INHIBITORY CAPABILITIES OF TEN MEDICINAL PLANTS USED BY TRADITIONAL HEALERS ON MAMMALIAN CARBOHYDRATE DIGESTING ENZYMES (ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE)

by

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[redacted]

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Biochemistry has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

Miss VP Ntini — 18 March 2014

Initials & Surname (Title) — Date

[redacted]

Signature: _____

DEDICATION

I dedicate this work to my family and people who believed in me and always encouraged me throughout this project.

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A

ACE	acetone
ADP	adenosine diphosphate
AIDS	Acquired Immunodeficiency virus

AMPK	adenosine monophosphate activated protein kinase
APK	activate protein kinase
ATCC	American type culture collection
ATP	adenosine triphosphate
α	alpha

B

β	beta

C

^{o}C	degrees Celsius
Ca^{2+}	calcium protons
CE	catechin equivalence
CEF	chloroform ethyl acetate formic acid
CHℓ	chloroform
CI	cell index
^{13}C-NMR	carbon-13 nuclear magnetic resonance
CNPG3	2-chloro-4-nitrophenyl-α-D-maltotrioside
CO_2	carbon dioxide

D

DCM	dichloromethane
DMEM	Dulbecco's minimum-eagle medium
DPPH	2,2-diphenyl-1-picrylhydrazyl

E

EMW	ethyl acetate methanol water
EC	enzyme commission
EtoAc	ethyl acetate

F

FBS	foetal bovine serum

Fig	figure

G

g	gram
GAE	gallic acid equivalence
Gluts	glucose transporters
Glut1	glucose transporter1
Glut2	glucose transporter2
Glut3	glucose transporter3
Glut4	glucose transporter4
Gluts5-12	glucose transporters5-12

H

H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HCℓ	hydrochloric acid
Hex	hexane
HIV	human deficiency virus
hr	hour
^{1}H-NMR	proton nuclear magnetic resonance

I

IC_{50}	inhibitory concentration
IDDM	insulin dependent diabetes mellitus
IDF	international diabetes federation
IRS	insulin receptor substrate

K

kDa	kilodaltons

L

l	litre

ℓ	litre

M

M	molar
MEME	Minimum Essential Medium Eagle
MeOH	methanol
mg	milligram
MHz	megahertz
min	minutes
mℓ	millilitre
mM	millimolar
MTT	3-(4,5-dimethylthiozyl-2-yl)-2,5-diphenyl tetrazolium bromide

N

NaOH	sodium hydroxide
nm	nanometer
NMR	nuclear magnetic resonance
NIDDM	non-insulin dependent insulin diabetes mellitus

P

PSN	penicillin, streptomycin, neomycin
PI3-kinase	phosphotydilinositol 3-kinase
$PPAY_y$	perixome proliferator $receptor_y$

Q

QE	quercitin equivalence

R

ROS	reactive oxygen species

S

[S]	substrate concentration
Sec	second
sec	section
$SGLT^1$	sodium glucose co-transporters
SH2	Src-homology-2
STD	sexually transmitted diseases

T

TB	tuberculosis
TEA	toluene ethyl acetate ammonia hydroxide
TLC	thin layer chromatography
TZDs	thiazolidinediones

U

UV	ultraviolet
µℓ	microlitre
µg	microgram

V

VDCC	voltage dependent Ca^{2+} channel
v/v	volume to volume

W

WHO	World Health Organization

X

XTT	(2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide)

Abstract

Diabetes mellitus is one of the fast growing chronic metabolic disorders throughout the world. It has become a life threatening disease and health burden. So far it can only be managed with commercial therapeutic agents, proper diet and exercise. People particularly from developing countries use medicinal plants to treat this condition. According to WHO, about 80% of the population in developing countries are dependable on medicinal plants. This prompted many researchers to explore the effectiveness and safety of these plants. In the current study ten medicinal plants were randomly chosen, screened for antidiabetic activity by testing their ability to inhibit α-amylase and α-glucosidase enzymes. The plants were tested using *in vitro* assays. The finely powdered leaves of each plant were extracted with hexane, chloroform, acetone and ethyl acetate. Phytoconstituents of each plant extracts were analyzed using both qualitative and quantitative methods. All plant extracts tested positive for phenols, flavonoids and all negative for starch. Their compounds were better separated in the TEA mobile system on the TLC plates. All plant extracts had more of total phenolics ranging between 0.1-400 GAE/mg than total flavonoids and condensed tannins. Antioxidant activity of the plant extracts was tested quantitatively at various concentrations using DPPH. Most plant extracts were able to scavenge the radicals produced by DPPH at highest concentration of 2.5 mg/mℓ. Not all plant extracts with the highest number of total phenolics had the highest antioxidant activity. For antidiabetic *in vitro* assays, plant extracts inhibited various percentages of both α-amylase and α-glucosidase activity at concentrations ranging between 0.019- 2.5 mg/mℓ. The best overall activity against both enzymes was observed in acetone and ethyl acetate plant extracts. *Cassia abbreviata* and *Helinus integrifolius* were even more active than acarbose which was used as positive control. These plant extracts inhibited both the enzymes in a dose dependent and non-competitive manner. Seeing that both extracts of *C. abbreviata* and *H. integrifolius* were consistent when inhibiting both enzymes, they were further evaluated for their effect on glucose uptake by the C2C12 muscle and H-II-4-E liver cells. All the plant extracts tested were able to increase glucose uptake in the muscle cells. However optimal increase was seen in the liver cells when treated with 250 μg/mℓ of acetone and ethyl acetate extracts of *C. abbreviata*.

The cytotoxicity effects of both acetone and ethyl acetate of *C. abbreviata* and *H. integrifolius* was tested using the xCelligence system on RAW 264.7 cells. Different cell indexes were obtained after treating the cells with different concentrations (0.05, 0.1 and 0.25 mg/mℓ) of each plant extracts respectively. The system was run for three days but the toxic effects of plant extracts were analyzed for the first ten hours. The results obtained shows that cell index decreased as the concentration of the plant extracts was increased. All the plant extracts were less toxic as compared to positive control, Actinomycin D.

The leaves of *H. integrifolius* were further exhaustively extracted with hexane, dichloromethane, acetone, ethyl acetate and methanol respectively. Since the DCM extracts yielded the highest mass in quantity, it was further used for isolation of active compounds. Column chromatography and bioassay guided fraction led to isolation of a mixture of triterpenes identified as α and β-amyrin. The structure was elucidated using nuclear magnetic resonance technique. The inhibitory capability of the isolated compound against α-amylase enzyme was less than the crude extract which inhibited more than 50% of the activity at a concentration of 1 mg/mℓ.

Based on the enzymes assays and cell culture work it can be concluded that *C. abbreviata* and *H. integrifolius* species are the best inhibitors of carbohydrate digesting enzymes, and therefore be used to manage postprandial hyperglycemia in the people with type 2 diabetes. However more work still need to be conducted for further isolation of more active compounds.

Chapter 1

1.1. Introduction

Plants have been used throughout ages for different purposes. Recently they are paving a comeback as a major source of medicines (Joy *et al*., 1998). The resurgence in the use of plants as traditional medicines is becoming popular in most parts of the world in recent years. According to the World Health Organization (WHO, 2002), over three quarters of the world's population rely mainly on plants for primary healthcare purposes. Green plants can synthesize and store biochemical and secondary compounds. These compounds have different pharmacological activities that are effective against various diseases and can be extracted or isolated from plants. They also serve as template in new drug design to enhance efficacy and lower their toxicity levels (Joy *et al*., 1998).

In Africa, the use of medicinal plant extracts is common and prevalent in traditional health practices. South Africa has a large rural population that depends on natural products for treatment of various ailments. This is mainly due to population growth, inadequate drugs, prohibitive cost of treatments and outbreak of different chronic diseases (Joy *et al*., 1998).

Chronic diseases are a major health problem globally. They account for a larger proportion of mortality, morbidity and disability in the world. Almost fifty percent (50%) of deaths globally are due to chronic diseases (Graeme, 2010). Diabetes mellitus is a chronic metabolic disorder characterised by abnormal glucose homeostasis together with disturbances of carbohydrate, fat, and protein metabolism resulting from insufficient insulin secretion and action or both. When this condition is untreated or poorly managed, permanent damage to organs such as kidneys, eyes, nerves, heart, blood vessels and sometimes death may occur from complications. Diseases like diabetes mellitus also have a direct impact on socio-economic issues. According to the International Diabetes Federation (IDF) (2007), diabetes mellitus has recently emerged as the fourth leading cause of death globally. Its prevalence has reached epidemic proportions in both developed and developing countries. Numerous studies showed that current diabetes mellitus treatments are associated with negative side effects such as diarrhoea, abdominal pains and weight gain

(Kumar *et al*., 2012). Moreover, majority of people from developing countries face difficult challenges of affordability and access to the treatment. As such, the tendency to rely on medicinal plants for treatment increases. People use different parts of various plants to treat diseases. Different medicinal plants use in the treatment or management of diabetes elicits their therapeutic effect through different mode of action. With the complications associated with available chemotherapeutic, the need for alternative strategies for prevention and treatment of diabetes mellitus is crucial. In this study ten medicinal plants used by traditional healers to treat various diseases were screened for their antidiabetic potential.

1.2. Literature review

1.2.1. History of medicinal plants

Plants are a very useful source of life and have been used throughout the years for different purposes. The use of plants for medical purpose started way back in history (Wondimu *et al*., 2007) and thousands of drugs have been derived from them (Rukangira, 2000). Previously, medicinal plants were popular in developing countries, however recently they are widely used in the whole world (Westaway, 2009). Majority of people days use medicinal plants products daily to treat different diseases and to maintain a good health.

Wondimu *et al*., (2007) reported that the past educated and modern society has been ignoring traditional medicine. However, recent studies show that there is an overwhelming interest in the use of indigenous plants (Wondimu *et al*., 2007). This is supported by the derivation of modern medicines from plants and also the immense resources engaged in conducting a number of ethnobotanical and ethnomedicinal studies. Since recognition by the WHO, the use of medicinal plants in primary health care has increased tremendously (Rukangira, 2001). So far, out of the 250,000 plant species on earth, it is reported that more than 80,000 are used for treatment of diseases and as possible leads for development of new drugs (Joy *et al*., 1998). Although some cultures use them as an alternative medication, in other countries, plants play a significant role in primary health care and also as part of their traditional values (Gurib-Fakim, 2006; WHO, 2002).

1.2.2. Traditional medicines

In most developing countries people use medicinal plants as part of their culture and as such referred to as traditional medicines. Since time immemorial, Asia and Africa were best known for the use of traditional medicines to treat diseases (Joy *et al*., 1998). Different countries and regions have their own form of traditional medicine that is different to others (Rukangira, 2001). Their approach to health, diseases and therapeutic methods also differs. For example, Indian traditional medicine is based on various systems such as *Ayurveda, Siddha* and *Unani* used by different communities (Gadgil, 1996). Amongst all, *Ayurveda* is considered the most ancient and widely practised (Joy *et al*., 1998). It is a set of guidelines to maintain healthy balance and harmony in the human body. Magner (2002) reported that it is based on body humours and internal life that maintain digestion and mental activity just like *Galenical* medicine. China also has its own traditional medicine system based on supporting good health and longevity (Kapoor, 1990; Patwardhan, 2005).

Africa is one of the continents best known for medicinal plants use globally. It is one of the developing continents with a large population relying on medicinal plants for primary health care. According to Zirihi *et al*. (2005) and WHO (2002) about 80% of rural communities in Africa depend on traditional medicinal plants forming part of their socio-economic and socio-cultural heritage. Medicinal plants are the preferred form of healthcare because of availability and affordability as compared to western medication (Elujoba *et al*., 2005). Plants are used for prevention, diagnosis, treatment of social, mental, physical illness and life-threatening diseases.

South Africa as in many African countries has a history of making full use of its diverse plant resources as medication for the treatment of diseases by different ethnic groups. Light *et al*. (2005) reported that out of 47 million South Africans, 57% use traditional medicines. It was estimated that about 20 000 tons of plants is consumed per year for medicinal purposes (Mander, 1998). Most of the medicinal plants users differ in terms of age, level of education, religion and occupation. Some of the people visit traditional healers where they are given medicinal plants to treat different diseases (Van Wyk *et al*., 1997). The South African government is now reviving the agricultural production of medicinal plants through research, nurseries, database and extension, because of its high demand (Mander *et al.*, 1995).

1.2.3. Demand for medicinal plants

The demand for medicinal plants and plant based therapeutics is escalating in both developed and developing countries. This is because many people have realised that natural products have less negative side effects, are affordable and easily accessible. The demand for medicinal plants also increases as the population growth increases. High rate of urbanisation together with escalating rate of unemployment also cause high demand for medicinal plants for commercialization. Cunningham (1993) observed that thousands of people depend on the trade of medicinal plants to generate income. For example, South Africa has 28 million medicinal plants consumers that utilise 19 500 tonnes of plant material and generating an expenditure of R273 million annually. Apart from that, the burden of pandemics and growth of life-style related diseases also increases the demand for medicinal plants. In South Africa, most medicinal plants are prescribed for treatment of different diseases, especially chronic diseases that cannot be cured (Mander *et al*., 2005).

1.2.4. Chronic diseases

Life span is shortened due to chronic diseases. Many of people are affected by these diseases at a young age, suffer for a long period and eventually die. In 2005, the World Health Organisation reported that out of 58 million deaths worldwide, 38 million are caused by chronic diseases. The prevalence of these diseases is not easily recognised because the symptoms are sometimes invisible, progressively slow and are under diagnosed. Change in lifestyle, unhealthy diet, smoking, obesity, lack of exercise and poor health are reported to be the main cause of chronic diseases. The most common chronic diseases include stroke, diabetes mellitus, cancer and heart diseases (WHO, 2002).

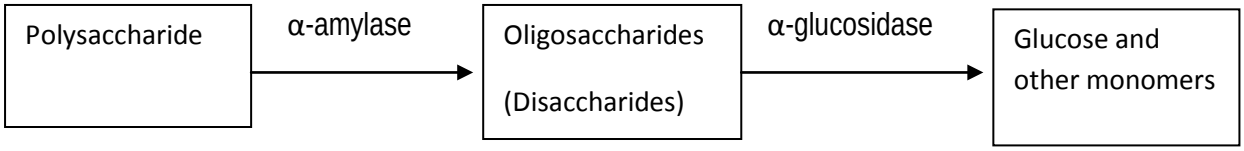
1.2.5. Pathophysiology of diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder characterised by chronic hyperglycemia and hypoglycemia. It occurs as a result of altered carbohydrate, fat and protein metabolism, linked to insufficient insulin secretion and action (Schenk *et al*., 2008). Relative or total lack of insulin in the body also alters carbohydrate metabolism. According to WHO standards, diabetes is confirmed when fasting

glucose level is > 6.1 mmol/ℓ (110 mg/dl) and 2 hour postprandial glycemia is ≥ 7.8 mmol/l (140 mg/dl) WHO (2006).

During normal blood glucose level and food digestion, the food passes through the digestive system and eventually turns into proteins, fats and carbohydrates. In the presence of carbohydrates, digestive enzymes are secreted in order to degrade the carbohydrates into smaller pieces. α-Amylase and α-glucosidase enzymes are mostly important for breakdown of carbohydrates to glucose. The salivary and pancreatic α-amylases are glycoside hydrolase enzymes responsible for breaking down polysaccharides into oligosaccharides. Further digestion is carried out by α-glucosidase enzyme in the small intestine, whereby the resulting disaccharides are hydrolysed into monosaccharides to enable their easy absorption, as shown below (Hanhineva *et al*., 2010; Collins *et al*., 1997).

Breaking down of carbohydrates by enzymes into small sugars



After digestion of carbohydrates, other monosaccharides such as fructose and galactose together with glucose will get absorbed in the hepatic portal vein through the small intestine. This results in an increase in the postprandial blood glucose level. An increase in blood glucose level, signal the β cells of the inlets of langerhans in the pancreas to release insulin.

1.2.5.1. Insulin secretion from pancreas

The secretion of insulin from pancreatic cells is a very complex process. According to Seino (2002), it involves the combination, interaction and stimulation from both inside and outside the pancreatic cells. The process is regulated by an interaction of metabolic and electronic events which induces secretion repeatedly (MacDonald and Wheeler, 2003). A schematic diagram is presented in figure1. When intracellular glucose is metabolised to adenosine triphosphate (ATP), glucose is transported into the beta cells. An increase in ATP/ADP ratio enlarges the cell surface for ATP and closing K^+ (K_{ATP}) channels, thus allowing the membrane to depolarize. The membrane is depolarised by decrease in ADP and increase in ATP to -40Mv or less. When the voltage dependent Ca^{2+} channel (VDCC) opens, it allows the influx of

extracellular Ca^{2+} to enter the beta cell and induces the release of insulin (Seino, 2002). All these electrogenic events leading to secretion of insulin are collectively called the K_{ATP} channel dependent stimulus secretion coupling (MacDonald and Wheeler, 2003).

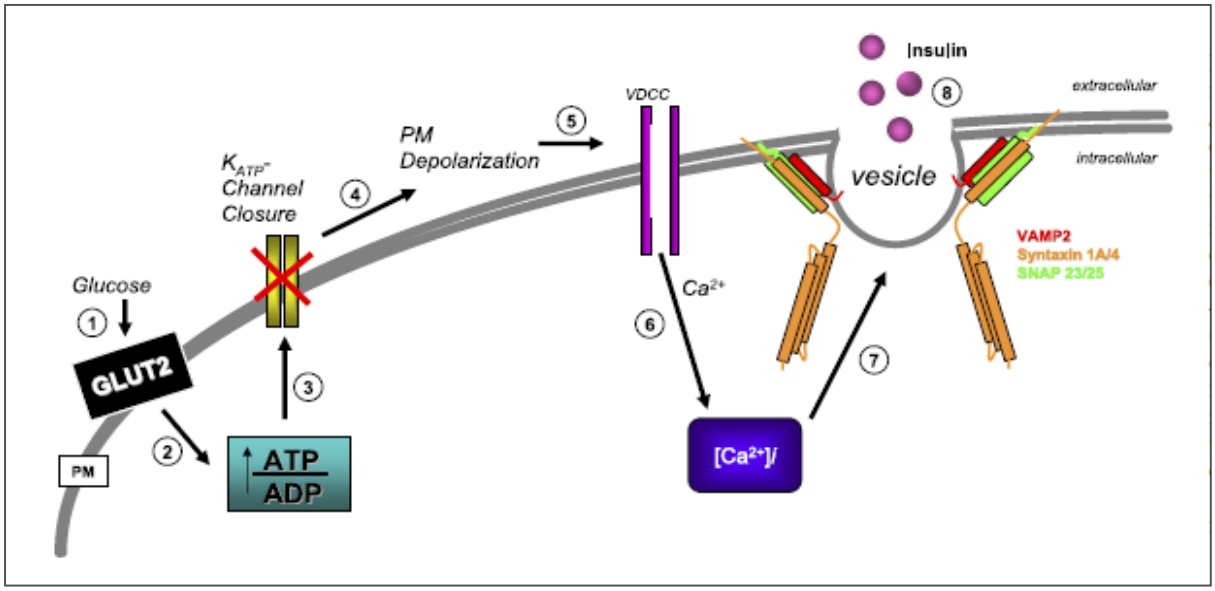


Figure 1: Sequential ionic control of insulin secretion (Jewell *et al*., 2010)

1.2.5.2. Role of Insulin action

Insulin is a hormone that regulates blood glucose levels. It activates the glucose transport mechanism and glucose utilizing pathways in different tissues of the body. It is a protein which consists of 51 amino acids and has a molecular weight of 6 kDa. Insulin has two polypeptide chains A and B that are linked by two disulphide bonds (Blundell *et al.,* 1972). This hormone is secreted in response to glucose and amino acids circulating after the intake of a meal. Rhodes and White (2002) described the principal role of insulin as to regulate homeostasis and stimulate glucose uptake by the cells, glycogen synthesis and also suppress glycogenolysis and gluconeogenosis as detailed in figure 2. These insulin actions are essential for maintaining normal cell homeostasis and also promote cell growth in all living cells.

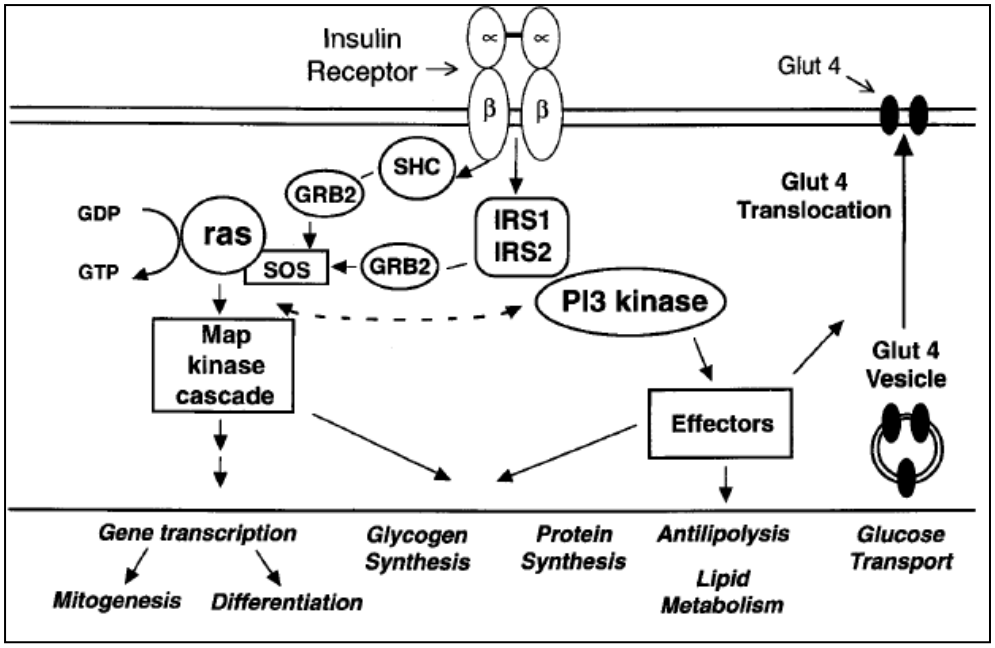


Figure 2: Central role of insulin actions (Houseknetch and Kahn, 1997)

Insulin action starts when it binds to its cell-surface receptor, thereby transmitting a message across the plasma membrane. Insulin receptor is made up of α and β subunit that are linked by disulphide bonds. When insulin binds to the α-subunit, the receptor activates tyrosine kinase in the β subunit. By so doing, the insulin receptor substrate (IRS) becomes phosphorylated and starts to interact with other proteins and lipids (Pessin, 2000). Following phosphorylation, they bind to the Src-homology-2 (SH2) domains, stimulate phosphatidylinositol 3-kinase (PI 3-kinase), (Grb-2) and tyrosine phosphatise-2 containing SH2. Saltiel and Pessin (2000) demonstrated that interaction between IRS proteins and PI 3-kinase induces the expression of serine/threonine kinase Akt and protein kinase C isoforms. Their expression signals the activity of Glut proteins in their specific cells for absorption of glucose.

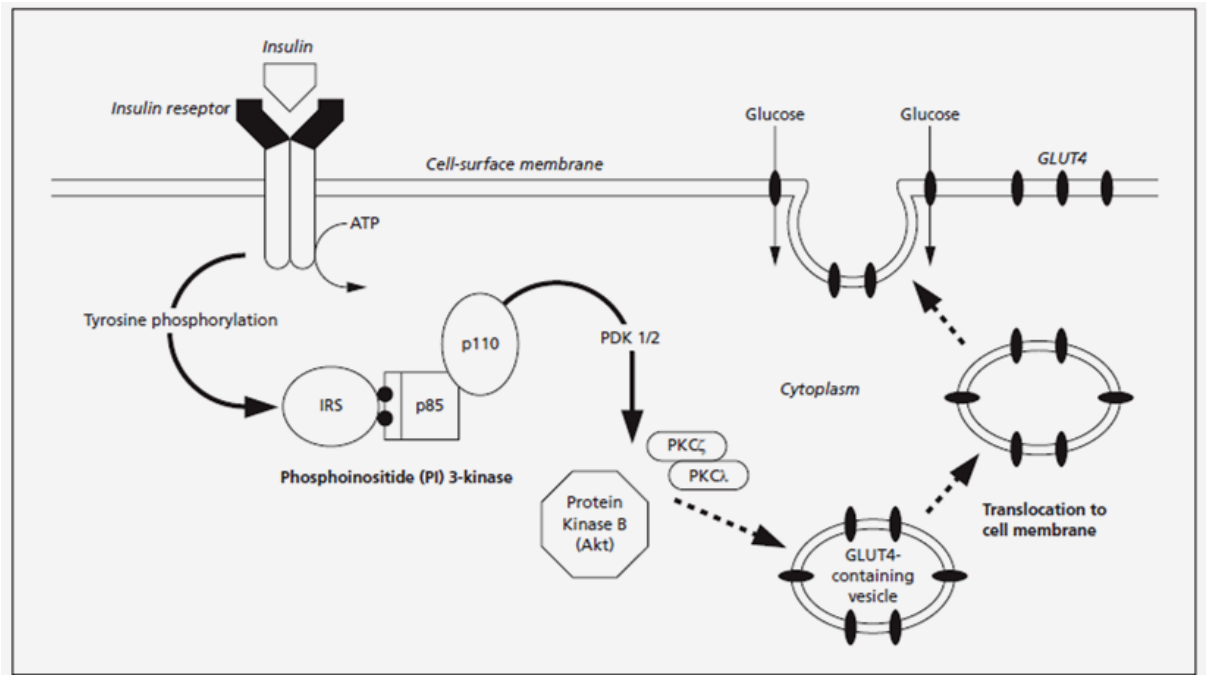


Figure 3: Insulin signalling transduction upon binding to its receptors (Nielsen, 2008)

1.2.5.3. Glucose absorption by cells

Glucose is the main source of energy and carbon for all cells. Its entry into the cell is a very important step which depends on different factors such as expression of suitable glucose transporters in the target tissue and hormonal regulation of their function (Gorovintis and Charron, 2003). The continuous supply of tissues by glucose is important for maintaining glucose homeostasis in the body. This flow of glucose across plasma membrane is accomplished by sodium glucose co-transporters ($SGLT^1$ 1-3) and facilitative glucose transporters (Gluts) (Brown, 2000). During the process, glucose transporters change their shapes such that glucose will bind to both the intracellular and extracellular sides of the cell. There are four different steps which glucose employs to enter the cell (Lienhard *et al*., 1992). Firstly, it occupies the outward facing binding side. Secondly, glucose and the transporter form a complex which will further change conformation to allow glucose to bind to the binding site of the cell. Thirdly, glucose will be released into the cytoplasm and fourthly, the unoccupied transporter changes conformation making the glucose binding site to face outward. Glut transporters are the main proteins that facilitate the movement of glucose across the cell by forming a large pore in which glucose can easily move through.

