THE EFFECTS OF *Clausena anisata* (WILLD) HOOK [RUTACEAE] LEAF EXTRACTS ON SELECTED DIABETIC RELATED CARBOHYDRATE METABOLIZING ENZYMES

by

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Declaration:

"I, **Mfana Henry Mkhombo,** hereby declare that the work on which this research is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being or is to be submitted for another degree at this or any other university or tertiary education institution or any examining body."

Signed ……………………………………

Date ……… of ……………………, 2010

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Abstract

Background:

Clausena anisata (*C. anisata*) (Willd) Hook [Family: Rutaceae] is one of the South African indigenous medicinal plant whose blood glucose lowering effect has been demonstrated in animal models of diabetes mellitus. However, the mechanism(s) by which extracts of these plants exert their blood glucose lowering effect have not been investigated

Objectives of the study:

The objectives of the current study were to investigate *in vitro* inhibitory effects of different *C. anisata* leaf extracts on the activities of human urinary α-amylase, *Bacillus stearothermophilus* α-glucosidase and rat hepatic glucose 6-phosphatase and to characterize the enzyme inhibitory effect of the extracts on these enzymes with respect to the mode of inhibition (type of inhibition) and IC_{50} values (measure of inhibition potency). Another objective of the study was to confirm the enzyme inhibitory effects of *C. anisata in vivo* by measuring postprandial glucose levels in alloxan induced diabetic rats after oral administration of sucrose.

Results:

Aqueous and methanolic extracts of *C. anisata* leaves inhibited human urinary α-amylase with IC_{50} values of 1947 ± 50 and 2436 ± 62 µg/ml respectively. Inhibitions of α-amylase by these extracts were however, significantly less than that of the reference drug acarbose (84 ± 11 µg/ml) ($P < 0.001$). The mode of inhibition of these extracts on human urinary α-amylase appears to be a reversible non-competitive one. Acetone and hexane extracts of *C. anisata* inhibited *Bacillus stearothermophilus* α-glucosidase with IC_{50} values of 1020 ± 32 and 2068 ± 59 µg/ml respectively. Inhibition of *Bacillus stearothermophilus* α-glucosidase by these two extracts was also significantly less than that produced by acarbose (36 ± 11 µg/ml) ($P < 0.001$). The mode of inhibition of *Bacillus stearothermophilus* α-glucosidase by the acetone extract was found to be reversible competitive in this case. Aqueous and methanolic extracts of *C. anisata* leaves also inhibited hepatic glucose 6-phosphatase with IC_{50} values of 493.6 ± 12 and 1012 ± 58 µg/ml respectively. In this case the effect of the aqueous extract was significantly less than that of the reference inhibitor of glucose 6-phosphatase, sodium vanadate (1651 ± 46 µg/ml) ($P < 0.05$). The mode of inhibition of glucose 6-phosphatase by *C. anisata* aqueous leaf extract was found to be irreversible. Furthermore, oral administration of *C. anisata* acetone leaf extract to normal and alloxan induced diabetic rats, 30 minutes before sucrose loading failed to prevent the rise in postprandial glucose levels in these animals.

Conclusions:

Although both *C. anisata* aqueous and hexane leaf extract inhibited human urinary α-amylase and *Bacillus stearothermophilus* α-glucosidase *in vitro*, this does not appear to be the case *in vivo. C. anisata* aqueous leaf extract appeared to be a more potent inhibitor of rat hepatic glucose 6-phosphatase than the known inhibitor of the catalytic subunit of this multi-component enzyme system.

Non-standard abbreviations

AGI	:	Alpha glucosidase inhibitors
DM	:	Diabetes mellitus
MetOH	:	Methanol
EtOH	:	Ethanol
G-6-P	:	Glucose 6-Phosphate
G6Pase	:	Glucose 6-Phosphatase
STZ	:	Streptozotocin
TLC	:	Thin layer chromatography
IC_{50}	:	50 % inhibitory concentration
pNPG	:	p-nitrophenylglucopyranoside
DNS	:	Dinitrosalisylic acid

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CHAPTER 1

INTRODUCTION, AIMS AND STUDY OBJECTIVES

1.1. Introduction

Diabetes mellitus (DM) is a chronic disorder characterized by both postprandial and fasting hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism (Bell, 1991; Balkau *et al*., 2000; Njombeni *et al*., 2006; Ahmed and Goldstein, 2006; Zanatta *et al*., 2007). Diabetic hyperglycaemia results either from an absolute deficiency in insulin secretion (type 1 diabetes mellitus) or insulin action (type 2 diabetes mellitus) or both (WHO, 1999; Ahmed and Goldstein, 2006; Andrade-Cetto *et al*., 2007). Approximately 171 million people worldwide suffer from diabetes mellitus (WHO, 1999; Balkau *et al*., 2000; Njombeni *et al*., 2006) If not controlled, hyperglycaemia can lead to the development of microvascular disorders such as nephropathy and retinopathy and macrovascular complications including cardiomyopathy, neuropathy, atherosclerosis and myocardial infarction (Randle *et al*., 1963; Taskinen *et al*., 1993; WHO, 1999).

One therapeutic approach to prevent postprandial hyperglycaemia is to retard the digestion and absorption of carbohydrates in the gastrointestinal tract through inhibition of enzymes such as α-amylase and α-glucosidase (Swanston-Flatt, 1990; Puls *et al*., 1977; Lebovitz, 1998; Singh *et al*., 2001; Ojewole, 2002). Alpha amylases hydrolyze complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α-glucosidase to monosaccharides which are absorbed through the small intestines into the hepatic portal vein (Smith *et al*., 2005). Inhibitors of both α-amylase and α-glucosidase delay digestion and subsequent absorption of carbohydrates thereby lowering postprandial glucose levels (Puls *et al*., 1977). Examples of such inhibitors which are currently used as anti-diabetic agents include acarbose (Schmidit *et al*., 1977; miglitol (Standl *et al*., 1999) and voglibose (Saito *et al*., 1998).

The liver is the main source of glucose during fasting state. In the fasting state and between meals, the liver releases glucose into the blood circulation through the metabolic pathways of glycogenolysis and gluconeogenesis (Nordlie *et al*., 1985). Studies with inhibitors of enzymes involved in both glycogenolysis and gluconeogenesis suggest that inhibition of these enzymes may be one of the mechanisms whereby some anti-diabetic agents exert their blood glucose lowering effect (Marles and Farnsworth, 1995). In particular, inhibitors of glucose-6-phosphatase (G6Pase),

which catalyzes the terminal steps in both glycogenolysis and gluconeogenesis have been shown to lower fasting blood glucose levels in experimental animal model of diabetes (Singh *et al*., 1992). Furthermore the widely used efficacious drug metformin (Glucophage) is reported to exert its blood glucose lowering effect, in part by inhibiting the expression and synthesis of G6Pase (Goldfine, 2001).

Plants continue to play an important role in the treatment of type 2 diabetes mellitus particularly in developing countries where most people have limited resources and do not have access to conventional anti-diabetic drugs (Ojewole, 2005). Many of the plants that are traditionally used as anti-diabetic remedies have been evaluated and their blood glucose lowering effect confirmed in experimental animal models of diabetes mellitus (Ojewole 2000; Frode and Medeiros, 2006) and clinical studies (Jayawardena *et al*., 2005; Day and Bailey, 2006). However the nature of the bioactive principles in these plants as well as their mechanism of hypoglycaemic action remains largely unknown. Some of the medicinal plants with anti-diabetic properties have been shown to have α-amylase (*Phaseolus actifolium*) and α-glucosidase (*Morus alba*, *Commelina communis*) and glucose 6-phosphatase inhibitory activities, an indication that these plants may exert their blood glucose lowering effects in part through activation or inhibition of these enzymes (Marles and Farnsworth, 1995).

One plant which is used traditionally as an anti-diabetic agent (ADA) and whose blood glucose lowering effect has been confirmed in streptozotocin (STZ)-induced diabetic rats is *C. anisata* (Ojewole, 2002). *C. anisata* also known as maggot killer (English), perdepis (Afrikaans) and isifudu (Zulu) is a South African indigenous plant about 10 m in height that belong the Rutaceae family of plants. In addition to anti-diabetic properties, the leaves of *C. anisata* are reported to possess anti-hypertensive anti-nociceptic and anti-inflammatory activities (Watt and Breyer-Brandwijk; 1962; Adesina, 1982; Hutchings *et al*., 1996; Ojewole, 2002).

An anti-diabetic agent may lower blood glucose levels by stimulating insulin secretion, improving insulin sensitivity or inhibiting glucose absorption from the small intestines and suppressing hepatic glucose production and by mimicking the action of insulin (Cheng and Funtus, 2005). Since the mechanism of hypoglycaemic action of *C. anisata* leaf extracts is currently unknown, it was hypothesized in the current study that the leaf extracts of *C. anisata* have inhibitory effect on some diabetic related carbohydrate metabolizing enzymes.

1.2. Aims and Objectives

The aim of the current study was to study the *in vitro* inhibitory effects of *Clausena anisata* leaf extracts on the activities of selected diabetic related carbohydrates metabolizing enzymes (α-amylase, α-glucosidase and glucose 6-phophatase) and to confirm where possible the result of these *in vitro* studies *in vivo* using normal and STZ-induced diabetic rats. The study had the following objectives

1. To extract dried leaves of *C. anisata* with solvents of different polarities and to screen the resultant crude extracts for inhibitory activity against selected diabetes related carbohydrate metabolizing enzymes (α-amylase, α-glucosidase and glucose 6-phophatase) using standard *in vitro* enzyme inhibition bioassays for these enzymes.

2. To determine and compare the IC_{50} (50 % maximal inhibitory concentration of an inhibitor) values of the crude *C. anisata* leaf extract(s) demonstrating appreciable enzyme inhibitory activities with those of known and commercially available enzyme inhibitors.

3. To determine, by means of Lineweaver-Burk double reciprocal plot the mode of enzyme inhibition of the crude extracts of *C. anisata* demonstrating appreciable enzyme inhibitory activities.

4. To confirm the *in vitro* effect of *C. anisata* on α-amylase and α-glucosidase *in vivo* by measuring postprandial glucose levels after an oral ingestion of sucrose in normal and streptozotocin-induced diabetic rats.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The aim of this literature review is summarize the current knowledge regarding the nature, properties, and characterization of enzymes inhibitors that are used as drugs. Information about diabetes mellitus as well as selected carbohydrate metabolizing enzymes that can be targeted in the treatment of type 2 diabetes mellitus is also included in this review. The review begins with some explanations of why most drugs in clinical use today are enzyme inhibitors, followed by a brief description of the different types of enzyme inhibitors as well as a description of procedures that are used to characterize enzyme inhibitors.

Next, follows a brief description of diabetes mellitus, its classification, pathogenesis and management, as well as descriptions of procedures used to assess and study the activities of α-amylase, α-glucosidase and glucose 6-phosphatase. These three enzymes have been described as possible targets for the treatment of type 2 diabetes mellitus. This is then followed by a brief description of the medicinal plant *Clausena anisata* as well as research that has been conducted into this plant. Lastly, the general procedures used to investigate anti-diabetic medicinal plants are also described.

2.2. Enzyme inhibitors

Enzymes are biological catalysts that are central and key to the life process. In animals, plants, bacteria and viruses, enzymes catalyze biochemical reactions, shuttle intermediate compounds along directed metabolic pathways and provide control over the entire biological processes (Mathews and van Holde, 1998; Smith *et al.*, 2005). Enzyme inhibitors are molecules that bind to enzymes and decrease their activity. Since blocking an enzyme's activity can kill a pathogen or correct a disease-induced metabolic imbalance, many enzyme inhibitors are used as therapeutic agents (Rich, 2005; Copeland, 2005; 2007). Enzyme inhibitors are also used as herbicides and pesticides (Hopkings & Groom, 2002).

2.2.1. Enzyme inhibitor drugs

Medicine in the twenty-first century has largely become a molecular science in which drug molecules are directed towards specific macromolecular targets whose bioactivity is pathogenic or at least associated with disease (Copeland, 2005). Among the biological macromolecules that one can envisage as drug targets, enzymes hold a prominent role because of their essential roles in the life process and pathophysiology. Furthermore, the structure of the enzyme active sites and their ligand binding pockets are ideally suited for affinity interactions with drug-like inhibitors (Rich, 2005; Copeland, 2007). For these reasons, most of the pharmacological drugs used today are enzyme inhibitors. Indeed, a survey conducted by Hopkings and Groom (2002) found that nearly half (47 %) of the therapeutic drugs used in modern clinical practice are enzyme inhibitors (Figure 2.1)

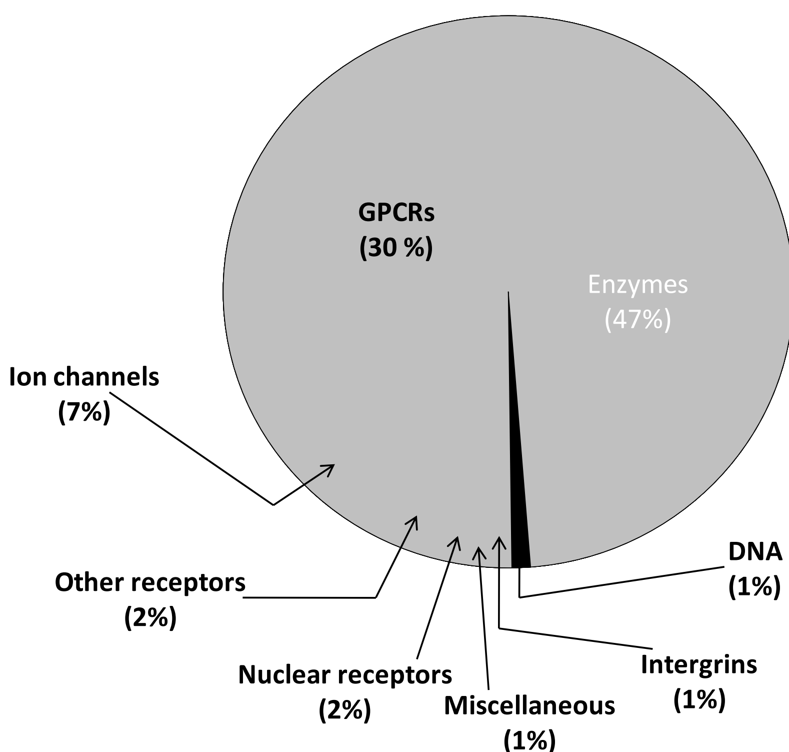


Figure 2.1: Distribution of marketed drugs by biochemical target class. GPCRs + G-protein coupled receptors (Hopkins & Groom, 2002).

A number of natural and synthetic enzyme inhibitors, from the old benchmarks of modern medicine (such as aspirin and penicillin) to newer drugs used to treat HIV and AIDS (e.g Saquinivir), have become over the years successful therapeutic drugs. Some of these drugs, which are in clinical use, are listed in Table 2.1 (Hopkings and Groom, 2002; Copeland, 2005).

Table 2.1: Examples of enzyme inhibitors that have become successful drugs

Inhibitor	Target enzyme	Clinical use
Azetazolamide	Carbonic anhydrase	Treatment of glaucoma
Acarbose	Alpha glucosidase	Anti-diabetic agent
Allopurinol	Xanthine oxidase	Treatment of gout
Aspirin	Cyclooxygenase 2 (Prostaglandin synthase	Anti-inflammatory agent
Captopril	Angiotensin converting enzyme (ACE)	Anti-hypertensive drug
Lovastatin and other statins	HMG-CoA reductase	Cholesterol lowering drug
Methotrexate	Dihydrofolate reductase	Anti-cancer drug
Penicillin	Bacterial transpeptidase	Treatment of bacterial infections
Saquinivir	HIV protease	Treatment of HIV and AIDS
Viagra	Phosphodiesterase	Treatment of erectile dysfunction

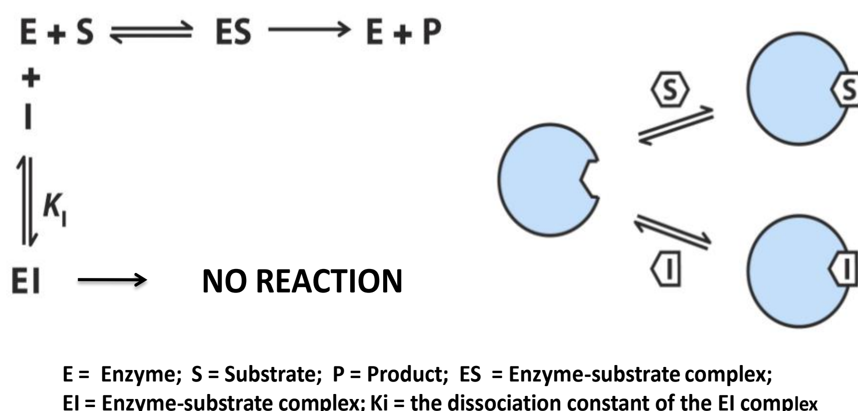
2.1.2 Types of enzyme inhibition

Enzyme inhibition may be irreversible or reversible. Irreversible inhibitors usually form a covalent bond to and permanently modify key amino acid residues needed for enzymatic activity (Smith et al., 2005). Susceptible amino acid residues include Ser and Cys residues, which have reactive -OH, and –SH groups, respectively. Reversible inhibitors bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. In contrast to substrates and irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis (Stryer, 1990; Mathews and van Holde, 1998). There are four different types of reversible enzyme inhibition: competitive,

mixed, non-competitive and uncompetitive. These four types differ in how or when they bind to the enzyme (Stryer, 1990; Mathews and van Holde, 1998, Smith *et al*., 2007).

