated diabetic mice (Student's t-test).*

compounds are concentrated in the BF from which they were isolated, as can be seen in the BF chromatogram (Fig. 2B).

Hypoglycemic Activity of LJ, BF, and Flavonoids

First, we analyzed the effect of LJ (400 mg/kg), its enriched flavonoid fraction BF (40 mg/kg), and F1 (kaempferitrin: 4 mg/kg) on glucose levels of normal and STZ-induced diabetic mice. The results are shown in Fig. 3. Human insulin was administered as positive control.

LJ had a discreet hypoglycemic effect in normal mice after 120 min (Fig. 3A). The same effect was not observed, however, for normal mice treated with BF and F1, whose glycemic levels were not different from control at any time assayed (Fig. 3A). On the other hand, LJ, BF, and the most abundant flavonoid (1) produced a consistent decrease in the glucose levels of diabetic mice after 4-h fasting, which were statistically different from nontreated diabetic animals at 60 and 120 min after treatment (Fig. 3B). The reduction rates after 120 min were 52, 53, and 60%, respectively. The decreased concentration of blood glucose observed in all normal groups throughout the treatment appears to be related to fasting.

To test this hypothesis, these experiments were performed after 8-h fasting, when there is no more intestinal content remaining to be absorbed. After this long fasting period, we observed no change in glycemia 120 min after treatment for both control groups (treated with saline solution or F1) and for STZ group treated with saline solution (Fig. 3C, black and green bars). However, STZ group treated with F1 presented a significant reduction on glycemia 120 min after treatment (Fig. 3C). Insulin (2 U/kg) treatment promptly decreased glycemia of both normal and diabetic mice showing that they are responsive to this hormone. This relative high dosage of insulin was necessary as the protocol we used to induce DM with STZ produces animals that are slightly resistant to insulin and has been considered as a mixed model of DM1 and DM2. Aiming at testing whether F1 presents hypoglycemiant effect by promoting insulin secretion, we evaluated the plasma insulin levels in these groups. Our results show that F1 does not alter the insulinemia of both control and STZ groups (Fig. 3C, red and blue bars).

In the second step, we investigated the hypoglycemic activity of **2** (4 mg/kg) and **3** (4 mg/kg), which are the second and the third major flavonoids of SD, respectively. Flavonoid **2** exhibited much lower hypoglycemic activity in diabetic mice when compared with **1**. The glycemic levels of treated diabetic animals were different from control only after 120 min. At this time, the glycemia reduction rate was only 28%. This flavonoid was not active in normal mice. Meanwhile, flavonoid **3** had no hypoglycemic activity in either normal or diabetic mice (data not shown).

The three major flavonoids tested are structurally similar. They have the kaempferol skeleton and a rhamnosyl residue at position 7 in common. The difference among them is solely in the glycosyl residues at C-3: a rhamnosyl in **1**, a glucosyl in **2**, and a neohesperidosyl residue in **3**. F1 (kaempferitrin), which has a rhamnosyl unit at C-3 and a second one at C-7, was the only one with expressive hypoglycemic activity at the dose tested.

To determine the importance of these rhamnosyl residues, we examined the hypoglycemic activity of two minor kaempferol glycosides also found in SD: flavonoid **5**, which has a rhamnosyl residue at C-7 but without a glycosyl residue at C-3, and flavonoid **6**, which in the opposite way has a rhamnosyl residue at C-3 and no substituent at C-7. These flavonoids were tested at the same dose (4 mg/kg) using the same experimental design described for the previous flavonoids.

Flavonoid **5** exhibited statistically significant hypoglycemic activity in diabetic mice after 120 min, with a glycemia reduction rate of 34%. Flavonoid **6** produced a statistically significant hypoglycemic effect in diabetic mice only after 60 min (27% of glycemia reduction); however, this effect was lost in 120 min. Neither flavonoids (**5** and **6**) had any hypoglycemic activity in normal mice (data not shown).

