Chapter V - Evaluation of the effect of *Coreopsis tinctoria* extracts and pure compounds on pancreatic beta-cells

The information obtained from the previous *in vivo* experiments is relevant; *Coreopsis tinctoria* extracts showed antihyperglycemic activity in STZ-induced glucose-intolerant rats and plasma biochemical analysis supported pancreatic function recovery when administering the flavonoid-rich AcOEt fraction. These results point either to a strong antioxidant-mediated protective activity against ROS-injury of pancreatic beta-cells protection, or to a mechanism that involved either an inhibition of apoptosis or promotion of proliferation.

So in this chapter possible pancreatic effects were explored: *C. tinctoria*’s extracts were therefore tested for insulin secretion capacity; beta-cell protection, whether by direct radical scavenging of ROS or by beta-cell mass preservation through apoptosis inhibition.

### 5.1. Evaluating Insulin secretion *in vitro*

A number of *in vitro* models have been developed for studying the pancreatic secretion of insulin and to test the potential insulinotropic plant extracts. These include the perifused pancreas (Hannan et al., 2006), intact isolated islets (Hoa et al., 2004) and insulin-secreting cell lines (Jayaprakasam et al., 2005). Insulin release is frequently measured by radioimmunoassay (using $^{125}$I-labelled insulin) or enzyme-linked immunoassay (Govindarajan et al., 2007b; Esmaeili and Yazdanparast, 2004; Ruitton-Uglienco, 1981). In this study we have selected an insulin secreting cell line (MIN6) and insulin release was measured by radioimmunoassay.

Several insulin-secreting cell lines have been developed in an attempt to establish cell lines that retain the characteristic features of beta-cells. The cell lines are transformed using different techniques such as irradiation, viral transformation, and transgenic technology (Poitout et al., 1996). They can therefore be different from primary beta-cells in terms of their behaviour and responsiveness to insulin secretagogues (Persaud, 1999). The most widely used beta-cell lines are RINm5F, HIT-T15, MIN6, INS-1, and BRIN-BD11 cells (Poitout et al., 1996). The advantage of cell lines is that they can be used in rapid-
throughput experiments and are much less labour intensive and conservative of resources than the use of isolated islets. However, because these cells are a transformation of pancreatic beta-cells, some characteristic features of beta-cells may not be faithfully represented by the cell lines (Persaud, 1999).

In measuring insulin secretion either in islets or cell lines, two practical factors need to be considered. First, any increase in the permeability of cell membranes will result in a release of insulin by non-specific mechanisms. It is important to test for this effect when assessing insulin release data. Integrity of cell membrane can be determined by trypan blue uptake which is an established test of cell viability that allows rapid visual readout of compromised plasma membrane integrity (Liu et al., 2009; Persaud et al., 1999) or by measuring the release of lactate dehydrogenase (LDH). Cell viability assays were therefore performed to evaluate *C. tinctoria* extracts effect on beta cell membrane integrity. Another fact to consider is that glucose, which can be present in polar plant extracts, can act as stimulant to insulin secretion. When insulinotropic action is actually verified it is important to remove glucose from extracts prior to testing them again.

Islet function *in vitro* is widely assessed by testing glucose stimulated insulin release using static incubation methods which consist in the sequential incubation periods of low and high levels of glucose followed by measurement insulin concentration present in the supernatants (Berney et al., 2002). In the present study we have successively adapted this method to monolayer MIN6 cell line (Liu et al., 2009).

Insulin secretion from MIN6 cells, cultured as monolayers, differs substantially from pancreatic islets, at least partly due to missing beta-to-beta cell contacts. These cellular contacts are abundant in MIN6 pseudoislets, which are islet-like structures that show a more pronounced glucose-induced insulin release (Kitsou-Mylona et al., 2008; Jones, 2008; Brereton et al., 2006; Brenner and Mest, 2004; Hauge-Evans et al., 1999).

So to further investigate *C. tinctoria* extracts effect on insulin secretion, pseudoislets were created and a perifusion method was applied. The islet perifusion method is a well-known method to investigate the kinetics of insulin secretion. It provides a dynamic picture of islet endocrine function but is more complex to perform than static incubation. In perifusion method islets are placed in milipore chambers and perifused
continuously with the same low and high glucose containing solutions, and in this case, in the presence or absence of \textit{C. tinctoria} extracts.

