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Evaluation of the anti-diabetic potential of *Catharanthus roseus* extracts

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Mestrado em Biotecnologia e Bio-Empreendedorismo
em Plantas Aromáticas e Medicinais

Trabalho efectuado sob a orientação da
Professora Doutora Cristina Pereira-Wilson
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Abstract

Type 2 DM is a metabolic disorder that results from genetic and environmental factors and affects 100 million people worldwide. It is characterized by hyperglycemia due to: i) decreased secretion of insulin by the pancreas and ii) resistance to the action of insulin in tissues as muscle, liver and adipocytes, leading to impaired glucose uptake.
The objectives of the present work were: i) the characterization of the HIT-T15 cell line (hamster pancreatic β-cell line) as an *in vitro* model to assess the anti-diabetic potential of plant extracts/compounds; ii) the evaluation of the anti-diabetic potential of *C. roseus* aqueous extracts as well as that of their major flavonoids (quercetin and kaempferol) in the β-cell context, through the assessment of parameters such as insulin secretion and insulin gene expression, β-cell protection against oxidative stress induced by dRib and other oxidant compounds, GSH levels and apoptosis.

Concerning the effects of *C. roseus* extracts on glucose-induced insulin secretion, we observed no effects on insulin secretion. However, the aglycones of the major flavonoid glycosides present in the extracts, Q and K at 20 μM, exhibited a strong induction of insulin secretion to levels above those achieved by glb (10 nM). That stimulatory effect was inhibited by both nifedipine and diazoxide indicating a mechanism similar to that of the pharmaceutical drug, which is targeting the insulin exocytosis process.
Under conditions of oxidative stress induced by dRib (30 mM) cell viability was decreased in approximately 50%, a condition that was not prevented by the co-incubation of dRib with the three *C. roseus* extracts. Despite this fact, Q, K and NAC (200 μM) exhibited protective effects against dRib-induced damage, in a concentration-dependent manner, except for K (40 μM).

Regarding GSH levels, a significant diminishment was observed in the presence of 30 mM dRib when compared to control, a situation that was rescued by NAC, Q and K. The increase of GSH levels induced by NAC was mainly due to its role as precursor of GSH shynthesis, since the effect was totally abolished in the presence of BSO, an inhibitor of γ-GCS. Q and K alone also increased GSH levels of HIT-T15 cells indicating the potential of these flavonoids for maintain GSH homeostasis and prevention of oxidative stress, particularly beneficial in a diabetic condition.

Taken together, these results indicate that *C. roseus* extracts might exert their attributed anti-diabetic activity in other organs like liver or skeletal muscle and that both Q and K, due to their antioxidant and remarkable insulin secretagogue effects, are good candidates for the prevention and/or management of type 2 DM.

Resumo

DM tipo 2 é uma desordem metabólica resultante de factores genéticos e ambientais que afecta 100 milhões de pessoas em todo o mundo. É caracterizada por uma condição de hiperglicémia devido a: i) um défice na secreção de insulina pelo pancreas e ii) resistência à acção da insulina em tecidos como o músculo, fígado e adipócitos, comprometendo a absorção de glucose.

Os objectivos deste trabalho experimental consistiram na: i) caracterização da linha celular HIT-T15 (células β-pancreáticas de hamster) como modelo *in vitro* para avaliar o potencial anti-diabético de extractos/compostos de plantas e ii) avaliação do potencial anti-diabético de extractos aquosos de *C. roseus* e flavonóides maioritários (quercetina e canferol), ao nível das células beta, através da avaliação de parâmetros tais como indução da secreção de insulina e expressão do gene da insulina, protecção das células beta contra o stresse oxidativo induzido pela 2-desoxi-D-ribose (dRib) e outros compostos oxidantes, níveis de GSH e apoptose.

Relativamente aos efeitos dos extractos de *C. roseus* na secreção de insulina induzida pela glucose, não se observaram efeitos a esse nível. Contudo, as agliconas dos principais flavonóides presentes nos extractos, Q e K a 20 μM, exibiram uma forte indução da secreção de insulina para níveis superiores aos alcançados pela glb (10 nM). Este efeito estimulatório foi inibido pela nifedipina e diazoxida, indicando um mecanismo de acção semelhante ao do fármaco, atingindo o processo de exocitose da insulina.

Em condições de stresse oxidativo induzido pela dRib (30 mM), a viabilidade celular foi diminuida em cerca de 50%, uma condição que não foi prevenida pela co-incubação da dRib com nenhum dos três extractos de *C. roseus*. Apesar deste facto, foi observado um efeito protector contra os danos induzidos pela dRib por parte da Q, K e NAC (200 μM), de uma forma dependente da concentração, excepto para o K (40 μM).

No que diz respeito aos níveis de GSH, ocorreu uma diminuição significativa destes na presença de 30 mM de dRib quando comparado com o controlo, sendo esta situação revertida pelo NAC, Q e K. O efeito do NAC no aumento dos níveis de GSH deveu-se maioritariamente ao facto deste ser um precursor da síntese de GSH pois o seu efeito foi totalmente inibido na presença do BSO, um inibidor da γ-GCS. Por si só, Q e K também aumentaram os níveis de GSH nas células HIT-T15, indicando o potencial destes flavonóides na manutenção da homeostasia da GSH e na prevenção do stresse oxidativo, uma situação particularmente benéfica numa condição diabética.

No conjunto, estes resultados indicam que os extractos de *C. roseus* podem exercer o seu potencial anti-diabético em outros órgãos, como no fígado ou músculo esquelético, e que a Q e o K, devido à actividade antioxidante e efeitos como secretagogos da insulina, são bons candidatos para a prevenção e/ou controlo da DM tipo 2.

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Abbreviations

AGE	Advanced Glycation End-products
ATP	Adenosine Triphosphate
CAT	Catalase
DM	Diabete Mellitus
dRib	2-deoxy-D-ribose
ER	Endoplasmic Reticulum
FFA	Free Fatty Acids
γ-GCS	gamma-glutamylcysteine synthetase
GIP	Glucose-dependent Insulinotropic Peptide
GIIS	Glucose-Induced Insulin Secretion
GK	Glucokinase
GLP-1	Glucagon-Like Peptide-1
GLUT	Facilitative Glucose Transporter (GLUT2, GLUT4)
GOX	Glucose Oxidase
GPX	Glutathione Peroxidase
GSH	Glutathione – reduced form
GSSG	Glutathione – oxidised form
H_2O_2	Hydrogen Peroxide
HGO	Hepatic Glucose Output
IFG	Impaired Fasting Glycaemia
IGT	Impaired Glucose Tolerance
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
K	Kaempferol

K_{ATP}	ATP-sensitive potassium channel
NAC	*N*-acetylcysteine
OGTT	Oral Glucose Tolerance Test
Q	Quercetin
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STZ	Streptozotocin
SUR	Sulfonylurea receptor
tBHP	*tert*-Butyl Hydroperoxide
WHO	World Health Organisation

Introduction

1. Diabetes mellitus – general considerations

Diabetes mellitus (DM) is a clinically and genetically heterogeneous group of disorders characterized by abnormally high levels of glucose in the blood (hyperglycemia). Hyperglycemic states are caused by absolute or relative insulin deficiency associated or not with insulin resistance that often leads to a disturbance in glucose homeostasis and in fat and protein metabolism (LeRoith *et al.*, 2004; Robertson, 2004). The prevalence of DM has reached epidemic proportions worldwide and is one of the worst global threats to mankind. The increase in diabetes prevalence seems to be associated with the spread of the western lifestyle, which is linked with increasing overweight and sedentary populations. Statistics revealed a prevalence of the disease of 2.8 % in 2000; this is estimated to be 4.4% in 2030, a scenario that is translated in an increase of 171 million people in 2000 to 366 million in 2030, making the human and economic costs derived from this situation enormous (Wild *et al.*, 2004).

The first observation that DM is not a single disorder remounts to Chakrata and Susruta (600 B.C.), two Indian physicians that differentiated two forms of the disease. During the 18^{th} and 19^{th} centuries a variety of the disorder was identified. This form, which today is recognized as type 2 DM, was less symptomatic, detected in later life, associated with overweight and identified by heavy glycosuria (increase of glucose levels in urine as a result of saturation of glucose reabsorption by the kidney). In the mid-1930s, Himsworth differentiated two clinical types of DM, with regard to the sensitivity and insensitivity of peripheral tissues to insulin (LeRoith *et al.*, 2004). Modern classification systems set by the National Diabetes Data Group of USA (1979) and by Word Health Organization (WHO, 1980) have distinguished two main types of DM. **Type 1 DM** also known as insulin-dependent DM, accounts for 5 to 10 % of the cases of DM, is characterized by auto-immune destruction of beta-cells of the endocrine pancreas leading to loss of insulin secretion. Type 1 diabetic patients dramatically depend on insulin administration for survival (LeRoith *et al.*, 2004; Robertson, 2004). **Type 2 DM** or non-insulin-dependent DM, comprising 90 to 95 % of cases in the diabetic syndrome, is a combination of genetic and nongenetic (age, high caloric diet, overweight or sedentary lifestyle) factors that cause insulin resistance and a relative insufficiency in insulin secretion (LeRoith *et al.*, 2004; Surampudi *et al.*, 2009). Among type 2 DM patients, there is a minority that exhibits a

form of the disease that results from mutations in a single gene. The most common subtype is the inherited maturity-onset diabetes of the young (MODY) characterized by early onset, usually before 25 years of age (McCarthy, 2004). Other type of DM, gestational DM, occurs in less than 5 % of all pregnancies, can also be distinguished from the former groups and is caused by insulin resistance and relative insulin deficiency during pregnancy (LeRoith *et al.*, 2004; McCarthy, 2004).

Diabetes mellitus could persist as an asymptomatic disease for a long period of time and secondary complications may arise before hyperglycemia is detected. For that reason, surveillance/prevention has to be a priority (Wallander *et al.*, 2008). Therefore, classification of glucose abnormalities in an asymptomatic person should, according to WHO, be based on the following diagnostic observations: i) occasional plasma glycemia ≥200 mg/dL (11.1 mmol/L), obtained at any time of day and without regard to when food was last ingested; ii) fasting plasma glycemia ≥126 mg/dL (7.0 mmol/L), fasting being a period of at least 8 hours without ingestion of food, or iii) plasma glycemia ≥200 mg/dL (11.1 mmol/L) at 2 hours after an oral glucose tolerance test (OGTT). The test must be carried out according to WHO criteria, with 75 g of anhydrous glucose dissolved in water (Conget, 2002). Diabetes is preceded by gradual stages of increasing glucose intolerance as listed in table I.

Table I – Diagnostic criteria of diabetes mellitus and other categories of hyperglycemia (from Stumvoll *et al.*, 2005).

	Glucose concentration in venous plasma (mmol/L)
Diabetes mellitus	Fasting ⩾7·0 or 2-h post-glucose load ⩾11·1
Impaired glucose tolerance	Fasting (if measured) <7·0 and 2-h post-glucose load ⩾7·8 and <11·1
Impaired fasting glucose	Fasting ⩾6·1 and <7·0 and 2 h post-glucose load (if measured) <7·8

Glucose load=75 g glucose orally.[10]

In the present work we will focus on type 2 DM, attempting to characterize potentially preventive effects that may result from the use of plants and particular attention will be paid to their action in the pancreatic beta cells.

1.1 Type 2 Diabetes mellitus

Type 2 DM affects 100 million people worldwide and is characterized by hyperglycemia, resulting from: i) decreased secretion of insulin by the pancreas and ii) resistance to the action of insulin in peripheral tissues as muscle, liver and adipocytes, leading to impaired glucose uptake (Cheng and Fantus, 2005; Montecucco *et al.*, 2008). Insulin resistance can be defined as the state of reduced responsiveness to normal circulating levels of insulin (Montecucco *et al.*, 2008*).* The convergence of factors (fig.1) such as obesity, physical inactivity, age and poor dietary habits that cause impaired insulin signaling in association with genetic susceptibility increases the risks of developing type 2 DM (Muoio and Newgard, 2008; Surampudi *et al.*, 2009).

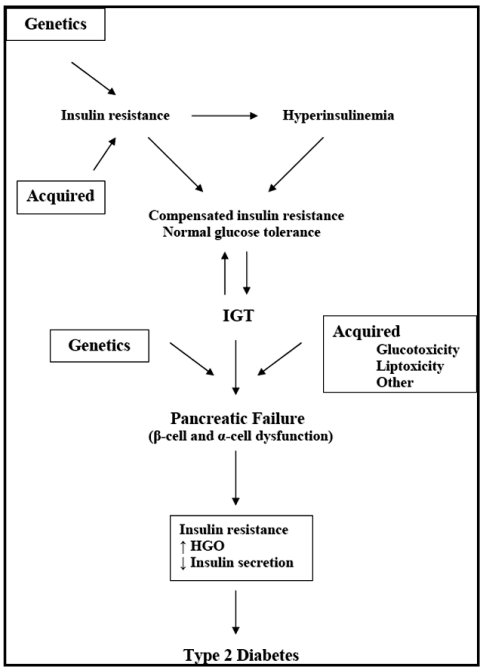

Figure 1 – Proposed etiology for type 2 diabetes mellitus (from Surampudi *et al.*, 2009). IGT: impaired glucose tolerance; HGO: hepatic glucose output.

In healthy subjects, the maintenance of glucose tolerance is a process achieved by different factors. After glucose ingestion, an increase in plasma glucose concentration occurs and stimulates insulin secretion by pancreatic beta cells. This combined situation of hyperinsulinemia and hyperglycemia leads to glucose uptake by the liver, gut and peripheral tissues and suppresses endogenous glucose production (DeFronzo, 2004). Glucose uptake by peripheral tissues occurs in mainly by muscle (80-85 %) and to a smaller degree (4-5 %) by adipocytes. These, have an important role on body glucose homeostasis as they regulate the release of free fatty acids (FFA) from stored triglycerides and the production of adipocytokines that influence insulin sensitivity in muscle and liver.

In a non-diabetic situation, the increased plasma insulin in response to glucose ingestion, will promote a decline in plasma FFA, which, in turn, increase muscle glucose uptake and contributes to the inhibition of hepatic glucose production. Endogenous glucose production is responsible for maintaining basal glucose levels in a fasting state and is derived from liver (85 %) through glycogenolysis and gluconeogenesis and also from kidney (remaining 15 %). Glucagon, another hormone from the endocrine pancreas, also plays a central role in glucose homeostasis through the stimulation of hepatic glucose output, under postabsorptive conditions. Any disturbance in this process can contribute to hyperglycemia in diabetic patients (DeFronzo, 2004).

1.1.1 Cellular response to insulin

Under a condition of type 2 DM the glucose homeostasis suffers disturbances. Insulin resistance, usually present before the onset of diabetes, is associated with dyslipidemia, hypertension increased platelet aggregation, vascular inflammation, endothelial dysfunction and premature atherosclerosis (Cheng and Fantus, 2005), which explain the circulatory diabetes related complications.

The cellular mechanisms that contribute to insulin resistance are related with defects in insulin receptor and/or signal transduction cascade (DeFronzo, 2004). The insulin receptor is necessary to mediate insulin action and it consists of a heterotetrameric membrane glycoprotein composed of two α- and two β-subunits, linked together by disulfide bonds. The binding of insulin molecule to the receptor's extracellular α –subunit brings α and β subunits closer together, inducing a conformational change that enables ATP bind to the β-subunit's intracellular domain. ATP binding results in receptor autophosphorylation and tyrosine phosphorylation of insulin receptor substrates - IRS (Kido *et al.,* 2001). The activation of insulin signal transduction in insulin target tissues, such as muscle and adipose tissue, ends with the translocation of glucose transporters to the plasma membrane. In muscle cells and adipocytes, GLUT4 is responsible for glucose transporter. Individuals with type 2 DM exhibit a 20 to 30 % reduction in insulin binding to adipocytes, caused by a reduction in the number of insulin receptors without change in insulin receptor affinity whereas, in muscle, a severe impairment in IRS-1 tyrosine phosphorylation occurs (DeFronzo, 2004).

In early stages of the disease process, to compensate insulin resistance, pancreatic beta-cells increases insulin secretion and this is sufficient to maintain normoglycemia. The

progression from normal to impaired glucose tolerance (IGT) is due to a decrease in tissue sensitivity to insulin and an increase in both fasting and glucose-stimulated plasma insulin levels occurs due to β-cell compensation (DeFronzo, 2004; Surampudi *et al.,* 2009). However, the subsequent decline in beta-cell function leads to a hyperglycemic condition and the establishment of the diabetic condition. This hyperglycemic condition (fig. 2), *per se*, is responsible to aggravate both insulin resistance and impaired insulin secretion – glucotoxicity (Cheng and Fantus, 2005; Surampudi *et al.*, 2009).

