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# Evaluation of six plant species used traditionally in the treatment and control of diabetes mellitus

By

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# DECLARATION

I declare that the dissertation hereby submitted to the University of Pretoria for the degree *Magister Scientiae* has not been previously submitted by me for a degree at this University or any other University, that it is my own work in design and execution and that all assistance in the study has been duly acknowledged.

_____

Mr. NKK Boaduo

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# ABSTRACT

Diabetes mellitus is becoming an increasing concern all over the world. Many people especially in poor communities have been using medicinal plants to treat diabetes and its complications. Much work has been done to find scientific evidence to support the use of medicinal plants in many cases with good evidence to support the traditional use. There has been an increase in research on the use of botanicals for either the treatment and/or management of diabetes in many parts of the world.

To start this study an informal survey on plant species used to treat diabetes was carried out with local inhabitants and herbal traders in the Newcastle region (KwaZulu Natal). The plant species were chosen based on their wide use by traditional healers and local inhabitants. The efficacy of the selected plant (*Senna alexandrina, Cymbopogon citrates, Cucurbita pepo, Nuxia floribunda, Hypoxis hemerocallidea* and *Cinnamomum cassia*) used to treat diabetes mellitus by traditional healers in KwaZulu Natal province of South Africa was evaluated under controlled laboratory conditions. With the exception of *Senna alexandrina* and *Nuxia floribunda*, there has been some independent evidence of the efficacy of these plant species

In this study three relevant *in vitro and semi in-vivo* assays were selected to test the efficacy of different extracts on alpha amylase (carbohydrate digestive enzyme) activity, alpha glucosidase (glucose absorption) activity and islets of Langerhans insulin secretory activity. Hexane, ethyl acetate, acetone and methanol extracts were examined and screened for their phytochemical properties and activity in the selected assays

## Alpha amylase inhibitory assay

Not all extracts of the plant species had α-amylase enzyme inhibitory activity. The acetone extracts of *C. pepo* and *H. hemerocallidea* had enzyme inhibition less than that of acarbose positive control ($EC_{50}$ = 1.82, 0.92 and 0.56 mg/ml respectively). The other plant species that had substantial α-amylase inhibitory activity was the methanol extracts of *C. citratus* and *C. cassia* ($EC_{50}$ = 0.313 and 0.12 mg/ml respectively), ethyl acetate extracts of *C. citratus* and *N. floribunda* ($EC_{50}$ = 1.20 and 1.60 mg/ml respectively). The hexane extracts of *C. cassia* (0.72 mg/ml), *N. floribunda* (0.88 mg/ml), *C. pepo* (0.70 mg/ml) and *S. alexandrina* (0.083 mg/ml) all had α-amylase inhibitory activity.The best activity was present in the intermediate polarity extracts. If these more apolar plant extracts are not toxic or do not have negative side effects they may be much more efficient than acarbose in managing α-amylase activity.

## Alpha glucosidase inhibitory assay

In contrast to the alpha amylase activity, the inhibitory activity of the non-polar (hexane and ethyl acetate) plant extracts was in general higher than that of polar extracts. With the methanol and acetone

extracts the inhibitory activity varied from no activity in the methanol extract of *C. cassia* to highly active methanol extract of *C. pepo* (70.3%) and acetone extract of *H. hemerocallidea* (84.35%). Among the plants studied *C. cassia* and *N. floribunda* (bark) had the highest inhibitory activity in the hexane and ethyl acetate extracts, the acetone extract of *H. hemerocallidea* had the highest inhibitory activity. The hexane crude extracts of *N. floribunda* and *C. citratus* had very high inhibitory activity at the highest concentration tested (1 mg/ml). The ethyl acetate crude extracts of all the plant species used in this study had an inhibitory activity above 90% against α-glucosidase at 1 mg/ml. When compared to acarbose all the plant species used in this screening study had good activity against the α-glucosidase enzyme with the exception of the methanol extract of *C. cassia*. The inhibitory activity of hexane and ethyl acetate extracts was close to that of the positive control. If the more non-polar plant extracts are not toxic or do not have negative side effects (not tested) it appears that they may be more or less efficient than acarbose in managing α-glucosidase activity.

Islets of Langerhans as a target site

Only with the *H. hemerocallidea* acetone extract was there an increase in insulin secretion of 2.5 mIU/L (Table 8) at 8 ug/ml. With all the other extracts the insulin levels were less than 0.2 mIU/L. The positive controls of acarbose and glibenclamide at a concentration of 1 mg/ml stimulated insulin secretion to 11.5 and 19.8 mIU/L respectively. In comparison, the positive controls acarbose and glibenclamide control produce a 5-8 fold greater increase in insulin secretion although the exposure was at a 100-fold higher concentration. This would indicate that the *H. hemerocallidea* acetone crude extract contains a very potent secretogogue compound. It is possible that higher concentrations of the other plant extracts may also have led to stimulation of insulin production. If the more non-polar plant extracts are not toxic or do not have negative side effects and are biologically available, it appears that they may be much more efficient than acarbose and glibenclamide in managing insulin secretion.

Conclusion

The best overall activity was observed in the non-polar and intermediate solvents (hexane and ethyl acetate). Although the organic extracts had good activity, it does not explain the use of aqueous extracts by traditional healers because water extracts were not active in the assays. The activity of the *C. pepo* acetone leaf extract and *N. floribunda* ethyl acetate bark extract is the first reported evidence of activity with regard to diabetes mellitus. From the *in vitro* results, it can be concluded some extracts of all the traditionally used species have some merit in the management of diabetes mellitus type II, as suggested by the ethnomedicinal leads. In may be worthwhile following up on this work by isolating the compounds responsible for the biological activities.

# TABLE OF CONTENTS

DECLARATION ..... ii
ACKNOWLEDGEMENTS ..... iii
ABSTRACT ..... iv
TABLE OF CONTENTS ..... vi
LIST OF TABLES ..... ix
TABLE OF FIGURES ..... x
LIST OF ABBREVIATIONS ..... xii
CHAPTER 1 ..... 1
1.1 Introduction ..... 1
1.2 Aim ..... 2
1.3 Objectives ..... 2
CHAPTER 2 ..... 3
Literature review ..... 3
2.1 Herbal remedies in general ..... 3
2.2 Traditional African Medicines ..... 3
2.2.1 Overview ..... 3
2.2.2 South African Herbalism ..... 6
2.2.3 Future prospects of African traditional medicine ..... 7
2.3 Diabetes Mellitus (DM) ..... 7
2.3.1 Introduction ..... 7
2.3.2 Classification of diabetes mellitus ..... 8
2.3.3 Other Forms ..... 10
2.3.4 Factors influencing the prevalence of diabetes ..... 10
2.3.4 Genetics of type II Diabetes mellitus ..... 12
2.4 Physiology of Glucose metabolism ..... 13
2.4.1 Glucose metabolism ..... 13
2.4.2 Pathophysiology ..... 15
2.5 Clinical recognition and diagnosis of diabetes mellitus ..... 17
2.6 Treatment ..... 19
2.6.1 Insulin therapy ..... 19
2.6.2 Other forms of diabetic therapies ..... 21
2.6.3 Concurrent non-drug therapy ..... 22

2.7 Complications of diabetes ..... 22
2.8 Herbal remedies and diabetes mellitus ..... 23
2.9 Diabetes research in South Africa ..... 26
2.10 Preliminary Screening ..... 26
2.10.1 Antioxidants and diabetes ..... 26
2.10.2 Total Polyphenolic content and diabetes ..... 27
2.12 Conclusion ..... 28
CHAPTER 3 ..... 29
Collection of plant material and preliminary work on extracts ..... 29
3.1. Collection and preparation of plant material ..... 29
3.1.2 Extraction ..... 34
3.1.3 TLC fingerprinting ..... 35
3.1.3.1 Thin layer chromatography (TLC) ..... 35
3.2 Total polyphenolic content ..... 36
3.3 Results and discussion ..... 36
3.3.1 Quantity extracted ..... 36
3.3.2 TLC fingerprints ..... 37
3.3.2.1 Vanillin and p-anisaldehyde sulphuric acid spray ..... 37
3.3.3 Antioxidant assay ..... 38
3.3.4 Total polyphenolic content ..... 42
CHAPTER 4 ..... 45
Alpha amylase inhibitory assay ..... 45
4.1 Introduction ..... 45
4.2 Materials and methods ..... 48
4.3 Results and discussion ..... 49
4.4 Conclusion ..... 54
CHAPTER 5 ..... 55
Alpha glucosidase inhibitory assay ..... 55
5.1 Introduction ..... 55
5.2 Materials and methods ..... 56
5.3 Results and discussion ..... 57
CHAPTER 6 ..... 60
Islets of Langerhans as a target site ..... 60
6.1 Introduction ..... 60

6.2 Materials and methods ..... 61
6.3 Results and discussion ..... 63
CHAPTER 7 ..... 65
7.1 Conclusion ..... 65
7.2 Recommendations ..... 68
APPENDIX A ..... 69
CHAPTER 8 ..... 70
8.1 References ..... 70

# LIST OF TABLES

Table 1: Classification of type I and type 2 diabetes……..10
Table 2: (i) Fasting Plasma Glucose test and (ii) Oral Glucose test……..19
Table 3: Plant species used to treat diabetes mellitus in Newcastle (Kwa-Zulu Natal)……29
Table 4: The alpha amylase family covers about 20 different enzymes specificities. They are all given EC numbers and are ordered in the following way: the members with crystallographically known three-dimensional structure, hydrolases, transferases, glucanotransferase that has been proposed to contain a circularly permuted version of the $(\alpha/\beta)_8$ barrel and proteins without catalytic function……46
Table 5: The minimum and maximum inhibitory α-amylase activity (%) of each plant at a minimum and maximum concentration with $EC_{50}$ (mg/ml) for each of the solvents used in this study……52
Table 6: The glucose amount (mg) present after treatment of intestinal rat acetone powder with six South African plants used to treat and manage diabetes……..57
Table 7: Alpha glucosidase inhibitory activity (%) of six South African plants used to treat and manage diabetes mellitus…… 58
Table 8: Table showing the amount of insulin stimulated by each plant species……..63
Table 9: Summery of results for all the plant species and the procedures performed ……..67

# TABLE OF FIGURES

Figure 1: Some of the animal parts used in African traditional healing systems indicating the skin and bones collected from numerous animal species believed to have healing properties (own picture) .....5

Figure 2: Dried herbal medicine available for sale in a South African herbal market, many of the medicinal plants come in different forms some are finely ground and sold while others are sold as whole plant parts (own picture) .....6

Figure 3: The extraction of energy from foodstuffs. In the first stage large molecules in food are broken down into smaller units, in stage two the small units are degraded to a few simple units that play a role i metabolism and the third stage consists of the citric acid cycle and oxidative phosphorylation (Stryer, 1988) .....14

Figure 4: Insulin binding and activation of GLUT4. The binding of insulin to insulin receptor stimulate the release of glucose which leads to glucose utilization in the cell (http://www.dolly.biochem.arizona.edu). .....16

Figure 5: Phosphorylation and stimulation of IRS1 with P85 subunit. This diagram shows all the signal transduction and activation subunits that are responsible in the oxidative Phosphorylation in the metabolic and growth pathway in cell (http://www.dolly.biochem.aizona.edu).....17

Figure 6: Schematic diagram for the investigation of antidiabetic/hypoglycaemic activity in medicinal plants.....25

Figure 7: Parts of the six plants species used in this study. A- *C. citratus*, B- *N. floribunda*, C- *C. pepo*, D- *S. alexandrina*, E- *C. cassia* and F- *H. hemerocallidea* (www.beautifulbotany.com).....33

Figure 8: Quantity of extracts produced (% yield) by six plant species using four different solvents; acetone, methanol, hexane and ethyl acetate. .....37

Figure 9: TLC chromatograms of six plant species *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea*(Hh), *Cinnamomum cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp), extracted with acetone and methanol (left to right) developed in CEF, EMW and BEA (top to bottom) and sprayed with vanillin sulphuric acid in methanol. The circled bands were florescent under ultraviolet light.....38

Figure 10: TLC chromatograms of six plant species {(left to right) *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea* (Hh), *Cinnamomum Cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp)} extracted with acetone, methanol, ethyl acetate and hexane (left to right) developed in BEA, CEF and EMW (top to bottom) and sprayed with *p*-anisaldehyde sulphuric acid in ethanol.....40

Figure 11: Antioxidant TLC chromatograms of six plant species *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea* (Hh), *Cinnamomum Cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp) extracted in acetone and methanol (left to right) developed in BAW, EMW, CEF (top to bottom). The ethyl acetate and hexane extracts did not show any qualitative antioxidant activity. ..... 41
Figure 12: Polyphenol quantity of six plant species extracted with four solvents (values are given as means ± SEM)..... 42
Figure 13: A ribbon diagram of human alpha amylase structure showing the three domains (Pink & Gray- A domain, Blue- B domain & Red- C domain) with the chloride ion (green) and calcium ion (yellow) (www.wikipedia.org) ..... 47
Figure 14: Alpha amylase inhibitory activity of the acetone extracts. Only *C. pepo, H. hemerocallidea & C. citratus* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated..... 50
Figure 15: Alpha amylase inhibitory activity of methanol extracts. Only *H. hemerocallidea, C. citratus & C. cassia* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated..... 50
Figure 16: Alpha amylase inhibitory activity of ethyl acetate extracts. Only *H. hemerocallidea, N. floribunda & C. citratus* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated ..... 51
Figure 17: Alpha amylase inhibitory activity of six plant species extracted with hexane. *S. alexandrina, C. cassia, C. pepo, H. hemerocallidea, C. citratus, N. floribunda. C. pepo* and *C. citratus* had a dose related inhibition at the different concentrations. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated ..... 51
Figure 18: Alpha glucosidase inhibitory activity (%) of six South African plants ..... 59
Figure 19: The pancreas is made up of many clusters of cells called islets of langerhans. Within each islet are different types of cells, including the alpha cells, which secrete glucagon, the beta cells, which secrete insulin and the delta cells, which secrete somatostatin (Krall & Beaser, 1988). ..... 61
Figure 20: Pancreatic Islet cell stain (hematoxylin and eosin) no. 2 illustrating the morphology of pancreatic islet cells as seen under a light microscope at high magnification (Eroschenko, 2005). These cells can be seen in exactly the same formation when evaluated unstained under an inverted microscope. ..... 62
Figure 21: Standard curve for polyphenols..... 69
Figure 22: Standard curve for maltose..... 69
Figure 23: Standard curve for glucose..... 69

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AGIs | Alpha glucosidase inhibitors |
| Anti-GAD | Anti-glutamic acid decarboxylase |
| ATP | Adenosine triphosphate |
| BAW | Butanol, glacial acetic acid, water |
| BEA | Benzene, ethanol, ammonia hydroxide |
| BSA | Bovine serum albumin |
| CAT | Catalase |
| CAM | Complementary and alternative medicine |
| CEF | Chloroform, ethyl acetate, formic acid |
| DM | Diabetes mellitus |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl suphoxide |
| DNS | 3.5-dinitrosalicylic acid |
| DPPH | 1, 1-diphenl-2-picrylhydroazyl |
| $EC_{50}$ | Concentration that will produce 50% of the maximum effect |
| EMW | Ethyl acetate, methanol, water |
| FAD | Flavin adenine dinucleotide |
| FPG | Fasting plasma glucose |
| GDM | Gestational diabetes mellitus |
| GIP | Gastric inhibitory polypeptide |
| GLP-1 | Glucagon like peptide |
| GLUT | Glucose transporter |
| GSH | Px- glutathione peroxidase |
| HBSS | Hank's balance salt solution |
| 3-HMX | 3-hydroxymethyl xylitol |
| IDDM | Insulin dependent diabetes mellitus |
| IRS1 | Insulin receptor site 1 |
| JOD | Juvenile-onset diabetes |
| KPBS | Krebs-ringer bicarbonate solution |
| LEPR | Leptin receptor |

| | |
|---|---|
| MOD | Maturity-onset diabetes |
| MRDM | Malnutrition-related diabetes mellitus |
| mRNA | Messenger ribonucleic acid |
| NAD | Nicotinamide adenine dinucleotide |
| NIDDM | Non-insulin dependent diabetes mellitus |
| OGT (T) | Oral glucose tolerance (test) |
| PPAR | y- Peroxisome proliferators activated receptors |
| SADA | South African diabetes association |
| SANBI | South African national botanical institute |
| SOD | Superoxide dismutase |
| Sp | Species |
| STI | Soybean tyrosine inhibitor |
| SUR | Sulphonylurea receptor |
| TLC | Thin layer chromatography |
| WHO | World Health Organization |

# CHAPTER 1

## 1.1 Introduction

The human population has always been plagued by diseases that have adversely affected health and well-being. Whilst for hundred years these ailments were caused by infectious agents, non-communicable diseases have become the main public health concern in the 21st century (Zimmet *et al*, 2001). Of these, one particular disease that is increasing causing greater morbidity and mortality, in both young and old, is diabetes mellitus. Diabetes mellitus is a metabolic disease characterized by hyperglycaemia resulting from defects in insulin secretion and/or action. This disease, which has been considered a disease of minor significance to world health until a few decades ago, is now seen as one of the main threats to human health in the 21st century (Zimmet *et al*, 2001). In the past three decades the number of people diagnosed with diabetes has exploded to record highs with further increases expected. The global figures according to Eastman *et al*, (1996) is set to increase from the current estimate of 150 million to 220 million by 2010 and to 300 million by 2025, representing an overall increase of about 46% worldwide.

Several forms of diabetes mellitus are known to occur but type I and II predominat. Type I diabetes is the autoimmune-mediated form of the disease and is characterized by the destruction of pancreatic beta cell islets resulting in absolute insulin deficiency whilst type II diabetes is characterized by insulin resistance or the abnormal secretion of insulin. In comparison, people inflicted with type I diabetes are wholly dependent on exogenous insulin for survival, whilst people with type II produce insufficient amounts of endogenous insulin and may at times require insulin supplementation for the control of blood glucose concentrations either directly or indirectly through the use of hypoglycaemic medication (Shafrir, 1997). According to Zimmet *et al* (2001), the diabetes epidemic relates particularly to type II diabetes and results in both the developed and developing world and is more prevalent in the general population than type I diabetes (the chronic form of the disease). Most worryingly there has been an increase in the number of cases in children with type II diabetes in developed countries like Japan, United States of America, Pacific Islands, Hong Kong, Australia and the United Kingdom i.e. all first world countries associated with high calorie-intake diets (Zimmet, *et al*, 2001)

To manage the medical catastrophe that diabetes has become, numerous treatments have been developed. In most cases treatment involves the use of exogenous insulin in the case of type I diabetes while for type II diabetes treatments usually involves a combination of drug therapy and life style

interventions such as diet modification and physical exercise (Zimmet *et al*, 2001). With regards to drug therapy type II diabetes is best managed through the use of drugs that increase insulin release, increase insulin effect, or modulate the glucose absorptive pathways. Recently there has also been a surge in the use of botanicals to treat and control diabetes, due to the common perception that the pharmaceutical products on the market induce severe complications following long term use (Hanefeld, 1998). To this extent, there has been a tremendous surge in research on the use of botanicals for either the treatment and/or management of diabetes. At present, several studies have been dedicated to surveys of these botanicals from across the globe. With much of this documentation being obtained through formal and informal discussions with local communities, traditional healers and spiritual leaders, many of the identified remedies need to be validated using validated scientific methods to confirm their efficacy.

## 1.2 Aim

To evaluate the efficacy of plant extracts derived from *Senna alexandrina, Cymbopogon citrates, Cucurbita pepo, Nuxia floribunda, Hypoxis hemerocallidea* or *Cinnamomum cassia* used to treat diabetes mellitus by traditional healers in KwaZulu Natal province of South Africa.

## 1.3 Objectives

1. To conduct a survey and literature study on the plants used to treat diabetes
2. To collect and validate plant material and prepare plant extracts
3. To conduct phytochemical screening for each of the plant extracts
4. To establish, through the use of *in vitro* (alpha amylase and alpha glucosidase assays) *and semi in-vivo* (Islets of langerhans as a target) screening methods, if crude extracts of *Senna alexandrina, Cymbopogon citrates, Cucurbita pepo, Nuxia floribunda, Hypoxis hemerocallidea* and *Cinnamomum cassia* have an impact on alpha amylase, alpha glucosidase activity and/or the ability of the pancreatic beta cells to secrete insulin.
5. To evaluate the potential use of the different plant species in treating diabetes mellitus

# CHAPTER 2

## Literature review

### 2.1 Herbal remedies in general

Ethnopharmacology is the study of plants used in traditional medicine and is therefore heavily reliant on interactions between researchers and indigenous communities who passed on the traditional knowledge over generations. Whilst in the main, ethnopharmacology focuses on the presence or absence of evidence for specific therapeutic responses through the use of herbal remedies, the field also extends into phytochemistry where the aim is to identify the chemical constituent of the plant or plant extract that is responsible for the pharmacological activities inherent to a specific plant. Ethnopharmacology may be best described as the multidisciplinary science aimed at studying herbal remedies (Mulholland, 2005).

There are four major types of herbal medicines in existence today according to Elvin-Lewis (2001); Asian, European, Indigenous African Medicines and Neo-western. The Asian herbal medicinal system has its origins in India, China, and Japan with the Aryuvedic, Unani and Siddha practice from India, Wu-Hsing from China and Kampo from Japan. (Feng *et al*, 2006). Asian and African indigenous herbal systems are the most diverse forms of medicines and still practised by various cultures where they are still intact. European herbalism has its roots in the Mediterranean civilization and has since evolved to include plants from all over the world. Neo-western herbal medicine is the latest branch in the herbal industry and constitutes a combination of the European medicinal system and indigenous African herbal systems. (Feng *et al*, 2006).

### 2.2 Traditional African Medicines

#### 2.2.1 Overview

The African continent has an enormous wealth of plant resources and traditional medicines play a vital role in the lives of millions of people throughout Africa (Iwu, 1993 & Botha *et al.*, 2001). The practice of African Traditional Medicine was largely suppressed during the colonial era and denigrated as pagan and unchristian and thus the word ‘witch doctor’ was coined by the missionaries from Europe when referring to traditional health care practitioners (Chavunduka, 1994). During the latter part of the 20$^{th}$ century and the dawn of post-colonial African societies the use of herbal medicines has again become more important and preferred choice for both many rural and urban dwellers in many African countries (Chavunduka, 1994).

According to Halberstein (2005) some of the earliest known written records from the eastern hemisphere also deal with the subject of healing with medicinal plant substances. The ancient Egyptians are credited with developing an elaborate and effective pharmacological collection of vast numbers of natural resources (Nunn, 1996). The plant extracts were prepared and taken internally, applied topically or administered by fumigation and vapour inhalation, with the oral intake being the most common. The Egyptians were also the first to use a number of medicines (wine, castor oil, marijuana, opium, mints and barley) which would have been validated by studies to be medically effective (Halberstein, 2005).

