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"MitoTea": *Geranium robertianum* L. decoctions decrease blood glucose levels and improve liver mitochondrial oxidative phosphorylation in diabetic Goto–Kakizaki rats★

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Several chemical compounds found in plant products have proven to possess beneficial properties, being currently pointed out due to their pharmacological potential in type 2 diabetes mellitus complications. In this context, we studied the effect of *Geranium robertianum* L. (herb Robert) leaf decoctions in Goto–Kakizaki (GK) rats, a model of type 2 diabetes. Our results showed that oral administration of *G. robertianum* leaf decoctions over a period of four weeks lowered the plasma glucose levels in diabetic rats. Furthermore, the treatment with *G. robertianum* extracts improved liver mitochondrial respiratory parameters (state 3, state 4 and FCCP-stimulated respiration) and increased oxidative phosphorylation efficiency.

Keywords: Goto–Kakizaki (GK) rats, type 2 diabetes mellitus, *Geranium robertianum* L., *Vaccinium myrtillus* L., herbal medicine, oxidative phosphorylation

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INTRODUCTION

In the last decades, globalization has lead to Western lifestyle generalization and to the subsequent increasing rate of childhood and adult obesity, metabolic syndrome and type 2 diabetes mellitus that have attained pandemic dimensions (WHO, 2006).

Diabetes mellitus is a complex and multifarious group of disorders that disturb the metabolism of carbohydrates, fat and protein, with one common manifestation — hyperglycaemia (WHO, 1980). Hyperglycaemia causes severe complications associated with diabetes, such as nephropathy, retinopathy, neuropathy and cardiovascular diseases, which severely impair the diabetic patients' life quality (Engelgau & Geiss, 2000; Reusch, 2003; Yorek, 2003). Hence, the major purpose of diabetes therapy is to attain normal glycaemic levels (Agius, 2007; Yu *et al.*, 2010). Nevertheless, due to the complex nature of the disease, it usually remains unreachable by using regular therapies, including chemical anti-hyperglycaemiant agents.

Medicinal plants, used in the folk medicine since ancient times, are being rediscovered for diabetes therapy and seem to be an important and useful alternative (or complementation) to the synthetic drugs used in type 2 diabetes' therapy. Despite the increasing use of medicinal plants in diabetes mellitus treatment (Ryan *et al.*, 2001), there is little knowledge about the mechanism of action and the therapeutic effects of several medicinal plants with anti-diabetic action attributed by folk medicine (Jayakumar, 2010).

Infusions and decoctions prepared from leaves of *Geranium robertianum* L., known as herb Robert or red Robin, are described as anti-hyperglycaemiant and commonly used in Portuguese herbal medicine (Castro, 1998; Cunha *et al.*, 2009). *In vivo* studies performed with this plant extracts are sparse.

To evaluate the anti-hyperglycaemiant effect of this plant, we used the Goto–Kakizaki (GK) rat, a non-obese spontaneous animal model of type 2 diabetes mellitus (Goto & Kakizaki, 1981). Several studies indicate that GK rat is a good model to study the events at the onset of the disease (Ferreira *et al.*, 1999c; Portha *et al.*, 2009). Therefore, young GK rats presenting moderate hyperglycaemia were selected and used in this study, to evaluate the anti-hyperglycaemic effects of *G. robertianum* decocts.

Additionally, the effects of these water extracts on mitochondrial efficiency were also assessed, since mitochondrial impairment is a common feature of several metabolic alterations, including diabetes mellitus (Lagouge *et al.*, 2006).

The major purposes of the present research were to evaluate the effects of *G. robertianum* decocts in blood glucose levels as well as their influence on mitochondrial activity.

MATERIALS AND METHODS

Materials. All reagents and chemicals used were of the highest grade of purity commercially available. Inhibitors and drugs were dissolved in water or ethanol. In control experiments, solvents were added to isolated mitochondria at concentrations not exceeding 0.2%.

Plant material. *G. robertianum* was collected in Santarém region (Portugal), dried in the dark and obtained directly from "11 anos — Segredo da plan-

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GK, Goto–Kakizaki; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio

ta - Produtos naturais e biológicos, Lda" (Seixal, Portugal). A voucher *G. robertianum* (No. 12751), after its botanical identification, has been deposited at the Herbarium of the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal).