Some studies have already described the use of pseudoislets in perifusion method to analyse the time-course reversibility of insulin secretion in response to plant extracts and to complement static incubation information (Menichini et al. 2011; Govindarajan et al., 2008; Govindarajan et al., 2007b).

\subsection*{5.2. Oxidative stress on beta-cells}

Reactive oxygen species play a critical role in the pathogenesis of many diseases, namely diabetes (Baynes, 1991), where glucose toxicity has been directly related to oxidative stress (Bonora, 2008; Robertson, 2004). It is known that pancreatic beta-cells are particularly vulnerable to oxidative stress when compared to other tissues, mainly because of their relatively low level of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Evans et al., 2002; Lapidot et al., 2002; Lenzen et al., 1996). This fact makes protection of beta pancreatic cells from oxidative stress an interesting therapeutic target and numerous reports have already demonstrated protection of beta-cells by antioxidant drugs in beta-cell lines and isolated pancreatic islets, rodents and humans (Hussain, 2007; Kaneto et al., 1999; Lortz et al., 2003; Tanaka et al., 1999; Yamamoto et al., 2008). Several plant extracts are rich in antioxidants and have also showed to provide that kind of protection (Lee et al., 2010; Kim et al., 2007c; Prince and Kamalakkannan, 2006; Coskun et al., 2005). With an amelioration of oxidative stress and repair of existing beta-cells with a return to full function, normalization of insulin secretion and blood glucose levels will be achieved. So, antioxidant-rich extracts such as \textit{C. tinctoria} extracts (Appendix I) represent potential candidates for beta-cell protection against oxidative stress.

In the present work we have evaluated cytoprotection against oxidative stress by pre-treating MIN6 cells with \textit{C. tinctoria} extracts and major flavonoids prior to oxidant treatment (tert-Butyl Hydroperoxide) after which viability was determined through
mitochondrial lactate-dehydrogenase activity (MTT assay) (Mossman, 1983) and/or ATP measurements (Crouch et al., 1993).

The pro-oxidant chosen, tert-Butyl Hydroperoxide (tBHP), is a membrane-permeant short chain analogue of lipid hydroperoxides widely used as a pro-oxidant agent to study oxidative stress in vitro (Fernandes et al., 2010; Sardão et al., 2007). Once inside the cells tBHP generates tert-butoxy radicals, which induce several physiological alterations such as lipid peroxidation, depletion of intracellular glutathione and DNA damage and, depending on the concentration, have been reported to cause apoptosis or necrosis in many cell types (Kweon et al., 2004).

Among several reactive oxygen species the superoxide anion is one of the most important, not only because of its rapid reactions, but also because it initiates the production of several other reactive oxygen species (ROS) (Munzel et al., 2002). The current available methods to quantify reactive oxygen species most commonly include chemiluminescent assays (Zhang et al., 2009; Armann et al., 2007), fluorescence-based techniques (Kang et al., 2010; Lin et al., 2008; Latha et al., 2004) and enzymatic assays (Afanas’ev et al., 2001).

Chemiluminescence is frequently used to detect superoxide anion in neutrophils (Gyllenhammar, 1987) and vascular cells (Munzel, et al., 2002). On exposure to superoxide anion, chemiluminescent probes release a photon, which in turn can be detected by a scintillation counter or a luminometer. Because most of these compounds are cell permeable, the superoxide anion measured reflects extracellular as well as intracellular anion production. Among chemiluminescent compounds, bis-N-methylacridinium nitrate (Lucigenin) is highly specific and sensitive to superoxide anion and therefore remains the most widely used probe (Bartosz, 2006; Tarpey et al., 2004; Munzel et al., 2002).

In the present work we used a lucigenin-based chemiluminescent method in order to detect superoxide anion generation by MIN6 cells pre-incubated with C. tinctoria extracts prior to oxidant (tBHP) treatment.

5.3. Beta-cell mass preservation
Decrease in beta-cell mass, either through an increase in apoptosis or a decrease in proliferation, is believed to be one of the factors responsible for the progression of type 2 diabetes (Baggio and Drucker, 2006; Weir and Bonner-Weir, 2004).