While traditional African medicine is probably one of the oldest and most diverse of all medicine systems since the origin of mankind, the use of remedies by the majority of Africa is poorly recorded. More importantly knowledge in this system is being lost due to poor documentation, urbanization and the destruction of biodiversity due to farming practices that increasingly destroy natural habitats (Halberstein, 2005). Traditional African medicine of some practitioners can be characterized by certain attributes such as the belief in the supernatural as a cause of illness, divination as a diagnostic procedure and the ritualized use of plant and animal derived preparations (Fig 1) in the treatment of diseases (Okpako, 1999). According to Mshiu and Chahabra (1982) African traditional healing system, in general may be divided into four main groups viz. herbalist who uses herbs (plants), minerals and at times animal parts to treat patients, the herbalist-ritualist who uses rituals in addition to herbal remedies to treat patients, ritualist-herbalist who base healing on specific attributes of spirits deemed responsible for the cause of the patient's illness and finally the spiritualist who performs rituals and divinations and whose healing expertise extend beyond that caused by daily living (Gessler *et al*, 1995). While illnesses may be managed with herbal remedies, supernatural causes involve the use of oracles, omens and/or contact with super natural beings or divine sources (Okpako, 1999).

Figure 1: Some of the animal parts used in African traditional healing systems indicating the skin and bones collected from numerous animal species believed to have healing properties (own picture)

An important feature in the use of African traditional medicines is the psychological component which also needs to be considered. The rituals with which herbal remedies are combined enhance the belief of therapeutic cure (Okpako 1999). As such it may even be possible to manage internal ailments through the application of external medications worn around the waist, neck and ankle or even placed under the pillow. African traditional healing system appear to take advantage of the placebo effect which is non-specific and triggered by the emotional state that may stimulate a cascade of events leading to immune stimulation , healing and other clinical benefits to the patient (Okpako, 1999).

In many traditional healing systems herbal medications are classified according to their actual or potential biodynamic effects on the body (Halberstein, 2005). Many of the samples are labelled according to their vernacular names, which leads to misidentification and misuse by people. Vernacular or common names pose a huge problem in that many of the names indicate the cultural importance and usage of the plant and more than one name may be widely known for one plant species i.e. *Clivia miniata* has two English common names, bush lily and orange lily, in Sotho *Asclepias fruticosa* is known as lebegana and lereke-la-ntja and in Afrikaans the same plant species is called melkbos and tontelbos (Van Wyk *et al*, 1997). According to Dounias (2000) cited in Grace *et al* (2002), vernacular names may refer to a number of unrelated plant species, especially when they are used for a common purpose. According to Grace *et al* (2002) synonyms of names given to different plant species and the application of multiple names to a single plant species, plant identification using vernacular names is notably very difficult. She goes on to say that vernacular names cited in literature may be erroneous or recorded for incorrect plant species. Only about 71% of the vernacular names encountered in most markets dealing

in medicinal plants accounted for 176 medicinal plant species that were identified (Botha *et al*, 2001). Poisoning from traditional medicines is usually a consequence of misidentification and it is not uncommon in South Africa (Fennell *et al*, 2004).

### 2.2.2 South African Herbalism

Medicinal plants are still an important part of South African cultural heritage as a large number (over 60%) of the population in urban and rural communities of South Africa are reliant on herbal medicines for their health care needs, mostly because of their affordability and easier accessibility (Manders, 1998). A large number of plant species are used by Zulu traditional healers, with approximately 3000 plant species known to have medicinal values and 1032 of these plant species; from 147 plant families are currently used as herbal medicines (Hutchings *et al.*, 1996 & van Wyk *et al.,* 1997). In South Africa the trade in traditional medicinal plants is dominated by material with a long shelf life (Fig 2) i.e. bark, roots, bulbs, whole plants, seeds and fruits which are dried and stored in cardboard boxes or sacks (Grace *et al*, 2002). There are several books and published works of traditional plant medicines in South Africa (Watt and Breyer-Brandwijk 1962, Cunningham 1988, Hutchings 1996, Van Wyk 1997 and Williams *et al* 2001). Volume 119 of the Journal of Ethnopharmacology in 2008 was dedicated to South African medicinal plant resources. The prescription and the use of traditional medicines in South Africa are currently not regulated and this always presents the danger of misadministration of potentially toxic plants (Fennell *et al*, 2004).

Figure 2: Dried herbal medicine available for sale in a South African herbal market, many of the medicinal plants come in different forms some are finely ground and sold while others are sold as whole plant parts (own picture)

### 2.2.3 Future prospects of African traditional medicine

During the last two decades traditional systems of medicines and medicinal plant research have become topics of global interest and importance. In developing nations many people are still heavily reliant on traditional healers and medicinal plants to meet their daily primary health care needs (Ojewole, 2002). The use of complementary and alternative medicine (CAM) is on the increase in the industrialized world. According to Ernst and White (2000), two surveys conducted telephonically in the UK showed that one fifth of the respondents have used some form of complementary and alternative medicine and are continuing to use them with the most common therapies being herbalism, aromatherapy and homoeopathy. It is generally presumed that they are safe based on their long usage in the treatment of disease according to knowledge accumulated over centuries (Fennell *et al*, 2004).

## 2.3 Diabetes Mellitus (DM)

### 2.3.1 Introduction

The first recorded description of diabetes mellitus dates back to the Ebers papyrus in Egypt around 1500 BC and it has also been noted in almost all of the ancient culture from Asia to Europe to the Americas (Soumyanath, 2005). The term diabetes mellitus describes a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbance of carbohydrates, fat and protein metabolism (Alberti *et al*, 1998). Diabetes is a chronic disease that occurs when the pancreas fails to produce sufficient insulin, or when the body cannot effectively use the insulin it produces. Since insulin is the main hormone that regulates blood glucose levels, hyperglycaemia is a common result of uncontrolled diabetes, which over time can lead to serious damage to many of the body's systems especially the nerves and blood vessels (WHO, 2006).

According to the World Health Organization (WHO) (2006), there are more than 180 million people worldwide suffering from diabetes and this number is likely to double by 2030. In 2005, an estimated 1.1 million people died from diabetes, with approximately 80% of these deaths occurring in poor and developing countries. Almost half of these deaths were in people under the age of 70 years with 55% being women. The WHO estimates that diabetes related deaths will increase by more than 50% in the next 10 years if the disease is not given urgent attention. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015. Diabetes and its complications therefore pose significant economic and public health consequences for individuals, families, health systems and countries (WHO, 2006).

### 2.3.2 Classification of diabetes mellitus

The first widely accepted classification of diabetes mellitus was published by WHO in 1980 and modified in 1985 (Alberti *et al*, 1998). An expert committee proposed two major classes of diabetes mellitus and named them insulin dependent diabetes mellitus (IDDM) or type I and non-insulin dependent diabetes mellitus (NIDDM) or type II. A new class of malnutrition-related diabetes mellitus (MRDM) was introduced including other types like impaired glucose tolerance and gestational diabetes mellitus (GDM) (Alberti *et al*, 1998)(Table 1). The proposed classification encompasses both clinical stages and aetiological types of diabetes mellitus and other categories of hyperglycaemia. The clinical stages reflect that diabetes, regardless of its aetiology, progresses through several clinical stages during its natural history. People who have or who are developing diabetes mellitus can be categorized by a stage according to the clinical characteristics, even in the absence of information concerning the aetiology (Alberti *et al*, 1998).

Diabetes mellitus is best classified into four major disease syndromes.

1. Type I diabetes (previously known as insulin-dependent or childhood-onset) is characterized by a lack of insulin production. It indicates the processes of beta-cell (β-cell) destruction that may ultimately lead to full diabetes where exogenous insulin is required to prevent the development of ketoacidosis, coma and death. It is usually characterized by the presence of anti-glutamic acid decarboxylase (anti-GAD), islet cell or insulin antibodies which point to the autoimmune processes that led to beta-cell destruction (Alberti *et al*, 1998). Without daily administration of insulin, Type I diabetes is rapidly fatal. Symptoms include excessive urine production (polyuria), thirst (polydipsia), constant hunger despite patients having a voracious appetite (polyphagia), weight loss, vision changes and fatigue (WHO, 2006). There are no clear symptoms of diabetes and many people may not even know they are suffering from the disease until it is too late.

2. Type II diabetes (formerly called non-insulin-dependent or maturity/adult-onset) results from the body's inability to use insulin which results in its accumulation in the fat cells (WHO, 2006). It is the most common form of diabetes and is characterized by disorders of insulin action and insulin secretion and either one can be the predominant feature (Alberti *et al*, 1998). Type II diabetes comprises 90% of the cases of diabetes in people around the world, and it is largely the result of excessive body weight and physical inactivity. Symptoms may be similar to those of Type I diabetes, but are often less marked. As a result, the disease tends to go undiagnosed

for several years, until severe complications have already arisen. Until recently, this type of diabetes was seen only in adults but it is now worryingly also occurring in obese children due to the lack of physical activity and an increase intake of high sugar diets (WHO, 2006).

Type II Diabetes is characterized by fasting and postprandial hyperglycemia and relative insulin insufficiency. If left untreated hyperglycaemia may cause long-term microvascular and macrovascular complications, such as nephropathy, neuropathy, retinopathy and atherosclerosis (Jain & Saraf, 2008). In the early stages of the disease, insulin resistance in the peripheral tissues like the muscle and fat is associated with a compensatory increase in insulin production by pancreatic β-cells. This increase in secretion of insulin initially promotes glucose utilization in the peripheral tissues and decreases hepatic gluconeogenesis. Unfortunately fasting insulin levels progressively increase in a step-wise manner until the β-cells can no longer compensate for the increased insulin resistance and subsequently fail viz. It is the combination of β-cell failure and associated loss of insulin that eventually promotes the severe hyperglycaemia evident in these patients (Jain & Saraf, 2008).

Treatment of a type-II diabetic patient may be simple i.e. increased physical activity to reduce weight (and consequently insulin resistance), reduce intake of dietary fat and adequate intake of complex carbohydrates and fibre which improves insulin action and secretion or more complex through medical intervention i.e. several commercially pharmaceuticals that either enhances insulin action or secretion. Of the two, the first treatment option is the most beneficial as drug therapy may be associated with the long term underlying and undesired effects like weight gain or hypoglycaemia (Jain & Saraf, 2008).

Type I and type II diabetes were clearly thought to represent genetically independent diseases but various lines of evidence suggest that there is a stronger genetic component in the etiology of type II diabetes (Creutzfeldt & Lefebvre, 1988). It is known that environmental factors play a part in the manifestation of the disease as well (van Tilburg *et al*, 2008).

Table 1: Classification of type I and type II diabetes

| Mode of classification | Type I diabetes | Type II diabetes |
|---|---|---|
| Age at onset | *Juvenile-onset diabetes* (JOD) Occurs predominantly in children and young adults | *Maturity-onset diabetes* (MOD) occurs predominantly in middle-aged or old people |
| Insulin dependence | *Insulin-dependent diabetes* (IDDM) usually, with periods without insulin dependence are not frequently observed shortly after the onset of diabetes | *Non-insulin dependent diabetes* (NIDDM) give insulin treatment for better control often advisable especially in younger patients |

3. Type III or Gestational diabetes; this type of diabetes first occurs during pregnancy. During pregnancy the need for insulin appears to increase and gestational diabetes occurs at the late stages of pregnancy. This type of diabetes may go away once the baby has been born but type II diabetes may develop later in life, in woman who has had gestational diabetes.

4. Secondary diabetes; diabetes may develop as a consequence of other diseases or medication. Davidson (1991) listed some causes of secondary diabetes a term coined by Davidson (1991) as “other types” of diabetes, these include pancreatic diseases (pancreatitis, surgery, cystic fibrosis), the use of other drugs not prescribed for the condition (contraceptive pills, diuretics, steroids), genetic syndromes (extremely rare but many have been described) and endocrine diseases (Cushing’s, acromegaly) (Davidson, 1991).

### 2.3.3 Other Forms

The other types of diabetes (some are not true diabetes, but may progress into diabetes with time) arise as a result of complications and are very uncommon forms of diabetes mellitus, but their underlying defects and disease process can be identified in a relatively specific manner and include genetic defects of the beta-cell, diseases of the exocrine pancreas, endocrinopathies, drug or chemically induced diabetes and uncommon forms of immune-mediated diabetes (WHO, 2002).

### 2.3.4 Factors influencing the prevalence of diabetes

Many risk factors have been identified which influence the prevalence or incidence of diabetes type II (van Tilburg *et al*, 2008). Factors of particular importance are:

- Age

  Age is the single most important variable influencing the prevalence of diabetes. Epidemiological studies show that prevalence increases with ages above 40. Oddly there has also been recorded evident of declining incidence with old age in some countries in Europe and the Americas (Jain & Saraf, 2008).

- Gender

  While diabetes was believed to be more common in females than males, recent trends have shown an equal prevalence for both males and females. However, there may be an increase in the prevalence of the disease in men in the last decade (Jain & Saraf, 2008).

- Country and place of residence

  While there appears to a country-associated distribution of the disease, this is most likely skewed due to the population age i.e. due to the fact that diabetes is an age related disorder; countries with elderly populations have more cases of diabetes compared to developing countries with younger populations. In some traditional communities in developing countries diabetes is rare (Jain & Saraf, 2008). Diabetes is considered as a disease of urbanization and several studies have found that the prevalence is significantly higher in urban populations than in rural communities within the same country (Jain & Saraf, 2008).

- Ethnicity

  Many studies have shown the impact of ethnicity on the prevalence of diabetes e.g. studies have shown that Mexican Americans have a 1.9 times higher prevalence of diabetes than Native Americans. A locally based study showed that diabetes was 4 times higher in Indian men than white men and twice as high in Indian women than white women (Jain & Saraf, 2008). When the traditional lifestyle among blacks was followed in the past, diabetes was virtually absent. Since the 1960s studies in South Africa indicated that the majority of the diabetic cases were all entirely hospital based. A study conducted in Cape Town showed a prevalence rate of 3% in 1969 of the black community, since then WHO has indicated an increase of up to 8% in 1985 (Bourne *et al*, 2002). Currently in South Africa the prevalence rate has risen to almost 30% of the population due to urbanization (Bourne *et al*, 2002).

- Socio-economic status and lifestyle

  Socio-economic deprivation associated with poor diet (unhealthy diets- high calorific / saturated fats) and other adverse lifestyle factors are linked to high rates for diabetes (Jain & Saraf, 2008).

- Obesity

  It is clear that obesity is a risk factor associated with diabetes type II diabetes (Jain & Saraf, 2008), as the prevalence of both obesity and diabetes have grown concurrently in many developing and developed countries.

### 2.3.4 Genetics of type II Diabetes mellitus

The occurrence of type II DM is also genetically linked. However, unlike other genetic disorders, type two diabetes, is a multi-factorial disease with many gene loci (genotype- genetic constitution of a cell, an organism or an individual), each with a small to moderate effect contributing to the overall disease. With any genetic disease, expression of these genes are dependent on environmental factors (Phenotype- overall characteristics of an organism e.g. morphology, biochemical or physiological properties) (van Tilburg *et al*, 2008).

Defects in the gene involved in insulin secretion or insulin action, such as insulin receptor substrate 1 (IRS1), the glucagons receptor, the sulphonylurea receptor (SUR), the peroxisome proliferators activated receptor-γ (PPAR-γ) and the MAPKBIP1 have all been observed. The mutations in these genes seem to be limited to a small percentage of type II diabetes patients or to a specific population (van Tilburg *et al*, 2008).

Qu *et al* (2008) described and identified a haplotype (genetic constitution of an individual's chromosome) in the leptin receptor (LEPR) gene which is associated with type II diabetes among the northern Chinese population. The LEPR gene has been considered as one of the genes associated with type II diabetes in many population studies in the last decade. The LEPR is a member of the cytokine receptor family, which plays a critical role in the regulation of energy balance including glucose metabolism and body weight by activating transcription (STAT) proteins STAT3, 5 and 6.

Most recently genome wide scans in four American populations have indicated suggestive linkage to type II diabetes or impaired glucose homeostasis on chromosome 5, 12 and X in Caucasians, on

chromosome 3 in Mexican Americans and chromosome 10 in African Americans. In the eastern and south eastern Chinese Han population two loci in a region on chromosome 9 showed suggestive evidence for linkage to type II diabetes (Jain & Saraf, 2008).

## 2.4 Physiology of Glucose metabolism

### 2.4.1 Glucose metabolism

Like any machine the body requires fuel to provide/produce energy for it to function. The fuels the body needs come from the food we eat, which are made up of carbohydrates (sugars and starch), proteins and fats. These carbohydrates, proteins and fats are split apart by digestive enzymes into their basic building blocks glucose, amino acids and fatty acids respectively (Krall & Beaser, 1989). There is a series of chemical reactions in the metabolic pathway which produces energy from glucose and this pathway is called the Krebs cycle coupled to the electron transfer chain which is present in practically all cells (Krall & Beaser, 1989).

From the three main sources of energy, carbohydrates appear to be the most important. Once carbohydrates are broken down into glucose, the glucose enters the blood stream and comes into contact with all cells including those in the pancreas (pancreatic β-cells). Here it causes the secretion of insulin in response to increased glucose levels. The insulin produced and subsequently excreted then stimulates the cells, through the attachment to insulin receptors on the cell surface, which allows glucose to enter the cells and to be used as a direct energy source or if in excess for the remaining energy is stored as glycogen in the liver or muscles (Krall & Beaser, 1989). (For more detail, please see 2.4.2. below)

There are three stages that must occur in order to generate energy from these foodstuffs (Fig 3);

- The first stage

  Involves the breakdown of large molecules into smaller units, i.e. proteins are hydrolyzed to their twenty kinds of constituent amino acids, polysaccharides are hydrolyzed to simple sugars and fats are hydrolyzed to glycerol and fatty acids (See 2.10.2 for more details on starch metabolism).

- The second stage

  The small units from the first stage are degraded to a few simple units that play a pivotal role in metabolism. Here most of the sugars, amino acids and fatty acids are converted into acetyl units of acetyl CoA and a small amount of adenosine triphosphate (ATP) is generated.

- The third stage

  The third stage consists of the citric acid cycle and oxidative phosphorylation, the final pathway in the oxidation of fuel molecules. Acetyl units are completely oxidized at this stage to $CO_2$ and four pairs of electrons are transferred to NAD+ and FAD for each acetyl group that is oxidized. ATP is then generated as electrons flow from the reduced forms of these carriers to $O_2$ in a process known as oxidative phosphorylation. The bulk of ATP is generated in this stage (Stryer, 1988).

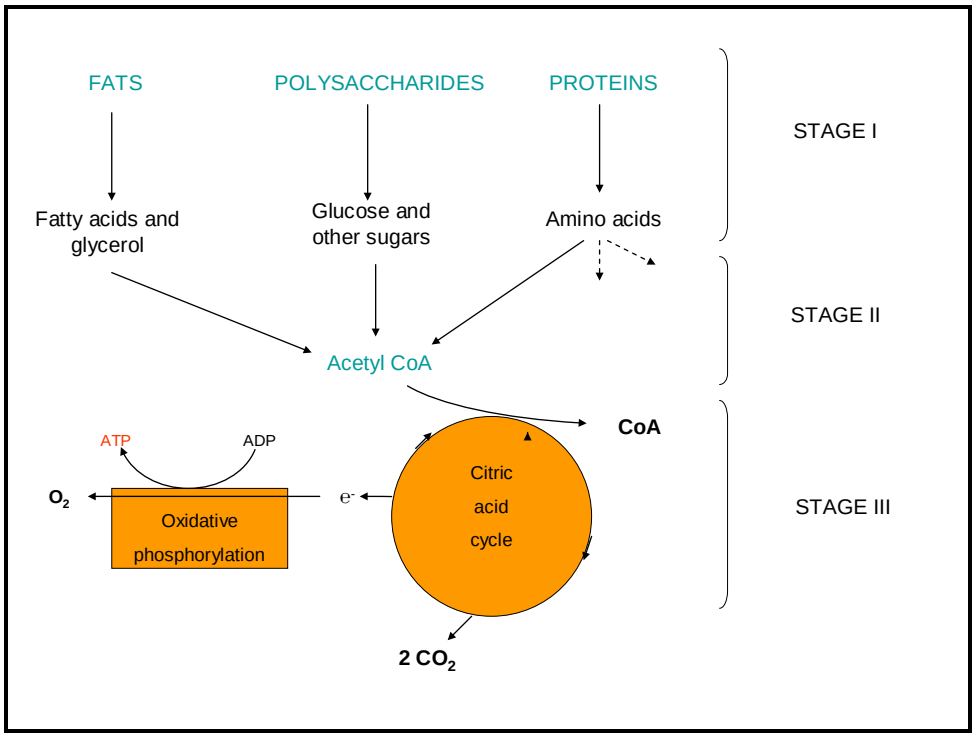



Figure 3: The extraction of energy from foodstuffs. In the first stage large molecules in food are broken down into smaller units, in stage two the small units are degraded to a few simple units that play a role in metabolism and the third stage consists of the citric acid cycle and oxidative phosphorylation (Stryer, 1988)

### 2.4.2 Pathophysiology

Appropriate treatment of Type II diabetes is dependent on the knowledge of the pathophysiology of the disease, the mechanisms underlying hyperglycaemia and the efficacy of various oral agents and insulin to improve fasting or postprandial hyperglycaemia (Codario, 2005). There is no single genetic defect that has been elucidated to explain the aetiology of the disease and thus it is said that the disease may result from a combination of multigenic, heterogeneous, complex and related causes (See 2.3.4 above). A small percentage (<10%) of individuals with monogenic causes of type II diabetes inherit two mutant genes from both parents (Codario, 2005).

To understand the disease, one needs to first understand the normal physiology of insulin action, which is its effect on glucose. Insulin is a protein made up of a long chain of smaller building blocks called amino acids. The β-cells have the ability to take 86 amino acids and hook them together to form a long chain of amino acids called proinsulin (Krall & Beaser, 1988). The proinsulin is packaged as secretory granules and within these granules the proinsulin is cleaved resulting in the production of insulin and a C-peptide which are released into the blood stream through the stimulation of β-cells. The C-peptide has no known function but it is released by β-cells into circulation and it has been useful as a marker to determine how much insulin is released (Krall & Beaser, 1988).

Insulin is released in two stages, during the first stage, which is very rapid and occurs within 10 minutes after the signal is received, insulin is released from the β-cells (Krall & Beaser, 1988). The second stage is more complicated, a signal is sent to the nucleus of the β-cells which stimulates the insulin gene which in turn turns on the transcription of mRNA which transports the signal to the part of the cell that is required to stimulate the production of insulin (Krall & Beaser, 1988). In people without diabetes it is not always possible to raise the levels of blood glucose no matter what they eat because the insulin reserve is plentiful and is secreted in exactly the correct amount, this is however not the case with people suffering from diabetes, the reverse is quite true (Krall & Beaser, 1988).

