6.1. The importance of medicinal plant research for safety and efficacy assessment of herbal medicines and drug discovery

The adverse effects of synthetic chemical drugs, their increasing costs and greater public access to information on safety and efficacy of medicinal plants has led to an increased interest in medicinal plants (WHO, 2002).

Ethnopharmacological leads have already resulted in the introduction of new single drug entities but have a greater role to play if crude extracts are accepted for clinical use in the West (Rawat, 2006). Unlike conventional medicines, plant-derived drugs do not follow exactly the same path as new chemical entities. Due to their chemical complexity, their development has always been a multi-step procedure starting with a crude extract followed by the standardized extract and ending up with isolated constituents.

This work was based on the ethnopharmacological report of use of Coreopsis tinctoria flowering tops infusion in Portugal’s traditional medicine. As it happens quite often, sufficient quality control and drug standardization is lacking for traditional recipes and in the case of Coreopsis tinctoria it was no exception as there were none. Therefore, this project “Phytochemical study of Coreopsis tinctoria and evaluation of its potential antidiabetic properties” is of great importance as it intends to contribute to this plant’s use validation and eventually the identification of a new chemical scaffold for diabetes therapy. To achieve that, two parallel approaches were followed: the phytochemical study and the pharmacological approach using in vivo and in vitro models for antidiabetic activity assessment.

Preclinical testing is an integral part of the modern drug discovery process that helps gathering important efficacy and safety data before clinical trials can be carried out. It is thus a vital step towards sources of new, effective and safe drugs. In the case of medicinal plants the preclinical evaluation involves documentation and testing of their biological efficacy, studies of toxicology and chemical profiling (Brijesh et al., 2006).
6.2. Phytochemical study of *Coreopsis tinctoria*

Plant extracts used in traditional medicine are chemically complex and might contain one or various structurally related active compounds that produce a combined effect. The aims of phytochemical studies are: i) to identify the bioactive constituents in the extracts ii) develop suitable methods for their extraction and, iii) contribute to standardization and quality control of herbal extracts/preparations.

Proper botanical identification, storage, isolation of marker compounds (preferably the therapeutic ones) and validated analytical methodologies for the quantification of the extracts/fractions are important milestones in herbal drug standardization.

As for *Coreopsis tinctoria*, commercial available samples of the flowering tops were used, from which the described traditional preparation was formulated: an infusion. In order to chemically characterize this preparation a chromatographic profile was elaborated and main compounds were identified and quantified.

*Coreopsis tinctoria* is quite abundant and widely distributed plant with described traditional uses in America, in China and in Portugal, so it would be expectable that there was phytochemical information available. There was merely one reference reporting from the late 1950’s. It was not until the beginning of this project in 2006 that another study from a Chinese group arose. Both studies claim the presence of flavonoids in the flowering tops and especially an abundant chalcone, okanin 4’-O-beta-glucoside, also known as marein. A fact that was confirmed in the present study by successfully isolation and identification of marein from the Portuguese samples of *C. tinctoria* flowering tops aqueous extract.

Marein (78) was readily detected by TLC, after eluting a sample of the aqueous extract. The chromatogram showed an intense purple spot after revelation with NP-PEG reagent. But it was not until the compound was isolated and submitted to NMR, that structure elucidation became complete. Marein’s aglycone, okanin (79), and correspondent flavanone (3’,4’,7,8-tetrahydroxyflavanone) (88) were also isolated from the less polar Hex:AcOEt 50% fraction and identified through NMR. All three compounds had already been described for this species (Zhang et al., 2006;
Shimokoriyama, 1957). This result also leads to the conclusion that there is no great chemical variation between the species cultivated and commercialized in Portugal and the one collected in China (Zhang et al., 2006), at least concerning the identity of the main constituents.

The knowledge of the chemical composition of an extract is essential for the evaluation and interpretation of the pharmacological activity as well as toxicity of the extract. But isolation of all phytoconstituents in a crude extract is time consuming and costly so other techniques such as HPLC coupled with a diode array detector and mass spectrometer have proven to be valuable tools in compound identification reducing such an overwhelming task.

Thus, for better undersating of *C. tinctoria* flowering tops aqueous extract chemical profile, an HPLC-DAD tandem mass spectrometry method has been developed, which allowed the separation and identification of flavonoid glycosides and aglycones, as well as phenylpropanoic acids. The MS analysis was achieved using the negative ion mode of detection as it provides better ion signal for acidic compounds, such as flavonoids, allowing the detection and identification of minor constituents in extracts (Fabre et al., 2001).

From this method a total of fourteen compounds were identified, seven were flavanones, two chalcones, two aurones, two caffeoylquinic acids and one flavonol, hence it permitted the establishment of a chromatographic profile of the aqueous extract.