Stimuli such as pro-inflammatory cytokines and/or chronic elevation of blood glucose promote oxidative stress generation in pancreatic islets to provoke beta-cell death by apoptosis (Baggio and Drucker, 2006; Le May et al., 2006; Amrani et al., 2000; Tiedge et al., 1997; Corbet et al., 1992).

Therefore, one approach to preventing and treating diabetes could be through the enhancement of beta-cell mass. This may occur through increased beta-cell replication, decreased beta-cell death or differentiation of possibly existing beta-cell progenitors (Lipsett and Finegood, 2002). Currently available therapeutic agents lower blood glucose through multiple mechanisms but do not directly reverse the decline in beta-cell mass (Baggio and Drucker, 2006).

Reliable methods for the assessment of the beta-cell mass are still lacking. The development and implementation of non-invasive techniques for the quantitative measurement of the functional beta-cell mass and the spatial visualization of its component would help in the early diagnosis of beta-cell loss or dysfunction in pre-clinical phases of diabetes. Furthermore, it would enable evaluation of emerging therapeutic approaches which specifically focus on preservation or regeneration of beta-cells (Saudek et al., 2008).

Screening studies that aim to determine plant extracts effect on beta-cell mass preservation frequently use histochemical staining of pancreatic beta-cells namely aldehyde fuchsin or propidium iodine (fluorescent dye) (Kim et al., 2009; Latha et al., 2004). Other studies use more specific techniques such as immunocytochemical staining of insulin containing beta-cells (Subash-Babu et al., 2009) others use fluorescence immunohistochemistry coupled with tunnel staining in order to measure apoptosis (Luo and Luo, 2006).

Alterations in beta-cell mass can also be determined by measuring the expression of molecules responsible for beta-cell survival such as uncoupling protein-2 (UCP-2) (Luo and Luo, 2006) or on the other hand, expression of molecules involved in beta-cell apoptosis such as nuclear factor kappa B (NFkB) downregulation (Menegazzia et al.,
Chapter V – Evaluation of the effect of *Coreopsis tinctoria* extracts and pure compounds on pancreatic beta-cells

2008), inhibition on p53/p21 expression, and inhibition on cleavage of caspases and poly(ADP-ribose) polymerase (PARP) (Kim and Kim, 2007).

Proliferation has also been measured in pancreatic beta cells in order to explain beta-cell mass preservation using radiolabelled \(^3\)H-thymidine incorporation in TC-tet beta-cells (Martineau et al., 2006) or synthetic nucleoside (thymidine analog) Bromodeoxyuridine (BrdU) incorporation in human islets and INS-1 cells (Fu et al., 2010).

Molecules with flavonoid structure have previously been described to increase beta-cell mass either by inhibiting apoptosis or promoting proliferation of beta cells after being exposed to an oxidizing agent (Lapidot et al., 2002, Pinent et al., 2008). This has led us to believe that *C. tinctoria* extracts and major compounds, flavonoids, could be involved in beta-cell cytoprotection and preservation through apoptosis inhibition. Therefore, MIN6 cells were pre-incubated with *C. tinctoria* extracts, marein and flavanomarein on a cytokine-induced apoptotic cell death model. A cytokine mixture composed by IL-1beta, TNF-alpha and IFN-gamma, was chosen for being known to contribute to beta-cell apoptosis through NFkB activation and endoplasmatic reticulum (ER) stress (Kharroubi et al, 2004). Apoptosis was later determined by caspase 3/7 activity using a kit as described in the experimental section.