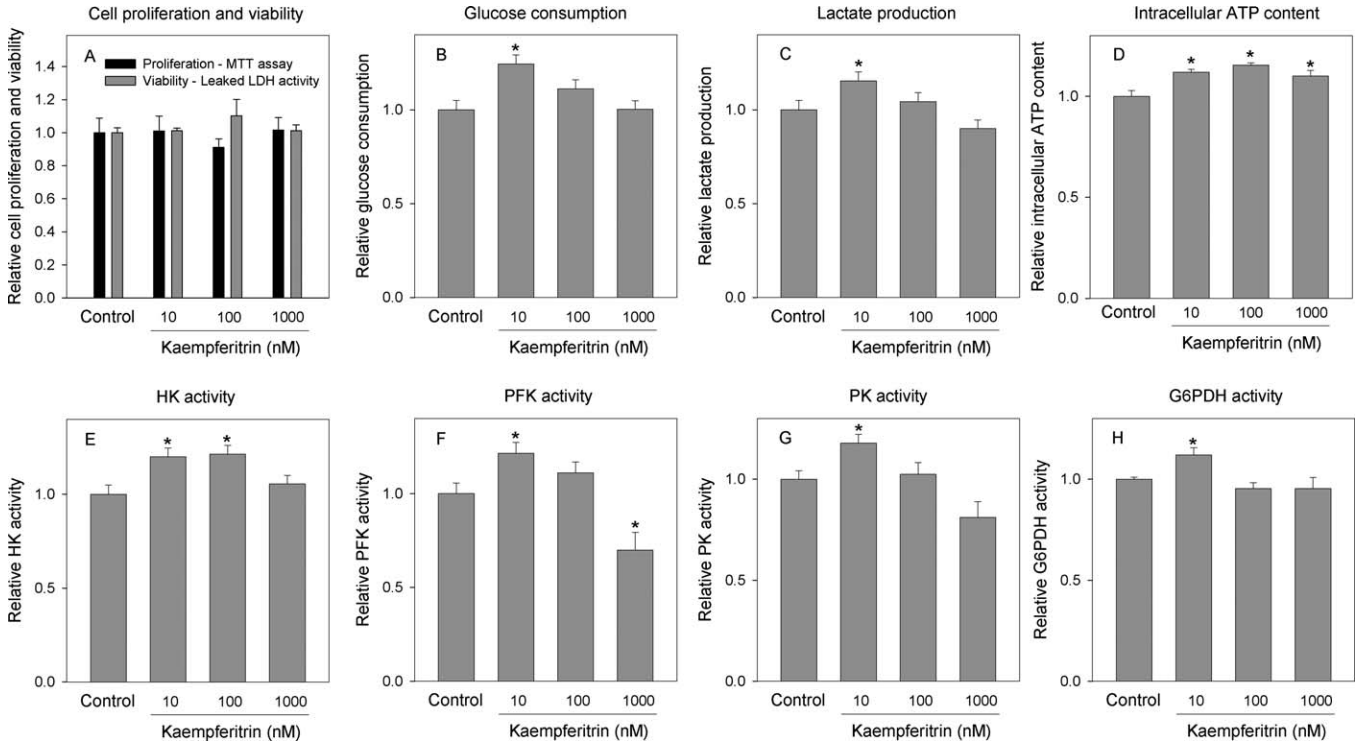

Fig 5

*Effects of kaempferitrin on metabolic parameters of C2C12 cells. Cell growth conditions, cell treatment, and experimental details are as described in the "Materials and Methods" section. All experiments were performed in triplicate, and data are presented as mean ± standard errors of four independent experiments (n = 4). *Differences were considered as statistically significant (P < 0.05, ANOVA with Bonferroni's post-test).*

Effect of Flavonoid 1 (Kaempferitrin) on Mouse PFK

The effect of kaempferitrin on the PFK activity of the main insulin-sensitive tissues was evaluated. PFK activity was reduced in all the tested tissues from diabetic mice when compared with controls (Fig. 4). The activity measured was 66, 58, and 68% lower in skeletal muscle, liver, and adipose tissue, respectively. Treatment with kaempferitrin (4 mg/kg) for 2 h resulted in a complete reversion of the decreased PFK activity in liver. However, the enzymatic activity was partially reversed in adipose tissue and presented a mild effect on skeletal muscle.

