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Traditional antidiabetic plants might provide new oral hypoglycemic compounds, which can counter the high cost and poor availability of the current medicines for many rural populations in developing countries. However, detailed studies on the efficacy, mechanism of action and safety of plant extracts are needed (Prasad et al., 2009).

4.1. Preclinical methods for evaluating antidiabetic activity and safety of plant extracts

Safety and efficacy evaluation of herbal medicines must rely on an adequate pharmaceutical documentation and should therefore follow both World Health Organization’s (WHO) research guidelines as well as European Medicines Agency (EMEA) guidelines (EMEA, 2005; World Health Organization, 1993).

The primary objectives of non-clinical studies are: (i) to determine whether they support the clinical use of a herbal medicine; (ii) to characterise the range of pharmacological actions; (iii) to define the chemical characteristics of pharmacologically active natural products and their mechanisms of actions.

Pharmacodynamic and general pharmacological investigations use animal models or bioassays that closely relate to human disease. Test assays can use whole animals, isolated organ or tissues, blood and its components, \textit{ex vivo} and tissue culture cells, and subcellular constituents (Matteucci and Giampietro, 2008).

The guidelines that apply to pre-clinical testing of a new chemical entity do not necessarily apply to a traditional plant medicine. If the purpose of testing is to assess claimed antidiabetic efficacy of the plant as a normal dietary adjunct, a comprehensive program of tests designed for a new chemical entity would not be appropriate. If it is proposed to consume inordinately large quantities of an unrefined extract, chronic toxicity assessments are indicated. Although toxicity tests are normally undertaken in normal, nondiabetic animals, efficacy studies are most useful when undertaken in models that closely represent the clinical target population. When understanding the mode of action is the main objective, efficacy studies ought to be done in more than one model. The selection of non-insulin-dependent models to assess efficacy of antidiabetic plants can also provide
relevant information about mechanism of action. Human type 2 diabetes arises from the combination of two pathogenic features: insulin resistance and beta-cell dysfunction and it is advantageous if a model designed to test efficacy exhibits both of these features (Day and Bailey, 2006).

In order to test possible antidiabetic activity, it is advisable to accurately replicate the traditional method of preparation of the plant, because slight deviations in the method may alter activity. The vast majority of traditional antidiabetic plant materials are taken orally, so initial tests with plant extracts usually involve oral administration in the diet. Oral gavage may be necessary if the smell or taste of the plant creates an aversion to feeding or drinking. In general, parenteral route of administration is unadvisable due to the risk of local adverse reaction. Adverse effects might not occur after oral administration. Thus the enteral route of administration provides a natural barrier against unnecessary toxicity and facilitates the initial dose-ranging studies. Plant extracts are very lightly to be modified chemically as they pass along the alimentary tract; thus enteral and parenteral administration may give rise to a different profile of effects. The efficacy of antidiabetic plant materials may vary with time; this is normally investigated through a sequence of acute, subchronic and chronic studies (Day and Bailey, 2006).

Selection of doses for animal studies should be established by means of a dose–response relationship and in accordance with traditional clinical doses. All studies should include a negative control group (vehicle only) and a positive control group (known drug) (Matteucci and Giampietro, 2008).

The typical measures of glucose homeostasis to be undertaken usually include basal or random blood/plasma glucose, insulin concentrations and oral (or intravenous, or interperitoneal) glucose tolerance tests (OGTT). This test gives information about the reactivity of the organism and the handling of elevated glucose when a test compound is present. The lowering of glucose can be better seen in this type of assay of glucose tolerance (Verspohl, 2002). Mean values of blood glucose for each time point are calculated and time-course of control and treated groups are compared. Area under the blood glucose curve (AUC) can be calculated and compared for both groups (Vogel, 2006).
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Toxicological methods include systemic, local, and special toxicity tests. Acute toxicity test needs a sufficient number of dose levels to determine the lethal dose and an observation interval of at least 7–14 days. In long-term toxicity tests the administration period may range from 2 weeks to 12 months as a function of the expected period of clinical use. Blood or tissue chemistry should include those determinations that may be useful to elucidate the biological activity of an herbal medicine (Matteucci and Giampietro, 2008).