The information from the extract’s chromatographic profile and the knowledge that polyphenols can interact with different biological targets through the carbohydrate metabolism (Chapter 2, Section 2.5.2), helped in the selection of the fraction to be tested. A more concentrated and flavonoid-rich fraction was therefore needed, and was achieved with AcOEt, in order to evaluate whether the activity persisted.

The chromatographic profile of the AcOEt fraction was obtained by successfully applying the method developed above for the aqueous extract. Apart from the flavonoids present in the aqueous extract, one pentahydroxyflavanone-glucoside (94), identified in this species for the first time, and chalcone coreopsin (76) were additionally found in the fraction, raising the number of total compounds found in *C. tinctoria*, to sixteen. Besides,
increased flavonoid concentration was expected and was indeed observed upon injection into the HPLC-DAD-MS/MS where the total ion chromatogram of the fraction revealed that marein was, again, the predominant flavonoid (Chapter 3, Section 3.3).

By coupling the information provided from the MS with the UV-Vis compound absorbance data, a preliminary identification of the flavonoids present in the extracts was achieved. When possible, compound’s retention times and fragmentation patterns were compared with reference standards for structural confirmation. From the compounds tentatively identified a dicaffeoylquinic acid (97), 3,4’,5,6,7-pentahydroxyflavanone glucoside (93), 3,3’,5,5’,7-pentahydroxyflavanone glucoside (94), 3’,5,5’,7-tetrahydroxyflavanone-O-glucoside (96), and flavanocoreopsin (95) were observed for the first time in this species.

Overall in this study, 9 out of 16 of the flavonoids present in *C. tinctoria* extracts were identified as glycosides. In ESI-MS/MS conditions the \([M-H]^-\) ions readily eliminated the sugar moieties to produce the corresponding \([Aglycone-H]^+\) ions. Loss of 162 a.m.u. revealed the presence of compounds with an hexose, putatively identified as glucose on the basis of biosynthetic knowledge.

Through this method an interesting and novel fragmentation pattern for chalcone-flavanone pairs was suggested (Chapter 3, Figure 3.12) concerning the fragments m/z 151 and 135. In a study developed by Sanz and colleagues in 2010, a similar molecule eriodictyol (3’,4’,5,7-tetrahydroxyflavanone) presented both fragments which were considered to derive directly from a retro Diels-Alder reaction. In this study it appears that the smaller fragment m/z 135 originates from fragment m/z 151 (event observed by increasing collision energies on the original marein sample) and therefore fragment (\(^{1,3}B’\)) would be missing. The absence of the typical (\(^{1,3}B’\)) fragment has already been previously stated when using negative ion mode (Zhang et al., 2008), due to a charge driven mechanism that would render (\(^{1,3}B’\)) fragment neutral and as consequence undetectable.

In order to evaluate marein’s concentration in *C. tinctoria* (*CTDI*) samples, a simple and reproducible HPLC-UV method (method 1) was developed and validated. By this method marein’s content in the aqueous extract was determined as 4% (w/w of dry extract) while in the AcOEt fraction used for *in vivo* assays, marein’s content nearly
reached 17% (w/w dry fraction), more than 4 times more concentrated than the aqueous extract.

Through method 2, using different plant material (CTD2), the aqueous extract presented low variation in terms of marein concentration (varied from 4.08 % (Sample D) to 4.60 % (Sample C) in the fresh aqueous extract when comparing with the values presented using method 1. The same proportion of marein was not verified in the AcOEt fraction that presented much higher values (marein content varied from 43% in sample C to 51% in sample B). The difference between marein content in the AcOEt fraction (Samples A, B and C) from method 1 to method 2 was most probably related to the fact that the samples might have been differently prepared because the extraction method “until exhaustion” consisted in a qualitative evaluation.

According to European Medicines Agency guidelines (EMEA, 2006), quantification of substances with known therapeutic activity or markers is mandatory and quantification of multiple compounds in crude extracts is advisable in order to obtain a more accurate evaluation of the extracts quality. Therefore, other commercially available compounds, known to be present in the samples of C. tinctoria, were submitted to quantification. Thus, besides marein, flavanomarein, chlorogenic acid and cynarin (1,3-dicaffeoylquinic acid) were chosen to complete C. tinctoria products standardization.