2.1.2.1. *Competitive inhibition*

A competitive inhibitor is a molecule that resembles the substrate and occupies the catalytic site because of its similarity in structure, but is completely unreactive. By occupying the active site, the inhibitor prevents normal substrates from binding and being catalyzed. Operationally, competitive inhibitors bind reversibly to the active site. Hence, inhibition can be reversed by (1) diluting the inhibitor, or (2) swamping the system with excess substrate (Mathews and van Holde, 1998; Smith *et al*., 2005). The general reaction mechanism for competitive reversible enzyme inhibition is shown on figure 2.2.



E = Enzyme; S = Substrate; P = Product; ES = Enzyme-substrate complex;
EI = Enzyme-substrate complex; Ki = the dissociation constant of the EI complex

Figure 2.2. A general reaction mechanism for competitive reversible enzyme inhibition

The Michaelis Menten equation for competitive inhibition is:

$$V = \frac{v_{max}[S]}{k_m(1 + \frac{[I]}{k_i}) + [S]}$$

Where [S] is the concentration of the substrate, [I] is the concentration of the inhibitor; Km is the Michaelis-Menten constant, Vmax is the maximum velocity and K_i is the dissociation constant of the EI complex (Mathews and van Holde, 1998).

2.1.2.2. *Mixed inhibition*

In mixed inhibition, the inhibitor bind to both the free enzyme [E] and enzyme substrate-complex (ES), but their affinities for these two forms of the enzyme are different ($K_i \neq K_i'$) (Stryker, 1990; Smith *et al*., 2005; Copeland, 2005). The inhibitor binds to the enzyme at a location other than the

active site in such a way that the inhibitor and substrate can simultaneously be attached to the enzyme. The binding of the inhibitor can affect the binding of the substrate, and vice versa. This type of inhibition can be reduced, but not overcome by increasing concentrations of substrate. The general reaction scheme for mixed inhibition is shown below.

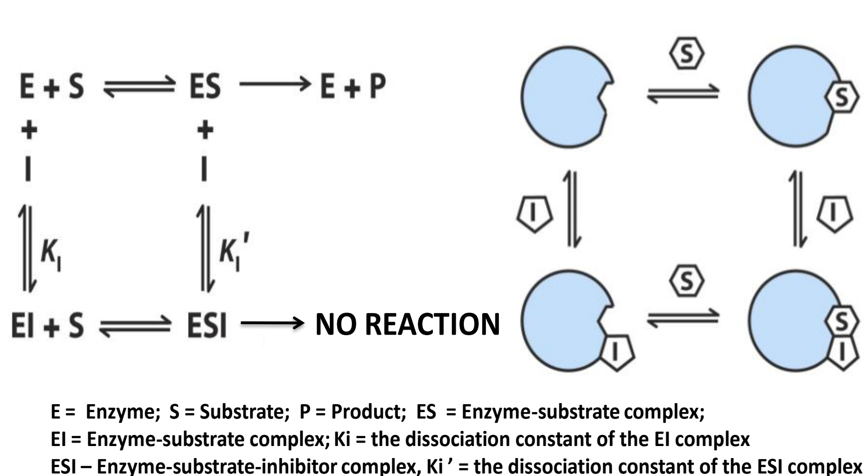


Figure 2.3. A general reaction for mixed inhibition

2.1.2.3. *Non-competitive inhibition*

Non-competitive inhibition is a form of mixed inhibition where the inhibitor has equal affinities for the free enzyme [E] and ES ($K_i = K_i'$) and does not affect the binding of the substrate (Stryker, 1990; Smith *et al.*, 2005; Copeland, 2005). In non-competitive inhibition, the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. As a result, the extent of inhibition depends only on the concentration of the inhibitor. The Michaelis-Menten equation for both mixed and non-competitive inhibition is

$$V = \frac{v_{max}[S]}{k_m(1 + \frac{[I]}{k_i}) + [S](1 + \frac{[I]}{k_i'})}$$

k_i is the dissociation constant of the enzyme-inhibitor (EI) complex and $k_{i'}$ is the dissociation constant of the enzyme-substrate-inhibitor (ESI) complex. In non-competitive inhibition $k_i = k_{i'}$.

2.1.2.4. *Uncompetitive inhibition*

In uncompetitive inhibition, the inhibitor binds to the enzyme only after the substrate has bound. The general reaction scheme for competitive inhibition is as follows

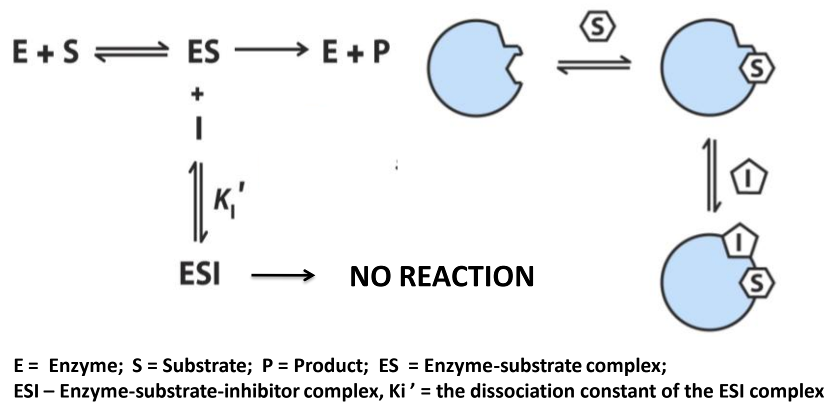


Figure 2.4. A general reaction scheme for competitive inhibition

The Michaelis Menten equation for uncompetitive inhibition is

$$V = \frac{v_{max}[S]}{k_m + [S](1 + \frac{[I]}{k_i})}$$

[I] is the concentration of uncompetitive inhibitor and *ki* is the dissociation constant of the ESI complex.

2.1.3. Characterization of enzyme inhibitors

Enzyme inhibitors are often characterized in terms of their mode of inhibition (inhibitor type) and potency (Smith *et al.*, 2005). Inhibitors of enzymes obeying Michaelis-Menten kinetics are often identified and differentiated from one another by means of their effect on the kinetic constants, K_m (the Michaelis-Menten constant) and V_{max} (maximum velocity of an enzyme) using Lineweaver-Burk plots. In this case, reaction rates of the biochemical reaction catalyzed by the enzyme of interest (*v*) are measured at different concentration of the substrate [S] in the presence and absence of the inhibitor under investigation. Lineweaver-Burk double reciprocal plots of 1/*v* versus 1/[S] are then plotted and both K_m and V_{max} obtained in the presence of the inhibitor are compared with those of the un-inhibited enzyme catalyzed reaction (Figure 2.5)

As it can be seen from Figure 2.5, a competitive inhibitor changes (increases) the apparent value of K_m (reciprocal of the x-intercept) but does not affect V_{max} (reciprocal of the y-intercept). A mixed enzyme inhibitor increases the apparent value of K_m and decreases the apparent value of V_{max}. A

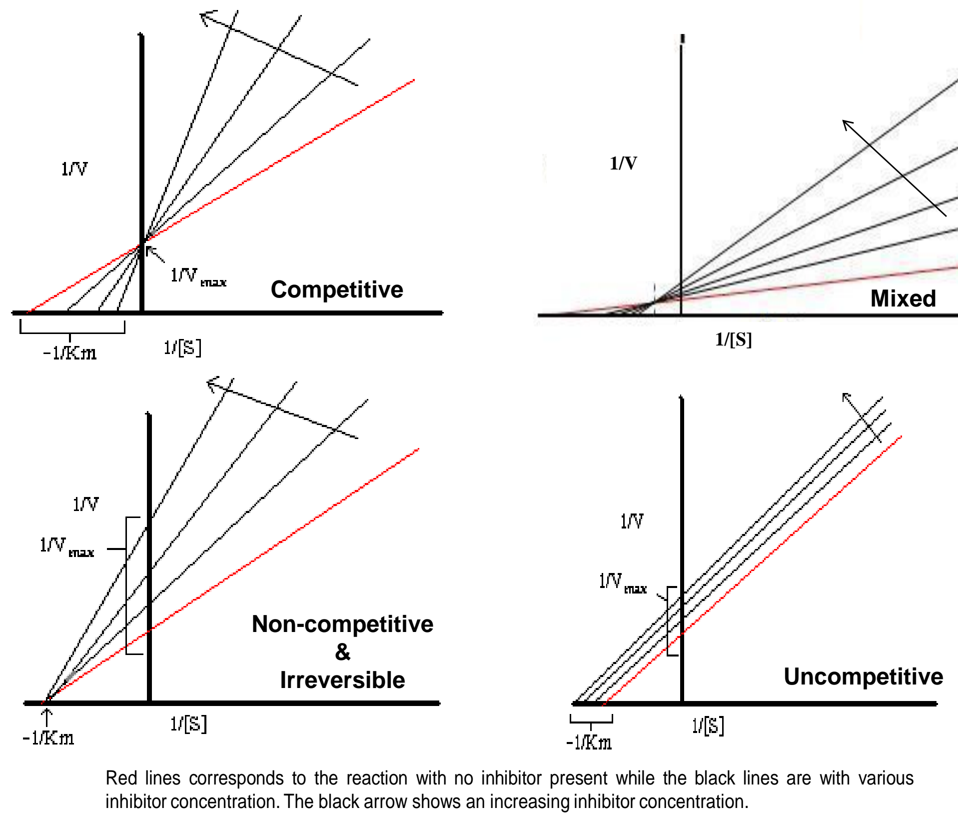


Figure 2.5. Lineweaver-Burk double reciprocal plots of 1/v versus 1/[S] plotted and both Km and Vmax obtained in the presence of the inhibitor are compared with those of the un-inhibited enzyme catalyzed reaction (Mathews and van Holde, 1998)

non-competitive inhibitor decreases the apparent value of V_{max}, but does not affect K_m. An uncompetitive inhibitor decreases both the apparent values of K_m and V_{max}. The Lineweaver-Burk double reciprocal plot of an irreversible inhibitor is similar to that of a reversible non-competitive inhibitor. The two types of inhibitors can, however, be differentiated from each other by diluting the reaction mixtures, re-measuring reaction velocities and re-plotting the double reciprocal plots (Mathews and van Holde, 1998; Smith *et al.*, 2005). If the inhibitor under investigation is a reversible non-competitive one, a change in the double reciprocal plot will be observed after dilution of reaction mixtures, otherwise one will be dealing with an irreversible enzyme inhibitor.

In Biochemistry, the potency (inhibitor strength) of an enzyme inhibitor is usually determined and reported in terms of dissociation equilibrium constants (K_i values) (Benjamin *et al.*, 2003). Pharmacologists on the other hand, often determine and report the potency of a medicinal enzyme inhibitor in terms of its IC_{50} (inhibitor concentration that reduces enzyme activity by 50 %) value.

An IC_{50} value depends on concentrations of the enzyme (or target molecule), the inhibitor, and the substrate along with other experimental conditions whereas inhibition constants are independent of the substrate but depends on the enzyme (target) and type of inhibitor (Cheng and Prusoff, 1973). IC_{50} values and inhibition constants are however, inter-convertible (Cheng and Prusoff, 1973; Benjamin *et al.*, 2003). A simple way of estimating IC_{50} value of an enzyme inhibitor is to determine the reaction rate of an enzyme at different concentration of the inhibitor. Calculate and plot percentage inhibition against inhibitor concentration (or the logarithm of inhibitor concentration) and determine IC_{50} by interpolation (Cheng and Prusoff, 1973)

IC_{50} values, inhibition constants and type of enzyme inhibition can be simultaneously determined graphically by means of Dixon plots. In a Dixon plot the reciprocal velocity (1/*v*) is plotted against concentration of inhibitor at two or more substrate concentrations, and over a range of inhibitor concentrations. A Dixon plot gives a family of intersecting lines. For a competitive inhibitor, the lines converge above the x-axis, and the value of [I] where they intersect is $-K_i$ (Figure 2.6). For a non-competitive inhibitor, the lines converge on x axis, and the value of [I] where they intersect is $-K_i$ (Figure 2.6). For uncompetitive inhibition, the lines are parallel. IC_{50} values are given by the reciprocal of the intersection of the Dixon plot lines with the x-axis (Masataka and Keiko, 2009).

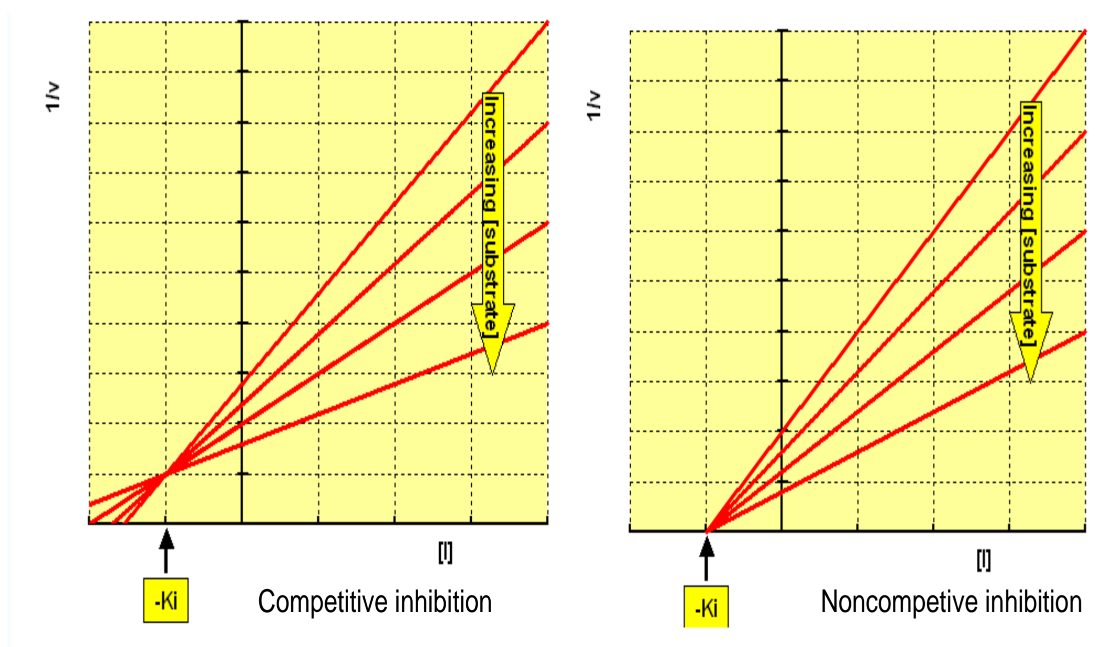


Figure 2.6. Dixon plots for competitive and noncompetitive inhibition (Masataka and Keiko, 2009)

2.3. Diabetes Mellitus

2.3.1. Definition, classification and prevalence of diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose level resulting from either absolute deficiency of insulin or insulin resistance (WHO, 1999; Zanatta *et al*., 2007). Besides hyperglycaemia, there is severe derangement in carbohydrate, lipid and protein metabolism (Ahmed and Goldstein, 2006). Currently, worldwide, it is estimated to affect 151 million people, and this figure is expected to reach 324 million in the year 2025 (Engelgau *et al*., 2004; Ahmed and Goldstein, 2006; Fatima, 2007).

Diabetes mellitus can be classified as type 1, type 2, gestational diabetes (hyperglycaemia of pregnancy) and maturity onset diabetes of the young (MODY) (Ahmed and Goldstein, 2006; Harris, 2000; Scheen and Lefebvre, 2000). Type 1 diabetes mellitus is caused by inadequate or absolute absence of insulin. It is brought about as a result of the organ specific autoimmune destruction of the insulin secreting β-cells of the pancreas by cytotoxic T-cells (Ahmed and Goldstein, 2006). It can also be idiopathic, when there is no evidence of immune-mediated β-cells destruction, but still characterised by inadequate insulin and progressive β-cells loss. The autoimmune destruction on β-cells compromises the production of insulin and the functions associated with it. The primary risk factor of type 1 diabetes is family history. It is also referred to as juvenile onset diabetes mellitus as it affects mostly younger individuals (Harris, 2000; Scheen and Lefebvre, 2000).

Type 2 diabetes mellitus is characterized by progressive deterioration of normal β-cells function (Ahmed and Goldstein, 2006). Individuals have sufficiently high levels of insulin in the blood, however, glucose levels remain elevated, putting a heavy burden to β-cells, eventually β-cells become exhausted and undergo apoptosis (Butler *et al*., 2004). Consequently, insulin production diminishes and glucose is unable to enter the cells and remains in circulation (Rosak, 2001). High risk individuals for type 2 diabetes are senior citizens (adult-onset diabetes mellitus), overweight and physically disabled individuals.

Gestational diabetes is defined as any degree of glucose intolerance which develops or recognized during the 2nd and 3rd trimester of pregnancy, it is therefore considered as diabetes mellitus unmasked by pregnancy (Bottalico, 2007). MODY is associated with autosomal dominant inheritance and is characterized by onset of hyperglycaemia to at least one family member younger than 25 years, correction of fasting hyperglycaemia without insulin for at least 2 years, and the absence of ketosis. At least 6 genetically different types of MODY have been described (Bottalico,

2007). In severe cases, patients ultimately require insulin to control glycaemia. Studies have revealed that more than 80 % of patients presenting with diabetes mellitus suffer from type 2 diabetes (Mycek *et al*., 2000; Maiti *et al*., 2004; Ahmed and Goldstein, 2006). About 15-20 % of patients present with type 1 diabetes mellitus. It has also been reported, though uncommon, that 2-5 % of pregnant woman suffer from gestational diabetes (Urger and Foster, 1998; Maiti *et al*., 2004).