The need for glucose transporters differs from cell to cell and their specific function. According to Medina and Owen (2002), Gluts are translocated in the plasma membrane in response to insulin and are expressed in all cells that transport glucose down a concentration gradient. All Gluts contain both amino and carboxyl termini and amino acids that are responsible for substrate selectivity. When glucose binds to the cell, Glut changes its orientation from an exofacial to endofacial orientation in order to release substrate into the cell. Different Gluts with various functions are expressed in various tissues and organelles like the liver, kidney, heart, brain, skeletal muscle, adipose cells and β pancreatic cells. According to Gorovitis and Charron (2003), there are 12 Gluts that have been identified up to date, however only functions of glut1-5 are known (Olson and Pessin, 1996). Glucose transporter1 (Glut1) transports basal glucose and also provides stable flow of glucose to the brain. Glut2 is found in organs which release glucose into blood and it transports glucose depending on the blood glucose level. Glut3 is found in the neural cells of the brain and works similar to Glut1 and it maintains steady flow of glucose into the cells. Glut5 absorbs fructose in the small intestine and kidneys (Katzung, 1995).

Out of all the glucose transporters, Glut4 is the major transporter which is found in many cells. It facilitates glucose uptake by the cells depending on the stimulatory insulin. During low concentration of glucose, Glut4 recycles between the plasma membrane and vesicular compartments in the cell. As detailed in the Holman *et al* (1994) study, when insulin binds to its receptor, Glut4 translocate from the extracellular to the inner surface of the plasma membrane. This action causes small vesicles containing Glut4 to move from the inner surface of the cell and fuse with endosomes. The fusing of Glut4 with cell membrane depends on the availability of insulin. Low levels of insulin stops the process, resulting in accumulation of glucose transporter in the intracellular vesicle (Lienhard *et al*., 1992). This process results in an increase uptake of glucose by the cells. Although PI 3-kinase is necessary for Glut4 translocation, binding of insulin to its receptors signals other molecules responsible for trafficking, docking and fusion of Glut4 (see figure4) (Pessin and saltiel, 2000). Defects in Glut4 trafficking and translocation leads to insulin resistance by cells (Garvey *et al*., 1998) and also contributes to impaired glucose transport in type2 diabetic patients (Ryder *et al*., 2000). Moreover, if the gene

expression of Glut4 is down regulated in tissues, insulin resistance might occur subsequently leading to development of diabetes.

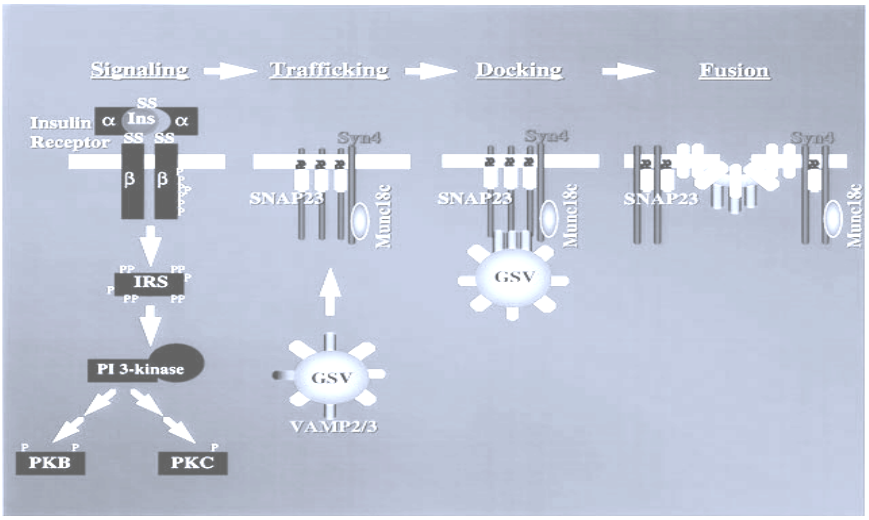


Figure 4: Schematic model showing the four main stages of translocation (Thurmond and Pessin, 2000)

1.2.6. Classification of diabetes mellitus

Diabetes mellitus is classified into three major types namely, type 1, type 2 and gestational diabetes.

1.2.6.1. Type 1 diabetes

Type 1 diabetes is an autoimmune related disease that occurs due to destruction of T-lymphocytes of pancreatic islet's beta cells (Gianani and Eisenbaith, 2005). It is commonly known as Insulin dependent diabetes mellitus (IDDM) and occurs in young children and adults. Environmental risk factors and genetic prediposition are believed to be the main factors that contribute to the development of this disease. They either alter the pancreas directly or indirectly by producing an abnormal immune response in the cell thereby preventing it from producing insulin. Since type 1 diabetes also develops in young people, it is suspected that viruses and infant nutrition can also desrupt the β cells of the pancreas. People with type 1 diabetes depend mainly on insulin for stabilizing blood glucose levels and to prevent ketoacidosis. Type 1 diabetes patients usually suffer from polydipsia, polyuria, weight loss, dry mouth, slow healing of skin wounds, cramps and infections on genitalia. It accounts for 10% of diabetic patients globally and its prevalence is still on the rise when compared to type 2 (Sierra, 2009).

1.2.6.2. Type 2 diabetes

Type 2 is a non-insulin dependent diabetes mellitus (NIDDM). It is the most prevalent type, accounting for 90-95% of diabetic cases worldwide (Sierra, 2009). NIDDM is mainly caused by insulin resistance, impaired regulation of hepatic glucose production, malfunction of β-cell and glucose intolerance (Gloyn, 2003). It occurs when the body does not respond to the available insulin or there is shortage of insulin production in the body due to failure of β-cell function. According to Riaz (2009), type 2 diabetes may take years to develop and its symptoms are not severe enough for patients to notice. This leads to development of micro and macrovascular complications when undiagnosed at an early stage. Type 2 diabetic patients are independent of exogenous insulin but mostly control their glucose levels with healthy diet and oral hypoglycaemic agents. Apart from hyperglycemia, this disease is associated with a group of cardiovascular diseases including dyslipidaemia, hypertension, hypercoagulability and other metabolic disorders. The International Diabetes Federation (IDF) and the World Health Organization (WHO) (1994) observed that the prevalence of type 2 diabetes is increasing because of rapid cultural and social changes, aging population and increase in urbanization (Zhang *et al*., *2009*). Factors such as obesity, unhealthy diet and genetic susceptibility seem to be the main causes of type 2 diabetes.

1.2.6.3. Gestational diabetes

Gestational diabetes is a metabolic disorder that any previous non-diabetic woman can develop temporarily during pregnancy. It develops mainly in women who are glucose intolerance and have susceptible genes to type 1 and type 2 diabetes (Riaz, 2009). Hormonal changes and weight gained during pregnancy also contribute to this condition. Undiagnosed gestational diabetes can lead to giving birth to larger babies than normal, maternal and foetal abnormalities and infant death. In most cases this condition ends after delivery but put the mother and baby at a high risk of developing type 2 diabetes at a later stage (IDF, 2010).

1.2.7. Causes of diabetes mellitus

Diabetes mellitus is a metabolic disorder that develops due to different factors. Numerous studies show that insulin resistance, obesity, genetic susceptibility and

environmental factors are the main factors that contribute to the development of this disease (Riaz, 2009; Akerblom *et al*., 1997).

1.2.7.1. Insulin resistance

Insulin resistance occurs when the body cannot use the secreted insulin that is available in the body to regulate blood glucose levels (Mlinar *et al*., 2007). It occurs due to hyperinsulinemia caused by excess β-cells which may disrupt insulin's mechanism of controlling glucose and lipid homeostasis (Mlinar *et al*., 2007). Insulin resistance contributes to the development of type 2 diabetes by increasing the need for insulin in people whose β-cells do not function properly.

1.2.7.2. Obesity

Lack of physical activity and obesity are the major contributors to the development of type 2 diabetes mellitus. Obesity is caused by overindulgence in unhealthy diet, genetics and other environmental factors. During an obese state, abdominal fat and adipokines secreted by adipose tissue prevent the body from responding to insulin secreted, hence resulting into insulin resistance. It also increases secretion of chemokines from adipocytes which have a negative impact on insulin sensitivity (Weisberg, 2003). Combination of both central and upper body obesity results in an increase in free fatty acids which causes hyperglycemia, hyperinsulinemia, hepatic insulin resistance and also inhibits glucose uptake mechanisms regulated by Glut4 (Arner, 1996) as shown in figure 5.

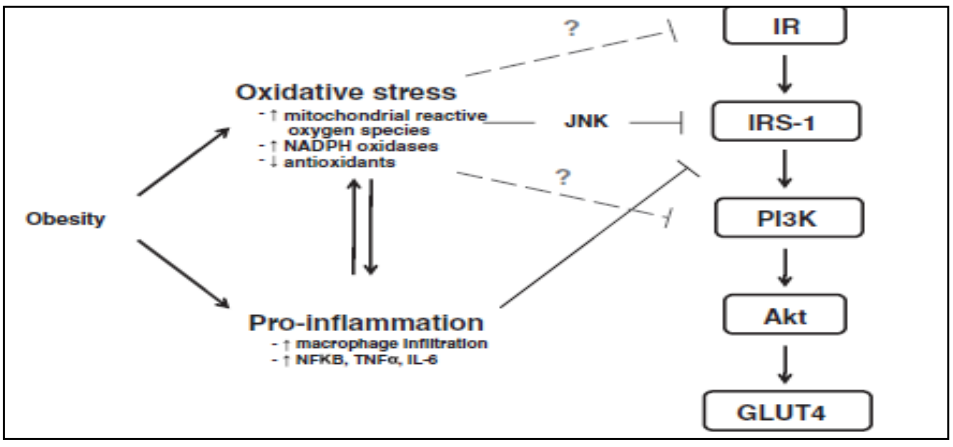


Figure 5: Mechanism by which obesity inhibits translocation of Glut4 (Styskal *et al*., 2012)

1.2.7.3. Environmental factors

Although evidence is limited, it is assumed that environmental factors such as toxins, food, viruses and free radicals cause diabetes mellitus, particularly type1. Most of these factors are assumed to trigger the immune system to disrupt the beta cells (Riaz, 2009). There are several viruses that are reported to play a role in the pathogenesis of type1 diabetes. In 2002, Akerblom and his colleagues reported that viruses such as cytomegalovirus, adenovirus, congenital rubella and mumps were commonly found in type1 diabetic patients. They damage the beta cell and induce pancreatitis. Dietary factors such as exposure to cow milk at an early age (infant stage) also increase the risk of developing type1 diabetes. According to Akerblom *et al* (1997), the impact of a specific environmental factor in inducing an autoimmune response depends on an individual's genetic susceptibility.

1.2.7.4. Oxidative stress

Free radicals resulting from reactive biochemicals, air pollution and smoke can also trigger the development of diabetes. When these radicals build up in the body, they damage the insulin producing cells, causes cancer, cardiovascular diseases and oxidative stress (Riaz, 2009). Oxidative stress plays a major role in the development of type 1 and type 2 diabetes and the complications associated with them. Oxidative stress negatively affects glucose homeostasis as a result of decrease in antioxidant defence or production of more free radicals in the body (Ruhe and MacDonald, 2001). It also impairs insulin signalling pathway causing insulin resistance. High concentration of insulin stimulates oxidative stress by inducing hydrogen peroxide (H_2O_2) during the activation of insulin receptors. When blood glucose level increases, non enzymatic glycation of proteins occur which result in more oxidative stress thereby inhibiting insulin-induced Glut4 transportation (Sailaya *et al*, 2004).

1.2.7.5. Genetic susceptibility

Diabetes mellitus is believed to be a hereditary disease. Genetic makeup of an individual may initiate the pathogenesis process either in accelerating or delaying the autoimmune response. Studies show that people with a history of certain genes like TCF7/L2 are at high risk of developing diabetes than others (Grant *et al*., 2009).

Some genes increase the chance of one to become obese leading to insulin resistance.

1.2.8. Global statistics of diabetes mellitus

Diabetes mellitus is a common and serious global health problem. At first, the prevalence was high in high income western countries, with a change in dynamics in the past decades to all countries around the world (WHO, 1994). According to (IDF, 2010) there are currently 285 million people living with diabetes and the number is expected to rise to 385 million people by 2025. More people from low-income countries are expected to be mostly affected by the year 2025. High prevalence of this disease is found within ethnic groups which are urbanized and are changing their lifestyles. Diabetes morbidity is higher in some countries than others because of demographics, social, cultural, environmental factors and economic development. For example 60% of the diabetic population are Asians and the number has increased due to urbanization and nutritional transition that took place in Asia (Hu, 2011). A study conducted by Sicree and Zimmet (2009) shown that type 2 diabetes incidence is high in the age group of 40-59, with more females affected than males. Due to ageing the number is expected to be high in the age group of 60-79 by 2025.

Africa is also one of the continents faced with a high incident of diabetes and other non-communicable diseases. In 2009, 12.1 million people in Africa were diabetic and the number is expected to rise to 23.9 million by 2030 (Sicree and Zimmet, 2009). South Africa is among the sub-Saharan countries experiencing a mounting rate of diabetes fuelled by lifestyle changes. Currently there are 6.5 million people living with diabetes in South Africa (IDF, 2010). Type 2 diabetes is the most prevalent type amongst those infected in urban areas. Relocation to urban areas leads to decrease in physical activity, easy access to transportation, changes in diet and increase in obesity. All the ethnic groups are affected. However, the Black and Indian communities are mostly affected due to lifestyle changes and culture (Ngobeni, 2008).

1.2.9. Global economic impact of diabetes mellitus

Increase in diabetes prevalence is not only a health burden but also an economic burden. Since type 2 diabetes affect people of the working class who are between

the ages of 20-49, it impacts negatively on production and economic growth of countries (Hall *et al*., 2011). Most diabetic patients loose earnings due to lost in work days, restricted activity and lower productivity at work. As a result, more people are pushed into poverty.

Treatment of this disease can be very costly such that it affects the national health budget negatively. In 2010, $376 billion was used for global health expenditures: a figure that is expected to rise to $490 billion in 2030 as reported by Zhang *et al*., (2010). Some people cannot afford or even access the treatment and as a consequence, they become disabled and develop macrovascular complications associated with the disease such, as:

i. Diabetic angiopathy- cardiovascular diseases such as stroke, abnormal heart conditions, and atherosclerosis that might cause death in diabetic people
ii. Diabetic nephropathy- kidney failures which might result in kidney transplant
iii. Diabetic retinopathy- eye disease which causes visual impairment and sometimes blindness
iv. Diabetic neuropathy- pain and numbness of limbs which is caused by damaged nerves
v. Diabetic ketoacidosis- when the body uses fat for energy due to lack of insulin which is responsible to transport of glucose to the cells

1.2.10. Pharmacological treatment and traditional medicines

1.2.10.1. Pharmacological agents

There are different oral agents used to treat diabetes, particularly type 2. These drugs were developed looking at the mechanism and pathophysiology of diabetes. Prescription of the treatment depends on age, predictable years of survival, other illness that the patient might have and patient's compliance in taking the medication (Lebovitz, 1999). Oral agents are usually given to newly diagnosed patients and are combined, if the disease is in an advanced stage. Different drugs are developed to target different sites and organs responsible for diabetes as detailed in figure6.

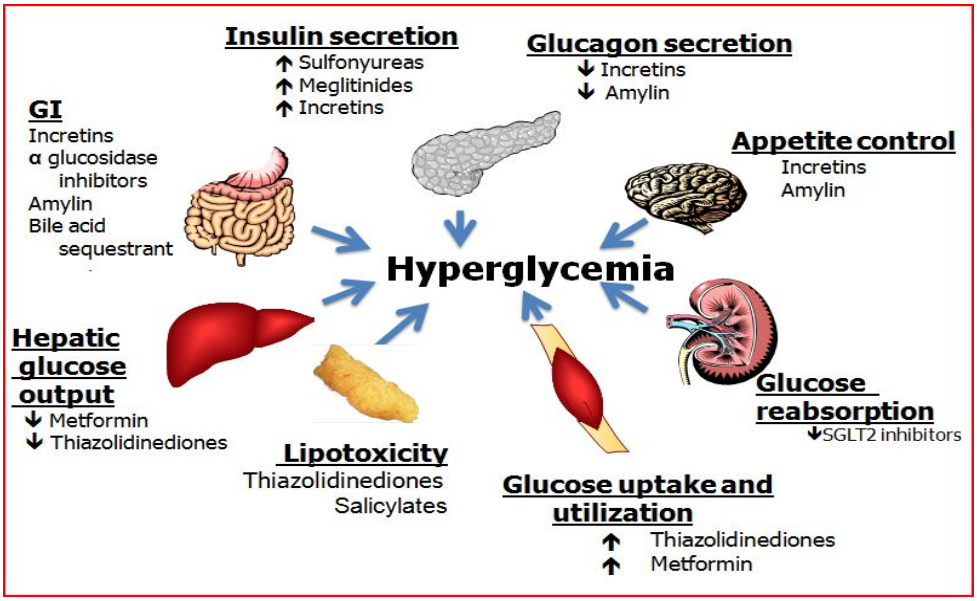


Figure 6: Sites of action of the current pharmacological therapy for treatment of type 2 diabetes (Evans and Rushakoff, 2010)

1.2.10.1.1. Sulfonylureas

Sulfonylureas are derived from sulfonic acid and urea, hence the name. They mainly work by inhibiting K_{ATP} channel in plasma membrane thereby increasing the release of insulin from pancreatic β-cell. They are divided into first and second generation agents. The first generation agents include acetohexomide, chloropropamide, tolazamide and tolbumide. This group have a lower binding affinity for ATP sensitive potassium channel. The first generation act slowly and as such, required in higher doses to be effective. Oral agents like glibenclamide, glipzide and glimepiride are regarded as second generation agents. Although these drugs are used by patients who are unable to respond optimally to glucose stimulus, they can also cause hypoglycaemia due to their ability to increase the release of insulin (Modi, 2007).

1.2.10.1.2. Biguanides

Metformin is one of the biguanides drugs effectively used to treat type2 diabetes. This drug is known to alter insulin action. It inhibits the liver to produce glucose and stimulate transport of glucose into the muscles. Although the exact mechanism of action of this drug is not yet known, numerous studies show that it increases insulin sensitivity (Fowler, 2007). It is also reported to activate the AMP-activate protein

kinase (AMPK) which regulates lipid and glucose metabolism (Kilo *et al*., 1980). It is very useful to people with obesity since it does not promote weight gain.

1.2.10.1.3. Thiazolidinediones (TZDs)

Thiazolidinediones are drugs that decrease insulin resistance. They enhance the body's sensitivity to both endogenous and exogenous insulin. Proglitazone and rosiglitazone are types of TZDs that influence the binding of the perixome proliferator receptor$_y$ (PPAY$_y$) (Willson *et al*., 2001). These receptors regulate the transcription of insulin responsive genes to control the production and function of glucose. When administered they reduce the fasting plasma glucose insulin and free fatty acids. Thiazolidinediones have a negative impact on patients whereby they increase subcutaneous deposition of fat and decrease visceral fat resulting in weight gain. Despite these shortcomings they are reported to be more effective than other antidiabetic medication (Fowler, 2007).

1.2.10.1.4. Alpha glucosidase inhibitors

According to Mondi (2007), alpha glucosidase inhibitors have been in the market since 1996. They act by inhibiting the activity of α-glucosidase enzyme. They block and delay the breakdown and absorption rate of carbohydrates in the small intestines thereby reducing postprandial hyperglycemia. Postprandial hyperglycemia is determined by factors such as timing quality and composition of meals as well as insulin secretion. Proper control of postprandial glucose levels is obtained when combined with dietary regulation and exercise (Ceriello, 2006). Drugs like acarbose and miglitol are used for reduction of postprandial hyperglycemia. These drugs are also associated with negative side effects such as bloating, diarrhoea and abdominal discomfort (Fujisawa *et al*., 2005).

1.2.10.1.5. Insulin

Insulin is a hormone responsible for maintaining normal blood sugar level in the body. Insulin therapy is usually used by type 1 diabetic patients who cannot produce insulin. It can only be recommended to type 2 diabetic patients during severe hyperglycemia and when oral agents have failed. The exogenous insulin is designed such that it has a similar structure and mode of action as the normal one secreted by

the pancreas (Novak and Meltelko, 2003). Different analogues of insulin have been in trial, however only four insulin analogues are in use (Mahler and Adler, 1999).

1.2.10.1.6. Antioxidants

Since oxidative stress contributes to the pathophysiology of diabetes, antioxidants play a major role in diabetes. Antioxidants are free radical scavenging molecules that inhibits oxidation of other molecules. There are two types of antioxidants namely enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants includes catalase, glutathione reductase and glutathione peroxidase while vitamins fall under non enzymatic antioxidants. When antioxidants are combined with antidiabetic drugs they reduce oxidative stress and also delay the development of diabetic complications (Ruhe and MacDonald, 2001). Antioxidants are also capable of reducing insulin resistance and protecting the vascular endothelium (Paolisso *et al.*, 1999). Consumption of fruits, vegetables and supplementation with antioxidant reduces the risk of type 2 diabetes (Hanhineva *et al*., 2010).

1.2.11. Medicinal plants as treatment

1.2.11.1. Antidiabetic plants

Plants have been used as a source of treatment for many diseases including diabetes. In most poor countries, plants are the only source of treatment available for treating diabetes (Garau *et al*., 2003). There is a large number of medicinal plants that are used to treat diabetes worldwide. The ethno-botanical information reports that there are more than 800 plant species that possess antidiabetic potential (Alarcon *et al*., 1998). These are very large and widely distributed families and species. Some of the few plant species that show antidiabetic potential from previous studies are *Panax species, Phyllanthus, Acacia Arabica, Aloe vera*, *Aloe barbandensis*, *Mormodica charantiia, Alium cepa* (Marles and Farnsworth, 1995; Perez, *et al*., 1998). A number of investigators have shown that these plants with antidiabetic activity are highly concentrated with complex polysaccharides and active constituents that are able to improve diabetic conditions. These plants use different mechanism in order to improve diabetic conditions. Some are able to regenerate the beta cells, others induce the release of insulin and fight insulin resistance, inhibit carbohydrate digesting enzymes, whereas other medicinal plants enhance glucose

uptake by adipose and muscle tissues etc. Most of the active constituents with antidiabetic activity are secondary metabolites of the plants (Marles and Farnsworth, 1995; Ross, 2001).

1.2.11.2. Secondary metabolites

Secondary metabolites are compounds synthesized by the plant for specific functions. They are synthesized using enzyme-mediated chemical reactions called metabolic pathways. These metabolites are used by the plant as a form of defence against pathogens and herbivores (Luckner, 1990). Moreover, they also contribute to the plant's colour, flavour and smell for pollination. According to Gurib-Fakim (2006), secondary metabolites of plants are expressed differently depending on the ecology, taxonomy, biochemical differentiation and diversity. Their major difference is observed in their molecular structure and function. According to Wagner and Farnsworth (1994), the activity of the plant is proportional to the percentage of the active constituents and mechanism of action. Plant's secondary metabolites have chemical and pharmaceutical properties that can be used for human health and in synthesis of new drugs. Furthermore some are used as dietary supplements to cure and prevent other diseases (Raskin *et al*., 2002). Different groups of secondary metabolites include glycosides, alkaloids, phenolics, tannins and terpenoids.