Considering flavanomarein, the chromatograms presented a double peak, at Rt 25.91 minutes, of similar absorption and equal MS spectra. When compared to the standard, the later peak was identified as the natural isomer 2S-flavanomarein (Rt 25.91 min) whereas the other identified as 2R-flavanomarein. Due to deficient peak resolution, for quantification purposes and in terms of potential biological activity, both peaks were considered and quantified as one. When considering both isomers, flavanomarein’s content has shown to be as abundant as marein’s. Together they make up more than 8% of the aqueous extract (sample D) and reach 84% in the AcOEt fraction (sample C). If we were to consider the presence of the same proportion between marein and flavanomarein in the sample of AcOEt fraction (sample A) used for in vivo testing, where marein content reached only 16.84%, then both would constitute nearly 34% (w/w dry fraction) of the AcOEt fraction, still both metabolites make up an important portion of the
administered fraction.

As for the dicaffeoylquinic acid, the commercially available standard used (cynarin; 1,3- dicaffeoylquinic acid) was not the dicaffeoylquinic present in the sample, but peak area is not believed to differ (dicaffeoylquinic acids share the same UV absorption maximum) so, the dicaffeoylquinic acid was quantified based on that standard. Overall, phenylpropanoic acids were present but in quite small amounts when comparing with the major compounds marein and flavanomarein (less than 10 fold) reaching around 0.63% (w/w dry extract) in the aqueous extract. Although, caffeoylquinic acids contribute with good antioxidant capacity and some of them, e.g. chlorogenic acid, have shown to promote an inhibitory effect on S-GLUT-1 mediated glucose transport (Welsh, 1989), they are less likely to be the chemical entities responsible for claimed antidiabetic activity due to low concentration in the extract.

The phytochemical approach developed herein has contributed to the identification of the major chemical structures present in C. tinctoria flowering tops bioactive extracts. These structures, mainly of chalcone and flavanone skeleton, that have already shown to exert antidiabetic activities at several targets are also likely to be involved in C. tinctoria's claimed antidiabetic activity. Thus they are interesting leads in drug discovery and development of new drugs for diabetes therapeutic.

6.3. Pharmacological approach for the evaluation of claimed antidiabetic activity

In order to evaluate the claimed antidiabetic activity of C. tinctoria flowering tops, in vitro and in vivo bioassays were needed and are nowadays considered a necessary step towards medicinal plants efficacy and safety assessment.

Diabetes is a complex multifactorial disease characterized by insulin deficiency and insulin resistance or both. The fasting and post-prandial blood glucose is elevated, exposing the patient to acute and chronic complications (micro- and macro-vascular) leading to blindness, kidney failure and heart disease. Improving glycemic control has been demonstrated to lower the risk of these complications (Modi, 2007).

Such a wide array of features are hard to replicate among non-primate animals, which often requires that efficacy studies should be done in more than one model,
especially to investigate the mode of action (Day and Bailey, 2006).

Accounts of the traditional use of an antidiabetic plant in type 1 or type 2 diabetic patients provide an indication of the type of model suitable for initial investigation (Day and Bailey, 2006). The traditional use in Portugal merely mentions *C. tinctoria*’s capacity to decrease hyperglycemia, thus the infusion could be applicable to both types of diabetes.

In this study, it seemed reasonable to start by targeting post-prandial hyperglycemia (PPHG) a deficiency present in type 2 diabetic and pre-diabetic subjects. In order to do so, two *in vivo* models of PPHG were developed.

The first model included the use of normoglycemic male Wistar rats and administration of the infusion (prepared according to the traditional use) at several doses (25–300 mg/Kg), 10 minutes prior to the oral glucose tolerance test (OGTT). This model was designed to evaluate the possible acute antihyperglycemic activity of aqueous extract by inhibiting the intestinal glucose absorption, a known target of some commercial antidiabetic drugs, e.g., guar-gum and acarbose (Krentz and Bailey, 2005). Flavonoid phloridzin, a known SGLUT-1 inhibitor, was used as a reference compound and it showed its characteristic glycemic peak with a 30 min delay without area under glycemic curve (AUC) variation compared to control, which is consistent with a reversible intestinal glucose absorption inhibition (Paulo et al., 2008).

However, the profile and AUC of glycemic curves obtained after the oral single administration of *C. tinctoria* aqueous extract were no different from the control. These results ruled out the hypothesis of glucose transport inhibition by metabolites of *C. tinctoria*, at least at the tested doses, even though there is a clear structural similarity between the chalcone glucoside marein, the major flavonoidal constituent in *C. tinctoria*, and the reference compound, dihydrochalcone glucoside phloridzin.

The efficacy of antidiabetic plant materials can vary with time, so after having preformed an acute study, subchronic administration was in order. Therefore a second model was developed so that the effect of a continuous oral administration of the extract on PPHG of glucose-tolerant and -intolerant male Wistar rats could be evaluated.