Glucose transport is rate limiting for overall disposal under most normal physiological conditions. Out of the five types of glucose transporters (GLUT) identified, GLUT4 is the only protein referred to as insulin-sensitive glucose transporter (Codario, 2005). Patients with type II diabetes usually have normal GLUT4 levels but impaired glucose transport and this may indicate that there is a flaw that exists in the insulin-influenced translocation of GLUT4 to the cell surface (Fig 4). This defective signalling pathway

between the receptor and the transport stimulation results in insulin resistance in type II diabetes patients (Codario, 2005).

Fasting glucose levels are dependent on hepatic glucose production, basal insulin levels, insulin sensitivity and the level and duration of the previous prandial glucose. After a meal, elevated glucose levels stimulate insulin release from β-cells. The secreted insulin binds to cell surface receptors and two extracellular α subunits bind to the insulin and transmit a signal to the two identical β subunits via the cell membrane (Codario, 2005). After the binding process the β subunit is phosphorylated increasing tyrosine kinase activity enhancing the phosphorylation of the various endogenous protein substrates (Codario, 2005). This results in a cascading sequence of reactions responsible for the synthesis of proteins and intracellular enzymes, which suppress glucose output and glucose uptake in the peripheral tissues is enhanced. Type II diabetic patients have multiple intracellular deficiencies related to the phosphorylation e.g. impaired ability to phosphorylate and to stimulate the association of insulin receptor stimulator-1 (IRS-1) with the P85 subunit of the PI-3 kinase, impaired phosphorylation of PI-3 kinase and impaired induction of GLUT4 translocation by PI-3 kinase (Fig 5). The glucose molecules than bind to the GLUT4 protein which facilitate the transport of glucose into or out of the cell (Fig 4).

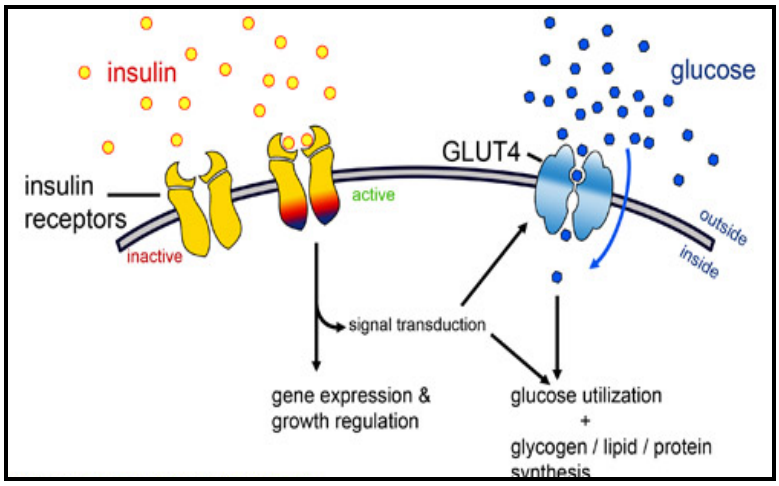



Figure 4: Insulin binding and activation of GLUT4. The binding of insulin to insulin receptor stimulate the release of glucose which leads to glucose utilization in the cell (http://www.dolly.biochem.arizona.edu).

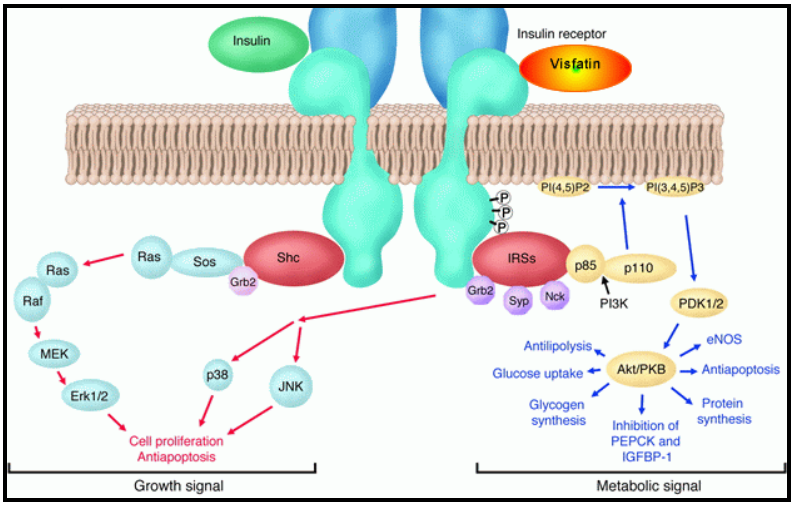



Figure 5: Phosphorylation and stimulation of IRS1 with P85 subunit. This diagram shows all the signal transduction and activation subunits that are responsible in the transduction of the insulin signal in the metabolic and growth pathway in cell (http://www.dolly.biochem.aizona.edu).

## 2.5 Clinical recognition and diagnosis of diabetes mellitus

It is very difficult to physically diagnose diabetes mellitus because there are no external symptoms during the early stages and the clinician must feel very confident that the diagnosis is fully established since the consequences for the individual will be long term and life long. The requirements for proper diagnostic confirmation for a person presenting with severe symptoms and gross hyperglycaemia differ from those of an asymptomatic person with blood glucose levels just above that of the diagnostic cut-off level (7.0 mmol $l^{-1}$ of fasting plasma glucose or 6.1 mmol $l^{-1}$ for whole blood) (Alberti *et al*, 1998).

The diabetic association of South Africa (2001) lists the following as signs that a person may be diabetic:

- Excessive appetite and thirst
- Increased amount and need of urine passed
- Weight loss ( in the case of type I diabetes)
- Feeling of weakness and tiredness
- Itchiness of the skin
- Slow healing of cuts and wounds
- Frequent infections
- Hampered sight
- Pricking and dead feeling sensations in hands and feet
- Dizziness and occasional loss of balance

- Erectile dysfunction.

Diagnosing type II diabetes is often more problematic because the onset is very gradual and a person can have diabetes for many years before presenting with complication of the disease (McDowell *et al*, 2007). In order to diagnose type II diabetes one must first understand the sequence of events that lead to the disease development (Diagram 1).

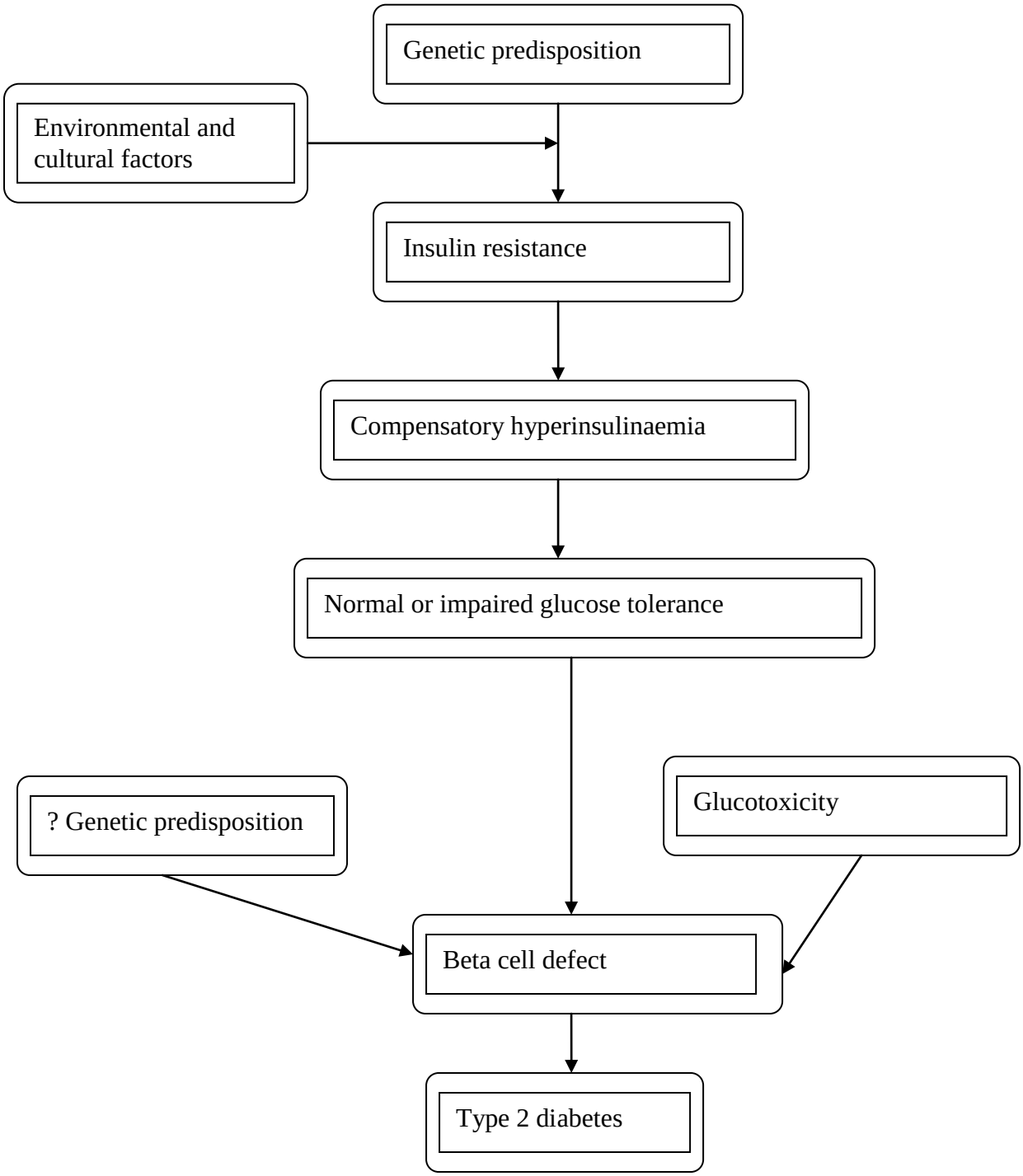



Diagram 1: The sequence of events leading to the diagnosis of type II diabetes mellitus (McDowell *et al*, 2007).

According to the report of the expert committee on the diagnosis and classification of diabetes mellitus (WHO, 2002), there are three ways/criteria to diagnose diabetes and each stage must be confirmed. The first criterion looks at symptoms of diabetes and cause (defined as any time of the day without regard to time since last meal) plasma glucose concentration ≥ of 200 mg/dl, the second looks at fasting plasma glucose (FPG) which must be ≥ 126 mg/dl (table 2i) and the last criteria look at the two hour plasma (PG) ≥ 200 mg/dl during an oral glucose tolerance test (OGTT) (WHO, 2002) (Table 2ii). The clinician should take into consideration such additional factors as family history, age, adiposity and concomitant disorders before deciding on a diagnostic or therapeutic course of action (Alberti, *et al*, 1998). If the FPG and OGT tests are positive, the test must be reconfirmed on a different day by repeating the both tests.

Table 2: (i) Fasting Plasma Glucose test and (ii) Oral Glucose test

| (i) Plasma Glucose results (mg/dL) | Diagnosis |
|---|---|
| 99 or below | Normal |
| 100 to 125 | Pre-diabetes (impaired fasting glucose) |
| 126 or above | Diabetes |

| (ii) Two hour plasma glucose result (mg/dL) | Diagnosis |
|---|---|
| 139 or below | Normal |
| 140 to 199 | Pre-diabetes (impaired glucose tolerance) |
| 200 or above | Diabetes |

## 2.6 Treatment

### 2.6.1 Insulin therapy

The main treatment of type I and more severe forms of type II diabetes mellitus is still dependent on insulin treatment. At present a number of different formulations of insulin are available for use in people. The differences are due to dose, source (animal or transgenic), purity and type (based on formulation) (rapid acting type e.g. “Toronto” insulin and slow/intermediate type e.g. protamine zinc insulin) (Krall & Beaser, 1989). In addition from a clinical perspective, the formulation may be characterised by the onset of action (how quickly it works), time of peak activity (time to best effect) and duration of action. Irrespective of the type of insulin in use, the aim of insulin therapy for patients with type I diabetes is to achieve normal glycaemia levels with minimal pathophysiological side effects (Davidson, 1991).

While the advent of modern insulin formulations has changed the lives of many diabetic sufferers for the better, this has come with the occurrence of numerous adverse drug reactions:

- Delayed local reaction at site of injection

  The earlier impure preparations of insulin caused lesions at the site of injection mainly due to the impurities of the insulin preparations (Davidson, 1991).

- Insulin allergy

  True allergy to insulin also called systemic insulin allergy is rare and occurs in less than 0.1% of patients receiving insulin therapy. It is common in patients with a history of interrupted insulin therapy. The side effect begins as an immediate reaction at the injection site and spreads to the rest of the body in the form of a rash and may also cause oedema and anaphylactic shock (Davidson, 1991).

- Insulin resistance

  Insulin resistance is defined clinically as a situation where the patient requires more than 200 IU of insulin daily for more than two consecutive days. Most commonly this condition may be associated with an increased incidence in infections and gross obesity (Davidson, 1991)

- Insulin induced lipoatrophy

  This side effect causes the loss of subcutaneous fat at the site of insulin injection. This is more common in young females but it is not limited to insulin dependent patients (Davidson, 1991).

- Insulin induced lipohypertrophy

  This condition is due to a local lipogenic effect of insulin and the use of pure insulin at the affected sites i.e. there's an accumulation of subcutaneous fat at this site. The continual injection of insulin at one site also results in diminished central effect as the subcutaneous fat accumulates at the site of injection. Injections in these areas should be avoided (Davidson, 1991).

### 2.6.2 Other forms of diabetic therapies

Other treatments available for use have become known as the oral hypoglycaemic as they can be administered orally unlike parenteral insulin. As the name implies, the oral hypoglycaemic agents work by lowering the glucose levels in the blood. There are several antidiabetic medications available and their use depends on the nature of the diabetes, age, situation of the person and several other factors.

The most common forms of type II diabetic drugs are:

- Sulphonylurea e.g. glibenclamide, are established oral hypoglycaemic agents that act by stimulating insulin secretion by the pancreatic β-cells. These drugs effectively reduce blood glucose levels in type II diabetic patients in the short term. These drugs have, however, not proved to be of much benefit in reducing the long term complications of the disease and at times may be associated with increased weight gains which may eventually lead to hypertension (Hanefeld, 1998).

- Metformin is a relatively old antidiabetic drug that improves the peripheral insulin effect at the musculature and inhibits hepatic gluconeogenesis by enhancing the uptake of glucose into the peripheral cells. It does have a number of undesired side effects such as hepatic impairment, renal impairment and heart failure and it has an anorexic effect (Hanefeld, 1998).

- Acarbose is a novel antidiabetic drug that attenuates postprandial hyperglycemia by delaying carbohydrate digestion, without causing major side effects. Acarbose exerts its inhibitory effects on the alpha-glucosidases, a family of membrane bound enzymes in the intestine that are involved in the digestion and uptake of carbohydrate into the blood stream. Acarbose initiates a cascade of events which leads to improved metabolic control in type II diabetes of all stages, by stimulating both the synthesis and secretion of insulin and in addition improves glycemic control when administered concurrently with other antidiabetic agents. The most common side effect reported of acarbose therapy is meteorism and flatulence. There are rare cases of patients experiencing diarrhoea resulting from the bacterial fermentation in the colon (Hanefeld, 1998).

- Other therapies with a yet to be identified mechanism of effect are the plant extracts currently in use for the treatment and control both diabetes type I and 2 e.g. *Sutherlandia frutescens*, *H. hemerocallidea* and *Psidium guajava.*

### 2.6.3 Concurrent non-drug therapy

In addition to drug therapy, a proper diet is critical in the therapy of diabetes mellitus. Such diets should be balanced and nutritious, ensuring normal growth and development and the attainment of the ideal body weight. When these diets are formulated, the total caloric intake of the patient is designed specifically for the ideal weight of the patient. In addition these diets are designed so that the total caloric intake is divided between the categories of carbohydrates, protein and fat with the use of natural foods high in fibre being highly encouraged (McDowell *et al*, 2007).

Exercise in type II DM patients is usually beneficial as it is increases total energy expenditure, which when combined with a healthy diet, should assist in weight loss. Regular exercise also helps to maintain lean body mass (McDowell *et al*, 2007). However, in insulin dependent diabetic patients exercise may cause hypoglycaemia due to increased absorption of insulin from the injection site and enhanced effectiveness at the tissue level (Davidson, 1991).

## 2.7 Complications of diabetes

The most common complications of diabetes according to the American Diabetes Association (Touchette, 2005) are:

- Cardiovascular disease

  Several cardiovascular diseases occur and they are all due to problems in how the heart pumps blood or how the blood circulates throughout the body. Diabetes medication may cause certain chemical changes in some of the substances found in the blood and this may lead to blood vessels narrowing or clogging up completely, resulting in a condition known as atherosclerosis. Hypertension is also a contributor of cardiovascular disease associated with diabetes (Touchette, 2005).

- Retinopathy (cataracts)

  This is a very common complication of diabetes which affects the retina and more commonly affects type I diabetic patients. Two types are common, the nonproliferative type where the blood vessels are closed off or weaken in the eye and this leads to blurred vision without

blindness. The proliferative type causes a proliferation or sprouting of blood vessels in the retina which may lead to severe eye problem which may result in cataract formation (Touchette, 2005).

- Nephropathy

  In the people with this condition the nephrons are unable to filter out the impurities in the blood and these begin to leak and impurities that are supposed to be removed from the body end up re-circulating in the blood. Not everyone with diabetes develops this condition and it is more common in type I diabetic patients than in type II diabetic patients (Touchette, 2005).

- Neuropathy

  While diabetes does not impair the brain or spinal cord, the peripheral nerves in the rest of the body are affected. This may lead to signal transduction errors which are interpreted aberrantly as pain in hands and feet or as a loss of sensation. This is not a prolonged condition and occurs for short periods of time. Several types of neuropathies occurs and the treatment varies according to the type; Distal symmetric polyneuropathy, focal neuropathy and autonomic neuropathy. The later type occurs in 20 to 40% of people with long standing diabetes (Touchette, 2005).

- Secondary infections

  Bladder infections caused by neuropathy, gingival infections and periodontitis, influenza, pneumonia, foot problems and in some women vaginal disease may occur (Touchette, 2005).

## 2.8 Herbal remedies and diabetes mellitus

The use of herbal remedies and plant derivatives has gathered a lot of interest since the 1980s and the use of complementary and alternative medicine has grown in many countries around the world. Studies conducted in several developed countries such as Australia, United Kingdom and United States of America report that almost half to two thirds of the population affected with diabetes use complementary and alternative medicine to control the condition (Ceylen *et al*, 2008). The use of herbal remedies and plant derivatives to help in the treatment of diabetes should certainly not be discounted. Although numerous 'miracle herbal cure' companies exist, and champion the ability of herbal compounds to supplement insulin as a treatment, these should not be taken at face value without thorough research (Ceylan *et al*, 2008). A review of literature covering the area of diabetes research

revealed many procedures used by researchers to investigate the hypoglycaemic effects of medicinal plants. Each procedure takes into account the aim and objectives of the study, thus a generalised schematic procedure has been summarised in figure 6 to show the overview of the steps taken when investigating hypoglycaemic effects of medicinal plants (Heinrich *et al*, 2004, Harbone, 1998 & Kinghorn & Balandrin, 1993).

There has been a lot of success with the use of plant species to treat and control diabetes and its complications. Much of the success has only been observed experimentally in animal models. However, only a small number of these plants have been studied to evaluate the effect of the herbal therapies on their diabetic condition being managed. In many cases it has been observed and documented that the plants under study have the ability to regulate or contribute to the regulation and production of insulin and glucose to a degree (Ceylan *et al*, 2008). Sepici *et al* (2004) conducted experiments on alloxan-diabetic rabbits and found that the oil from the myrtle plant (*Myrtus communis*) exerts effectual hypoglycaemic activity in the diabetic animal models used without inducing any toxicity.

Alcoholic stem extracts of *Coscinium fenestratum* lowered blood glucose levels in diabetic rats with no toxic effects observed (Shirwaikar *et al* 2005). Ojewole and Adewunmi (2004) tested the effect of *Tetrapleura tetraptera* fruit aqueous extracts in rats and found it to be effective in the control of adult onset diabetes (type II diabetes). Dimo *et al*, 2007 investigated the activity of *Sclerocarya birrea* extract in streptozotocin-induced rats and found that the extracts were able to decrease blood glucose and plasma insulin levels. More recently van de Venter *et al* (2008) performed *in* vitro studies on 11 plant species including *S. birrea*, previously shown to have *in vivo* antidiabetic activity, confirming the *in vivo* work undertaken previously.

Wang *et al* (2007), demonstrated that *P. guajava* extracts had significant inhibition of α-glucosidase activity in the small intestines of diabetic mice while a study by van de Venter *et al* (2008) confirmed the inhibitory activity *in vitro*. *In vitro* assays on *Euclea undulate* and *Schkuhria pinnata* according to Deutschländer *et al* (2009) showed potential in lowering blood glucose levels in C2C12 myocytes cell lines at 50 µg/ml. The *in vitro* assay results from the same study also had α-glucosidase and α-amylase inhibitory activity of *Pteronia divaricata* and *Euclea undulate* at 50 µg/ml with $IC_{50}$ values of 31.22±0.154 and 49.95±0.007 µg/ml respectively.