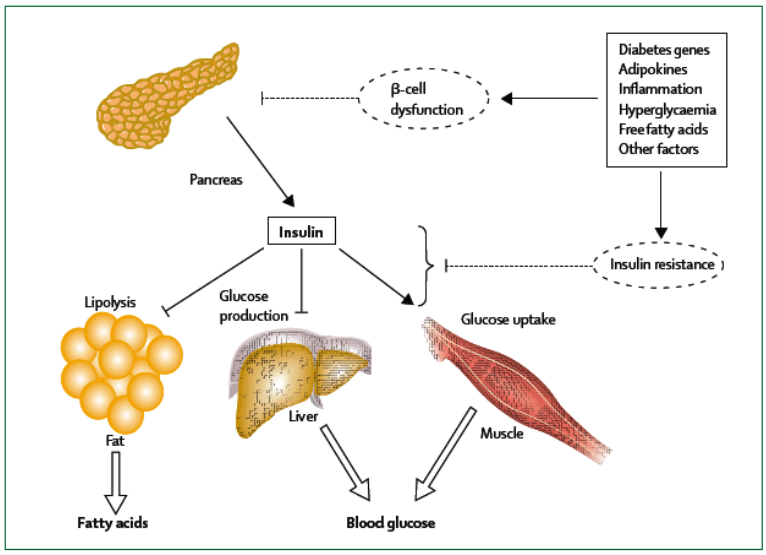

Figure 2 – Abnormalities in type 2 diabetes mellitus that contribute to hyperglycemia and circulating free fatty acids (from Stumvoll *et al.*,2005).

DM type 2 is established only in the presence of both insulin resistance and insulin secretion defects. The last parameter will be treated with more detail in subsequent sections, regarding the incidence of the present work on beta-cells defects and its potential protection by natural compounds.

2. The pancreas: structure and role in glucose homeostasis

The pancreas is an organ located in the abdominal cavity and is divided in two portions according to its functions: the exocrine and the endocrine pancreas. The exocrine pancreas is responsible for the secretion of enzymes involved in the process of digestion whereas the endocrine pancreas secretes hormones involved in regulation of energy metabolism and fuel homeostasis. The endocrine pancreas represents a small part of the

pancreas and consists of groups of cells – islet of Langerhans – that comprise about 1 to 2% of the total mass (i.e., approximately one million islets in the human pancreas) and are imbedded in the exocrine portion. Each islet is a structure highly irrigated by blood vessels that facilitates the secretion of the hormones directly to them and innervated by sympathetic, parasympathetic, and sensory nerves. There are four different cell types composing the islets, each of them responsible to synthesize and secrete distinct hormones (fig. 3).

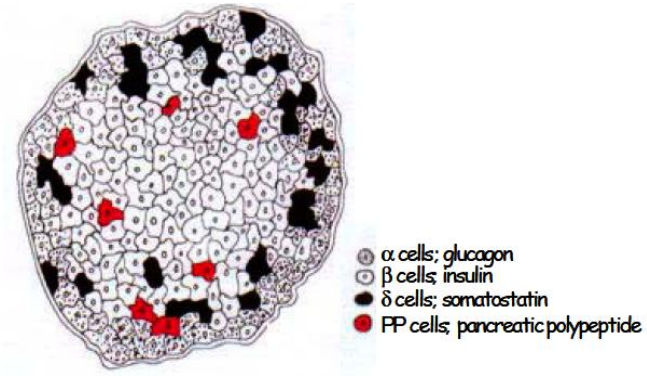

Figure 3 - Diagrammatic representation of the endocrine cell distribution in a typical mammalian islet of Langerhans (from Rodríguez, 2004).

The cell types are α, β, δ and F cells, which constitute 25%, 60%, 10% and 1% of total islet cells, and are responsible for the secretion of glucagon, insulin, somatostatin and pancreatic polypeptide (PP), respectively. The remaining 4 % consists of connective tissue and blood vessels. Both insulin and glucagon are involved in regulation of blood glucose concentration and are inhibited reciprocally. Somatostatin regulates hormone secretion by the α and β cells whereas the function of PP has not been fully characterized (Rhoades and Pflanzer, 2004).

Glucose homeostasis is maintained by the interaction of three different processes: insulin secretion by the beta cells, tissue glucose uptake and hepatic glucose production. Therefore, keeping blood glucose concentration constant regulates a constant supply of glucose by the organism. This normal level is achieved by a balance between glucose absorption from the gut, tissue utilization (glycolysis, pentose phosphate pathway activity, tricarboxilic acid cycle activity, glycogen synthesis) and endogenous production by glycogenolysis and gluconeogenesis. This complex process is regulated by the pancreatic hormones insulin and glucagon (Kawahito *et al.*, 2009).

2.1 Insulin

Biologically active human insulin is a protein that consists of two polypeptide chains, the A chain (21 amino acids) and the B chain (30 amino acids), joined by two interchain disulfide-linked bridges and this structure is highly conserved in higher vertebrate evolution (LeRoith *et al.*, 2004). In pancreatic β-cells the insulin gene is transcribed and translated as preproinsulin, the precursor molecule, which contains a signal peptide that facilitates its translocation from cytoplasm to rough endoplasmic reticulum, where the signal sequence is cleaved to originate proinsulin. This proinsulin molecule is packaged into vesicles (granules) in the Golgi apparatus and undergoes a maturation process (fig. 4) during which specific endopeptidases cleave the proinsulin originating an insulin molecule and a C-peptide molecule. It appears that this maturation process is highly affected by Ca^{2+} dependent proteases. Granules are then translocated to the cell surface and its content released by exocytosis (LeRoith *et al.*, 2004; Rodríguez, 2004).

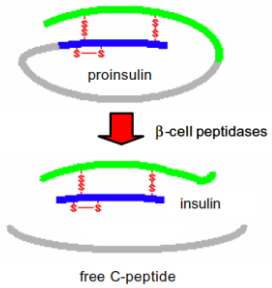

Figure 4 - Insulin maturation. Proinsulin is cleaved specifically by β-cell peptidases producing the mature insulin and the C-peptide (from Rodríguez, 2004).

2.2 Glucose-sensing in the β-cell

Blood glucose is maintained at concentrations very near 5 mM, with glucose transporter isoform 2 (GLUT2), liver glucokinase and pancreatic beta-cells being responsible to monitoring blood glucose levels (Im *et al.*, 2006). Glucose is the strongest natural stimulus for insulin secretion from β-cells, because it was most likely a principal food component during evolution (Suckale and Solimena, 2008). However, pancreatic beta cells also respond to other breakdown products from food, such as other monossacharides, amino acids and fatty acids. As examples, leucine, arginine and palmitate can increase insulin secretion of rat islets pre-cultured in glucose; fructose, alone, does not stimulates insulin secretion but increases its synthesis and potentiates glucose-induced insulin secretion (Suckale and Solimena, 2008). There are other molecules that potentiate glucose-induced insulin secretion such as glucagon-like peptide-1 (GLP-1), glucose-dependent

insulinotropic peptide (GIP) or pituitary adenylate cyclase-activating polypeptide (PACAP) that interact with G-protein-coupled receptors to generate second messengers such as cAMP and Ca^{2+} (Rutter, 2001; Surampudi *et al.*, 2009).

Glucose enters the beta-cell through GLUT2 and an equilibrium between intra- and extracellular media is rapidly obtained. GLUT2, unlike GLUT4, does not require mobilization by insulin and its low substrate affinity (K_m=17 mM) promotes a high rate of glucose influx, proportional to blood glucose concentrations up to 10 mM (Suckale and Solimena, 2008).

After entering the β-cell, glucose is phosphorylated, and thus entrapped inside the cell, by a glucokinase (GK), specific for beta and liver cells. GK unique properties distinguishes it from other hexokinases and allow it to function as glucose sensor: i) low affinity for glucose (K_m= 6 mM), which is in the middle range of blood glucose after a meal, whereas other hexokinases are already at its maximum speed at this concentration and ii) is not inhibited by its product – glucose-6-phosphate – which allows a continuous activity despite high glycolysis load (Suckale and Solimena, 2008).

Glucose transport through β-cell membrane is rapid and thus unlikely to be rate-limiting for glucose metabolism (Rutter, 2001). On the other hand, glucose phosphorylation by GK is the rate-limiting step in the glycolytic cascade that leads to insulin secretion, thus a small change in GK activity significantly alters the threshold for glucose-stimulated insulin secretion (Im *et al.*, 2006).

2.3 Insulin gene expression regulation

The regulation of insulin gene expression in β-cells is achieved by a variety of pancreatic transcription factors and the A3, C1 (RIPE3b, in rat) and E1 elements (fig. 5) in the insulin gene enhancer region are of great importance for the activation of insulin gene transcription (Kaneto *at al.*, 2009). Pancreatic and duodenal homeobox factor-1 (PDX-1) binds to the A3 element whereas NeuroD binds the E1 element and both proteins play critical roles in insulin gene expression and islet development and function. Recently, a transactivator, MafA, was identified, which controls β-cell-specific insulin gene transcription through binding to the C1 (RIPE3b) element (Kaneto *et al.*, 2009). In addition to insulin gene, PDX-1 is also responsible for the activation of other genes involved in glucose sensing and metabolism such as GLUT2 and GK. Both PDX-1 and NeuroD are expressed in various cell types in the pancreas while MafA expression is restricted to the β-cells (Kaneto *at al.*, 2009). MafA is a weak transactivator of the insulin promoter when

expressed alone as are PDX-1 and NeuroD. However, co-expression of these three factors, synergistically and strongly activate the insulin promoter. Furthermore, MafA, PDX-1 and NeuroD seem to be also involved in proliferation and survival of β-cells in the adult pancreas (Aramata *et al.,* 2007; Kaneto *at al.,* 2009).

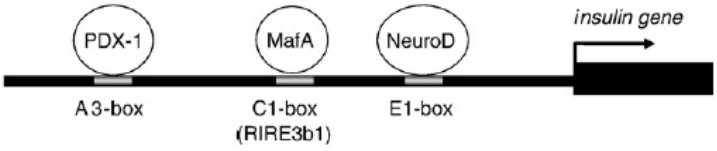

Figure 5 – Key transcription factors involved in insulin gene transcription (from Kaneto *et al.,* 2009).

In pancreatic beta-cells these processes are regulated by extracellular stimuli, such as glucose metabolism, under both physiological and pathological conditions. Insulin gene transcription is stimulated by short-term exposure to high glucose concentrations (Aramata *et al.,* 2007). However, under a diabetic condition, hyperglycemia and consequentially the production of oxidative stress and dyslipidemia provokes a decrease in insulin gene expression due to a decreased expression and/or DNA binding activities of MafA and PDX-1 (Kaneto *et al.,* 2009). These adverse effects may be prevented by treatment with antioxidants (Tanaka *et al.,* 1999; Tanaka *et al.,* 2002).

2.4 Signal transduction in insulin secretion

As many other endocrine cells and neurons electrical signals play a central role in the regulation of secretion and pancreatic beta cells which also have been reported to be electrically excitable by Dean and Matthews in 1968 (Rorsman *et al.,* 2000). An increase in extracellular glucose concentration from basal levels (5 mM) to a stimulatory concentration (10 mM) leads to a β-cell slow depolarization from the resting potential (-70 mV). This change in membrane potential is the central step in the signal transduction cascade, which generates an increase in intracellular Ca^{2+} concentration, leading to insulin secretion (Rorsman *et al.,* 2000).

Glucose-induced insulin secretion (GIIS), *in vivo*, exhibits a biphasic pattern of response (fig. 6). The "first phase secretion" consists of a rapid increase in insulin secretion shortly after an increase in glucose concentration, reaches a peak after 3 to 4 minutes and then rapidly declines to a nadir at 8 minutes (Rorsman *et al.,* 2000; Straub and

Sharp, 2002). The "second phase secretion" arises from the nadir and is characterized by a steadily increasing rate until it reaches a plateau after a further 25 to 30 minutes. Type 2 DM patients always have the first phase of insulin secretion compromised (Straub and Sharp, 2002). In 1984, the ATP-sensitive potassium channel (K_{ATP}) was identified and found to be responsive to glucose.

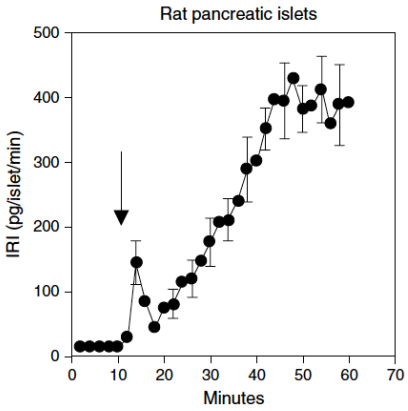

Figure 6 – Biphasic profile of glucose-induced insulin secretion (from Straub and Sharp, 2002).

The first and second phase of insulin secretion occur through different pathways, which are the K_{ATP} channel dependent pathway and K_{ATP} channel independent pathway that are associated with first phase insulin secretion and second phase secretion, respectively (Straub and Sharp, 2002).

2.4.1 K_{ATP} channel dependent pathway

After a meal, glucose enters beta-cell through GLUT2 and its metabolism, initiated and controlled by GK, leads to an increase in the ATP/ADP ratio, inducing K_{ATP} channel closure (fig. 7A). This situation provokes a depolarization of the β-cell, which, in turn, results in the opening of voltage-dependent calcium channels. The resulting increase in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_I$, modulates kinases or other effector systems involved in secretion, triggering the movement of secretory vesicles to the cell surface, their fusion with the plasma membrane and finally releasing stored insulin. The presence of aggregates of Ca^{2+} channels, granules and exocytotic machinery in specific locations of the membrane constitute the evidence for the location of "hot spots" for exocytotic activity (LeRoith *et al.*, 2004; Straub and Sharp, 2002).

Insulin-containing granules (fig. 7B) can be docked at the plasma membrane or free to move in the cytosol – reserve granules. After a glucose challenge only a small fraction of the docked granules will be released. On arrival at the membrane after translocation from the reserve pool, the granules dock and are primed for release. The priming process

involves chemical modifications between granules membrane and plasma membrane proteins, preparing them for fusion, in a process dependent of ATP (Eliasson *et al.*, 1997). Therefore, the granules could be primed or unprimed and the former may be divided into readily releasable and immediately releasable. The latter would be the ones that make up the first phase insulin secretion and are located in the specialized areas of the membrane (LeRoith *et al.*, 2004; Rorsman *et al.*, 2000; Straub and Sharp, 2002).

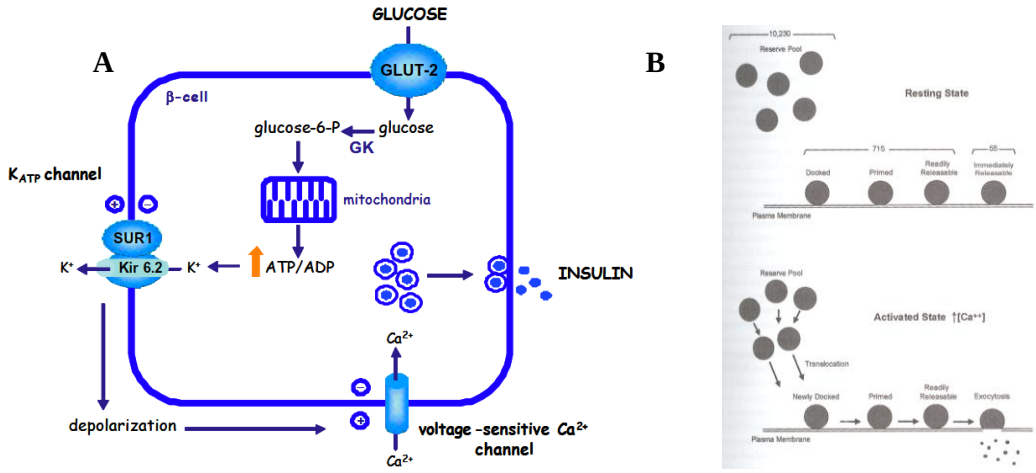

Figure 7 – A) K_{ATP} channel-dependent pathway of glucose sensing in the β-cell (from Rodríguez, 2004); B) β-cell granule pools in resting and activated states (from LeRoith *et al.*, 2004).

Sulfonylureas, as glibenclamide, used in the present work, are clinically approved therapeutic agents for the management of type 2 DM for more than 40 years. They exert their pharmacological activity by promoting the closure of K_{ATP} channels leading to membrane depolarization, opening of Ca^{2+} channels and initiation of insulin secretion. This effect is mediated by the binding to a sulfonylurea receptor (SUR1), which together with protein Kir6.2, forms functional K_{ATP} channels. Sulfonylurea's effect on this channel is independent of the cell's metabolic state (Rorsman *et al.*, 2000).

2.4.2 K_{ATP} channel independent pathway

The demonstration of a second pathway, K_{ATP} channel independent pathway, separate from K_{ATP} channel dependent pathway, which acts by the increase of secretory response to $[Ca^{2+}]_i$ was published in 1992. Since both pathways act in a synergistic form they are not strictly independent (fig. 8) (LeRoith *et al.*, 2004; Straub and Sharp, 2002).