Glucose intolerance was achieved by injecting a low dose of streptozotocin (40
mg/Kg i.p.). This substance has been frequently used to induce either type 1 or type 2 diabetes (Frode and Medeiros, 2008). It accumulates preferentially in pancreatic beta-cells via the GLUT-2 glucose transporter, where it targets mitochondrial DNA leading to impairment of mitochondrial signaling function and consequent induction of beta-cell apoptosis through several mechanisms, which includes caspases activation and ROS production (Lenzen, 2008; Steppel and Horton, 2004).

Even though, conscientious of its limitations, we have obtained a reliable method of induction of impaired glucose tolerance and/or mild insulin-deficient diabetes in the adult Wistar rat, characterized by presumably reduced beta-cell mass (destruction of some population of pancreatic beta-cells by low-dose STZ) and consequent disturbed residual insulin secretion leading to a decreased ability to handle with glucose leading to a modest hyperglycemia during an oral glucose tolerance test (OGTT). Therefore the resulting model presented fasting blood glucose levels similar to normal control, but higher area under the glycemic curves. This was probably due to a deficiency in the early-phase insulin secretion caused by the STZ-induced partial destruction of pancreatic cells, as evidenced by the normal glycemic levels reached after 180 min.

The oral administration of *C. tinctoria* infusion (500 mg/Kg/daily; 20 mg marein) to glucose-intolerant rats for three weeks restored the normal response to glucose load. Although the blood insulin levels were not determined, such recovery could result from normal glucose-induced insulin secretion from pancreas of experimental animals.

In order to determine whether the activity shown by the infusion was exclusively due to its major constituents, a flavonoid-rich AcOEt fraction was prepared and tested in a dose that reflected the same amount of marein present in the active infusion concentration (500 mg infusion sample A – 20 mg marein/Kg, equivalent to 125 mg/Kg AcOEt fraction sample A). Results indicated a glucose tolerance regain induced by this flavonoid-rich fraction together with a more rapid improvement of glucose tolerance, taking only 2 weeks for these values to normalize.

These data support the hypothesis that flavonoids and phenyl-propanoic acids are the active constituents of *C. tinctoria* flowering tops extracts. Taking into account that the amount of marein in both extract and fraction was the same, the faster glucose tolerance
effect (observed when the fraction was used to treat glucose-intolerant rats when compared with the infusion treated animals), can only be due to variation on the relative amount of polyphenols in both AcOEt fraction and infusion.

Regarding hepatotoxicity, when analyzing the biochemical data (liver transaminases, AST and ALT) the results suggest that both *C. tinctoria* aqueous extract (500 mg/Kg) and AcOEt fraction (125mg/Kg) administered for 21 days had no toxic effects on liver of glucose-intolerant and normal rats.

Considering pancreatic function, lipase values were also monitored at the end of the treatment with the AcOEt fraction. Lipase levels have already been reported to be increased in diabetes, namely in experimentally-induced diabetes using STZ (Duan et al., 1989) and, in this study, similar behavior was observed. Treatment with *C. tinctoria* flavonoid-rich AcOEt fraction (sample A) for 21 days was able to reverse the pancreatic function impairment. Curiously the above mentioned study showed that insulin replacement reversed the changes on activity and mRNA levels of lipase, therefore, they assumed insulin deficiency was responsible for the observed alteration.

In order to explain these results, two possible mechanisms of action were proposed: induction of insulin secretion by a direct effect of *C. tinctoria* flavonoids on beta-cells, or recovery of beta-cell mass. Although *C. tinctoria* extract up to 300 mg/Kg caused no change on glycemic curve in an OGTT in normal rats, the hypothesis of induction of beta-cell insulin secretion was not ruled out, since that experimental observation could be due to the short period of time (10 min.) between administration of extract and glucose load, comparing with other experimental models in which insulinotropic samples were administered 30 min. prior to glucose load (Govindarajan et al., 2008).

Several studies demonstrated that beta-cell mass reduction during the course of diabetes is due to an increased apoptotic rate of these cells whereas the neogenesis rate is unaltered (Butler et al., 2003). Oxidative stress, generated by hyperglycemia, is among the factors that can damage beta-cells (Brownlee, 2003) and so, antioxidant therapy has been postulated as a possible intervention to delay diabetes evolution and its complications (Maritim et al., 2003). Antioxidants, like N-acetyl-L-cysteine and
aminoguanidine, were shown to protect beta-cells from glucose toxicity and blunt progression of diabetes in a rodent model (Tanaka et al., 1999). Oral administration of vitamins E and C for a short period of time to STZ-induced diabetic rats decreased the oxidative stress condition but not the diabetic parameters (Rupérez et al., 2008).