Phenolics are aromatic compounds characterised by hydroxyl (OH) group that are directly attached to an aromatic ring. They are widely distributed in plants responsible for different colour development, pollination and protection against UV radiation (Heinrich *et al*., 2004; Bruneton, 1999). It has been proved by many scientific studies that phenolic compounds have antimicrobial activity since they inhibit the development and growth of pathogens (Okwu and Okwu, 2004). Phenolic compounds are mainly found in food like fruits, vegetables, cereals, olives, chocolate and beverages like coffee, tea and wine. They contribute to the colour and astringency of some foods. Among other pharmacological activities of phenolics are their ability to inactivate and inhibit the expression of mutagens and carcinogens. The two common types of phenolics are the non flavonoids and flavonoids phenolic compounds. Non flavonoid phenolics include simple phenols like catechol, euginol and benzoic acid like vannilic acid, gallic acid etc (Jadhav *et al*., 2004).

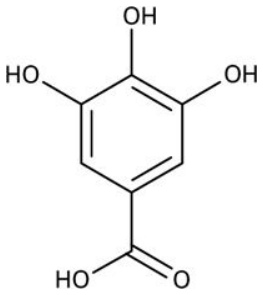


Figure 7: Basic structure of Gallic acid (http://mtc.aarrcjournal.org)

Flavonoids are a large class of polyphenols synthesized by vascular plants. They consist of two moieties which are benzopyran (A and C) and phenyl (B ring) as seen in figure 8. Different subclasses of flavonoids include flavonols, flavones, flavanones, flavanols, isoflavone and anthocyanins. These subclasses of flavonoids vary in the C ring and linkage between benzopyran and phenyl group (Tadera *et al*., 2005). Flavonoids are low in molecular weight and they usually present themselves in the form of pigments in plants. They are considered to be of good nutritional value because they play an important role in human health. Some of their pharmacological properties include free radical scavenging activity, strong antioxidant activity, anti-inflammatory action, inhibition of hydrolyte and oxidative enzymes (Zhishen *et al*., 1999). They are able to reduce blood lipid and glucose, heart diseases and also enhance human immunity. Some flavonoids are reported to be able to inhibit α-glucosidase and aldose reductase thereby reducing postprandial blood glucose level (Lee and Kim, 2001; Harguchi *et al.*, 1996).

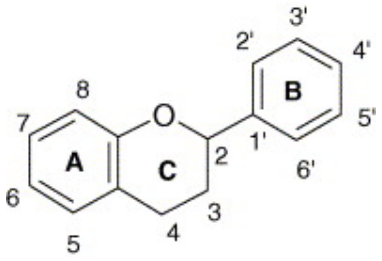


Figure 8: Basic structure of flavonoids (www.enzoprofessional.com)

Tannins are non-flavonoid secondary metabolites that are soluble in polar solutions. They are divided into two groups namely, hydrolyzed (proanthocyanidins) and condensed tannins (procyanidins) that differ structurally (Okigbo *et al*., 2009). Most plants use tannins to protect themselves against pathogenic microorganisms since they possess antimicromicrobial and antifungal properties (Provenza, 1995).

Procyanidins are mostly abundant in human diet. They contribute to both sensational and nutritional quality of food. Numerous studies conducted shows that tannins are un-nutritional in animals since they form complexes with proteins which have negative impact on appetite and nutrient utilization. They also inhibit digestive enzymes like lipases, proteases and glucosidases (MacDougall *et al*., 2005). When procyanidins interact with glucosidase enzyme, they inhibit the activity of the enzyme thus delaying the absorption of glucose. Some procyanidins are able to inhibit α-amylase enzyme activity (Gonclaves *et al*., 2001) and as such be regarded as having good antidiabetic potential. Condensed and hydrolysable tannins have a large number of free phenolic groups that form strong hydrogen bonds with other molecules as seen below (fig 9).

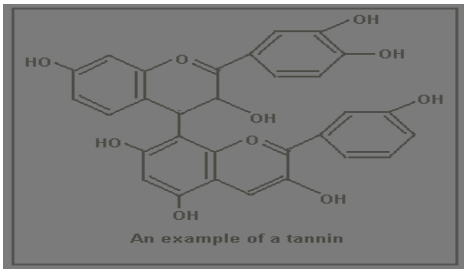


Figure 9: Basic structure of tannins. (http://www.theworldwidewine.com)

The table below (table 1), shows the various mechanisms of action of different secondary metabolites on reducing diabetic effects.

Table1: Mechanism of action of some secondary metabolites with antidiabetic activity

Secondary metabolites(names)	Mechanism of action	References
Catechins	-Inhibition of α-amylase and α-glucosidase enzymes, -Protection of pancreas and increase insulin secretion	Ishikawa *et al*., 2007 and Chakravarthy *et al*., 1982
Flavonoids	-Inhibition of α-amylase and α-glucosidase enzymes, -Increase insulin secretion, Improve glucose uptake by muscle and adipose tissues -Induce hepatic glucokinase activity	Jung *et al*., 2006; Coskun *et al*., 2005 and Johnston *et al*., 2005;
Phenolic acids	- Inhibition of α-amylase and α-glucosidase enzymes -Protect the pancreas ,Increase insulin secretion, -Improve glucose uptake by muscle and adipose tissues - Induce hepatic glucokinase activity	Jung *et al*., 2006; Coskun *et al*., 2005 and Welsch *et al*., 1989;
Anthocyanins	-Inhibition of α-amylase and α-glucosidase enzymes	Iwai *et al*., 2006
Proanthocyanidins	- Inhibition of α-amylase and α-glucosidase enzymes	Lee *et al*., 2007

1.2.10.3. Safety of medicinal plants

Medicinal plants are generally assumed to be safe because they are natural derivatives. Their safety is based on the knowledge accumulated since years immemorial, however recent studies have shown that medicinal plants that were considered to be safe are associated with negative side effects and toxicity. Some plants were found to be poisonous, mutagenic and carcinogenic (Schimmer *et al*., 1988). Poisoning from traditional medicinal plants arises from misidentification, incorrect preparation, inappropriate administration and dosage due to self-administration (Stewart and Steenkamp, 2002). Environmental conditions such as contaminated soil by microorganisms and heavy metals, inappropriate collection methods, processing and storage also contribute to the toxicity of medicinal plants (Tadmor *et al*., 2002). Medicinal plant might be toxic to any organ, but the most commonly affected are liver, skin and gastrointestinal tract. Though some medicinal plants are used for treating a particular disease, they end up causing another disease. For example a commonly Chinese medicinal herb ginseng, used for treatment of various diseases was found to cause hypertension and mastalgia (Haq, 2004).

Even though there is a tremendous growth in the use of medicinal plants globally, information on their safety is still very limited. Most plants that are used as medicines today have not been evaluated for toxicity and they have the potential to cause serious toxic effects. As such toxicological studies on these plants are important. Toxicological studies are an important part of the pre-clinical safety evaluation of potential medicinal plants. They are used to determine if the plant is safe for human consumption. Plants with medicinal properties can be evaluated for toxicity by firstly treating animal models under controlled conditions and observing the effects. *In vitro* studies can also be performed where cells, sub-cellular fractions and single celled organisms are treated with the plant extract to test its cytotoxicity effects. There are different normal cell lines such as macrophages that can be used to test cytotoxicity effects of plant extracts (Timbrell, 2000).

1.2.11. Plants used in the study

Ten medicinal plants were selected on the bases of their traditional use as represented in table 2 were investigated in this study. Many studies show that these plants possess different secondary metabolites and also have various pharmacological activities such as antimicrobial, antioxidant, antimalarial, antiproliferative, anticancer activities etc. Different parts of selected medicinal plants are used differently by different people to treat various diseases as detailed in table 2.

Table 2: Names and traditional uses of the plants under investigation

Plant names	Family	Traditional use of the plant	Plant parts	References
Albizia gummifera (J.F. Emel)	Euphorbiaceae	Gonorrhoea, fever, skin disease, malaria	Roots, bark and leaves	Kokwaro, 1976
Antidesma venosum (E. Mey. ex Tul.)	Mimosaceae	Gynaecological complaints, dysentery, malaria, dysmenorrehoea	Root, bark and leaves.	Hutchings *et al*., 1996; Chhabra *et al*., 1993
Barringtoina racemosa (L. Spreng)	Lecythidaceae	Ulcer, cancer, piles, fever, thyphoid	Seeds, leaves, and bark.	Hussin *et al*.,2009; Deraniyagala *et al*., 2003
Cassia abbreviata (H. Brenan)	Fabaceae	Malaria, pneumonia, chest complaints, toothache	Leaves, roots, bark and stem	Dharani and Yenesew, 2010; Kokwaro, 2009
Helinus integrifolius [(Lam) Kuntze)]	Rhamnaceae	Arthritis, paralysis, infertility, backache	Roots, bark and leaves	Hillton-Tayor, 1996
Kigelia africana [(Lam) Benth]	Bignoniaceae	Dysentery, verenal dieases,wounds, abscess	Roots, bark and leaves	Akunyili and Houghto, 1993
Maytenus undata (Thunb)	Celastraceae	Syphillis, TB, stomach problems	Roots	Lall and Meyer; 1995; Ghazanfar, 1994
Milletia stuhlmanni (Taub)	Fabaceae	Stomachache	Bark	Banzouzi, 2008
Senna italica (Mill)	Fabaceae	Laxative, STDs, constipation,	Leaves	Tshikalange *et al.*, 2005
Warbugia salutaris (Bertol. F)	Canellaceae	Dry cough, chest infections, sinusitis, malaria	Bark, stems and leaves	Mohanlall and Odhav, 2009; Jansen and de Groot, 1991

1.3. Purpose of the study

1.3.1. Aim

The study was aimed at the scientific evaluation of the *in vitro* antidiabetic and cytotoxicity effects of selected medicinal plants.

1.3.2. Objectives of the study

a. To extract crude plant material selected for the study using solvents of varying polarity
b. To test for the antioxidant activity of the different extracts of the plants
c. To determine the phenolic content of the different extracts of the plants
d. To determine the presence of flavonoids and tannins in the different extracts of the plants
e. To test for the cytotoxic effect of the different extracts of the plants on a macrophages cell line, Raw 264.7
f. To evaluate the ability of the different extracts of the plants in inhibiting α-glucosidase activity from rat intestines
g. To evaluate inhibitory activity of the plant extracts on α-amylase enzyme from hog pancreas
h. To evaluate the effects of plant extracts on glucose uptake by C2C12 muscle and H-4-II-E liver cells

Chapter 2

2. Materials and Methods

2.1a. Plants collection

Plants used in this study were collected from Ga-Mashishimale village in Phalaborwa, Limpopo province and Nelspruit botanical gardens, Mpumalanga province while others were provided by University of Limpopo, Department of Biochemistry, Microbiology and Biotechnology. The plants selected are *Albizia gummifera* (J.F. Emel)*, Antidesma venosum* (E. Mey. ex Tul.)*, Barringtonia racemosa* (L. Spreng)*, Cassia abbreviata* (H. Brenan)*, Helinus integrifolius* (Lam) Kuntze*, Kigelia africana* (Lam) Benth, *Maytenus undata* (Thunb)*, Milletia stuhlmanni* (Taub)*, Senna italica* (Mill) *and Warbugia salutaris* (Bertol. F). For bio-conservational reasons, only the leaves of the selected plants were used in the study.

2.1b. Plants storage

Following collection of plants, the leaves were separated from the stems and allowed to dry at room temperature. Dried leaves were ground into fine powder with a grinder, and stored in bottles in the dark until use.

2.2. Plant Extraction

Extraction is the separation of medicinally active portions of a plant**.** The quality of extracts depends on the quality of plant raw material, extraction procedure and the choice of solvents. In this study each dry plant material (2 g) was extracted with 20 mℓ of the solvents, namely: *n*-hexane, chloroform, ethyl acetate and acetone. The plant solvent mixture was tightly closed to avoid evaporation of solvents and left overnight at room temperature. The supernatant was filtered with a Whatman no.1 filter paper into pre-weighed glass vials. The solvent was removed under a stream of air. The quantity of plant material extracted by each solvent was determined. Prior to assaying, all extracts were dissolved in dimethylsulphoxide (DMSO) (Sigma-Aldrich) to yield a stock solution of 10 mg/mℓ.

2.3. Qualitative phytochemical analysis

2.3.1. Different tests

All plant extracts were qualitatively tested for the presence of bioactive compounds using the methods described by Yadav and Agarwala (2011) methods.

2.3.1a. Test for carbohydrates (Iodine test)

Each plant extract (2 mℓ) was mixed with 2 mℓ of iodine solution. A dark blue or purple colour indicated the presence of carbohydrate.

2.3.1b. Test for glycosides (Salkowski's test)

Briefly 100 µℓ of each plant extract was mixed with 2 mℓ of chloroform and 2 mℓ of concentrated H_2SO_4 and gently shaken. A reddish brown colour indicates the presence of steroidal ring which represents the glycone portion of the glycoside (Borokini and Omotayo, 2012).

2.3.1c. Test for steroids

About 2 mℓ of chloroform was added to 1 mℓ of each plant extract followed by adding 1 mℓ of concentrated sulphuric acid (H_2SO_4). The presence of a red colour is indicative of the presence of steroids (Borokini and Omotayo, 2012).

2.3.1d. Test for phenols

Each plant extracts (2 mℓ) was mixed with 2 mℓ of 2% solution of $FeCℓ_3$. The presence of blue /black colouration indicates the presence of phenols.

2.3.1e. Test for flavonoids (Shinoda test)

About 2 mℓ of each crude extract was mixed with 2 mℓ of 2% solution of NaOH. The presence of flavonoids is indicative when the yellow colour formed by the mixture turns colourless following the addition of 5-8 drops of diluted HCℓ.

2.3.2 Thin Layer Chromatography (TLC) fingerprinting

The method was performed according to (Kotzé and Eloff, 2000). Plant extracts dissolved in acetone were analysed on the TLC plates. About 10 µℓ of each plant extract was loaded on 10x10 cm TLC plates (Merck, Silica gel 60 F_{254}). The plates

were further developed in three different mobile systems viz: Toulene: Ethanol: Ammonia hydroxide (90:10:1), [TEA] (non-polar/basic), Chloroform: Ethyl acetate: Formic acid (5:4:1), [CEF] (intermediate polarity/acidic) and Ethyl acetate: Methanol: Water (40:5:4), [EMW] (Polar/neutral). The separated compounds were sprayed with vanillin-sulphuric acid and heated at 110^{o}C for optimal colour development (Stahl, 1969).

2.4. Quantitative phytochemical analysis

Phytochemicals are chemical constituents synthesised by plants as secondary metabolites. These are compounds that contribute to the medicinal value of a plant and are responsible for the pharmacological activity of the plant. They can be determined either quantitatively or qualitatively. Only total phenolic content, total flavonoids and total condensed tannins of all plant extracts were determined quantitatively in this study.

2.4.1. Total phenolic content

The total phenolic content of all plant extracts were determined using the method of Singleton *et al*. (1999). Twenty five (25 µℓ) of each plant extract was firstly oxidised with 250 µℓ of Folin-Ciacalteau's reagent (Sigma-Aldrich) in test tubes. The mixture was incubated at room temperature for five minutes in the dark. To stop the reaction, 750 µℓ of 20% sodium carbonate was added to the mixture. About 4 mℓ of distilled water was added into the tubes to make up a total volume of 5 mℓ and the mixture was incubated in the dark for 2 hours at room temperature. The mixture was transferred into cuvettes and absorbance was read at 760 nm using T60 UV-Visible spectrophotometer. The total phenolic content of each plant extracts were determined by extrapolation from a gallic acid calibration curve (0-500 µg/mℓ) and the results were expressed as gallic acid equivalent/ mg (GAE/mg).

2.4.2. Total flavonoids content

The total flavonoids in all plant extracts were determined using aluminium chloride colimetric assay adapted from Yadaw and Agarwala *et al*., (2011). Briefly 100 µℓ of each plant extract was mixed with 300 µℓ of methanol in test tubes. Ten percent of aluminium chloride (20 µℓ) was added to the mixture followed by 20 µℓ of 1 M potassium acetate. A total volume of up to 1 mℓ was made after adding 560 µℓ of

distilled water. The reaction was incubated for 30 min at room temperature and the absorbance was read at 420 nm with Anthos 2010 micro plate reader spectrophotometer. Total flavonoids were determined by extrapolation from quercitin (0-10 mM) and the results were expressed as QE/mg.

2.4.3. Total condensed tannins

Total condensed tannins in each of the plant extracts was determined by mixing 30 µℓ of plant extract with 540 µℓ of 4% vanillin-methanol according to the method of Sun *et al* (1998). Concentrated sulphuric acid (43 µℓ) was added to the mixture and incubated for 15 min at room temperature. Absorbance was read at 500 nm using T60 UV-Visible spectrophotometer. Standard curve was calibrated using catechin (0-10 mM) as a standard reference and the results were expressed as CE/mg.

2.5. Antioxidant activity (Quantitative DPPH assay)

The radical scavenging ability of all plant extracts was tested qualitatively using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma) method according to Katsube *et al* (2004). All the DMSO dissolved extracts (10 mg/mℓ) were serially diluted in a 96 well microtiter plate with 100 µℓ of methanol. The total volume of the dilutions was 100 µl. An equal amount of 1 mg /mℓ of vitamin C (ascorbic acid) was used as positive control. The wells without plant extracts and vitamin C were regarded as negative controls. To each well 100 µℓ of 0.025% DPPH-methanol was added, and incubated for 5 min at room temperature. Absorbance was determined at 595 nm using T60 UV-Visible spectrophotometer. Antioxidant activity was calculated as follows:

%Antioxidant activity (amount of DPPH reduced) = 100% - amount of DPPH oxidised

$$\text{DPPH oxidised} = \frac{(\text{Control } A_{595} - \text{Experiment } A_{595}) \times 100}{\text{Control } A_{595}}$$

2.6. Enzyme assays

2.6.1a. Alpha amylase inhibition assay (CNPG3 method)

Alpha amylase inhibition assay was performed using two chromogenic methods whereby inhibition was determined by the reaction colour change. The plant extracts

were tested for their potential to inhibit alpha amylase enzyme at various concentrations. The extracts were serially diluted with 0.5 M phosphate buffer (pH 6.8). About 100 µℓ of 0.5 M phosphate buffer (pH 6.8) was dispensed in all the wells of 96 well microtiter plates. A 100 µℓ of the different extracts dissolved in DMSO (10 mg/mℓ) were added to the first wells of microtiter plates and two-fold serially diluted. One hundred microliters of the amylase mono reagent (KAT) was added to all wells, except for the control wells followed by 20 µℓ of the alpha amylase enzyme (1 mg/mℓ) (Sigma). After 5 min incubation at 37^{o}C, absorbance was read at 405 nm using Anthos 2010 micro plate reader spectrophotometer. Negative control wells contained only plant extract, buffer and reagent whereas blank contained buffer and reagent only. Acarbose (1 mg/mℓ) (Sigma) was used as a positive control. The amount of glucose released during the reaction was determined by the intensity of yellow colour developed after incubation. This method was performed according to Zhang *et al* (2011) with slight modification. The activity of the enzyme inhibited was calculated using the following equation: %Enzyme inhibition= (100% - activity of test as percentage of control)

$$\%\text{Activity of test} = \frac{(\text{Control } A_{405} - \text{Experiment } A_{405}) \times 100}{\text{Control } A_{405}}$$

2.6.1b. Alpha amylase inhibition assay (DNS method)

Extracts dissolved in DMSO were tested for α-amylase enzyme inhibitory capability using a Dinitrosalicylic acid (DNS) method as described by Shai *et al* (2010). The plant extracts were serially diluted with 0.5 M phosphate buffer (6.8) to make up three different concentrations of 2.50, 1.25 and 0.625 mg/mℓ in test tubes. A 100 µℓ of 0.25% soluble starch was added to the mixture followed by 40 µℓ of α-amylase enzyme from (Sigma) to start the reaction. The mixture was incubated for 5 min at 37^{o}C. After incubation, 400 µℓ of dinitrosalysilic acid (DNS) (Sigma) was added to stop the reaction and boiled for 10 min for optimum colour development. Four hundred microliters of water was added to the mixture and the absorbance determined at 540 nm. The reaction mixtures without enzyme were used as blanks. Acarbose (Sigma) was used as positive control and the reaction mixtures with starch

only served as the negative controls. The percentage enzyme inhibited was calculated as in section 2.6.1a.

2.6.2. Alpha glucosidase inhibition assay

2.6.2a. Sucrase inhibition

Plant extracts were tested for enzyme inhibitory capability using α-glucosidase (Sigma). The mammalian intestinal glucosidase, form a complex of three individual enzymes, namely sucrase, maltase and isomaltase. In this study only the sucrase and maltase were tested for their activity in the presence of plant extracts according to Zhang *et al*., (2011). For sucrase inhibition, alpha glucosidase enzyme was prepared by dissolving 0.3 g of rat intestinal powder in 0.5 M sodium phosphate buffer (pH 6.9) and vortexed. The mixture was centrifuged at 200 rpm for 10 minutes and the supernatant was used as glucosidase enzyme. The extracts were two-fold serially diluted with 0.5 M sodium phosphate buffer (pH 6.9) in a 96 well plate. Hundred micro-litres of 2% sucrose was added to each well followed by 20 µℓ of α-glucosidase enzyme to start the reaction. The mixture was incubated for 1 hour at 37^{o}C. After incubation the reaction was stopped by adding 100 µℓ of glucose reagent (KAT) and incubated further for 5 minutes. Absorbance was read at 550 nm (Anthos 2010 micro plate reader spectrophotometer). Reaction mixtures without sucrose, enzyme and glucose reagent were used as blanks. Negative controls contained plant extracts and glucose reagent only whereas acarbose (1 mg/mℓ) was used as a positive control. The activity of the enzyme was determined by measuring the quinomine colour produced during the reaction. The percentage inhibition was calculated as follows:

% Enzyme inhibition= (100% - activity of test as percentage of control)

$$\% \text{ Activity of test} = \frac{(\text{Control } A_{500} - \text{Experiment } A_{500}) \times 100}{\text{Control } A_{550}}$$

2.6.2b. For the maltase inhibition, the method was repeated as in section 2.6.2a but using maltose as substrate instead of sucrose.

2.6.3. Enzyme kinetics

2.6.3a. Alpha amylase enzyme kinetics

Enzyme kinetics was performed to determine the type of inhibition exerted by the plant extracts on both α-amylase from hog pancreas and α-glucosidase from mammalian rat intestine enzymes as described by Zhang *et al*. (2011). Only plant extracts that inhibited more than 50% of the activity of both were tested. With the α-amylase enzyme kinetics, 200 µℓ of 3 mM amylase mono reagent (substrate) was serially diluted (0.02 - 3 Mm) with 0.5 M phosphate buffer (pH 6.8). About 20 µℓ of each plant extract was dispensed to all wells except for the control wells. The reaction was started by adding 20 µℓ of the enzyme and incubated for 20 minutes at 37^{o}C. The amount of glucose released was determined by taking absorbance at 405 nm using microtiter reader (Anthos 2010 micro plate reader spectrophotometer). A double reciprocal plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted. The type of inhibition was determined by plotting the results on a Lineweaver-Burk plot to calculate the V_{max} and km values.

2.6.3b. Alpha glucosidase enzyme kinetics

For α-glucosidase enzyme kinetics, the method was repeated as above (section 2.1.3a) except that the concentration of substrate (sucrose) varied (0.01 - 2%). The substrate was serially diluted with 0.5 M phosphate buffer (pH 6.9). Twenty microliters of each plant extracts was added to all experimental wells followed by 20 µℓ of α-glucosidase enzyme to start the reaction. Reaction mixture was incubated at 37^{o}C for 20 minutes and stopped by adding 100 µℓ of glucose reagent. The amount of glucose released was determined spectrophotometrically using a sucrose standard curve. A double reciprocal plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted. The type of inhibition was determined by plotting the results on a Lineweaver-Burk plot which showed the V_{max} and km values.

2.7. Glucose uptake assay on muscle and liver cells

2.7.1. Cell culture maintenance

The C2C12 muscle and H-4-II-E liver cell lines were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. The cells were incubated at 37^{o}C in a humid atmosphere of 5% CO_2. C2C12 muscle cells were maintained at a logarithmic phase in Minimum Essential Medium (MEM) medium (Sigma). The medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Auckland, New Zealand) and 1 mℓ of gentamycin. The H-4-II-E liver cells were maintained in Dulbecco's Minimum-Eagle Medium (DMEM) (Gibco, Auckland, New Zealand) supplemented with 1 mℓ of gentamycin and 10% FBS. The muscle cells were sub-cultured every 4 days while the liver cells were sub-cultured every 2 days to maintain a logarithmic growth.

2.7.2. Glucose uptake assay

Glucose uptake assay was performed to evaluate the effect of plant extracts on glucose uptake by cells. Only ethyl acetate and acetone extracts of *Cassia abbreviata* and *Helinus integrifolius* were assessed for their effect on glucose uptake by the muscle and liver cells. These are the plant extracts that consistently inhibited 50% of both α-amylase and α-glucosidase enzymes. The method was performed according to Deutschländer *et al* (2009). Plant extracts were tested at various concentrations ranging from 0.065 – 1.0 mg/mℓ. Cells (C2C12 and H-4-II-E) were firstly dislodged from the flasks by tripsinization, counted and further seeded in 96–well microtiter plates. Each well contained 25x10^3 of C2C12 cells and 14, 5 x10^3 H-4-II-E cells per well. The medium was aspirated and the cells were treated with 100 µℓ of each plant extract at various concentrations for 24 hours including the positive control (insulin). After 24 hr incubation, 50 µℓ of treated cells were transferred into new plates. One hundred microliters of glucose oxidase kit reagent (Sigma) was added to the wells and incubation was continued for 30 minutes at 37^{o}C. The reaction was terminated by adding 50 µℓ of H_2SO_4. Glucose uptake by the cells was determined by measuring absorbance at 540 nm using Anthos 2010 micro plate reader spectrophotometer.