The facts that under maximal activation of K_{ATP} channel-dependent pathway, glucose

still increases insulin secretion and that β-cells exhibited a partial secretory response to glucose without changes in $[Ca^{2+}]_i$ which is already elevated at low glucose concentration, suggests that an amplifying pathway - K_{ATP} channel-independent pathway- exists (Rutter *et al.*, 2001). This independent pathway is Ca^{2+} dependent, since $Ca^{2+}{}_i$ has to be elevated, but is not mediated by any further rise in $Ca^{2+}{}_i$ ((Henquin, 2000).

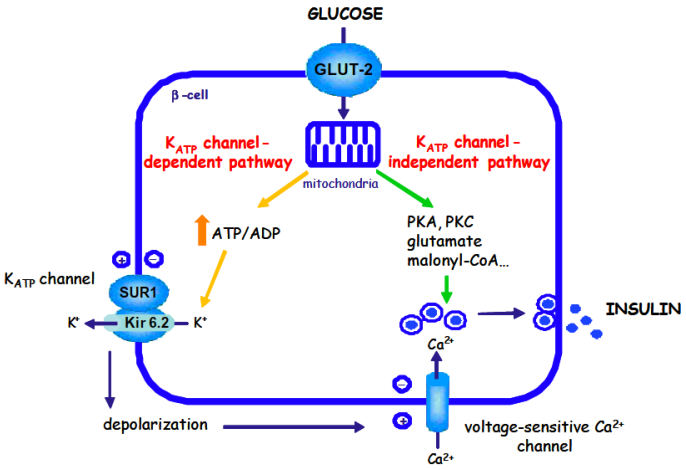

Figure 8 - Schematic representation of the K_{ATP} channel-dependent and -independent pathways of glucose sensing in the β-cell (from Rodríguez, 2004).

However, the mechanisms involved in this pathway are still under discussion, despite some mechanisms and second messengers have been suggested, such as malonyl-CoA, glutamate or the ratio between guanosine triphosphate (GTP)/ guanosine diphosphate (GDP) (Straub and Sharp, 2002).

In summary, K_{ATP} channel-independent pathway serves to optimize the secretory response induced by the K_{ATP} channel-dependent signal and remains functionally silent until a depolarization, induced by the last pathway, occurs. This hierarchy between the two pathways ensures that no insulin is inappropriately secreted in the presence of low glucose concentrations (Henquin, 2000).

3. β-cell failure in type 2 diabetes mellitus

The pathophysiology of type 2 DM, at the beta-cell level, is characterized by a decrease in β-cell mass and/or defects in the secretory machinery. Despite the controversy about the relative contribution of each factor for the development of the disease, it is now established that these factors are not dissociable, once the pathways regulating β-cell turnover are also implicated in β-cell insulin secretory function (Donath *et al*, 2005). In type 2 DM patients a decrease in β-cell mass of about 25-50 % was observed after

increased rates of β-cell apoptosis (Cnop *et al.,* 2005). These values suggest that β-cell dysfunction also contributes to the initial stages of disease process (Chang-Chen *et al.,* 2008).

In the early stages of the development of type 2 DM the scenario of insulin resistance is highly compensated by an increase in β-cell mass and function. However, the progression of hyperglycemia and elevated circulating free fatty acids leads to impaired β-cell function and, ultimately, β-cell failure and death by apoptosis (Chang-Chen *et al.,* 2008).

Pancreatic β-cell mass depends on the interplay between three major processes, that are proliferation, neogenesis and apoptosis and disturbances will result in loss of β-cell mass. Proliferation of pre-existing β-cell ensures the maintenance of β-cell mass during adult life, under normal conditions, and this process depends on three major groups of cell cycle proteins – cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors. Neogenesis is the process by which new β-cells are formed from precursor cells of the pancreatic ducts. Finally, β-cell apoptosis is a process directed by pro- and anti-apoptotic genes, extracellular signals and intracellular ATP levels, which appears to be a major contributor to β-cell failure in late stages of the development of type 2 DM (Chang-Chen *et al.,* 2008).

There are several mechanisms underlying β-cell failure, including the generation of reactive oxygen species (ROS), alterations in metabolic pathways, increases in intracellular calcium and the activation of endoplasmic reticulum (ER) stress (Chang-Chen *et al.,* 2008).

3.1.1 Glucotoxicity

Glucose concentration is the principal determinant for regulation of β-cell mass and function and transient increases in its plasma levels induce insulin secretion (Chang-Chen *et al.,* 2008). However, in a setting of chronic hyperglycemia, glucotoxicity occurs – the toxic effects on the beta-cell function and mass as the result of chronic exposure to supraphysiological glucose levels (Robertson *et al,* 2004). Glucotoxicity acts through mitochondrial dysfunction with production of ROS, ER stress and increased levels of intracellular calcium. The long-term increases in intracellular calcium leads to a proapoptotic situation (Chang-Chen *et al.,* 2008). Furthermore, glucotoxicity induce a reduced expression of genes involved in glucose-induced insulin secretion (GIIS), such as insulin, GLUT2, GK, voltage-dependent Ca^{2+} channels and the transcription factors that

regulate their expression (Cnop *et al.,* 2005).

3.1.2 Lipotoxicity

A diabetic profile is also characterized by dyslipidemia, which is an increase in circulating FFA. In healthy subjects, FFA induces stimulatory effects on insulin secretion, but in individuals with a genetic predisposition to diabetes, is a factor to progressive β-cell failure by apoptosis and impairment of insulin secretion (Chang-Chen *et al.,* 2008; Cnop *et al.,* 2005). Since lipotoxicity only adversely affects β-cells if hyperglycemia has been established (Robertson 2009), it constitutes an additional cause of beta-cell dysfunction and death in a set of type 2 DM.

3.1.3 ER stress

The ER of β-cell is a highly active and well-developed structure due to its high secretory demands and, because of that fact, ER stress plays a role in the pathogenesis of diabetes, contributing to pancreatic β-cell loss (Donath *et al.,* 2005). ER stressors are addressed to increased biosynthetic demands induced by chronic hyperglycemia, elevated FFA and chronic over-nutrition. When a stress situation is caused in the ER, provoked by misfolded proteins that aggregate in the ER lumen, FFA esterification that delay processing and export of newly synthesized proteins or precipitation of saturated triglycerides (Cnop *et al.,* 2005; Donath *et al.,* 2005), occurs the activation of a signal response – Unfolded Protein Response (UPR). The UPR alleviates ER stress, restores homeostasis and prevents cell death through: i) decrease new protein arrival to the ER, ii) increase the amount of ER chaperones to increase folding capacity and iii) increase the cell capacity to dispose of misfolded proteins. If the steps described above fail, the apoptotic cascade is triggered (Chang-Chen *et al.,* 2008; Eizirik *et al.,* 2008). However, UPR play a dual role in β-cells because its action as beneficial regulator under physiological conditions turns into a cause of β-cell dysfunction and apoptosis under stress situations such as hyperglycemia and dyslipidemia (Chang-Chen *et al.,* 2008; Eizirik *et al.,* 2008).

3.2 Hyperglycemia-induced chronic oxidative stress in β-cell dysfunction

Hyperglycemia and resulting glucotoxicity is recognized as the causal link between diabetes and diabetic complications (Rolo and Palmeira, 2006). Hyperglycemia-induced oxidative stress seems to be involved in the progression of pancreatic β-cell dysfunction

(Rolo and Palmeira, 2006; Kajimoto and Kaneto, 2004). Chronic hyperglycemia is the cause for secondary complications of type 2 DM such as retinopathy, kidney failure, neuropathies, micro and macrovascular disease (Robertson, 2004).

Several biochemical pathways and mechanisms of glucotoxicity have been suggested (fig. 9). These include glucose autoxidation, protein kinase C activation, methylglyoxal formation and glycation, hexosamine metabolism, sorbitol formation and oxidative phosphorilation (Robertson, 2004). All these pathways have in common the formation of ROS, which in physiological concentrations help to maintain homeostasis but when accumulated over time cause chronic oxidative stress, leading to defective insulin gene expression, insulin content, impaired insulin secretion and increased apoptosis (Robertson, 2004; Robertson *et al.*, 2007). This situation becomes particularly relevant to β-cell due to its low levels of intrinsic antioxidant defenses when compared with other cell types (Robertson, 2004; Robertson and Harmon, 2006; Robertson *et al.*, 2007; Kajimoto and Kaneto, 2004).

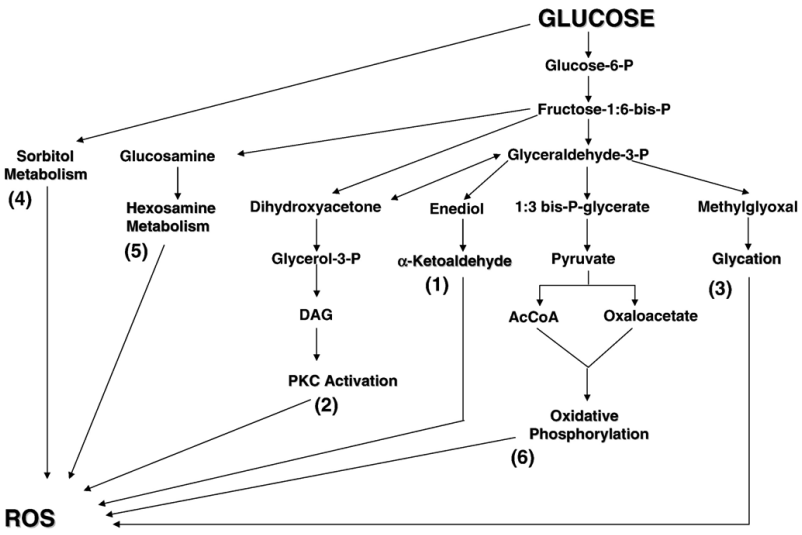

Figure 9 – Six biochemical pathways through which glucose metabolism can lead to the formation of reactive oxygen species (from Robertson and Harmon, 2006).

Under physiological conditions, glucose preferentially undergoes glycolisis and oxidative phosphorylation (fig. 9, pathway 6). In hyperglycemic conditions the excessive levels of glucose a high load in the glycolitic pathway and inhibit glyceraldehyde catabolism. This implies that substrates such as glucose, fructose-1,6-biphosphate and glyceraldehyde-3-P are pushed to alternative pathways. The progression through all these pathways leads to an excessive formation of ROS and advanced glycated end products (AGE's) (Robertson, 2004). Reducing sugars, like glucose, glucose-6-P, fructose or 2-deoxy-D-ribose form reversible early glycation products with proteins (for example,

glycated hemoglobin). These early glycation products may undergo further rearrangement and oxidations to produce AGE's, a process that alter proteins structure and function irreversibly. Aldehydes, such as glyoxal and methylglyoxal that are produced in excess under a hyperglycemic condition, can produce AGE's more efficiently than glucose (Han *et al.*, 2007; Mostafa *et al.*, 2007).

The molecular mechanisms of glucotoxicity and defective insulin gene expression involve the loss of two critical proteins that activate the insulin promoter (fig. 10), namely the PDX-1 and the RIPE-3b1 activator, recently identified as MafA (Robertson, 2004; Robertson *et al.*, 2007). In addiction, hyperglycemia induce superoxide production by mitochondria, which activates UCP-2 that is a subtype of mitochondrial carrier proteins which acts as proton carrier in the mitochondrial inner membrane and modulate the coupling between the respiratory electron transport chain and ATP synthesis. The activation of UCP-2 by superoxide decreases the ATP/ADP ratio thus reducing the insulin secretory response (Rolo and Palmeira, 2006).

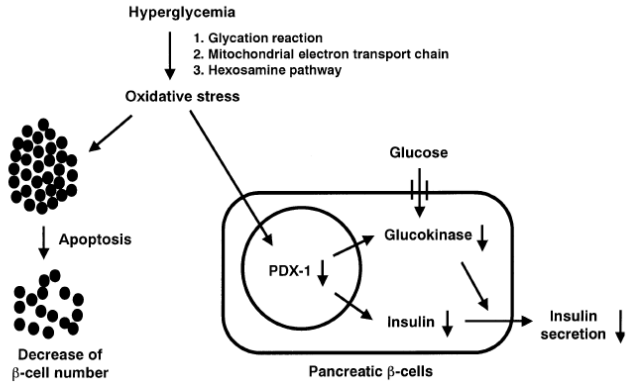

Figure 10 - A proposed molecular mechanism for β-cell glucose toxicity in diabetes (from Kajimoto and Kaneto, 2004).

The prevention of the deleterious effects of glucotoxicity, via oxidative stress, on β-cell function may be achieved by the use of antioxidants and overexpression of antioxidant enzymes (Kajimoto and Kaneto, 2004; Robertson and Harmon, 2006; Tanaka *et al.*,1999).

The antioxidant systems consist of antioxidant enzymes and diverse endogenous and dietary antioxidant compounds that react with and inactivate ROS (Roberts and Sindhu, 2009). The primary antioxidant enzymes include superoxide dismutases (SOD), which catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide, catalase (CAT) that converts H_2O_2 to water and molecular oxygen and glutathione peroxidase (GPX), involved in the reduction of H_2O_2, lipoperoxides and other hydroperoxides to their

corresponding hydroxylated compounds using glutathione as hydrogen donor. Among the nonenzymatic antioxidants there are vitamin C, vitamin E, β-carotene and reduced glutathione (GSH). The cell's antioxidant potential must be maintained either by dietary intake and/or *de novo* synthesis (Roberts and Sindhu, 2009).

The islet is among the least capable tissues in terms of intrinsic antioxidant enzyme expression and activities. For instance, the expression of SOD-1, SOD-2, CAT and GPX in the beta-cells was reported to be very low (Robertson, 2004; Robertson *et al.,* 2007). On the other hand, gene expression of γ-glutamylcysteine ligase (γ-GCL) that is the rate-limiting enzyme in the GSH synthesis is well represented in islets and GSH levels are closer to that of other tissues. However, it is known that γ-GCL expression is down-regulated in hyperglycemic scenarios that occur during the progression of type 2 diabetes (Robertson, 2004; Robertson *et al.,* 2007). Several studies revealed that overexpression of antioxidant enzymes in islets produce beneficial effects against oxidative damage. For instance, overexpression of SOD and CAT promote an enhanced β-cell tolerance to oxidative stress as well as protection against prooxidant toxicants and these effects were more expressive in a combinatorial rather than single overexpression. The protection of islet against increased levels of peroxide, induced by glucose and ribose, was attained by increasing islet GPX activity (Tanaka *et al.,* 2002). Additionally, overexpression of γ-GCL has been shown to protect against oxidative damage of β-cell insulin gene expression, insulin content and secretion (Robertson, 2004; Robertson *et al.,* 2007).

Antioxidant drugs have been used in the management of type 2 DM. Several studies have demonstrated that vitamins and supplements can help lower the markers of oxidative stress in diabetic subjects and animals (Rahimi *et al.,* 2005). N-acetylcisteyne (NAC), a ROS scavenger and a precursor of GSH (Rahimi *et al.,* 2005), exhibit protective effects against oxidative damage, preserving insulin gene expression, PDX-1 binding to the insulin promoter, insulin content, GIIS and β-cell apoptosis (Kho *et al.,* 2005; Robertson, 2004; Robertson *et al.,* 2007; Tanaka *et al.,* 1999). Also, sulphonylureas, like glibenclamide and glipizide, seem to exhibit antioxidant activities in diabetic animals (Rahimi *et al.,* 2005).

Recently new interest has emerged in natural antioxidants from plants to replace the synthetics that are utilized. Phytochemicals with antioxidant activity are present in all parts of higher plants and include phenolic acids, flavonoids, monoterpens, phenylpropanoids, tannins and triterpens (Rahimi *et al.,* 2005).

4. Phenolic compounds and antioxidant activity

Phenolic compounds are plant secondary metabolites that arose from the pentose phosphate, shikimate and phenylpropanoid pathways. This group of phytochemicals constitute the most widely occurring and its physiological and morphological importance in plants relies on their role in growth, reproduction, protection against pathogens and predators and color and sensory attributes of fruits and vegetables. Among a wide range of beneficial activities such as anti-allergenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory, it appears that the antioxidant activity is one of the major contributors to their beneficial effects. Taking this fact in consideration, phenolic compounds become a great natural source of antioxidants (Balasundram *et al.*, 2006).

With regard to the chemical structure, phenolic compounds possess an aromatic ring (benzene) with one or more hydroxyl group directly attached to it and the structural diversity implies that its classification into several classes of compounds (table II) (Balasundram *et al.*, 2006; Vermerris and Nicholson, 2006).

Table II – Classification of phenolic compounds based on the number of carbons in the molecule, according to Harborne and Simmonds, 1964 (from Vermerris and Nicholson, 2006).