5.4. Effect of *C. tinctoria* on insulin secretion from the MIN6 beta-cell line

5.4.1. Static Incubation

Extracts containing flavonoids, e.g. quercetin, have been shown to exert direct stimulatory effect on the exocytotic release of insulin from MIN6 beta-cells (Pinent et al, 2002). Thus, the possibility of *C. tinctoria* infusion and AcOEt fraction exerting its effects to reverse glucose-intolerance by directly stimulating insulin secretion was investigated. Graphics shown in Figure 5.1 represent the effect of *C. tinctoria* (0.1; 0.3 and 1 mg/mL) aqueous extract (Figure 5.1 A) and AcOEt fraction (Figure 5.1 B) on insulin secretion from MIN6 cells at 2 mM and 20 mM glucose using the static incubation method. From the chart we can observe that either at 2 mM or 20 mM there was no statistically significant increase in insulin secretion in MIN6 cells treated with *C. tinctoria* aqueous extract or AcOEt fraction when comparing to untreated control. Although MIN6 cells were not glucose
responsive at 20 mM, significantly elevated insulin secretion values were registered in the presence of the protein kinase C activator, PMA (Phorbol 12-myristate-13-acetate), or the adenylate cyclase activator, FSK (forskolin), which validates the experiment. The fact that the insulin-secretion in response to glucose is not observed in the MIN6 cells in this experiment could be related to the fact that this cell line has been maintained in 25 mM glucose for years and have adapted to high levels of glucose in the media, becoming less sensitive to glucose. Also, Dowling and colleagues (2006) state that the glucose-sensitive insulin-secretion (GSIS) phenotype is relatively unstable in long-term culture of beta-cells. The cells used in this experiment were high passage so it is possible that a loss of sensitivity might have occurred.
Figure 5.1. Effect of *C. tinctoria* (0.1; 0.3 and 1 mg/mL) aqueous extract (Sample D) (A) and AcOEt fraction (SampleC) (B) on insulin secretion from MIN6 cells in static incubations at 2 and 20 mM glucose. PMA (phorbol 12-myristate) and IBMX/FSK (3-isobutyl-1-methylxanthine / Forskolin) were used as positive controls. Data are expressed as percentage of basal insulin content of untreated control cells. Bars show mean ± SD (n = 8); * P<0.01.

5.4.2 Perifusion assay

As for the perifusion experiments, the results indicate that *C. tinctoria* extracts (both aqueous and AcOEt) in the concentration tested (1 mg/mL) (Figure 5.2) did not promote insulin secretion in the presence of either 2 mM or 20 mM glucose. In contrast, insulin secretion from MIN6 pseudoislets was generally significantly elevated by the presence of the protein kinase C activator, PMA. It has been previously stated that MIN6 cells cultured in these 3D structures present an increase in the amplitude of the responses when comparing to the equivalent monolayer cells (Hauge-Evans et al., 2002). Using the perifusion method applied to the pseudoislets (Chapter 8; Section 8.8.3) a more dynamic and intense response to glucose was therefore expected and was in fact observed in this experiment.
Chapter V – Evaluation of the effect of *Coreopsis tinctoria* extracts and pure compounds on pancreatic beta-cells

![Graph A](image1.png)

**B**

![Graph B](image2.png)

**C**

![Graph C](image3.png)

**D**
Chapter V – Evaluation of the effect of *Coreopsis tinctoria* extracts and pure compounds on pancreatic beta-cells

**Figure 5.2.** Time course of the effect of *C. tinctoria* aqueous extract and AcOEt fraction (1 mg/mL) on insulin secretion from perifused MIN6 pseudoislets at 2 mM and 20 mM glucose. PMA (phorbol 12-myristate acetate) was used as positive control. Data are expressed as percentage of insulin content of untreated control cells. Points show mean ± SD (n = 4). Legend: 2G = 2 mM glucose; 20G = 20 mM glucose.

Observing channels A and B (Figure 5.2, A and B), when the *C. tinctoria* aqueous extract and AcOEt fraction (1mg/mL) are respectively added to pseudoislets at basal glucose levels (2 mM glucose), there is a reduction in insulin secretion which seems to be reversible on removal of the extract/fraction, although later in time pseudoislets have shown to be poorly responsive to PMA.

In channels C and D (Figure 5.2, C and D), pseudoislets seem to respond to 20 mM glucose with a slight increase in insulin secretion although when *C. tinctoria* aqueous extract and AcOEt fraction (1mg/mL) are added, there seems to be a reduction in insulin secretion even in the presence of 20 mM glucose although there might be a small transient increase (specially in channel D). Again this inhibition of insulin secretion seems to be transient and in channels, C and D, where there is full recovery when extract and fraction are removed and positive control (PMA) is added.