Diabetes mellitus is a serious epidemic affecting both male and female as well as across racial lines. The vast majority of people suffering from diabetes mellitus live in developed countries, and very few and unreported cases occur in developing (Amos, 1997). In the World's highly industrialized countries the prevalence is very high owing to the lush life-style the people are leading (Amos, 1997). In Africa, Latin America and the Asian subcontinents incidences reach high proportions as a result of poor socio-economic status of the people in those areas. (Levitt, 1993; Mollentze, 1995). In South Africa, estimates of adult-onset diabetes mellitus range from about 5% in the white communities, 13 % in those people of Indian descent (Omar, 1985). In the black population, the prevalence is 6-8 % (Levitt, 1993; Mollentze, 1995), and the coloured population mainly in the Western Cape is 8 % (Levitt, 1997).

2.3.2. Pathophysiology of type 2 diabetes mellitus

Type two diabetes mellitus is believed to be caused by both genetic and environmental factors (Cheng and Funtus, 2005). The most important risk factor for type 2 diabetes mellitus is believed to be obesity, in particular central obesity. Research has established that free fatty acids and adipokines from white adipose tissue associated with central obesity mediate insulin resistance (Smith *et al*., 2005). Initially, insulin resistance is compensated for, by increased insulin secretion by pancreatic beta-cell, but with time, these become exhausted and type 2 diabetes results (Cheng and Funtus, 2005; Zanatta *et al*., 2007). The pathogenesis of type 2 diabetes is summarized Figure 2.7.

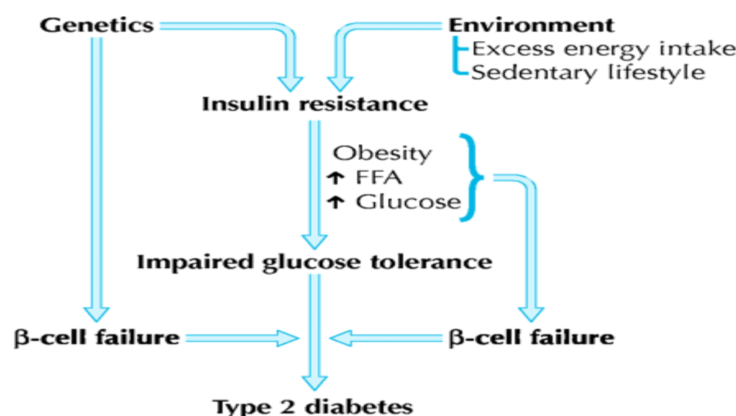


Figure. 2.7 : Overview of the pathogenesis of type 2 diabetes mellitus. FFA = free fatty acids (Cheng and Funtus, 2005)

If not controlled, hyperglycaemia can cause various physiological alterations including disturbances in carbohydrate, lipid, protein and nucleic acid metabolism (Alberti, 1998), This leads to severe metabolic derangements with macrovascular complications including possible amputation of limbs or toes, blindness and chronic fatigue, (Genuth, 2006). If managed properly, diabetes mellitus can be well controlled but if not managed daily, serious medical emergencies like heart attack, strokes, renal failure and comas can arise (Nathan, 2005). Successful management of diabetes mellitus comes with tremendous economic burden on patients, their immediate families and health care providers (Albert, 1997). Diabetic ketoacidosis, retinopathy, nephropathy and neuropathy are associated complications of persistent high levels of blood glucose. Such complications result in microvascular and macrovascular disorders which lead to cardiovascular diseases mainly by accelerating atherosclerosis. As a result coronary and myocardial infarction, angina, stroke and peripheral vascular disease and ultimately death results (Stratton, 2000).

2.3.3. Management of type 2 diabetes mellitus

The major goal in the management of diabetes mellitus is to bring blood glucose within normal physiological levels (Bailey, 2000). Type 2 diabetes patients are advised to eat an appropriate diet, reduce weight and do physical activities. If hyperglycaemia persists, oral anti-diabetic drugs or traditional medicines (anti-diabetic medicinal plants) can be used to control type 2 diabetes (Bailey, 2000). A summary of how type 2 diabetes is managed is given in Figure 2.8. In practice, timely adjustment to dosage or type of both of orally administered antihyperglycaemic agent or insulin or both are made to attain target haemoglobin A_{1c} concentration within 6 to 12 months in order to prevent development of both microvascular and macrovascular diabetic complications (Genuth, 2006).

2.3.4. Mechanism of action of anti-diabetic agents

Antihyperglycaemic agents used to manage and control type 2 diabetes mellitus exert their glucose lowering effects via a variety of mechanisms (Figure 2.9). These mechanisms of action include: reduction of hepatic glucose production, (metformin, a biguanide), enhancement of insulin secretion by pancreatic β-cells, (insulin secretagoges) improvement of insulin sensitivity (TZDs and metformin) and inhibition of intestinal glucose digestion and absorption (α-glucosidase inhibitors). The use of these drugs is however, limited by the fact that they have adverse side effects, such as potential hypoglycaemia (e.g. sulfonylurea), weight gain, (meglitinides, sulfonylurea and thiazolidinesdiones), gastro-intestinal discomforts (α-glucosidase inhibitors, and alpha amylase inhibitors) and lactoacidosis (metformin) (Joe *et al*., 1999 and Evans, 2002; Gahagan *et al*., 2003).

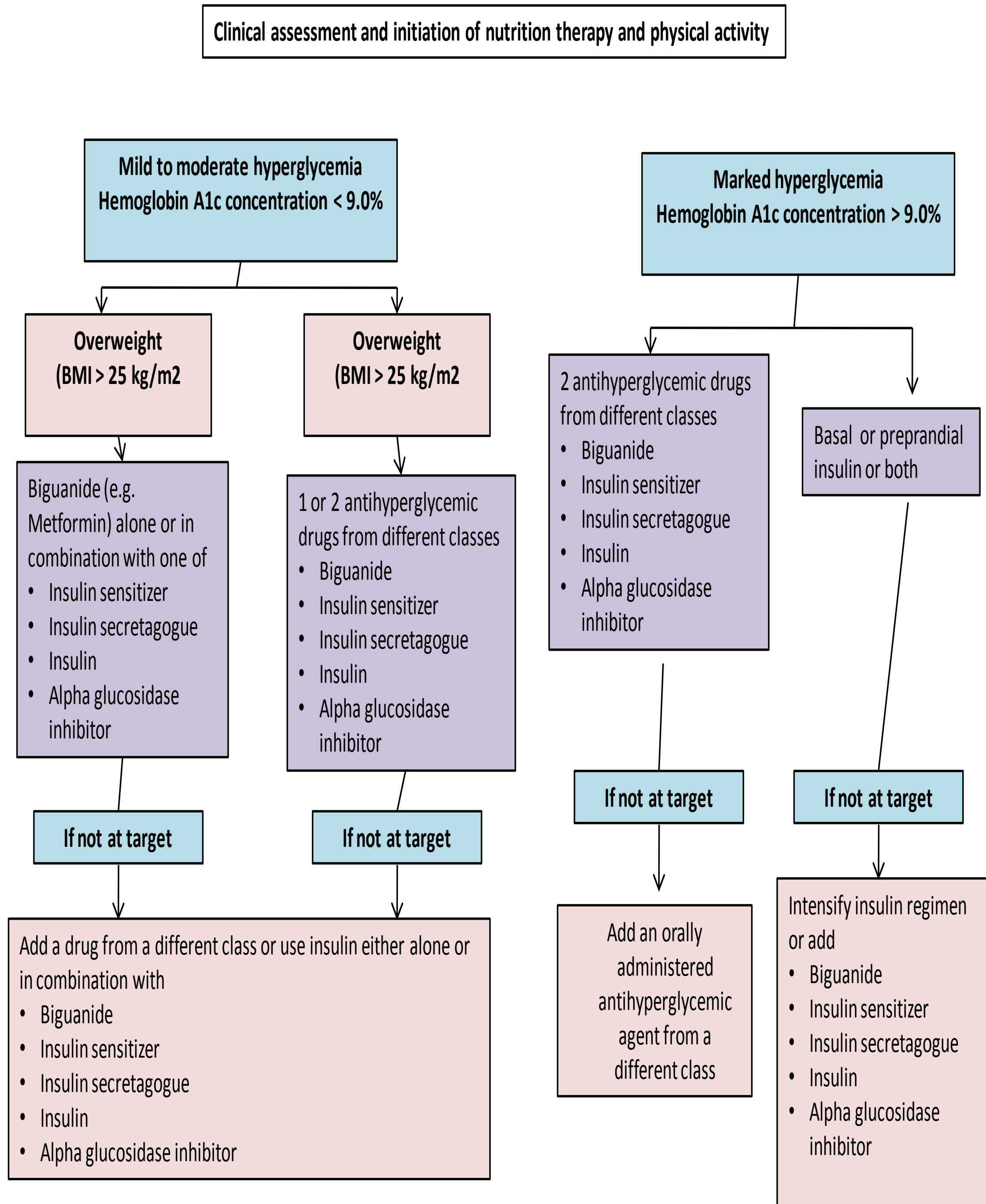


Figure 2.8. Management of type 2 diabetes mellitus

In light of the above mentioned side effects, high secondary failure rates and high costs of conventional type 2 diabetes antihyperglycemic agents, many researchers all over the world have turned their focus towards traditional medicinal plants with the hope of discovering new and save antihyperglycemic agents which exert their blood glucose lowering effects through novel mechanisms.

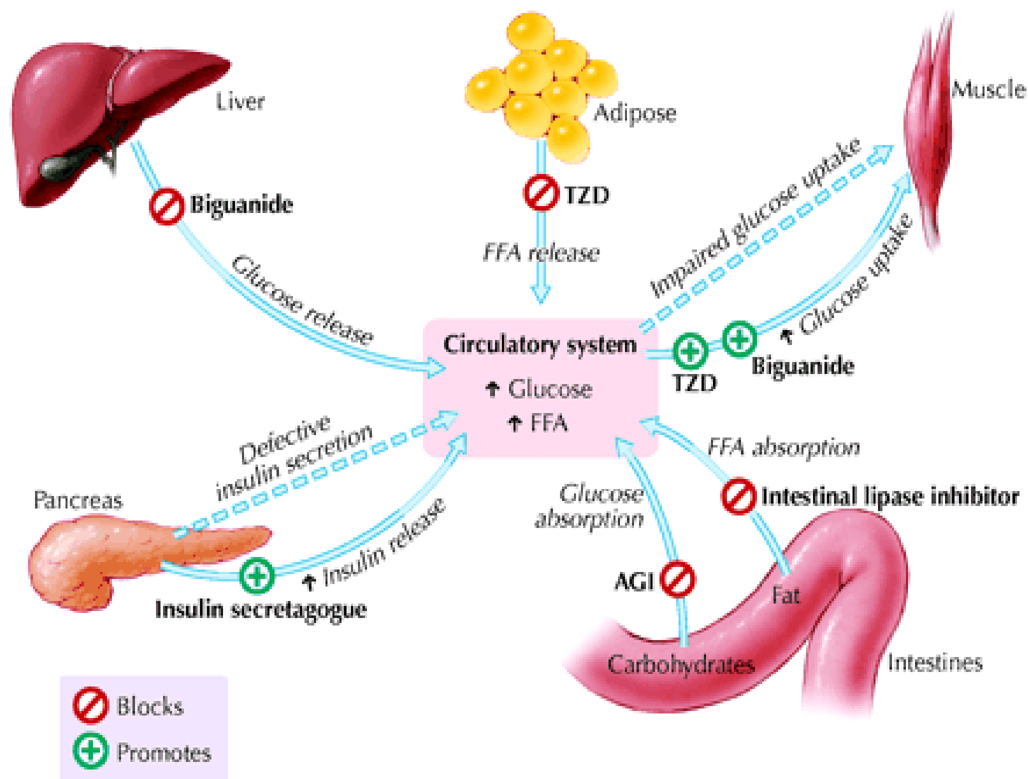


Figure 2.9. Major target organs and actions of orally administered antihyperglycemic agents in type 2 diabetes mellitus. TZD = thiazolidinedione; FFA = free fatty acid; AGI = α-glucosidase inhibitor (Cheng and Funtus, 2005).

A large number of plants that are traditionally used as anti-diabetic remedies have been evaluated and their blood glucose lowering effect confirmed in experimental animal models of diabetes mellitus (Ojewole 2000; Frode and Medeiros, 2006) and clinical studies (Jayawardena *et al*., 2005; Day and Bailey, 2006). However the nature of the bioactive principles in these plants as well as their mechanism of hypoglycaemic action remains largely unknown.

2.3.5. Selected diabetic related enzymes and their inhibitors

In light of the fact that the majority of drugs in clinical use today are enzyme inhibitors (Copeland, 2005), it is reasonable to expect that enzyme inhibitors could also be useful in the treatment of diabetes mellitus. Indeed, research conducted during the last few decades has identified a number of carbohydrate metabolizing enzymes as possible targets for antidiabetic treatment. These carbohydrate metabolizing enzymes include the digestive enzymes α-amylase and α-glucosidase and gluconeogenic enzymes, in particular glucose 6-phosphatase that are reviewed in the next section.

2.3.5.1. Amylases

2.3.5.1.1. Definition, distribution and metabolic roles

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers (oligosaccharides) composed of glucose units (Gupta *et al*., 2003). Amylases are widely distributed in microorganisms, plant and animals. In plants amylases are involved in seed germination and fruit ripening (Muralikrishna and Nirmala, 2005). In animals amylases work in concert with α-glucosidases to digest complex carbohydrates to simpler sugars which are then absorbed (Figure 2.10).

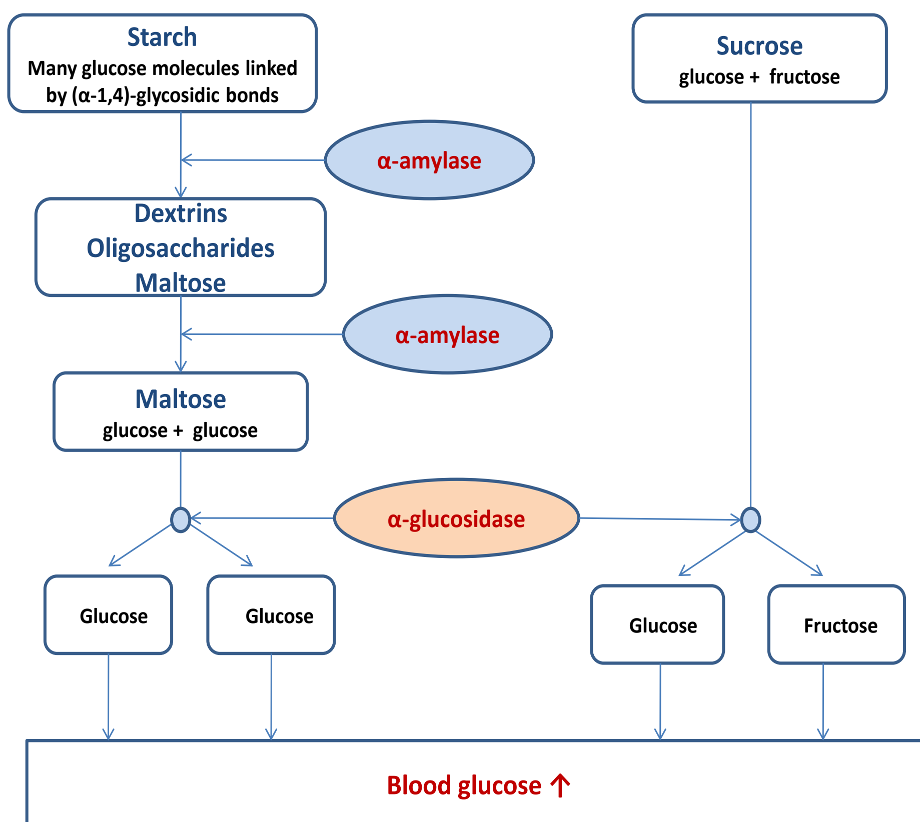


Figure 2.10: Carbohydrate digestion by α-amylases and α-glucosidases

2.3.5.1.2. Classification of amylases

Amylases are classified into three major groups on the basis of their mechanism of action: endoamylases, exoamylases and de-branching amylases (Gupta *et al*., 2003; Muralikrishna and Nirmala, 2005).

a) Endoamylases (1,4 - α-D-glucan-glucanhydrolase, EC. 3.2.1.1)

Endoamylases, also known as α-amylases or "liquefying amylases" hydrolyses internal α-(1,4)-glycosidic bonds in amylase, amylopectin and related polysaccharides such as glycogen to produce oligosaccharides of varying chain length. The products of starch hydrolysis by endoamylases have an α-configuration at C-1(carbon atom number one) of the reducing glucose unit (hence the name α-amylase). Starch hydrolysis by endoamylases result in a rapid decrease in the viscosity and iodine-staining power (Hill and MacGregor, 1988). In animals α-amylases exist in two major isomeric forms, namely salivary amylase and pancreatic amylase. These amylase isoforms are reported differ in their physical properties, amino acid sequence as well as substrate specificity (Smith *et al.*, 2005).

b) Exoamylases (1,4-α-Dglucan maltohydrolase EC 3.2.1.2)

Exoamylases, also known as β-amylases or "saccharifying" amylases, they cleave α-1,4-glycosidic bonds in amylose, amylopectin and glycogen from the non-reducing end by successive removal of maltose or glucose in a stepwise manner. The products of starch by exoamylases have the β-configuration at C-1 of the reducing glucose unit due to the inversion of the product (hence the name β-amylase). In contrast to the action of endoamylases these result in a slow decrease in the viscosity and iodine staining power of starch (Hill and MacGregor, 1988).

c) De-branching amylases (1,4-α-D-glucan glucohydrolase EC 3.2.1.3)

De-branching amylases, also known as γ-amylases hydrolyses mainly α-1,6-glycosidic bonds in branched starches such as amylopectin and glycogen. The product of starch hydrolysis by γ-amylases in concert with α-amylases and β-amylases are α- and β- limit dextrins (Muralikrishna and Nirmala, 2005)

2.3.5.1.3. Determination of α-amylase activity

Various methods are available for the determination of α-amylase activity (Priest, 1977; Gupta *et al.*, 2003; (Muralikrishna and Nirmala, 2005). These methods are based on either the colourimetric measurement of reducing sugars released by the action of amylase on starch, decrease in the starch-iodine colour intensity, degradation of colour-complexed (chromogenic) substrate or the decrease in the viscosity of the starch suspension.

a) Colourimetric measurement of reducing sugars.