Effects of Kaempferitrin on C2C12 Myoblast Metabolism

To better understand the mechanism by which the F1 (kaempferitrin) is promoting hypoglycemiant effects, we assessed the effects of the flavonoid on several metabolic parameters of the mouse myoblast cell line C2C12. Three concentrations of kaempferitrin were used in these experiments: 10, 100, and 1,000 nM. These concentrations were chosen based on previous publication where this range influences muscle metabolism (35). Initially, we evaluated whether kaempferitrin was able to affect C2C12 proliferation and viability. Assessing MTT assay for cell proliferation and the activity of LDH in the culture media as an assessment for the enzyme leaked from the cells to evaluated cell viability, we concluded that kaempferitrin was affecting neither the proliferation nor the membrane integrity of C2C12 cell in the range of concentrations used (Fig. 5A). These results are in agreement with those obtained in 3T3-L1 cell (36). However, the flavonoid-stimulated glucose consumption and lactate production when C2C12 cell were incubated for 120 min in the presence of 10 nM kaempferitrin (Figs. 5B and 5C, respectively). This stimulation was not observed in the presence of 100 or 1,000 nM kaempferitrin. These results were also observed testing the regulatory glycolytic enzymes, PFK and PK (Figs. 5F and 5G, respectively), and whether HK was activated by 10 and 100 nM kaempferitrin (Fig. 5E). Additionally, G6PDH, the first enzyme on pentose-phosphate shunt, was also activated only by 10 nM kaempferitrin (Fig. 5H). On the other hand, all the concentrations tested for kaempferitrin augmented the intracellular ATP content (Fig. 5D).

Discussion

Kaempferitrin at the i.p. dose of 4 mg/kg has shown an activity quantitatively similar to that presented by the extract (LJ) and the BF at 400 and 40 mg/kg, respectively. Flavonoids **2** and **3**, the second and third major flavonoids, respectively, however, were inactive or exhibited low activity in diabetic mice. These

flavonoids do not seem to play an important role as hypoglycemic agents, suggesting that kaempferitrin is the main hypoglycemic compound in SD, responsible for the activity of LJ and BF.

Flavonoids without rhamnosyl groups in positions 3 and 7 are inactive or have lower activity when intraperitoneally administered. This finding implies that both rhamnose residues are important structural requirements for hypoglycemic activity, as the lack of the residues in those positions or the replacement of rhamnose in position 3 by another glycosyl moiety leads to impaired activity. It is worth pointing out that different results can be expected for *in vivo* oral administration of these glycosylated flavonoids as they may be extensively metabolized in the gastrointestinal tract (37).

A large number of studies have focused on the structure/activity relationship of flavonoids acting on various molecular targets—receptors, transporters, and especially enzymes—related to glycemic control (38–43). Despite the structure–activity relationship established in the current study for *in vivo* hypoglycemic activity of the kaempferol glycosides presented here, the mechanism responsible for this action is still unknown. Therefore, it was not possible to outline a comparison with structural requirements for action on specific targets.

Kaempferitrin has shown hypoglycemic activity when administered orally to alloxan-induced diabetic rats (17,18). The authors proposed that the flavonoid activates the insulin-signaling pathways, thus increasing glucose uptake in peripheral tissues (18). In contribution to this hypothesis, we can mention the study of Tzeng et al. (36). These authors demonstrated that kaempferitrin stimulates the classical insulin-signaling transduction pathways in 3T3-L1 cells: phosphorylation of insulin receptor, insulin receptor substrate 1, and other intracellular regulatory proteins, thus increasing GLUT-4 translocation, a transmembrane-facilitative glucose transporter, responsible for glucose uptake in insulin-sensitive tissues (44). In the same study, kaempferitrin also showed an ability to stimulate secretion of adiponectin, a protein hormone that enhances peripheral insulin sensitivity (45).