Besides, there is evidence that flavonoids (as presented in Chapter 2, Section 2.5.2.6) like the antioxidants mentioned above, can increase viability of beta-cells exposed to STZ or other oxidative stress conditions (Thomas et al., 2007; Prince and Kamalakkannan, 2006; Coskun et al., 2005) and they can act as well on intracellular ROS and in that way improve beta-cell function (Lee et al., 2010; Kim et al., 2007b). There are even studies that correlate flavonoid intake to increase in beta-cell mass (Chapter 2, section 2.5.2.4) either by inhibiting apoptosis or promoting proliferation of beta-cells after being exposed to an oxidizing agent (Pinent et al., 2008). Additionally, antioxidant defense system appears to be under-expressed in pancreatic cells (Hotta et al., 2000) but flavonoid-rich extracts administration to diabetic animal models has been shown to increase expression of enzymes like catalase and superoxide dismutase as well as glutathione peroxidase system (Kim et al., 2009; Bagri et al., 2009).

Although antioxidant therapy for type 2 diabetes treatment is controversial (Laviano, 2007), increased oxidative stress due either to fasting or postprandial hyperglycemia is accepted as a participant in the development and progression of diabetes due to increased beta-cell damage (Bonora, 2008).

Therefore, the logical choice for the positive control in the experiment was an antioxidant. However, daily oral administration for 21 days of the standard antioxidant Trolox (50mg/Kg) was not sufficient to reverse the glucose-intolerance state of Wistar rats. These results can have one or more of the following explanations:

(i) the antioxidant capacity of 125 mg of *Coreopsis tinctoria* AcOEt fraction (sample A) was higher than that of 50 mg of Trolox, which was indeed verified later (Appendix I).

(ii) the active flavonoids of *Coreopsis tinctoria* extract have better intestinal absorption than Trolox,

(iii) *Coreopsis tinctoria* flavonoid metabolites are better antioxidants than Trolox
metabolites, or,

(iv) *Coreopsis tinctoria* chemical constituents and Trolox act differently on glucose-intolerant rats.

### 6.4. Search for active extracts constituents and investigation of the mechanism of action

To further understand *C. tinctoria*’s extracts antihyperglycemic activity, *in vitro* studies were developed. Being aware of the dangers of such a reductionist approach when looking for new compounds to treat a particular disease state a portfolio of tests backed up by *in vivo* studies are frequently used (Houghton, 2001).

In this study, *in vivo* experiments pointed to glucose tolerance regain possibly through pancreatic function recovery. This means that the extracts could be exerting their effect by acting directly on the pancreas, either though an insulinotropic effect or through a cytoprotective effect. These hypotheses were tested using a mouse insulinoma cell line (MIN6 pancreatic beta-cells).

Several extracts containing flavonoids have been shown to be able to induce insulin secretion from MIN6 pancreatic beta-cell line (Sharma et al., 2008), which is a well-characterized experimental model for *in vitro* studies of insulin secretion that facilitates the study of agents, which are thought to have direct effects on the beta-cell (Persaud, 1999).

In order to exclude non-specific release of insulin caused by *C. tinctoria* extracts cytotoxicity, cell viability was assessed by trypan blue exclusion assay, and no cytotoxicity was detected at concentrations up to 1 mg/mL.

Results from static incubation experiments indicated that both *C. tinctoria* aqueous extract and AcOEt fraction, up to 1 mg/mL, had no effect on insulin secretion. Previous reports (Hauge-Evans et al., 1999; Josefsen et al., 1999) stated that MIN6 cells lose responsiveness to glucose in later passages as monolayer cultures. However, this loss of responsiveness to nutrients does not impair the general insulin secretory mechanism, and MIN6 cells respond appropriately to other non-nutrient stimuli (Hauge-Evans et al., 1999; Govindajaran et al., 2008). This was confirmed in the present study using insulin
secretagogues PMA (phorbol 12-myristate-13-acetate) a PKC activator and FSK+IBMX (Forskolin + Isobutyl-methylxanthine) adenyl cyclase activators.

In the subsequent experiment, the cells were configured as three-dimensional pseudoislets, which have shown to greatly increase their response to glucose (Hauge-Evans et al., 2002). In this experiment MIN6 pseudoislets also responded better both to 20 mM glucose and to the insulin secretagogues used as control but not to C. tinctoria aqueous extract nor to the AcOEt fraction at the tested concentration (1mg/mL). From these experiments, static incubation and perifusion, it seems that C. tinctoria does not possess an insulinotropic effect, at least at the tested concentrations.

This new information, along with the in vivo data, pointed to a stronger than Trolox antioxidant-mediated mechanism of pancreatic beta-cells ROS-injury protection, or to any other mechanism like inhibition of ROS-independent apoptosis or proliferation promotion, possibilities that were considered and were subsequently tested.