Reducing sugars released by the action of α-amylase on starch are generally measured by either alkaline copper, alkaline ferricyanide or alkaline dinitrosalicylic (DNS) acid (Robyt, Ackerman and Keng, 1972; Benfeld, 1955). Among these methods, the DNS method is the most commonly used, because of its reliability and simplicity (Miller, 1957). The major drawbacks of the DNS methods are the slow loss in colour produced and the destruction of glucose by the constituents of the reagents. To overcome these limitations, a modified method for the estimation of reducing sugars was developed (Miller, 1959). Rochelle salts were excluded and 0.05% sodium sulphate was added to prevent the oxidation of the reagent. Since then the modified method has been used extensively to measure reducing sugars without any further modification in the procedure (Gupta *et al*., 2003).

b) Decrease in starch-iodine colour intensity

Starch forms a deep blue complex with iodine and with progressive hydrolysis by α-amylases it changes to a red brown colour (Hollo and Szeitli, 1968; Gupta et al., 2003). The disappearance of the blue is proportional to the activity of α-amylases and it is commonly used as a semi-quantitative estimate of α-amylase activity. However, this procedure measures only endoamylase activity (Muralikrishna and Nirmala, 2005).

c) Degradation of chromogenic substrate

Chromogenic substrates were developed for α-amylase assays especially for clinical samples. The dye is covalently linked to starch or one of its constituents (amylose and amylopectin) to give an insoluble material (azure derivative) (Rinderkneet *et al*., 1967). When these substrates are acted on by α-amylases, fragments containing dye is solubilized, the remaining insoluble substrates removed by centrifugation and absorbance of the supernatant is taken as a measure of amylase activity. *p*-Nitrophenyl derivative of oligosaccharide also has been used to detect both α- and β- amylase activities. The amount of *p*-nitrophenol released correlates with the activity. Even though this method is very simple and accurate to differentiate between α-and β-amylase activities, it is not used for routine analysis due to its high cost.

2.3.5.1.4. Factors influencing α-amylase activity

As is the case with most enzymes, the activity of α-amylases is influenced by factors such as enzyme and substrate concentration, temperature and pH of the reaction mixture as well as the presence of activators and inhibitors. The effects of enzyme concentration, substrate concentration, pH and temperature on enzyme activity are described elsewhere in most standard Biochemistry text books. Below is a brief description of substances that are known to activate or inhibit the activity of α-amylases.

a) Activators of α-amylases

Alpha-amylases are metalloenzymes which contain a covalently bound Ca^{2+}, act as allosteric activators. Enhancement of amylase activity by Ca^{2+} is based on its ability to interact and stabilize the negatively charged amino acids (aspartate and glutamate) in the active site of the enzyme. In addition to Ca^{2+} ions, other metal ions such as Ba^{2+} ,Co^{2+} ,Mg^{2+} ,Zn^{2+} ,Cu^{2+} ,Al^{3+} ,Fe^{2+} and Hg^{2+} at low concentration (approx. 5 mM) are known to activate amylases (Muralikrishna and Nirmala, 2005; Gupta *et al*., 2003).

b) Inhibitors of α-amylases

The activity of α-amylases is reported to be inhibited by metal chelators, organic acids, heavy inorganic metal ions, pure natural products and crude plant extracts (Muralikrishna and Nirmala, 2005; Gupta *et al*., 2003).

i) Metal chelators, organic acids and heavy inorganic metal ions.

All metal chelators are strong inhibitors of amylases as they are metalloenzymes. In certain α-amylases such as cereal amylases Ca^{2+} is loosely bound to the enzyme and can be removed by treatment with metal chelators such EDTA, EGTA, etc (Bush *et al*.,1989). Of the organic acids, citric acid and oxalic acid are reported to be the most potent inhibitors of amylase. Heavy metal ions such as Al^{3+}, Fe^{2+} and Hg^{2+} are known to inhibit amylases at high concentrations (Muralikrishna and Nirmala, 2005).

ii) Pure natural products

Acarbose, a synthetic pseudotetrasaccharide originally isolated from actinoplane species of microorganisms is an established inhibitor of both alpha amylase and alpha glucosidase (Yoon and

Robyt, 2003; Kim *et al*., 2005). Arcabose and its structural analogues were found to be a mixed noncompetitive inhibitor of *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, human salivary, and porcine pancreatic alpha-amylases (Yoon and Robyt, 2003).

iii) Crude plant extracts

A number of researchers have investigated the α-amylase inhibitory activity of crude plant extracts. Although most extract investigated failed to demonstrate appreciable α-amylase inhibition, few of the extract investigated did show α-amylase inhibitory activity. The information from studies which reported appreciable α-amylase inhibition is summarized in Table 2.2.

Table 2.2. Medicinal plants that were reported to have appreciable α-amylase inhibitory activity

Plant species	Type of extract	Ext. concentration (mg/ml)	Inhibition (%)	IC_{50} (mg/ml)	Source of amylase	Mode of inhibition /comment	References
Bergenia ciliata	MetOH (50%)	0.2	93.5±2.1	(A) 739 (B) 401	p. p. amylase	Active principles (A)(-)-3-O-galloylepicatechin (B)(-)-3-O-galloylcatechin	Bandari *et al*., 2008
Murraya koenigii	$CHCl_3$ Aqueous	0.5 0.4	83.94±1.18	1.96 1.06	p. p. amylase m.p. amylase	Significantly higher than that of acarbose	Narayan *et al*., 2005
Morus alba	EtOH (90%)	23	75.58±1.18	17.60	Not specified	Less potent than acarbose	Nickavar & Mosazadeh, 2009
Morus alba var nigra	EtOH (90%)	23	73.91±0.35	13.28	Not specified	Acts by increasing glucose uptake Less potent than acarbose	Chen *et al*., 1995
Ocinum tenuiflorum	Aqueous	0.5	76.27±3.6	1.55 9.86	p. p. amylase m.p. amylase	Significantly more than acarbose	Bhat *et al*.,2008
Phyllanthus amarus	Hexane	1	100	0.032	p. p. amylase	Inhibitory effect attributed to the presence of pentacyclic terpenoids	Ali *et al*.,(2006)
Pine (bark and needle)	EtOH (70%)	0.06	58.00	1.7 1.69	h. s. amylase p. p. amylase	Inhibitory activity similar to that of acarbose Competitive mode of inhibition Suppressed postprandial blood glucose level in vivo	Kim *et al*., (2005)
Salvia verticillata	EtOH (90%)	36	99.52±0.16	18.34	Not specified		Nickavar *et al*., (2008)
Salvia virgata	EtOH (90%)	36	76.67±1.15	19.73	Not specified		Nickavar *et al*., (2008)
Andrographis paniculata	Leaf EtOH	62.5	80	50.9	p. p. amylase	mode of inhibition not specified, but more potent than acarbose	Subramanian *et al*.,2008

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EtOH=Ethanol extract, MetOH=Methanol extract, $CHCl_3$=Chloroform,
p. p. amylase= porcine pancreatic amylase, m.p. amylase=mice pancreatic amylase,
h.p. amylase= human pancreatic amylase

From the information provided in Table 2.2 it can be concluded that α-amylases are inhibited by both polar and non-polar extracts. The majority of the plant extracts investigated showed strong α-amylase inhibitory effects and were more potent than the standard drug acarbose. The mode of

inhibition of most these extracts is not specified and the *in vitro* inhibitory effects were not confirmed *in vivo*. The source of α-amylase used in most of the studies was porcine pancreatic α-amylase and only one study investigated human derived α-amylase (Kim *et al*., 2005).

2.3.5.1.5. Pharmaceutical/Medicinal significance of α-amylase inhibitors

Alpha amylase inhibitors inhibit the digestion and the production of glucose from complex polysaccharides. Thus, these inhibitors have the potential to suppress postprandial blood glucose level in diabetic patients. Acarbose which lowers blood glucose by inhibiting α-amylases and α-glucosidases is currently used as an antidiabetic drug. Other potential α-amylase inhibitors (starch blockers) such as phaseolamin (from white kidney beans) still need to undergo clinical trials ( ).

2.3.5.2. Alpha-glucosidases

2.3.5.2.1. Definition, distributions and roles in metabolism

In general α-glucosidases (glycosidases) are enzymes that catalyze the hydrolysis of α-(1,4) glycosidic linkages in starch and related compounds. However, the term α-glucosidase is usually reserved for dissacharidases which hydrolyze disaccharides into respective monosaccharides (Smith et al., 2005). In animals α-glucosidases are found in the intestinal brush border and comprises of sucrase, maltase and isomaltase. The major function of these disaccharidases is to hydrolyze disaccharides ingested or produced by the action of alpha amylases on starch to monosaccharides which are then absorbed (Figure 2.10). Interspecies variations in α-glucosidases have been reported (Gupta *et al*., 2003). Studies conducted by Chiba (1979) and Kato *et al*. (2002) indicated that α-glucosidase also catalyzes transglycosylation reactions to synthesize glycosylated compounds. Hyperactivity of α-glucosidase can lead to rapid increase in postprandial glucose concentrations, and deficiency in humans may lead to hypoglycaemia and difficulty in glycogen storage by the liver (Soro *et al*., 2007).

2.3.5.2.2. Determination of α-glucosidase activity

There are several methods used in the determination of α-glucosidase activity. The most commonly used methods for the determination of α-glucosidase activity are the measurement of reducing sugars released by action α-glucosidases and degradation of chromogenic substrates.

2.3.5.2.3. Measurement of reducing sugars.

Alpha-glucosidase activity can be measured colourimetrically in terms of the amount of reducing sugars it releases from its substrates. As in the case of the measurement of α-amylase activity, reducing sugars released by the action of α-glucosidase enzymes are generally measured by means of the DNS procedure.

2.3.5.2.4. Degradation of chromogenic substrates

Several α-glycoside substrates have been developed for the measurement of α-glucosidase activity (Gupta *et al*., 2003). The most commonly used of these substrates is *p*-nitrophenyl-α-D-glucopyranoside (PNPG) (Muralikrishna and Nirmala, 2005). α-Glucosidase enzymes hydrolyses this substrate to liberate *p*-nitrophenol which under alkaline condition has a yellowish colour. The intensity of the resultant yellow colour is proportional to the activity of α-glucosidase enzymes.

2.3.5.2.5. Factors influencing α-glucosidase activity

The activity of α-glucosidase enzymes has been shown to be inhibited by several natural products isolated from microorganisms and plant materials (Kim *et al*., 2000; Gupta *et al*., 2003). Acarbose (Schmidt *et al*., 1977), miglitol (Standl *et al*., 1999) and voglibose (Saito *et al*., 1998) isolated from microoganisms are known inhibitors of α-glucosidase enzymes (Kim *et al*., 2005). These substances, in particular, acarbose is currently used as an antidiabetic agent which suppresses postprandial hyperglycaemia in diabetic patients through inhibition of intestinal brush border α-glucosidase enzymes (Lebovitz, 1998, Singh *et al*., 2002). Acarbose, in addition, is also used as a standard α-glucosidase inhibitor for comparing newly discovered α-glucosidase inhibitors (Deutschlander *et al*., 2009).

Natural products from plants (secondary plant metabolites) with α-glucosidase inhibitory activity include polyphenols and flavonoids (Kim *et al*., 2000, Jung *et al*., 2006), tannins (Van Wyk *et al*., 1997; Hutchings *et al*., 1996) and terpenoids (Akah and Okafor, 1992; Marles and Farnsworth, 1995; 2001, Ojewole, 2002). Polyphenols, flavonoids and tannins are able to inhibit the activities of digestive enzymes such as α-glucosidases due to their ability to form complexes with proteins (Nickavar *et al*., 2008). Crude plant extracts have also been shown to inhibit α-glucosidase enzymes. Research studies that have investigated the inhibitory effect of crude plant extracts on α-glucosidase enzymes are summarized in Table 2.3. As in the case of α-amylases, it can be seen that α-glucosidases are inhibited by different types of extracts including tea infusions. The extracts showed significant α-glucosidase inhibition with % inhibitions above 60 % for other extracts. The

sources of α-glucosidase enzyme were mammalian small intestines and microorganisms such as yeast, *B. stearothermophilus* and *S. cerevisiae.* The effects of the extracts were investigated in both *in vitro* and *in vivo* studies. The extracts significantly suppressed postprandial blood glucose levels *in vivo*, and in other studies they were reported to be more potent and in others less potent when compared with acarbose.

Table 2.3**.** Medicinal plants that were reported to have appreciable α-glucosidase inhibitory activity

Plant species	**Type of extract**	**Ext concentration (mg/ml)**	**Inhibition (%)**	**IC_{50} (mg/ml)**	**Source of α- glucosidase**	**Mode of inhibition/comment**	**References**
Pinus sylvestris	Pine bark EtOH	0.2	N S	1.5 0.025	p. s. intestine *S. cerevisiae*	Mixed type inhibition and less potent inhibitor than acarbose	Kim *et al*., 2005
Alstonia scholaris	Leaf MeOH	N S	74 51	1.72 (Maltose) 1.96 (Sucrose)	rat intestine	Active principles: flavonoids Not compared with acarbose	Jong-Anurakkhun *et al*., 2007
Morus alba	Tea infusion	1.72	67	7.7 (Maltose) 1.7 (Sucrose)	rat intestine	*In vitro* cell culture studies Not compared with acarbose	Hansawasdi & Kawabata, 2006
Cecropia obtusifolia	BuOH whole plant	2.00	N S	0.014	rat intestinal (in vivo)	Suppressed a rise in postprandial glucose level More potent than acarbose	Andrade-Cetto *et* al., 2007
Andrographis paniculata	Leaf EtOH	62.5	80	17.2	Yeast	mode of inhibition not specified, but more potent than acarbose	Subramanian *et al*.,2008
Euclea undulata	Whole plant ethanol extracts	0.2	62	49.5	Chang liver cell lines	Less potent than acarbose	Deutschlander *et* al., 2009
Bergenia ciliata	MetOH	N S	69	33.3	rat intestine	Active principles (-)-3-O-galloylepicatechin & (-)-3-O-galloylcatechin	Bhandari *et al*., 2008
Commelina communis	Aqueous extract of dried leaf leaves	10	77.4	N S	STZ-diabetic mice	Mixed inhibition, more potent than acarbose	Youn *et al*., 2004
Syagrus romanzoffiana	Seeds MetOH	0.1	55	6.5	*Bacillus stearothermophil us*	Less potent than acarbose	Lam *et al*., 2008
Grateloupia Elliptica	Whole plant ethyl acetate	0.8	64	4.8	rat intestinal	Bioactive compounds Less potent than acarbose	Kim *et al*., 2008

EtOH=Ethanol extract, MetOH=Methanol extract, BuOH= Butanol extract, p. s. intestine= porcine small inestine, *S. cerevisiae = Saccaromyces cerevisiae* , STZ-diabetic mice = Streptozotocin-induced diabetic mice, *B. stearothermophilus = Bacillus stearothermophilus*
N S= Not Specified

2. 3.5.3. Glucose-6-phosphatase (G6Pase)

2.3.5.3.1. Definition, distributions and roles in metabolism

Glucose 6-phosphatase (G6Pase) (EC 3.1.3.9) is a multi-component enzyme system that is an integral constituent of the membrane of the endoplasmic reticulum with its active site directed towards the endoplasmic reticulum lumen (Figure 2.15). The G6Pase enzyme system comprises of: 1) a p46 transport protein, T1, that permits the entry of glucose 6-phosphate (G6P) into the endoplasmic reticulum; 2) a p36 catalytic subunit that cleaves the substrate (G6P) into glucose and

inorganic phosphate; 3) two additional transporters, T2 and T3, that return the products phosphate and glucose respectively, to the cytosol and 4) a calcium binding regulatory subunit on the cytosolic side of the endoplasmic reticulum membrane.