Structure	Class
C_6	simple phenolics
C_6 - C_1	phenolic acids and related compounds
C_6 - C_2	acetophenones and phenylacetic acids
C_6 - C_3	cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C_6 - C_3	coumarins, isocoumarins, and chromones
C_{15}	chalcones, aurones, dihydrochalcones
C_{15}	flavans
C_{15}	flavones
C_{15}	flavanones
C_{15}	flavanonols
C_{15}	anthocyanidins
C_{15}	anthocyanins
C_{30}	biflavonyls
C_6-C_1-C_6, C_6-C_2-C_6	benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	quinones
C_{18}	betacyanins
Lignans, neolignans	dimers or oligomers
Lignin	polymers
Tannins	oligomers or polymers
Phlobaphenes	polymers

Among these classes, phenolic acids, flavonoids and tannins are considered the main dietary phenolic compounds (Balasundram *et al.*, 2006). In the context of the present work we will focus on the flavonoids.

Flavonoids encompass the largest group of plant phenolics, accounting for half of the 8,000 naturally occurring phenolic compounds. They are low molecular weight

compounds, consisting of 15 atoms of carbon and their basic chemical structure are represented in figure 11 (Balasundram *et al.*, 2006).

The flavonoid basic structure consists of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring, the C ring. Variations in substitution patterns in C ring are used to classify as flavonols, flavones, flavonones, flavonols, isoflavones, flavanonols and anthocyanidins (Balasundram *et al.*, 2006; Vermerris and Nicholson, 2006).

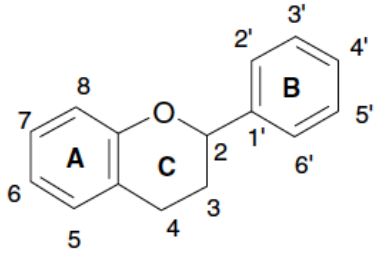

Figure 11 - Generic structure of a flavonoid molecule (from Balasundram *et al.*, 2006).

Substitutions in A and B rings, including oxygenation, alkylation, glycosilation, methylation or hydroxylation originate different compounds within each class of flavonoids, conferring to this class wide chemical, physical and biological properties (Balasundram *et al.*, 2006; Pinent *et al.*, 2008).

These compounds are present in vegetables, nuts, fruits and beverages such as coffee, tee and red wine and are reported as beneficial due to their antioxidant effects, which makes them good candidates for the development of functional foods with potential protective/preventive properties against several diseases, including type 2 DM (Pinent *et al.*, 2008). Also, these compounds exert their beneficial actions on cells through their modulation of proteins (and enzymes), gene expression and cell signalling cascades.

The antioxidant properties of flavonoids are due to their capacity to i) scavenge free radicals by donating a hydrogen atom from an aromatic OH group to a free radical, because they are able to stabilize an unpaired electron through its delocalization, ii) chelate metal cations and iii) inhibit the activities of enzymes that have oxidant activities such as lipoxygenase, cyclooxygenase or monooxygenase (Balasundram *et al.*, 2006; Moon *et al.*, 2005; Nijveldt *et al.*, 2001; Rice-Evans *et al.*, 1997).

Plants have been utilized as medicines for thousands of years. These medicines initially took the form of crude drugs but, recently, the use of plants as medicines has involved the isolation and characterization of pharmacologically active compounds

(Balunas and Kinghorn, 2005). A great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties. However, scientific information on antioxidant properties of those plants that are less widely used in culinary and medicine, is still scarce (Miliauskas *et al.,* 2004). Quercetin (fig. 12A), one of the best-described flavonoids, and kaempferol (fig. 12B) were used in the present work, since they represent the major constituents (aglycones) found in *C. roseus* aqueous extracts (Pereira *et al.*, 2009).

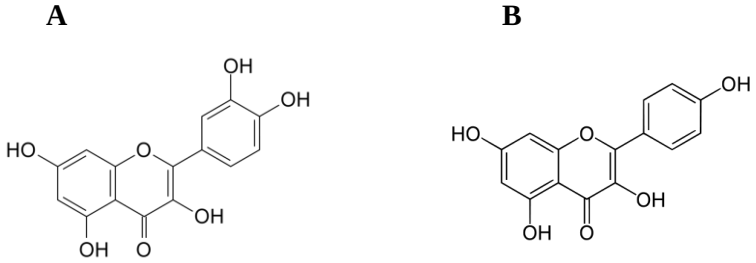

Figure 12 – Structure of the flavonoids quercetin (A) and kaempferol (B).

Several *in vivo* studies (table III) concerning the effects of several flavonoids, quercetin among them, on pancreatic β-cell function has been performed. These studies showed the effects of flavonoids on insulin secretion, maintenance of plasma glucose levels, beta-cell apoptosis and as well as oxidative stress (Pinent *et al.,* 2008).

In vitro studies, using insulin-secreting cell lines have also been performed, corroborating the same beneficial effects described above (Pinent *et al.,* 2008). Quercetin has been associated with enhanced insulin secretion *in vitro* (Hii and Howell, 1985) whereas kaempferol effects at the beta-cell level have not been established.

Table III – *In vivo* effects of flavonoids on insulin secreting capacity, beta-cell apoptosis and oxidative stress (from Pinent *et al.*, 2008). EGCG: epigallocatechin gallate; GSPE: grape seed procyanidin extract.

Flavonoid	Treatment	Model	Effect
Soy protein	30% in diet	Sprague-Dawley rats fed normal chow and high-fat diet	
Equol	2.55 μM in plasma		Inhibition of insulin secretion
Genistein Daidzein	0.4 μM in plasma 0.15 μM in plasma	Sprague–Dawley rats	
	0.2 g/kg diet	Nonobese diabetic (NOD) mice	Reduction of plasma glucose levels Increase in plasma insulin levels Increase in insulin-positive beta cells
Quercetin	10 to 15 mg/kg·day (10 d)	Normoglycemic rats	Increase in the number of pancreatic islet cells
		STZ-diabetic rats	Increase in the number of pancreatic islet cells Reduction of plasma glucose levels
	15 mg/kg·day (4 wk)	STZ-diabetic rats	Increase in plasma insulin levels Reduction of plasma glucose levels Protection of pancreatic beta-cell degeneration Reduction of oxidative stress markers and increase in the activities of antioxidant enzymes in pancreas
	10 mg/kg·day (14 d)	STZ-diabetic rats	Increase in plasma glucose levels
Rutin	100 mg/kg·day (45 d)	STZ-diabetic rats	Increase in plasma insulin levels Reduction of plasma glucose levels Reduction of lipid peroxidative products and increase in the activities of antioxidant enzymes in pancreas
EGCG	5 mg/kg·day (4 d)	STZ-diabetic rats	Reduction of the plasma insulin levels Reduction of the size of pancreatic islets
	100 mg/kg·day (10 d)	STZ-diabetic rats	Suppression of the iNOS expression and prevention of islet damage
Epicatechin	30 mg/kg twice a day (6 d)	STZ-diabetic rats	Prevention of hyperglycemia Prevention of hyperglycemia Prevention of pancreas morphology Increase in insulin release from isolated islets Reduction of nitrite production
Silymarin	200 mg/kg (8 doses, 30 d)	Alloxan-diabetic rats	Increase in plasma insulin levels Reduction of plasma glucose levels Induction of pancreas recovery
GSPE	50 to 100 mg/kg·day (1 to 3 d)	Alloxan-diabetic rats	Prevention of hyperglycemia and hypoinsulinemia Restoration of oxidative status in the pancreas
Catechins	0.1% solution	Hamsters	Protection against N-nitrosobis(2-oxopropyl)amine-induced oxidative damage
	600 mg/kg·day (10 d)	STZ-diabetic rats	Increase in serum insulin levels Reduction of serum glucose levels Reduction of serum lipid peroxides

4.1 *Catharanthus roseus* (L.) G. Don

Catharanthus roseus (L.) G. Don belongs to the Apocynaceae family and it is known by several names such as Madagascar periwinkle, *Vinca rosea* and *Lochnera rosea*.

C. roseus, originally an endemic specie of Madagascar, is an erect herb or subshrub with either pink or white flowers that has now a wide tropical distribution. It is cultivated as an ornamental plant in gardens throughout the world (Svoboda, 1983).

Figure 13 – *Catharanthus roseus* (L.) G. Don flower.

Traditionally, hot water decoction of the leaves and/or the whole plant is used as household remedy for treatment of diabetes, as an oral hypoglycemic agent, in several countries such as Brazil, Cook Islands, Dominica, England, Jamaica, Mozambique, Pakistan, Taiwan, Thailand and West Indies (Nammi *et al.*, 2003; Ferreres *et al.*, 2008; Singh *et al.*, 2001). The fresh leaf juice of *C. roseus* has been used by Ayurvedic practices in India with beneficial action. With the study of the plant's hypoglycemic activity, the two terpenoid indole alkaloids – vinblastine and vincristine – the first anticancer agents clinically used in chemotherapy were discovered (Ferreres *et al.*, 2008; Sottomayor *et al.*, 2004). Since these alkaloids are produced at very low levels in *C. roseus* leaves a great interest directed by the investigation of their biosynthetic pathways arose. However, information about other natural compounds produced by *C. roseus* remains relatively limited and water extracts of this plant are used for purposes like controlling bleeding, diabetes, fever and rheumatism without the active compounds being known (Ferreres *et al.*, 2008). In fact, several studies using *C. roseus* leaves extracts show a blood sugar lowering capacity (Chattopadhyay, 1999; Nammi *et al.*, 2003; Singh *et al.*, 2001) but the exact mechanism by which hypoglycemic action was performed have not been elucidated.

Recently, studies regarding the phenolic composition of *C. roseus* seeds, stems, leaves and petals were performed by Ferreres and colleagues (2008) and Pereira and co-workers (2009) leading to the characterization of caffeoylquinic acids and flavonol glycosides (di- and trisaccharides of kaempferol, quercetin and isorhamnetin). Also, the scavenging ability against DPPH• radical and against reactive oxygen (superoxide radical) and reactive nitrogen (nitric oxide) species was evaluated and an antioxidant effect was observed. These results highlight the potential of *C. roseus* extracts to function as natural

antioxidants in the prevention/management of type 2 DM and lead us to further investigate their effects in the beta-cell context.

5. Objectives

The objectives of the present work are:

- Characterization of the HIT-T15 cell line (hamster pancreatic β-cell line) as an *in vitro* model to assess the anti-diabetic potential of plant extracts/compounds.

- Evaluation of anti-diabetic potential of *C. roseus* aqueous extracts as well as their major flavonoids (quercetin and kaempferol), in the β-cell context, through the assessment of parameters such as insulin secretion and insulin gene expression, β-cell protection against oxidative stress induced by dRib and other oxidant compounds, GSH levels and apoptosis.

Materials and Methods

1. Chemicals

Roswell Park Memorial Institute medium (RPMI), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), *tert*-butyl hydroperoxide (tBHP), 2-deoxy-D-ribose (dRib), N-Acetyl-L-cysteine (NAC), glucose oxidase (GOX; EC 1.1.3.4), glibenclamide (Glb), quercetin (Q), kaempferol (K; kindly supplied by Dr. Alberto Dias), trypsin, bovine serum albumin (BSA), HEPES (N-[2-hy-droxyethyl]piperazine-N'-[2-ethane-sulfonic acid]), glutathione reductase (EC 1.6.4.2.), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), buthionine sulfoximine (BSO), diazoxide and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glyoxal was purchased from Fluka (Switzerland). Fetal Bovine Serum (FBS) was obtained from Lonza (Switzerland).

2. Cell culture

HIT-T15 cells (hamster pancreatic β-cell line), obtained from ATCC Global Bioresource Center, were maintained in culture in 75 or 25 cm^2 polystyrene flasks (TPP, Switzerland) with RPMI 1640 media containing 5.6 mM glucose with 10% FBS and 1% antibiotic-antimycotic solution under an atmosphere of 5% CO_2 at 37ºC. Assays were performed in complete media except with dRib where FBS was decreased to 0.5%. Media was changed every 2-3 days and cells were split once a week with a 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution. Cell number and viability was determined by counting in a Neubauer chamber using trypan blue staining. The cells were subcultured at a density of 3.0×10^6 cells/75 cm^2 flask or 1.0×10^6 cells/25 cm^2 flask.

3. Plant material: *Catharanthus roseus*

Plants of *Catharanthus roseus* (L.) G. Don cv. Little Bright Eye were grown at 25°C in a growth chamber, under a 16 h photoperiod, using white fluorescent light at a photon flux density of 70 μmol/m2/s. Seeds were acquired from AustraHort (Australia) and voucher specimens were deposited at the Herbarium of the Department of Botany of the

Faculty of Sciences of the University of Porto (PO 61912). Stems, leaves and petals were separated, frozen and lyophilized. The dried materials were powdered and kept in a desiccator, in the dark.

3.1 Aqueous extracts of *Catharanthus roseus*

Aqueous extracts were prepared by boiling 1.5 g of dried material for 20 min in 300 mL of water and filtering through a Büchner funnel. The resulting extracts were then lyophilized. The lyophilized extracts were kept in a desiccator, in the dark (Pereira *et al*., 2009). E1 represents aqueous extract of *C. roseus* leaves, E2 refers to aqueous extract of *C. roseus* senescent leaves and E3 to aqueous extract of *C. roseus* flowers. The quantification of the phenolics, present in different parts of *C. roseus*, was performed by Pereira and colleagues (2009) and it is described in table IV. They also assessed the organic acids and amino acids profiles.

Table IV – Quantification of phenolic compounds in *C. roseus* plant parts aqueous extract (adapted from Pereira *et al.*, 2009).

Compounds	**Concentration (mg/kg dry basis)**		
	Petals	**Leaves**	**Senescent Leaves**
3-O-caffeoylquinic acid	nd	2971.6	5692,3
Kaempferol-3-O-(2,6-di-O-rhamnosyl-galactoside)-7-O-hexoside	nd	52.7	nd
4-O-caffeoylquinic acid	11153.2	5156.8	11892,3
5-O-caffeoylquinic acid	nd	187.7	742,7
Quercetin-3-O-(2,6-di-O-rhamnosyl-galactoside)	1027.9	310.9	549,1
Kaempferol-3-O-(2,6-di-O-rhamnosyl-galactoside)	8120.8	8.5	218,0
Kaempferol-3-O-(2,6-di-O-rhamnosyl-glucoside)	4296.3	nd	nd
Kaempferol-3-O-(6-O-rhamnosyl-galactoside)	9567.2	nd	nd
Kaempferol-3-O-(6-O-rhamnosyl-glucoside)	4639.8	nd	nd
Isorhamnetin-3-O-(6-O-rhamnosyl-galactoside)	989.2	nd	nd
Isorhamnetin-3-O-(6-O-rhamnosyl-glucoside)	1330.4	nd	nd
Σ	41125	8688	19094

nd – not detected.

4. MTT reduction test

A MTT reduction assay was performed in order to: i) select a range of concentrations of *C. roseus* aqueous extracts that were not cytotoxic to HIT-T15 cells; ii) determine concentrations of oxidative toxicants – dRib, glyoxal, glucose oxidase and tBHP – that induce 50% of cell damage in these cells and iii) assess protective effects by extracts, Q and K against oxidative-induced toxicity in the same cells.

The MTT assay was used as marker of cell metabolic capacity in order to estimate cell viability. It is based on the reduction of a tetrazolium salt to formazan by cellular dehydrogenases. Expansion in the number of viable cells results in an increase in the overall activity of the dehydrogenases and subsequently an increase in the amount of formazan dye formed. For that, two hours before the end of the incubation period, MTT (final concentration 0.5 mg/mL) was added to each well. Hydrogen chloride 0.04 M in isopropanol was then added to dissolve the formazan crystals. The dye produced by viable cells was quantified with a spectrophotometer by measuring the absorbance at 570 nm against a blank.

4.1 Assessment of *C. roseus* extracts toxicity

HIT-T15 cells were plated in 24-multiwell culture plates at 2.0×10^5 cells per well and grown for 2 days. Cells were then treated with different concentrations of *C. roseus* aqueous extracts for 48 h. The number of viable cells was estimated by the cell capacity to reduce MTT, and the results were expressed as percentage relative to the control (cells without test compound).

4.2 Assessment of dRib, glyoxal, GOX and tBHP tocixity

In order to determine the concentration of toxicant that induced 50% of cell death (IC_{50}) a MTT assay was performed. Cells were plated in 24-multiwell culture plates at 2.0×10^5 cells per well, grown for 2 days and incubated with different concentrations of glyoxal, glucose oxidase (GOX), 2-deoxy-D-ribose (dRib) and *tert*-butyl hydroperoxide

(tBHP) for 48, 48, 24 and 2 h, respectively. The Hill slope was calculated graphically using a computer program (GraphPad Prism, version 4.00, GraphPad Software Inc.). Based on the dose–response curves of cell viability, the IC_{50} concentrations were estimated and used in the following experiments to evaluate the protective potential of the *C. roseus* extracts as well as that of quercetin (Q) and kaempferol (K) as described below.