2.7.3. Cytotoxicity test using real-time xCelligence system

2.7.3a. Cell culture maintenance

The RAW 267.4 macrophage cells were maintained at a logarithmic growth phase in DMEM. The medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Auckland, New Zealand) and 1% antibiotic [penicillin, streptomycin and neomycin, (PSN)] mixture. Cells were incubated at $37^{o}C$, 5% $CO_{2,}$ and 95% humidity.

2.7.3b. Cytotoxicity test

Based on the enzyme assays, only acetone and ethyl acetate of *C. Abbreviata* and *H. Integrifolius* were tested for their cytotoxicity effects on macrophages Raw 264.7 cells. The cytotoxicity effects were tested using real-time xCelligence system (Roche). The method was performed according to the instructions of the manufacture. Briefly 100 µℓ of DMEM was added to the E-plate 96. About 300 sweeps were ran in an interval of 15 minutes. During the sweeps the electrode impedance was determined by the ion environment both at the electrode solution interface and the solution (results shown on the table below). Following the sweeps, about 2500 cells were added to each well and subsequently treated with various concentrations (0.05, 0.1 and 0.25 mg/mℓ) of each plant extract respectively. The system was allowed to run for 3 days and cytotoxicity effects were determined by measuring the cell index after the first 10 hours of treatment in an interval of 15 minutes. Control cells were treated with 100% DMSO, while Actinomycin D was used as positive control. Cell index was calculated as follows:

$$\text{Cell index} = \frac{\text{Control - Experiment}}{\text{Control}}$$

2.8. Isolation of bioactive compounds

2.8.1. Serial fractionation

The finely grounded powder leaves of *Helinus integrifolius* weighing 433 g was exhaustively extracted with five different solvents namely; hexane, dichloromethane,

acetone, ethyl acetate and methanol respectively. The plant material was extracted with 4 litres of each solvent for 24 hours. The extracts were filtered into pre-weighed 250 mℓ beakers, dried and weighed.

2.8.2. Column chromatography

About 10 g of dichloromethane (DCM) extracts was re-dissolved in 100 mℓ of DCM together with 30 g of silica gel (Merck). The solvent was evaporated with Stuart rotary evaporator (Lasec) rotating at 150 rpm under reduced pressure and water bath temperature of 52^{o}C. Silica gel (400 g) was mixed with 1000 mℓ of DCM to make slurry and packed in a glass column. Twenty five grams (25 g) of the DCM sample was thinly loaded on top of the silica gel and covered with cotton wool. The column was eluted with 100 % DCM and subsequently, the polarity of eluting solvent was increased with 1000 mℓ of 10 % ethyl acetate per fraction. The fractions were collected in 250 mℓ beakers and further concentrated using a rotary evaporator at 52^{o}C. Ten microliters of each collected fraction was loaded on 10x10 cm TLC plates (Merck). The plates were developed under saturated conditions in hexane: ethyl acetate (9:1) systems to separate compounds in each fraction. Each plate was sprayed with vanillin-sulphuric acid reagent (0.1 g vanillin (Sigma): 28 g methanol: 1 mℓ sulphuric acid) to visualize UV active compounds. Thereafter, the chromatograms were heated for 5 min in a 110^{o}C oven for optimal colour development (Stahl, 1969). The fractions with the same TLC profiles were pooled together and transferred into pre-weighed vials and allowed to dry. A total of 13 sub-fractions were collected. Each set of fractions was re-dissolved in DMSO to make a concentration of 10 mg/mℓ and tested for alpha amylase inhibition capability as described in section 2.6.1a. The fraction with the high inhibitory capability was further subjected to small column chromatography.

2.8.3. Isolation of compound from fraction C

Silica gel 60 (50 g) was suspended in 150 mℓ of hexane and subsequently packed in a glass column. Fraction C (0.5 g) eluted with 100% DCM was mixed with hexane and 5 g of silica gel 60. The mixture was dried and finely spread on top of the gel and covered with cotton wool. The column was eluted using 1 ℓ of 100% hexane followed by polarity increments with 1 ℓ of 10% ethyl acetate, 1 ℓ of 20% ethyl acetate respectively. Fractions were collected in 20 mℓ test tubes.

2.8.4. Characterisation of compounds using nuclear magnetic resonance (NMR)

Nuclear magnetic resonance is a technique used to determine the chemical structure of compounds in detail. The samples were run with a Varian 400 MH_t spectrophotometer and benzene was used as a solvent reference. Carbon, proton and 2D spectra were generated.

Chapter 3

Qualitative and quantitative phytochemical analysis of ten medicinal plants

3.1. Introduction

Plants play a major role in treating different diseases since time immemorial. Their medicinal activity and value lie within the bioactive chemicals called phytochemicals. These are secondary metabolites synthesized by plants as a form of defense mechanism and are capable of stimulating a distinct physiological action in the human body (Manjamaki *et al*., 2010). Secondary metabolites such as tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids can be derived from bark, leaves, flowers, roots, fruits and seeds. Many of these metabolites cannot be synthesized commercially but can only be obtained from wild or culture plants (Rates, 2001). The presence of secondary metabolites has driven a growing interest in the use of natural products from plants as an alternative source of therapy and also development of new drugs (Gurib-Fakim, 2006). Although a plant might contain hundreds and thousands of compounds, only a few of them are responsible for the medicinal activity of the plants. According to Edeoga *et al*., (2005) flavonoids, tannins and phenolic compounds are the most important bioactive constituents.

Bioactive phytochemicals differ from plant to plant and from part to part. They vary in composition and concentration depending on the ecological changes (Moure *et al*., 2001). The problem with evaluating pharmacological activity of plants is that extracts from a single plant are mixture of several compounds (Farnsworth, 1993). In order to evaluate a plant for its biological activity, the compounds must first be extracted. There are a number of extraction techniques that can be employed such as soxhlet extraction, maceration, percolation, digestion and steam distillation (Tiwari *et al*., 2011).

The quality of extracts depends on the quality of plant raw material, surface area of material to be extracted and extraction method used. The finely powdered material increases the surface area and allows the extracting solvents to penetrate into the plant cell components thereby increasing the yield of biological compounds present in them. Extraction also depends on the choice of solvents with varying polarities. Polar biological compounds readily dissolve in polar solvents whilst non-polar

dissolves in non-polar solvents (Tiwari *et al*., 2011). In this study, four solvents were used namely: *n*-hexane, chloroform, acetone and ethyl acetate. The presence of chemical constituents present in each plant extracts were determined quantitatively and qualitatively using thin layer chromatography and different tests.

3.2. Objectives

a. To extract different compounds from plants using four different solvents
b. To determine the total phenolic content of the plant extracts
c. To determine the presence of other secondary metabolites qualitatively
d. To determine the total flavonoids present in the plant extracts
e. To determine the total condensed tannins present in the plant extracts
f. To determine the phytochemical profile of plant extracts using the TLC method

3.3. Materials and methods

3.3.1. Plants Extraction

Each dry plant material (2 g) was extracted with 20 mℓ of four different solvents namely: hexane, chloroform, ethyl acetate and acetone (SMM Instruments). The mixture was tightly covered to avoid evaporation of solvents and left overnight at room temperature. The supernatant was filtered with a Whatman no.1 filter paper into pre-weighed glass vials. The solvent was removed under a stream of air and the remainings were quantified.

3.3.2. Qualitative phytochemical analysis

3.3.2.1. Different Tests

All plant extracts were qualitatively tested for the presence of phytochemical constituent described by Borokini and Omotayo (2012) and Yadav and Agarwala (2011) methods.

3.3.2.2. Thin Layer Chromatography

The method was performed according to (Kotzé and Eloff, 2000) as elaborated in section 2.3.2. The phytochemicals present in acetone dissolved plant extracts were analysed on the TLC plates.

3.3.3. Quantitative phytochemical analysis

Phytochemicals are chemical constituents synthesized by plants as secondary metabolites. Only total phenolic content, total flavonoids and total condensed tannins of all plant extracts were determined quantitatively in this study.

3.3.3.1. Total phenolic content

The total phenolic content of all plant extracts were determined using the Singleton *et al*., (1999) method as explained in section 2.4.1.

3.3.3.2. Total flavonoids content

The total flavonoids in all plant extracts were determined using aluminium chloride colimetric assay adapted from Yadaw and Agarwala *et al*., (2011) as elaborated in section 2.4.2.

3.3.3.3. Total condensed tannins

Total condensed tannins in each of the plant extracts were determined by mixing 30 µℓ of plant extract with 540 µℓ of 4% vanillin-methanol according to the method of Sun *et al*., (1998). The method is explained in detail in section 2.4.3.

3.4. Results

3.4.1. Mass extracted from plant material

The amount of plant extracts obtained after extracting all plant materials with hexane, chloroform, acetone and ethyl acetate is presented in figure 3.1. Each plant material (2 g) was extracted with 20 mℓ of each solvent. Chloroform extracts had the highest mass of extracts than other solvents. Hexane extracted the least amount of extracts weighing about 0.01 g. Chloroform extract of *Warbugia salutaris* and ethyl acetate extract of *Milletia stuhlmanni* yielded the highest mass of 0.5 g. All *Cassia abbreviata* and *Senna italica* plant extracts weighed less than 0.1 g.

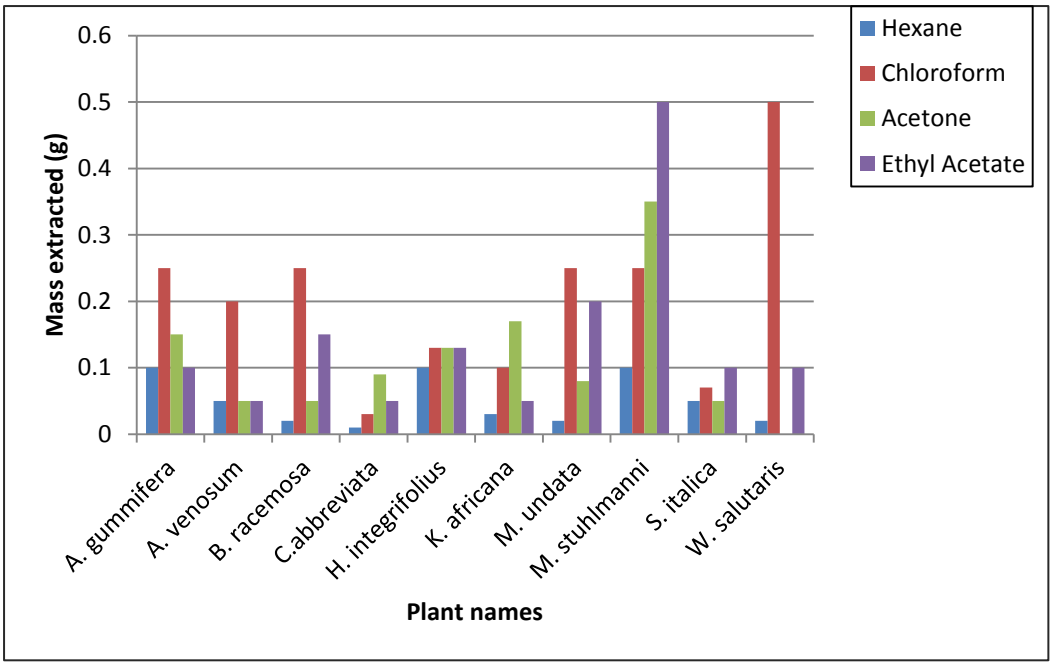


Figure 3.1: Total mass of extracts obtained from 2 g of each plant material extracted with 20 mℓ of four different solvents.

3.4.2. Phytochemical analysis

Phytochemicals present in all plant extracts were determined both quantitatively and qualitatively. Different standard curves were obtained using different compounds as standard reference for quantitative analysis. Total phenolics, flavonoids and condensed tannins were determined by extrapolating from the standard curves. All plant extracts had more phenolics and flavonoids than other phytochemicals tested.

3.4.2.1. Qualitative analysis

Qualitative analysis of the phytochemicals was performed in order to determine the presence of phenols, flavonoids, glycosides, steroids and starch in all plant extracts. All plant extracts contained phenols (indicated by +). Only hexane extracts of *K. africana*, *M. undata* and *M. stuhlmanni* as well as chloroform extracts of *K. africana* and *M. undata* tested negative for flavonoids. Only a few plants tested positive for glycosides and steroids particularly in the acetone and ethyl acetate extracts. All plant extracts tested negative for the presence of starch (table 3.1).

Table 3.1: Phytochemical analysis of each plant extract from ten medicinal plants studied.

Plant names	**Phenols**				**Flavonoids**				**Glycosides**				**Steroids**				**Starch**			
	H	**C**	**A**	**E**	**H**	**C**	**A**	**E**	**H**	**C**	**A**	**E**	**H**	**C**	**A**	**E**	**H**	**C**	**A**	**E**
A. gummifera	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-
A. venosum	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-
B. racemosa	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-
C. abbreviata	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-
H. integrifolius	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-
K. africana	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
M. undata	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
M. stuhlmanni	+	+	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-
S. italica	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
W. salutaris	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

(**+** = Present and **-** = Absent)

3.4.2.2. Quantitative analysis

3.4.2.2a. Total phenolic content

The total phenolics were determined from gallic acid standard curve, represented as GAE/mg (fig 3.2). Both acetone and ethyl acetate extracts had the highest amount of phenolics. The highest amount of total phenolics were obtained from ethyl acetate extracts of *C. abbreviata* with 400 GAE/mg followed by acetone extracts with 392 GAE/mg total phenolics. The hexane extracts of *S. italica* measured the least

amount of about 0.3 GAE/mg. Overall *C. abbreviata* extracts had the highest number of total phenolics (table 3.2).

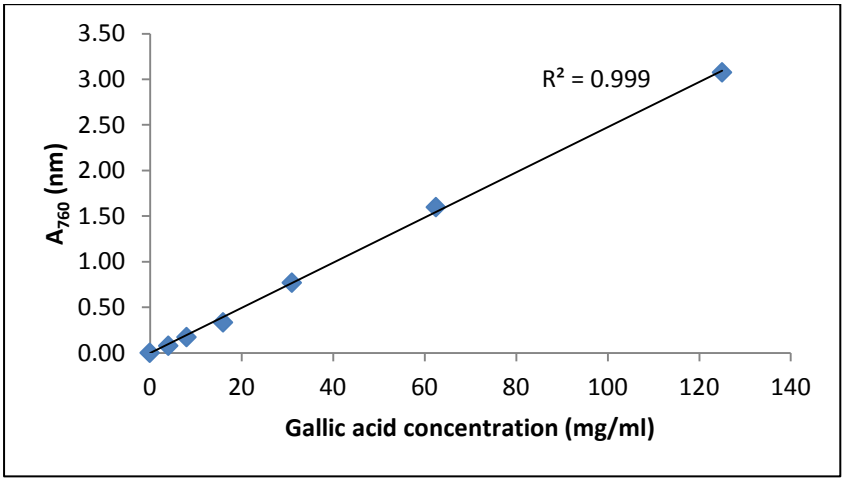


Figure 3.2: Standard curve of gallic acid used as a standard reference for total phenolic content.

Table 3.2: Total phenolic content of all plant extracts

Plant names	Hexane (GAE/mg)	Chloroform (GAE/mg)	Acetone (GAE/mg)	Ethyl acetate(GAE/mg)
A. gummifera	0.4	26	52	4
A.venosum	14	30	22	76
B. racemosa	52	28	64	28
C. abbreviata	28	92	392	400
H. integrifolius	32	32.8	79.2	27
K. africana	6	14	4	20
M. stuhlmanni	0.6	0.9	0.6	12
M. undata	24	26	28	30
S. italica	0.3	52.8	14	16
W. salutaris	0.4	0.8	20	0.5

3.4.2.2b. Total flavonoids

Different amount of total flavonoids were obtained. The total flavonoids content were determined by extrapolating from quercitin standard curve (fig 3.3). Ethyl acetate extracts of *A. gummifera* had the least amount of flavonoids of about 0.488 QE/mg.

All *H. integrifolius* plant extracts had the highest amount of flavonoids with ethyl acetate having 5.565 QE/mg. Majority of hexane plant extracts had the highest amount of flavonoids ranging between 1.035-4.507 QE/mg. Overall acetone extracts had the highest amount of total flavonoids (table 3.3).

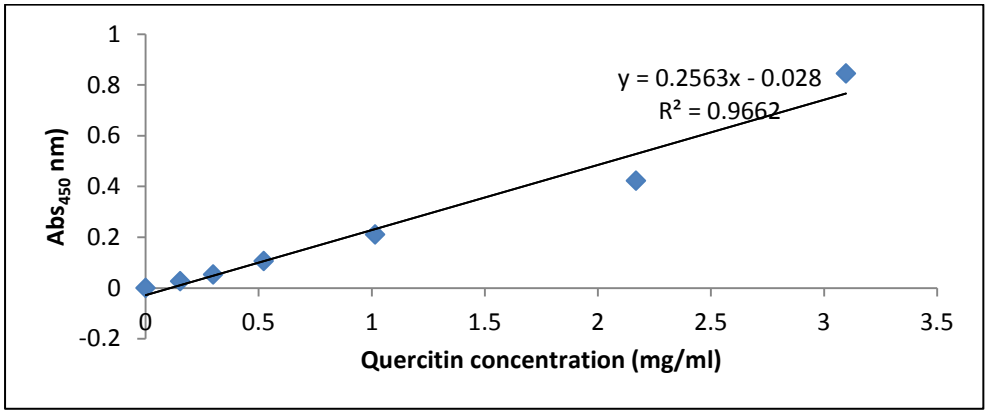


Figure 3.3: Standard curve of quercitin used as standard reference for total flavonoids.

Table 3.3: Total flavonoids of all plant extracts

Plant names	Hexane (QE/mg)	Chloroform(QE/mg)	Acetone (QE/mg)	Ethyl acetate(QE/mg)
A. gummifera	1.638002	0.565714	1.070341	0.488783
A. venosum	1.095054	2.05038	2.946986	2.360849
B. racemosa	1.638002	0.565714	1.070341	0.488783
C. abbreviata	2.433789	3.727189	1.226949	0.891752
H. integrifolius	4.386168	4.967234	4.12741	5.565277
K. africana	1.545511	0.681109	0.818485	0.853287
M. undata	4.507138	1.348847	2.805946	1.910256
M. stuhlmanni	1.03553	2.090676	2.140132	2.211567
S. italica	2.969594	3.922354	4.082626	2.699709
W. salutaris	2.67747	0.700433	1.502708	1.268253

3.4.2.2c. Total condensed tannins

Total condensed tannins were determined by extrapolating from catechin standard curve (figure 3.4). All plant extracts had the low amount of tannins ranging between -0.00-0.139 CA/mg. Only acetone and ethyl acetate extracts of *C. abbreviata* had

the highest amount of tannins measuring 0.139 and 0.137 CA/mg respectively. Hexane extracts of *K. africana* and acetone extracts of *M. undata* measured -0.00 CA/mg for total condensed tannins (table 3.4).

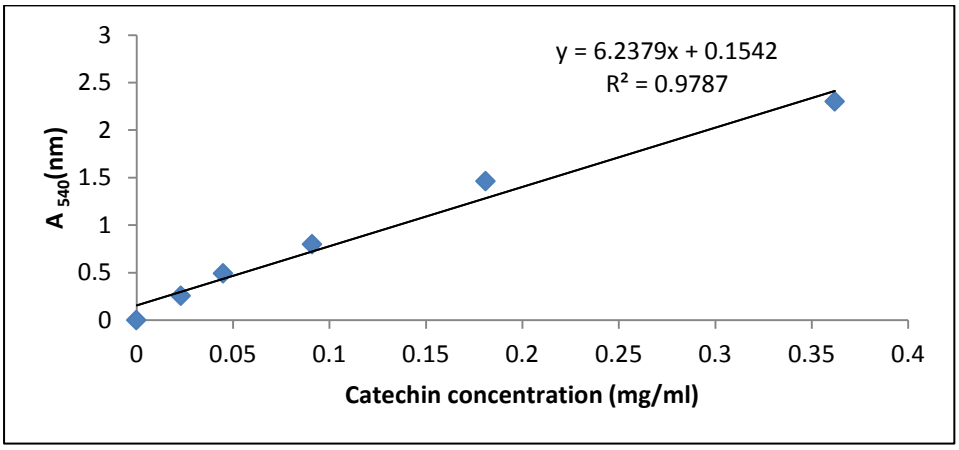


Figure 3.4: Standard curve of catechin used as a standard reference for total condensed tannins.

Table 3.4: Total condensed tannins of all plant extracts

Plant names	Hexane (CA/mg)	Chloroform(CA/mg)	Acetone (CA/mg)	Ethyl acetate(CA/mg)
A. gummifera	0.017127	0.01036	0.019566	0.020196
A. venosum	0.025546	0.016812	0.113515	0.058594
B. racemosa	0.015632	0.008786	0.040417	0.010596
C. abbreviata	0.031369	0.036562	0.139245	0.137829
H. integrifolius	0.032864	0.03491	0.077871	0.09471
K. africana	-0.00105	0.017678	0.020117	0.014058
M. undata	0.003121	0.01752	-0.00231	0.023028
M. stuhlmanni	0.025704	0.026648	0.026962	0.007921
S. italica	0.022635	0.017127	0.046555	0.005639
W. salutaris	0.046948	0.007055	0.017442	0.041912

3.4.2.3 Thin layer chromatography

Phytochemical analysis was performed using TLC plates developed in BEA, CEF and EMW. All the extracts contained chemical compounds which were represented by a number of distinct coloured bands on the TLC plates (figure 3.5a-d). More compounds were observed in chloroform and acetone extracts developed in the TEA system followed by CEF. Hexane extracts had the least amount of bands

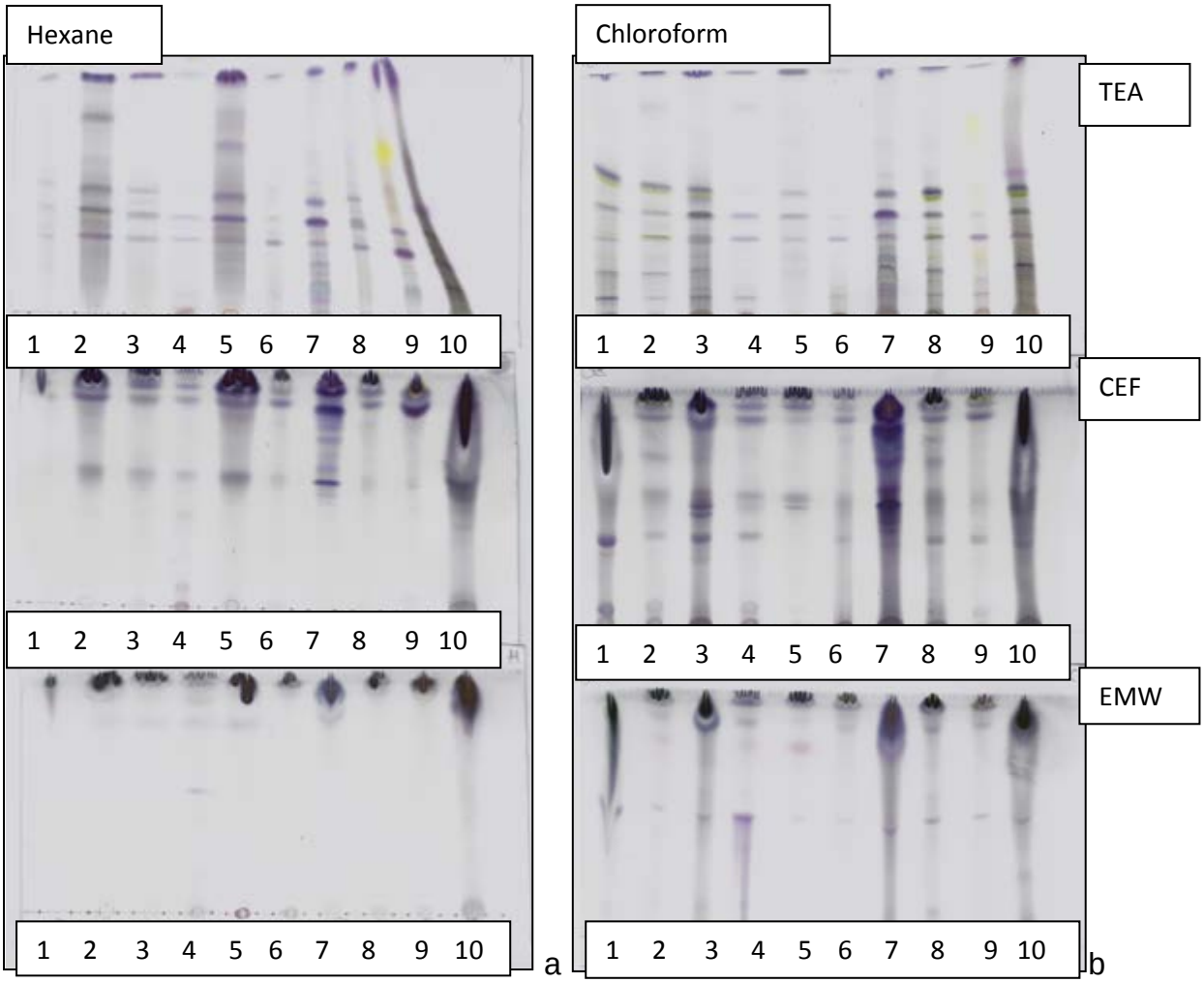


Figure 3.5a-b. TLC profile of hexane and chloroform extracts of investigated plant species. Presence of compounds was detected by spraying with vanillin-sulphuric reagent. 1- *Albizia gummifera*, 2- *Antidesma venosum*, 3-*Barringtonia racemosa*, 4-*Cassia abbreviata*, 5-*Helinus integrifolius,* 6-*Kigelia africana,* 7-*Maytenus undata, 8-Milletia stuhlmanni,* 9-*Senna italica* and 10-*Warbugia salutaris*