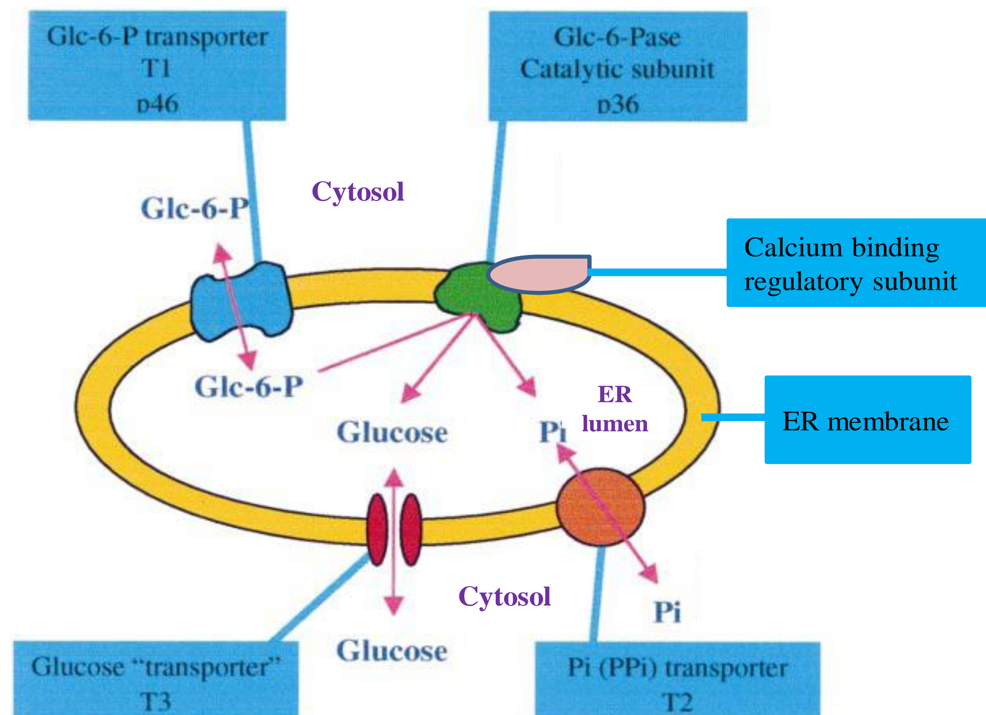

Figure 2.11: Glucose 6-phosphatase enzyme system Glc-6-Pase: glucose 6-phosphatase; Glc-6-P: glucose 6-phosphate; ER:

G6Pase is found mainly in the endoplasmic reticulum of the liver and kidney cortex (Shaftingen and Gerin, 2002; Maiti *et al*., 2001; Berg *et al*., 2001). It is also expressed in the β-cells of the pancreatic islets (Khan *et al*., 1995) and in the intestinal mucosa of the human, mouse (Ockerman, 1995) and rat (Chatelain *et al*., 1998). G6Pase catalyzes the final step in both the glycogenolytic and the gluconeogenic pathways, cleaving phosphate from glucose 6-phosphate to liberate free glucose into the circulation (Smith *et al*, 2005). It is, thus, uniquely situated to regulate both circulating concentrations of glucose and the storage of excess glucose as glycogen. Low Glucose 6-phosphatase activities have also been described in skeletal muscles and in astrocytes (Forsyth *et al*., 1993). G6Pase act as a hydrolase and as a translocase in biological systems, it catalyzes the hydrolytic conversion of glucose-6-phosphate (G6P) to glucose and inorganic phosphate (P_i) (Surholt and Newsholme, 1981). It is a key enzyme in the final step of both glycogenolysis and gluconeogenesis, where its major goal is to increase free glucose in blood circulation (Surholt and Newsholme, 1981; Baquer *et al*., 1998). G6Pase also catalyzes potent transphosphorylations from carbamoyl phosphate, hexose phosphates, diphosphate, phosphoenolpyruvate and nucleoside di-

and triphosphates, to D-glucose, D-mannose, 3-methyl-D-glucose, or 2-deoxy-D-glucose (Surholt and Newsholme, 1981).

2.3.5.3.2. Determination of glucose-6-phosphatase activity

The G6Pase system is present in high quantities in the ER of hepatocytes. To study G6Pase activity, the ER must be isolated from other cellular components including the plasma membrane and other cytoplasmic organelles. Due to the expansive nature of the ER in cells, it is sheared into small vesicles called microsomes under standard homogenization procedures. To assay catalysis without complication from the involvement of transporters, the microsomes are treated with Triton X-100. Triton X-100 is a mild non-ionic detergent that permeabilizes the microsomal membranes without denaturing proteins. It is essential that the detergent is added to the microsomal preparation prior to the enzyme assay. The G6Pase activity is measured by determining the production of P_i generated by the hydrolysis of G6P. Each assay is initiated by the addition of microsomes to experimental buffer containing G6P. The presence of P_i is determined through a modified spectrophotometric P_i assay (Smith *et al*., 2005).

2.3.5.3.3. Clinical significance of glucose 6-phosphatase hyperactivity and hypoactivity

Hyperactivity of G6Pase in a diabetic condition will elevate rather than alleviate the symptoms associated with extremely high blood glucose concentrations. Deficiency or hypoactivity of G6Pase can lead to glycogen storage disease type 1 called Von Gierke's disease which results in the enlargement of the liver and kidney due to the accumulation of excess glycogen (Surholt and Newsholme, 1981).

2.4. *Clausena anisata*

Ethnopharmacological studies indicate that more than 1200 plants are used to control diabetes mellitus in traditional medicinal systems of different cultures worldwide (Marles and Farnsworth, 1995; Grover *et al*., 2002). The hypoglycaemic activity of a large number of these plants/plant products has been evaluated and confirmed in animal models (Day and Bailey, 2006; Frode and Medeiros, 2006) as well as in clinical studies (Jayawardena *et al*., 2005; Day and Bailey, 2006). However, many of the plants extracts with confirmed hypoglycaemic activities still need to be characterized in terms of toxicity, active principles and hypoglycaemic mechanism of action. One such anti-diabetic medicinal plant whose blood glucose lowering effect has been demonstrated in

animal model of diabetes (Ojewole, 2002) and still need to be investigated further is *Clausena anisata (C. anisata)*. A brief review of this plant is given below.

2.4.1. Description of *Clausena anisata*

Clausena anisata (Willd) Hook (Family Rutaceae) is a small tree, about 10m in height belonging to the Rutaceae family of plants. (Figure 2.12). It is also known as *Perdepis* (Afrikaans, because the crushed leaves has an unpleasant smell, characteristic of horse's urine), *isifudu* (Zulu), *umNukelambiba* (Swazi). This plant has been used by South African traditional healers to treat a variety of ailments including diabetes mellitus (van Wyk and Griecke, 2000; van Wyk *et. al*., 2002).

Figure 2.12. Tree of *C. anisata* showing branches, leaves and ripe fruit (Photo by Bart Wursten)

It is abundant in forests and forest margins, riverine thickets and bushveld, from Western Cape up the eastern coast of KwaZulu-Natal, Mpumalanga, eastern Zimbabwe, Mozambique, northwards to Ethiopia and the Sudan and westwards to Sierra Leone where it plays a major role in the ecosystem (Watt and Breyer-Brandwijk, 1962; van Wyk and Gericke, 2000; van Wyk *et. al*., 2002). The main stem is smooth, grey to light brown and is without thorns, the leaves alternate and crowded towards the ends of the branches (Figure 2.12 and Figure 2.14), each leaf has 4-10 pairs of leaflets. The flowers have white petals with numerous yellow stamens.

Figure 2.13. Twigs of *C. anisata* showing fresh flowers and young fruit (Photo by Bart Wursten)

The fruit are berries, initially are green, then reddish-black when fully ripe (Figure 2.14) providing a source of nourishment to many bird and insect species. The wood is yellow, hard, strong and is used for hut-building and making of knobkerries (van Wyk *et al*., 2002).

Figure 2.14. *C. anisata* leaf and ripe fruit (initially green then reddish-black) (Photo by Bart Wursten)

2.4.2. Medicinal uses

This South African flowering plant have been reported to be an effective remedy against epilepsy or convulsions, diabetes mellitus, arthritis, rheumatism and other inflammatory conditions. It has also been reported to treat hypertension, heart failure and cardiovascular ailments (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996 and Ojewole, 2002). The roots and leaf infusion are effective remedies against internal parasites especially flatworms infestations, such as schistozomiasis and taeniasis (Ojewole, 2002). Other studies have reported the use of the plant to relieve constipation and abdominal pains. Research conducted on certain rural communities

revealed the use of this plant to treat malaria and other febrile conditions (Watt and Breyer-Brandwijk, 1962). It was also observed that the roots infusion of the plant offer an effective relief against headaches, body pains, eye complaints, influenza and respiratory ailments (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996). Some indigenous tribes like the Venda people, use the leaf concoction to treat toothaches, swollen gums, mental disorders, impotence and sterility (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996 and Ojewole, 2002)

2.4.3. Phytochemical composition

Different phytochemicals have been isolated from various morphological parts of the plant (Hutchings *et al*., 1996). *C. anisata* has been widely reported to contain large amounts of coumarins, (especially scopoletin, chalepin, helietin, osthole, coumarrayin, xanthoxyletin, heliettin, imperatorin, furanocoumarin derivatives) and volatile oil containing phenylpropanoids (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996 and Ojewole, 2002).

Also present in the diverse mixture of chemical compounds contained in the plant extracts of *C. anisata* include terpenoids, sesquiterpenoids, fatty acids, methylchavicol, limonin, limonene, myrcene, zapoteterin, umbelliferon, xanthotoxin, pimpenellin, limonoids and the alkaloids clausanitine, clausenolide and mupamine (Mester and Reisch, 1977; Mester *et al*., 1977 Adesina and Ette, 1982; Lakshmi *et al*., 1984; Hutchings *et al*., 1996; Ojewole, 2002)

2.4.4. Demonstration of hypoglycaemic and anti-diabetic action

Studies on the root methanolic extracts of this plant showed a significant reduction in postprandial and fasting blood glucose levels of streptozotocin and alloxan induced diabetic rats, however, the root methanolic extracts were found to be not as potent as arcabose or metformin (Ojewole 2000). Studies conducted by Ojewole (2002) revealed that when methanolic extracts of *C. anisata* were administered on fasted normal, fasted STZ-induced diabetic rats produced a profound decrease in blood glucose concentration of the experimental animals.

2.4.5. Mode of hypoglycaemic and anti-diabetic action

The active hypoglycaemic ingredient of *C. anisata* has not been isolated and identified. However, the blood glucose lowering effect of *C. anisata* is believed to be associated with the presence of secondary metabolites (Okorie, 1975; Adesina, 1982). Such metabolites are terpenoids, coumarins,

phenylpropanoids and alkaloids found in the leaf extract of this plant (Okorie, 1975). According to Ojewole (2002) it was difficult to draw any logical conclusions on the mechanism of action of the diverse mixture of compounds contained in the methanolic extract of *C. anisata*.

However, Marles and Farnsworth (1995) indicated that terpenoids and coumarins present in this plant extracts may possess hypoglycaemic activities in part at least. To date the mechanism of hypoglycaemic action of *C. anisata* remains speculative. Studies have revealed that the mechanism of antidiabetic action of *C. anisata* extracts appears to involve the stimulation of β-cells of the pancreas to secrete insulin and probably hepatotoxicity (Marles and Farnsworth, 1995). Toxins are chemical agents capable of causing cellular damage at molecular level without causing any significant histopathological changes. Coumarins are hepatotoxic in rats and dogs and no hepatotoxicity was reported in humans and primates. In addition the plant extract may possess an enzyme inducing effect (Marles and Farnsworth, 1995; Ojewole, 2002).

2.5. Investigation of medicinal plants for hypoglycaemic activity

During the last few decades, a large number of medicinal plants and herbs have been investigated for their hypoglycaemic and antidiabetic properties. A review of literature of literature covering this area of research suggest that procedures used by most researchers to investigate the hypoglycaemic effect of medicinal plants and herbs can be summarized as in Figure 2.15.

2.5.1. Selection, of plant material for investigation

The plant material to be investigated can be selected on the basis of some specific traditional uses (ethnobotanical bioprospecting approach). Extract prepared from plant used as traditional remedies to treat certain diseases are more likely to contain biologically active compounds of medicinal interest (Heinrich *et al*., 2004). Alternatively, the plant can be selected based on chemotaxonomical data. In the chemotaxonomic approach, knowledge that a particular group of plants contain a certain class of natural products may be used to predict that taxonomically related plant may contain structurally similar compounds (Heinrich *et al*., 2005). The use of literature data base early in the selection process can provide some preliminary information on the type of natural products already isolated from the plant and the extraction methods employed to isolate them (Heinrich *et al*., 2005). Another approach known as the information driven approach, utilizes a combination of ethobotanical, chemotaxonomic and random approaches together with a data base that contains all relevant information concerning a particular plant species (Kinghorn and Balandrin, 1993; Harborne, 1998; Heinrich *et al*., 2005). The database is used to prioritize which plants should be

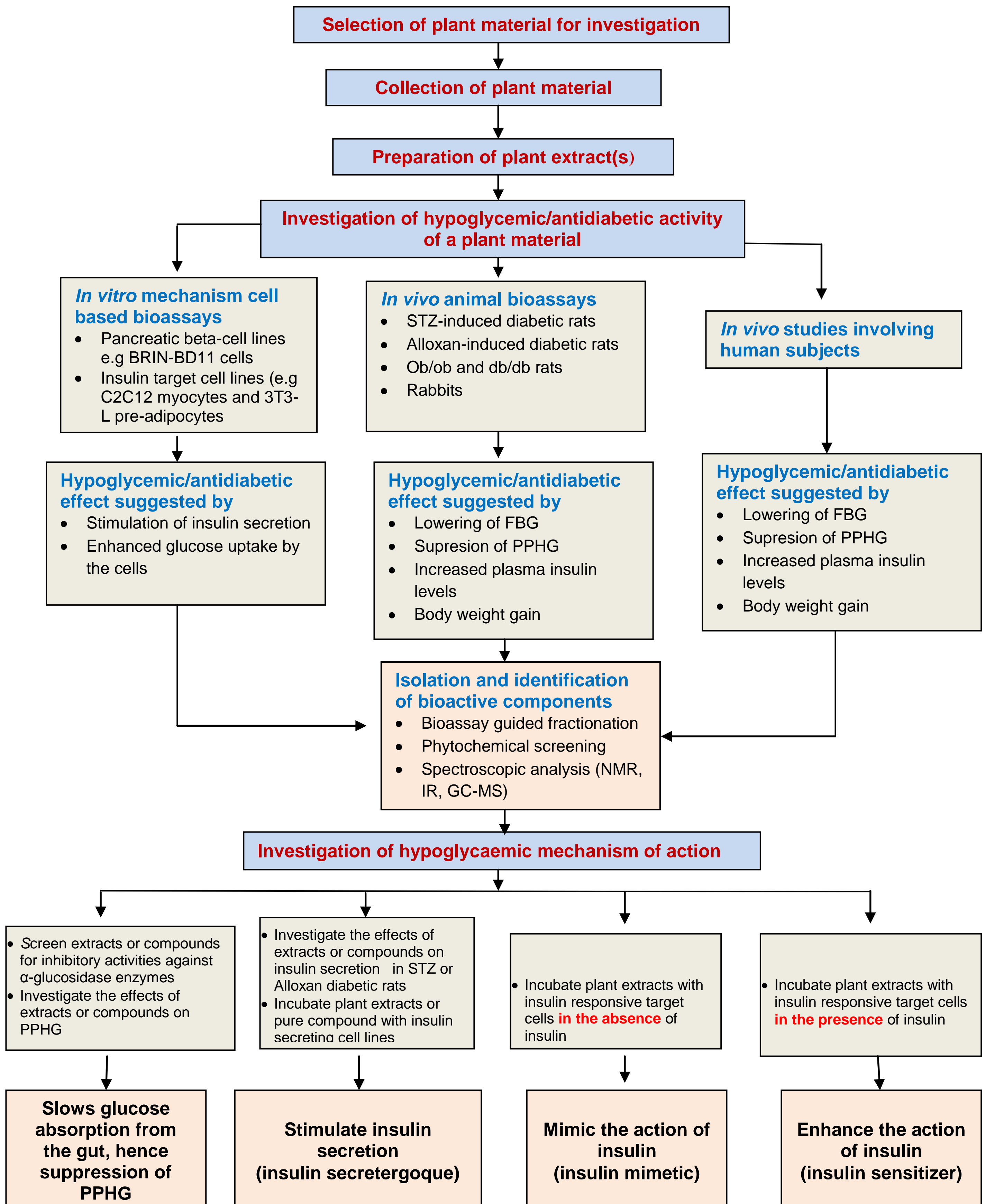

Figure 2.15: A general scheme for investigating the hypoglycaemic/antidiabetic properties of plant materials; FBG – Fasting blood glucose level; PPHG – Postprandial hyperglycemia

extracted and screened for biological activity. This approach is favoured by large organizations (particularly pharmacological companies) interested in screening thousand of samples for bioactivity as it may reduce costs by a process known as de-replication - the process of avoiding the repeated discovery of common or known drugs (Heinrich *et al*., 2005).

2.5.2. Collection of plant material

The whole plant or a particular part can be collected depending on where the metabolites of interest (if they are known) accumulate. Hence aerial (e.g. leaves, stems, flowering tops, fruit, seed, bark) and underground (e.g. tubers, bulbs, roots) parts can be collected separately. Collection of plant materials can be influenced by factors such as the age of the plant and environmental conditions (e.g. temperature, rainfall, amount of daylight, soil characteristics and altitude) (Williams *et al*., 1996; Harborne, 1998). Thus, it is important to take this into consideration for the re-collection purpose, in order to ensure reproducible profile (nature and amount) of metabolites (Satyajit *et al*., 2006). The plant from which the material is collected must also be identified correctly. A plant taxonomist or a botanist should be involved in the detailed authentication of the plant (i.e. classification into its class, order, family, genus and species) (Satyajit *et al*., 2006). Any feature related to the collection, such as the name of the plant, the identity of the parts collected, the place and date of collection, should be recorded as part of the voucher (a dried specimen pressed between sheets of paper) deposited in a herbarium for future reference (Harborne, 1998; Satyajit *et al*., 2006).Extraction of plant materials

Plant materials are commonly extracted by means of liquid solvents in what is known as the “solid-liquid solvent extraction”. A typical solid-liquid solvent extraction process for plant materials involve drying and grinding of the plant material, choosing a suitable extraction solvent and extraction procedure (Starmans and Nijhuis, 1996; Jones and Kinghorn, 2005). Once the plant material has been collected, it needs to be dried as soon as possible. A common practice is to leave the sample to dry on trays at ambient temperature and in a room with adequate ventilation (Heinrich *et al*., 2005; Satyajit *et al*., 2006). Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. Plant materials should be sliced into small pieces and distributed evenly to facilitate homogeneous drying. Protection from direct sunlight is advised to minimize chemical reactions (and formation of artefacts) induced by ultraviolet rays (Satyajit *et al*., 2006). To facilitate the drying process, the material can be dried in an oven. This can also minimize reactions (e.g. hydrolysis of glycosides) that can occur as long as there is some residual moisture present in the plant material. The dried material should be stored in sealed containers in a dry and cool place. Storage for prolonged periods should be avoided as some constituents may be decomposed (Heinrich *et al*., 2004; Jones and Kinghorn, 2005).