Preparation of extracts. Plant leaves were washed in deionised water, dried and ground to a powder. The decoction was prepared by boiling 125 g of the dried material in 1000 ml of deionised water for 15 min, filtered and centrifuged at $7000 \times g$ for 5 min and kept at –20 °C.

Animals. Male spontaneously diabetic GK rats were obtained from a local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra), established in 1995 with breeding couples from the colony at the Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K. Suzuki). Animals were kept under controlled light and humidity conditions and with free access to powdered rodent chow (diet C.R.F. 20, Charles Rivers, France) and water (or plant decoct) in accordance with European Community guidelines. GK rats were randomly divided and housed in two separated groups, one of them drinking *ad libitum* the *G. robertianum* decoct and the other, used as a control, drinking distilled water. The experiments lasted for 4 weeks and were carried out in accordance with the National Requirements for Vertebrate Animal Research and the European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes.

Glycaemia. Glycaemia in a non-fasting condition (occasional) was determined twice a week. Blood glucose levels were determined through the glucose oxidase reaction by using a glucometer (Glucometer Elite — Bayer SA, Portugal) and compatible reactive test strips. Blood samples were collected from the tail vein.

Intraperitoneal glucose tolerance test. Intraperitoneal glucose tolerance test (IPGTT — 1.8 g glucose/kg body mass, i.p.) was carried out after fasting glycaemia determination (fasting period 16–18 h). The concentration of blood glucose was measured 30, 60, 90 and 120 min after glucose load by the method described above.

Preparation of mitochondria. GK rats were maintained *ad libitum* for at least 12 h, before being sacrificed by cervical displacement, according to a pre-established method (Gazotti *et al.*, 1979), with slight modifications (Ferreira *et al.*, 1997). Protein was determined by the biuret method, using BSA (bovine serum albumin) as a standard (Gornall *et al.*, 1949).

Mitochondrial respiration. Oxygen consumption of isolated mitochondria was determined polarographically at 25 °C with a Clark oxygen electrode connected to a suitable recorder in a closed chamber with magnetic stirring (Estabrook, 1967). Mitochondria equivalent to 1.0 mg protein, 2 μM rotenone and succinate (5 mM), as respiratory substrate, were added to 1 ml of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM Hepes, pH 7.2). To induce state 3 respiration, 300 nmol of ADP (magnesium salt) was added. The respiratory control ratio (RCR) was calculated according to Chance and Williams (1956). FCCP-uncoupled respiration was performed by adding 1.5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) to mitochondria energized with succinate (Ferreira *et al.*, 1999b), after a phosphorylative cycle. In order to validate respiratory activity assays, 1 mM KCN was added and the slope due to O_2 diffusion was subtracted from all traces.

Statistics. The results are presented as mean ± SEM (standard error of mean) of the number of experiments shown in table and figure legends. Statistical significance was determined using paired Student's *t*-test. $P < 0.05$ was considered significant.

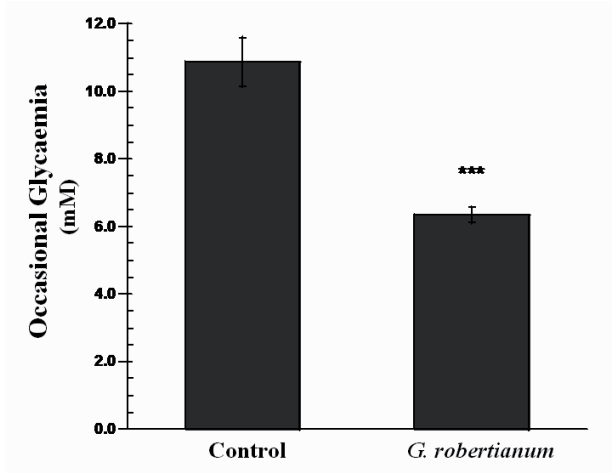

Figure 1. Effect of *G. robertianum* leaf decoction on GK rat occasional glycaemia.
Data are presented as means ± SEM for four rats in each group. Values statistically different from control (distilled water): $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Our results showed that *G. robertianum* leaf decocts led to a significant decrease of occasional glycaemia by 35% in diabetic Goto–Kakizaki rats when compared to a control group drinking distilled water during the same period of time (four weeks) (Fig. 1). These results corroborate the popular anti-diabetic properties attributed to this plant extract. The amount of liquid ingested was significantly lower, probably related to the lower glycaemia (Table 1) and the quantity of ingested food was also decreased, as compared with the control GK group. However, no significant mass changes were observed between the *G. robertianum* and control groups during the four weeks of the experiment.