An appreciation of the mode of action of an antidiabetic plant material indicates the types of diabetes against which the material is most likely to be effective. For instance, plant materials that slow intestinal glucose absorption are most effective in the prandial and early postprandial periods and can be used as dietary adjuncts to all types of diabetes. An improvement of glucose tolerance on an oral glucose tolerance test (OGTT) in normal animals can be caused by candidate compounds which inhibit gastric emptying and thereby delay the glucose absorption from the gut (Vogel, 2006). Knowledge of the mode of action also indicates the risk of “over-lowering” blood glucose. Substances that stimulate insulin secretion at low glucose concentrations strongly inhibit hepatic glucose output, or block counter-regulatory mechanisms to raise blood glucose can induce overt hypoglycemia and are often referred as “hypoglycemic”. Substances that enhance or partially mimic insulin action or reduce intestinal glucose absorption are not likely to lower glucose to the extent of hypoglycemia and are considered to be “antihyperglycemic” (Day and Bailey, 2006).

Data obtained from changes in blood glucose is small due to the lack of information concerning the underlying mechanism (pancreatic or extrapancreatic effect). The next step, therefore, could be to look at pancreatic effects (insulin secretion) (Verspohl, 2002). It is relevant to note that measures of beta-cell function (eg. insulin secretion) insulin action (glucose disposal), glucose handling by key glucoregulatory tissues (eg. muscle and liver), and intestinal absorption are likely to yield valuable information (Day and Bailey, 2006). Guidelines underlie the importance of a good animal experiment design, analysing the data correctly, and using the minimum number of animals necessary to achieve the scientific objectives (Festing and Altman, 2002).
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4.1.1. Animal models of diabetes mellitus

Animal models have been extensively used to investigate the *in vivo* efficacy, mode of action and side effects of antidiabetic plants and their active principles. Due to the heterogeneity of diabetic conditions in man, no single animal model is entirely representative of a particular type of human diabetes.

The animal models most widely used are small rodents, because they are less expensive to maintain than larger animals and generally show a more rapid onset of their diabetic conditions consistent with their short lifespan (Bailey and Flat, 2003).

**Animal models of insulin-dependent diabetes** provide a valuable insight into the efficacy of potential adjuncts to insulin therapy in severely hypoinsulinemic states. The main insulin-dependent models are: spontaneous syndromes (Bio Breeding rat; Non Obese Diabetic mouse) and experimentally induced (chemically with streptozotocin (STZ), alloxan or surgically by near-total pancreatectomy). These incur extensive or complete loss of pancreatic beta-cells, and the consequent lack of insulin causes extreme hyperglycemia with glycosuria, polyuria, polydipsia, hyperfagia and weight loss (Day and Bailey, 2006).

**Animal models of Type 2 diabetes** are likely to be as complex and heterogeneous as the human condition. Thus, in some animals, insulin resistance predominates, whilst in others beta-cell failure is more persistent.

Selective inbreeding of animals that spontaneously develop a type 2 diabetes-like phenotype has generated many of the strains used today. Such as the:

i) **Goto Kakizaki (GK) rat**, that develops relatively stable hyperglycemia in adult life and typically presents mildly elevated fasting blood glucose but rises further on challenge with glucose presenting both insulin resistance and impaired insulin secretion;

ii) **the KK mouse**, that gradually becomes obese in adult life, which is associated with insulin resistance, compensatory hyperinsulinemia and islet cell hyperplasia. Eventually, mild hyperglycemia supervenes;

iii) **and the Nagoya–Shibata–Yasuda (NSY) mouse** which spontaneously develops diabetes in an age-dependent manner. Key features include impaired insulin secretion in the face of mild insulin resistance (Rees and Alcolado, 2005).
Much can also be learnt from animals with single gene mutations, such as:

i) the ob/ob: monogenic model of obesity (leptin deficient),
ii) db/db: monogenic model of obesity (leptin resistant),
iii) and the Zucker (fa/fa) rat monogenic model of obesity (leptin resistant).