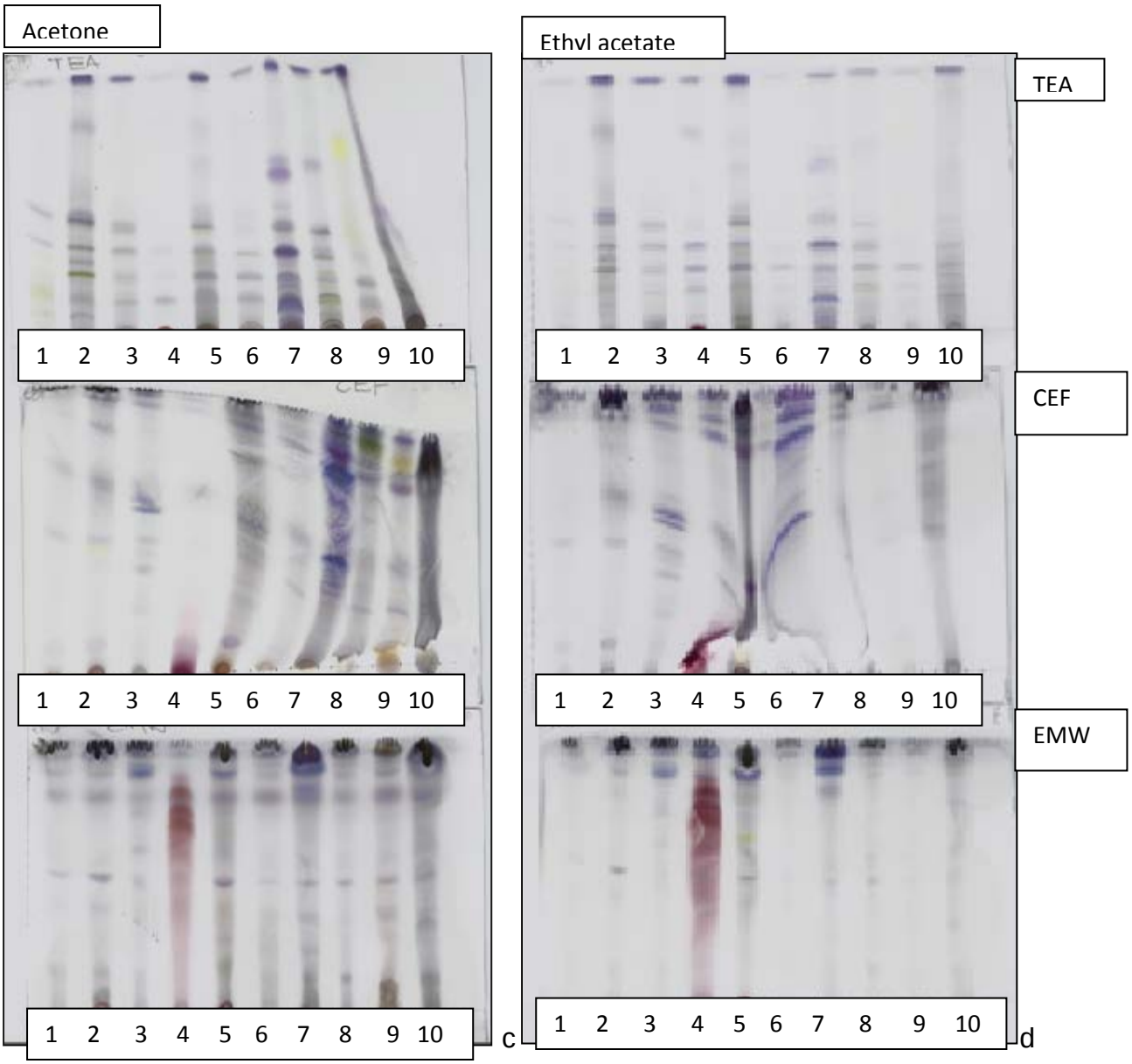


Figure 3.5c-d. TLC profile of acetone and ethyl acetate extracts of investigated plant species. Presence of compounds was detected by spraying with vanillin-sulphuric reagent. 1- *Albizia gummifera*, 2- *Antidesma venosum*, 3-*Barringtonia racemosa*, 4-*Cassia abbreviata*, 5-*Helinus integrifolius,* 6-*Kigelia africana,* 7-*Maytenus undata, 8-Milletia stuhlmanni,* 9-*Senna italica* and 10-*Warbugia salutaris*

3.5. Discussion and conclusion

The plant kingdom is well known for producing drugs. Drugs derived from plants are said to be easily accessible, affordable, less harmful and efficient (Yadav and Agarwala, 2011). Most drugs are derived from plants that contain organic compounds such as tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids. Some of these organic compounds are also used widely in human therapy, veterinary, agriculture and scientific research. Information about chemical constituents of plants is necessary for synthesis of drugs and other therapeutic agents. In the present study, phytochemical constituents of ten medicinal plants were analysed both qualitatively and quantitatively. The powdered leaves of all plants were extracted with four solvents. Different masses of extracts ranging between 0.01-0.5 g were obtained after extraction. Chloroform yielded the highest mass of extracts in most plants compared to other extractants. Out of the four solvents used, hexane extracted the least amount of extracts than other extractants showing that the plants had less of non-polar compounds. When comparing extraction from different plant leaves, *H. integrifolius* yielded an almost equivalent mass of 0.13 g for all solvents. *Milletia stuhlmanni* yielded the highest mass in all solvents than other plants. In general, different masses of extracts were obtained from different leaves which shows that different plant leaves posses different phytochemicals.

The qualitative analysis of all plant leaves are summarized in table 3.2. From the table it was observed that all plant extracts contains phenolic content. The presence of flavonoids was observed in all plant extracts except for hexane and chloroform extracts of *K. africana, M. undata* and *M. stuhlmanni*. The results contradicts with the total flavonoids test which revealed the presence of flavonoids in all plant extracts. Only the plant extracts of *A. gummifera, M. undata*, and *W. salutaris* revealed the presence of glycosides while only few plant extracts tested positive for steroids. Starch was absent in all plant extracts, this was observed when the brown color of iodine did not change to blue/black when added to the crude extracts.

Phenolic compounds are the largest group of plant metabolites that are found everywhere in the plant. They include phenolic acids, flavonoids, tannins, stilbenes and lignans (Dai and Mumper, 2010). Total phenolic content expressed as GAE/mg

was determined in all the plant extracts. Different amount of total phenolics were measured in all plant extracts. *Milletia stuhlmanni* plant extracts had the least amount of total phenolics compared to other plant extracts as shown on table 3.2. Overall acetone, ethyl acetate and chloroform extracts of *C. abbreviata* had the highest amount of total phenolics of 400, 392 and 92 GAE/mg respectively followed by acetone extracts of *H. integrifolius* (79.2 GAE/mg). In general, the highest amount of phenolics were found in acetone and ethyl acetate extracts implying that the plants under investigation contains phenolics that are medium to highly in polarity.

Total flavonoid content of all plant extracts were also measured. Flavonoids are a large class of hydroxylated polyphenols divided into different subclasses synthesized by plants. Quercitin is one of the flavonoids found in plants. In the present study, total flavonoids were determined by extrapolating from quercitin standard curve measured in QE/mg. Data from table 3.3 revealed that all plant extracts had flavonoids ranging between 0.488783–5.565277 QE/mg. Out of all the plants tested, *H. integrifolius* extracts had the highest number of total flavonoids followed by *S. italica* extracts. Ethyl acetate extracts of *B. racemosa* and *A. gummifera* had the least number of flavonoids 0.488783 GAE/mg. The results correspond with other studies conducted showing that the plants under investigation contains flavonoids which attributes to their antioxidant activity. High amount of total flavonoids content were found in hexane plant extracts when comparing all the extracts.

Another group of secondary metabolites present in the plant leaves measured quantitatively was the total condensed tannins. It is well known that tannins are subdivided into subgroups, namely condensed and hydrolysable tannins. In this study total condensed tannins were tested using the vanillin assay with catechin used to generate a standard curve. The results revealed that all plant extracts did not contain much of condensed tannins. This was also observed when most of the plant extracts turned light pink instead of dark pink. Among all the plant extracts tested, only acetone and ethyl acetate of *C. abbreviata* had the highest number of condensed tannins (0.1339245 and 0.137829 CA/mg) respectively. The results correspond with the study conducted by Kokwaro (1993) which showed that *C. abbreviata* is rich in tannins.

Thin layer chromatography was used to separate and view different compounds present in the plant extracts. According to McGaw (2002), TLC is a rapid and effective method to obtain fingerprints of plant extracts. Different compounds are represented by different coloured bands on the TLC plates (figures 3.5a-d). TEA was the best mobile phase since more compounds of all plant extracts were separated efficiently than in CEF and EMW. In general, the separation of compounds in both the CEF and EMW systems were not efficient because the majority of extracted compounds were non-polar for these systems. Hence most of the compounds moved at the rate of the mobile phase, making it difficult to count the number of extracted compounds even after spraying (figure 3.5a-d). In TEA it appeared that ethyl acetate extracts contained the highest number of compounds followed by chloroform. The EMW system separated more compounds of the acetone extracts while hexane and chloroform extracts had the least number of bands respectively.

Both qualitative and quantitative phytochemical analysis results revealed that the leaves of different plant species contain various amounts of phytochemicals with varying polarities. These results provide preliminary information on some important groups of bioactive compounds present in the investigated plant species. The results also gives an idea of which bioactive compounds might be responsible for particular biological activity that the plant exhibit. Further identification of active plant extracts with certain properties are reported in the next chapters.

Chapter 4

Screening for antioxidant and *in vitro* enzyme inhibition of ten selected medicinal plants

4.1. Introduction

Diabetes mellitus is a fast growing metabolic disorder that is characterized by abnormal blood glucose level in the body. It is a common and very prevalent disease in both developed and developing countries. Although drugs are available for the management of the disease, its prevalence still increases every year. Diabetes is a disease that is caused by different factors such as obesity, eating of unhealthy diet, environmental factors or can be genetically inherited. Oxidative stress is also reported to play an important role in the development and progression of the disease (Henriksen *et al*., 2010). In diabetes, glucose oxidation causes excessive production of free radicals. The production of high levels of free radicals in the body decreases antioxidant defense mechanism resulting in damage of cellular organelles as well as the induction of beta-cell apoptotic pathways (Valko *et al*., 2007; Henriksen *et al*., 2010). Diabetes mellitus also causes an imbalance in the body's natural equilibrium (Maritim *et al*., 2003). This equilibrium shift causes glucose to chemically combine with the proteins in the blood stream leading to the formation of free radicals. Tiwari and Rao (2002) indicated that persistent hyperglycemia promotes the production of ROS inside the cells through activation of the polyol pathway which increases glucose auto-oxidation causing depletion of free radical scavenging enzymes. The level of antioxidant enzymes influences the susceptibility of various tissues to oxidative stress and development of macrovascular complications. The more free radicals that are produced in diabetic patients, the more cells are damaged leading to the onset of diabetes-related diseases and death (Ruhe and McDonald, 2001). It is thus important to find therapies that can help reduce oxidative stress either directly or indirectly.

Antioxidants are very important in diabetic patients due to their ability to scavenge free radicals. Apart from their ability in delaying the progression of diabetes and macrovascular complications, they also help in boosting the body's defense. Some of the currently available antidiabetic agents have antioxidant activity. Plants on the

other hand are considered to be a rich source of antioxidants. Plants contain large amounts of antioxidants such as tocopherols (vitamin E), carotenoids, ascorbic acid, flavonoids and tannins (Pratt, 1992; Larson, 1988).

Stabilization of postprandial hyperglycemia is one of the dietary therapies for diabetic patients. As such, plants rich in antioxidants play a useful role in the inhibition of carbohydrate digesting enzymes (Kumar *et al*., 2012). Carbohydrate digesting enzymes break down starch and other carbohydrates into small glucose molecules for easy absorption. When inhibited, absorption is delayed leading to reduction in postprandial hyperglycemia (Nilupa *et al*., 2008). The current commercial agents for the management of diabetes are associated with their non-specific nature and as a consequence cause the unnecessary accumulation of undigested carbohydrates in the large intestine (Murai *et al*., 2002). According to Gurib-Fakim (2006), the relevance of ethnobotanicals in the search for new drugs is very important. Antidiabetic agents with fewer side effects originating from plants are of interest in the pharmaceutical industry for the management of diabetes. To date, more than 800 plants have been identified as possible sources of therapy for the management or treatment of diabetes in particular non-insulin dependent diabetes mellitus (NIDDM). Despite this, only a fraction of these plants have been evaluated scientifically (Bailey and Day, 1989; Perez *et al*., 1984). Some of the plants with antidiabetic activity have been found to contain antioxidant constituents suggesting their relevance in free radical scavenging and prevention of macrovascular complications associated with diabetes (Adefegha and Oboh, 2012). In this study, the antidiabetic potential of selected plants were evaluated *in vitro* using various parameters.

4.2. Objectives of the study

a. To test for the presence of antioxidant constituents in selected plant extracts
b. To evaluate the inhibitory activity of the plant extracts on α-amylase enzyme from hog pancreas
c. To evaluate the inhibitory activity of the plant extracts on α-glucosidase from rat intestines
d. To determine the type of inhibition exerted by the plant extracts on α-amylase and α-glucosidase enzymes

4.3. Materials and methods

4.3.1. Antioxidant activity (Quantitative DPPH assay)

Plants with antioxidant activity are capable of scavenging free radical molecules. The radical scavenging ability of all plant extracts was tested quantitatively using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma), using the method of Katsube *et al*. (2004) as described in section 2.5.

4.3.2. Alpha amylase inhibition assay

Alpha amylase inhibition assay was performed using two chromogenic methods. Both methods depend on the colour change in the reaction which is determined by the activity of α-amylase.

4.3.2a. Alpha amylase inhibition assay (CNPG3 reagent method)

The 2-Chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) is a chromogenic method which is used to measure the amount of glucose produced by α-amylase in the presence of plant extracts. It involves the hydrolysis of the substrate 2-chloro-p-nitrophenol linked with maltotriose by α-amylase to produce glucose and other products.

$$10CPNG_3 \xrightarrow{\text{α- amylase}} 9\ CNP + 1\ CNPG_2 + G_3 + G$$

The amount of glucose produce is observed when there is a colour change to yellow in the reaction mixture which is then measured spectrophotometrically. This method was performed according to Zhang *et al*. (2011) with slight modification as described in section 2.6.1a.

4.3.2b. Alpha amylase inhibition assay (DNS method)

Another colorimetric method used for α-amylase inhibition assay was DNS method as described by Ali *et al*. (2006), with slight modification. Dinitrosalicylic acid (DNS) is a reagent used to detect the presence of reducing sugars in a reaction. It involves the reduction of yellow to a brown colour product caused by the sugars present in the reaction mixture.

3,5-dinitrosalicylic acid + glucose ⟶ 3-amino-5-nitrosalicylic acid + glu conic acid

In this study the method is elaborated in detail in section 2.6.1b.

4.3.2c. Alpha glucosidase inhibition assay

Plant extracts were tested for enzyme inhibitory capability using α-glucosidase from mammalian rat intestine (Sigma). The mammalian intestine glucosidase is a complex of individual enzymes, namely sucrase and maltase. However in this study these enzymes were tested for their activity in the presence of plant extracts according to the method described by Zhang *et al*. (2011). For sucrase inhibition assay the method was performed as described in section 2.6.2a while that for maltase was as in section 3.2.3.2a using maltose as substrate.

4.3.3. Enzyme kinetics

4.3.3a. Alpha amylase enzyme kinetics

Enzyme kinetics was performed to determine the type of inhibition exerted by the plant extracts on both α-amylase and α-glucosidase enzymes as described by Zhang *et al*., (2011) (section 2.6.3a). Only plant extracts that inhibited more than 50% of the activity of α-amylase enzymes were tested for the type of inhibition (competitive or non-competitive inhibition) they exert on the enzymes. The type of inhibition was determined by plotting the results on a Lineweaver-Burk plot which show the V_{max} and km values.

4.3.3b. Alpha glucosidase enzyme kinetics

For α-glucosidase enzyme kinetics, the method was performed as in section 2.6.3b.

4.4. Results

4.4.1. Antioxidant activity (Quantitative DPPH assay)

Antioxidant activities of all plant extracts were determined quantitatively at various concentrations. Only the IC_{50} of each plant extract was determined (Table 4.1). Plants with good antioxidant activity had an IC_{50} of less than 2.5 mg/mℓ. All plant extracts showed antioxidant activity at various concentrations. Majority of the hexane and chloroform extracts did not show antioxidant activity below 2.5 mg/mℓ. The highest antioxidant activity was observed with the acetone followed by ethyl acetate, while the lowest activity was observed with chloroform extracts of all the plants. This is because compounds with high polarity are effective radical scavengers. *Cassia abbreviata* and *H. integrifolius* had the highest antioxidant activity than other plants. Acetone extracts of *H. integrifolius* showed the best antioxidant activity at lower concentration of 0.019 mg/mℓ followed by the acetone and ethyl acetate extracts of *C. abbreviata* with 0.020 and 0.025 mg/mℓ respectively. Antioxidant activity of all plant extracts increased in a concentration dependant manner. When compared according to the solvents, hexane extracts of *M. stuhlmanni* (IC_{50} 0.019 mg/mℓ) had the best antioxidant activity among all hexane extracts followed by *H. integrifolius* with IC_{50} 0.4 mg/mℓ. The chloroform extract of *C. abbreviata* had the best antioxidant activity among all chloroform extracts of the different plants with IC_{50} of 0.078 mg/mℓ. Acetone extracts of *H. integrifolius* (IC_{50} 0.019 mg/mℓ) on the other hand, had the best antioxidant activity among all extracts followed by *C. abbreviata* with an IC_{50} of 0.020 mg/mℓ. For ethyl acetate extracts, *C. abbreviata* (IC_{50} 0.025 mg/mℓ) had the best antioxidant activity compared to all plant extracts.

Table 4.1: Free radical scavenging activity of extracts of selected plants (IC_{50} mg/mℓ).

Name of plant	*Hexane* IC_{50} (mg/mℓ)	*Chloroform* IC_{50} (mg/mℓ)	*Acetone* IC_{50} (mg/mℓ)	*Ethyl acetate* IC_{50} (mg/mℓ)
A. gummifera	>2.5	>2.5	0.625	>2.5
A. venosum	>2.5	>2.5	0.091	>2.5
B. racemosa	>2.5	>2.5	0.156	>2.5
C. abbreviata	>2.5	0.078	0.020	0.025
H. integrifolius	0.4	>2.5	0.019	0.156
K. africana	>2.5	>2.5	1.25	>2.5
M. stuhlmanni	0.019	>2.5	>2.5	1.25
M. undata	>2.5	>2.5	1.25	>2.5
S. italica	>2.5	2.50	0.206	0.312
W. salutaris	>2.5	>2.5	>2.5	>2.5

4.4.2. Enzyme assays

The inhibitory capability of plant extracts on carbohydrate digesting enzyme was determined by inhibiting α-amylase and α-glucosidase enzymes. Their inhibition capabilities were determined colorimetrically after serial dilution of the plant extracts with phosphate buffer. Acarbose was used as positive control.

4.4.2a. Alpha amylase inhibition assay

In vitro α-amylase enzyme inhibition assay was performed to determine the effect of all plant extracts on the enzyme. Inhibitory capability of each plant extract was determined at various concentrations following serial dilution. A positive reaction is evident when the colour of glucose mono reagent changes to yellow due to presence of glucose in the reaction. Absorbance was read at 450 nm. Most of the plant extracts did not inhibit 50% of the enzyme activity at concentrations below 2.5 mg/mℓ. The lowest IC_{50} were observed when using CNPG3 (A in table 4.2) method than the DNS method. The acetone extracts of *C. abbreviata* was the highest inhibitors at a concentration of 0.5 mg/mℓ. When using DNS reagent (B) method, the highest enzyme inhibition was obtained at the lowest IC_{50} of 0.625 mg/mℓ. Both the

acetone and ethyl acetate extracts were better inhibitors of the enzyme compared to hexane and chloroform extracts. When using CNPG3 ("A" method) hexane extract of *S. italica* had the best inhibitory activity with IC_{50} of 1.7 mg/mℓ. With chloroform extracts, *H. integrifolius* had the best activity with an IC_{50} of 1.2 mg/mℓ. Acetone extracts of *C. abbreviata, A. venosum and S. italica* had the best inhibitory activity with IC_{50} 0.5, 1.6 and 1.7 mg/mℓ respectively while the ethyl acetate extracts of *A. venosum* was the best inhibitor with IC_{50} 1.6 mg/mℓ followed by *H. integrifolius* with an IC_{50} 1.7 mg/mℓ. When using DNS ("B" method), most plant extracts showed inhibitory activity. *Cassia abbreviata* and *W. salutaris* hexane extracts inhibited α-amylase enzyme at a low IC_{50} of 0.625 mg/mℓ. *Maytenus undata* was the second best with IC_{50} of 1.25 mg/mℓ among all other extracts. The lowest concentration at which chloroform extracts (*C. abbreviata*, *H. integrifolius*, *M. undata* and *S. italica*) inhibited 50% of the enzyme activity was at IC_{50} of 0.625 mg/mℓ. The acetone and ethyl acetate extracts of *A. venosum*, *H. integrifolius*, *M. undata* and *S. italica* also had the lowest IC_{50} of 0.625 mg/mℓ. Only hexane extracts of *A. gummifera*, *A. venosum*, *B. racemosa*, *K. africana*, *M. stuhlmanni* had the same IC_{50} when using both assays. The same effect was observed with chloroform and acetone extracts of *B. racemosa* and *K. africana* when using both assays. The results obtained for ethyl acetate of *A. gummifera*, *B. racemosa*, *C. abbreviata*, *K. africana* was similar when using both assays. The results also indicated that increase in concentration may result in further inhibition of the enzyme.

Table 4.2: Minimum concentrations (IC_{50}) at which selected plant extracts inhibited at least 50% of the alpha amylase enzyme activity.

Plant names	Hexane (mg/mℓ)		Chloroform (mg/mℓ)		Acetone (mg/mℓ)		Ethyl acetate (mg/mℓ)	
	A	B	A	B	A	B	A	B
A. gummifera	>2.5	>2.5	>2.5	2.5	>2.5	2.5	2.5	2.5
A. venosum	>2.5	>2.5	>2.5	2.5	1.6	0.625	1.6	0.625
B. racemosa	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
C. abbreviata	2.5	0.625	>2.5	0.625	0.5	>2.5	>2.5	>2.5
H. integrifolius	>2.5	2.5	1.2	0.625	2.5	0.625	1.7	0.625
K. africana	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
M. undata	>2.5	1.25	>2.5	0.625	>2.5	2.5	>2.5	0.625
M. stuhlmanni	2.5	2.5	>2.5	1.25	>2.5	2.5	>2.5	2.5
S. italica	1.7	2.5	>2.5	0.625	1.7	0.625	>2.5	0.625
W. salutaris	>2.5	0.625	>2.5	1.25	>2.5	1.25	2.5	1.25

A= Alpha amylase enzyme inhibition assay using reagent kit (KAT) (CNPG3), B= Alpha amylase enzyme inhibition assay using DNS method.

4.2.2b. Alpha glucosidase inhibition assay

For alpha glucosidase inhibition assay, all plant extracts were serially diluted with phosphate buffer and tested for α-glucosidase inhibitory activity at concentrations between 0.019–2.5 mg/mℓ. Two percent (2%) maltose and sucrose were used as substrates for the enzyme. Absorbance was read after incubation at 37^{o}C for 1 hour. Inhibition was determined by the intensity of quinone colour produced in the reaction. The more intense the colour, the higher the enzyme activity which is an indication of more glucose produced. For all the plant extracts tested, ethyl acetate and acetone plant extracts were the most active extracts against the enzyme when using both maltose and sucrose as substrates (figures 4.4.1a to 4.4.1h). Hexane and chloroform plant extracts did not show potential inhibition. The inhibitory capability of all plant extracts increased as their concentration was increased. Acarbose used as positive control inhibited the enzyme far better than all plant extracts. Figure 4.4.1a–4.4.1d show the inhibitory activity of the extracts when maltose was used as substrate for the α-glucosidase enzyme. Hexane extracts (figure 4.4.1a), had less

inhibitory activity than that of acarbose. The highest inhibition obtained was about 25% with *M. undata* and *H. integrifolius.* None of the extracts inhibited more than 30% of the enzyme activity. *Antidesma venosum* was the lowest inhibitor with less than 10% at 2.5 mg/mℓ.