Nevertheless, intraperitoneal glucose load performed before and after the treatment with the plant decocts did not show significant differences between the two groups (Table 2).

Further, our results showed that liver mitochondria isolated from GK rats treated for four weeks with leaf

Table 1. Food and liquid intake, and mass gain of GK rats

Condition	Plant extract	
	Control	*Geranium robertianum*
Food ingested (g/day per rat)	23.7 ± 0.4	19.1 ± 0.5*
Liquid ingested (ml/day per rat)	57.0 ± 1.8	29.0 ± 0.8**
Final mass (%)	106.2 ± 0.4	105.4 ± 1.2

The amount of food and liquid ingested was recorded 3 times a week. The final mass of each rat was expressed as the percentage of its initial mass, to limit inaccuracies. Data are presented as means ± SEM for four rats in each group. Values statistically different from control (distilled water): $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 2. Glycaemia in fasting condition and after glucose loading

		Glycaemia (mM)	
		Control	*G. robertianum*
initial	fasting	6.0 ± 0.3	5.4 ± 0.0
	30 min	22.6 ± 2.3	22.5 ± 0.0
	60 min	21.2 ± 1.7	21.8 ± 0.0
	90 min	17.9 ± 1.2	17.9 ± 0.1
	120 min	15.6 ± 1.0	13.8 ± 0.0
final	fasting	5.7 ± 0.3	5.9 ± 0.0*
	30 min	21.2 ± 1.2	17.9 ± 1.0*
	60 min	19.3 ± 0.8	18.0 ± 0.0*
	90 min	20.0 ± 0.8	15.4 ± 0.0
	120 min	16.8 ± 0.6	13.7 ± 0.1

Initial glycaemias was evaluated following glucose intraperitoneal load prior to *G. robertianum* (or distilled water) ingestion and final glycaemia was evaluated 4 weeks later. Data are presented as means ± SEM for three rats in each group. Values statistically different from glycaemias evaluated before plant extract treatment: *$P<0.05$.

preparations presented a higher respiratory chain activity, since the respiratory rates evaluated in the presence of succinate as a respiratory substrate or in the presence of FCCP, a classic uncoupler, were significantly increased (Table 3). Moreover, mitochondria isolated from GK rats treated with the plant extract showed an improved phosphorylative efficiency, as the respiratory control ratio evaluated in the presence of succinate was significantly higher in those rats than in controls (Table 3). These results indicate both an increased activity of respiratory complexes and a higher coupling between the oxidative and phosphorylative systems.

DISCUSSION

In the present study, the effects of *G. robertianum* leaf decoctions were evaluated on blood glucose levels. This plant extract is commonly used in popular medicine in Portugal due to its attributed anti-diabetic properties (Castro, 1998; Cunha *et al.*, 2009). However, *in vivo* studies to support this empirical data are missing.

To evaluate the *G. robertianum* anti-hyperglycaemic potential, studies were performed using a type 2 diabetes animal model widely studied in our laboratory, the Goto–Kakizaki rat (Ferreira *et al.*, 1999a; 1999b; Moreira *et al.*, 2003; Oliveira *et al.*, 2004; Sena *et al.*, 2008). Our results revealed that *G. robertianum* decoction significantly decreased GK rats' occasional glycaemias, as compared with a control group. Nevertheless, evaluation of glycaemia after intraperitoneal glucose administration revealed that the *G. robertianum* decoct induced a significant decrease only 60 min after glucose load. Similar results were obtained for a *Vaccinium myrtillus* decoct, another plant extract also used in Portugal due to its supposed anti-diabetic properties (Ferreira *et al.*, 2010). These results, apparently contradictory, probably reflect specific effects in carbohydrate absorption or metabolism induced by these two plant extracts, rather than the stimulation of insulin release and/or glucose uptake by peripheral tissues. Furthermore, these plant extracts also decreased the amount of food ingested, which is in good agreement with the lower occasional glycaemia.

Our previous work with this type diabetes 2 animal model showed that liver mitochondria exhibited enhanced respiratory activity and improved coupling of the phosphorylative and oxidative systems in rats in their first year of life, as compared with non-diabetic Wistar rats of similar ages (Ferreira *et al.*, 1999b; 1999c; 2003), associated with differences in mitochondrial composition (Ferreira *et al.*, 1999a). Also, the liver mitochondria of GK rats presented an improved antioxidant capacity, compared with Wistar rats (Ferreira *et al.*, 1999a; 2003).