Some strains maintain euglycemia by mounting a robust and persistent compensatory beta-cell response, matching the insulin resistance with hyper-insulinemia. The ob/ob mouse and fa/fa rats are good examples of this phenomenon. Others, such as the db/db mouse and Psammomys obesus rapidly develop hyperglycemia as their beta-cells are unable to maintain the high levels of insulin secretion required throughout life (Rees and Alcolado, 2005).

In recent years, diabetic transgenic models have been produced by overexpression, knockout of selected genes or replacement with an altered form (knock-in) of a gene affecting beta-cell function and insulin action (Bailey and Flatt, 2003). These last models have the advantage that the modified gene’s expression can be controlled so that it is only expressed in certain tissues or in certain time in the animal development promoting the viability of the model.

Non-insulin-dependent forms of diabetes can also be produced by low dose or neonatal administration of alloxan or STZ (Bailey and Flatt, 2003). These kinds of models of diabetes are considered a screening step in the search for drugs for the treatment of diabetes (Kecskemeti et al., 2002). Streptozotocin (STZ) is an antibiotic derived from Streptomyces achromogenes and structurally is a glucosamine derivative of nitrosourea. Its structural similarity to glucose allows it to enter the pancreatic beta-cell via a glucose transporter-GLUT-2 and causes alkylation of deoxyribonucleic acid (DNA). Furthermore, STZ induces activation of poly adenosine diphosphate ribosylation and nitric oxide release. As a result of STZ action, pancreatic beta-cells are destroyed by necrosis (Mythili et al., 2004). The diabetes induced by STZ is associated with polydipsia and loss in body weight (Kim et al, 2009). Although high-dose STZ severely impairs insulin secretion mimicking type 1 diabetes, low-dose STZ has been known to induce a mild impairment of insulin secretion which is similar to the feature of the later stage of type 2 diabetes (Srinivasan et al., 2005; Reed et al., 2000). Researchers have also developed a rat model that combined a
high-fat diet following low-dose STZ which closely mimics the natural history of the disease (Zhang et al., 2008; Sahin et al., 2007; Srinivasan et al., 2005; Reed et al., 2000). There are other models such as the neonatal STZ (n-STZ) model for type 2 diabetes, that exhibit various stages of type 2 diabetes mellitus, such as impaired glucose tolerance, mild, moderate and severe hyperglycemia. The n-STZ rats exhibit slightly lowered plasma insulin levels, slightly elevated plasma glucose levels and lowered pancreatic insulin content (Lee et al., 2003; Frantus et al., 1987). The beta-cells in the n-STZ rats bear a resemblance to the insulin secretory characteristics found in type 2 diabetic patients (Tormo et al., 1997; Shinde et al., 2001; Morin et al., 1997; Dachicourt et al., 1997; Arulmozhi, 2004).

The potential problem with STZ is that its toxic effects are not restricted to pancreatic beta-cells since it may cause renal injury (Valentovic et al., 2006), oxidative stress inflammation, and endothelial dysfunction (Lei et al., 2005). Despite its widespread use, there is a wide variability in the extent of diabetes depending on species, strain, age, and laboratory, thus limiting the predictability of its effects. Furthermore, the efficacy of STZ varies even in an apparently uniform group of animals receiving the same dose of the compound (Rodrigues et al., 1999).

In general, by using these models of diabetes induced by chemical drugs, the majority of published studies report the amount of reduction of blood glucose that is always evaluated after a period of fasting following acute or chronic treatment with a specific natural product. Comparative studies are carried out with nondiabetic and/or diabetic animal groups treated with known antidiabetic drugs, but results do not permit to further explore the mechanism of action of the studied natural product (Frode and Medeiros, 2006).