The diabetic animal model used in the current work, a single, relatively low, i.p. injection of STZ, shows a low ability to secrete insulin even at high glycemia and also a slight resistance to insulin and has been characterized as a mixed model of DM1 and DM2 (23,46–48). This model is different from the alloxan-induced diabetic rats described above as the latter is exclusively a DM1 model, normally responsive to insulin. The fact that kaempferitrin promotes its hypoglycemic effects on the current diabetic model, a mix of DM1 and DM2 models, reveals that this drug is able to bypass insulin resistance and lower glycemia even when insulin resistance is established. This is an advantage for a hypoglycemic activity, as it allows its use for reducing glycemia in both DM1 and DM2. Nonetheless, kaempferitrin is probably not improving the responsiveness to insulin, as if it was the case, one would expect to observe a decrease in insulin levels with no alteration in glycemia. The current work shows that kaempferitrin presents no effect on insulinemia for both control and STZ groups. In addition, the effects of kaempferitrin cannot be attributed to a reduction in carbohydrate digestion and absorption as the flavonoid was also able to reduce glycemia after 8-h fasting, when there is no content in the digestive tube to be digested or absorbed.

To look further into the hypoglycemic mechanism of kaempferitrin, we analyzed whether the flavonoid is able to modulate PFK activity in the major tissues responsible for the control of glycemia: skeletal muscle, liver, and adipose tissue from normal and STZ-induced diabetic mice. PFK is the main rate-limiting enzyme for glycolysis, the principal metabolic pathway for cell glucose consumption (23,25,29,30,48). Kaempferitrin increased PFK activity in adipose tissue and especially in the liver of diabetic mice, in which impaired enzymatic activity was totally reversed. This result shows that the flavonoid is probably able to stimulate cellular glucose utilization in these tissues. Stimulation of glycolysis *per se* is a possible mechanism for the hypoglycemic effects, although one might keep in mind that other mechanisms should be involved for an effective and persistent reduction on glycemia. These results were corroborated by the experiments using C2C12 myoblast cells in culture. In these experiments, we observed that kaempferitrin stimulates not only PFK but also the other rate-limiting glycolytic enzymes, HK and PK. Moreover, the flavonoid also stimulated G6PDH, suggesting that not only glycolysis but also the pentose-phosphate shunt, another important glucose-metabolizing pathway, is stimulated on treatment. Although the flavonoid does not modify the cells proliferation and viability, it increases glucose consumption and lactate production, as well as the intracellular ATP content. Altogether, these results reveal that kaempferitrin is able to ameliorate the complete glucose utilization by the cell, improving catabolic and anabolic pathways and the overall energy homeostasis. However, an intriguing observation was that intracellular ATP content augmented under all concentrations used, whereas the glucose catabolism (glucose consumption, lactate production, and metabolizing enzyme activity) was augmented only by 10 nM kaempferitrin. This effect was reversed with 100 and 1,000 nM (except HK, which was stimulated by 10 and 100 nM). Metabolism activation by insulin presents a similar pattern where higher concentrations of the hormone reverse its stimulatory properties (25,26,31).

As far as we know there is only one study reporting a stimulatory effect of a flavonoid on PFK. Genistein was able to increase the enzyme activity in a worm (*Raillietinae chinobothrida*) submitted to an anthelmintic stress produced by this isoflavone (32). Studies that correlate flavonoids to increasing action on PFK activity and diabetes, however, were not found (49). Many hypoglycemic flavonoids, on the other hand, have shown activity on other glucose-metabolizing enzymes. The glycosylated flavonols rutin and quercitrin were able to modulate the activity of HK, glucose 6-phosphatase, and fructose 1,6-bisphosphatase (49,50), whereas fisetin, another glycosylated flavonol, was reported to modulate the enzymes above

mentioned as well as PK, LDH, G6PDH, glycogen synthase, and glycogen phosphorylase (51). Similar action is documented for the flavanones naringenin and diosmin (52,53). This shows that the regulation of key enzymes of carbohydrate metabolism is a frequent mechanism involved in the glucose-lowering action of flavonoids.

Conclusions

Our findings demonstrate that SD has hypoglycemic potential in both DM1 and DM2. Its hypoglycemic activity, reported for the first time in this study, is probably due to the glycosylated flavonol kaempferitrin, previously known to possess a glucose-lowering effect. The rhamnosyl units at positions 3 and 7 of the flavonoid skeleton seem to be important for this effect. We also demonstrated that kaempferitrin stimulates glucose-metabolizing enzymes. This is the first report on the stimulatory activity of a flavonoid on PFK in a diabetes model. Taken together, these findings contribute to the knowledge of the mechanisms by which kaempferitrin exerts hypoglycemic activity.

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