In any event, if *C. tinctoria* aqueous extract and AcOEt fraction are affecting beta-cells it appears as though they cause a reversible inhibition of basal insulin secretion, which backs up the static incubation data suggesting that the compounds do not stimulate insulin secretion.

5.5 *C. tinctoria* extracts, marein (78) and flavanomarein’s (85) cytotoxicity – Cell viability assays

5.5.1. Trypan Blue Exclusion assay

After incubation in the absence or presence of *C. tinctoria* extracts (aqueous extract sample D and AcOEt fraction sample C), MIN6 cells were exposed to membrane-impermeant dye, trypan blue (0.1% w/v) for 15 min at 37°C. The presence of dye within cell nuclei was determined by light microscopy and the proportion of stained or unstained cells was estimated. Membrane integrity was preserved as the results show that there was
no dye uptake. Thus, we can conclude that the extracts at the maximal concentration of 3 mg/mL were not toxic to MIN6 cells.

5.5.2. Luminescent assay - Celltiter-Glo

To determine whether *C. tinctoria* extracts, marein and flavanomarein had any effect on cell viability, ATP measurements (Crouch et al., 1993) was carried out on cultured MIN6 cells treated with a range of concentrations. As shown in Figure 5.3, *C. tinctoria* extracts (aqueous extract sample D and AcOEt fraction sample C), marein (78) and flavanomarein (85) had no significant effect on the viability of MIN6 cells under experimental conditions.

![Cell viability graph](image)

**Figure 5.3.** MIN6 cell viability when pre-treated for 24h with *Coreopsis tinctoria* extracts; (aqueous extract sample D, AcOEt fraction sample C) and pure compounds (marein (78) and flavanomarein (85)). Cell viability was assessed through measurement of total ATP. Data are expressed as percentage of ATP content of untreated control cells. Bars show mean ± SD.

150
5.6. Effect of *C. tinctoria* extracts and pure compounds on tBHP-induced cytotoxicity in MIN6 cells

In this subsequent experiment, as an oxidative stress model, we used tBHP as a surrogate for high glucose-induced ROS, to study the effect of *C. tinctoria* extracts, marein and flavanomarein on MIN6 cell damage due to oxidative stress. tBHP is known to induce a dose- and time-dependent decrease in cell viability (Kweon et al., 2004; Zhao et al., 2004). In this study we have chosen to use 400 µM tBHP for 2h where reduction of the viability of cells was approximately 50% that of controls for MTT assay and 70-80% for the ATP measurement assay.

To assess the protective potential of *C. tinctoria* extracts and the two flavonoids against tBHP-induced cell death, cells were pre-treated with *C. tinctoria* aqueous extract (sample D) (50 and 100 µg/mL), the ethyl acetate fraction (sample C) (50 and 25 µg/mL) marein and flavanomarein (200 and 400 µM) for 24 h before adding 400 µM tBHP, and subsequently assaying cell viability. As shown in Figures 5.4 and 5.5, *C. tinctoria* extracts, marein and flavanomarein exerted a dose-dependent cytoprotective effect by increasing cell viability. As seen in Figures 5.4 and 5.5, there was an overall significant increase (ranging from 20% to 30% increase) in viability in tBHP-challenged MIN6 cells (400 µM in 2 hours) when pre-incubated with *C. tinctoria* aqueous extract (100 µg/mL, p<0.01), AcOEt fraction (50 µg/mL; p<0.01), marein (400, 200 µM; p<0.01), and flavanomarein (400 µM, 200 µM; p<0.01). These data indicate that tBHP induces cell death in MIN6 cells, and demonstrate that this effect is attenuated by *C. tinctoria* extracts, marein and flavanomarein.
Figure 5.4. MIN6 cell viability when pretreated for 24h with *Coreopsis tinctoria* extracts: aqueous extract sample D (Aq. Ext.) and AcOEt fraction sample C (AcOEt Fr.) and challenged with oxidant tBHP (2h). Cell viability was assessed through A) lactate dehydrogenase activity (MTT viability test) and, B) ATP measurement. Data are expressed as percentage of untreated normal cells. Bars show mean ± SD; ** p<0.01; * p<0.05 compared to tBHP-challenged cells.
Figure 5.5. MIN6 cell viability when pretreated for 24h with marein and flavanomar, and challenged with oxidant tBHP (2h). Cell viability was assessed through measurement of total ATP. Data expressed as a percentage of the ATP content of untreated control cells. Bars show mean ± SD; ** p<0.01 compared to tBHP-challenged cells.