The decision about which model to choose, for a particular experiment, usually is multifactorial. Ideally, the experiments should be carried out in several different models, taking into account that none of them completely reflects the complexity of human diabetes mellitus type 2 and that precautions should be taken when trying to extrapolate the findings to the clinical practice (Arias-Dias and Balibrea, 2007).

4.2. Effect of a single administration of C. tinctoria aqueous extract on the blood glucose levels of normal Wistar rats in a situation of oral glucose challenge
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The OGTT was used as a screening method for acute antihyperglycemic activity since the results give the overall effect of the tested sample on the organism handling of elevated blood glucose (Verspohl, 2002). Although this method does not indicate the precise mechanism of action, the profile of glycemic curve is altered in the presence of S-GLUT-1 inhibitors, like phlorizin and isoquercitrin, when administered 10 min prior to glucose load (Paulo et al., 2008). Phlorizin (100 mg/Kg), the reference S-GLUT-1 inhibitor, showed time-dependent antihyperglycemic activity by delaying the glycemic peak in 30 min (Figure 4.1), but the oral single administration of 25, 50, 100 or 300 mg/Kg of aqueous extract (sample A) (corresponding to 1 - 12 mg/Kg of marein) had no effect on profile or area under the glycemic curves (Figure 4.2).

**Figure 4.1.** Effect of phlorizin on blood glucose levels of normal Wistar rats submitted to an oral glucose tolerance test, compared to control. Graphic shows mean ± SEM (n = 6); *p < 0.001 (at 30 minutes).
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Figure 4.2. C. tinctoria aqueous extract effect on blood glucose levels of normal Wistar rats submitted to an oral glucose tolerance test. A- 25 mg/Kg; B- 50 mg/Kg; C- 100 mg/Kg and D- 300 mg/Kg. Graphics shows mean ± SEM (n = 3-6). No statistically significant differences between control and treated groups were found.

4.3. Development of glucose-intolerant animal model

Subjects with impaired glucose tolerance have a fasting glucose level in the nondiabetic range (< 126 mg/dL) but show hyperglycemia (>140 mg/dL) after an OGTT or a meal (O’Keefe et al., 2008). This is due to the loss of the first phase insulin secretion following a meal, an early defect also present in the development of type 2 diabetes.

Injection of streptozotcin (STZ) to animals can cause direct beta-cell destruction resulting in insulin deficiency. High doses of STZ have been used to produce a completely insulin-deficient type 1 diabetes animal model, while low doses can produce a mild insulin deficient state similar to type 2 diabetes (Section 4.1). On day 15 of a high fat diet, intraperitoneal (i.p.) injection of a low dose of streptozotcin (40 mg/Kg) dissolved in citrate buffer (pH 4.5) for five consecutive days produced metabolic changes in mice
resembling a type 2 diabetic state. These animals showed fasting hyperglycemia and increased body weight comparing to non-diabetic control (Arulmozhi et al., 2008). In our model, a single intraperitoneal injection of 40 mg/Kg of STZ dissolved in aqueous saline solution produced, after 7 days, a glucose-intolerant state in male Wistar rats, characterized by normal fasting blood glucose levels (65 ±10.8 mg/dL) but increased AUC\textsubscript{0-180min} in a OGTT (AUC glucose-tolerant control = 20048±1752 vs. AUC glucose-intolerant group = 25646±2499; \( p < 0.05 \)) (Figure 4.3). Additionally, the degree of post-oral-glucose hyperglycemia did not change over the three week period of experiment, i.e. AUC\textsubscript{0-180min} glucose-intolerant control after 3 weeks (26248±1081) was not statistically different (\( p > 0.05 \)) from AUC of the same group at the beginning of experiment (25646±2499). This observation indicates that the animals could not by themselves reverse the STZ-induced damage to pancreas and therefore constitutes a stable and reliable model.