In epidemiologic studies some flavonoids are found to relate to aging disease prevention. Such protection could be due to their antioxidant action (Thabrew et al., 1998). As antioxidants, polyphenols can protect cells against oxidative damage and, therefore, limit the risk of oxidative stress-related degenerative diseases, like cardiovascular and neurodegenerative diseases and diabetes (Giugliano et al., 1996).

In the present study, another in vitro model was developed in order to assess C. tinctoria extracts cytoprotective capacity against oxidative stress using a pancreatic beta-cell line (MIN6). The chosen oxidative agent, tBHP, is a known potent oxidant that can induce strong oxidative damage. As a result, treatment with this agent substantially decreased MIN6 cell survival rate in a time-dependent manner (Kweon et al., 2004). In our experimental conditions, exposure to 400 µM tBHP for 2 hours mainly induced cell death by necrosis, which is in accordance with the decrease in viability.

Pre-incubation with C. tinctoria extract and fraction significantly prevented tBHP-induced cell damage and so did C. tinctoria isolated compounds and main flavonoids, marein and flavanomarein.

In pre-incubation experiments, in which cells are exposed to the toxic agent in fresh
medium after 24h pre-incubation with the test compound, it is expected that tBHP-induced damage will be due to cellularly mediated effects, such as e.g. increased enzymatic and non-enzymatic cellular antioxidants (Ramos et al., 2008). Therefore an extracellular mode of protection would be less likely to happen in this experimental model, where extracts or tested compounds are removed from the culture medium before tBHP treatment.

The observed cell viability increase promoted by C. tinctoria extracts could be due to the antioxidant properties of its constituents, mainly chalcones and flavanones. Flavonoids and polyphenols compounds in general are known antioxidants and thus are potential candidates that have been shown to protect cells from oxidative injury and prevent cell death (Lee et al., 2010; Kim et al., 2007a; Prince and Kamalakkannan, 2006; Coskun et al., 2005; Choi et al., 2003). Several studies have shown that many flavonoids including quercetin can increase cell GSH content, the activity of antioxidant and phase 2 enzymes as well as inhibit cytochromes P450 (Kang et al., 1999; Ferguson, 2001; Alía et al., 2005; Moon et al., 2006). Also treatment with a chalcone derivative, panduratin A, has shown to protect against elevated ROS levels, lipid peroxidation and disruption of intracellular antioxidant systems, induced by tBHP, which was probably due to the compound’s capacity to quench radical species (Sohn et al., 2005).

The antioxidant activities of C. tinctoria aqueous extract, AcOEt fraction and their main flavonoid marein, have been previously determined through the in vitro cell-free DPPH method. C. tinctoria infusion revealed good radical scavenging activity (EC$_{50}$ = 21.0 µg/mL) and C. tinctoria AcOEt fraction (EC$_{50}$ = 7.2 µg/mL) just like marein (EC$_{50}$ = 16.7 µM or 7.5 µg/mL) were approximately three times better radical scavenger than the food antioxidant BHT (EC$_{50}$ = 47.3 µM or 10.4 µg/mL) (Appendix I). Additionally, results from the ORAC assay (Appendix I), using Trolox as antioxidant reference, show that the antioxidant capacity of freshly prepared aqueous extract is equal to that of the common antioxidant Trolox (1.1 g TEAC / g of aqueous extract) whereas the antioxidant capacity of AcOEt fraction (3.4 g TEAC / g AcOEt fraction) is three times higher.

Overall these results show that contrary to aqueous extract (500mg/Kg/day) and AcOEt fraction (125 mg/Kg/day) Trolox at 50mg/Kg/day was unable to reverse the
glucose-intolerance in Wistar rats, as antioxidant capacity of Trolox at that daily dose was, at least, 10-fold inferior to that necessary to be as effective as the aqueous extract after three weeks of treatment. Also, during the in vivo studies, Trolox’s solubility in water (at the applied concentration) was low, which did not facilitate higher concentrations to be tested.

In the present study *C. tinctoria* extracts and main compounds, chalcone and flavanone glucosides, have proven to protect MIN6 cells from tBHP-induced cell damage, but whether the protection provided was due to decreased ROS levels it was still to be determined.

Therefore, in order to evaluate if *C. tinctoria* extracts were involved in decreasing radical oxygen species, superoxide anion production was measured through a lucigenin-enhanced chemiluminescent method. The results show that, under the experimental conditions used, there was a significant increase in that particular radical oxygen species when MIN6 cells were incubated with the pro-oxidant tBHP, and hence an increase in luminescence was verified. Data from this experiment also showed that the ROS-dependent luminescence was quenched by the addition of SOD enzyme, but not by *C. tinctoria* extracts. Unlike many phenolic-rich extracts, which have shown to scavenge ROS in vitro (Kweon et al., 2004), pre-incubation of MIN6 cells with *C. tinctoria* extracts could not abrogate tBHP-mediated intracellular ROS, although it still yielded protection to cells from tBHP damage.