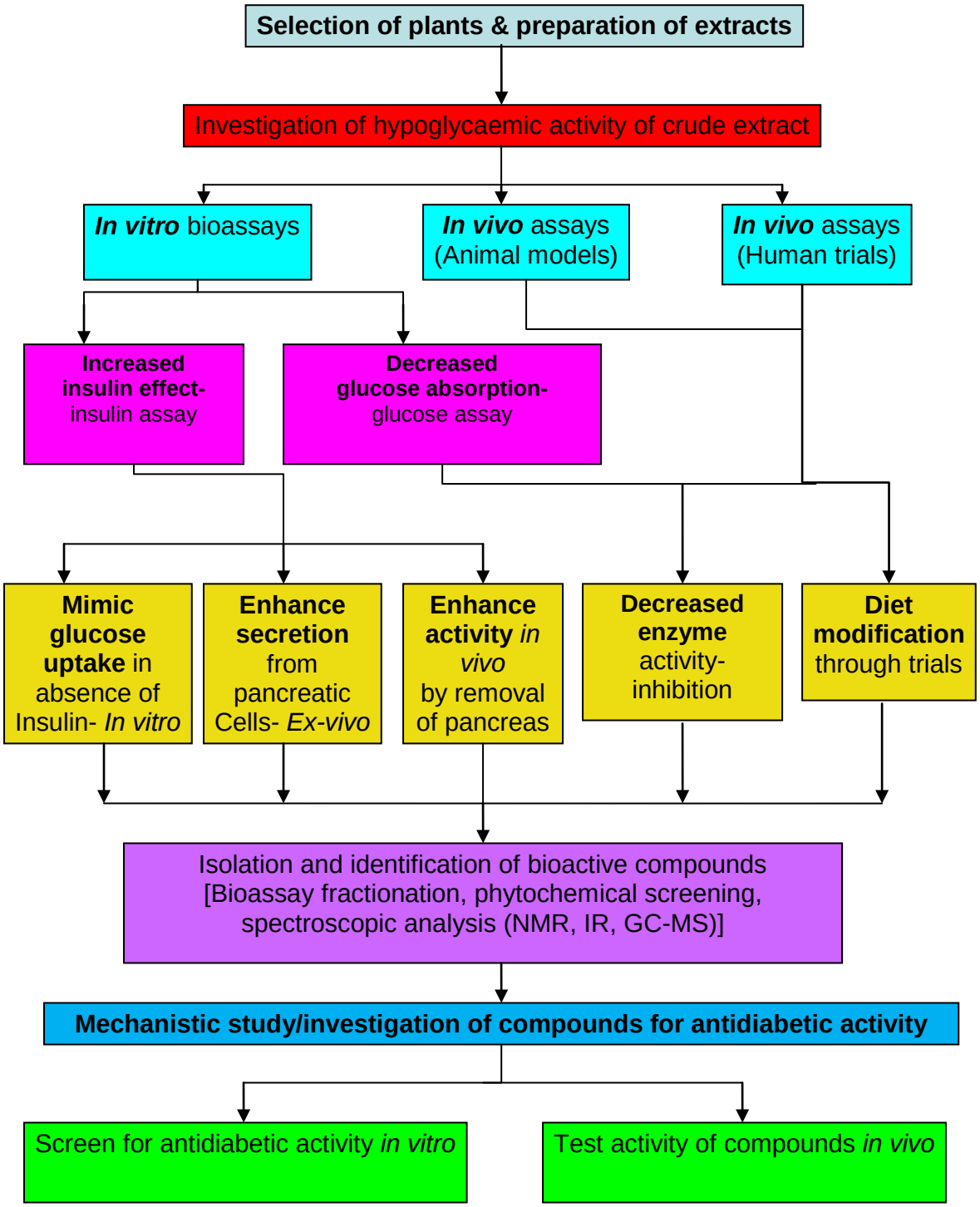



Figure 6: Schematic diagram for the investigation of antidiabetic/hypoglycaemic activity in medicinal plants.

## 2.9 Diabetes research in South Africa

In South Africa several surveys have been conducted over years and have yielded various plant species used in the treatment and control of diabetes and its complications. A recent study survey conducted in the Eastern Cape province of South Africa revealed 14 plant species belonging to six families used in the treatment and management of diabetes (Erasto *et al*, 2005). The most notable been, *Artemisia afra, Psidium guajava, Terminalia sericea, Hypoxis hemerocallidea* and all of these species had some degree of anti-diabetic activity. An inventory conducted by Deutschländer *et al* (2009) identified 32 plant species from 20 plant families traditionally used by traditional health care practitioners in the treatment and control of diabetes including descriptions on the phytochemistry and bioactivity.

Recent literature reports have given great consideration to the use of *Sutherlandia frutescens* in the treatment and control of diabetes and the possibility of it being used as a preventative measure in diabetes (Sia 2004). Chadwick *et al* (2007) found that a *S. frutescens* extract is able to reinstate serum insulin levels to near-normal, in addition to reversing insulin resistance at the level of muscle and fat tissue. While mechanistic studies are still ongoing it is believed that *S. frutescens* extracts bring about their beneficial effects by enhancing the secretory functions of pancreatic α and β cells much like the sulfonylurea compounds. There's also speculation that the effect may be due to an ability to reduce reactive oxygen species within the pancreas (Sia, 2004).

## 2.10 Preliminary Screening

Plants have played an important role in the treatment and control or many infectious and chronic diseases for centuries, mainly in rural populations. According to Gullo and Hughes (2005) in general 75% of new drugs originate from natural sources and are very useful in combating disease. In order to ascertain the potential ability of the selected plants to promote health in people with diabetes, the following factors were considered;

### 2.10.1 Antioxidants and diabetes

Glucose oxidation during intermediate metabolism is believed to be the main source of free radicals (Maritim *et al*, 2003). When glucose is oxidized in a transition metal dependent reaction it produces enediol radical anions that are converted into reactive ketoaldehydes and superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which when not degraded by catalase (CAT) or glutathione peroxidase (GSH-Px) can lead to the production of highly reactive

hydroxyl radicals in the presence of transition metals (Maritim *et al*, 2003). High levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acid which eventually leads to cell death.

There are several lines of evidence to suggest that antioxidant defence levels may be low in diabetes. The activities of the antioxidant enzymes CAT, superoxide dismutase (SOD) and GSH-Px are all reduced in diabetic patients (Laight *et al*, 2000).

Antioxidants may counter the action of free radicals in three ways:

- Increase in levels of enzymes that degrade free radicals (Rahimi *et al*, 2005)
- Increase in levels of proteins such as transferrin that can bind the metals which stimulate the production of free radicals (Rahimi *et al*, 2005)
- Chemical antioxidants such as vitamin C and E which act as direct free radical scavengers (Rahimi *et al*, 2005).

Antioxidant therapy can be achieved by supplementation with pharmaceutical preparations of antioxidant nutrients which may confer both cardiovascular and metabolic benefits in diabetes (Laight *et al*, 2000). Plants have numerous/substantial amounts of antioxidants and their action is an important property of plant medicines associated with diabetes (McCune & Johns, 2002).

### 2.10.2 Total Polyphenolic content and diabetes

Polyphenols have the ability to interfere with cell/tissue based assays in diabetes research by causing damage to cellular proteins, membrane lipids and nucleic acid. During the past decade the interest in polyphenols, including flavonoids has increased considerably with nutritionist, epidemiologist and food manufacturers taking a special interest in polyphenols, this is mainly due to their various biological properties like their anti-oxidant effects. Polyphenols include other subclasses such as phenolic acids, stilbenes, lignans, tannins and oxidized polyphenols (George *et al*, 2005). The importance of antioxidants in preventative medicine has been recognized for several years and polyphenols have become the focus of research interest due to the perceived health-beneficial effects (Wollgast & Anklam, 2000). Diseases that are believed to be caused or partially enhanced by oxidative stress include cardiovascular and cerebrovascular disease, some forms of cancer and other disorders such as diabetes and rheumatoid disease (Wollgast & Anklam, 2000).

Polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently being identified (Wollgast & Anklam,

2000). Polyphenols are products of the secondary metabolism of plants and originate from two main synthetic pathways; the shikimate pathway and the acetate pathway, both of which are derived or occur from glucose metabolism within plants (Wollgast & Anklam, 2000). A group of polyphenolic compounds are also called tannins at times as they are powerful reducing agents, and have been known to inhibit the activity of digestive enzymes including that of trypsin and amylase (Thompson *et al*, 1984). Polyphenols may also exert their effect as chelators of divalent cations.

## 2.12 Conclusion

Diabetes mellitus is a metabolic disease which is classified into two major types (type I and II). Other forms do occur and with that come a whole of complications which are all manageable if the gruelling treatment regimes can be followed. The two types of diabetes have very different implications on the lifestyle of the patient. The complications associated with diabetes have life changing effects on a person and may affect different parts of the body. Several pharmaceutical drugs are available for the control and management of diabetes, but with lifestyle changes and ever growing environmental conditions many of these drugs have lost the effectiveness over time due loss of efficacy and the development of toxicity, which leads to more physiological conditions which at times, if not seen early in patients, may lead to death.

In recent years much work has been directed to evaluate the evidence that medicinal plants used traditionally all over the world to treat and control diabetes can be safely used to treat and control this metabolic disease and its complications effectively. There have been numerous success stories using animal models in the testing of these plant extracts and this has encouraged wide-spread research into phytotherapy for diabetes mellitus. In this study six plants species that have been used with good effect by a traditional healer Mr Radebe were collected from the Newcastle region in the KwaZulu Natal province and they were subjected to several phytochemical screenings and evaluated for their *in vitro* and semi *in vivo* anti-diabetic activity, using the α-amylase, α-glucosidase and pancreatic-beta cell assays.

# CHAPTER 3

## Collection of plant material and preliminary work on extracts

### 3.1. Collection and preparation of plant material

To start this study an informal verbal survey on plant species used to treat diabetes was carried out with local inhabitants and herbal traders in the Newcastle region (KwaZulu Natal) at the local herbal market. Mr Radebe was friendly enough to introduce me to different traditional healers and to accompany me in my discussions with them. From the information gathered, six plant species were selected based on the popularity of use by locals, the ease at which they can be obtained and the efficacy according to the traditional healers and the community that uses them. All the traditional healers were chosen at random and on their willingness to share information for this study. The data collected included the local names, plant parts used and the methods of preparation for each plant species. The scientific names were obtained from literature and the identity of the species was verified at the Pretoria National Herbarium of SANBI (South African National Biodiversity Institute) in Pretoria. The six plant species were from six different families (table 3) (Fig. 7).

Table 3: Plant species used to treat diabetes mellitus in Newcastle (Kwa-Zulu Natal).

| Family | Scientific name | Local name | Parts used and mode of preparation |
|---|---|---|---|
| *Caesalpiniaceae* | *Senna alexandrina (Cassia angustifolia)* | Senna leaves | Leaves are boiled in water and taken orally |
| *Poaceae/Gramineae* | *Cymbopogon citrates* | Isiqunga (lemon grass) | An infusion of the whole plant is taken orally |
| *Curcuribitaceae* | *Cucurbita pepo* | Intshunga (pumpkin leaves) | Infusion of the upper parts (leaves and stem) taken orally |
| *Loganiaceae* | *Nuxia floribunda* | Umlulama (forest elder) | Infusion of bark and roots taken orally |
| *Hypoxidaceae* | *Hypoxis hemerocallidea* | Inongwe (African potato) | Infusion of the corms boiled and taken orally |
| *Lauraceae* | *Cinnamomum cassia* | Cinnamon | Infusion of the bark taken orally |

*Cassia angustifolia* Vahl. (*Senna alexandrina*) is a shrub to about 1 m in height, it is indigenous to hot barren regions and cultivated commercially in the Middle East. This plant has yellow flowers and papery pods containing six to eight seeds. The plant is used medicinally in Mozambique as a purgative and it is also believed to have anti-diabetic activity (Watt & Breyer-Brandwijk, 1962). There has been no scientific work done on this plant in connection to diabetes.

*Cymbopogon citratus* Stapf; is also known as lemon grass. It is an aromatic tropical grass with clumped, bulbous stems that gives origin to the leaf blades. Lemon grass oil is used as an antiseptic to treat athlete's foot. The stem and leaves can be used as cooking ingredients. This grass is also used in an array of cosmetics and it's helpful in treating coughs, cuts, asthma, bladder disorders and headaches (Watt & Breyer-Brandwijk, 1962). This plant is native to Asia (Sri Lanka, Indochina, etc.) This plant is known to have anti-diabetic activity by traditional healers. Recent studies have investigated the hypoglycaemic and hypolipidemic effects of fresh *C. citratus* aqueous leaf extracts in animal models and it has the ability to lower fasting plasma glucose levels dose-dependently (Adeneye & Agbaje, 2007). To date the most significant hypoglycaemic effect is seen at the oral dose of 500 mg/kg/ per day of the extract (Adeneye & Agbaje, 2007). The extract contains saponins, tannins, alkaloids and simple sugars (Onabanjo *et al*, 1993).The plant extract also reduces serum cholesterol levels *in vivo*. (Agbafor and Akubugwo, 2007)

*Cucurbita pepo,* is also known as summer squash or pumpkin. It has a long prickly stem bears large downy leaves and deep yellow coloured funnel-shaped flowers and it bears orange fruit containing numerous flat white seeds. The seeds have been prescribed for the treatment of ailments of the prostate gland and the oil from the seeds is used in the treatment of urinary ailments (Watt & Breyer-Brandwijk, 1962). This plant family contains biologically active components such as polysaccharides, fixed oils, para-aminobenzoic acid, sterols, proteins and peptides (Bechbauer *et al*, 1998; Kuhlman *et al*, 1999 and Appendino *et al*, 1999). Many of the phytochemicals isolated are from the seed extract. Pumpkin seed and fruit has been widely studied with regard to diabetes and proven to reduce blood glucose levels in type II diabetic patients after 30 g of pumpkin powder made from the fruit was given to them (Caili *et al*, 2006 & Quanhong *et al*, 2005). The fruit and seeds of pumpkin have hypoglycaemic activity in normal and alloxan-induced rats and rabbits, while water-soluble polysaccharides hypoglycaemic activity has been shown to excel that of glibenclamide in alloxan–induced diabetic rats (Zhang, 2001 & 2004 & Peng, 2002). The seed extract has high levels of antioxidants (Xanthopoulou *et al*, 2009). To date the protein bound polysaccharide isolated from pumpkin increases levels of serum

insulin, reduces glucose levels and improve glucose tolerance *in vivo* but a large quantity of the extract is required to elicit hypoglycaemic effect (Quanhong *et al*, 2005). It appears that pumpkin leaves have not yet been investigated in detail.

*Nuxia floribunda* Benth also known as forest elder is a small to medium sized tree 3 to 10 m tall and has sweetly scented cream-white flowers. The forest elder has a number of uses as traditional Zulu medicine. Several parts of the plant are used to an array of ailments from coughs to colds and influenza (Hutchings *et al*, 1996). No literature was found on the use of this plant as an anti-diabetic agent.

*Hypoxis hemerocallidea* Fisch & Mey also known as the African potato and is believed to be a wonder plant in terms of its medicinal value. It is used by the Botswana people as a charm against thunder and storms (Watt & Breyer-Brandwijk, 1962). The plant grows in the wild and in South Africa it is distributed along the Eastern Cape Province and stretches into the KwaZulu natal province to Lesotho, Gauteng, Limpopo and Zimbabwe. Traditional western ailments treated by the corm extract include urinary infections, hypertension, testicular tumours and HIV/AIDS. Its traditional use dates back many generations (Watt & Breyer-Brandwijk, 1962 & Drewes *et al*, 2008). The major biologically active chemical components of this plant are hypoxoside and rooperol. It has been reported to also contain phytosterol glycosides (β-sitosterol) and some sterolins (Drewes *et al*, 1984 & van Wyk *et al*, 2002). There are several published *in vivo* rodent studies on the ability of the aqueous plant extract as an anti-diabetic agent (Ojewole, 2002 & 2006; Mahomed & Ojewole, 2003; Drewes *et al*, 2008). The antioxidant activity has been studied in the leaves and corms. The leaf extract also has high antioxidant activity (Katerere & Eloff, 2008).

*Cinnamomum cassia* Blume is a slender evergreen tree that grows up to 20 metres high. Young branches are smooth and brown. It has small white flowers and green fleshly fruits with one seed and turns dark purple or black when mature. This plant is endemic to China. The leaves and bark are used as by Africans and Asians as flavouring in foods and beverages. The bark is also used as an aromatic bitter (Watt & Breyer-Brandwijk, 1962). The plant is used as an astringent, germicide, chronic bronchitis and many other ailments (Barceloux, 2009). Much of the work done with this plant involves the use or the extraction of volatile oils from the leaves and bark. The chemical composition of the plant varies depending on the plant part used. The main chemical constituents isolated are monoterpenes and sesquiterpenes (Barceloux, 2009). It has been reported that bark extracts of *C. cassia* contains an insulin-like peptide, cinnanaldehyde known to inhibit aldose reductase and is more active than other derivatives of the compound (Lee, 2002). The efficacy of *C. cassia* has been proven in several clinical

trails suggesting its effectiveness in lowering plasma glucose levels in patients (Mang *et al*, 2006; Khan *et al*, 2003; Pham *et al*, 2007 & Dugoua *et al*, 2007). Ranjbar *et al* (2006) conducted a comparative cross-sectional pharmacological study on patients using cinnamon tea and found that it increased total serum antioxidant status. In clinical trails no adverse effects were reported but the possible long-term effects are not known which places a question mark on the true efficacy of the plant as an antidiabetic supplement (Pham *et al*, 2007).

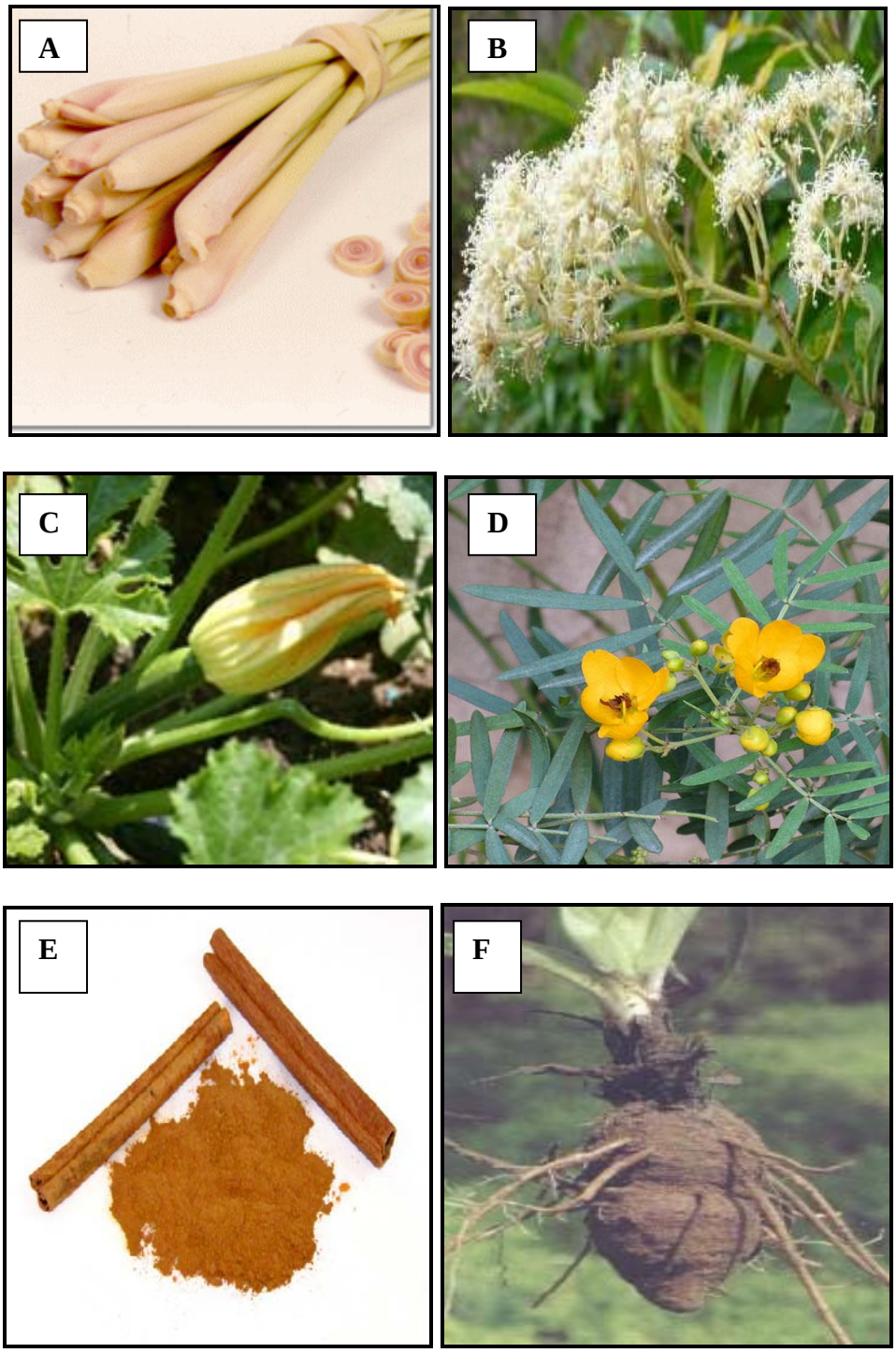



Figure 7: Parts of the six plants species used in this study. A- *C. citratus*, B- *N. floribunda*, C- *C. pepo*, D- *S. alexandrina*, E- *C. cassia* and F- *H. hemerocallidea* (www.beautifulbotany.com).

From the informal discussions with traditional healers it was found that the preparation of these plant species differed from community to community. Many of herbal traders had their own modes of preparation with some preferring to dry and then finely grind the ingredients together, while others use fresh material homogenized together. In all cases after boiling, the solutions are allowed to stand and cool then sieved and taken orally. Even though a large body of information had been generated on the use and preparation of medicinal products, no clinical information supporting their use was available. This, unfortunately, makes it difficult to verify the success achieved with the various therapeutic preparations. For this reasons, these species were evaluated for semi-*in vitro* anti-diabetic activity.

Raw samples of each plant (leaves and bark) (see table 3) were bought from local herbal traders in Newcastle (Kwa-zulu Natal) during October-December 2007. The scientific names were verified by SANBI (South African National Biodiversity Institute) at the National Herbarium in Pretoria. Six plant species from six different families were identified; *Senna alexandrina* (*Caesalpiniaceae*), *Cymbopogon citratus* (Stapf) (*Poaceae/Gramineae*), *Cucurbita pepo* (*Curcuribitaceae*), *Nuxia floribunda* (Benth) (*Loganiaceae*), *Hypoxis hemerocallidea* (Fisch & Mey) (*Hypoxidaceae*) and *Cinnamomum cassia* (Blume) (*Lauraceae*). All plant materials were placed in brown paper bags and dried in the Phytomedicine Programme drying room and ground into fine powder for extraction with a Jankel and Kunkel grinder to a size of less than 1 mm.

### 3.1.2 Extraction

For extraction, the method of Kumaran and Karunakaran (2005) and Eloff (1998b) was adapted. One gram of powdered extract of each plant material was dissolved in 10 ml acetone, methanol, hexane and ethyl acetate at room temperature and placed on a Labotec shaker for 20 minutes and then centrifuged at 30000 rpm for 5 minutes. The extract was filtered using Whatman No. 1 filter paper and the filtrate was collected in pre-weighed vials and allowed to dry under fan, in a well ventilated room. The amount extracted was determined by the change in weight of the vials following drying. The dried residue of the extract was re-dissolved in acetone and used for the subsequent *in vitro* experiments. All extracts used was freshly extracted and dried prior to use for each procedure/experiment. The total quantity extracted for each solvent system for all the plant was determined, as a sum of the mass extracted for each of the solvent system. Total extractability was determined as a percentage of total quantity extracted.