4.3 Protection by *C. roseus* extracts, quercetin and kaempferol against oxidative-induced toxicity

The prevention of cell death induced by the oxidative compounds was measured by MTT assay by i) co-incubations of the extracts or Q or K (Q and K dissolved in DMSO 0.5 % v/v final concentration) at several concentrations with dRib for 24 h, with glyoxal and GOX for 6 h and with tBHP for 2 h and ii) pre-incubation of the extracts or Q or K, at different concentrations for 24 h followed by a 24 h incubation with dRib, a 6 h incubation with glyoxal and GOX and a 2 h incubation with tBHP.

5. Insulin Secretion

Since HIT-T15 cells are known to lose their ability to produce insulin over time, the ability of plant extracts/isolated compounds to induce the secretion of insulin were assessed between passages 65 and 80.

HIT-T15 cells were seeded into 24-well plates at a density of $3x10^5$ cells per well and grown for 2 days. The cells were washed twice with Krebs–Ringers Bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16 mM HEPES, pH 7.4, and 0.2% bovine serum albumin), pre-incubated for 30 min in KRB buffer and washed again with the same buffer. Then, cells were incubated for 1 h at 37 ºC with KRB buffer containing 4 mM glucose in the presence or absence of the plant extracts or isolated compounds. After incubation, aliquots of the media were stored at -20°C until insulin measurement. The content of insulin in the samples was measured using an ELISA-based commercial kit – Rat Insulin ELISA Kit TMB (Shibayagi - Gunma, Japan) – following the manufacturer's specifications.

6. Quantification of total protein

Total protein was measured in cell lysates and used for normalization of other measured parameters. Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hércules, CA, USA) and bovine serum albumin was used as a protein standard.

7. Glutathione content

HIT-T15 cells were plated in 12-multiwell culture plates at 4.0×10^5 cells per well and grown for 2 days. Cells were then treated with the test compounds for 24 h. After washing with PBS, cells were scrapped in 300 µl of 5% (w/v) 5-sulfosalicylic acid for protein precipitation and centrifuged 2 min at 12,000 rpm. The glutathione content of HIT-T15 cells was determined spectrophotometrically by the DTNB-GSSG reductase recycling assay as described in Anderson (1985), with some modifications (Lima *et al.*, 2004). The final concentration of the assay reagents was 0.6 mM DTNB, 0.21 mM NADPH and 2 U/ml glutathione reductase.

8. Assessment of apoptosis by nuclear condensation assay

Cells treated with the test compounds at chosen concentrations for 48 h were collected and fixed with 4% paraformaldehyde for 15 min at room temperature and attached to a polylysine treated slide using a Shandon Cytospin. Cells were then washed in PBS and incubated with Hoechst for nuclei staining. Stained cells were observed under the fluorescent microscope and the percentage of cells with condensated DNA were obtained from a counting of at least 400 cells.

9. Western blotting

After treatment with the chosen concentration of test compounds, cells were washed with PBS and lysed for 15 min at 4ºC with ice cold RIPA buffer (1 % NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.2), 2 mM EDTA), supplemented with 20 mM NaF, 1 mM

phenylmethylsulfonyl fluoride (PMSF), 20 mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Twenty five micrograms of total protein from each cell lysate were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05 % Tween-20) containing 5 % (w/v) non-fat dry milk , washed in TPBS and then incubated with primary antibody. 3After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). β-actin was used as loading control.

10. Quantitative Real-Time PCR

Total RNA was extracted from the cells using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions, and total RNA was reverse transcribed with the iScript cDNA synthesis kit (BioRad) using a My Cycler^{TM} Thermal Cycler (BioRad); the thermal profile consisted of 5 min at 25ºC, 60 min at 42ºC and 5 min at 85ºC. Real-time PCR for quantification of hamster insulin and acidic ribosomal phosphoprotein P0 (36B4), as housekeeping gene, was carried out with SsoFast EvaGreen supermix (BioRad) in the CFX96 Real-Time system (BioRad). The thermal profile consisted of 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles of 30 sec at 95ºC, 30 sec at 68ºC, and 30 sec at 72ºC. Primers sequences, according to Kawamura and colleagues (2006), used for the amplification, were:

hamster insulin forward primer – AGAAGCCATCAGCAAGCAGG;
hamster insulin reverse primer – AGAGTGCCTCCACAAGGTGG;
36B4 forward primer – CAATGGCAGCATTTACAACCC;
36B4 reverse primer – CCCATTGATAATGGAGTGTGG.

11. Statistical analysis

Data are expressed as means ± SEM. Significant differences between control and treated cells were determined by Student's *t*-test. Differences were considered significant when $P \leq 0.05$.

Results and Discussion

1. Evaluation of *Catharanthus roseus* aqueous extracts cytotoxicity

Regarding the objectives of this work, the assessment of HIT-T15 cell line susceptibility to the *C. roseus* aqueous extracts had to be done firstly, in order to choose a range of concentrations that did not induce cell death for the following experimental work. For that purpose, the effects of extracts on cell viability and growth was measured by the MTT assay, which determines the metabolically active cells. In figure 14 is represented the cell viability as percentage of the control in the presence of increasing concentrations of the *C. roseus* extracts.

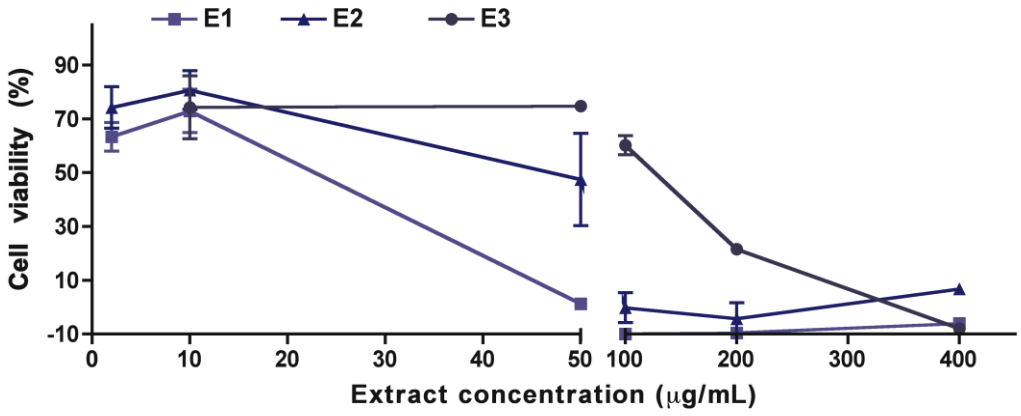

Figure 14 – Effect of different concentrations of *C. roseus* aqueous extracts in HIT-T15 cells, for 48 h, measured by MTT reduction assay. E1: leaves aqueous extract; E2: senescent leaves aqueous extract; E3: flower aqueous extract. Values are mean±SEM of 3 independent experiments. Cell viability was calculated as percentage of the control (without extracts).

Since the assay was done after 48 h of treatment, cells grow in between, and therefore the MTT reduction capacity in the beginning of the assay was subtracted from all the results after 48 h of treatment (including the control). In this way, we can distinguish between inhibition of cell growth (values between 0 and 100%) and cell death (values above 0%). As shown in Fig. 14, cell toxicity was induced by higher concentrations than 50 ug/ml for E1, 100 ug/ml for E2 and 400 ug/ml for E3. The differential effect of the three extracts is well evidenced at 50 ug/ml being the potential to inhibit cell growth ordered as E1>E2>E3. The IC_{50} for cell growth inhibition of the extracts was 22 µg/mL, 45

μg/mL and 125 μg/mL for E1, E2 and E3, respectively. This behavior could be explained by differences in extracts phytochemical profile, described in table IV. The characterization of *C. roseus* extracts and quantification of phenolic compounds, organic acids and amino acids was performed by Pereira and co-workers (2009), pointing out the differences between distinct plant parts, namely, flowers, leaves and senescent leaves. The phenolic profile of the extracts demonstrated their richness in these compounds, namely in caffeoylquinic acids and flavonoid glycosides. Interestingly, comparing the three extracts, the E3 flower less cytotoxic extract present the higher content of phenolic compounds (in addition to a great variety), whereas E2 leaves extract were more cytotoxic and presented the lower content and variety of phenolic compounds. It seems, therefore, there are an indirect relationship between extract phenolic content and its induced toxicity (or inhibition of proliferation) in HIT-T15, indicating that they may not be involved. Other compounds, besides phenolics, organic acids and amino acids, present in the extracts may account for the toxic effects since more than 80% of the crude extracts were not characterized.

2. Assessment of glucose-induced insulin secretion on HIT-T15 cells

A diabetic condition occurs when there is an absolute or relative lack in insulin production by the endocrine pancreas in response to glucose associated or not with a deficient response of target tissue cells to insulin (Robertson, 2004; Klover and Mooney, 2004). This impaired glucose-stimulated insulin secretion is the cause of hyperglycemia, a characteristic of type 2 diabetes patients, that leads to secondary complications (Robertson, 2004; Robertson and Harmon, 2006; Robertson, 2009). In this way, when a study of anti-diabetic potential of new compounds is performed in the beta-cell context, the parameter of glucose-stimulated insulin secretion appears to be one of the most important to be evaluated.

The HIT-T15 cell line is a clonal line of pancreatic β-cells that secretes insulin in response to a glucose stimulus. However, this cell line loses this ability in a passage-dependent manner, a effect that is accelerated culturing the cells in medium with a high concentration of glucose (Santerre *et al.*, 1981; Zhang *et al.*, 1989). For this reason all the experiments were performed between passages 65 and 80, as recommended by Zhang and

co-workers (1989) for glucose-induced insulin secretion assays and cells cultured in medium with relatively low concentration of glucose (5.6 mM).

Figure 15 represents the insulin secretion in response to different glucose concentrations. We can observe a marked increase in insulin secretion at 0.5 mM of glucose, which stabilizes between 1 and 4 mM and tends to slightly increase again for glucose concentrations above 4 mM.

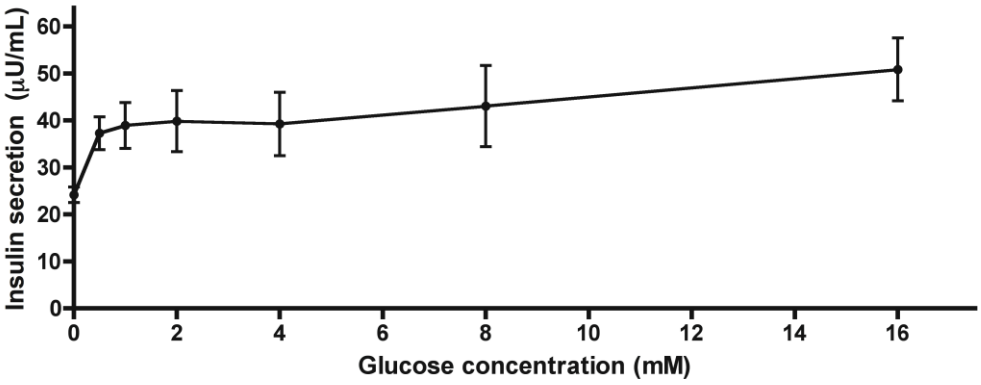

Figure 15 – Insulin secretion in response to glucose in HIT-T15 cells after 1 h incubation with the designated glucose concentration. Values are mean±SEM of 3 independent experiments.

This pattern of response was consistent with that reported by Zhang and co-workers (1989) when testing the same range of glucose concentrations, despite that they achieved higher values of insulin concentration for all the tested concentrations of glucose. The higher insulin concentration achieved by them was possibly because they use the double of the cells we used here. In addition, another reason may be to the fact they grow cells in medium containing 11.1 mM of glucose, once they wanted to reproduce a hyperglycemic condition, which may increase insulin secretion at first, but will decrease it faster along cell passage as we discussed previously. In our case, the preventive effects of *C. roseus* on beta cells directed our work, supporting once more culturing the cells in normoglycemic conditions (5.6 mM) as we did. This preliminary assay allowed us to choose 4 mM glucose as stimulatory concentration to proceed testing the stimulatory potential of *C. roseus* aqueous extracts, Q and K on glucose-induced insulin secretion. Park and co-workers (2008) and Yoon and collaborators (2007) also used a similar glucose concentration (5 mM) to assess the insulin-stimulating potential of natural compounds in the HIT-T15 cell line. From the first work resulted a response in terms of insulin secretion slightly higher

(≈100 μU/mL) than that obtained by us (≈ 40 μU/mL) and from the former a similar insulin concentration (≈ 30 μU/mL) was achieved.

2.1 Effects of *Catharanthus roseus* aqueous extracts, Q and K on glucose-induced insulin secretion

The pathogenesis of type 2 diabetes is associated with defective sensing of glucose stimulus by the β cells (Robertson and Harmon, 2006). Although lifestyle modifications as diet, physical exercise, weight loss and stop smoking should be present in both diabetes prevention and therapy, most of the times pharmacotherapy is also needed in order to achieve a better glucose regulation, and in some cases exogenous insulin administration is needed (Triplitt, 2007; Robertson, 2009). Sulfonylureas, like glibenclamide (glb), a group of drugs that target the pancreatic β cells, increase insulin secretion by binding to sulfonylurea receptor on β-cell, leading to closure of ATP-sensitive K^+-channels. This action causes membrane depolarization and opening the voltage-dependent Ca^{2+} channels leading to a calcium influx and exocytosis of insulin contained in vesicles (Cheng & Fantus, 2005).

In this work, glibenclamide 10 nM was used as positive control (fig. 16 and 17) and its known stimulatory effect on insulin secretion was observed. Glb significantly increased insulin secretion in HIT-T15 cells from 101.2 μU/mL to 143.8 μU/mL (42% increase), a similar result as previously described by Leu *et al.* (2009) with a 140% increase and as occurred in human β cells (Guerra *et al.*, 2009) with an increase from 24.3 μU/mL to 41.2 μU/mL, in the presence of 1 μM glb.

Regarding the effects of *C. roseus* (fig. 16) as insulin secretagogue, any of the aqueous extracts increased insulin secretion at the tested concentrations. We did not test higher concentrations since they affect cell growth and viability as shown in Fig. 14 and Fig. 19 (see in section 3.1 below). These results indicate that the anti-diabetic potential of *C. roseus* aqueous extracts may not be at the beta-cells increasing insulin secretion.

The screening of phenolic compounds present in these extracts was performed by Ferreres and co-workers (2008) and Pereira and colleagues (2009), which allowed the identification of three caffeoylquinic acids and flavonol glycosides (di- and trisaccharides of kaempferol, quercetin and isorhamnetin). Once the acquisition of these glycosides was not possible and their purification is difficult to achieve, the aglycones quercetin and kaempferol were tested in this study for their ability to affect insulin secretion.

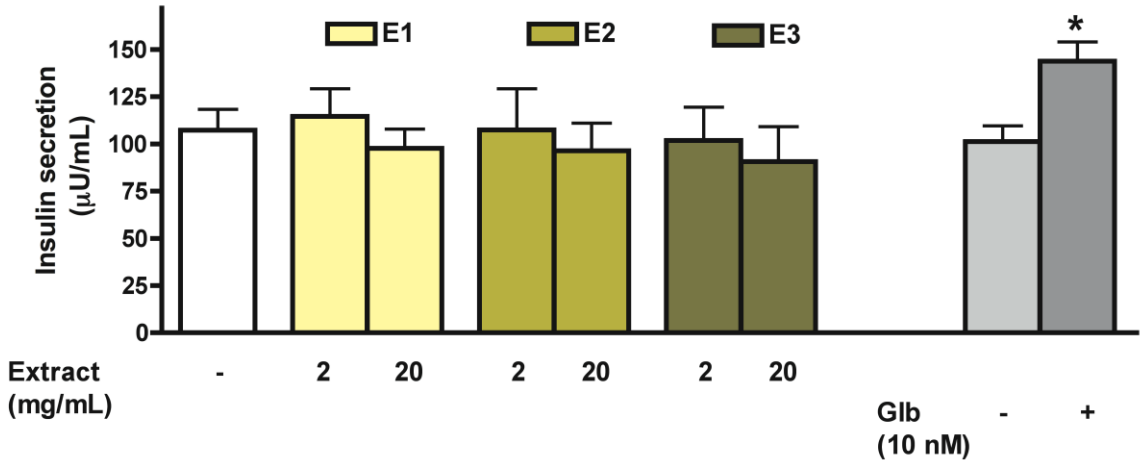

Figure 16 – Insulin secretion in response to *C. roseus* aqueous extracts and glibenclamide in HIT-T15 cells in the presence of 4 mM glucose after 1 h incubation. E1: leaves aqueous extract; E2: senescent leaves aqueous extract; E3: flowers aqueous extract. Values are mean±SEM of 4 independent experiments. *$P \leq 0.05$ when compared to control.