After drying, plant materials are grounded into a fine powder. Grinding of plant materials into smaller particles facilitates subsequent extraction procedures by rendering the sample more homogeneous, increasing the surface area, and facilitating the penetration of solvents into cells (Harborne, 1998; Satyajit *et al.*, 2006). Mechanical grinders (e.g. hammer and cutting mills) are employed to shred the plant material into various particle sizes. Potential problems of grinding include the fact that some material (e.g. seeds and fruits rich in fats and volatile oils) may clog up the sieves and the heat generated may degrade thermolable metabolites (Harborne, 1998). The choice of the extraction solvent depends mainly on the polarity and hence the solubility of the bioactive compound(s) of interest. Although water is generally used as an extractant in many traditional protocols, organic solvents of varying polarities are often used (either alone or in different combinations) in modern methods of extraction to exploit the various solubilities of plant constituents. The choice of the extraction procedure depends on the nature of the source material and the compound to be isolated. Solvent extraction procedures applied to plant natural products include but not limited to maceration, percolation, soxhlet extraction, steam distillation and sequential solvent extraction (Starmans and Nijhuis, 1996; Harborne, 1998; Jones and Kinghorn, 2005).

If the polarity and solubility of compounds that are isolated is not known, a convenient and frequently used procedure is sequential solvent extraction. In sequential solvent extraction, the plant material is extracted with a series of solvents of different polarity. The usual way is to start with a non-polar solvent and exhaustively extract the plant material followed by a series of more polar solvents until several extracts are obtained of increasing solute polarity. For example, a first step, with dichloromethane, will extract terpenoids, less polar flavonoids (flavones, flavonols, flavonones) and other less polar materials (Starmans and Nijhuis, 1996; Jones and Kinghorn, 2005). A subsequent step with acetone or ethyl acetate will extract flavonoid glycosides and other medium polar constituents. A subsequent extraction with an alcohol or water will extract highly polar constituents (Jones and Kinghorn, 2005). Once the extraction is complete, the extractant is usually concentrated under vacuum, for large volumes or solvents and blown down under nitrogen for small volumes, ensuring at the same time that volatiles are not lost. Aqueous extracts are generally freeze-dried and stored at –20 ^{o}C as this low temperature reduces the degradation of the bioactive natural product. Extraction protocols may sometimes be modified depending on the type of molecules being extracted, e.g. acids may be added to extract alkaloids as their salts (Jones and Kinghorn, 2005).

2.5.4. Determination of the hypoglycaemic/antidiabetic activity of a plant material

A review of literature suggest that the blood glucose lowering effect or the antidiabetic properties of a plant material may be determined by either of three procedures: 1) *In vitro* mechanism based cell bioassays; 2) *In vivo* animal bioassays and 3) In vivo studies involving the use of human subjects.

2.5.4.1. *In vitro* mechanism based bioassays

Cell based assays commonly used to screen or evaluate the antidiabetic activity of medicinal plants belongs to a class of *in vitro* bioassays known as "mechanism based assay" (Benjamin, *et al*., 1994; Soumyanath and Srijayanta, 2006). A mechanism-based bioassay differ from an ordinary cell culture bioassay in that it can provide a possible mechanism of action at the same time that the plant material is screened for biological activity (Benjamin, *et al*., 1994). Two different types of mechanism based *in vitro* bioassays are commonly used to assess the antidiabetic/hypoglycaemic of medicinal plants and/or products: the insulin secretion stimulation (Gray and Flatt, 1997; 1998; Soumyanath and Srijayanta, 2006) and the glucose uptake (utilization) bioassays (Soumyanath and Srijayanta, 2006; van de Venter *et al*., 2008). Insulin secretion stimulation bioassays in general, assess the ability of a plant extract or natural product to stimulate either perfused pancreas, isolated pancreatic islets cells or clonal pancreatic beta cell–lines (e.g. BRIN-BD11 cells) to secrete insulin (Hannan, *et al*., 2007). Glucose uptake (utilization) These types of bioassays assess the ability of plant materials to enhance glucose uptake by insulin target cell-lines (e.g. C2C12 myocytes, 3T3-L1 preadipocytes and human Chang liver cells) (Soumyanath and Srijayanta, 2006; van de Venter *et al*., 2008).

2.5.4.2. *In vivo* animal bioassays

In vivo bioassay screening tests for antidiabetic activity of medicinal plant extracts and other antidiabetic remedies are usually carried out in normal or diabetic animals (mostly rats and mice) in which diabetes has been induced either by chemical, dietary, surgical or genetic manipulations (Rees and Alcolado; 2005; Masiello, 2006; Day and Bailey, 2006; Fröde and Medeiros, 2008). By far the most commonly used animal models for screening plants for antidiabetic activity are the chemically (alloxan and streptozotocin) induced diabetic animal models (Fröde and Medeiros, 2008). Alloxan and streptozotocin exert their diabetogenic action when administered parenterally: intravenously, intraperitoneally or subcutaneously (Fröde and Medeiros, 2008). The dose of these agents required for inducing diabetes depends on the animal species, route of administration and nutritional status. According to the administered dose of these agents, syndromes similar to either

type 1, type 2 diabetes mellitus or glucose intolerance can be induced (Lenzen *et al*., 1996 and Mythili *et al*., 2004; Federiuk *et al*., 2004).

In general, the majority of published studies which evaluated the antidiabetic activity of medicinal plants using alloxan or streptozotocin-induced animal models of diabetes 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 (Grover *et al*., 2002; Bnouham *et al*., 2006; Day and Bailey, 2006). Comparative studies are carried out with nondiabetic and/or diabetic animal groups treated with known antidiabetic drugs (Day and Bailey, 2006). Glucose is measured by standard glucose-oxidase or dehydrogenase assays, mainly by means of commercial meters available everywhere (Matsui *et al*., 2006; Fröde and Medeiros, 2008). Animal models of diabetes appear to be more useful in screening plants for their antidiabetic activity than *in vitro* bioassay screening techniques, but ethical and practical considerations make it impossible to screen large numbers of samples (Day and Bailey, 2006; Fröde and Medeiros, 2008).

2.5.5. Isolation and identification of bioactive components

Bioactive fractions are fractionated using a bioassay guided fractionation. In bioassay-guided fractionation, a crude mixture is fractionated into its fraction components using chromatographic procedures, followed by biological evaluation (bioassay) of each fraction. Only fractions which display biological activity in the bioassay are selected for further fractionation. The cycle of fractionation and testing and further fractionation is repeated until a pure compound with the desired activity is isolated (Rimando *et al*., 2000). Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will try to attempt the elucidation of the compound. Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D and 2-D Proton NMR as well as C-13 NMR, Infra Red (IR), Mass Spectrometry (MS) and X-Ray analysis (Harborne, 1998).Investigation of the mechanism of action of antidiabetic plant extracts

An antidiabetic agent may exert its blood glucose lowering effect by stimulating insulin secretion from pancreatic beta-cells, enhancing glucose uptake by fat and muscle cells, altering the activity of some enzymes that are involved in glucose metabolism or slowing down the absorption of sugars from the gut (Tanira, 1994; Cheng and Funtus, 2005). Mechanism based *in vitro* procedures for investigating the effects of plant materials on insulin secretion, glucose uptake and activities of enzymes involved in glucose metabolism have been described above.

2.5.6.1. Effect on insulin secretion

In most published studies, investigation of the effect of medicinal plant extract on insulin secretion *in vivo* has involved the use of streptozotocin or alloxan induced animal models of diabetes (Sheela *et al*., 1996; Kesari *et al*., 2006; Eidi *et al*., 2006;). Both alloxan and streptozotocin causes destruction of pancreatic beta cells of the pancreas, resulting in reduced insulin secretion (Szuldelski, 2001, Fröde and Medeiros, 2008). In streptozotocin and alloxan induced animal models of diabetes, insulin is markedly depleted but not absent (Fröde and Medeiros, 2008). For this reasons these animal models have been widely used to study the effect of antidiabetic remedies on insulin secretion *in vivo.*

In order to investigate the effect of a plant extract on insulin secretion *in vivo*, the majority of published studies have divided normal animal and diabetic animals into at least five groups: normal control rats, normal rats treated with plant extract, diabetic control; diabetic rats treated with plant extract and diabetic rats treated with a conventional insulin secretagogues (Sheela *et al*., 1996; Kesari *et al*., 2005; Pari and Satheesh, 2006). Experimental animals are then treated with the plant extract for a given period of time while control groups receive vehicle during the experimental period. At the end of the experimental period blood is withdrawn for the measurement of plasma insulin. A significant increase in the plasma insulin level of experimental rats compared to those of control rats would suggest the insulinotropic effect of the plant extract, whereas a significant increase in the plasma insulin level of extract treated diabetic animal compared with the diabetic control but no difference between the plasma levels of extract treated normal animal and normal control would suggest a regenerative effect of the plant extract on pancreatic beta cells (Kusano and Abe, 2000; Bnouham *et al*., 2006).

2.5.6.2. Intestinal digestion and absorption of carbohydrates

In order to investigate the effect of an antidiabetic plant extract on intestinal digestion and/or absorption of carbohydrates, study animals are usually divided into experimental and control groups. Experimental animals are given a plant extract under investigation while control animals are given a vehicle. An hour later, both groups of animals are given a fixed amount of glucose, sucrose or starch. Thereafter, blood glucose levels are measured at 0.5, 1, 2 and 3 hrs after administration of the carbohydrate. Areas below the oral glucose tolerance curves of experimental groups are then calculated and compared with those of control groups (Dimo *et al*., 2007; Hannan *et al*., 2007). Alternatively, a glucose tolerance test can be determined in the same group of animals before and after oral administration of the plant extract (Karato *et al*., 2006). A comparison of the glucose

tolerance curve before and after oral administration of the plant extract will indicate whether or not the plant extract contribute to the delay in carbohydrate digestion and subsequent lowering of the blood glucose level glucose.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials, apparatus and instruments

3.1.1. Plant material

Fresh, dark-green leaves of *C. anisata* were collected from the Lowveld Botanical Garden in Neilspruit, Mpumalanga Province. The identity of the plant tree was confirmed by the South African National Botanical Institute (SANBI). Twigs were cut from the top branches of the tree using a light weight, rope operated garden shears. The leaf samples were harvested on the same day and from the same tree to avoid any possible variations that may be brought about the changes in environmental and climatological conditions.

3.1.2. Reagents

3.1.2.1. Chemicals

General chemicals used include organic solvents, ammonium chloride, hydrochloric acid, potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium acetate, sodium carbonate, sodium hydroxide, sodium chloride, sodium potassium tartrate, sodium azide (NaN_3), sodium bisulphite and sulphuric acid. Other special chemicals were 3, 5-Dinitrosalicilate reagent (DNS), Molybdate reagent (2. 5 g of $(NH_4)_6Mo_7.4H_2O$ in 2. 45 M H_2SO_4 w/v), Reducing agent (1. 0 % Elon, p-methyl aminophenol sulphate in 3. 0 % sodium bisulphite ($NaHSO_3$), w/v). All were obtained from Merck and were of analytical grade.

3.1.2.2. Enzymes

The enzymes used in the study were α-amylase, α-glucosidase and glucose-6-phosphatase. Alpha glucosidase (from Bacillus stearothermophilus, 289 mg solid, 103 units/mg protein) and glucose-6-phosphatase (Crude microsomal preparation from rabbit liver (279 mg solid, 0.28 units/mg protein) were obtained from SIGMA-ALDRICH Chemical Company, St. Louis M.O., USA.

Alpha amylase was also obtained from urine samples taken from the National Health Laboratory Services (NHLS), other sources of α-amylase and glucokinase were bovine and porcine pancreas (obtained fresh from a local abattoir) and pancreas from rats experimental models obtained from the Department of Physiology, University of Limpopo (Medunsa Campus).

3.1.2.3. Substrates

Substrates used for α-amylase include soluble starch, potato starch and corn starch. Substrates for α-glucosidase and glucose 6-phosphatase respectively were *p*-Nitrophenyl glycopyranoside and glucose 6-phosphate (1.17 M aqueous solution, 304 mg/ml), all were purchased from SIGMA-ALDRICH Chemical Company, St. Louis M.O., USA.

3.1.3. Apparatus and instruments

Specific equipments that were used include analytical and preparative TLC plates, TLC tanks, fraction collectors, and accessories, water bath (labcon), vortex mixer (Heidolph), weighing balance (PB303, Mettler Toledo), glassware (Schott Mainz, Durban) and magnetic stirrer (all purchased from Bio-Rad Laboratories, Richmond, USA) as well as the Beckman DU-7 UV/Visible spectrophotometer from Germany.

3.2. Methods and techniques

3.2.1. Extraction procedure

The leaves of *C. anisata* were washed with tap water after collection and stored in a dark enclosure at room temperatures for dryness. The dried leaves Approximately 700 g were carefully separated from their twigs and mortar and pestle were used in the grinding process, and 250 g of soft, green powdery material was obtained. The green powder sample of *C. anisata* was sequentially extracted using solvents of varying polarity. Firstly it was twice extracted successively with hot distilled water for 24 hours. The greenish-yellow substance obtained was filtered and concentrated to dryness in a rotavapor. The procedure was repeated with the residue further extracted with acetone, methanol and finally with hexane. The dry extracts of each solvent were stored between 1-4 ˚C until they were ready for use in experiments.

3.4.2. Screening of leaf extracts of *C. anisata* for enzyme inhibitory effect

3.4.2.1. Alpha amylase

Inhibition assay for the enzyme α-amylase was performed in accordance with the standard, chromogenic *in vitro* inhibition procedures modified by Benfeld (1955). Porcine pancreatic amylase (Sigma-Aldrich) or urine amylase was dissolved in ice-cold 20 mM phosphate buffer (pH 6.7) containing 6.7 mM sodium chloride to give a concentration of 5 unit/ml. Seven duplicate test tubes including the blank and control were prepared. In each test tube 250 µl of the enzyme preparation was mixed with 100 µl of each of the leaf extracts of *C. anisata* except the blank (hexane, acetone, methanol and water, 2 mg/ml). The mixtures were stirred in a vortex and pre-incubated in a water bath at 37 °C for 20 minutes.

After incubation, 250 µl of the substrate preparation (0.5 % w/v starch, in 20 mM phosphate buffer, pH 6.7) was transferred into each test tube to start the reaction. The mixture was vortexed and then incubated at 37 °C for 15 minutes. Two millilitres (2 ml) of DNS colour reagent was added and the mixture was stirred in a vortex and boiled in a water bath at 100°C for 10 minutes, thereafter the mixture was cooled down in a running tap water and the absorbance was read at 540 nm using a spectrophotometer.

Inhibition rates were calculated as percentage controls using the formula:
% inhibition = 100 – % reaction (where % reaction = mean product in sample/ mean product in control x 100)

3.4.2.2. Alpha glucosidase

In separate test tubes 100 µl of the enzyme solution (5 units/ml, in phosphate buffer, pH 6.7) was mixed with 50 µl of the plant extract of *C. anisata* (hexane, acetone, methanol and water, 2 mg/ml). Blank and control was also prepared. The mixture was then pre-incubated in a water bath at 37 ˚C for 20 minutes. Hundred microlitres (100 µl) of pNPG (3 mM in phosphate buffer pH 7) substrate solution was added in each test tube to start the reaction, the mixture was further incubated for a 15 minutes after which 2 ml of 0.1 M Na_2CO_3 was added in each tube to stop the reaction. The reaction mixture was then cooled down in running tap water. The absorbance of the yellow coloured solution was read at 400 nm. Absorbance readings and percentage inhibitions were measured and calculated respectively as in 3.4.2.1 above.

3.4.2.3. Glucose 6-phosphatase.

Fifty micro litres (50 µl) of each of the plant extract of *C. anisata* (hexane, acetone, methanol and water, 2 mg/ml) were transferred into separate test tubes followed by 100 µl of enzyme solution (glucose 6-phosphatase in 0. 25 M sodium acetate buffer, pH 6.7). The mixture was then pre-incubated in a water bath at 37 ˚C for 20 minutes. Thereafter 100 µl of the substrate solution (glucose-6-phosphate in 0. 25 M sodium acetate buffer pH 6. 7) was then added into each of the test tube to start the reaction. The mixture was again incubated at 37 ˚C for 15 minutes. When the reaction is complete, 500 µl of Molybdate reagent (2.5 g of $(NH_4)_6Mo_7.4H_2O$ in 2. 45 M H_2SO_4 w/v) and 500 µl of reducing agent (1, 0 % Elon (p-methyl aminophenol sulphate) in 3. 0 % sodium bisulphite ($NaHSO_3$), w/v) were added. The intensity of the blue solution was measured with a spectrophotometer at 660 nm.

3.4.3. Determination of 50 % inhibitory concentration (IC_{50})

3.4.3.1. Alpha amylase inhibition.

The extract was serial diluted to give different concentrations. Seven duplicate test tubes including blank and control were prepared. In each test tube containing 50 µl of the dilutions of the plant extract, 100 µl of the enzyme was added. The mixture was pre-incubated at 37 ˚C for 20 minutes.
The mixture was the removed from the water bath and 100 µl of the starch substrate solution was added in each test tube, the mixture were thoroughly stirred in a vortex mixer and incubated at 37 ˚C for 15 minutes. When the reaction was complete, 2ml of DNS reagent was added. The mixture was boiled for 10 minutes and then cooled down in a running tap water. The absorbance of the solution was read at 540 nm. The graph of % inhibition was plotted against extract concentration to determine the IC_{50} of the water extract of *C. anisata*.

3.4.3.2. Glucose 6-phosphatase inhibition.

The procedure that was followed in this assay is similar to that of α-amylase in 3.4.3.1.1, except where different reagents and plant extracts were used. The graph of % inhibition was plotted against extract concentration to determine the concentration of the water extracts of *C. anisata* giving 50 % inhibition (IC_{50}).