### 3.1.3 TLC fingerprinting

#### 3.1.3.1 Thin layer chromatography (TLC)

- Solvent system

  Four solvent systems were used, the first was made up of ethyl acetate, methanol and water (EMW 10:1.35:1- polar/neutral), the second was made up of chloroform, ethyl acetate and formic acid (CEF 10:8:2- intermediate polarity/ acidic), the third was made up of benzene, ethanol and ammonia hydroxide (BEA 18:2:0.2- non polar/ basic) and the last solvent system was made up of butanol, glacial acetic acid and water (BAW 4;1;5- highly polar ) and was used for the antioxidant assay test instead of BEA. All solvent systems were prepared freshly ( Kotze & Eloff, 2002).

- Stationary phase and spray reagents

  The chemical constituents of the extracts were analyzed by thin layer chromatography using an aluminium backed thin layer chromatography plates ($F_{254}$ Merck). On each plate 100 µg (10 µl of 10 mg/ml) of the redissolved extracts was loaded. The compounds on the plates were visualized under UV fluorescent light to detect UV absorption spots/plant constituents and subsequently sprayed with vanillin and *p*-anisaldehyde for visualization of compounds (Eloff, 1998b). The vanillin spray reagent was prepared using 0.1 g of vanillin dissolved in 28 ml of methanol and 1 ml of sulphuric acid ($H_2SO_4$) was carefully added. The *p*-anisaldehyde spray reagent was prepared using 1 ml of *p*-anisaldehyde solution in 18 ml of ethanol and 1 ml of sulphuric acid. After spraying the plates, they were incubated at $100^0$C for optimal colour development.

- Free radical scavenging activity

  The qualitative antioxidant activity was assayed as free radical scavenging activity using TLC plates developed in BAW, CEF and EMW for acetone and methanol extracts as stated above. 1, 1 diphenyl-2-picrylhydroazyl (DPPH) (0.2 g of DPPH dissolved in 100 ml of methanol) was used (Parejo *et al*, 2002).

## 3.2 Total polyphenolic content

The total polyphenol content was determined using the Folin-Ciocalteu method (Singleton & Rossi Jr, 1965). A calibration curve of gallic acid was prepared and the quantity of the polyphenols present in each crude extract was determined using the regression equation from the calibration curve ($y= 2.4145x + 0.0425$, $R^2= 0.97$) (see appendix A Fig. 20), which was linear from 0 mg/ml to 60 mg/ml. The polyphenolic concentration in the final mixture was determined applying the Beer-Lambert's law (Parejo *et al*, 2002).

A stock solution of 20 mg of gallic acid was made using 70% acetone in water. Aliquots of gallic acid were placed in each test tube starting from 0 ml to 1 ml (with increments of 0.2 ml) and water was added to each test tube to make up a final volume of 1 ml in each test tube. One millilitre of Folin-Ciocalteu solution was added to each test tube, followed by the addition of 2 ml of sodium carbonate solution for the standards. For the determination of the polyphenolic content of the plant extracts, 20 mg of the crude extracts was dissolved in 70% acetone in water. One millilitre (0.2 mg/ml) of the crude extract was subsequently incubated with Folin-Ciocalteu solution and $Na_2CO_3$. The test tubes with the standards and extracts were incubated for exactly two hours for the colour to develop and each test tube was read at 760 nm in a plastic cuvette on Helios Beta spectrophotometer with a path length of 1 cm (Thermo Electron Cooperation).The procedure was repeated three times for both the standards and extracts.

## 3.3 Results and discussion

### 3.3.1 Quantity extracted

Acetone was the best extraction solvent out of the other three solvents (methanol, hexane and ethyl acetate) used; it extracted the highest quantity of plant material with an overall extraction efficacy of 24%. This corroborated results found in other plant species (Eloff, 1998b). Methanol was the second best with an overall extraction efficacy of 20%. Hexane and ethyl acetate extracted the lowest overall quantities at 5% and 4% respectively. The acetone extract of *C. cassia* produced the highest quantity of extract (33%) out all the plant species. The hexane and ethyl acetate extracts of *H. hemerocallidea*, *N. floribunda* and *C. pepo* produced the lowest quantity of extracts (1%) (Figure 8).

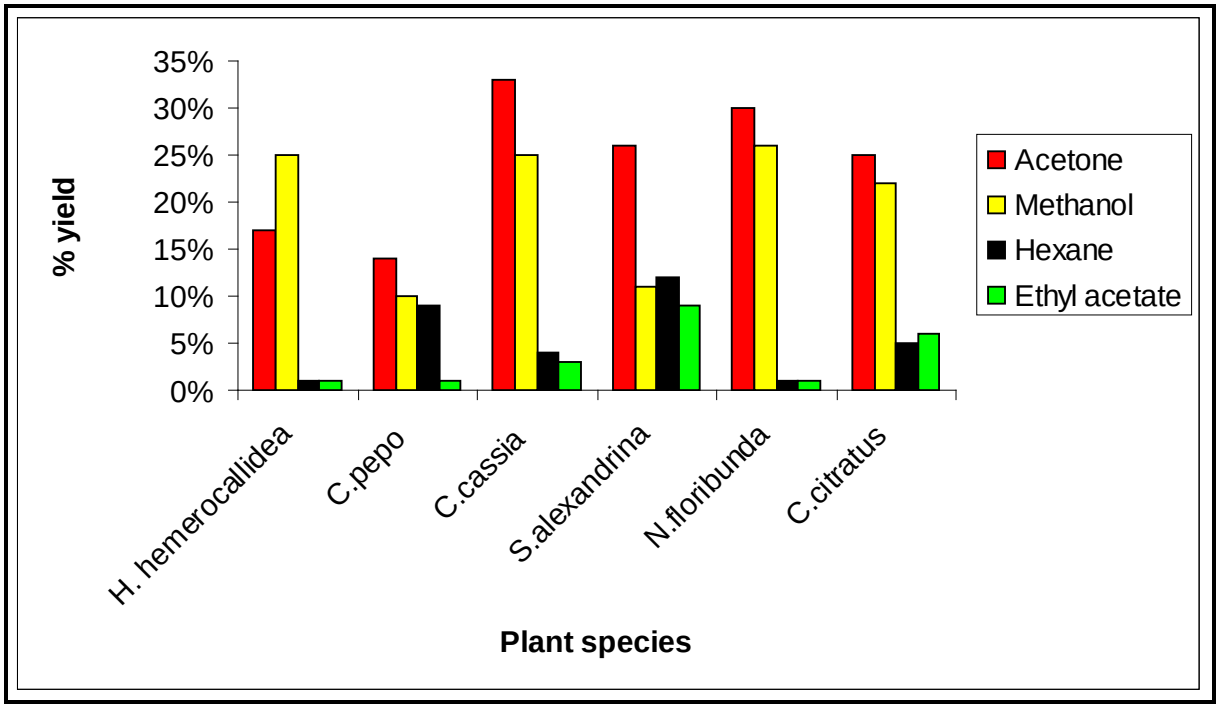



Figure 8: Quantity of extracts produced (% yield) by six plant species using four different solvents; acetone, methanol, hexane and ethyl acetate (This is an average quantity of all the extracts (four) obtained).

### 3.3.2 TLC fingerprints

#### 3.3.2.1 Vanillin and p-anisaldehyde sulphuric acid spray

To investigate the number of components in the extracts several TLC systems were investigated. For the plates developed with vanillin, CEF and EMW mobile phase were the best solvent systems for the separation of almost all the extracted compounds, in comparison to BEA. Many of the extracted compounds were distributed or separated efficiently using CEF and EMW. The separation of the extracted compounds in BEA was not very efficient (Fig 9).

The TLC chromatogram of the six plant species sprayed with *p*-anisaldehyde sulphuric acid spray also produced a number of bands. In CEF it appeared that the acetone, methanol and ethyl acetate extracts produced the best separation with hexane extracts contained the lowest number of bands. For the BEA mobile phase the ethyl acetate and hexane extracts were not separated (results not shown) and in EMW the separated bands were too non-polar and migrated to the top of the TLC plate (Fig 10). With the two spray reagents used, the compounds were more visible when the TLC plates developed in CEF were sprayed with *p*-anisaldehyde sulphuric acid spray for all the four extractants.

### 3.3.3 Antioxidant assay

No activity was evident in the hexane and the ethyl acetate extracts for any of the elution systems. The CEF elution system failed to elute bands with significant free radical scavenging activity. The acetone and methanol extracts, however, showed good activity in the BAW and EMW solvent systems (Fig 11). *C. cassia* and *N. floribunda* acetone and methanol extracts had the best activity; with the *C. citratus* methanol extract also being fairly active.

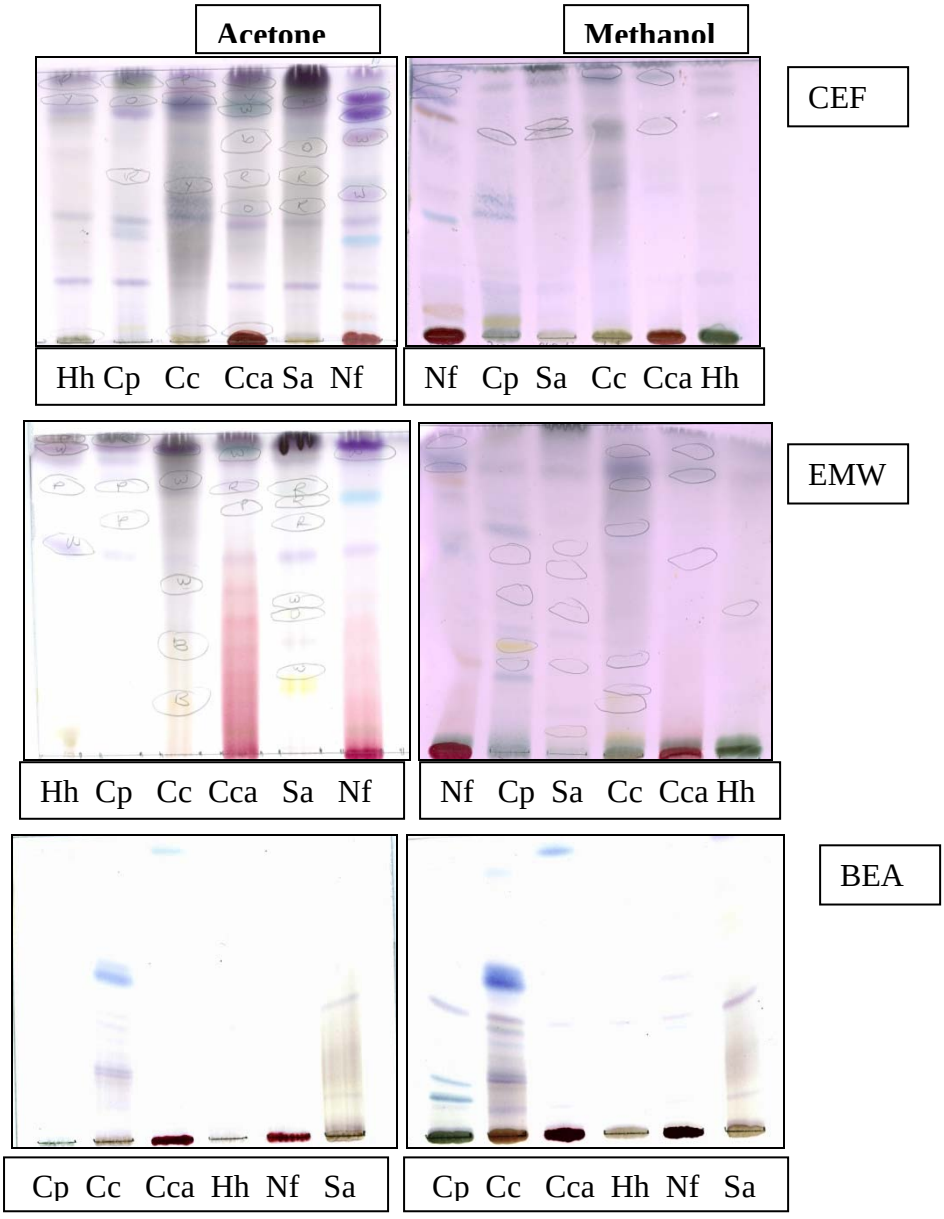
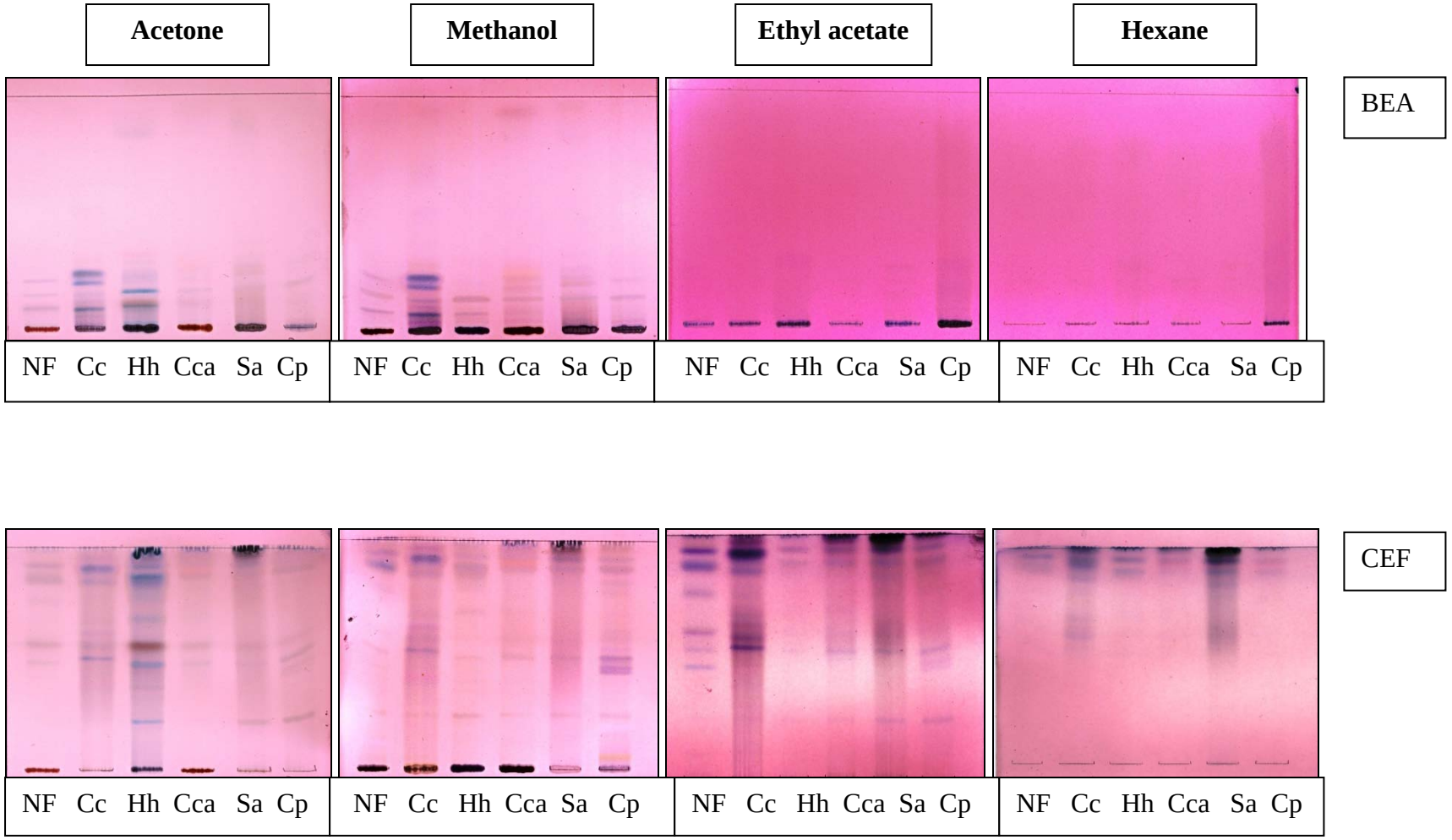



Figure 9: TLC chromatograms of six plant species *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea*(Hh), *Cinnamomum cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp), extracted with acetone and methanol (left to right) developed in CEF, EMW and BEA (top to bottom) and sprayed with vanillin sulphuric acid in methanol. The circled bands were florescent under ultraviolet light


Acetone
Methanol
Ethyl acetate
Hexane
BEA
CEF
NF Cc Hh Cca Sa Cp
NF Cc Hh Cca Sa Cp
NF Cc Hh Cca Sa Cp
NF Cc Hh Cca Sa Cp
NF Cc Hh Cca Sa Cp
NF Cc Hh Cca Sa Cp
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NF Cc Hh Cca Sa Cp

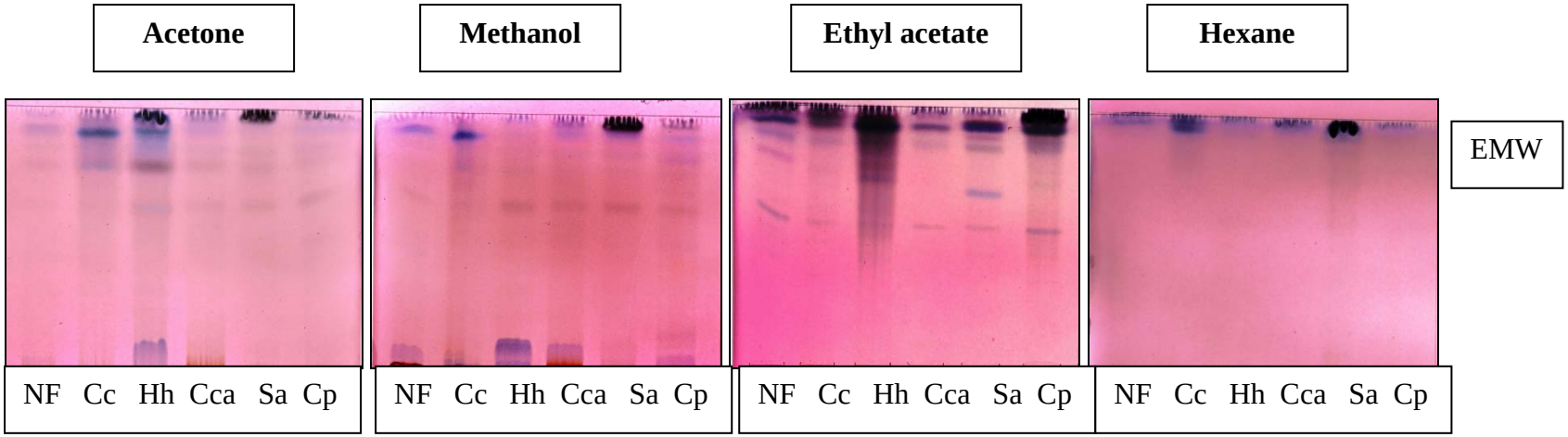


Figure 10: TLC chromatograms of six plant species {(left to right) *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea* (Hh), *Cinnamomum Cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp)} extracted with acetone, methanol, ethyl acetate and hexane (left to right) developed in BEA, CEF and EMW (top to bottom) and sprayed with *p*-anisaldehyde sulphuric acid in ethanol.

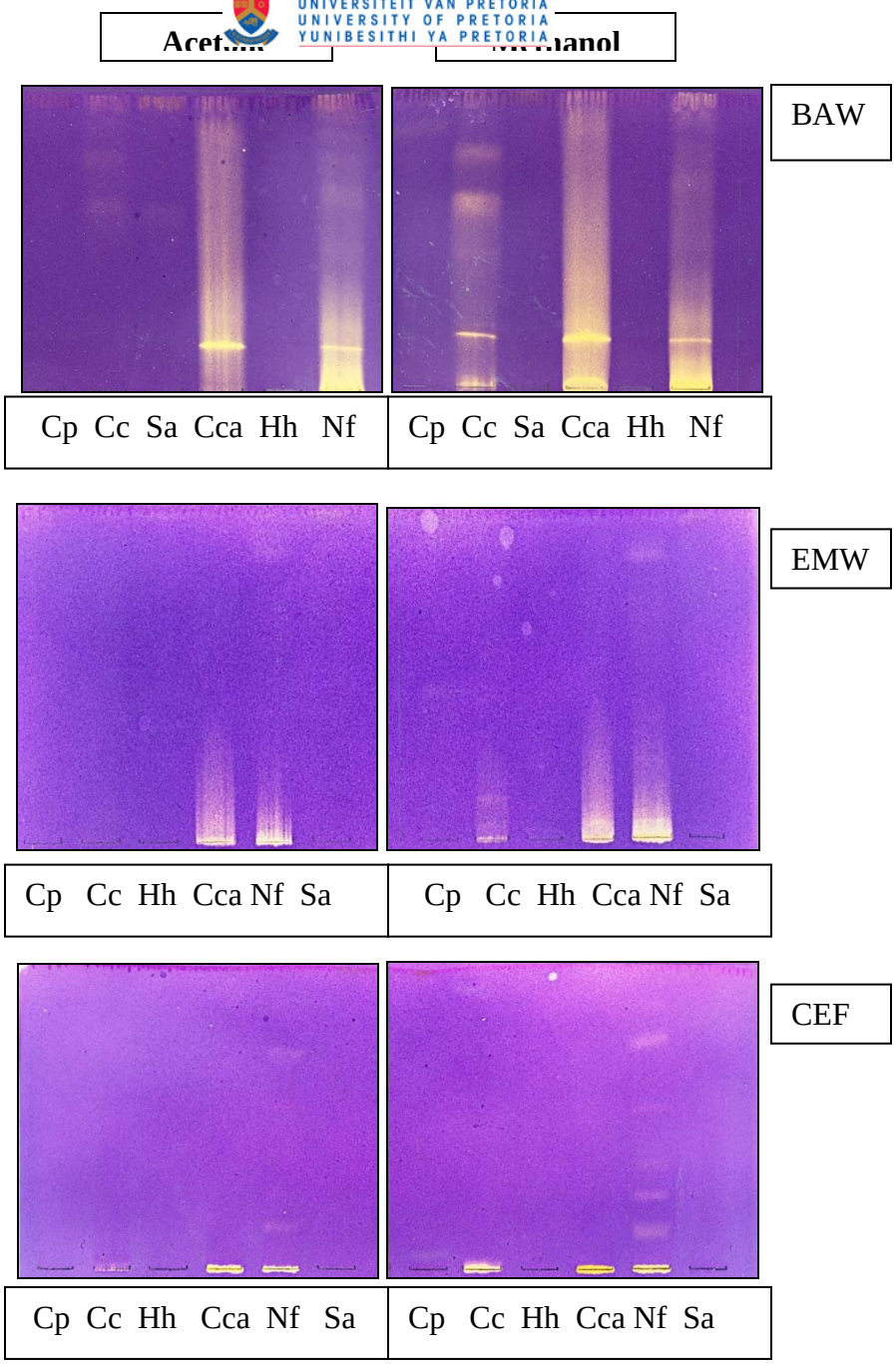


Figure 11: Antioxidant TLC chromatograms of six plant species *Nuxia floribunda* (Nf), *Cymbopogon citratus* (Cc), *Hypoxis hemerocallidea* (Hh), *Cinnamomum Cassia* (Cca), *Senna alexandrina* (Sa), *Cucurbita pepo* (Cp) extracted in acetone and methanol (left to right) developed in BAW, EMW, CEF (top to bottom). The ethyl acetate and hexane extracts did not show any qualitative antioxidant activity.