*Figure 4.3.* Glucose-tolerant (Gluc. tol.) and glucose-intolerant (Gluc. intol.) Wistar rat behaviour on OGTT throughout the 21 day subchronical study. Graphics show mean ± SEM (n = 5); * \( p<0.05 \), ** \( p<0.01 \).
Body weight of glucose-intolerant rats followed the same slight increase as the normoglycemic rats (glucose-tolerant control) over the same 3 week period of experiment (Figure 4.4), which is consistent with a pre-diabetic state.

Figure 4.4. Weight variations of glucose-tolerant and glucose-intolerant controls during 21-day subchronical study. Graphic shows mean ± SD (n = 5). No statistically significant differences between glucose-tolerant and -intolerant groups were found.

4.4 Effect of continuous oral administration of *Coreopsis tinctoria* flowering tops aqueous extract on glucose-intolerant rats

The efficacy of antidiabetic plant extracts can vary with time, therefore a subchronical study using glucose-intolerant was developed.

*C. tinctoria* infusion at 100 and 500 mg/Kg was administered daily to glucose intolerant rats, for 21 days by gavage. The results show a tendency in glycemic AUC reduction of glucose-intolerant group treated with 500 mg/Kg of extract, since week 1. After 3 weeks treatment with extract (500 mg/Kg/day), corresponding to a daily dose of 20 mg/Kg of marcin (78) (Quantified by HPLC-UV quantification method 1 in Chapter 3, section 3.2.3), the animals are no longer glucose-intolerant: AUC = 20091±831 mg.min/dL vs AUC (glucose-tolerant control) = 19069±276 mg.min/dL, p > 0.05 but vs AUC (glucose-intolerant control) = 26248±1081 mg.min/dL, p < 0.01 (Figure 4.5). There was no mortality or any toxic reactions observed up to the end of the study period.
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Additionally, twenty one days oral treatment with *C. tinctoria* extract (500 mg/Kg) caused no significant change in hepatotoxicity biomarkers levels, alanine and aspartate transaminases (ALT and AST), comparing with control group (Figure 4.6).

**Figure 4.5.** Blood glucose levels in response to the oral glucose challenge in each group at the end of week 1, 2 and 3 of the treatment. Graphics show the mean of blood glucose AUC’s of test groups ± SEM (n = 5-6); **p > 0.05 ; * p < 0.01.

**Figure 4.6.** Hepatotoxicity biomarkers levels, alanine and aspartate transaminases (ALT and AST) comparing with glucose-intolerant control group. Data express mean ± SEM (n = 5-6). No statistically significant differences were registered between the treated groups and the glucose–intolerant group.
4.5 Effect of continuous oral administration of *C. tinctoria* flowering tops AcOEt fraction on glucose-tolerant and intolerant rats

In order to understand whether flavonoids were the compounds responsible for glucose tolerance regain observed previously, a flavonoid-rich AcOEt fraction (Sample A) was prepared (Chapter 8; Section 8.2.2) and administered to both glucose-tolerant and glucose-intolerant animals.

The results (Figure 4.7) show that after 2 weeks treatment with the AcOEt fraction (125 mg/Kg), containing 20 mg of marein (78) (Quantified by HPLC-UV quantification method 1 in chapter 3, section 3.2.3), was sufficient to revert glucose-intolerance: AUC = 20907±449 mg.min/dL vs. AUC (glucose-tolerant control) = 19782±435 mg.min/dL (P>0.05) and the glucose-tolerance remained unchanged until the end of the experiment.

Additionally, glucose-tolerant rats treated with AcOEt fraction didn’t change significantly the glycemia values on OGTT throughout the three-weeks of experiment, which indicates an antihyperglycemic effect rather than a hypoglycemic one.

Trolox, a known and potent antioxidant, was used for comparative purposes since it has already been reported that mitochondrion-dependent apoptosis in both diabetic rats cardiac tissue and pancreatic cells could be suppressed with antioxidant treatment (Bojunga et al., 2004; Thomas et al., 2007). Besides it has also been suggested that polyphenols, namely flavonoids, can improve glucose-tolerance through a beta pancreatic function recovery whether mediated by an antioxidant mechanism (Chapter 2, section 2.5.2.6) or by an increase in beta-cell mass (Chapter 2, section 2.5.2.4). Even though, in this experiment Trolox at 50 mg/Kg (maximal concentration achieved in aqueous solution) was not able to reverse the glucose-intolerance state after 21 days treatment.