Several authors support the view that most flavonoids must be modified, namely deglycosilated, in order to be absorbed in the form of aglycones; intestinal enzymes or colonic microflora in the intestine makes these modifications (Crespy *et al.*, 2003; Pinent *et al.*, 2008; Williamson *et al.*, 2000). Under these conditions, the aglycone form of parent compounds would be one of the forms that internal organs, such as the endocrine pancreas (β-cells in particular), would become exposed to, after ingestion of plant foods or extracts (such as herbal teas).

Q and K (the aglycone flavonoids present in *C. roseus*) at 20 µM exhibited a remarkable effect potentiating insulin secretion (fig. 17) above those achieved by glb. The effect was concentration-dependent, being the result similar to that of gbl when flavonoids were tested at 5 µM. The insulin release was not due to cell death as confirmed by trypan blue assay (data not shown) and by MTT assay (see fig. 20 in section 3.1 below).

The insulin secretagogue effect of Q was already reported in literature. Hii and Howell (1985) reported that exposure of isolated rat islets at Q enhances insulin release by 44-70%. Also a study of Coskun and colleagues (2005) showed that Q-treated diabetic rats has increased insulin immunohistochemical staining as well as preservation of islet cells. Regarding K results, this compound had been associated to an increase in insulin staining in islets of STZ-induced diabetic rats only due to its presence in the extract of *Eucommia ulmoides* Oliv. (Lee *et al.*, 2005). However, it seems there are no reports of a marked increased in insulin secretion as the observed in our present study.

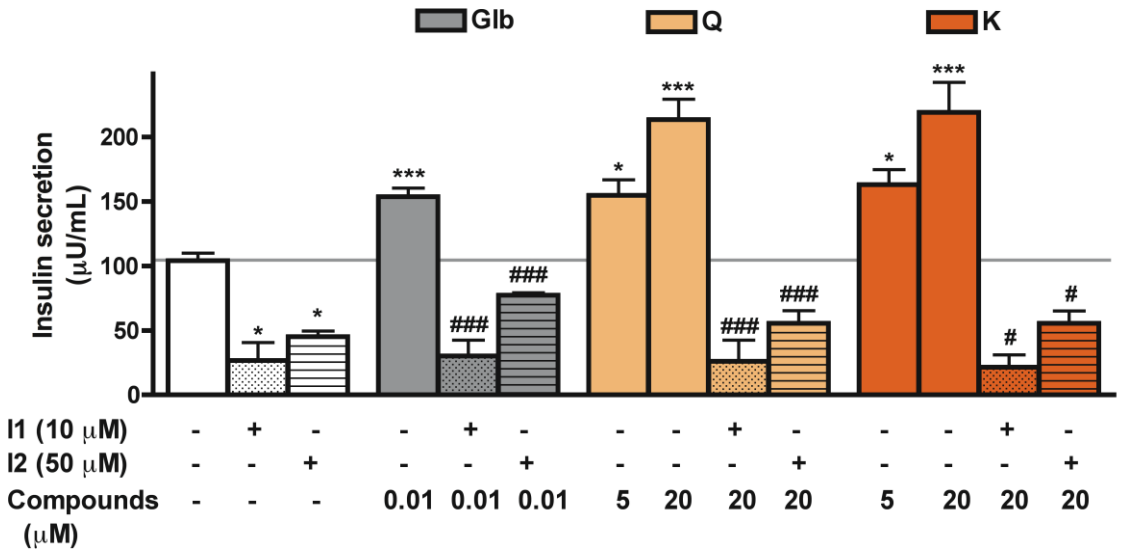

Figure 17 – Insulin secretion in response to quercetin, kaempferol and glibenclamide in HIT-T15 cells, stimulated by 4 mM glucose, in the presence or absence of Ca^{2+} channel blocker (I1-nifedipine) and of K^{+} channel activator (I2-diazoxide) after 1 h incubation. Values are mean±SEM of 4 independent experiments. **P*≤0.05; ****P*≤0.001 when compared to control; #*P*≤0.05; ### *P*≤0.001 when compared to the respective compound.

Next, to examine by what mechanism Q and K enhance the glucose-stimulated insulin secretion, nifedipine (I1) that opens K^{+} channels and diazoxide (I2), a L-type of Ca^{2+} channel blocker were used. These inhibitors allowed us to understand if the Q and K mechanism of action relied on insulin degranulation process, that is, a sulfonylurea-like mechanism. In this way, in the presence of either I1 or I2 it is expected that the whole process of membrane depolarization and Ca^{2+} influx would be compromised, consequently leading to insulin secretion cessation. Figure 4 demonstrates a significant decrease in insulin secretion compared to control when I1 or I2 were present. The same inhibitory effect was observed in the presence of glb, Q or K; in other words, the insulin stimulatory effect of these compounds was significantly reverted by I1 and I2. Taken together, these results indicate that both Q and K may exert their action in beta cells through the same mechanism that glb, targeting the insulin exocytosis process. The use of I1 and I2 also corroborates that insulin secretion induced Q and K was not due to cell death or loss of membrane integrity.

Hii and Howell (1985) also associate the performance of certain flavonoids (quercetin being one of them) in beta cells to their ability, at least in part, to alter Ca^{2+} fluxes. Yoon and colleagues (2007), as well as Park and co-workers (2008), also reported the same pattern of response by HIT-T15 cells to nifedipine and diazoxide, indicating an

association between the insulin secreting potential of their tested compounds (ginsenosides) and the insulin exocytosis.

3. Effects of dRib-induced damage in HIT-T15 cells

Hyperglycemia is a hallmark of diabetes mellitus and may lead β-cell failure. This state of chronic exposure to supraphysiologic levels of glucose cause irreversible β-cell damage – glucose toxicity – and oxidative stress is known as the main mechanism underlying it (Kho *et al.,* 2005; Tanaka *et al.,* 2002).

Sugars that contain aldehyde groups that are oxidized to carboxylic acids are reducing sugars and they can produce reactive oxygen species (ROS) through autoxidation and protein glycosylation (Kaneto *et al.,* 1999; Kho *et al.,* 2005). Glucose is the sugar with lower reducing capacity, therefore, long-term exposure to high glucose concentrations is needed in order to exert toxic effects in β-cells (Kho *et al.,* 2005). However, it has been reported that 2-deoxy-D-ribose (dRib), a reducing sugar with high reactivity with proteins, promotes apoptosis by increasing oxidative stress within 24 h in a HIT-T15 cell line, being a good *in vitro* surrogate for glucose toxicity and diabetic complications studies (Kho *et al.,* 2005; Kho and Woo, 2008). Therefore, we choose dRib to induce oxidative damage in cells. It is known that, as glucose, dRib toxicity causes decreased insulin mRNA levels, diminished β-cell insulin content and defective glucose-induced insulin secretion (Tanaka *et al.,* 2005).

A preliminary assay was performed in order to assess the effects of different dRib concentrations in HIT-T15 viability, through a MTT assay (figure 18).

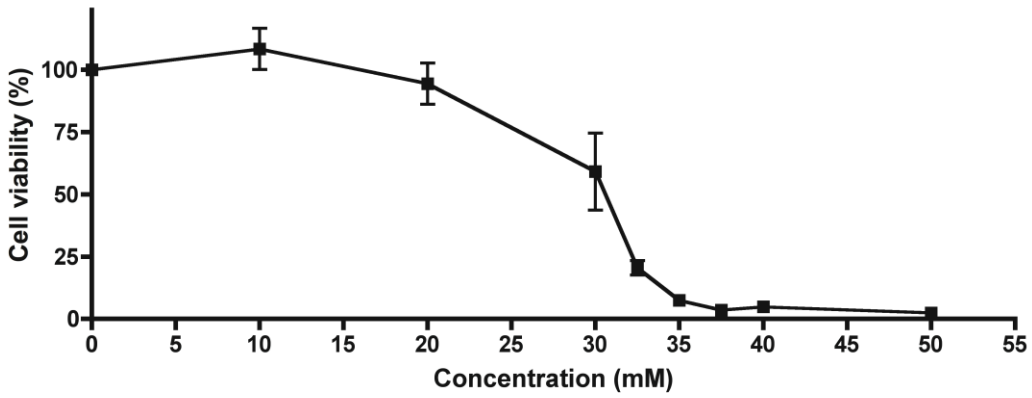

Figure 18 – Viability of HIT-T15 cells after treatment with various concentrations of dRib for 24 h, by a MTT assay. Cells were cultured in RPMI medium containing 5.6 mM glucose and 0.5% FBS.

This pattern of response was consistent with that reported by Kho *et al*. (2005), which demonstrates that 24 h incubation with 30 mM dRib decreases approximately 50% cell viability. So, this concentration was chosen to proceed testing its effects on other parameters such as insulin secretion, GSH levels, apoptosis, insulin mRNA levels, as well as Q and K protection against dRib-induced β-cell toxicity.

3.1 Effects of extracts, Q, K and NAC on prevention of dRib toxicity to β-cells

The effects of dRib-induced toxicity in HIT-T15 were measured by the MTT assay. The protective effects of *C. roseus* aqueous extracts (fig. 19), Q and K (fig. 20), as well as NAC (a reference antioxidant; fig. 20), on the prevention of this type of toxicity were also evaluated.

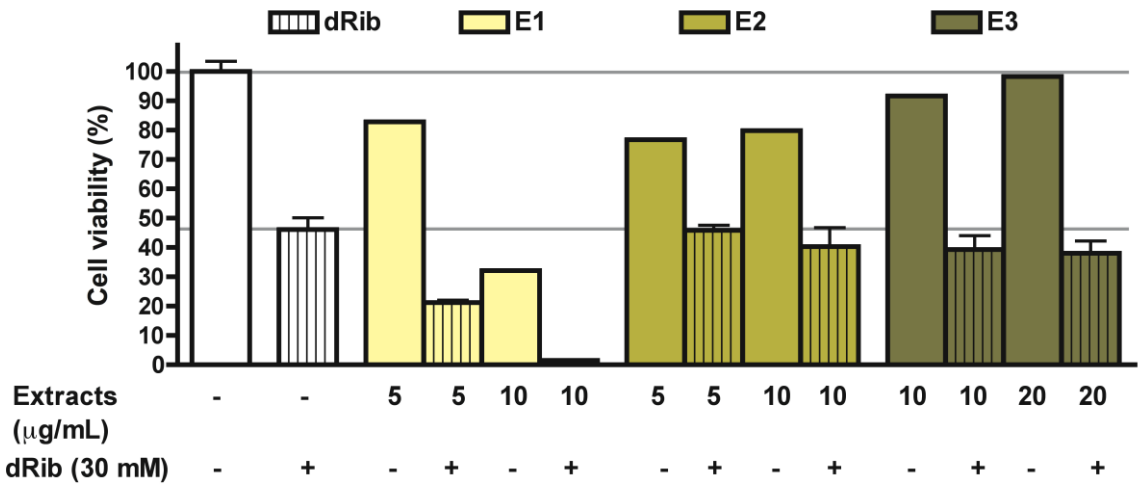

Figure 19 – Effect of *C. roseus* aqueous extracts in dRib-induced toxicity (30 mM) in HIT-T15 cells, after 24 h exposure. E1: leaves aqueous extract; E2: senescent leaves aqueous extract; E3: flowers aqueous extract. Values are mean±SEM of 2 independent experiments.

The toxicity induced by dRib 30 mM affected HIT-T15 cells inducing approximately 50% of cell death. However, co-incubation of dRib with the three *C. roseus* extracts did not prevent toxicity. In fact, the extracts alone, with the exception of E3, induced some decrease of reduction of MTT, most probably due to inhibition of cell growth during the 24 h of treatment (see Fig. 14). Once again it seems that the reported hypoglycaemic potential of *C. roseus* (Chattopadhyay, 1999; Li *et al.*, 2004; Singh *et al.*, 2001) is not targeted for β-cell protection against oxidative stress, or at least not in this tested concentrations and extracts.

In figure 20, the toxic effects of dRib is again observed, being reverted by NAC 200 μM. This protective effect of NAC against glucose and dRib-induced toxicity was already described by Tanaka and colleagues (2002) but using NAC 10 mM, a considerably higher concentration that the one used in the present study. They had reported that both glucose and dRib increased intracellular peroxide levels in human and rat pancreatic islets and NAC, as a potent antioxidant, reverted that situation. In this work, Q and K also exhibited some protective effect against dRib-induced damage, in a concentration-dependent manner, except for K (40 μM). The possible cytoxicity of the compounds alone was also assessed and shown to be not present in the tested concentrations (Fig. 20).

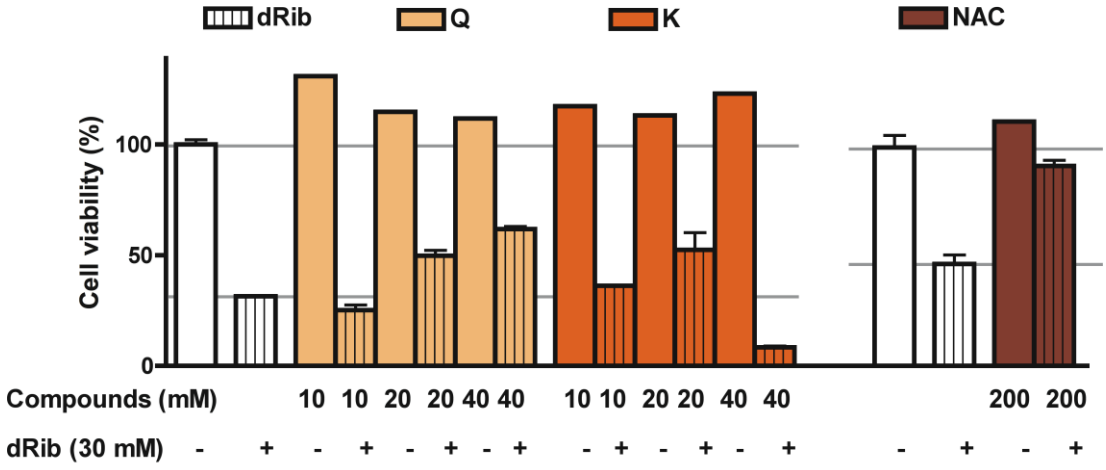

Figure 20 – Effects of Q and K, as well as the antioxidant NAC, in dRib-induced toxicity (30 mM) in HIT-T15 cells, after 24 h exposure. Values are mean±SEM of 2 independent experiments.

However, more replicates will be necessary to evaluate Q and K potential in protecting beta-cells in order to get statistically significance and to ensure their efficacy as a preventive or adjuvant to type 2 diabetes mellitus.

3.2 Effect of dRib-induced damage in insulin secretion

The effects of dRib and tBHP, another oxidant compound, in the insulin release by HIT-T15 and the protective effect of Q and K on dRib-induced toxicity can be observed in figure 21. Insulin secretion was not significantly decreased by dRib 30 mM, contrarily to what was expected. Tanaka and colleagues (2002) observed a significant decrease but used a longer incubation period with dRib, which may explain the lack of effect by us. In our assay dRib was incubated for 1 h, with and without Q or K, whereas Tanaka and co-

workers performed a 72 h preincubation before measuring insulin release. Despite the weak dRib-induced

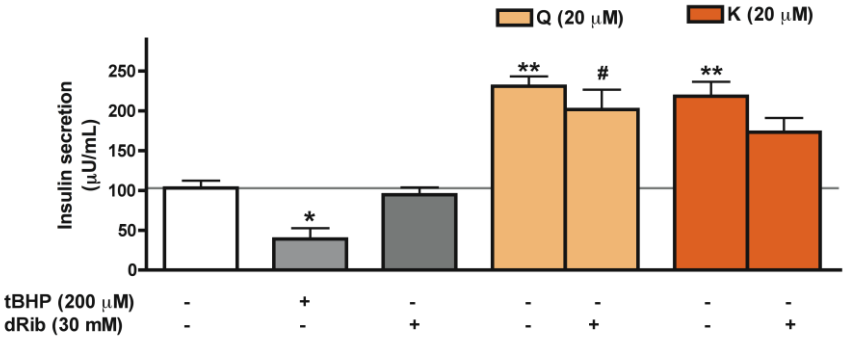

Figure 21 - Insulin secretion in response to the oxidants tBHP and dRib in HIT-T15 cells, stimulated by 4 mM glucose, in the presence or absence of quercetin and kaempferol, after 1 h incubation. Values are mean±SEM of 3 independent experiments. *$P \leq 0.05$; **$P \leq 0.01$ when compared to control; #$P \leq 0.05$ when compared to dRib alone.

toxicity, when this compound was co-incubated with Q or K an increase in insulin secretion continued to occur when compared to the levels of dRib alone. This increase was statistically significant for Q ($P \leq 0.05$) but not for K. The insulin secretion attained was, however, below than the one obtained by incubating with the flavonoids alone, which may indicate some oxidant effect of dRib in these conditions. Incubation conditions similar to the ones used by Tanaka *et al.* (2002) may be necessary to test whether Q and K are in fact protective against glucotoxic-induced decrease in insulin secretion. That may be probable in view of the antioxidant potential already reported for Q and K (Lima *et al.*, 2006; Wang *et al.*, 2006). Tanaka *et al.* (2002) also reported that NAC, a potent antioxidant, could revert dRib-induced toxicity in insulin secretion to control levels.