### 3.3.4 Total polyphenolic content

The average quantity of the polyphenols collectively for all plant species was, as expected, highest in the methanol extracts at 35.93%: The methanol extract with the highest quantity of polyphenols was *N. floribunda* (69.06%), *C. cassia* (61.19%) and *C. citratus* (44.21%). The remaining methanol extracts all had lower polyphenol quantities ranging from 10 to 20%. The average quantity of polyphenols collectively for all the ethyl acetate extracts was 28.34%. The ethyl acetate extracts had a high quantity of polyphenols for *N. floribunda* (45.04%), *S. alexandrina* (43.38%), *C. citratus* (34.69) and *C. cassia* (33.03) with *H. hemerocallidea* being the lowest at 0.72%. The average quantity of the polyphenols collectively for all plant species extracted in acetone was 25.09%. The acetone extracts of *C. cassia* had the highest amount of polyphenols followed by *N. floribunda*, *S. alexandrina, and C. citratus*. *H. hemerocallidea* and *C. pepo* had amounts lower than 5%. The hexane extracts had the least amount of polyphenols extracted the overall average of the polyphenol amount/quantity was less than 1% (0.59%) (Fig.12).

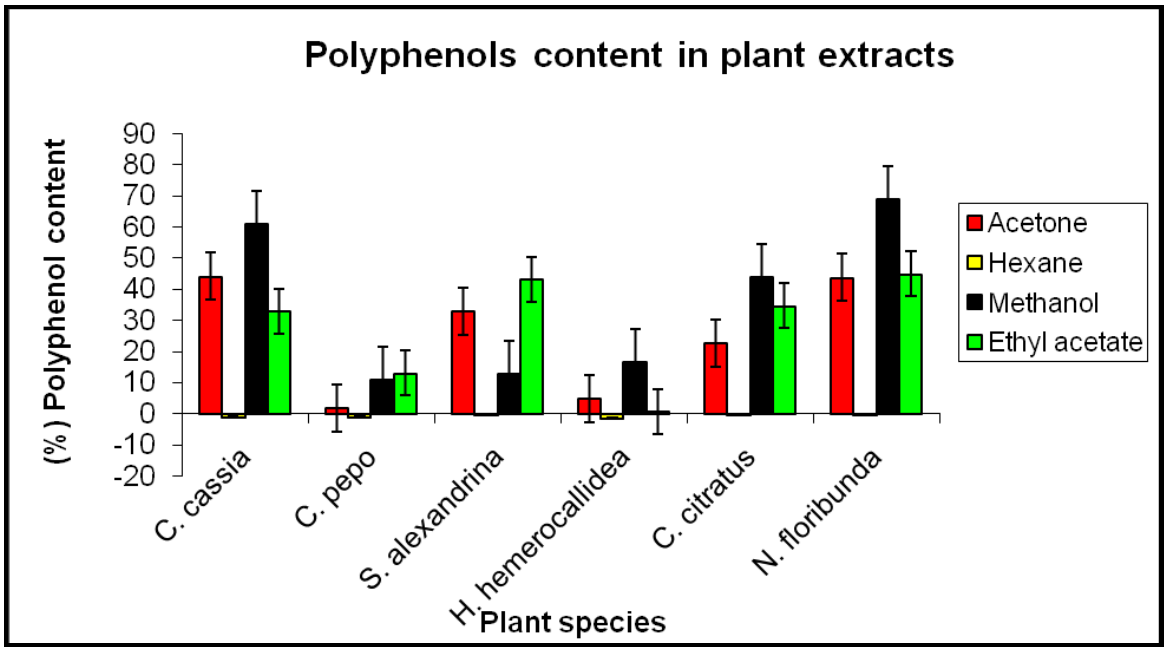


Figure 12: Polyphenol quantity of six plant species extracted with four solvents (experiment was done in triplicates; values are given as means ± SEM).

The results indicate that all six plant species were best extracted in solvents with intermediate polarity namely acetone and methanol (Fig. 8).The non-polar solvents hexane and ethyl acetate had an overall extraction amount was less than 6% respectively. *C. cassia* and *N. floribunda had* the highest extraction yield in acetone. The rest of the plants delivered extraction yields between 14% and 25%. Compounds from *N. floribunda* were most soluble in methanol followed by *C. cassia* and *H. hemerocallidea*. The remaining plants extracted in methanol had extraction yields between 10-22%. *S. alexandrina* had the highest yield of all the hexane extracts and the remaining plants all had extraction yields between 1% and 9%. *S. alexandrina* was best extracted in ethyl acetate and the other plant species had extraction yields between 1% and 6%, the lowest yield observed in all the four solvents used. This showed that the polarity of the extracting solvent plays an important role in the quantity extracted and that the polar and intermediate polar solvents (methanol and acetone) have higher extracting abilities than non-polar solvents. Extract yields increases with solvent polarity with the exception of acetone (Martini & Eloff, 1998). The results show that the extraction yields obtained by all the solvents used in this study were not that different from that of other researchers under relatively similar extraction conditions.

The compounds present in the acetone and methanol extracts were separated better than the non-polar compounds present in the hexane and ethyl acetate extracts (Fig. 9). The chromatograms developed in CEF and EMW had the best results when sprayed with the vanillin spray reagent. The BEA solvent system was not effective in separating compounds in the extracts indicating that the extracts did not contain many non-polar compounds. By TLC one gets an idea of what is in the extract and the chromatograms are important for reproducibility. Comparing the results of the TLC chromatograms, Kotzé and Eloff (2002) found in their study that there was a similarity in the chemical composition of the non-polar components of extracts when using extractants of widely varying polarity and belonging to different selectivity groups. They explained this by stating that if there were large quantities of saponins or other soap-like compounds that could solubilise very non-polar compounds into polar extractants (Kotzé and Eloff, 2002)

The qualitative antioxidant activities of these plants were tested by spraying chromatograms with DPPH (Fig.11). The purple-coloured DPPH is a stable free radical which is reduced to α, α-diphenyl-ß-picrylhydrazine (yellow colour) by reacting with an antioxidant. Antioxidants interrupt free radical chain oxidation by donating hydrogen from the hydroxyl groups to form a stable end

product (Akowuah *et al*, 2005). The acetone extracts TLC chromatogram developed in CEF, BAW and EMW of *C. cassia* and *N. floribunda* had antioxidant activity (Fig. 11). The plate developed in CEF spotted with acetone extracts did not move from the base indicating the compounds present were generally very polar and/or basic. The methanol extracts of C.*cassia, C. citratus* and *N. floribunda* all had free-radical scavenging activity.

The results therefore suggest, that the antioxidant activity of the plant could be related to the hydroxyl group due to their polar nature (Prasad *et al*, 2004). According the results presented the acetone extracts of *C. cassia* and *N. floribunda* had of 44% and 43% polyphenols respectively (Fig.12). The methanol extracts of *N. floribunda*, *C. cassia* and *C. citratus* had 69%, 61% and 44% polyphenols respectively, which correlates with their observed antioxidant activity, suggesting that polyphenols are likely to contribute to the free-radical scavenging activity of these plant extracts as seen with *N. floribunda, C. cassia* and *C. citratus*. Similar results were also obtained by other researchers (Hayouni *et al*, 2007 & Ranjbar *et al*, 2006). The other plant species had high levels of polyphenols but no antioxidant activity. Katerere & Eloff (2008) studied the antioxidant activity of *H. hemerocallidea* leaves and corms and found that both extracts had antioxidant activity but the corm activity was confined to the origin on the TLC plate due to its highly polar nature. The antioxidant activity of *H. hemerocallidea* in this study did not correspond to that of Katerere & Eloff (2008). This may be explained either by changes in the chemical composition due to different genetic or environmental parameters for plants originating from KwaZulu Natal and Gauteng.

# CHAPTER 4

## Alpha amylase inhibitory assay

### 4.1 Introduction

Starches rank amongst some of the most abundant complex carbohydrate polymers on earth. It is an important source of energy for animals, higher plants and microorganisms. Starch has a complex structure and thus requires a series of enzymes to break it down (Janecek, 1997). There are three amylases, α-amylase, β-amylase and glucoamylase, which are the best known enzymes that work on starch (Janecek, 1997). These enzymes are all closely related in function but differ structurally and the main distinguishing factor in their action is their mechanism of glucosidic bond cleaving. The α-amylases use a mechanism involved in hydrolysis of glucosidic bonds by this enzyme retains the α-configuration of the product while the other two enzymes inverts the anomeric atom configuration to β. There are over 20 different enzyme specificities in the α-amylase family (Table.4) (Janecek, 1997).

The α-amylase family enzymes exhibit, in general, a very low degree of sequence similarity and they contain several well defined regions in their amino acid sequences that are highly conserved (Janecek, 1997). Alpha-amylases consists of three domains; the catalytic core domain, comprising of a (β/α) 8 barrel and containing an extended loop inserted between the third β-strand and the third α-helix which is known as domain B. The C terminal domain forms a distinct globular unit (Fig.13) (Pyan, 2004). Elements from domain A and B are involved in the architecture of the three most functionally important sites i.e. the active site, the calcium binding site and the chloride binding site. Both mammalian and insect enzymes require one calcium ion to maintain their structural integrity (Payan, 2004).

Table 4: The alpha amylase family covers about 20 different enzymes specificities. They are all given EC numbers and are ordered in the following way: the members with crystallographically known three-dimensional structure, hydrolases, transferases, glucanotransferase that has been proposed to contain a circularly permuted version of the $(\alpha/\beta)_8$ barrel and proteins without catalytic function (Janecek, 1997)

| EC | Enzyme/Protein |
|---|---|
| 3.2.I.1 | α-amylase |
| 3.2.I.10 | Oligo-1,6-glucosidase |
| 3.2.I.60 | Maltotetraohydrolase |
| 2.4.I.19 | Cyclodextrin glycosyltransferase |
| 3.2.I.20 | α -glucosidase |
| 3.2.I.41 | Pullulanase |
| 3.2.I.1/41 | Amylopullulanase |
| 3.2.I.54 | Cyclomaltodextrinase |
| 3.2.I.68 | Isoamylase |
| 3.2.I.70 | Dextran glucosidase |
| 3.2.I.93 | Trehalose-6-phosphate hydrolase |
| 3.2.I.98 | Maltohexaohydrolase |
| 3.2.I.116 | Maltotriohydrolase |
| 3.2.I.133 | Maltogenic amylase |
| 3.2.I.135 | Neopullulanase |
| | Maltopemntaohydrolase |
| | Maltooligosyltrealose hydrolase |
| 2.4.I.18 | Glucan branching enzyme |
| 2.4.I.25 | Amylomaltose |
| 2.4.I.25/ 3.2.I.33 | Glucan debranching enzyme |
| | Maltooligosyltrehalose synthase |
| 2.4.I.5 | Glucosyltransferase |
| | Amino acid transport-related protein |
| | 4F2 heavy-chain cell surface antigen |

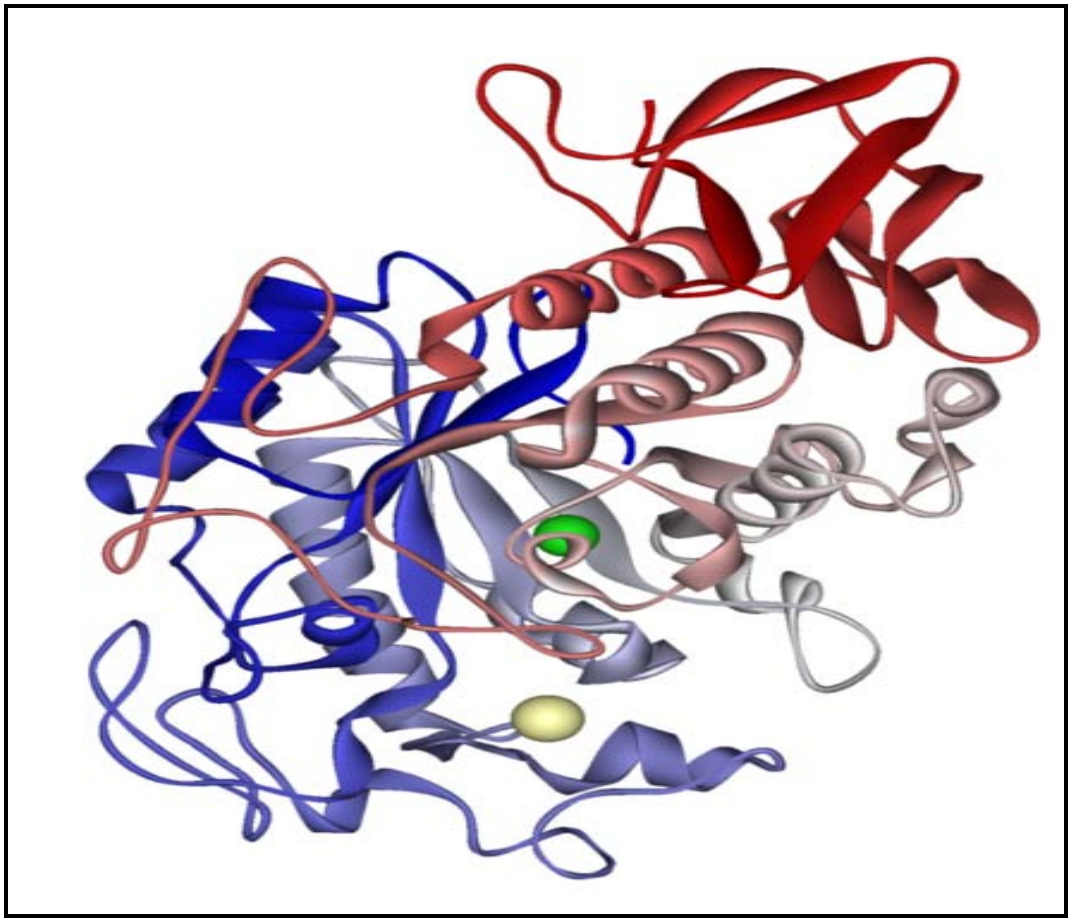

Figure 13: A ribbon diagram of human alpha amylase structure showing the three domains (Pink & Gray- A domain, Blue- B domain & Red- C domain) with the chloride ion (green) and calcium ion (yellow) (www.wikipedia.org)

Two kinds of α-amylase are produced by mammals, salivary α-amylase from the parotid gland and pancreatic α-amylase from the pancreas (Yoon & Robyt, 2003). The digestion of food begins with the salivary α-amylase in the mouth and stopped by the low pH of the stomach. When the food passes from the stomach into the small intestine it is neutralized and the digestion of starch is completed by α-amylase secreted into the small intestine from the pancreas (Yoon & Robyt, 2003).

Therefore any drug or chemical that inhibits these enzymes will delay or prolonged the time period of carbohydrate digestion. While this will not decrease the actual amount of glucose formed, it does reduce the rate of glucose formation and subsequent slows the rate of absorption leading

ultimately to a decrease in the maximum post-prandial plasma glucose. Plant seeds are a rich source for a large number of different inhibitors acting on α-amylases or other polysaccharides processing enzymes (Svensson *et al*, 2004). Some α-amylase inhibitors show strict target enzyme specificity and recognize only one out of the several closely related isozymes or enzymes from different species. Inhibitors from bean have been studied and are known to influence human and animal nutrition. They have also been used as starch blockers in obesity and diabetes therapy (Svensson *et al*, 2004).

Several α-amylase inhibitors have been isolated from plant seeds. The α-amylase inhibitor (α-AI) isolated from the common bean *Phaseolus vulgaris* has three different isoforms (α-AI-1, α-AI-2, α-AI-3). Alpha-AI-1 is known to inhibit porcine pancreatic α-amylase as well α-amylase present in the digestive tract of humans (Guzman-Partida *et al*, 2007). Ali *et al* (2006) isolated α-amylase inhibitors from *Phyllanthus amarus* which they observed to have a significant inhibition of the human α-amylase enzyme. They also mention that the plant contains lipophilic, potential α-amylase inhibitor compounds which contribute to its *in vivo* antidiabetic activity. A-amylase inhibitors have also been isolated from *Hibiscus sabdarifa* Linn (Roselle tea) by Hansawasdi *et al* (2000).

Acarbose is a natural product produced by several species of the *Actinoplanes* bacteria during fermentation that has recently become an important component in the management of diabetes mellitus. It is a pseudotetrasccharide with an unsaturated cyclitol attached to the nitrogen of the 4-amino-4, 6-dideoxy-D-glucopyranose, which is linked α-(1→4) to maltose (Yoon & Robyt, 2003). Acarbose is a strong competitive inhibitor of α-glucosidase, α-amylase, cyclomaltodextrin glucanytransferase (CGTase), glucoamylase and glucansucrases (Yoon & Robyt, 2003).

## 4.2 Materials and methods

The method employed for this assay was adapted from Hansawasdi *et al* (2000). This method was slightly modified by changing the concentrations of substrates and plant extracts used for this study. Starch azure (1 mg) (Sigma-Aldrich) was dissolved in 75 ml of sodium phosphate buffer, pH 6.9 by placing the container into a water bath at 60-70$^0$C for solution within 20 minutes. The plant extracts were set at predetermined concentrations for each plant (0.2 to 1 mg/ml) (0.25ml) and were mixed with 50% DMSO (0.25 ml), 1 ml of distilled water and 1.25 ml of porcine pancreatic

(EC-3.2.1.1) solution (10 U/ml)(Sigma-Aldrich) into separate test tubes, and incubated for 5 minutes at 25$^0$C. A sample blank was prepared in a similar manner, with 0.25 ml of 50% DMSO in the place of the plant extract. Following incubation, the starch solution (2.5 ml) was added to each test tube to make up a final volume of 5 ml. This final mixture was thoroughly mixed on a vortex, prior to a second incubation at 37$^0$C for 3 minutes.

After the second incubation, 1 ml of 3, 5-dinitrosalicylic acid (DNS) (Sigma-Aldrich) solution (made up of 2M NaOH (0.8 g), 12 g of potassium sodium tartrate and 0.547 mg of DNS) and 1 ml of the incubated samples was added together and vortexed in a new tube. These tubes were placed in a water bath and heated for 15 minutes at 85$^O$C. After 15 minutes 900 µl of distilled water was added to each tube to dilute the mixture.

The amylase activity was determined by measuring the absorbance of each test tube at 690 nm on a Helios Beta spectrophotometer (Thermo Electron Cooperation). A calibration curve of maltose was prepared and the amount of maltose in each crude extract was determined using the regression equation obtained from the calibration curve ($y= 0.7105x$, $R^2= 0.9844$) (see appendix A Fig. 22), which was linear from 0µg/ml to 1.2µg/ml. The concentration of maltose in the final mixture was determined according to Beer-Lambert's law (Ali *et al*, 2006). The enzyme activity was therefore measured by the rate of formation of maltose from starch. The $EC_{50}$ was determined using Kinatica 4.4 (Thermo Scientific). All data was fit to an Emax model except for acarbose in hexane which was fitted using Hills equation (Gabrielsson & Weiner, 2006). All experiments were conducted in triplicates.

## 4.3 Results and discussion

Twenty four extracts were prepared from six plants using the four different solvents (acetone, methanol, hexane and ethyl acetate) as extractants. The concentration versus percentage amylase inhibition of each plant extract is displayed in the graphs below for each solvent used, with the acarbose control (Fig 14-17) and the $EC_{50}$ (mg/ml) values are presented in table 5. Only three of the plants extracted with the acetone (Fig. 14), methanol (Fig. 15) and ethyl acetate (Fig. 16) had α-amylase enzyme inhibitory activity, while hexane extracts of all six plant species (Fig. 17) had α-amylase enzyme inhibitory activity.

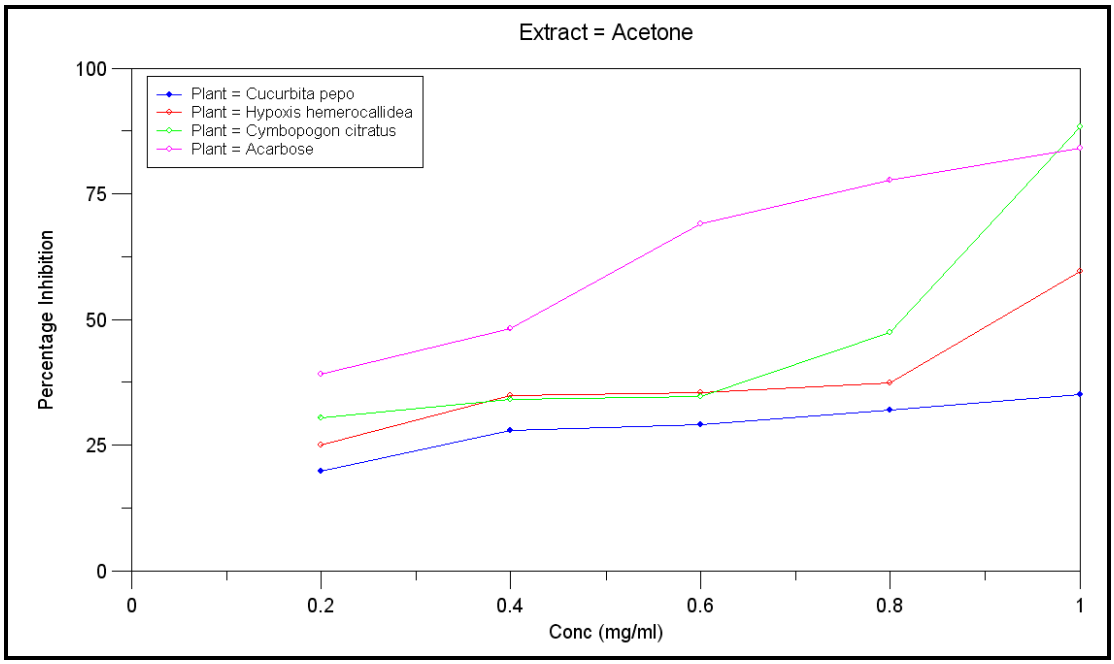


Figure 14: Alpha amylase inhibitory activity of the acetone extracts. Only *C. pepo, H. hemerocallidea* & *C. citratus* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated.