As recent evidence suggests that certain flavonoids as kaempferol, present in *G. robertianum* leaves (Amaral *et al.*, 2009), can improve mitochondrial biogenesis (Rasbach & Schnellmann, 2008), the GK rat seemed to us a suitable animal model for evaluation of these plants' extracts effects on OXPHOS by an *in vivo* assay.

Our results showed that the mitochondrial respiratory activity, assessed in the presence of succinate as a respiratory substrate and ADP (state 4 and state 3, respectively) or in the presence of the uncoupler FCCP, was significantly augmented in GK rats supplied with *G. robertianum* decocts. These results point to a higher expression of respiratory complex proteins, which is in good agreement with previous reports indicating an enhancement of mitochondrial biogenesis in the presence of flavonoids (Lagouge *et al.*, 2006; Rasbach & Schnellmann, 2008; Csiszar *et al.*, 2009).

G. robertianum extract treatment increased the efficiency of coupling between oxidative and phosphorylative systems, since RCR was considerably higher in GK rats consuming this plant extract. Therefore, this higher coupling is probably related with the high content of respiratory complexes and F_0F_1-ATPsynthase and also with mitochondrial membrane lipid adjustments (Ames *et al.*, 1995).

Similar results were obtained for a *V. myrtillus* decoct (Ferreira *et al.*, 2010), probably due to the presence of quercetins in *V. myrtillus* leaves (Rasbach & Schnellmann, 2008; Riihinen *et al.*, 2008; Davis *et al.*, 2009).

Table 3. Effect of *G. robertianum* on GK rat liver mitochondrial parameters

Condition	V_3 nmol $O_2 \times mg^{-1} \times min^{-1}$ (%)	V_4 nmol $O_2 \times mg^{-1} \times min^{-1}$ (%)	FCCP nmol $O_2 \times mg^{-1} \times min^{-1}$ (%)	RCR (%)
Control	36.4 ± 4.1 (100.0 ± 11.2)	9.0 ± 1.0 (100.0 ± 9.2)	42.3± 5.7 (100.0 ± 13.4)	4.2± 0.6 (100.0 ± 14.0)
G. robertianum	100.2 ± 6.3 (275.0 ± 17.6)***	15.5 ± 1.7 (172.1 ± 18.4)**	99.1 ± 5.0 (234.1 ± 11.7)***	6.8 ± 0.7 (161.9 ± 15.7)**

Values of respiratory rates in state 3, 4 and FCCP-stimulated respiration (respectively V_3, V_4 and FCCP) are expressed as nmol $O_2 \times$(mg protein)$^{-1} \times min^{-1}$ and as percentage of control (GK rats drinking distilled water). Results are presented as mean ± SEM of triplicates of experiments performed with mitochondrial preparations from three rats in each group. Statistics: ***$P<0.0001$; **$P<0.001$ as compared to controls.

Indeed, the high content of polyphenolic compounds is also correlated to several medicinal properties attributed to these plants. Anthocyanosides and quercetins found in *V. myrtillus* constitute important therapeutic agents in diabetes, delaying the onset of diabetic complications, generally related to vascular damage induced by oxidative stress (Duke, 1992; Roy *et al.*, 2002; Riihinen *et al.*, 2008), and can be suitable for diabetic patients. Regarding its chemical composition, *G. robertianum* also has a high content of polyphenols and flavones with a high antioxidant activity, the most significant being 3,4-dimethoxyflavone, homoeriodictyol and kaempferol (Amaral *et al.*, 2009; Neagu *et al.*, 2010).

Due to their essential function in aerobic metabolism, mitochondria are associated with the pathophysiology of diabetes. Indeed, qualitative, quantitative and functional mitochondrial perturbations affecting both the onset of diabetes and associated complications have been identified (Sivitz & Yorek, 2010). Thus, for all the stated reasons, the valuable effects of *G. robertianum* and *V. myrtillus* leaf decoctions that we named "MitoTeas", particularly their high antioxidant activity and the improvement of mitochondrial OXPHOS, associated with lowering of occasional glycaemia, make these medicinal plants valuable candidates for diabetes therapy. Nevertheless, further studies are required in order to fully explain the mechanisms involved in OXPHOS efficiency improvement by these "MitoTeas".

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