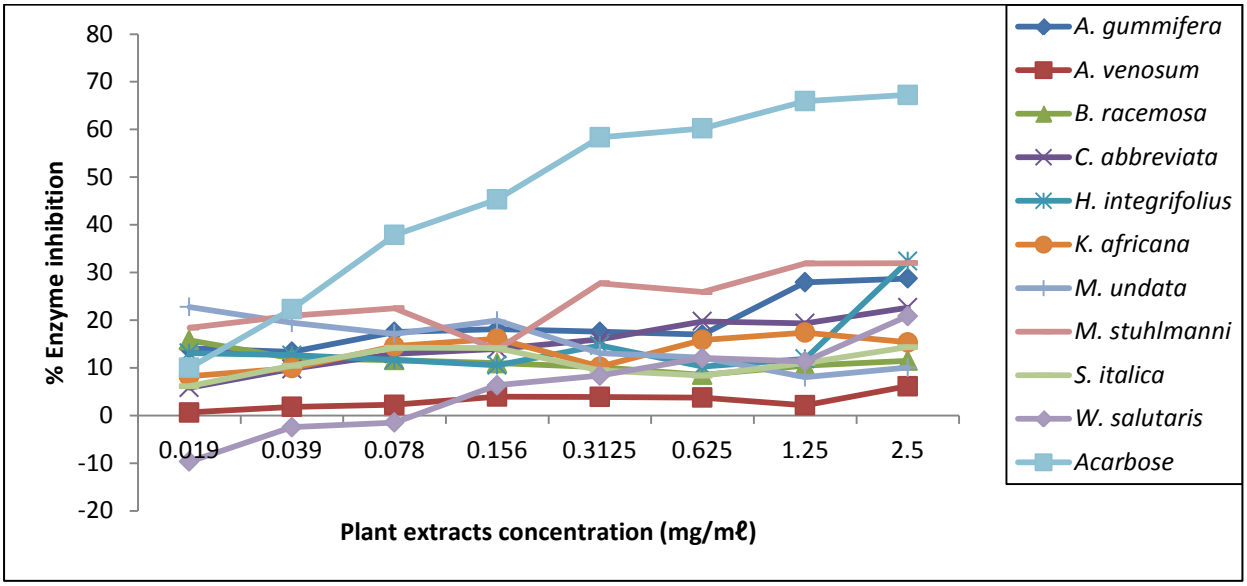


Figure 4.4.1a: Effect of hexane extracts of selected plants on α-glucosidase enzyme activity using 2% maltose as a substrate.

Chloroform extracts on the other hand inhibited less than 50% of the enzyme activity even at high concentration of 2.5 mg/mℓ. *Cassia abbreviata* was the highest inhibitor than other plant species inhibiting 25% at IC_{50} of 2.5 mg/mℓ. All other plant species inhibited between 5-20% of the enzyme activity when compared to acarbose which inhibited about 70% of the enzyme activity.

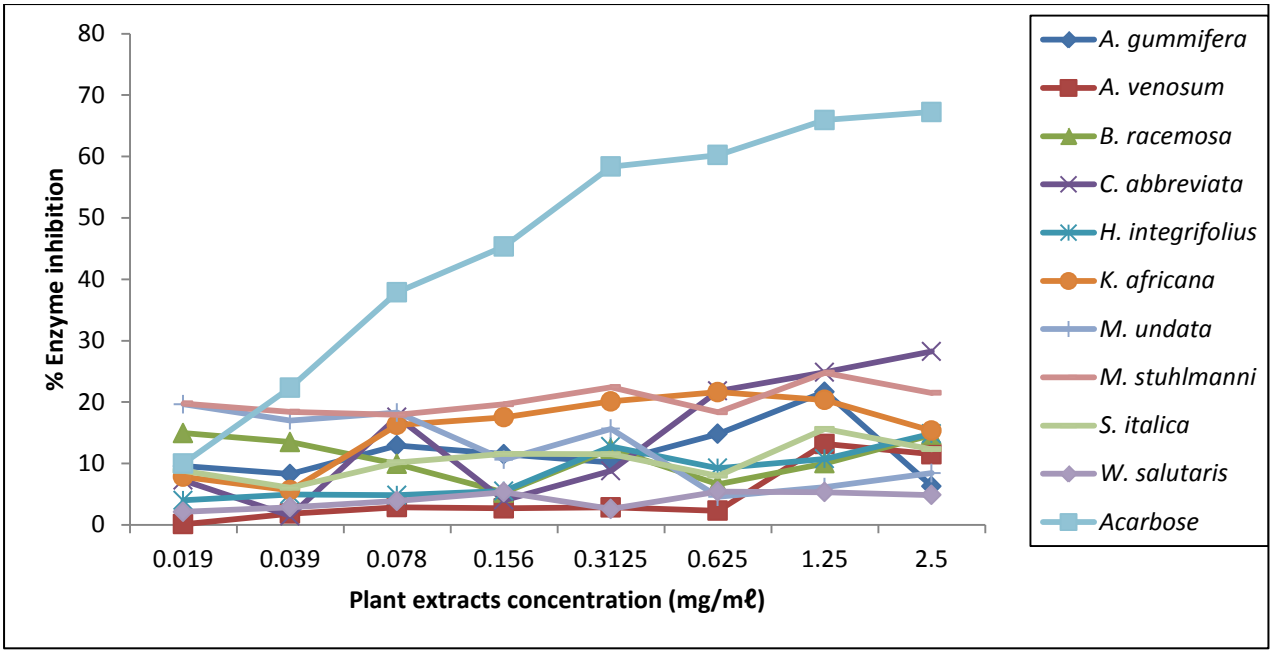


Figure 4.4.1b: Effect of chloroform extracts of selected plants on α-glucosidase enzyme activity using 2% maltose as a substrate.

The highest inhibitory activity was obtained with two plant extracts (*C. abbreviata* and *H. integrifolius*). These plants showed greater inhibition than acarbose, inhibiting more than 70% of the enzyme activity at 2.5 mg/mℓ. The lowest inhibition was obtained with *W. salutaris* (<5% at 2.5 mg/mℓ).

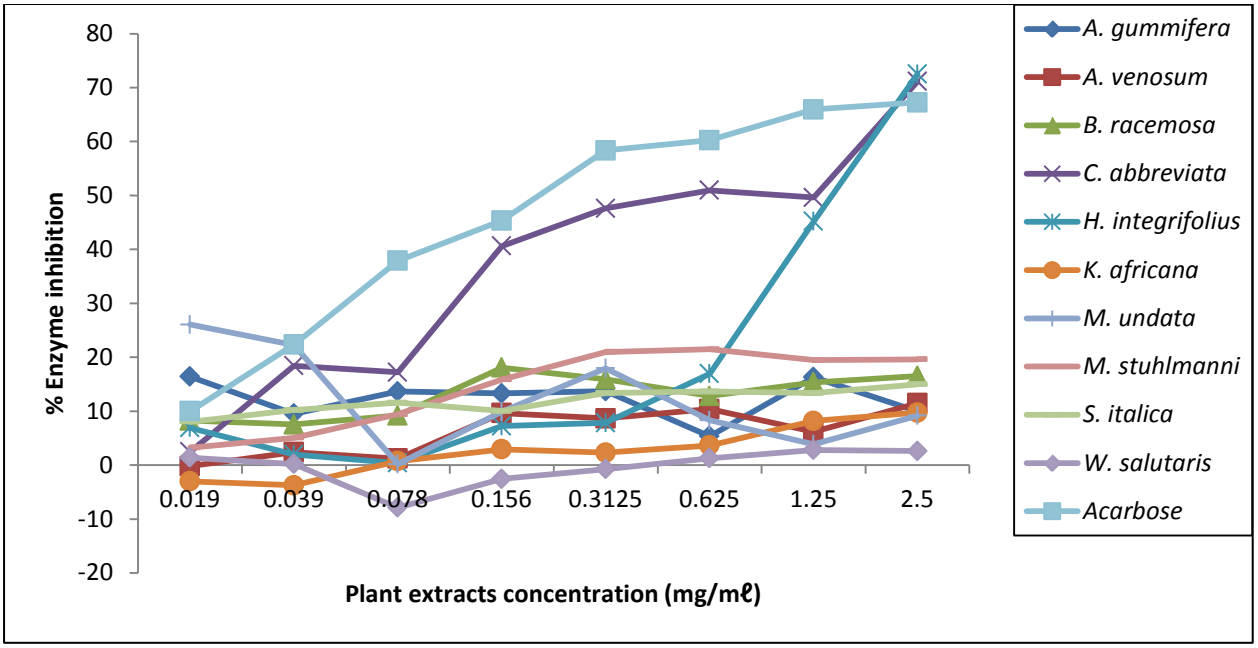


Figure 4.4.1c: Effect of acetone extracts of selected plants on α-glucosidase enzyme activity using 2% maltose as a substrate.

The figure (4.4.1d) below show a similar trend of inhibition when enzyme was treated with ethyl acetate extracts. *Helinus integrifolius* and acarbose inhibited 60% at the highest concentration of 2.5 mg/mℓ followed by *C. abbreviata* at 58%. These plants inhibited enzyme activity comparable to acarbose. Both *B. racemosa* and *W. salutaris* did not show any inhibitory capability. The rest of the other plant extracts inhibited less than 20% of the enzyme activity.

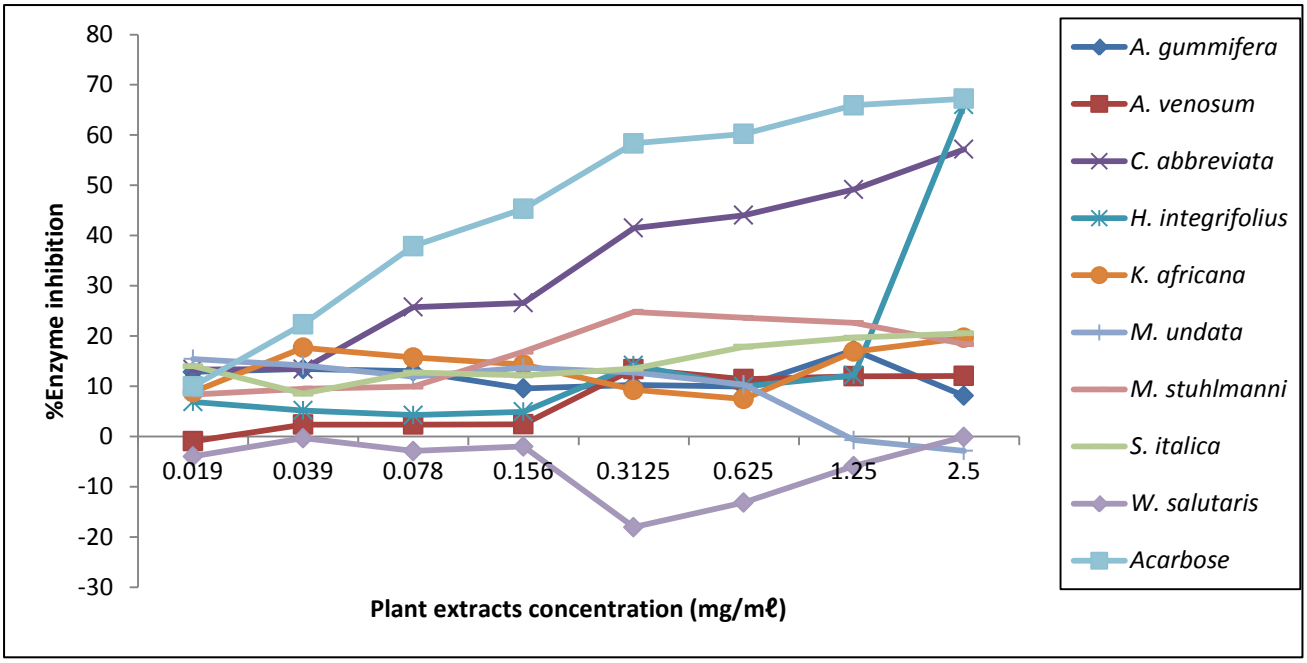


Figure 4.4.1d: Effect of ethyl acetate extracts of selected plants on α-glucosidase enzyme activity using 2% maltose as a substrate.

Figures 4.4.1e–4.4.1h illustrate the results obtained during α-glucosidase enzyme inhibition when using sucrose as a substrate. All the plant extracts inhibited the enzyme in a concentration dependant manner. The inhibitory potential of the plant extracts was in general better than when using maltose as a substrate. Hexane extracts in figure 4.4.1e inhibited less than 50% of the enzyme activity. *Helinus integrifolius* was the highest inhibitor with an activity comparable to that of acarbose. *Barringtonia racemosa*, *M. undata*, and *K. africana* were the second most active plants inhibiting 45% enzyme activity at 2.5 mg/mℓ. *Antidesma venosum* and *M. stuhlmanni* showed a similar trend when inhibiting α-glucosidase enzyme activity. The least active plant was *A. gummifera.*

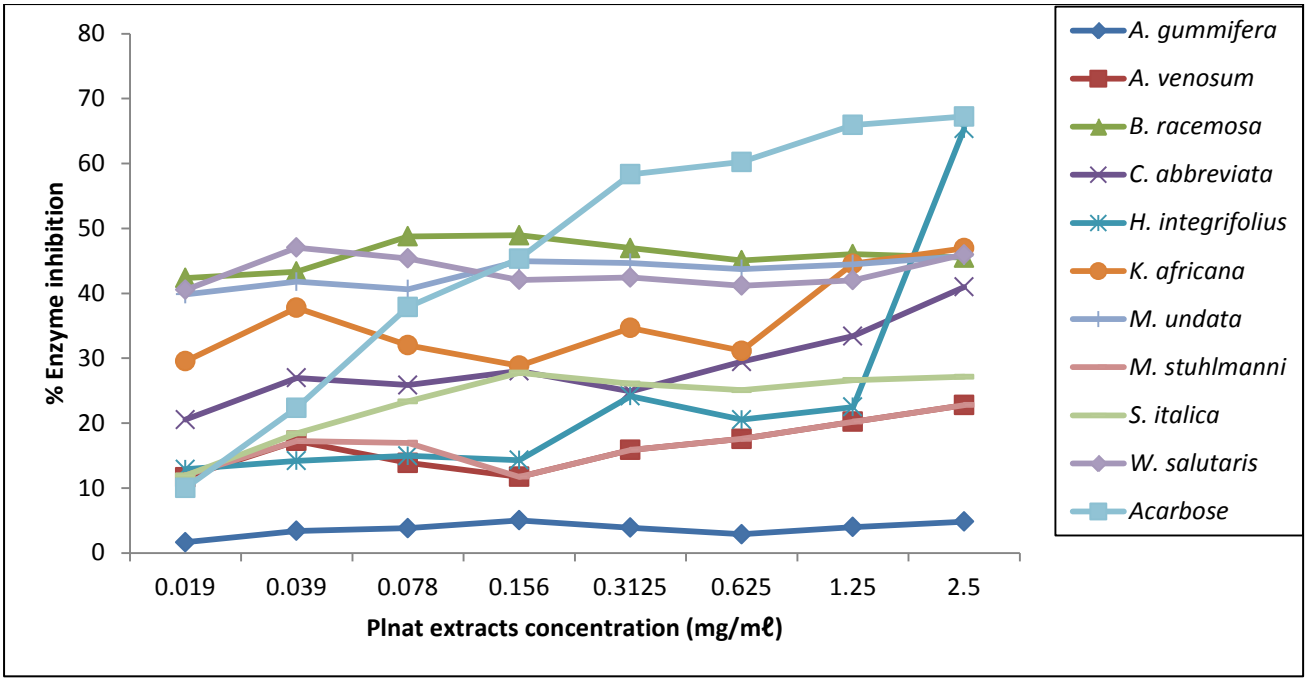


Figure 4.4.1e: Effect of hexane extracts of selected plants on α-glucosidase enzyme activity using 2% sucrose as a substrate.

Chloroform extracts inhibited between 10-90% of the enzyme activity. *Cassia abbreviata* was the strongest inhibitor inhibiting 90% at IC_{50} of 2.5 mg/mℓ followed by acarbose and *H. integrifolius* inhibiting 65% at IC_{50} of 2.5 mg/mℓ. Unlike with maltose, most plants were able to inhibit more than 20% of the enzyme activity. *Albizia gummifera* inhibited the least percentage of enzyme activity.

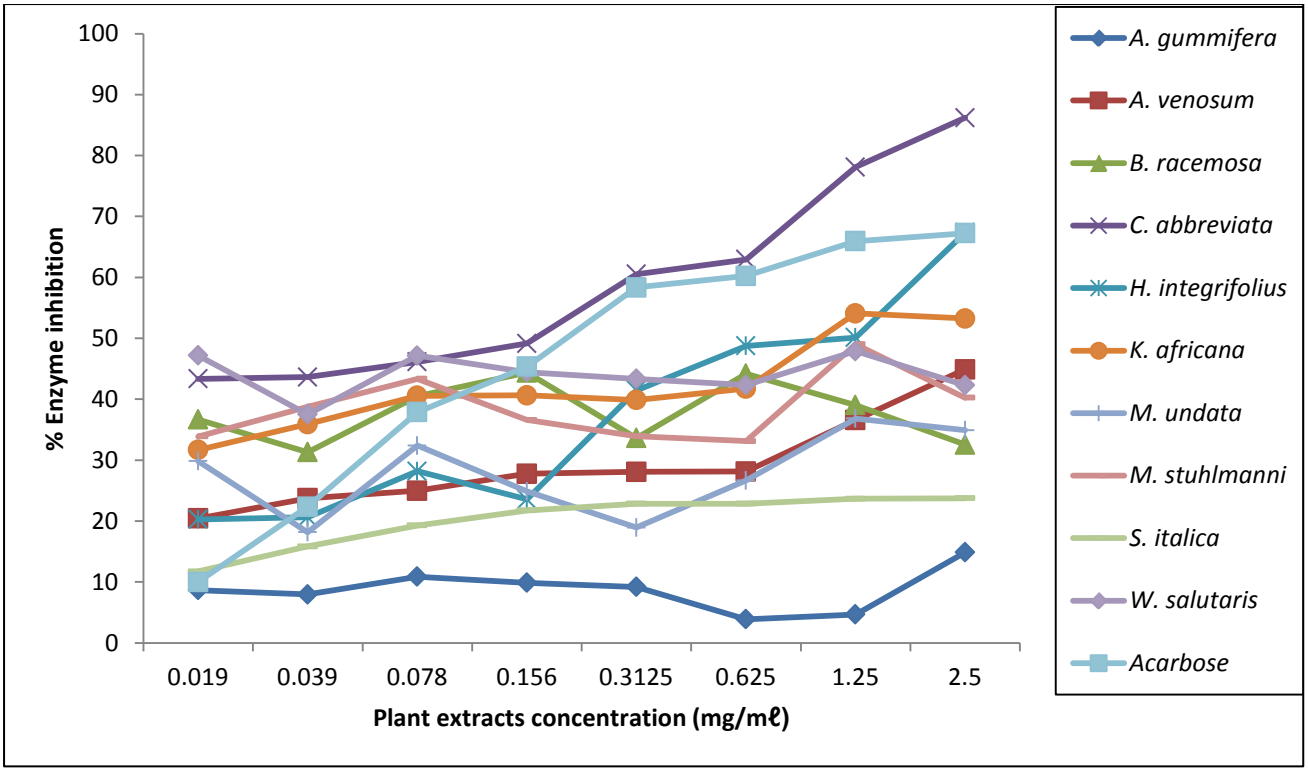


Figure 4.4.1f: Effect of chloroform extracts of selected plants on α-glucosidase enzyme activity using 2% sucrose as a substrate.

Most intermediate polar extracts, overall, showed better inhibitory activity than non-polar extracts. The figure (figure 4.4.1g) below shows inhibitory activity of acetone extracts. Amongst the plants, *H. integrifolius* had the highest inhibitory activity, inhibiting almost 100% of the enzyme activity. *Maytenus undata* was the second best inhibitor, followed by acarbose (70 and 65% respectively). A fifty percent inhibition was obtained for *C. abbreviata* and *K. africana* extracts at an IC_{50} of 2.5 mg/mℓ while *A. gummifera* inhibited only 10% of the enzyme activity.

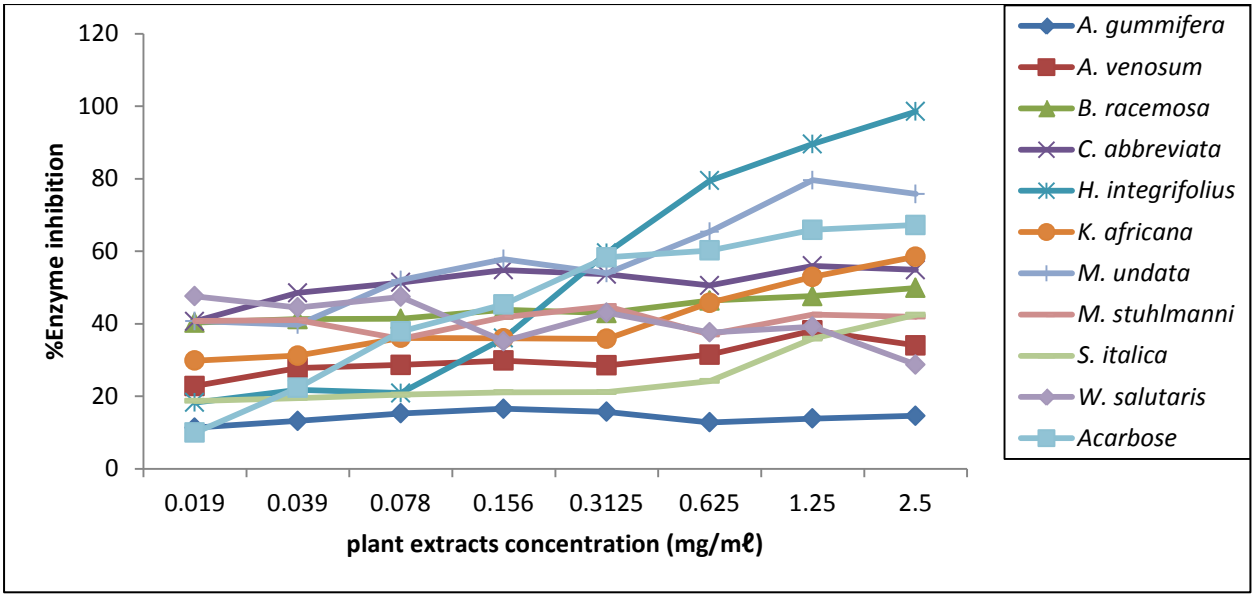


Figure 4.4.1g: Effect of acetone extracts of selected plants on α-glucosidase enzyme activity using 2% sucrose as a substrate.

The inhibitory capability of ethyl acetate extracts on α-glucosidase enzyme is represented on figure 4.4.1h. These extracts inhibited enzyme activity similar to that obtained for acetone extracts. *Helinus integrifolius* had an excellent α-glucosidase inhibitory activity (>90% at 2.5 mg/mℓ). The second best extracts were *C. abbreviata* and *K, africana* inhibiting about 60% of the enzyme activity. Only *A. venosum*, *A. gummifera* and *M. stuhlmanni* inhibited less than 20% of the glucosidase activity.

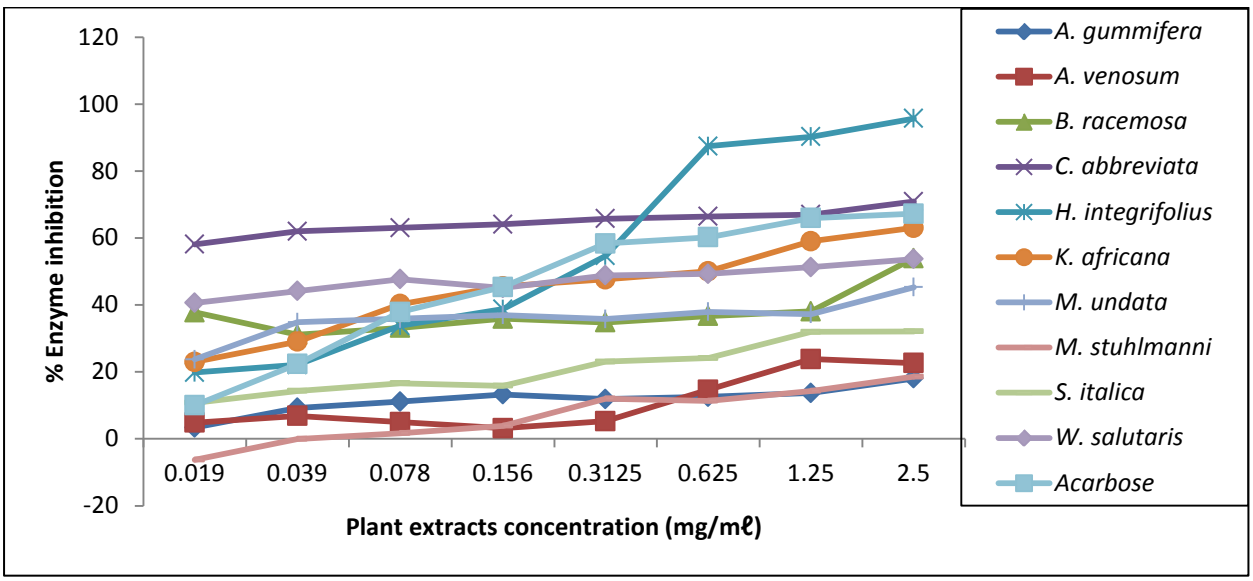


Figure 4.4.1h: Effect of ethyl acetate extracts of selected plants on α-glucosidase enzyme activity using 2% sucrose as a substrate.