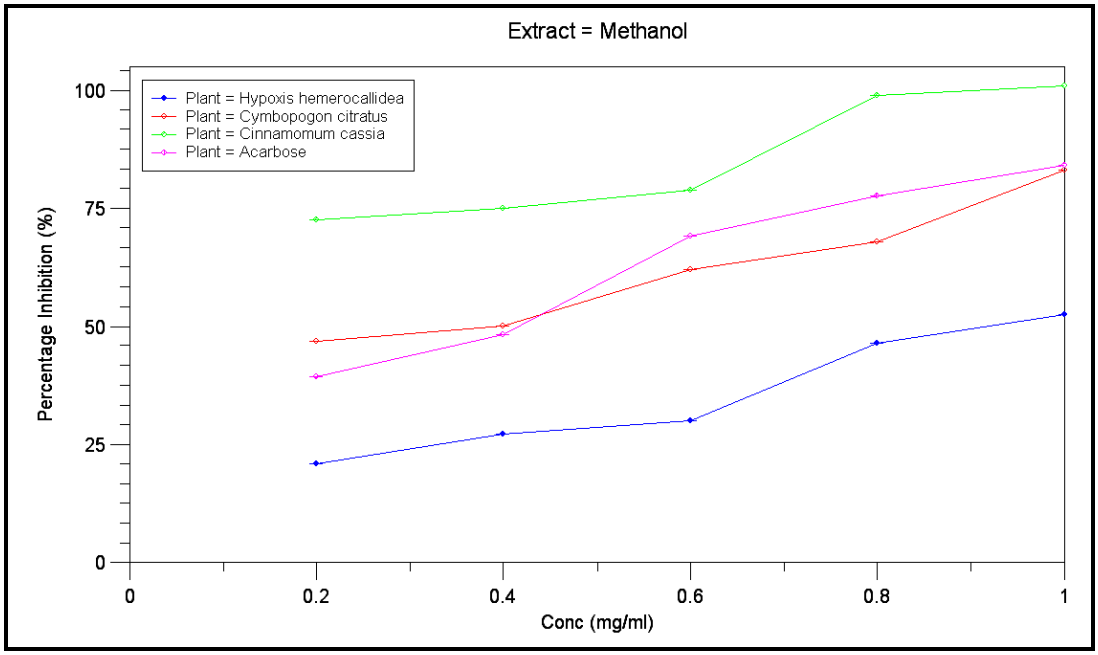


Figure 15: Alpha amylase inhibitory activity of methanol extracts. Only *H. hemerocallidea, C. citratus & C. cassia* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated.

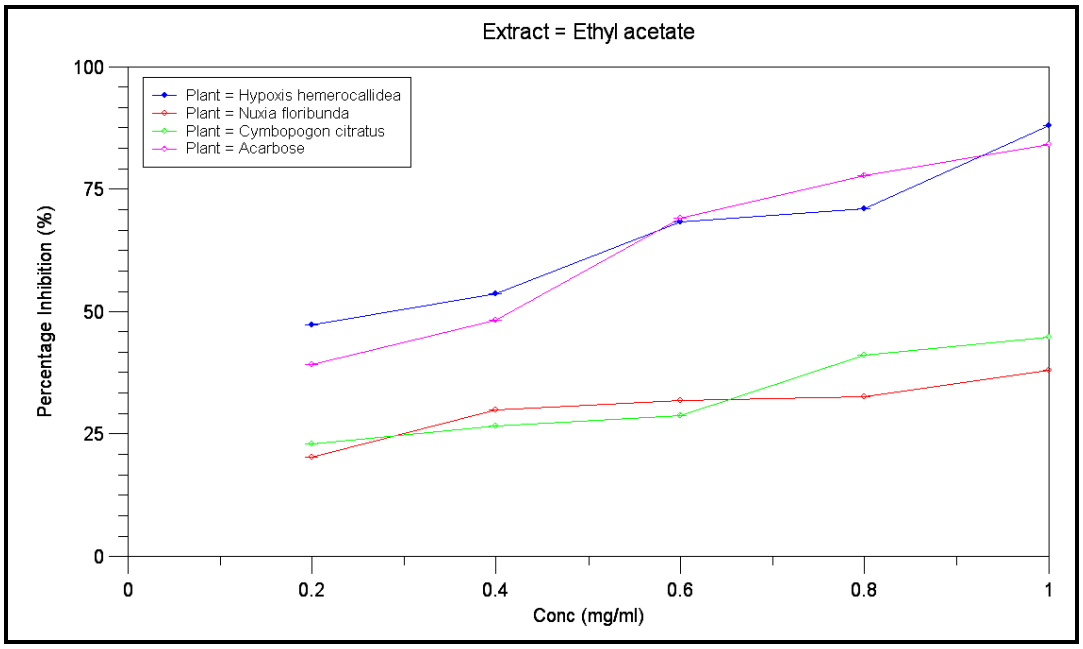


Figure 16: Alpha amylase inhibitory activity of ethyl acetate extracts. Only *H. hemerocallidea, N. floribunda & C. citratus* showed inhibitory activity. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated.

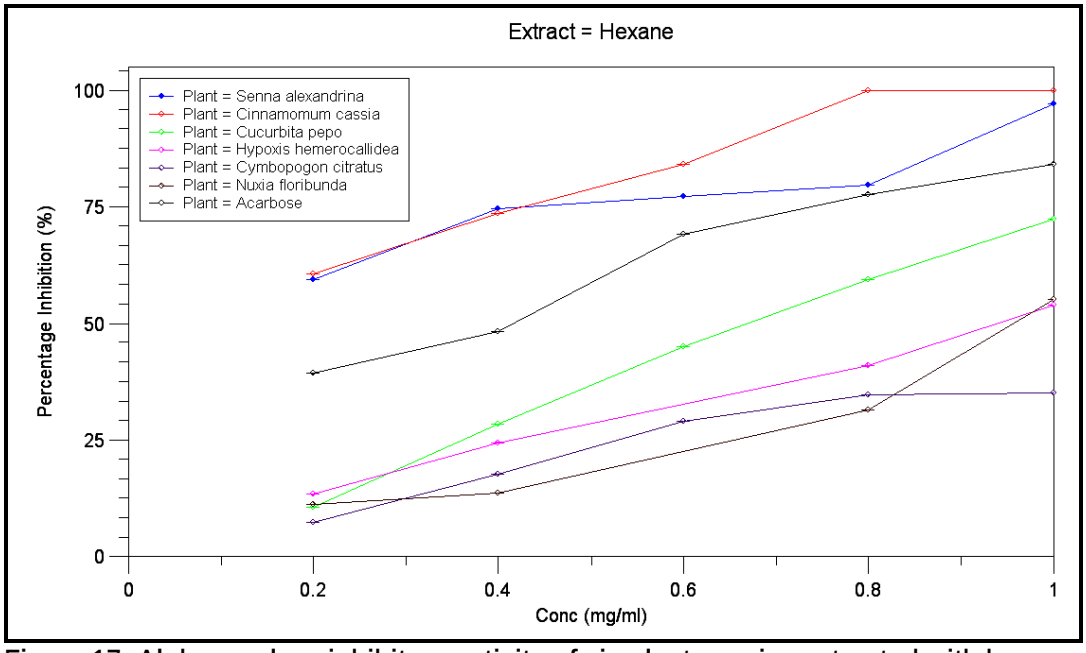


Figure 17: Alpha amylase inhibitory activity of six plant species extracted with hexane. *S. alexandrina, C. cassia, C. pepo, H. hemerocallidea, C. citratus, N. floribunda. C. pepo* and *C. citratus* had a dose related inhibition at the different concentrations. Acarbose the positive control is a metabolite a microorganism and not a plant as indicated.

Table 5: The inhibition of α-amylase activity (%) and $EC_{50}$ (mg/ml) for each of the solvents used in this study at the minimum and maximum dose.

| Solvents | Acetone | | | Methanol | | | Ethyl acetate | | | Hexane | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentrations (mg/ml) | 0.2 | 1 | $EC_{50}$ (mg/ml) | 0.2 | 1 | $EC_{50}$ (mg/ml) | 0.2 | 1 | $EC_{50}$ (mg/ml) | 0.2 | 1 | $EC_{50}$ (mg/ml) |
| Acarbose in water | 39.22 | 84.06 | 0.56 | 39.22 | 84.06 | 0.56 | 39.22 | 84.06 | 0.56 | 39.22 | 84.06 | 0.50 |
| *H. hemerocallidea* | 25.01 | 59.62 | 0.92 | 20.84 | 52.33 | 1.32 | 47.17 | 87.82 | 0.29 | 13.53 | 54.00 | 0.960 |
| *C. citratus* | 30.46 | 88.21 | 0.65 | 46.85 | 88.07 | 0.31 | 22.97 | 44.81 | 1.2 | 7.31 | 34.99 | 1.30 |
| *C. cassia* | NI* | NI | >1 | 72.54 | 100.88 | 0.12 | NI | NI | >1 | 60.52 | 99.93 | 0.72 |
| *C. pepo* | 19.87 | 35.10 | 1.82 | NI | NI | >1 | NI | NI | >1 | 10.64 | 72.29 | 0.70 |
| *N. floribunda* | NI | NI | >1 | NI | NI | >1 | 20.28 | 37.91 | 1.6 | 11.25 | 55.11 | 0.88 |
| *S. alexandrina* | NI | NI | >1 | NI | NI | >1 | NI | NI | >1 | 59.28 | 97.10 | 0.083 |

$EC_{50}$: Concentration that produces 50% of the maximum response. Although five different concentrations were tested only two are represented here.

NI: No inhibition

In this study six plants were studied for their potential anti-diabetic activity. The results from this study showed that not all the plant species had enzyme inhibition activity. The acetone extracts of *C. pepo* and *H. hemerocallidea* (Fig.14) had enzyme inhibition less than that of acarbose. The methanol extracts of *C. citratus* and *C. cassia* ($EC_{50}$= 0.29 and 0.12 mg/ml respectively) (Fig.15) also had enzyme inhibition greater than acarbose ($EC_{50}$= 0.56 mg/ml). The ethyl acetate extracts of *H. hemerocallidea, N. floribunda* and *C. citratus* (Fig.16) had enzyme inhibition greater than acarbose (Table 5). This indicated that an increase in the exposure concentration may result in further inhibition. All the hexane extracts (Fig.17) had enzyme inhibitory activity and the lowest activity was observed for *N. floribunda* extract ($EC_{50}$ = 10.00 mg/ml). The highest observed enzyme inhibitory activity was in observed in *C. cassia* and *C. pepo* ($EC_{50}$ = 0.23 and 0.50 mg/ml respectively).The best activity was seen for the intermediate polarity solvent system (ethyl acetate). It has been previously argued that plants high in polyphenolic compounds may produce an artificial α-amylase activity in vitro, as a result of enzyme precipitation instead of enzyme inactivation (Ali *et al*, 2006). The absence of significant polyphenols in the ethyl acetate extracts indicates that the obtained result is not just polyphenolic induced precipitation of proteins.

The treatment goal of diabetes is to maintain near normal levels of glycaemic control in both the fasting and post-prandial states. Many natural resources have been investigated with respect to the suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestines (Bhandari *et al*, 2008). Alpha-amylase catalyses the hydrolysis of α-1.4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for intestinal absorption (Bhandari *et al*, 2008). The inhibition of their activity in the digestive tract of humans is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from the starch by these enzymes (Bhandari *et al*, 2008).

The result of this study lends credence to the use of these plant species in the treatment and control of diabetes, and adds support to the results found *in vivo* by other researchers who showed a direct decrease in blood glucose concentration for *H. hemerocallidea* acetone and ethyl acetate extracts (Ojewole, 2002 & 2006; Mahomed & Ojewole, 2003; Drewes *et al*, 2000); *C. cassia*

methanol and hexane extracts (Mang *et al*, 2006; Khan *et al*, 2003; Pham *et al*, 2007 & Dugoua *et al*, 2007) and *C. citratus* methanol and ethyl acetate extracts (Adeneye & Agbaje, 2007). While *C. pepo* seeds and fruits have been reported to effectively lower blood glucose concentrations directly (Caili *et al*, 2006 & Quanhong *et al*, 2005), this is the first demonstration that the activity lies partially in the acetone and hexane leaf extracts. The activity of the ethyl acetate extract *of Nuxia floribunda* has not been previously reported.

## 4.4 Conclusion

The different extracts obtained differed substantially in activity against α-amylase. Initially water was also tried as an extractant to approximate the conditions used traditionally because rural inhabitants do not have sophisticated extractants available. There was hardly any activity in the aqueous extracts. This makes it difficult to understand the efficacy of aqueous extracts used traditionally. Possibly there is substantial particulate matter in the traditional preparations and non-polar compounds may be released from this in the intestine.

If the more non-polar plant extracts are not toxic or do not have negative side effects it appears that they may be much more efficient than acarbose in managing α-amylase activity. In several cases the activity was two to three times higher on a mass base. If one keeps in mind that the crude extract contains many different compounds it appears that the active compound(s) present in some plant extracts have at least one order of magnitude higher activity than the commercially available positive control. This aspect could be investigated in more detail but it is beyond the scope of an MSc study.

In the next chapter the efficacy of extracts against another enzyme alpha glucosidase will be examined.

# CHAPTER 5

## Alpha glucosidase inhibitory assay

### 5.1 Introduction

The ingestion of carbohydrate-rich meals causes a series of complex physiological events which result in the use or storage of carbohydrates. The initial entry of food into the mouth activates salivary gland amylase which has a small effect in the cleaving of starches (Lebovitz, 1997). In the duodenum complex carbohydrates are cleaved by pancreatic amylase into oligosaccharides which are too large to be readily absorbed. Once the oligosaccharides bind to the enzymes (α-glucosidase) located on the brush border of the enterocytes of the jejunum the complex oligosaccharides are cleaved into monosaccharides and absorbed immediately (Lebovitz, 1997). When glucose is transported across the enterocytes of the duodenum and jejunum, it stimulates the release of gastric inhibitory polypeptide (GIP) into the plasma. The presence of carbohydrates in the lumen of the duodenum and jejunum also results in the stimulation of the ileal mucosal cells to release glucagonlike peptide 1 (GLP-1). The elevation of both GIP and GLP-1 cause a delay in gastric emptying and amplify glucose mediated insulin secretion (Lebovitz, 1997).

There are three commercial alpha-glucosidase inhibitors (AGIs) available worldwide; they are acarbose, miglitol and voglibose (Cheng & Josse, 2004). These inhibitors are one of the alternative therapeutic approaches in the management of diabetes.

- Acarbose is a polar nitrogen-containing pseudotetrasaccharide, inhibits intestinal brush border gluco-amylase, maltase, sucrase and dextrinase and pancreatic amylase, with minimal effects on beta-glucosidase. In addition, acarbose binds to intestinal sucrase with a greater affinity than sucrose. Acarbose does not interfere with intestinal absorption of glucose (Lebovitz, 1997).
- Voglibose is a valiolamine derivative and is a potent inhibitor of most α-glucosidase enzymes, with weaker activity compared to acarbose and little effect on the pancreatic α-amylase enzyme (Lebovitz, 1997).
- Migitol is a deoxynojirimycin derivative and differs from acarbose and voglibose because it inhibits beta-glucosidase enzymes and to some degree and also interacts with intestinal

sodium-dependent glucose transporters i.e. interferes with glucose absorption as well (Lebovitz, 1997).

The primary benefits achieved by AGIs in diabetic patients would be a reduction in postprandial glycaemia and a decrease in the extremes between maximal and minimal postprandial glucose levels (Lebovitz, 1997). According to Cheng and Josse (2004), the glucose-lowering effect of AGIs is generally less than that of the other oral anti-hyperglycaemic agents. A reduction in postprandial glycaemia should be beneficial in type II diabetic patients (Lebovitz, 1997). AGIs are used in combination with other glucose-lowering agents and although they are considered safe, do not cause hypoglycaemia when used as monotherapy, there are associated adverse effects resulting from the colonic metabolism of carbohydrates (Cheng & Josse, 2004).

## 5.2 Materials and methods

For the assay, 0.2 ml of 56 mM sucrose (Sigma-Aldrich) was dissolved in 0.1M potassium phosphate buffer, pH 7, (0.2 ml), and mixed with 0.1ml (1 mg/ml) of the plant extract in 50% dimethyl suphoxide (DMSO) in solution (Nishioka *et al*, 1998 & Bhandari *et al*, 2008). This mixture was subsequently incubated at $37^0$C for 5 minutes. A solution of α-glucosidase (1 mg/ml) was prepared from rat intestinal acetone powder (Sigma-Aldrich) and 1ml of this enzyme solution was subsequently added to the pre-incubated mixture of the sucrose and the plant extract. The reaction was stopped after 30 minutes by adding 0.75 ml of 2M Tris-HCL buffer at pH 6.9. The solution was then centrifuged for 20 minutes at 3000 rpm the supernatant was removed; filtered using a micro-pore filter (0.20 μm) and placed into test tubes. The resultant solution was tested for the percentage conversion of sucrose to glucose using a commercial glucose oxidase test kit (GAGO-20, Sigma). The negative control contained 0.1 ml of 50% DMSO instead of plant extract.

For the commercial glucose oxidase concentration; the test mixture (1 ml) of the filtrate was incubated with 2 ml of the glucose kit assay reagent solution at $37^0$C for 30 minutes and the reaction stopped by careful adding 2 ml of 12N $H_2SO_4$ into each test tube. After this incubation the optical density (OD) was determined at 540 nm on a microplate reader with a path length of 1cm (VERSA max, Labotec). The glucose calibration curve was prepared using the components supplied within the Glucose-B test kit (GAGO-20).The quantity of glucose present in the test

solution was determined using the regression equation from the calibration curve (y= 17.75x + 0.0162, $R^2$= 0.9979) (see appendix A Fig. 23), which was linear from 0 to 0.8µg/ml.

## 5.3 Results and discussion

The inhibitory activities (%) for the different solvent systems are presented in table 7 and represented graphically in figure 18 and the amount of sucrose converted to glucose is represented in table 6. The inhibitory activity of the plant extracts was in general much higher than that for the α-amylase activity (> 75%) for the hexane and ethyl acetate extracts. With the methanol and acetone extracts the inhibitory activity varied from no activity in the methanol extract of *C. cassia* to highly active methanol extract of *C. pepo* (70.3%) and acetone extract of *H. hemerocallidea* (84.35%). Among the plants studied *C. cassia* and *N. floribunda* (bark) had the highest inhibitory activity in the hexane and ethyl acetate extracts, the acetone extract of *H. hemerocallidea* had the highest inhibitory activity (Fig. 18). The acarbose control had excellent α-glucosidase inhibitory activity (100%) and this was in line with the inhibitory activity of hexane and ethyl acetate extracts (table 7).

Table 6: The glucose quantity (mg) present after treatment of intestinal rat acetone powder with six South African plants used to treat and manage diabetes

| Plant Species | Glucose quantity (mg)* | | | |
|---|---|---|---|---|
| | Ethyl acetate | Hexane | Methanol | Acetone |
| Acarbose | < 0.000001 | < 0.000001 | < 0.000001 | < 0.000001 |
| *C. pepo* | 0.004 | 0.018 | 0.297 | 0.654 |
| *C. cassia* | 0.004 | 0.126 | 1.066 | 0.624 |
| *S. alexandrina* | 0.009 | 0.021 | 0.387 | 0.350 |
| *N. floribunda* | 0.005 | < 0.000001 | 0.871 | 0.659 |
| *H. hemerocallidea* | 0.052 | 0.010 | 0.414 | 0.156 |
| *C. citratus* | 0.050 | < 0.000001 | 0.546 | 0.294 |

*amount of glucose produced was calculated as: Glucose (mg): y = (17.75x + 0.0162)*10 (y=OD; x= concentration (mg); 10= dilution factor made in sample preparation). Values are means of *n*=3.
**ND-not detectable

Table 7: Alpha glucosidase inhibitory activity (%) of different extracts of six South African plant species used to treat and manage diabetes mellitus at a concentration of 1 mg/ml

| Plant Species | Inhibition (%)* | | | |
|---|---|---|---|---|
| | Ethyl acetate | Hexane | Methanol | Acetone |
| Acarbose | 100 | 100 | 100 | 100 |
| *C. pepo* | 99.56 | 98.24 | 70.30 | 34.58 |
| *C. cassia* | 99.64 | 87.38 | NA* | 61.27 |
| *S. alexandrina* | 99.09 | 97.93 | 61.27 | 65.04 |
| *N. floribunda* | 99.53 | 100 | 12.85 | 34.11 |
| *H. hemerocallidea* | 94.83 | 99.01 | 58.61 | 84.35 |
| *C. citratus* | 95.02 | 100 | 45.42 | 70.55 |

*Percentage inhibition was calculated as follows: Inhibitory activity (%) = (100- $G_a$), $G_a$ is the percentage amount of glucose (table 6) that was left over after the reaction was stopped.
NA: No activity

The hexane crude extracts of *N. floribunda* and *C. citratus* had very high inhibitory activity at the highest concentration tested (1 mg/ml). The ethyl acetate crude extracts of all the plant species used in this study had an inhibitory activity above 90% against α-glucosidase at 1 mg/ml. When compared to acarbose all the plant species used in this screening study showed good activity against the α-glucosidase enzyme with the exception of the methanol extract of *C. cassia*. The exact mechanism of action of these plant extracts remains unknown, but it can be speculated that the activity is in part due to the presence of non polar compounds in the crude extracts of hexane and ethyl acetate, and most likely the same compounds responsible for the α-amylase activity. A similar result was found by Bhandari *et al* (2008) after partitioning *Bergenia ciliate* extract between water and ethyl acetate and found that the non-polar ethyl acetate fraction had a higher enzyme inhibitory activity. It is interesting that there was a major difference in the activity of two intermediate polarity extractants ethyl acetate and acetone. This could be very useful information in the isolation of the active compounds. The similar results found for different plant extracts may mean that the active compound(s) may be common intermediate metabolites and not a scarce plant secondary compound.

For the polar solvents (methanol and acetone) the activity may be also in part due to polyphenols and the antioxidant free radical scavenging activity of the crude extract. When the results of this study are compared to that of acarbose it was noted that the activity for acarbose was caused by its highly polar nature (Rodriguez *et al*, 2008). This therefore tends to suggest that the possible effective compound(s) yet to be identified and characterized may represent a new class of inhibitory molecules.