This experiment has demonstrated that *C. tinctoria* extracts were unable to decrease superoxide concentration caused by tBHP treatment. However, it is important to state that there are other factors involved in tBHP-induced oxidative damage (Kweon et al., 2004). Measurement of other parameters, such as antioxidant enzymes depletion and lipid peroxidation or even using a more inclusive probe e.g. Diclhlorofluorescein (DCF), that would measure the presence of other ROS, could help confirm these results.

Still, these results seemed to point to a ROS-independent mode of protection so the search for a mechanism responsible for *C. tinctoria* cytoprotection against tBHP-induced cell injury continued. Thus, another hypothesis for the cell viability preservation observed in this study was proposed: Could the *C. tinctoria* extracts and pure compounds provide
beta cell mass preservation through an antiapoptotic mechanism?

As previously mentioned, flavonoid intake has been correlated to an increase in beta-cell mass either by inhibiting apoptosis or promoting proliferation of beta cells (Chapter 2, section 2.5.2.4). Particularly, chalcones and flavanones, have already demonstrated therapeutic activity against cancer cells interfering with cell cycle progression, ROS production and apoptosis (Yadav et al., 2011; Kuo et al., 2010; Hsu et al., 2006; Singh and Agarwal, 2006). The action of chalcones in interfering in cell functions involved in inflammatory responses, and their antioxidant activities has also been previously documented (Nobre-Júnior et al, 2009, Herencia et al., 2001).

In order to verify the capacity of *C. tinctoria* extracts to protect MIN6 cells by preserving beta-cell mass through inhibition of apoptosis, cells were incubated with the extracts and pure compounds prior to treatment with a mixture of pro-inflammatory cytokines; IL1-beta, TNF-alpha and IFN-gamma.

Even though tBHP can also induce cell death through apoptosis (Zhao et al., 2005), pro-inflammatory cytokines (interleukin-1beta; interferon gamma; tumor necrosis factor alpha) are a more specific and reliable method for apoptosis induction in beta cells, since they have been shown to induce mitochondrial stress, cytochrome c release, activation of caspase-9 and -3, and DNA fragmentation in various types of pancreatic beta-cell lines, human and rat islets (Grunnet, 2009; Papaccio et al., 2005; Chang et al., 2004; Barbu et al., 2002) and have been implicated in the elimination of beta-cells in type 1 and 2 diabetes (Spranger et al., 2003; Eizirik and Mandrup-Poulsen, 2001).

Results confirm cytokine’s capacity to activate caspase 3/7 and thus induce apoptosis in MIN6 cells. And again, *C. tinctoria* extracts and pure compounds (at tested concentrations) presented no signs of toxicity, as values for caspase activation (translated as % of cell death through apoptosis compared to untreated cells) were not significantly different from untreated control.

Pre-incubation with *C. tinctoria* aqueous extract (Sample D) has presented significant apoptosis reduction at 50 µg/mL (2.04 µg/mL which corresponds to 4.53 µM of marein). An even more intense reduction was observed with the AcOEt fraction (Sample C) at 50 and 100 µg/mL (21.57 and 43.14 µg/mL which corresponds to 47.93 µM of marein).
µM to 95.87 µM of marein, respectively) showing a dose-dependent effect.

Searching for increased cytoprotection (with an even more significant reduction of apoptosis) pure marein and flavanomarein at increasing concentrations (200 and 400 µM) were independently pre-incubated with MIN6 cells. Results show significant reductions in caspase 3/7 activity although there was no dose-dependent effect. In this case reduction in apoptosis reached around 30% and didn’t go any further. A possible explanation for this behaviour could be that at elevated doses (more than 100 µM of pure compound) cell uptake was somehow blunted. But more tests regarding intracellular concentration of the flavonoids and related metabolites would be needed to confirm this statement. Also, the fact that incubating the AcOEt fraction (sample C), at 50 µg/mL, proved to be more efficient in apoptosis reduction (around 50% compared to untreated control) which could be explained by a synergistic effect of the other compounds present, namely flavanomarein and other flavanones, chalcones and phenylpropanoic acids.

Overall, these experiments show that the cytoprotective effect of *C. tinctoria*’s extracts and pure compounds is probably not directly due to an antioxidant capacity but most likely a consequence of their capacity to interact with the apoptotic signalling pathway in MIN6 cells, inhibiting it and therefore protecting pancreas against cell injury.

The discovery of the mechanism by which *C. tinctoria* extracts and major compounds prevent tBPH- and cytokine-induced cell damage by apoptosis inhibition can aid understanding the mechanisms involved, not only in beta-cell protection but also possibly explain beta-cell recovery after exposure to oxidative conditions.