On the other hand, 1 h incubation with tBHP 200 μM, also an oxidant compound, was enough to significantly decrease the insulin secretion in HIT-T15. Åkesson and Lundquist (1999) had demonstrated that glucose-induced insulin release was rapidly and markedly suppressed by 300 and 3000 μM of tBHP. They had addressed this fact to either the inhibition of glucose-induced influx of extracellular Ca^{2+} and to tBHP oxidative action in thiol groups situated in the membrane and intracellulary that suppresses totally the glucose-induced insulin secretion. The last hypothesis was supported by the observation that tBHP decreased the GSH/GSSG ratio in rat pancreatic islets, especially since GSH is assumed to hold the thiol groups "critical" for glucose-stimulated insulin release in the reduced state (Ammon *et al.*, 1977; Ammon *et al.*, 1985).

Contrarily to dRib, tBHP worked decreasing significantly glucose-induced insulin release in our testing conditions maybe because it induces oxidant effects in the cells much more rapidly than dRib.

3.3 Effect of dRib-induced damage on GSH levels

Chronic hyperglycemia lead to the formation of ROS that, when accumulated in excess for prolonged periods of time, cause chronic oxidative stress. This process is particularly relevant for the beta cells due to its characteristic low levels of antioxidant defenses (Tanaka *et al.*, 2002; Robertson *et al.*, 2007).

The importance of GSH in the insulin secretion process described above conjugated with its importance in the cellular system of antioxidant defenses led us to further investigate the effect of dRib in the GSH levels of HIT-T15 cells as well as the influence of Q, K and NAC (figure 22).

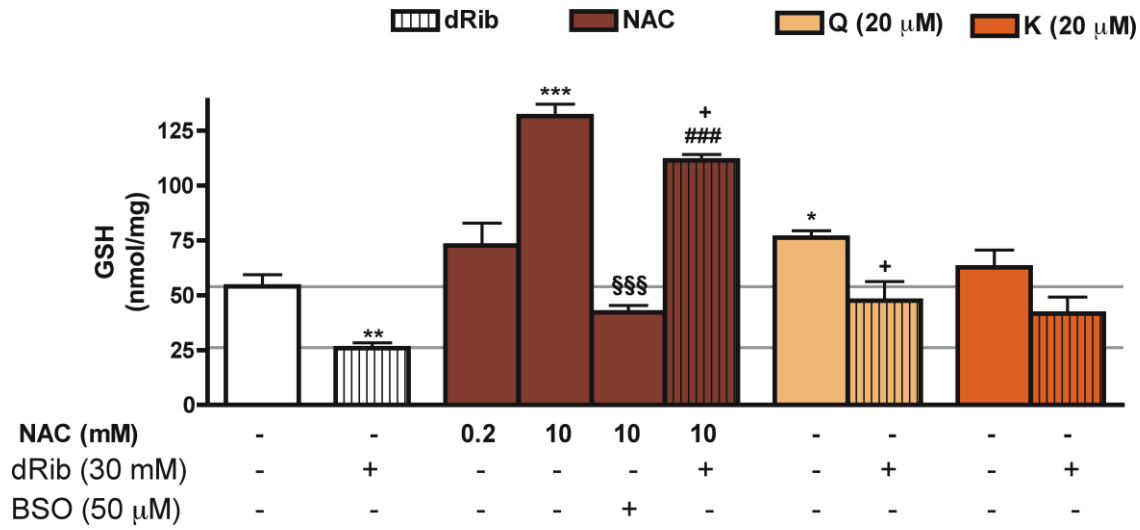

Figure 22 – Effect of quercetin, kaempferol as well as the antioxidant NAC in the decreased levels of reduced glutathione (GSH) induced by 30 mM dRib, in HIT-T15 cells, after 24 h exposure. BSO, the inhibitor of GSH synthesis, was used with NAC. Values are mean±SEM of 3 independent experiments. **P*≤0.05; ***P*≤0.01; ****P*≤0.001 when compared to negative control; ### *P*≤0.001 when compared to dRib; +*P*≤0.05 when compared with respective compound; §§§ *P*≤0.001 when compared to 10 mM NAC.

The GSH levels were significantly decreased (*P*≤0.01) in HIT-T15 cells incubated with 30 mM dRib for 24 h, when compared to control, a situation that was rescued by NAC (*P*≤0.001), Q (*P*≤0.05) and K. As mentioned before, the prevention of glucose and ribose toxicity by NAC in this cell line had been reported by Tanaka and colleagues (1999)

and this protection could be due both to its role as a stable precursor of cysteine, which can then enter in the glutathione synthesis cycle, and to its glutathione-sparing effect due to its own antioxidant properties (Rahimi *et al.,* 2005).

In fact, in the presence of BSO, an inhibitor of γ-glutamylcysteine synthetase (GCS) and therefore an inhibitor of GSH synthesis, the increase of GSH levels induced by NAC was totally abolished. As antioxidants, Q and K, also exhibited a protective effect in the presence of dRib. It has been well established that the exposure to dRib increase ROS levels during the first step of protein glycation while significantly lowering the GSH content, resulting in highly oxidative condition which leads to cell death (Fico *et al.,* 2008). A study performed in embryonic stem cells (Fico *et al.,* 2008) also elucidated the mechanism involved in dRib induced GSH depletion. The authors concluded that both inhibition of GSH synthesis, through inhibition of GCS, and an increase in GSH efflux were involved.

Besides the protection against dRib-induced toxicity, all the compounds alone increased the GSH levels, indicating the potential of Q and K for maintaining GSH homeostasis and prevention of oxidative stress, particularly beneficial in a diabetic condition. The increase of GSH levels by flavonoids, particularly in the case of Q, may also help to explain the protection afforded against dRib-induced toxicity (Fig. 20) in addition to flavonoids direct antioxidant activity.

3.4 Effects of dRib on apoptosis

A decrease in the number of functional insulin-producing beta cells contributes to the pathophysiology of type 2 diabetes (Donath *et al.,* 2005). Regulation of the beta- cell mass appears to involve a balance between beta-cell replication and apoptosis (Butler *et al.,* 2003). In general, the apoptotic cascade is triggered by various kinds of stimuli such as DNA damage, cell cycle perturbation, metabolic imbalance, cytokines as well as oxidative stress. In a diabetic condition, hyperglycemia induces an increased production of ROS from enhanced glycation that leads to necrosis and/or apoptosis of beta-cells (Yoon *et al.,* 2004).

The evaluation of beta-cell apoptosis induced by a range of concentrations of dRib and its possible protection by NAC or K was performed through the evaluation of two parameters: i) genomic DNA condensation and ii) expression of apoptosis markers. In order to assess nuclear condensation, cells were exposed at 20, 30 and 40 mM of dRib, for 24 or 48 hours, and dRib 30 mM was co-incubated with NAC 5mM and K 20 μM. The

observations showed no differences in DNA condensation between the tested dRib concentrations (with or without test compounds) in comparison to control (data not shown). The differences denoted were related to cell density, corroborating the above effects of dRib in cell viability and/or proliferation. There was a marked diminution in cell density as dRib concentration increases while NAC and K restored the density to control levels (data not shown). The absence of chromatin condensation despite the effect on cell density were not concordant with another previous study, where the pro-apoptotic effects of 24 h exposure with 30 mM dRib in HIT-T15 cells and its protection by 10 mM of NAC are documented (Koh *et al.,* 2005). As in this study, we also decrease serum concentration in the medium to 0.5% predispose cells for apoptosis; therefore, maybe other factors in the milieu of the cells were protecting cells from apoptosis. A different study using this cell line had demonstrated also induction of chromatin condensation and apoptotic bodies in the presence of hydrogen peroxide (Yoon *et al.,* 2004).

Corroborating our results of chromatin condensation, the expression of apoptosis markers, evaluated by western blotting, in particular the cleavage of PARP and caspase-9, as well as p53 and Bcl-2 expression levels, were not changed by dRib (data not shown). Nevertheless, more experiments have to be performed in order either to confirm these results and/or optimize the assay conditions.

3.5 Effects of dRib in insulin mRNA levels

The toxic effects of long-term exposure of HIT-T15 cells in media containing high glucose concentrations have been described (Harmon *et al.,* 2005; Moran *et al*., 1997; Olsen *et al.,* 1993; Robertson *et al.,* 1992; Robertson *et al.,* 2007) and are associated with reduction of insulin gene expression, content and secretion due to loss of binding of transcription factors – PDX-1 and RIPE-3b1 activator – to the promoter region of the insulin gene.

In the present study, we proposed to evaluate the effects of Q and K on dRib-induced disruption of insulin gene expression. To accomplish this objective a preliminary assay was preformed on determine the differences in insulin mRNA expression, in the presence or abence of dRib 30 mM, over time. To do so, total RNA was extracted from cells and quantified. Total RNA yielding ranged from 23.47 ng/µL to 196.9 ng/µL with a good level of purity ($A_{260}/A_{280}\approx2.05$). Then, RNA was reverse transcribed and the Real-Time PCR analysis performed (fig. 23) using the acidic ribosomal phosphoprotein P0 (36B4) as

housekeeping gene, because of its highly conserved regions that make it an effective standard for use in gene expression analysis (Akamine *et al*., 2007).

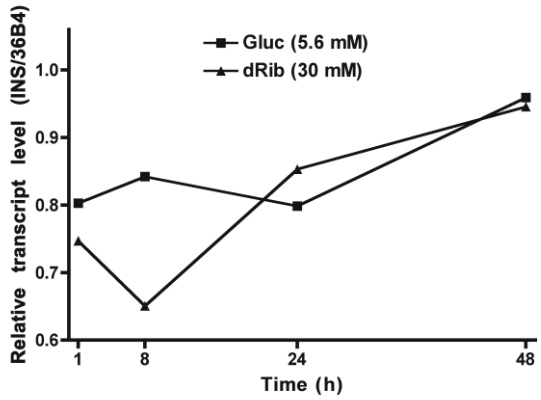

Figure 23 – Real-Time PCR quantification of insulin mRNA contents in HIT-T15 cells treated with glucose 5.6 mM with or without dRib (30 mM) for 1, 8, 24 and 48 hours.

The obtained results of this preliminary experiment did not allow to take any definitive conclusions. Due to time restrictions, the optimization of culture conditions as well as the experiment replication was not possible. Nonetheless, we would expect a decrease in insulin gene expression in the presence of dRib over time (Tanaka *et al.,* 2002). In our case, we observe a continuous decrease of insulin gene expression up to 8 h of incubation with dRib, apparently restoring control levels after 24 and 48 h of incubation. It is possible that, after an initial effect of dRib on insulin gene expression, cells were able to recover after 24h of treatment and return to initial levels of insulin transcripts.

In this way, work is needed to analyze Q and K effects in the insulin gene expression, being for this a point of awareness for future work in view of the interesting results here reported.

4. Susceptibility of HIT-T15 cells to other oxidant toxicants

As previously described, oxidative stress is the mechanism underlying glucose toxicity in beta cells and consequent impairment of insulin secretion and beta cell's apoptosis. Considering that, HIT-T15 susceptibility to other oxidant toxicants, namely glyoxal, glucose oxidase and *tert*-butyl hydroperoxide, was evaluated. Additionally, the protective effects of *C. roseus* aqueous extracts, Q and K, against the effects of these toxicants were also assessed in terms of co- and pre-incubation experiments.

4.1 Glyoxal and Glucose oxidase (GOX)

Glyoxal is a reactive α-oxoaldehyde and a physiologic metabolite, formed by lipid peroxidation, ascorbate autoxidation, oxidative degradation of glucose, and degradation of glycated proteins (Mlakar *et al.*, 1996; Wells-Knecht *et al.*, 1995). Glucose forms reversible early glycation products with proteins, that undergo further rearrangement and oxidation, originating advanced glycation end-products (AGEs), which alter protein structure irreversibly and, consequentially, their function (Han *et al.*, 2007; Shangari and O`Brien, 2004). In fact, aldehydes like glyoxal, are 20,000 times more reactive than glucose in glycation processes (Thornalley *et al.*, 2005; Mostafa *et al.*, 2007) by binding with free sulphydryl and amino groups of proteins (Han *et al.*, 2007). Under physiological states, glyoxal detoxification is made by GSH. A decrease in cellular GSH concentration during oxidative stress subsequently increases intracellular levels of glyoxal and AGE formation, potentially leading to apoptosis, necrosis or cell growth arrest (Shangari and O`Brien, 2004).

HIT-T15 susceptibility to glyoxal-induced toxicity was, in the present study, evaluated by the MTT assay (fig. 24A and 24C). In the presence of glyoxal, an abrupt decrease in cell viability was observed for concentrations between 1 and 5 mM. The calculated IC_{50} was 1.94 mM for 48 h incubation. Next, glyoxal 5 mM was tested in different incubation times (fig. 24C) and the calculated IC_{50} was 4.9 h. This allowed us to chose the 5 mM concentration for 6 h as the condition to perform the protection assays.

Glucose oxidase (GOX) is a flavoenzyme that in presence of glucose generates hydrogen peroxide (H_2O_2) – a central ROS molecule that can act over a wide range of targets while easily penetrating cell membranes, working also as a signaling molecule (Rost *et al.*, 2007). A range of GOX concentrations was tested in HIT-T15 cells (fig. 11B) and a rapid decrease in cell viability occurred between 5 and 10 mU/mL. The estimated IC_{50} was 6.5 mU/mL for a 48 h exposure. Using 10 mU/mL of GOX at different incubation times (fig. 24C) an IC_{50} of 5.8 h was obtained. The subsequent protection assays against GOX-induced toxicity was carried out using 10 mU/mL for 6 hours.

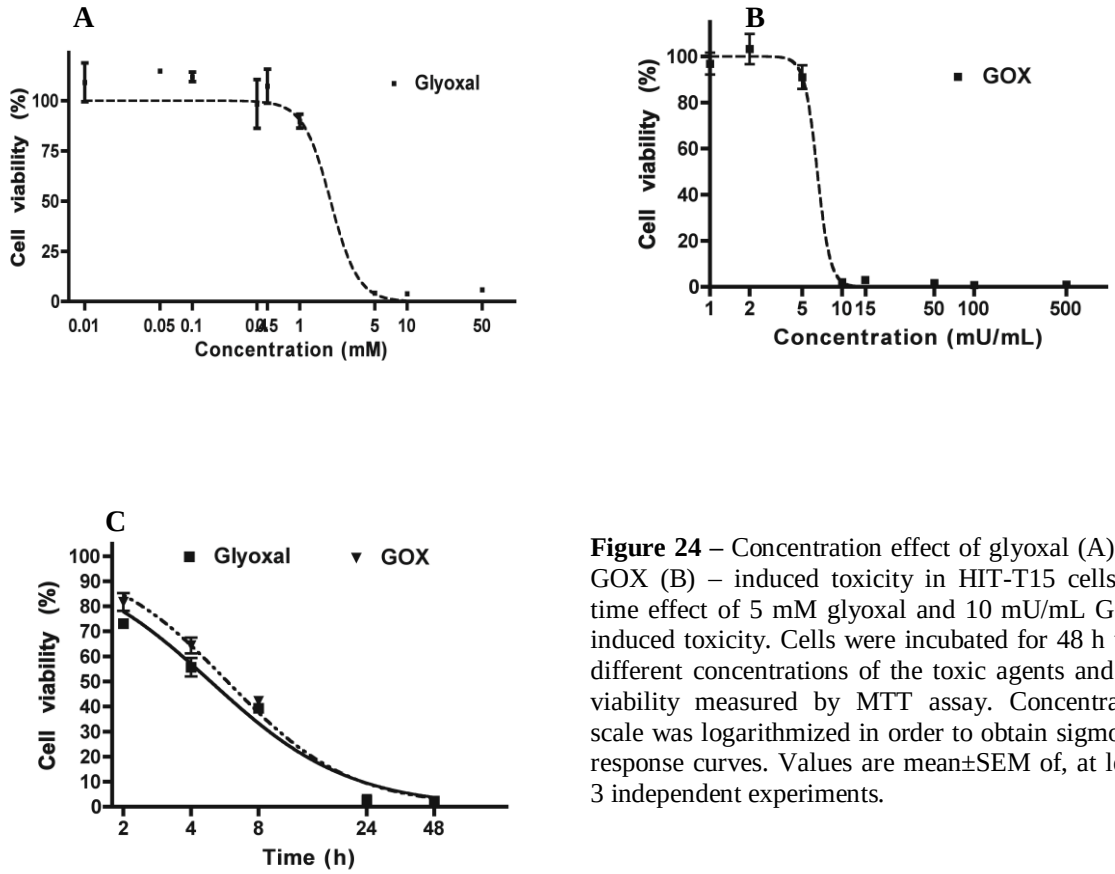

Figure 24 – Concentration effect of glyoxal (A) and GOX (B) – induced toxicity in HIT-T15 cells; C: time effect of 5 mM glyoxal and 10 mU/mL GOX-induced toxicity. Cells were incubated for 48 h with different concentrations of the toxic agents and cell viability measured by MTT assay. Concentration scale was logarithmized in order to obtain sigmoidal response curves. Values are mean±SEM of, at least, 3 independent experiments.