5.7. Effect of *Coreopsis tinctoria* extracts on superoxide anion production by pancreatic beta-cells

Tert-butyl hydroperoxide (tBHP) treatment provokes an increase in superoxide anion, and the resulting oxidative stress is known to be cytotoxic. To determine whether generation of superoxide anion occurred in tBHP-treated MIN6 cells and to examine the effect of *C. tinctoria* aqueous extract and ethyl acetate fraction on ROS formation, we used a chemiluminescent approach employing the luminescent probe, Lucigenin along with luminescence enhancer, NADPH (Baker et al., 2004). Stimulation of MIN6 cells with tBHP induced a time-dependent increase in lucigenin luminescence intensity (Figure 5.6 A) indicative of increased superoxide anions formation. The presence of superoxide dismutase (SOD) (added at the end of experiment as described in Chapter 8; Section 8.8.7) caused a marked decrease in the intensity of the chemiluminescent signal (Figure 5.6 A), confirming
the origin of the luminescence and validating the method. Pre-incubating the cells with the plant aqueous extract sample D or AcOEt fraction sample C caused no detectable decrease in the luminescence signal, suggesting that the extracts do not act primarily by decreasing superoxide anion concentrations in the cellular media. Note that the extract and fraction had no effect themselves on superoxide anion production (Figure 5.6 B).

Figure 5.6. Superoxide anion (O$_2^-$) measurements in A) MIN6 cells challenged with tBHP and tBHP plus SOD. B) MIN6 cells pretreated with C. tinctoria aqueous extract and AcOEt fraction and challenged with tBHP. Measurements are expressed in relative light units and area under the curve (relative light units/min) Points and bars show mean ± SD; **p<0.01 comparing to control cells; ## p<0.01 compared to tBHP-challenged cells.
5.8. Effect of *Coreopsis tinctoria* aqueous extract, AcOEt fraction, marein and flavanomarein on cytokine-induced apoptosis in MIN6 Cells.

Caspase-3 and 7 play key effector roles in the apoptotic cascade (Lakhani et al., 2006). To assess the ability of *C. tinctoria* extracts and pure compounds to inhibit this pathway, MIN6 cells were treated with the cytokine cocktail for 18 h, with or without pretreatment with *C. tinctoria* extracts or pure compounds. A cytokine cocktail was used as positive control for caspase activation. The data shown in Figures 5.7 and 5.8 demonstrate that after 18h incubation time, the cytokine mixture was clearly able to induce apoptosis in MIN6 cells (p<0.01) and that the *C. tinctoria* extracts or pure compounds did not themselves have any significant effect on apoptosis. However, as shown in Figure 5.7, cytokine-induced apoptosis, as assessed by caspase-3/7 activation was significantly inhibited by pre-treatment with *C. tinctoria* aqueous extract (sample D) and the ethyl acetate fraction (sample C) a dose-dependent manner with a maximal decrease in cytokine-induced apoptosis of approximately 50 % at the highest concentration tested (100 µg/mL).

![Figure 5.7. *C. tinctoria* extracts pre-treatment (24h) effect on untreated and on cytokine-induced MIN6 cells apoptosis. Data expressed as a percentage of the caspase activity content of untreated control cells. Bars show mean ± SD; * p<0.05; ** p<0.01 compared with cytokine-challenged cells.](image-url)
Similarly, as shown in Figure 5.8, the individual flavonoids, marein and flavanomarein, present in both extract (sample D) and fraction (sample C) (but more concentrated in the later) also reduced cytokine-induced apoptosis by approximately 30% confirming their involvement in the process, although not in a dose-dependent manner for concentrations ranging from 400-100 µM.

![Figure 5.8](image)

**Figure 5.8.** *C. tinctoria* pure compounds, marein and flavanomarein pretreatment (24h) effect on untreated and on cytokine-induced MIN6 cells apoptosis. Data expressed as a percentage of the caspase activity content of untreated control cells. Bars show mean ± SD; * p<0.01 compared to cytokine-challenged cells.