Such discovery is of great interest since it might be exploited therapeutically for type 2 diabetes by preventing the progressive loss of beta-cell mass.

Finally, when observing the “whole picture” of the pharmacological approach, concerning *C. tinctoria* claimed antidiabetic activity, there is a need to understand whether data obtained from *in vitro* experiments is translated to the *in vivo* results and vice-versa, in other words: Could cytoprotection, through an antiapoptotic effect, provided by *C. tinctoria* extracts major constituents, be the mechanism responsible for glucose tolerance regain and pancreatic function recovery verified in the STZ-induced
glucose intolerant Wistar rats submitted to prolonged treatment with those extracts?

To answer this question, a couple of issues immediately arise:

i) bioactive compounds bioavailability and,

ii) *in vitro* model’s limitations.

Firstly, there are no strict guidelines to be followed on the concentrations to be used *in vitro*: different studies have used concentrations that range from nanomolar to hundreds of micromolar or even millimolar levels though it’s quite clear that the concentration and chemical form of bioactive compounds *in vivo* need to be considered in any interpretation of *in vitro* data concerning potential beneficial or deleterious effects (Williamson, 2002).

It is relevant to know the absorption and metabolic fate of dietary flavonoids in order to better understand their *in vivo* function. Although the monitoring of flavonoid metabolites was not part of this research, there are already some studies in which it has been done. In an earlier study (Yoshimura et al., 2009) chalcone naringenin (20 mg/Kg) when orally administered to male Sprague Dawley rats presented one major plasma metabolite, identified as a naringenin-glucoronide. This metabolite presented a peak concentration of 5 µM at approximately 1 hour after administration.

Considering that the effective *in vivo* concentration of *C. tinctoria* extracts (500 mg/Kg of aqueous extract and 125 mg/Kg of AcOEt fraction) also had 20 mg/Kg of chalcone marein, a similar amount (in micromolar range) of marein is also expected to be available in the plasma of the rats used in our study. Additionally, as in the present study, *C. tinctoria* extracts were administered subchronically, for 21 days, so it is possible that accumulation in plasma did occur. Besides, *C. tinctoria* extracts are not only rich in chalcone marein but in other flavonoids as well. Extracts HPLC-DAD-MS/MS analysis has shown that flavanomarein is also present in significant amounts (Chapter III, section 3.5). Taking into account the factors above and that the amounts present in the plasma are the ones that reach the tissues, the pure compounds that were used for *in vitro* beta-cell experiments were tested at micromolar range (200-400 µM).

In the present study, glycosilated flavonoids known to be abundant in both *C. tinctoria* extract and fraction were pre-incubated with MIN6 cells and have presented
significant cytoprotective capacity possibly through an apoptosis prevention mechanism. There have been some studies where flavonoid glycosides, such as rutin, delphinidin and cyanidin glucosides and rutinosides, were less effective or even ineffective protecting cells from tBHP-induced cell injury (Ramos et al., 2008; Lazzé et al., 2003). Thus it seems that the presence of a sugar moiety could diminish the protective effect. A possible explanation for this behavior has been suggested by Youdim and colleagues in 2000, who proposed antocyanin glucosides cell uptake could be subject to hindrance, owing to the bulkier glucoside structure. However a recent study (Chen et al., 2010), reported the protective effect of a flavanone glycoside, hesperidin, preventing LDH release, ROS generation and lipid peroxidation, which makes consensus, on this matter, hard to reach.

Nevertheless, in an in vivo situation, dietary glucosides can still play a role in the chemoprevention, since it is known that they can be deglycosylated to yield the correspondent aglycones in the intestine by colon microflora (Ramos et al., 2008; Scalbert and Williamson, 2000). They are then absorbed into the intestinal cells by passive mechanisms. The absorbed aglycone may be efficiently conjugated as glucoronides, sulfates or methylated derivatives (Chen et al., 2006). These conjugates are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiologic behavior is likely to be different from that of the native compounds (Kroon et al., 2004).

Due to commercial unavailability, C. tinctoria main compounds aglycones neither the conjugates were additionally tested in vitro. However, it is possible that the flavonoid glucosides (marein and flavanomarein), tested in the beta-cells, have been subject to hydrolysis and thus loose the glucose moiety. This has been shown to happen before (Theron et al., 1994) due to the presence of beta-glucosidases in the fetal calf serum present in the culture medium.

Aware of in vitro experiments limitations and knowing that they are completely independent and do not exactly mimic the in vivo situation, these results still provide interesting and relevant information concerning possible mechanisms of action of the flavonoids present in C. tinctoria flowering tops extracts regarding it’s claimed antidiabetic effect.