4.4.3. Enzyme kinetics

Enzyme kinetics of α-amylase and α-glucosidase was performed to determine the type of inhibition the plant extracts exerted on both α-amylase and α-glucosidase enzymes. The concentration of substrate in both cases was varied and Lineweaver-burk graphs were plotted. The K_m remained unchanged and V_{max} differed in both α-amylase and α-glucosidase kinetics demonstrating a non-competitive type of inhibition.

4.4.3a. α-Amylase enzyme kinetics

Only plant extracts that inhibited 50% of both α-amylase and α-glucosidase enzymes were tested for the type of inhibition they exert on the enzymes. All plant extracts tested, displayed a pattern of non-competitive inhibition, with K_m remaining unchanged and V_{max} decreasing (figure 4.4.2a-4.4.2b). The V_{max} of the enzyme decreased from 1.73 mg.mℓ$^{-1}$ in the absence of plant extracts to 1.60, 0.47 and 0.33 mg/mℓ$^{-1}$ following treatment of enzyme with 10 mg/mℓ of chloroform, ethyl acetate and acetone extracts of *H. integrifolius* respectively (figure 4.4.2a). The K_m remained unchanged at about -0.1 mg/mg.S^{-1}. The same trend was also observed when *C. abbreviata* extracts were added to the reaction mixture. The V_{max} of the enzyme decreased from 1.639 mg.mℓ$^{-1}$ in the absence of plant extracts to 1.126 and 1.102 mg.mℓ$^{-1}$ in the presence of acetone and ethyl acetate extracts of *C. abbreviata* respectively (figure 4.4.2b). The K_m remained unchanged at about -0.1 mg/mg.S^{-1}.

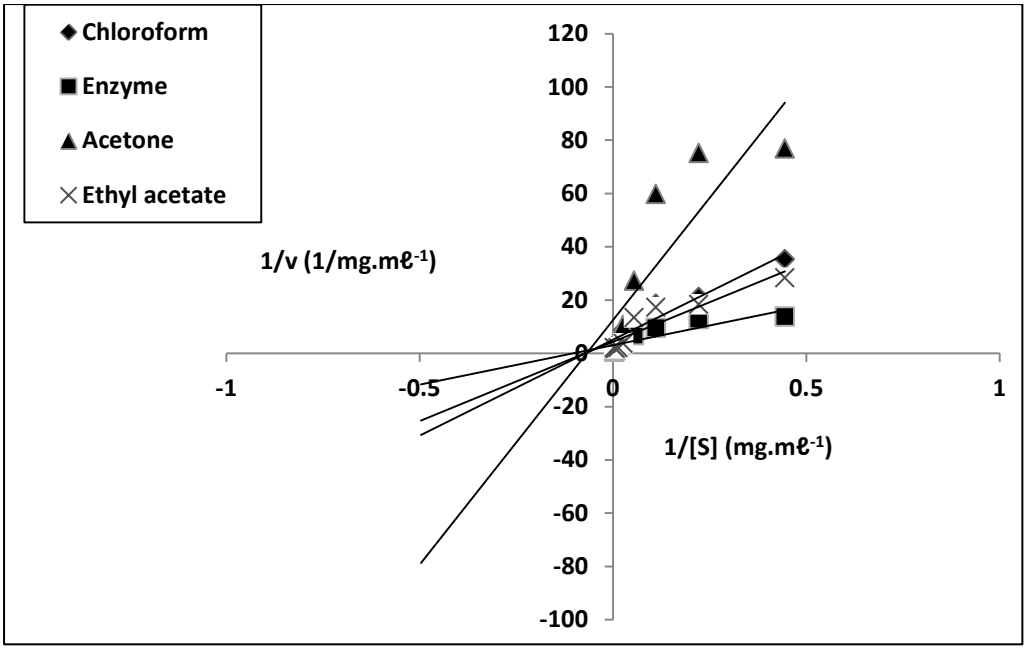


Figure 4.4.2a: Lineweaver-burk plot showing the type of inhibition exerted by chloroform, ethyl acetate and acetone extracts of *H. integrifolius* on α-amylase enzyme.

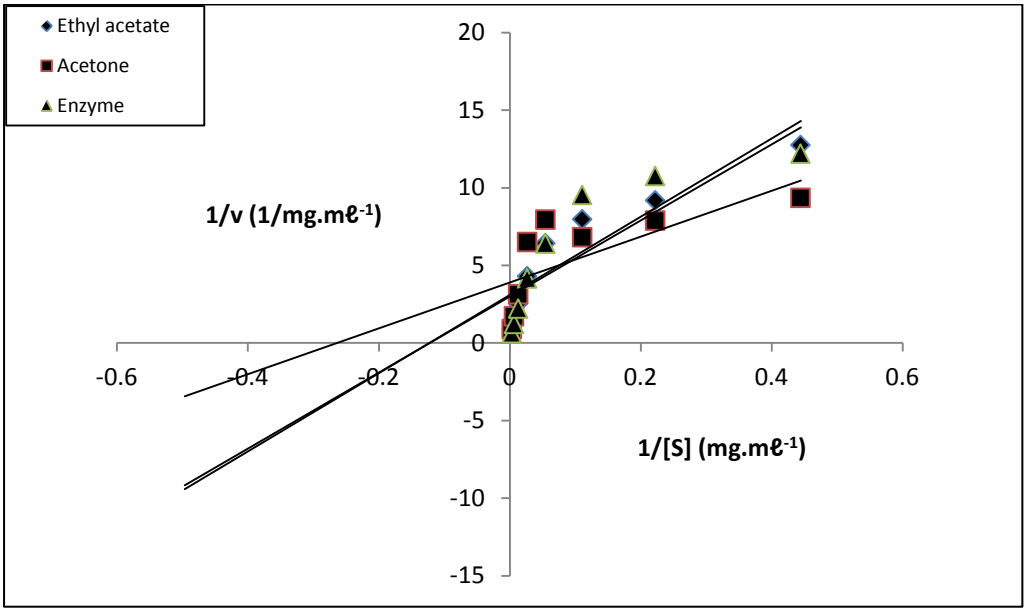


Figure 4.4.2b: Lineweaver-burk plot showing the type of inhibition exerted by acetone and ethyl acetate extracts of *C. abbreviata* on α-amylase enzyme.

4.4.3b. α-Glucosidase enzyme kinetics

Cassia abbreviata and *Helinus integrifolius* plant extracts were furthermore tested for the type of inhibition they exert on α-glucosidase enzyme. The concentration of the substrates (maltose and sucrose) for the α-glucosidase enzyme was varied. All the plant extracts (10 mg/mℓ), displayed a non-competitive inhibition towards the α-glucosidase enzyme (figure 4.43a-d). Figure 4.4.3a and 4.4.3b demonstrate the type of inhibition exerted by *H. integrifolius* while figure 4.4.3c and 4.4.3d demonstrate the type of inhibition exerted by *C. abbreviata.* The graph below (figure 4.4.3a) shows the type of inhibition exerted by acetone and ethyl acetate extracts of *H. integrifolius* on α-glucosidase enzyme. The tested extracts displayed a non-competitive type of inhibition. The K_m of the enzyme remained the same at -0.49 mg/mℓ.S^{-1} in the presence of both acetone and ethyl acetate extracts. The V_{max} was reduced from 0.13 mg.mℓ^{-1} to 0.06 and 0.03 mg.mℓ^{-1} after adding ethyl acetate and acetone extracts respectively.

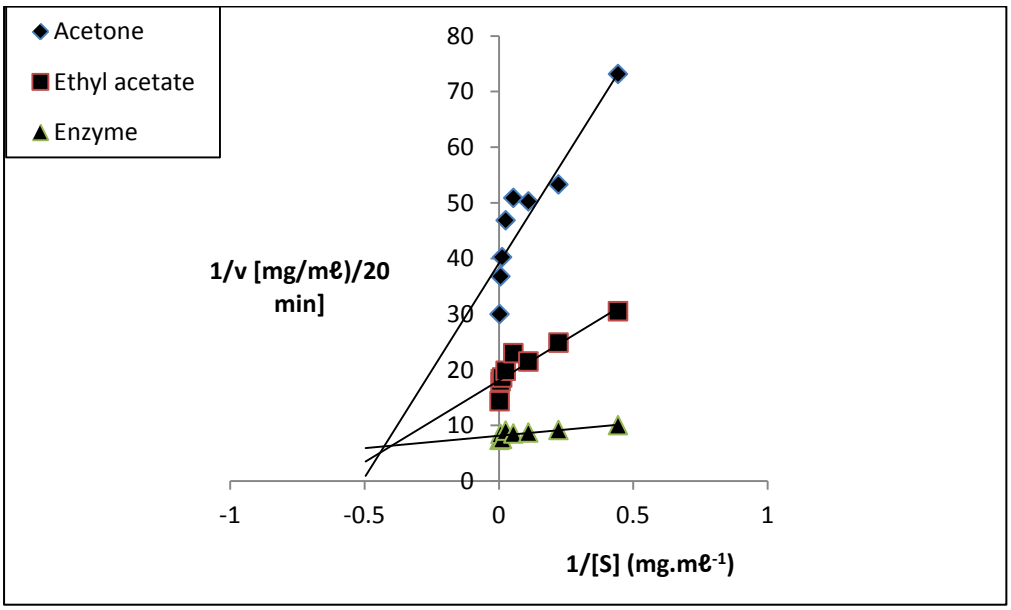


Figure 4.4.3a: Lineweaver-burk plot showing the type of inhibition exerted by acetone and ethyl acetate extracts of *H. integrifolius* on α-glucosidase enzyme when using sucrose as a substrate.

When using maltose as a substrate (figure 4.4.3b), K_m remained unchanged at 0.01 mg/mℓ.S^{-1}. Ethyl acetate extracts of *H. integrifolius* decreased the V_{max} of the

enzyme from 0.783 to 0.31mg.mℓ$^{-1}$ while the acetone extracts decreased the Vmax from 0.783 to 0.130 mg.mℓ$^{-1}$.

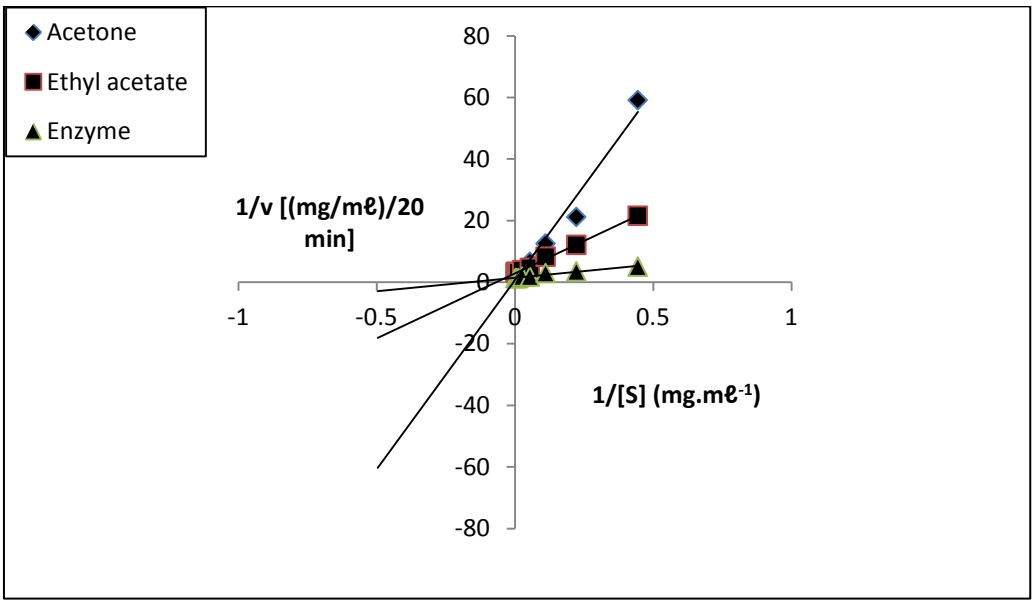


Figure 4.4.3b: Lineweaver-burk plot showing the type of inhibition exerted by acetone and ethyl acetate extracts of *H. integrifolius* on α-glucosidase enzyme when using maltose as a substrate.

Cassia abbreviata plant extracts (figure 4.4.3c and 4.4.3d) also displayed a type of inhibition similar to that of *H. integrifolius*. The V_{max} of the enzyme decreased from 0.111 mg.mℓ$^{-1}$ to 0.1095 mg.mℓ$^{-1}$ when treated with acetone extracts and to 0.05 mg.mℓ$^{-1}$ with ethyl acetate extracts.

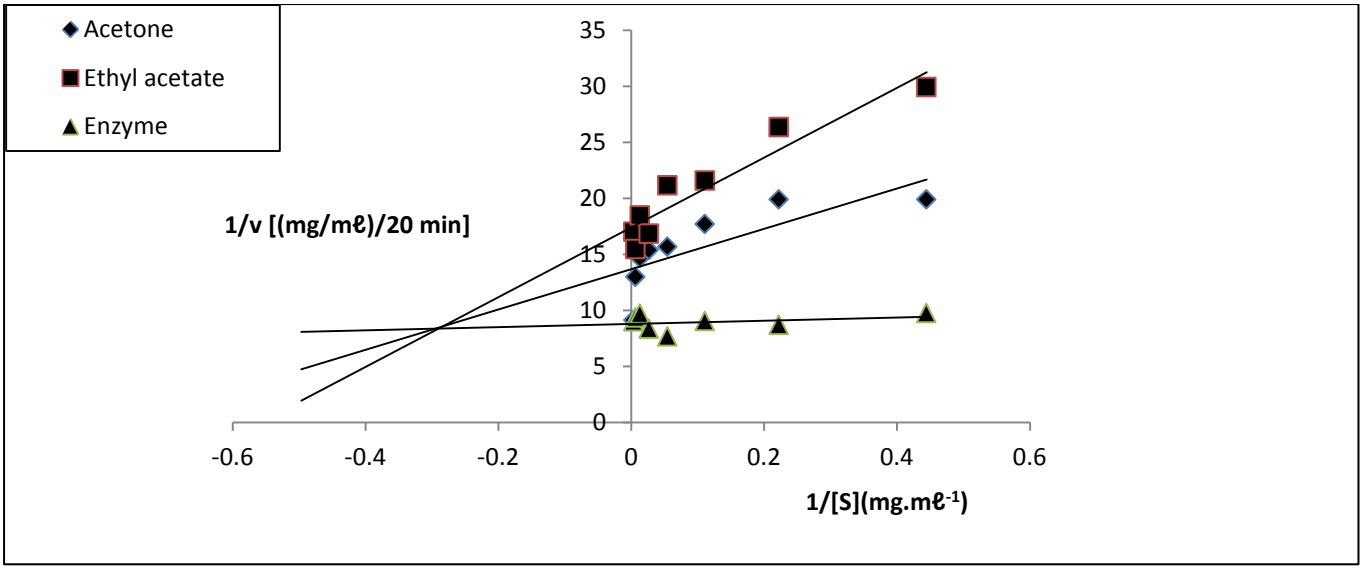


Figure 4.4.3c: Lineweaver-burk plot showing the type of inhibition exerted by acetone and ethyl acetate extracts of *C. abbreviata* on α-glucosidase enzyme when using sucrose as a substrate.

The same trend of inhibition was observed when *C. abbreviata* extracts were tested using maltose as substrate. The V_{max} of the enzyme decreased from 0.75 to 0.37 and 0.36 0.13 mg.mℓ$^{-1}$ following the treatment of the enzyme with ethyl acetate and acetone extracts respectively with K_m remaining the same at -0.25 mg/mℓ.S^{-1}.

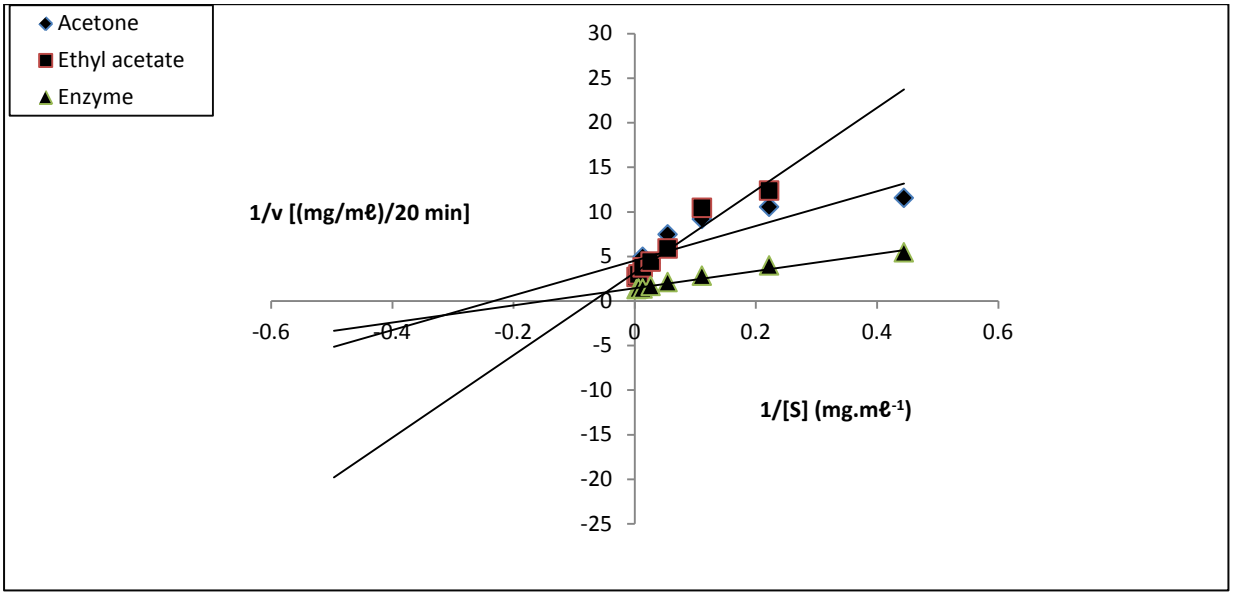


Figure 4.4.3d: Lineweaver-burk plot showing the type of inhibition exerted by acetone and ethyl acetate extracts of *C. abbreviata* on α-glucosidase enzyme when using maltose as a substrate.

4.5. Discussion and conclusion

Antioxidants which are present in plants, herbs and those from dietary sources help in preventing macro-vascular complications in diabetic patients (Buyukbalci and Sedef Nehir, 2008). Tannins and flavonoids are the secondary metabolites contained in plants that are considered to be rich natural source of antioxidants, which are capable of preventing the destruction of *β*-cells and diabetes-induced ROS formation (Aslan *et al*., 2010). As such the use of plants can be considered a good strategy to manage diabetes, especially those plants that exhibit good enzyme inhibitory activity and capable of scavenging free radicals in order to prevent oxidative stress and diabetic complications (Joshi *et al*., 1999).

In the present study, the selected plants were tested for antioxidant activity, quantitatively. The plants were tested at various concentrations after serial dilution and vitamin C was used as a positive control. Antioxidant activity of each plant extract was observed when the purple colour of DPPH was reduced to yellow in the presence of plant extracts.

In general, data revealed that most of the extracts of plants with high antioxidant activity were those of the acetone and ethyl acetate extracts. In this study, plant extracts with the lowest IC_{50} are considered to have the highest antioxidant activity. The acetone extract of *H. integrifolius* and hexane extract of *M. stuhlmanni* exhibited potent antioxidant activity with an IC_{50} of 0.019 mg/mℓ followed by acetone extracts of *S. italica* and *C. abbreviata* (0.020 and 0.0206 mg/mℓ) respectively. Most of the hexane and chloroform extracts did not show radical scavenging capability at concentrations lower than 2.5 mg/mℓ. The activity observed with acetone extracts maybe due to the presence of intermediate polar compounds present in the extracts. Previous reports have associated high antioxidant activity with the presence of intermediate to polar compounds in extracts (Li *et al*., 2005; Lee *et al*., 2000). It is also likely that the antioxidant activity may be associated with the total phenolic content of the extracts. Majority of phenolic compounds have been found to contain ascorbic acid, tocopherol and pigments which possess high antioxidant activity (Li *et al*., 2005). Arbianti *et al*., (2007) also reported that there is a correlation between total phenolic content of a plant and its antioxidant activity. Interestingly, in our findings, not all the plants exhibited that correlation, suggesting that not all the phenolics present in the plant extracts have antioxidant activity.

The antidiabetic activity of the plant extracts was determined by testing their inhibitory capabilities on carbohydrate digesting enzymes. Recent studies indicate that inhibition of these enzymes delay the absorption rate of glucose leading to suppression of postprandial hyperglycemia (Kumar *et al*., 2012). Currently in the market, acarbose and voglibose are used as α-amylase and α-glucosidase inhibitors. However these therapeutic agents are associated with undesirable side effects. Ten plants under investigation demonstrated different degrees of inhibitory activities against both α-amylase and α-glucosidase enzymes.

In the α-amylase inhibition assay, two colorimetric methods were employed namely DNS and CNPG3 reagent method. In both cases the qualities of enzymatic inhibition were determined by calculating IC_{50} of the plant extracts, with lower numbers indicating higher enzymatic inhibition. When using the CNPG3 reagent method, the highest inhibitory activity towards the enzyme was obtained with the acetone extracts of *C. abbreviata* (0.5 mg/mℓ) followed by chloroform extract of *H. integrifolius* (1.2 mg/mℓ), suggesting that an increase in concentration may result in further inhibition of the enzyme. The results also show that the majority of plant extracts were not able to inhibit α-amylase enzyme at concentration lower than 2.5 mg/mℓ. With the DNS method, the majority of plant extracts inhibited at least 50% of the enzyme activity at 0.625 mg/mℓ. The observed variation between the two assays might be due to different substrates used for α-amylase enzyme in both assays. α-Amylase enzyme breaks down maltose faster than starch. Therefore it was difficult for the extracts to inhibit the enzyme's activity when using maltose as a substrate than when using starch. However, some plant extracts such as hexane extracts of *A. gummifera, A. venosum, B. racemosa, K. africana* and *M. stuhlmanni* had the same IC_{50} when using both methods. Among all the plant extracts tested, none of them inhibited enzyme activity as compared to acarbose the positive control with an IC_{50} of 0.019 mg/mℓ. The best inhibitory activity was seen in the intermediate polar solvent (acetone and ethyl acetate) in both methods. From this observation it can be suggested that α-amylase enzyme is inhibited mostly by medium to highly polar compounds. These include compounds such as flavonols, flavonoids, triterpenes *etc* that are able to cause conformational changes in the structure of α-amylase enzyme, hence inhibiting its activity (Kim *et al*., 2000).

Alpha glucosidase is an enzyme also involved in the digestion of carbohydrates during metabolism. The enzyme is produced by the villi lining the small intestine of mammals which hydrolysis disaccharides to monosaccharides. It is divided into maltase-glucoamylase and sucrase–isomaltase which are both involved in the final hydrolysis of starch (Jones *et al*., 2011). Inhibition of this enzyme is considered to be one of the most effective approaches to treat type 2 diabetes mellitus (Kumar *et al*., 2012). In this study, ten medicinal plants were tested for their inhibitory capabilities against α-glucosidase enzyme. Both maltase and sucrase inhibition were tested using maltose and sucrose as substrates.

Different inhibitory capabilities of plant extracts against the enzyme were observed. During the maltase inhibition assay, the enzyme was weakly inhibited by hexane and chloroform extracts of tested plants. Majority of the plant extracts inhibited 30% of the enzyme activity at high concentration of 2.5 mg/mℓ. Chloroform extract of *B. racemosa* did not show any form of inhibition against the enzyme. The highest inhibition was observed with the acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* which inhibited more than 50% of the enzyme activity at a concentration of 1.25 mg/mℓ. The observed effect may be due to high number of total phenolics, flavonoids and tannins they contain than the rest of other plant species. This was observed during preliminary phytochemical tests conducted in this study. A number of total phenolics, flavonoids and tannins have been reported by Hanhineva and colleagues (2010) to have inhibitory capability against both α-amylase and α-glucosidase enzyme. Acarbose used as positive control was a potent inhibitor compared with the tested plant extracts inhibiting more than 70% of the enzyme. This concurs with the commercial use of this drug as one of the inhibitors of carbohydrate digesting enzymes in type2 diabetic patients.