Furthermore, the potential protective effect of *C. roseus* extracts against glyoxal-induced toxicity was assessed through the measure of cell viability (MTT) and is illustrated in figure 25.

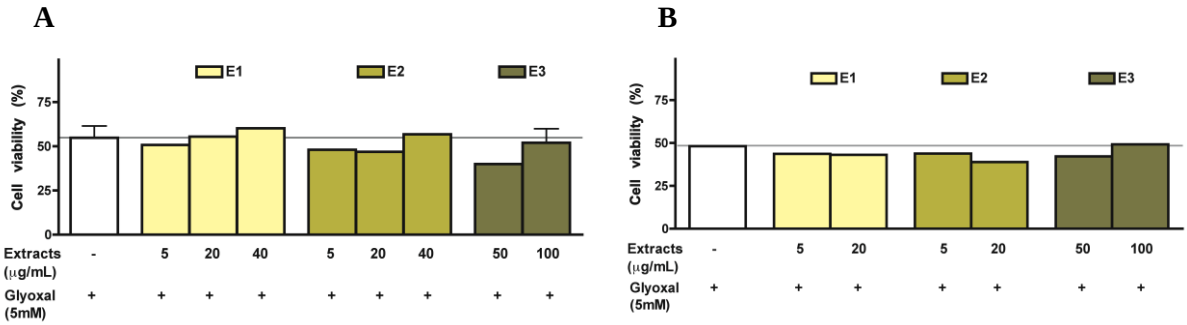

Figure 25 – Effects of glyoxal (5 mM) on HIT-T15 viability for 6 h and *C. roseus* aqueous extracts protection. A: co-incubation of glyoxal with extracts for 6 h; B: 24 h preincubation with extracts, followed by 6 h incubation with glyoxal.

The *C. roseus* extracts did not exhibit any protective effects against glyoxal toxicity, at the tested concentrations.

The same type of results were obtained using GOX (fig. 26) were the extracts did not exhibit a protective effect under GOX toxicity. Despite the fact that in some cases a slight protection was observed, that results were not reproducible in subsequent assays.

These results were not consistent with those published by Ferreres and co-workers (2008) which points out the ability of *C. roseus* extracts to scavenge superoxide radical and nitric oxide, reactive oxygen and nitrogen species, respectively. However, through the chemical assay (DPPH), used by the authors, the extracts presented, in fact, a concentration-dependent antioxidant capacity with the flowers presenting the strongest effects (EC_{50} at 197 μg/mL), followed by seeds (EC_{50} at 265 μg/mL) and leaves (EC_{50} at 447 μg/mL). These concentrations are significantly higher than those used in the present work and this may be the reason for the discrepant results.

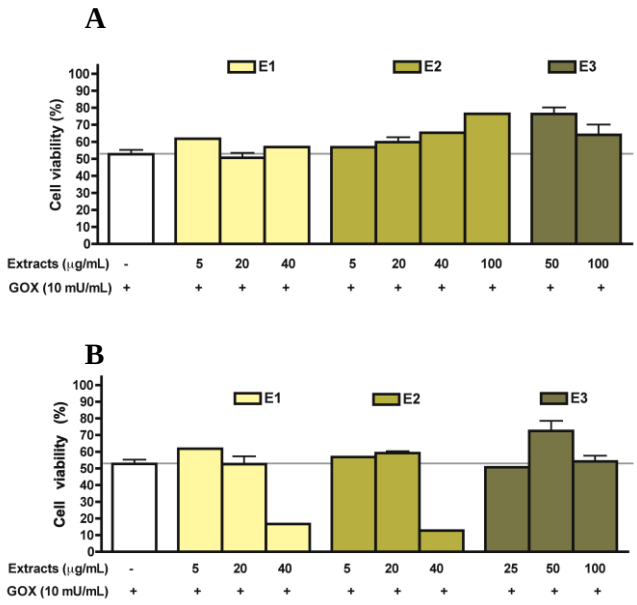

Figure 26 - Effects of GOX (10 mU/mL) on HIT-T15 viability for 6 h and *C. roseus* aqueous extracts protection. A: co-incubation of GOX with extracts for 6 h; B: 24 h preincubation with extracts, followed by 6 h incubation with GOX.

Another explanation could be that the mechanism by which the extracts exert their antioxidant action was not enough to protect the toxicity induced by glyoxal and GOX in the beta-cells. The damages induced by glyoxal, for example, in protein glycation may be not a target for the action of the antioxidant flavonoids present in the extracts. A protective effect may be exerted by extracts in scavenging the ROS but the effects on protein structure will be much more toxic to the cells and a protection may not be seen.

We had also to speculate about the aqueous nature of the extracts that may difficult their entry through the cell membrane, due to its low lipophilicity, and negatively affect their intracellular antioxidant activity.

4.2 *tert*-butylhydroperoxide (tBHP)

The susceptibility of HIT-T15 cell line to another prooxidant toxicant, tBHP that is widely used to induce oxidative stress in different biological systems, was also studied in the present work. tBHP is metabolized by the microsomal cytochrome P450 system to ROS which subsequently initiates lipid peroxidation and depletes cellular reduced glutathione (GSH) content (Yau *et al.*, 2002). Figure 27 shows the effects on HIT-T15 viability of different concentrations of tBHP, during 2 hours of exposure.

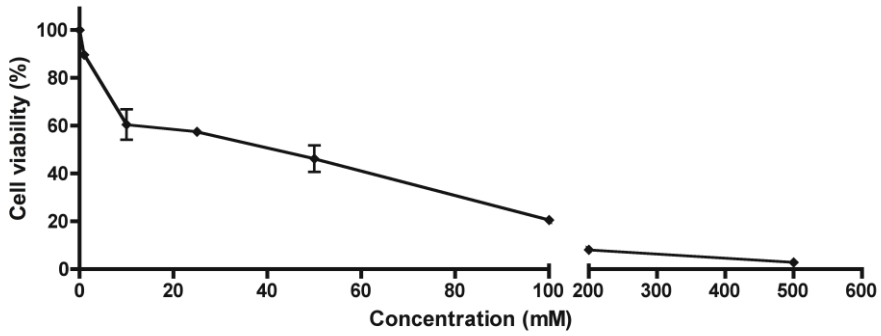

Figure 27 - Viability of HIT-T15 cells after treatment with various concentrations of tBHP for 2 h, by a MTT assay. Cells were cultured in RPMI medium containing 5.6 mM glucose and 10 % FBS.

During the incubation period a sequential decrease in cell viability was observed as the tBHP concentration increases. The concentration of 30 mM was chosen to evaluate the protective effects of *C. roseus* extracts, Q and K in tBHP-induced toxicity in this cell line. This concentration was chosen once it induces approximately 50% of cell death in 2 h.
The protective effects of *C. roseus* aqueous extracts were represented in figure 28. A clear pattern of protection has not been seen against tBHP- induced toxicity in HIT-T15. Co-incubation of tBHP with E2 40 mg/mL (fig. 28A) appears to induce a slight protection, however, this result was not reproducible.

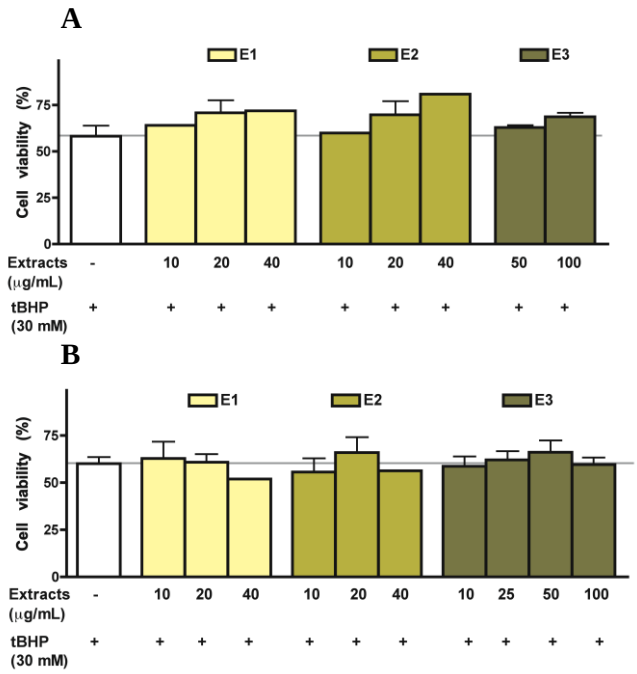

Figure 28 - Effects of tBHP (30 mM) in HIT-T15 viability for 2 h and *C. roseus* aqueous extracts protection. A: co-incubation of tBHP with extracts for 2 h; B: 24 h preincubation with extracts, followed by 2 h incubation with tBHP.

Regarding the effects of Q and K against tBHP-induced damage (fig. 29) and given the results previously obtained by our work group that strongly indicated the potential of Q on preventing cell death induced by tBHP in HepG2 cells (Lima *et al.*, 2007), a strong response by Q was expected. Furthermore ROS, tBHP radicals and intracellular iron ions are involved in the toxicity of tBHP (Davies, 1989) and direct effects on these parameters, found for Q in hepatocytes (Lima *et al.*, 2007), would be expected to reduce the level of damage also in HIT-T15 cells.

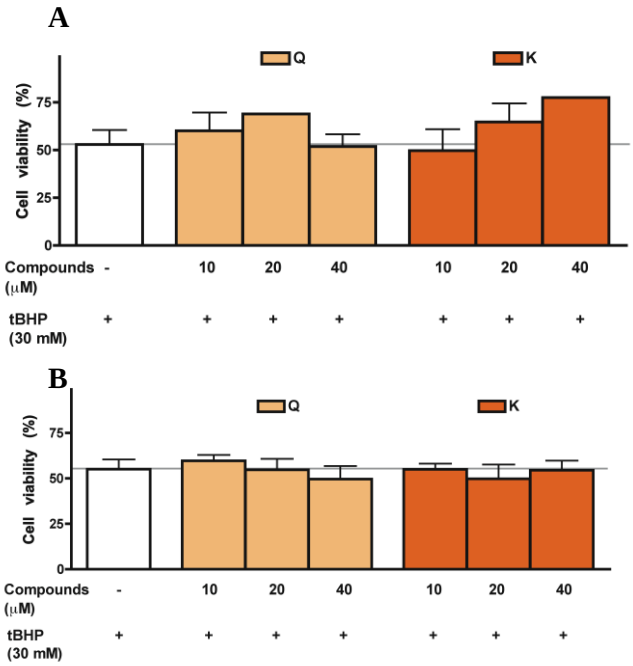

Figure 29 - Effects of tBHP (30 mM) in HIT-T15 viability for 2 h and Q and K protection. A: co-incubation of tBHP with compounds; B: 24 h preincubation with compounds, followed by 2 h incubation with tBHP.

Despite those facts, neither Q nor K protected HIT-T15 cells from tBHP damages. Another controversy is that Q alone significantly improves GSH levels (fig. 22) and that should revert, at least partially, the cytotoxicity induced by tBHP in depleting GSH levels (Yau *et al.*, 2002). Another work by Alía and colleagues (2005) stated that ROS generation induced by tBHP was significantly reduced when HepG2 cells were pretreated for 2 or 20 h with 10 μM and for 20 h with 5 μM quercetin and that some of the quercetin treatments prevented the significant increase of glutathione peroxidase, superoxide dismutase, glutathione reductase and catalase activities induced by tBHP.

Taken together these results regarding the protective effects of *C. roseus* extracts and isolated compounds, Q and K, against prooxidative toxicants in HIT-T15 were not conclusive and more optimizations should be done.

Conclusions and Future Perspectives

The main objective of the present work was to investigate the anti-diabetic potential of aqueous extracts of leaves, senescent leaves and flowers of *C. roseus*, as well as their major flavonoid aglycones Q and K at the level of beta-cells - insulin producing cells. For that purpose, we investigated the extracts´ effects on processes such as protection against oxidative damage induced by dRib, tBHP, glyoxal and GOX, glucose-induced insulin secretion, apoptosis, insulin gene expression and GSH levels using the β-cell line HIT-T15. In the studies of β-cell protection against dRib-induced toxicity and insulin secretion, NAC (a potent antioxidant) and glibenclamide (an oral hypoglycemic drug) were used as positive controls, respectively.

Concerning the effects of *C. roseus* extracts on glucose-induced insulin secretion, we observed no effects on insulin secretion. However, the aglycones of the major flavonoid glycosides present in the extracts, Q and K at 20 μM, exhibited a strong induction of insulin secretion to levels above those achieved by glb (10 nM). That stimulatory effect was inhibited by both nifedipine and diazoxide indicating a mechanism similar to that of the pharmaceutical drug, which is targeting the insulin exocytosis process. The lack of the effect of the *C. roseus* extracts in our experimental model did not invalidate possible action *in vivo* on β-cells, since biotranformations reactions that happen after absorption of the flavonoids glycosides may turn the resulting compounds (that may include the aglycones tested) active.

Under conditions of oxidative stress induced by dRib (30 mM) cell viability was decreased in approximately 50%, a condition that was not prevented by the co-incubation of dRib with the three *C. roseus* extracts. Despite this fact, Q, K and NAC (200 μM) exhibited protective effects against dRib-induced damage, in a concentration-dependent manner, except for K (40 μM). The possible cytoxicity of the compounds alone was also assessed and shown to be not present in the tested concentrations. As mentioned above, and considering that Q and K had protective effects, C. roseus extracts may have protective and antioxidant effects on β-cells in vivo, despite no effects was observed in vitro here.
Although the oxidant effects of dRib in insulin secretion have been described, using our experimental approach was not possible here to observe a significant decrease on insulin secretion. However we did observe a decrease on insulin secretion when dRib was co-incubated with Q or K, when compared to the levels obtained with the flavonoids alone.

Because of this experimental fatality, we were unable to conclude if Q and K were able to protect significantly from the decrease of insulin secretion induced by dRib, and that should be considered in further studies using a different experimental approach, such as incubating HIT-T15 cells with dRib for a longer time.

Regarding GSH levels, a significant diminishment was observed in the presence of 30 mM dRib when compared to control, a situation that was rescued by NAC, Q and K. The increase of GSH levels induced by NAC was mainly due to its role as precursor of GSH shynthesis, since the effect was totally abolished in the presence of BSO, an inhibitor of γ-GCS. Q and K alone also increased GSH levels of HIT-T15 cells indicating the potential of these flavonoids for maintain GSH homeostasis and prevention of oxidative stress, particularly beneficial in a diabetic condition. Therefore, in addition to their direct antioxidant activity, the increase of GSH levels by Q and K may also help to explain the protection afforded against dRib-induced toxicity. The induced toxicity by dRib in our tested conditions seemed to be not dependent on apoptosis, and therefore more studies will be necessary to investigate the mechanisms involved. With a preliminary experiment, dRib seemed also to affect the expression of insulin gene only in the first hours of incubation, but further investigation will be necessary to make final conclusions, and in particular the possible modulation by Q and K.

The toxic effects induced by glyoxal, glucose oxidase and *tert*-butylhydroperoxide was not prevented either by *C. roseus* extracts or by Q or K, probably due to the acute toxicity induced by these compounds (high toxicity in a shorten period of time) as compared with dRib, in cells that are believed to possess less cellular antioxidant defences.

Taken together, these results indicate that *C. roseus* extracts might exert their attributed anti-diabetic activity in other organs like liver or skeletal muscle and that both Q and K, due to their antioxidant and remarkable insulin secretagogue effects, are good candidates for the prevention and/or management of type 2 DM.

For purposes of future and complementary work, once the effects of Q and K on prevention of β-cell apoptosis and on glucotoxicity related decrease in insulin gene expression were not conclusive some other approaches should be tried. First, the assessment of β-cell apoptosis must be further evaluated probably using a lower concentration of dRib for a longer incubation time and performing western blotting experiments with more apoptotic markers. The evaluation of necrosis should also be done. The experiments on insulin gene expression should be repeated with longer incubation

times, and with smaller concentrations of dRib, in order to verify alterations over time as well as test the compounds Q and K for their protection of gene insulin expression.

Regarding the *C. roseus* extracts *per se* it would be interesting to evaluate other extracts obtained from plants raised under natural conditions, once that growing conditions are responsible for alterations in their phytochemical profile.

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