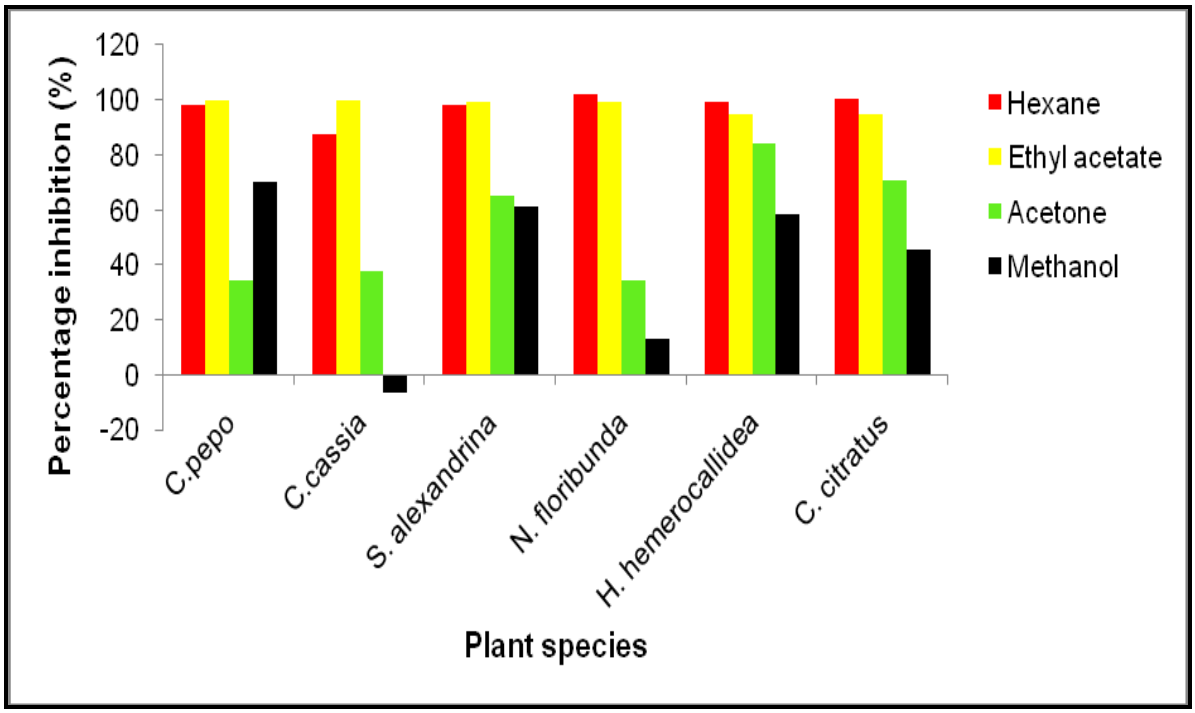


Figure 18: Alpha glucosidase inhibitory activity (%) of six South African plants (Representation of experiments conducted in triplicates).

In the next chapter the efficacy of extracts against the secretion of insulin from the pancreatic islet cells of Langerhans will be examined.

# CHAPTER 6

## Islets of Langerhans as a target site

### 6.1 Introduction

The pancreas is a gland situated below and behind the stomach. Within the pancreas are very small bits of tissue called islets of Langerhans. These islets contain β-cells, which manufacture, store, and eventually release insulin directly into the blood stream at the appropriate times (Krall & Beaser, 1988). Each normal pancreas has about 100,000 islets of Langerhans, which form clusters of various types of cells, the most important type been the β-cells (Fig. 19). There are usually between 1000 and 2000 β-cells in each islet (Krall & Beaser, 1988). In addition to producing and excreting insulin, these cells are also play an important role in measuring the blood glucose levels within seconds to a range within 2 mg/dL (Krall & Beaser, 1988).

Islet cells were first discovered in 1869 and have since been viewed as a possible *in vitro* system for a syndrome that cannot be mimicked very effectively using cell lines (Bhonde *et al*, 2007). Islets are miniature organ systems that retain their structure and differentiated state and have the ability to secrete insulin upon stimulation independent of the nervous control (Bhonde *et al*, 2007). Isolated islets *in vitro* respond to glucose stimulation and hence have immensely contributed to the study of various pharmacological aspects and for the screening of promising antidiabetic agents (Bhonde *et al*, 2007).

According to Norberg *et al* (2004) a large number of crude plant extracts and purified substances from plants have been tested in clinical trails for the treatment of diabetes. Many studies have been conducted to prove the mechanism of action of many of these crude plant extracts and compounds. The proposed mechanism for the action of most of these plants is believed to be the stimulation of the β-islet cells of Langerhans to secrete insulin (Qa'dan *et al*, 2009). Norberg et al (2004) showed how an ethanol extract of *Gynostemma pentaphyllum* Makino (Cucurbitacease), a herb widely used in Southeast Asia, has the ability to stimulate insulin secretion from isolated rat pancreatic islets. Chandramohan *et al* (2008) isolated 3-hydroxymethyl xylitol (3-HMX) from *Casearia esculenta* Roxb, which has a significant blood glucose lowering effect when the roots

were fed to streptozotocin-diabetic rats. *Retama raetam*, *Trigonella foenum* seeds, *Eriobotrya japonica* LINDL and *Hibiscus rosa sinensis* L all have insulin stimulating activity in diabetic *in vitro* and *in vivo* models.

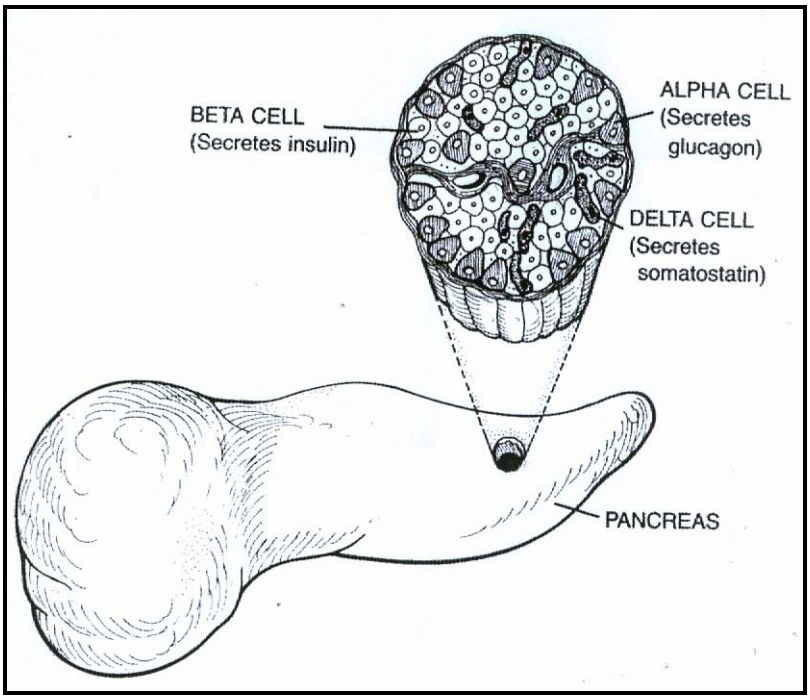


Figure 19: The pancreas is made up of many clusters of cells called islets of langerhans. Within each islet are different types of cells, including the alpha cells, which secrete glucagon, the beta cells, which secrete insulin and the delta cells, which secrete somatostatin (Krall & Beaser, 1988).

## 6.2 Materials and methods

For the isolation of islets of Langerhans, two Wister rats (approximately 8 weeks of age) were used. The study was approved by the Animal Use and Care Committee of the University of Pretoria, according to ethical standards of South Africa (SANS 10386-2008). After the animals were euthanized by cervical dislocation, their pancreases were aseptically removed, cut into small pieces and subsequently washed in an Erlenmeyer flask, three times with Hank's balanced salt solution (20 ml) (HBSS). The washed tissue was then subjected to 1 mg/ml of 0.1% collagenase digestion for 20 to 30 minutes, prior to suspension in dulbecco's modified eagle's medium (DMEM) supplemented with 2 mg/ml soybean tyrosine inhibitor (STI) and 2% (2 ml) bovine serum albumin (BSA) with 0.5 mg/ml of collagenase type V (Sigma-Aldrich) for another 30 minutes. The cells were

then pelleted at 1000 rpm for 10 minutes, prior to seeding into culture flasks (25 $cm^2$) containing 15 ml Dulbecco's modified Eagle's medium supplemented with 10% BSA (2.5 ml) with addition of 2.5 ml of RPMI-1640 and 2.5 ml HEPES, in 2.5 ml KPBS buffer (pH 7.4) and incubated at $37^0$C in 5% $CO_2$ in the air for 48 hours under high humidity (Shewade *at al*, 1999).

Following incubation, the presence of islet cells was confirmed by visual inspection with an inverted microscope (Fig. 20). The incubated cells were then transferred into tubes containing 1 ml of 10 mM Krebs-Ringer bicarbonate solution (KPBS) supplemented with HEPES at pH 7.4, 10% BSA and 50 mg of glucose and incubated for 1 hour at $37^0$C on a shaker at 30 rpm (Heidolph polymax 1040, Labotec). The islets were then re-incubated in 15 ml plastic centrifuge tubes for 60 minutes at $37^0$C with 2, 4, 6, 8 or 10 µg/ml of the plant extract (The predetermined concentrations were selected based on the principles of pharmacokinetics, where it is unusual for tissue concentrations to exceed 10 µg/ml) After 1 hour the mixture was centrifuged and the supernatant was stored at -$20^0$C for insulin assay (Shewade *at al*, 1999). For the positive controls acarbose (1 mg/ml) and glybenclamide (1 mg/ml) were used in the place of the plant extract. The quantity of insulin produced by the islets following stimulation by each plant was analysed by AMPATH (a commercial pathology laboratory on South Africa) using a chemoluminescence method.

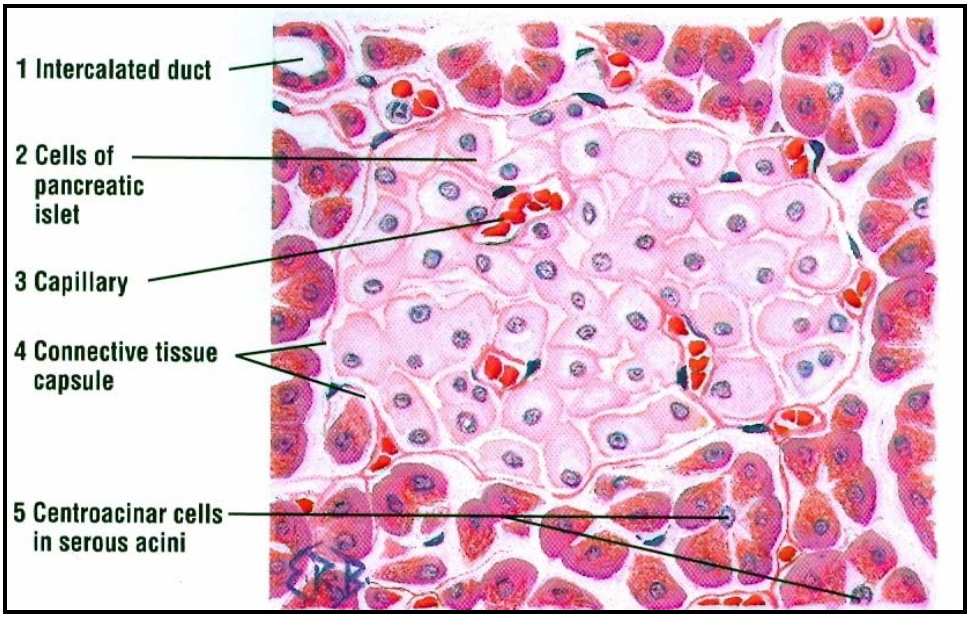


Figure 20: Pancreatic Islet cell stain (hematoxylin and eosin) no. 2 illustrating the morphology of pancreatic islet cells as seen under a light microscope at high magnification (Eroschenko, 2005). These cells can be seen in exactly the same formation when evaluated unstained under an inverted microscope.

## 6.3 Results and discussion

Only with the *H. hemerocallidea* acetone extract was there an increase in insulin secretion of 2.5 mIU/L (Table 8) at 8 ug/ml. With all the other extracts the insulin levels were less than 0.2 mIU/L. The positive controls of acarbose and glibenclamide at a concentration of 1 mg/ml stimulated insulin secretion to 11.5 and 19.8 mIU/L respectively. It is possible that higher concentrations of the plant extracts, especially *H. hemerocallidea*, may also have led to stimulation of insulin production.

Table 8: The quantity of insulin stimulated by different concentrations of extracts of different plant species.

| Plant species | Results | | | | |
|---|---|---|---|---|---|
| | Mass of plant extract used (µg/ml) | | | | |
| | 2 | 4 | 6 | 8 | 10 |
| *C. pepo* | <0.2 ml U/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L |
| *H. hemerocallidea* | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | 2.5 mIU/L | 2.5 mIU/L |
| *C. cassia* | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L |
| *C. citratus* | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L |
| *N. floribunda* | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L |
| *S. alexandrina* | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L | <0.2 mIU/L |

The isolated islet cells, according to Bhonde *et al*, (2007) provides a handy model system for ex-vivo determination of insulin secretory activity of beta cells, due to their independence from the somatic and nervous system i.e. secretion of insulin is a self regulatory process. From the results obtained in this section of the study only the *H. hemerocallidea* acetone crude extract had an effect on the islet cells which lead to a stimulation of insulin to 2.5 mIU/L for both the 8 and 10 ug/mL concentrations tested. The similarity in response in conjunction with a lack of response at 6ug/mL tends to suggest that concentrations tested are at the bottom of the sigmoid dose response curve (within the 20% effect level). It is therefore believed that increasing the dose will result in a proper dose-response relationship.

In comparison, the acarbose and glybenclamide control produce a 5-8 fold greater increase in insulin secretion despite the exposure being 100 fold higher in concentration. This would indicate that the *H. hemerocallidea* acetone crude extract has a very potent secretogogue compound therein. From previous studies in an induced rodent diabetic model, the ability of *H. hemerocallidea*

to decrease plasma glucose concentrations was well established (Ojewole, 2002 & 2006; Mahomed & Ojewole, 2003; Drewes *et al*, 2000). The ability of extract to induce insulin secretion therefore provides a mechanism for the activity seen *in vivo*. If one considers the insulin secretory activity in conjunction with the ability of the plant extract to inhibit both the alpha amylase and alpha glucosidase enzymes tends to suggest that *H. hemerocallidea* and acarbose share similar mechanisms of action.

When the activity of all the assays is looked at in combination, *H. hemerocallidea* had the widest activity, as the acetone extract had inhibitory activity in both the digestive enzyme assays in addition to stimulating the secretion of insulin, in the absence of significant anti-oxidant activity. In addition, if one considers the α-amylase enzyme inhibitory assay had an $EC_{50}$ of 0.92 mg/ml (which was higher to that of acarbose at 0.56 mg/ml), 85% α-glucosidase inhibitory activity at 1 mg/ml (compared to the 100% for acarbose) while stimulating insulin secretion at a low dose of 10 ug/ml, different compounds may be responsible for the activities of this plant extract.

# CHAPTER 7

## 7.1 Conclusion

The objectives of this study were:

1 To conduct a survey and literature study on the plants used to treat diabetes

The difficulties caused by diabetes mellitus and the relevant pharmacological background were discussed. In recent years much work has been directed to evaluate the evidence that medicinal plants used traditionally all over the world to treat and control diabetes can be used to treat and control this metabolic disease and its complications effectively. There have been numerous success stories using animal models in the testing of these plant extracts and this has encouraged wide-spread research into phytotherapy for diabetes mellitus. It could be shown that many plant species are used with very good effect to treat diabetes mellitus in humans.

2 To collect and validate plant material and prepare plant extracts

After discussions with different traditional healers in KwaZuluNatal under the guidance of Mr Radebe several plant species were indentified and plant material was bought and the identity verified by the Pretoria National herbarium. Preliminary work indicated that cold water extracts of finely ground plant material had no effect in the assays used. Solvents of varying polarity were used to extract the plant material.

3 To conduct phytochemical screening for each of the plant extracts

The plant species were best extracted in solvents with intermediate polarity namely acetone and methanol. The polarity of the extracting solvent plays an important role in the quantity extracted and that the polar and intermediate polar solvents (methanol and acetone) have higher extracting abilities than non-polar solvents. By TLC one gets an idea of what is in the extract and the chromatograms are important for reproducibility. The results of the qualitative

antioxidant activity therefore suggest polyphenols are likely to contribute to the free-radical scavenging activity of these plant extracts.

4 To establish, through the use of *in vitro* (alpha amylase and alpha glucosidase assays) *and semi in-vivo* (Islets of langerhans as a target) screening methods, if crude extracts of *Senna alexandrina, Cymbopogon citrates, Cucurbita pepo, Nuxia floribunda, Hypoxis hemerocallidea* and *Cinnamomum cassia* have an impact on alpha amylase, alpha glucosidase activity and/or the ability of the pancreatic beta cells to secrete insulin.

All the studied plant species had activity in at least one of the chosen assays (Table 9). The majority of the plant species had significant α-amylase and α-glucosidase inhibitory activity. In most cases the activity was present in the hexane extract and was non-related to the anti-oxidant activity of the extract i.e. activity is not due to polyphenolic driven enzyme precipitation. The best effect was seen with the acetone and ethyl acetate extract of *H. hemerocallidea* which had both digestive enzyme inhibitory and insulin secretory activity (only the acetone extract).

Table 9: Summary of results for all the plant species and the procedures performed.

| Plant species | Solvent | DPPH activity | Polyphenols present | Alpha amylase activity | Alpha glucosidase activity |
|---|---|---|---|---|---|
| *C. pepo* | Hexane | 0 | 0 | 2 | 3 |
| | Ethyl acetate | 0 | 1 | 0 | 3 |
| | Acetone | 0 | 1 | 2 | 2 |
| | Methanol | 0 | 1 | 0 | 3 |
| *C. cassia* | Hexane | 0 | 0 | 2 | 3 |
| | Ethyl acetate | 0 | 2 | 0 | 3 |
| | Acetone | 3 | 3 | 0 | 3 |
| | Methanol | 3 | 3 | 3 | 0 |
| *S. alexandrina* | Hexane | 0 | 0 | 3 | 3 |
| | Ethyl acetate | 0 | 3 | 0 | 3 |
| | Acetone | 0 | 1 | 0 | 3 |
| | Methanol | 0 | 1 | 0 | 3 |
| *N. floribunda* | Hexane | 0 | 0 | 2 | 3 |
| | Ethyl acetate | 0 | 2 | 2 | 3 |
| | Acetone | 3 | 2 | 0 | 2 |
| | Methanol | 3 | 3 | 0 | 1 |
| *H. hemerocallidea* | Hexane | 0 | 0 | 2 | 3 |
| | Ethyl acetate | 0 | 1 | 3 | 3 |
| | Acetone | 0 | 1 | 2 | 3 |
| | Methanol | 0 | 1 | 2 | 3 |
| *C. citratus* | Hexane | 0 | 0 | 2 | 3 |
| | Ethyl acetate | 0 | 2 | 2 | 3 |
| | Acetone | 0 | 2 | 2 | 3 |
| | Methanol | 2 | 3 | 3 | 2 |

0= no activity, 1=low, 2=medium, 3=high activity.

5. To evaluate the potential use of the different plant species in treating diabetes mellitus.

From the *in vitro* results, it can be concluded that all the tested plants have some merit in the management of diabetes mellitus type II, as suggested by the ethnomedicinal lead. However, when the use of water extracts by the community is taken in consideration, only the use of *H. hernerocallidea* may support the proper traditional use as the effective extract was in acetone. The effectivity of the hexane extracts tends to suggest that digestive enzyme inhibition may not be the reason for the use of these plants traditional. From the results, it is also evident that acetone and ethyl acetate extracts of *H. hernerocallidae*, methanol and ethyl acetate extracts of *C. citratus*, and methanol and hexane extracts of *C. cassia*, acetone and hexane extracts of *C. pepo* and ethyl acetate extract of *N. floribunda* may be more effective than acarbose which

has become a widely used medicine in the management of type-II diabetes, and therefore warrants further investigation.

## 7.2 Recommendations

1. The *in vivo* effects of the *H. hemerocallidea* extracts needs to be examined to determine the value of the plant extract in the management of type II diabetes in man.
2. Further work needs to be undertaken to isolate, identify and characterize the compound(s) responsible for the α-amylase, α-glucosidase inhibitory and insulin secretory activity of *H. hernerocallidae*.
3. If isolation is possible, the mechanism of action of the effective compounds needs to be elucidated.
4. To conduct extensive toxicity tests for each plant extract to establish the safety of the non-polar extracts of the plant species used.

# APPENDIX A

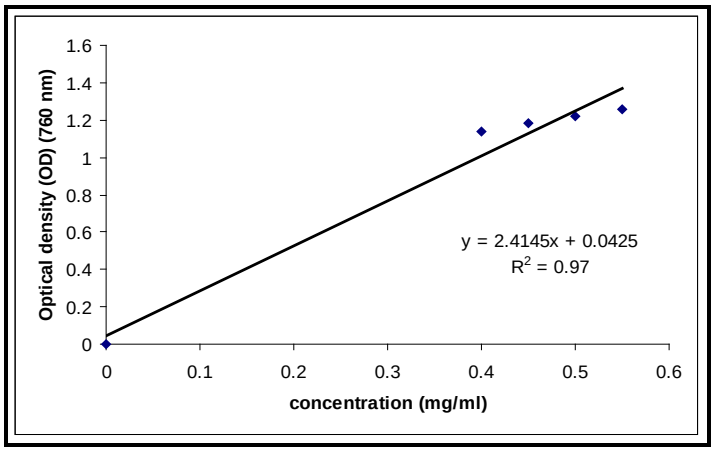


Figure 21: Standard curve for polyphenols

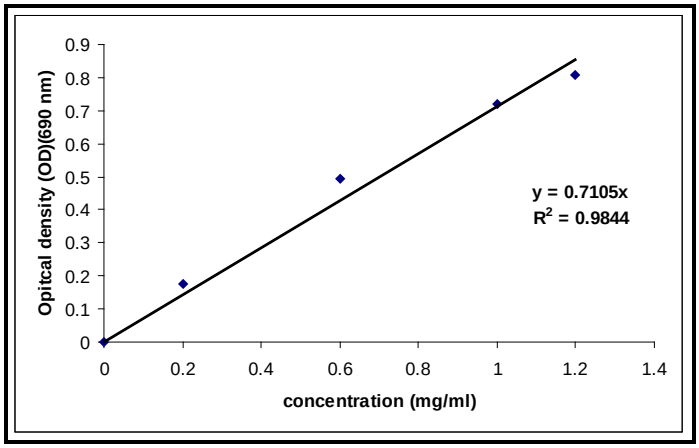


Figure 22: Standard curve for maltose

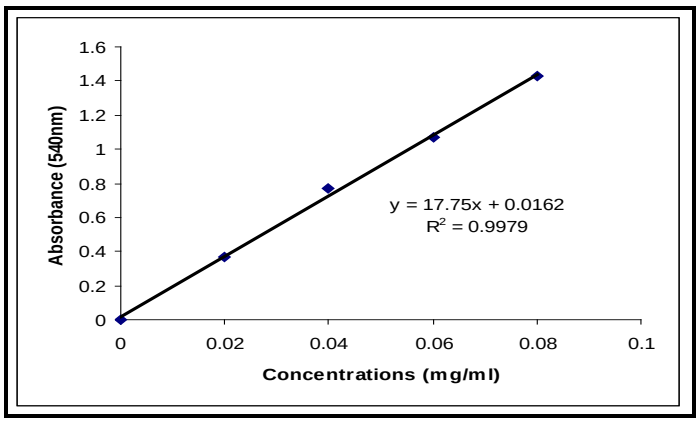


Figure 23: Standard curve for glucose

# CHAPTER 8

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