Body weight of animals of all groups in this section have followed the same slight increase observed previously, indicating the absence of a severe metabolic impairment.
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**Figure 4.7.** Area under the glycemic curve obtained through OGTT at the end of the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} weeks of treatment. Animals treated with *C. tinctoria* AcOEt fraction (125 mg/Kg) differ from glucose-intolerant non-treated animals from the end of the 2\textsuperscript{nd} week until the end of the treatment; Data express the mean of blood glucose AUC’s ± SEM (n =6-11); \* \( p<0.05 \).

Hepatotoxicity of AcOEt fraction was accessed at the end of the treatment by analysing the plasma aspartate and alanine transaminases (AST and ALT) and no significant differences were found between treated and non-treated animals neither between glucose-tolerant and intolerant controls (Figure 4.8).

**Figure 4.8.** Biochemical analysis of hepatotoxicity parameters at the end of the treatment with *C. tinctoria* AcOEt fraction (125 mg/Kg): Aspartate transaminases and Alanine transaminases (AST and ALT). Data express the mean of blood glucose AUC’s ± SEM; (n = 6-11). No differences were found between treated and non-treated animals and neither between glucose-tolerant and intolerant animals.
Pancreatic function was evaluated by measuring plasma lipase (Figure 4.9). As expected, glucose-intolerant control group showed significant increase in lipase values (34.6 ± 1.76 U/L; n = 5) due to pancreatic injury by STZ. The results clearly show that the intolerant animals treated with 125 mg/Kg AcOEt fraction (13.5 ± 0.84 U/L; n = 6), after 3-weeks treatment, almost re-established normal values (8.35 ± 0.69 U/L; n = 6) and were significantly different from glucose-intolerant control animals (P<0.001). Once again, AcOEt fraction had no effect on plasma lipase of glucose-tolerant animals.

![Figure 4.9](image)

Figure 4.9. Plasma lipase values at the end of the three-week experimental period. Data express the mean ± SEM; (n = 5-6). Glucose-intolerant animals treated with *C. tinctoria* AcOEt fraction (125 mg/Kg/day) showed lipase values significantly lower than those of glucose-intolerant control (*P<0.001*) but the extract had no effect on glucose-tolerant animals (#P>0.05).

In this chapter it has been shown that:

i) Unlike phlorizin (100 mg/Kg), a dihydrochalcone known for its S-GLUT-1 inhibition effect., *C. tinctoria* flowering tops aqueous extract, rich in chalcone marein (78), at concentration range (25-300 mg/Kg) had no effect on blood
glucose levels of normal Wistar rats when administered 10 minutes before an OGTT;

ii) A low dose of STZ (40 mg/Kg) administered to male Wistar rats (250-300 g) was able to induce a relatively stable and reliable glucose-intolerance model used for subchronical studies (no significant changes in fasting blood glucose levels nor body weight values were observed throughout the 21-day treatment).

iii) *Coreopsis tinctoria* flowering tops orally administered at 500 mg/Kg (20 mg/Kg of marein (78)) once a day for 21 days to glucose-intolerant Wistar rats promoted a significant improvement in blood glucose levels at the end of the 3 weeks of treatment;

iv) When testing the flavonoid-rich *Coreopsis tinctoria* flowering tops AcOEt fraction by daily administrating it at 125 mg/Kg (20 mg/Kg of marein (78)) by oral gavage to glucose-intolerant Wistar rats, not only glucose tolerance was regained already at the end of the second week of treatment but also pancreatic function was recovered after 21 days.

v) Finally, none of the *Coreopsis tinctoria* extracts, at the tested concentrations, has shown to be toxic to the animals (normal levels of liver transaminases: AST and ALT).