3.4.3.2. Alpha glucosidase

The reagents used in this procedure are similar to those used in 3.2.2.2 except that the acetone extracts of *C. anisata* were serial diluted to give different concentration. Five duplicate test tubes were prepared, blank and control test tubes were also prepared. In each test tube containing 50 µl of the serial dilutions, 100 µl of the enzyme was added and the mixture pre-incubated at 37 ˚C for 20 minutes. The mixture was then removed from the water bath and 100 µl of the *p*-nitrophenyl glycopyranoside (pNPG) substrate solution was added in each test tube, the mixture was stirred in vortex mixer and incubated at 37 ˚C for 15 minutes. When the reaction was complete, 2 ml of 0.1 M Na_2CO_3 was added in each tube. The mixture was boiled for 3-5 minutes after which they were cooled down in running tap water. The absorbance readings were measured with a spectrophotometer. The graph of % inhibition was plotted against different extract concentration to determine the IC_{50} of the acetone extract of *C. anisata*.

3.4.4. Determination of mode of inhibition

3.4.4.1. Alpha amylase inhibition.

Two sets of 7 duplicate test tubes were prepared, one set to determine the enzyme activity in the presence of an inhibitor (leaf extract) (labelled set A) and the other set to determine the activity of the enzyme in the absence of an inhibitor (set B), blank and control test tubes were also prepared. In set A, 100 µl of the inhibitor (water extract, 2 mg/ml) solution was added in each test tube except the blank and control, this was followed by 100 µl of the enzyme (Porcine or urine amylase (5 units/ml). In set B 100 µl of phosphate buffer (20 mM, pH 6.7 containing 6.7 mM sodium chloride) was added in each test tube followed by 100 µl of the enzyme solution. The mixtures in both sets of test tubes were thoroughly mixed in a vortex mixer after which the sets were pre- incubated in a water bath at 37 ˚C for 20 minutes. The mixtures were then removed from the water bath. Serial dilutions of the substrate solution were added in both sets of test tubes (A and B) with concentration ranging between 2. 5 mg/ml to 0. 03125 mg/ml. The two sets of test tubes were then incubated at 37 ˚C for 15 minutes. The two sets were then removed from the water bath and 2 ml of DNS (3, 5 - Dinitrosalicylic acid reagent) colour reagent was added and the mixtures were boiled for 10 minutes. Absorbance of the coloured solution was read at 540 nm. Double reciprocal curve **(1/V v/s 1/[S])** for both sets were plotted to determine the effect of the plant extract (inhibitor) on V_{max} and K_m of the enzyme, where V and [S] are respectively, the velocity of the reaction and substrate concentration.

3.4.4.2. Glucose 6-phosphatase inhibition

Two sets of 7 duplicate test tubes were prepared, one set to determine the enzyme activity in the presence of an inhibitor (plant extract)(set A) and the other set to determine the activity of the enzyme in the absence of an inhibitor (set B), blank and control test tubes were also prepared. In set A, 50 µl of the inhibitor solution (water extract, (2 mg/ml) was added in each test tube except the blank and control, this was followed by 100 µl of the enzyme solution (glucose-6-phosphatase in 0. 25 M sodium acetate buffer, pH 6.7).

In set B, 50 µl of phosphate buffer was added in each test tube followed by 100 µl of the enzyme solution. The mixtures in both sets of test tubes were thoroughly mixed in a vortex mixer after which the sets were pre-incubated in a water bath at 37 ˚C for 20 minutes. The mixtures were then removed from the water bath. Serial dilution of the substrate solution (Glucose-6-phosphate) with concentrations ranging between 2. 5 mg/ml - 0.3125 mg/ml was added in both sets of test tubes (A and B). The two sets of test tubes were then incubated at 37 ˚C for 15 minutes. The two sets were then removed from the water bath and 1 ml of Molybdate reagent (2.5 g of $(NH_4)_6Mo_7.4H_2O$ in 2.45 M H_2SO_4 w/v) and 1 ml of reducing agent (1.0 % Elon (p-methylaminophenol sulphate) in 3. 0 % sodium bisulphite ($NaHSO_3$), w/v) solutions were added and the mixtures boiled for 3-5 minutes. The mixtures were cooled in a running tap water. The absorbance of the blue solution was read at 660 nm. Double reciprocal graph **(1/V v/s 1/[S])** was plotted to determine the effect of the plant extract (inhibitor) on V_{max} and K_m of the enzyme.

3.4.4.3. Alpha glucosidase.

Two sets of 7 duplicate test tubes were prepared, one set to determine the enzyme activity in the presence of an inhibitor (plant extract) (set A) and the other set to determine the activity of the enzyme in the absence of an inhibitor (set B), blank and control test tubes were also prepared. In set A, 50 µl of the inhibitor solution was added in each test tube except the blank and control, this was followed by 100 µl of the enzyme (α- glucosidase in 0.1 M Phosphate buffer, pH 7.0) and in set B 50 µl of phosphate buffer (0.1 M Phosphate buffer, pH 7.0) was added in each test tube followed by 100 µl of the enzyme solution. The mixtures in both sets of test tubes were thoroughly stirred in a vortex mixer after which the sets were pre- incubated in a water bath at 37 ˚C for 20 minutes. The mixtures were then removed from the water bath. Serial dilution of the substrate solution 3 mM p-nitrophenyl glycopyranoside in phosphate buffer, pH 7.0 (pNPG) was added in both sets of test tubes (A and B) with concentration ranging between 2, 5 mg/ml - 0.3125 mg/ml. The two sets of test tubes were then incubated at 37 ˚C for 15 minutes. The two sets were then removed from the

water bath and 2ml of 0.1M Na_2CO_3 solution was added to stop the reaction, the contents of the mixtures were then boiled for 10 minutes. The mixtures were cooled in running tap water. The absorbance of the yellow coloured solution was read at 400 nm. Double reciprocal curve **(1/V v/s 1/[S])** was plotted to determine the effect of the plant extract (inhibitor) on V_{max} and K_m of the enzyme.

3.5. *In vivo* anti-diabetic effects of *C. anisata* leaf extracts

3.5.1. Induction of experimental diabetes

Diabetes mellitus was induced in 12 h fasted animals by intraperitoneal injection of alloxan monohydrate (Sigma, St. Louis, Mo., USA) dissolved in sterile normal saline at a dose of 140 mg/kg body. Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were administered with 20 % glucose solution intraperitoneally 6 h after alloxan treatment. The rats were then kept for the next 24 h on 5 % glucose solution bottles in their cages to prevent hypoglycaemia Diabetes was confirmed in alloxan-treated rats by measuring fasting blood glucose levels 72 h after alloxan treatment. Rats with marked hyperglycaemia (blood glucose level above 11.0 mM) were selected and used in the study.

3.5.2. Effect of *C. anisata* acetone leaf extract on postprandial blood glucose level

Effect of *C. anisata* acetone extract on postprandial blood glucose level in experimental and control rats was determined according to the method described by Hannan *et al*., (2007) with some modifications. Briefly, a group of six 18 h fasted normal rats and a group of six 18 h fasted alloxan-induced diabetic rats were given *C. anisata* stem bark acetone extract by gavages (500 mg/kg body weight). A control group of normal rats and a control group of diabetic rats received distilled water at the same time. 20 minutes later, each rat was given 1 ml of 80% (w/v) sucrose solution. Blood glucose levels were then measured at 30, 60, 90, 120, and 150 minutes after sucrose loading using Glucometer 4 Ames (Bayer Diagnostics). Blood glucose tolerance curves of experimental rats were plotted and compared with those of control rats.

3.6. Statistical Data

Data, expressed as mean ± SD were analyzed using the Sigma Stat statistical program (version 8.0). Comparisons were made between normal and alloxan-induced diabetic rats as well as between treated and untreated alloxan-induced diabetic rats by means of unpaired Student's t-test and their significance were established by ANOVA. Differences of $P < 0.05$ were considered statistically significant.

CHAPTER 4

RESULTS

4.1. Percentage yield of leaf extracts *C. anisata*.

Two hundred and fifty grams (250 g) of dried and powdered leaves of *C. anisata* was sequentially extracted with solvent of decreasing polarity (water, methanol, acetone and hexane). The percentage yields of extracts obtained with these solvents are shown in Figure 4.1.

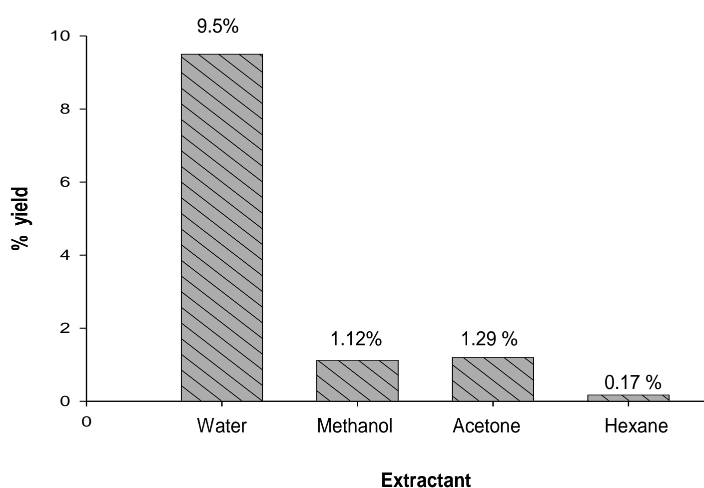

Figure 4.1. Percentage yield of *C. anisata* leaf extracts.

Figure 4.1 and table 4.1 indicate the different solvents used in the extraction, namely hexane, acetone, methanol and water. The mass and the percentage yield of each solvent extract are also shown. The mass of each extract was obtained after evaporation of the solvent (extractant).

Table 4.1. Mass and percentage yield of extractant.

Extractant	**Mass of extract of *C. anisata* leaves (g)**	**Percentage yield of extractant (%)**
Hexane	0. 43	0. 17
Acetone	3. 232	1. 29
Methanol	2. 81	1. 12
Water	23.74	9. 5

The highest percentage yield in mass was observed on the water extracts of *C. anisata,* followed by acetone, then methanol and hexane. Extraction with hexane produced the lowest percentage yield.
% yield of the extract is the total dry mass after evaporation of the solvent (e.g. hexane) and it was calculated as follows:

% Yield = mass of dry extract / total mass dry powdered sample x 100

4.2. Screening of *C. anisata* leaf extracts for enzyme inhibitory activities

Water, methanol, acetone and hexane extracts of *C. anisata* obtained by sequential extraction were screened for enzyme inhibitory activities against human urinary α-amylase, *Bacillus stearothermophilus* α-glucosidase and rat hepatic glucose 6-phosphatase. Results are shown in Figure 4.2.

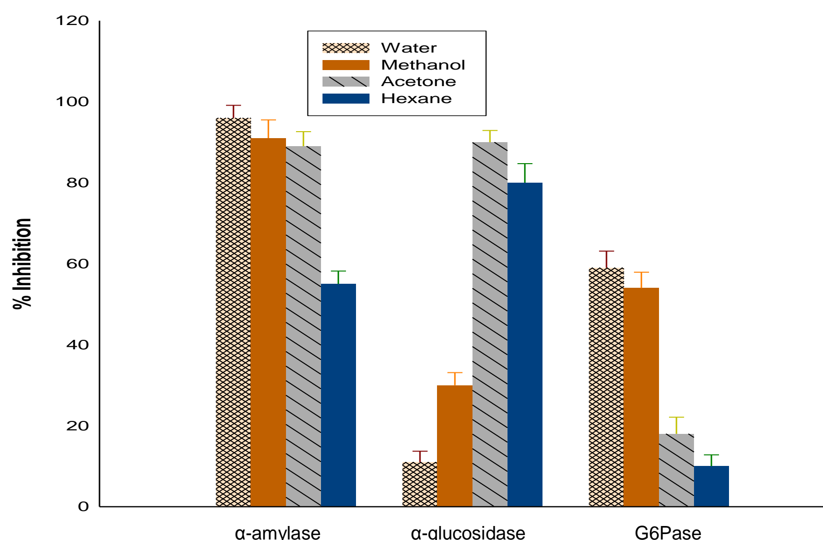

Figure 4.2: Enzyme inhibitory effects of *C. anisata* leaf extracts

The water, acetone and methanol extracts of *C. anisata* showed appreciable (> 80 %) enzyme inhibitory activity against human urinary α-amylase. Acetone and hexane extracts gave appreciable (> 80 %) inhibitory activity against *Bacillus stearothermophilus* α-glucosidase whereas water and methanol showed appreciable enzyme inhibitory activities against rat hepatic glucose 6-phosphatase.

4.3. IC_{50} values of *C. anisata* water and methanol extracts against α-amylase.

IC_{50} (concentration of inhibitor producing 50 % inhibition) values of *C. anisata* water and methanol extracts which demonstrated appreciable inhibitory activity against human urinary α- amylase during the screening test were determined and compared with that of acarbose (a known inhibitor of α- amylase). Results are tabulated in Table 4.2.

Table 4.2: IC_{50} values of *C. anisata* water and methanol extracts against α- amylase compared with that of acarbose.

Inhibitor	**IC_{50}** (μg/ml) **against human urinary amylase**
C. anisata water extract	1947 ± 50*
C. anisata methanol extract	2436 ± 62*
Acarbose	84 ± 11

* **Mean values are significantly different compared with acarbose ($P < 0.001$).**

Both water and methanol *C. anisata* leaf extracts inhibited human urinary amylase significantly less than acarbose ($p < 0.001$) (IC_{50} values significantly higher than that of acarbose).

4.4. IC_{50} values of *C. anisata* acetone and hexane extracts against α-glucosidase

IC_{50} values of *C. anisata* acetone and hexane extracts which demonstrated appreciable inhibitory activity against *Bacillus stearothermophilus* α-glucosidase during the screening test were determined and compared with that of acarbose (a known inhibitor of α- glucosidase). Results are tabulated in Table 4.3.

Table 4.3: IC_{50} values of *C. anisata* acetone and hexane extracts compared with that of acarbose

Inhibitor	**IC_{50} (μg/ml) against** *B. stearothermophilus* α-glucosidase
C. anisata acetone extract	1020 ± 32*
C. anisata hexane extract	2068 ± 59**
Acarbose	36 ± 12

* **Mean value significantly different compared with that of acarbose ($P < 0.01$).**
** **Mean value significantly different compared with that of acarbose ($P < 0.001$)**

C. anisata water and methanol leaf extracts demonstrated significant inhibitory activities ($P < 0.01$ and $P < 0.001$ respectively) against *Bacillus stearothermophilus* α-glucosidase, however, the extracts were less effective than acarbose.

4.5. IC_{50} values of *C. anisata* water and methanol extracts against glucose-6-phosphatase.

IC_{50} values of *C. anisata* water and methanol extracts which demonstrated appreciable inhibitory activity against rat hepatic glucose 6-phosphatase screening test were determined and compared with that of sodium vanadate (a known inhibitor of glucose 6-phosphatase). Results are tabulated in Table 4.4.

Table 4.4: IC_{50} values of *C. anisata* water and methanol extracts compared with that of sodium vanadate

Inhibitor	**IC_{50} (µg/ml)** **rat hepatic glucose-6-phosphatase**
C. anisata water extract	494 ± 12 *
C. anisata methanol extract	1012 ± 59*
Sodium vanadate	1651 ± 46

*Mean values are significantly different compared with metformin. P < 0.05

The results show that both water and methanol extracts of *C. anisata* leaves are more potent inhibitors of glucose-6-phosphatase than sodium vanadate (IC_{50} values lower than that of sodium vanadate).

4.6. Mode of inhibition of *C. anisata* water extract of on α- amylase.

The mode of inhibition (type of inhibition) of *C. anisata* water extract against human urinary amylase was determined by means of Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S]. Results are shown in Figure 4.3.

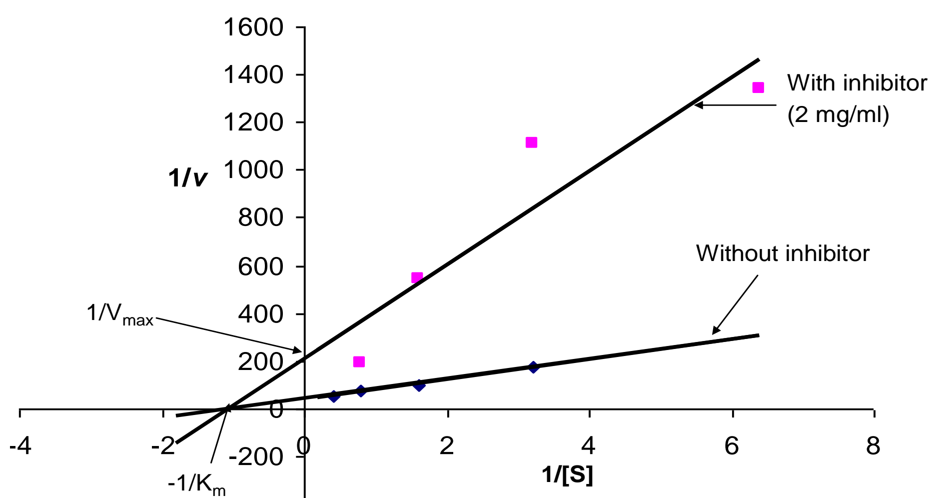

Figure 4.3: Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S] of water extracts of *C. anisata* against human urinary α-amylase

4.7. Mode of inhibition of *C. anisata* acetone extract of against α-glucosidase.

The mode of inhibition (type of inhibition) of *C. anisata* acetone extract against α-glucosidase was determined by means of Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S]. Results are shown in Figure 4.4.

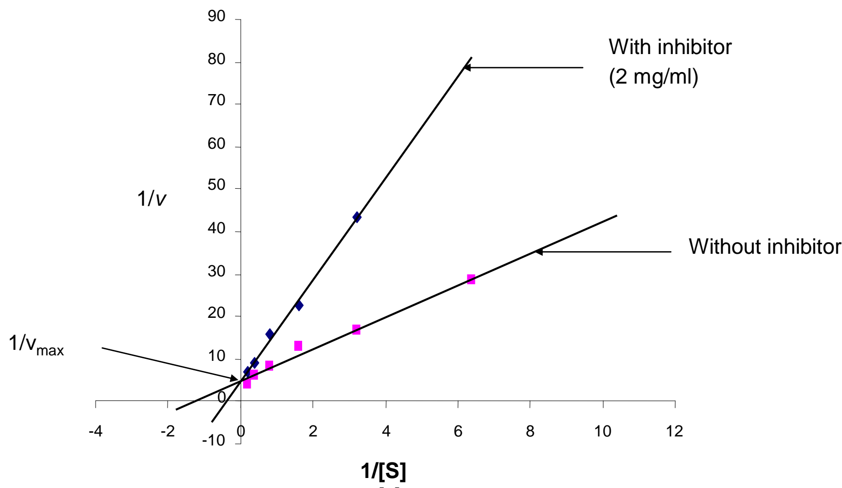

Figure 4.4: Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S] of acetone extracts of *C. anisata* against α-glucosidase

In the double reciprocal plot the x- intercept represent the K_m (rate constant) and the y- intercept represent the V_{max} (maximum velocity of the enzyme) of the enzyme. It is evident that the V_{max} of the enzyme has been altered whereas the K_m remains unchanged. This type of inhibition is called noncompetitive.

4.8. Mode of inhibition of *C. anisata* water extract against glucose-6-phosphatase.

The mode of inhibition (type of inhibition) of *C. anisata* water extract against glucose 6-phosphatase was determined by means of Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S]. Results are shown in Figure 4.5.

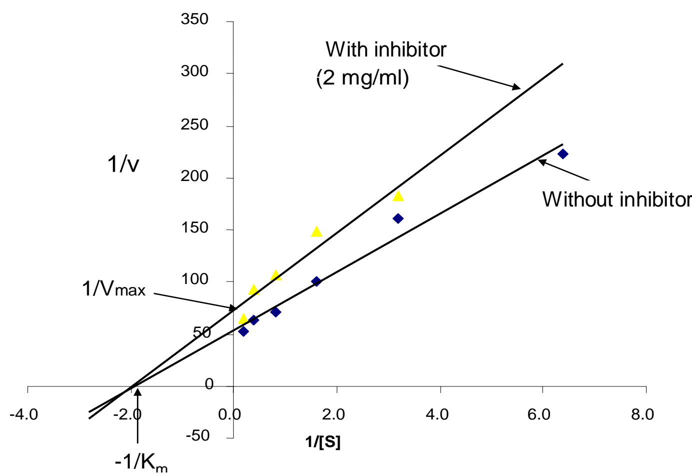

Figure 4.5: Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S] of water extracts of *C. anisata* against glucose-6-phosphatase

4.9 Effects of *C. anisata* acetone extract on postprandial blood glucose level

Administration of *C. anisata* leaf acetone extract in the fasting state 30 minutes before sucrose loading to either normal or alloxan – induced diabetic failed to suppress the postprandial increase in blood glucose levels in both the experimental and control groups rats (Figure 4.6)

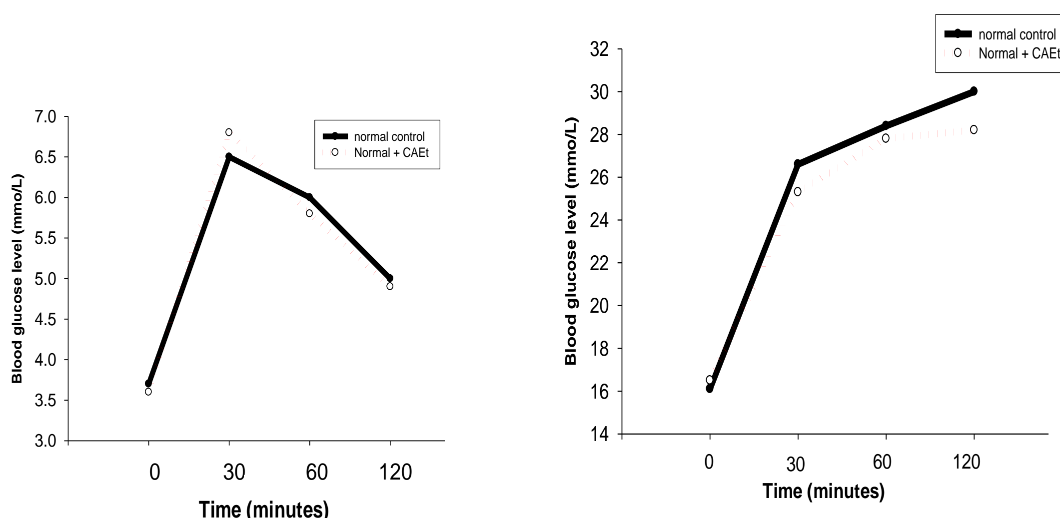

Figure 4.6. Effects of *C. anisata* on postprandial glucose level after sucrose loading in normal and alloxan induced diabetic rats.

4.10. Summary of the main findings of the study

Aqueous and methanolic extracts of *C. anisata* leaves inhibited human urinary α-amylase with IC_{50} values of 1947 ± 50 and 2436 ± 62 µg/ml respectively. Inhibitions of α-amylase by these extracts were however, significantly less than that of the reference drug acarbose (84 ± 11 µg/ml) ($P < 0.001$). The mode of inhibition of these extracts on human urinary amylase appears to be a reversible non-competitive one. Acetone and hexane extracts of *C. anisata* inhibited *Bacillus stearothermophilus* alpha glucosidase with an IC_{50} values of 1020 ± 32 and 2068 ± 59 µg/ml respectively. Inhibition of *Bacillus stearothermophilus* α-glucosidase by these two extracts was also significantly less than that produced by acarbose (36 ± 11 µg/ml) ($P < 0.001$). The mode of inhibition of *Bacillus stearothermophilus* α-glucosidase by the acetone extract was found to be reversible competitive in this case. Aqueous and methanolic extracts of *C. anisata* leaves also inhibited hepatic glucose 6-phosphatase with IC_{50} values of 493.6 ± 12 and 1012 ± 58 µg/ml respectively. In this case the effect of the aqueous extract was significantly less than that of the reference inhibitor of glucose 6-phosphatase, sodium vanadate (1651 ± 46 µg/ml) ($P < 0.05$). The mode of inhibition of glucose 6-phosphatase by *C. anisata* aqueous leaf extract was found to be

irreversible. Furthermore, oral administration of *C. anisata* acetone leaf extract to normal and alloxan induced diabetic rats, 30 minutes before sucrose loading failed to prevent the rise in postprandial glucose levels in these animals.

CHAPTER 5

DISCUSSION, LIMITATIONS AND CONCLUSIONS

5.1 Discussion

Antidiabetic medicinal plants are reported to exert their blood glucose lowering effects through a variety of mechanisms (Tanira, 1994; Bastaki, 2005; Cheng and Funtus, 2005; Bnouham *et al*., 2006). These mechanisms are more or less similar to those of the synthetic oral hypoglycemic drugs (Cheng and Funtus, 2005) and include: i) stimulation of insulin synthesis and/or secretion from pancreatic beta-cells; ii) regeneration/revitalization of damaged pancreatic beta cells; iii) improvement of insulin sensitivity (enhancement of glucose uptake by fat and muscle cells); iv) mimicking the action of insulin (acting like insulin); v) alteration of the activity of some enzymes that are involved in glucose metabolism and vi) slowing down the absorption of sugars from the gut (Tanira, 1994; Cheng and Funtus, 2005).

In the current study, the effects of *C. anisata* leaf extracts on the activities of human urinary α-amylase, *Bacillus stearothermophilus* α-glucosidase and rat hepatic glucose 6-phosphatase were investigate *in vitro*. Alpha amylases hydrolyze complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α-glucosidase to monosaccharides which are absorbed through the small intestines into the hepatic portal vein (Smith *et al*., 2005). Inhibitors of both α-amylase and α-glucosidase delay digestion and subsequent absorption of carbohydrates thereby lowering postprandial glucose levels (Puls *et al*., 1997). Examples of such inhibitors which are currently used as anti-diabetic agents include acarbose (Schmidit *et al*., 1977; Davis and Granner, 1996), miglitol (Standl *et al*., 1999) and voglibose (Saito *et al*., 1998). Hepatic, glucose 6-phosphatase catalyzes the terminal steps in both glycogenolysis and gluconeogenesis and its inhibitors are reported to lower fasting blood glucose levels in experimental animal model of diabetes (Gupta *et al*., 1969, 1971, Marles and Farnsworth, 1995, Singh *et al*., 1992, Akah and Okafor, 1992; Ojewole 2002, 2003, 2005).

Aqueous, methanolic and acetone extracts of *C. anisata* leaves demonstrated appreciable (> 80 %) inhibitory activities towards human urinary α-amylase. The inhibition of human urinary α-amylase by the hexane extract of *C. anisata* (56 %) could be regarded as insignificant when compared to the effect of the other three types of extracts. These observations suggest that human urinary α-amylase,

and hence human pancreatic α-amylase is inhibited by more polar constituents of *C. anisata* leaves. This is in agreement with the results of related studies which have reported α-amylase inhibitory activities in the more polar extracts of plant materials (Ortiz-Andrade *et al.*, 2007). Thus, the α-amylase inhibitory activity of *C. anisata* leaf extracts could be attributed to the presence of polyphenols, flavonoids and their glycosides which are known to be soluble in more polar solvents (Eloff, 1998).

On the other hand, *Bacillus stearothermophilus* α-glucosidase was found to be strongly inhibited by both acetone and hexane extracts of *C. anisata* leaves. In this case aqueous and methanolic extracts produced relatively very weak enzyme inhibitory activities, 12 % and 30 % respectively. This suggests that *Bacillus stearothermophilus* α-glucosidase, at least in the present study was inhibited by relatively non-polar constituents of *C. anisata* leaf extracts. Based on the findings of other similar studies, candidate phytochemicals responsible for α-glucosidase inhibition observed in the current study will include terpenoids and non-polar flavonoids (Okorie 1972). Rat hepatic glucose 6-phosphatase was found to be only moderately inhibited in the current study by both aqueous and methanolic extracts of *C. anisata*, 58 % and 56 % respectively. Thus, like human urinary α-amylase, rat hepatic α-glucosidase is inhibited by relatively polar, hydrophilic constituents of *C. anisata* leaf extracts

Enzyme inhibitors, including enzyme inhibitor drugs are often characterized in terms of their mode of inhibition (inhibitor type) and potency (Smith *et al.*, 2005). Inhibitors of enzymes obeying Michaelis-Menten kinetics are often identified and differentiated from one another by means of their effect on the kinetic constants, K_m (the Michaelis-Menten constant) and V_{max} (maximum velocity of the enzyme) using either Lineweaver-Burk plots or Dixon secondary plots (Stryer, 1990; Mathews and van Holde, 1998). The potency of an enzyme inhibitor is usually determined and reported either in terms of an IC_{50} value (inhibitor concentration corresponding to 50% inhibition) or inhibitor dissociation equilibrium constants (K_i value). A low IC_{50} value suggest a higher affinity of the enzyme for the inhibitor and vice versa, whereas, a high K_i value suggest a higher affinity of the enzyme for the inhibitor and vice versa (Stryer, 1990; Mathews and van Holde, 1998). IC_{50} values and dissociation constants are however, inter-convertible (Cheng and Prusoff, 1973; Benjamin *et al.*, 2003). An IC_{50} value can be determined by interpolation from a plot of percentage inhibition against inhibitor concentration (or the logarithm of inhibitor concentration). Both IC_{50} values and inhibitor dissociation equilibrium constant can be accurately estimated from secondary Dixon plots of the reciprocal of the reaction velocity (*1/v*) against inhibitor concentration [*i*] (Cheng and Prusoff, 1973).

In the current study, the potency of *C. anisata* leaf extracts as inhibitors of human urinary amylase, *Bacillus stearothermophilus* α-glucosidase and rat hepatic glucose 6-phosphatase was assessed and reported in terms of IC_{50} values determined by interpolation of plots of percentage inhibition versus inhibitor concentration. Although IC_{50} values determined in this way are less accurate than those obtained by Dixon plots, they nevertheless provide useful information. Also, the resultant IC_{50} values of the extracts under the study were compared with those determined for known inhibitors of α-glucosidase enzymes and glucose 6-phosphatase, acarbose and vanadate ions respectively.

Aqueous and methanolic extracts of *C. anisata* leaves were found to inhibit human urinary amylase with IC_{50} values of 1947 ± 50 and 2436 ± 62 μg/ml respectively. Inhibitions of α-amylase by these extracts were however, significantly less than that of the reference drug acarbose (84 ± 11 μg/ml) ($P < 0.001$). Acetone and hexane extracts of *C. anisata* were found to inhibit *Bacillus stearothermophilus* α-glucosidase with IC_{50} values of 1020 ± 32 and 2068 ± 59 μg/ml respectively. Inhibition of *Bacillus stearothermophilus* α-glucosidase by these two extracts was also significantly less than that produced by acarbose (36 ± 12 μg/ml) ($P < 0.001$). Aqueous and methanolic extracts of *C. anisata* leaves also inhibited hepatic glucose 6-phosphatase with IC_{50} values of 494 ± 12 and 1012 ± 59 μg/ml respectively. In this case the effect of the aqueous extract was significantly less than that of the reference inhibitor of glucose 6-phosphatase, sodium vanadate (1651 ± 46 μg/ml) ($P < 0.05$), an observation which suggest that these extracts are more potent inhibitors of glucose 6-phosphatase than sodium vanadate,

With regard to the mode of inhibition or type of inhibition, the aqueous *C. anisata* leaf extract demonstrated a reversible non-competitive mode of inhibition. This observation suggest that the α-amylase inhibitory components of *C. anisata* aqueous leaf extract do not resemble the normal substrates of amylase in structure (Smith *et al*., 2005; Venable and Aschenbrenner, 2007). Since the Lineweaver–Burk double reciprocal plot for irreversible enzyme inhibitors are similar to those of reversible non-competitive inhibitors and that the two types of inhibitors can be differentiated from each other through dilution of the reaction mixtures and re-determining the double reciprocal plots (Mathews and van Holde, 2003), dilution of reaction mixtures suggested that the mode of inhibition of *C. anisata* aqueous leaf extract was indeed a non-competitive one, and not an irreversible one. The mode of inhibition of *Bacillus stearothermophilus* α-glucosidase by *C. anisata* acetone extract was found to be a competitive one. This observation suggest that α-glucosidase inhibitory components present in the acetone extracts of *C. anisata* leaves could resemble the normal substrates of this enzyme in structure (Venable and Aschenbrenner, 2007). *C. anisata* aqueous leaf

extract also inhibited glucose 6-phosphatase. In this case the mode of inhibition by the extract initially appeared to be a reversible non-competitive. Dilution of the reaction mixture however, did not affect the appearance of the Lineweaver-Burk double reciprocal plot, an indication that the aqueous extract *C. anisata* contain components which might be irreversible inhibitors of glucose 6-phosphatase.

An attempt was also made in the current study to confirm the observed *in vitro* inhibitory effect of *C. anisata* leaf extracts on α-amylase and α-glucosidase *in vivo* by determining postprandial glucose levels in normal and alloxan-induced diabetic rats after oral administration of sucrose. The result of this experiment was however, disappointing in that the acetone extract of *C. anisata* leaves failed to suppress the rise in postprandial glucose level after carbohydrate ingestion. This observation suggests that the *in vitro* inhibitory activities of both the aqueous and acetone extracts of *C. anisata* leaves are not applicable *in vivo*. Acetone was chosen as an extractant of choice for *in vivo* experiments because it is known to extract both polar and non-polar compounds from plant materials (Eloff, 1998).

5.2. Limitations of the current study

It was an aim of the author to carry out bioassay guided fractionation and identification of bioactive compounds in the active extracts of *C. anisata* leaves using spectroscopic techniques such as NMR, HPLC and GS/MS. However, because of reasons beyond the author and researcher, this could not be accomplished. Without doubt this endeavor would have yielded additional valuable information. Also, the author/researcher wished to conduct more *in vivo* investigation including evaluation of the chronic effects of *C. anisata* leaf extracts on other diabetes mellitus related parameters such as fasting blood glucose levels, plasma insulin, plasma lipids, body weight and glycated hemoglobin ($H1_{ac}$) levels. However, due to the high mortality rate of alloxan-induced diabetic rats experienced in the current study, these could not be done. Again, this would have yielded additional valuable information

5.3. Conclusions

The two main conclusions that can be drawn from the results of the current study are:

1). Although both *C. anisata* aqueous and hexane leaf extract inhibited human urinary α-amylase and *Bacillus stearothermophilus* α-glucosidase in vitro, this does not appear to be the case in

vivo. Thus, the previously reported hypoglycaemic activity of *C. anisata* leaf extract (Ojewole, 2002) could not be attributed to its inhibition of digestive α-glucosidase enzymes, and hence suppression of postprandial glucose levels. Additional studies are needed in order to investigate other possible hypoglycaemic mechanism of action of *C. anisata* leaf extracts and to isolate and identify bioactive components responsible for the observed hypoglycaemic action of *C. anisata* leave extracts.

2. *C. anisata* aqueous leaf extract appeared to be a more potent inhibitor of rat hepatic glucose phosphatase than the known inhibitor of the catalytic subunit of this multi-component enzyme system. Further studies are needed to characterize this extract and to isolate glucose 6-phosphate inhibitory components of the extract.

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