When using sucrose as a substrate, different percentages of enzyme inhibitions were observed. Most of the hexane and chloroform plant extracts inhibited just above 40% of enzyme activity. A greater inhibitory capability was seen in the acetone and ethyl acetate extracts with *H. integrifolius* inhibiting more than 80% of the enzyme at 2.5 mg/mℓ. Although there are citations (Sobiecki, 2008; Ndawonde *et al*., 2009) about the medicinal use of this plant, there are no reports about its antihyperglycemic activity at least to our knowledge. Hence its activity is speculated

to be due to triterpenes it contains. Some plant extracts such as the ethyl acetate extract of *C. abbreviata* and *H. integrifolius* showed better activity than the positive control acarbose at high concentration of 2.5 mg/mℓ. Both the acetone and ethyl acetate crude extracts of *A. gummifera* were the least active against α-glucosidase. The exact mechanism of action of these plants remains unknown, but it may be likely that the observed activity is due to the slightly intermediate polar compounds in the acetone and ethyl acetate extracts. Overall both enzymes were inhibited appreciably as the concentration of plant extracts was increased suggesting that the enzyme was inhibited in a dose dependant manner. However judging by IC_{50} of the α-amylase and α-glucosidase enzyme inhibition, most plant extracts were better inhibitors of α-glucosidase enzyme. The results follow a similar trend with previous studies showing that most plant extracts inhibit α-glucosidase better than α-amylase (Ranilla *et al*., 2010; Kwon *et al*., 2007). The exact mechanisms of these plant extracts remains unknown but it can be speculated that α-amylase is able to break down the substrate faster than α-glucosidase enzyme. Most of the plant extracts exhibited mild inhibition (about 40%) of both enzymes. This shows that increase in the concentration of these extracts may result in further inhibition.

Different enzyme inhibitors inhibit enzyme using different modes of action. Some inhibit the enzyme competitively by binding to the active site of the substrate whilst others inhibit noncompetitively by binding to any site of the substrate. Only plant extracts that inhibited both α-amylase and α-glucosidase better than others, were evaluated for the type of inhibition they exerted on the enzyme. The type of inhibition was determined using Lineweaver-burk plot. Only acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* were tested. Since K_m remained the same and V_{max} differed when evaluating both plants, it can be concluded that the enzymes were inhibited in a non-competitive manner.

Antioxidant activity of plants also played a major role in the inhibitory activity of carbohydrate digesting enzymes since the plant extracts with high antioxidant activity were better inhibitors. A study conducted by Hanhineva and colleagues in 2010, have shown that polyphenolics with high antioxidant activity are also good inhibitors of carbohydrate digesting enzymes. *Cassia abbreviata* and *Helinus integrifolius* showed better inhibiting capability of both enzymes when compared to the other

plants. Although further tests needs to be conducted, these plants may be potentially useful for controlling type 2 diabetes from *in vitro* findings obtained in this study. The effect of these plant extracts on glucose uptake by cells is detailed in the next chapter.

Chapter 5

Effects of *Cassia abbreviata* and *Helinus integrifolius* extracts on glucose uptake by muscle and liver cells.

5.1. Introduction

The human body, just like any other machine needs energy in order to function well. Glucose is the main fundamental source of energy for the human body. Once polysaccharides are broken down into glucose, the glucose enters the blood stream where it signals the pancreas to secrete insulin. Insulin is a hormone responsible for maintaining homeostasis in the body. It induces Gltu4 to transport glucose to various cells of the body, particularly the muscles and adipocytes (Braynt *et al*., 2002). The liver is the main organ that comes into first contact with insulin. It assists the body to maintain normal glucose concentration during fasting and postprandial states (Pagliassotti and Horton, 1994). Skeletal muscle which accounts for about 75% of the whole body, stores glucose in the form of glycogen and oxidizes it when needed to produce energy. Adipose tissues are one of the major tissues in the human body that stores energy in the form of triglycerides (Huang and Crech, 2007). Glucose disposal into adipose tissues is the most common mechanism by which high blood glucose level is reduced after meal. Uptake of glucose by adipose tissues is insulin dependent. In the skeletal muscle, uptake of glucose is facilitated via a signaling cascade. The signaling activates PI3K and Akt to induce translocation of Glut4 (Kang *et al*., 2012). Following translocation, glucose is transported to the cells. It is reported by many investigators that exercise increases uptake of glucose by the muscles (Gorovits and Charron, 2003; Vishnu Prasad *et al*., 2009; Martineua *et al*., 2010). In liver cells, glucose uptake is regulated by Glut2 and kinase. Following binding of insulin to the receptors (IRS) and Glut2, the enzyme glucokinase is expressed. The presence of insulin inhibits gluconeogenesis via receptor-mediated phosphoinositide-3-kinase and Akt thereby regulating glycolysis (Withers *et al*., 1999). It also promotes glycogen synthesis and storage as well as preventing production of glucose from glycogen (Schenk *et al*., 2008).

Insufficient insulin in the body results in the failure of peripheral cells to absorb glucose with a subsequent increase in high level of glucose in the blood

(hyperglycemia). High blood glucose over a prolonged period of time results in long term damage of organs such as eyes, nerves, heart and blood vessels (Marieb, 2004). Type 2 diabetes is reported to occur as a result of hepatic and peripheral (muscle and adipose tissue) insulin resistance (Cerasi, 2000). Insulin resistance of the cells is caused by factors such as free fatty acids and complex network of adipokines which reduce the cellular sensitivity to insulin. There are available therapeutic agents such as metformin and troglitazone that are used to manage this condition. These drugs increase muscle insulin sensitivity (Modi, 2007).

However, some people use medicinal plants to improve the cell's sensitivity towards insulin because they are easily affordable, accessible and have less undesirable effects (Springfield *et al*., 2005). Unfortunately many of the plants are used without knowledge of their safety and efficacy. There is a misconception that medicinal plants are natural, pure and are therefore harmless. However, recent scientific research has shown that many plants used as medicines also have toxic effects (Fennell *et al*., 2004). For example in 1988 Venter and Joubert reported that 15% of acute poisoning incidents brought to hospitals in South Africa were due to traditional medicines. Another report (Kannappan and Sundaram, 2009) suggests that upon the administration of certain herbs, different interactions can occur depending on the concentration. Some interactions can produce useful and desirable effects while others can be harmful to patients. As such toxicological studies are required to validate the traditional use of a majority of medicinal plants. Hence, the cytotoxic effects of these plants were also evaluated *in vitro* on Raw 267.4 cells.

5.2. Objectives of the study

a. To evaluate the effects of active plant extracts on glucose uptake by C2C12 muscle and H-4-II-E liver cells.
b. To evaluate the cytotoxic effects of the plant extracts on Raw 267.4 cells

5.3. Materials and methods

5.3.1. Glucose uptake assay by muscle and liver cells

Glucose uptake assay was performed to evaluate the effect of extracts with better inhibiting activity of carbohydrate digesting enzymes on glucose uptake by cells. Only the ethyl acetate and acetone extracts of *Cassia abbreviata* and *Helinus integrifolius* were assessed for their effect on glucose uptake by the muscle and liver cells. The extracts were selected based on their 50% inhibitory activity of α-amylase and α-glucosidase enzymes in the previous chapter. The method was performed according to Deutschländer *et al*. (2009) as described in section 2.7.2.

5.3.2. Cytotoxicity test (xCelligence system)

The cytotoxicity test was performed using real-time xCelligence system. The method was carried out according to the instructions of the manufacture described in detail in section 2.7.3b. The viability of cells after treatment with plant extracts was determined by the Cell Index of the cells measured. High Cell Index indicates more viable cells.

5.4. Results

5.4.1. Glucose uptake assays

The C2C12 muscle and H-4-II-E liver cells were treated with varying concentrations of acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* extracts overnight at various concentrations (125, 65, 25, 4 and 1 μg/mℓ). The ability of the cells to absorb glucose was determined spectrophotometrically after adding glucose oxidase reagent. Insulin was used as a positive control for muscle cells, while metformin was used for the liver cells only. Both extracts increased the uptake of glucose in the muscle and liver cells (figure 5.4a and b). Figure 5.4a represents the effect that *C. abbreviata* and *H. integrifolius* extracts exerted on C2C12 muscle cells. Acetone and ethyl acetate extracts of *C. abbreviata* significantly increased uptake of glucose by C2C12 cells at 65 and 125 μg/mℓ respectively. Unlike *C. abbreviata, H integrifolius* extracts did not show any effect on these cells. The positive control (insulin) increased glucose uptake by at least 2-4%. When the H-4-II-E liver cells were treated with ethyl acetate and dichloromethane extracts of *H. integrifolius*, there seemed to be an increase in glucose uptake by the cells (figure 5.4b). Glucose uptake was observed to be increased by extracts of *Cassia abbreviata* when compared to *H. integrifolius* extracts and the positive control metformin. Optimum uptake was observed when the cells were treated with 250 μg/mℓ of acetone and ethyl acetate extracts of *C. abbreviata* extracts. Dimethylsulphoxide was not shown to have any effect on the cells. Cells treated with insulin exhibited a 2% increase in glucose uptake while those treated with metformin had about 3% glucose uptake following treatment at 125 μg/mℓ.

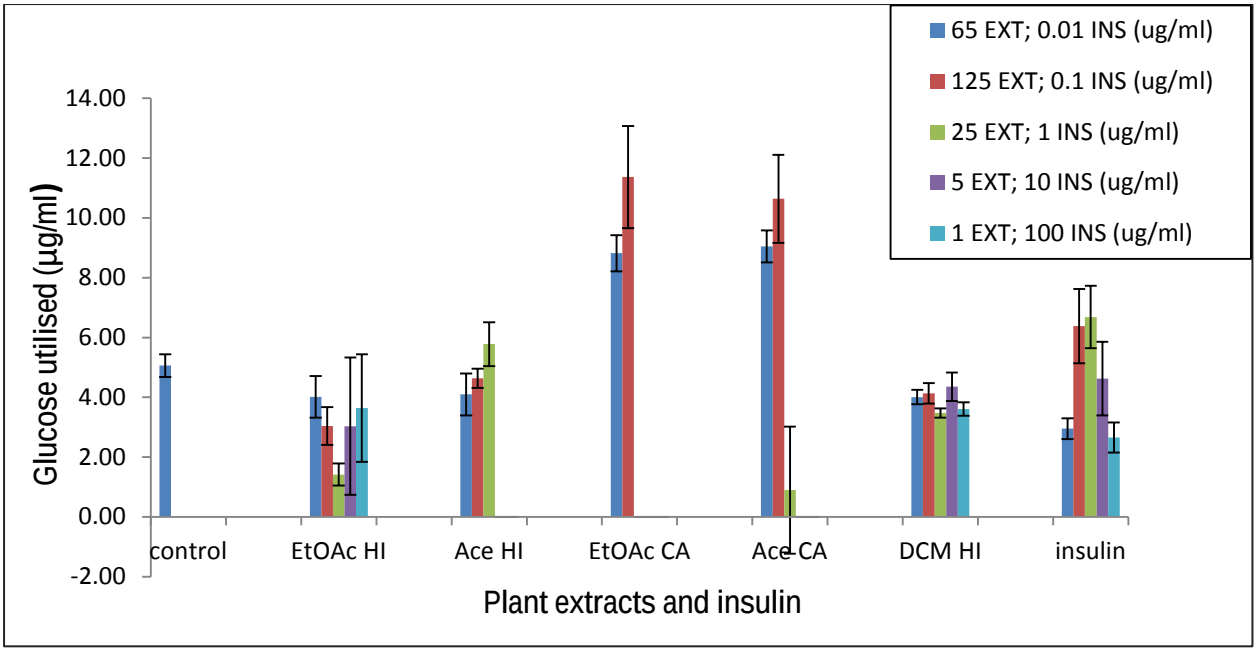


Figure 5.4.1a: The percentage of glucose uptake by the C2C12 muscle cells when treated with acetone and ethyl acetate extracts of *H. integrifolius* and *C. abbreviata* at various concentrations. Insulin was used as a positive control. HI = *Helinus integrifolius*, CA= *Cassia abbreviata,* EXT= extract, MET= metformin, INS= insulin.

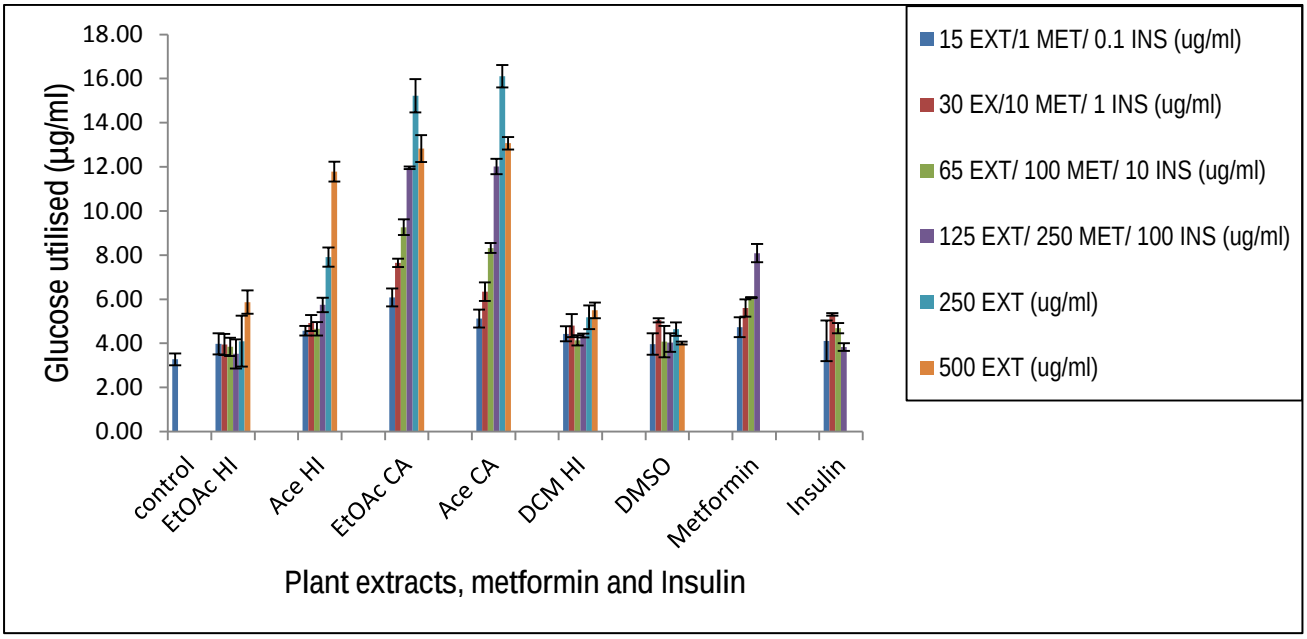


Figure 5.4.1b: The percentage of glucose uptake by the H-4-II-E liver cells when treated with acetone and ethyl acetate of *C. abbreviata* and *H. integrifolius* extracts at various concentration. Insulin and metformin were used as positive controls. HI= *H. integrifolius*, CA= *C. abbreviata,* EXT= extract, MET= metformin, INS= insulin.

5.4.2. Cytotoxicity tests

Different cell index were obtained using the real-time xCelligence system after treating the cells with different concentrations of plant extracts. The highest Cell index was obtained when the cells were treated with the lowest concentration of 0.05 mg/mℓ of all plant extracts (figure 5.4.2c-f). Most of the cell indexes were lower than 0.4 suggesting that most of the cells were still viable following treatment with 0.25 mg/mℓ of the plant extracts. Actinomycin D, the positive control was the most toxic to the cells since the cell index was very low compared to cells treated with plant extracts. When the cells were treated with acetone (figure 5.4.2a) and ethyl acetate (5.4.2b) extracts of *H. integrifolius*, had the highest cell index of about 0.4 at the lowest concentration of 0.05 mg/mℓ. The cell index decreased as the concentration of the plant extracts increased. This indicates that as the concentration of plant extracts increased, the cells became less viable.

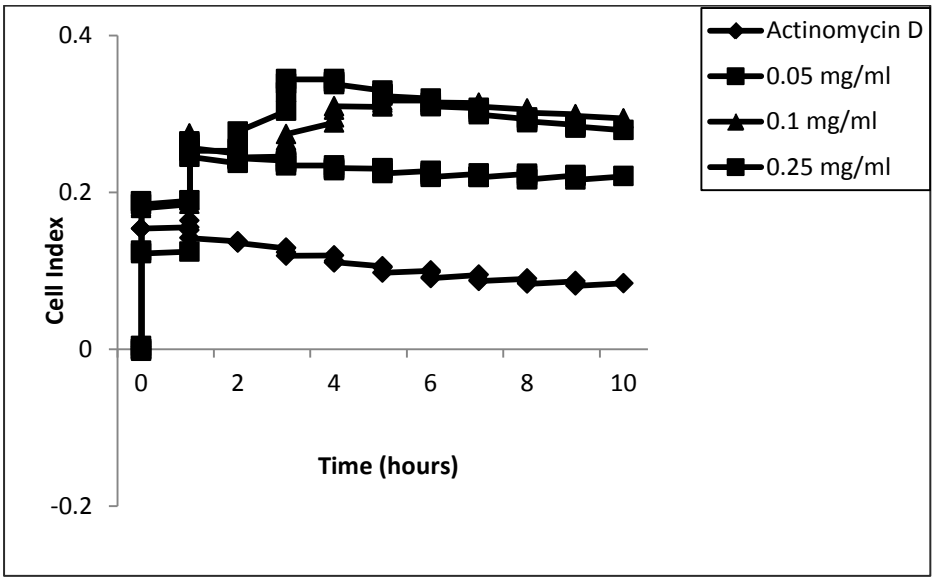


Figure 5.4.2a: Cell indexes obtained when Raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mℓ of the acetone extracts of *H. integrifolius* for 10 hrs. Positive control = Actinomycin D.

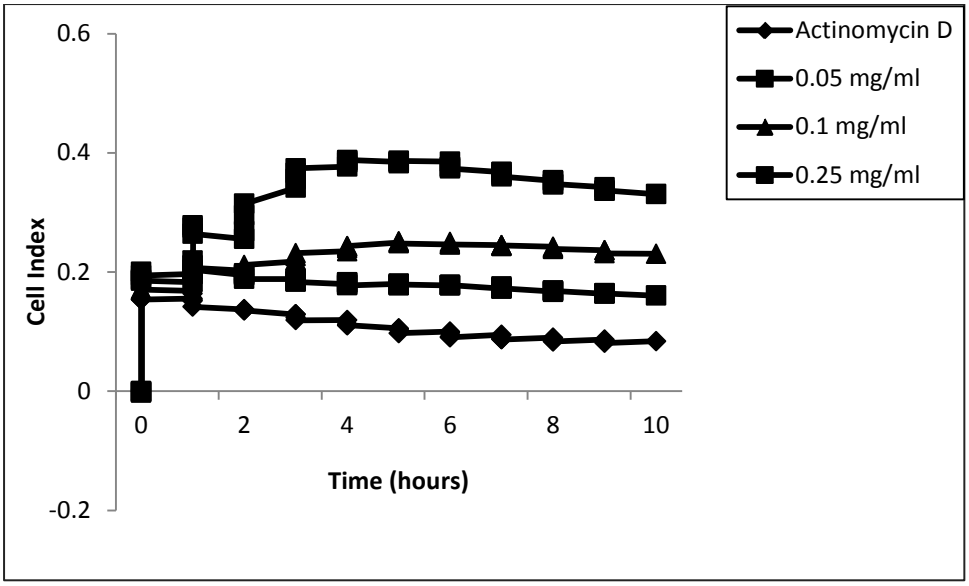


Figure 5.4.2b: Cell indexes obtained when Raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mℓ of the ethyl acetate extracts of *H. integrifolius* for 10 hrs. Positive control = Actinomycin D.

Figures 5.4.2c and 5.4.2d show different cell indexes obtained when the cells were treated with acetone and ethyl acetate extracts of *C. abbreviata*. When treated with 0.05, 0.1 and 0.25 mg/mℓ of plant extracts, the cell index decreased respectively from about 0.4, to 0.35 and 0.19. The observed effect of *C. abbreviata* was consistent with that of *H. integrifolius*. The cell index decreased as the concentration of all plant extracts increased. The highest cell index of about 0.4 was obtained following ten hours of exposure of the cells to acetone and ethyl acetate extracts of *C. abbreviata*. Cells treated with Actinomycin D yielded the lowest cell index of about 0.1, suggesting the less toxic effect of the extracts when compared to the positive control.

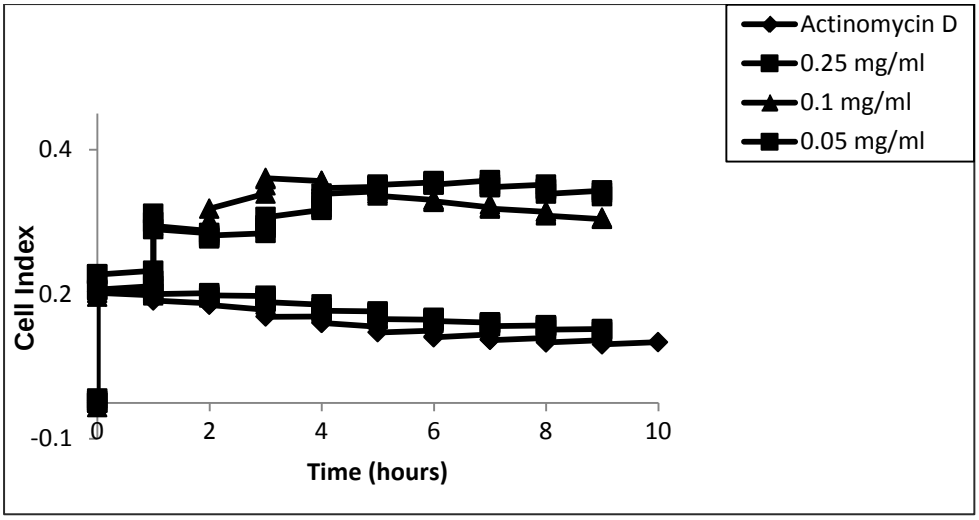


Figure 5.4.2c: Cell indexes obtained when Raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mℓ of the acetone extracts of *C. abbreviata* for 10 hrs. Positive control = Actinomycin D.

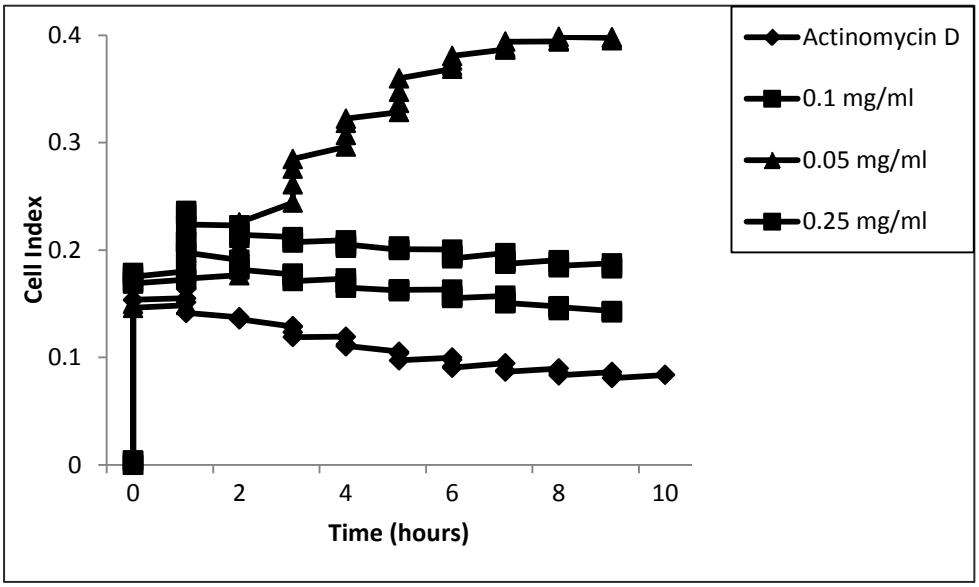


Figure 5.4.2d: Cell indexes obtained when Raw 264.7 cells were treated with 0.05, 0.1 and 0.25 mg/mℓ of the ethyl acetate extracts of *C. abbreviata* for 10 hrs. Positive control = Actinomycin D.

5.5. Discussion and conclusion

Peripheral tissues are the major sites of glucose disposal. Skeletal muscles accounts for about ±80% of insulin mediated glucose uptake. They also play a huge role in maintaining normal glucose level in the system. Decrease in glucose uptake by these cells is caused by various factors such as insulin resistance that results in high blood glucose level (Kemp *et al*,. 1997). During this condition, various oral agents that can reduce blood glucose level in different ways are used together with recommended diets and exercise. In the present study, only extracts which exhibited consistent inhibitory activity against carbohydrate digesting enzymes were evaluated for their effect on glucose uptake by the cells. The effect of each plant extract was tested at different concentrations following serial dilution with medium.

From the data obtained, plant extracts were observed to increase uptake of glucose by the cells dissimilarly at various concentrations. The effect of both plant extracts was compared with that of insulin and untreated cells. The acetone and ethyl acetate extracts of *C. abbreviata* increased glucose uptake by muscle cells significantly at 65 and 125 µg/mℓ respectively. The finding with extract of this plant is in agreement with those obtained from toxicity studies were extracts are shown to be toxic to the cells at 0.25 mg/mℓ. *Helinus integrifolius* extracts did not seem to have any effect on the uptake by muscle cells particularly at lower concentrations. This might be due to the inability of constituents in *H. integrifolius* to enhance glucose utilization at lower concentrations. As such increasing the extracts concentration may cause toxic effects to other cells which may raise concerns about toxic effect of these extracts at higher concentrations. Glucose uptake was increased notably by 10% when the liver cells were treated with both *C. abbreviata* and *H. integrifolius* extracts than muscle cells. However at 125 µg/mℓ, *C. abbreviata* extracts increased glucose uptake of muscle and liver cells correspondingly. Optimal uptake was observed as the concentration of *C. abbreviata* was increased to 250 µg/mℓ in the liver cells. Extracts of *C. abbreviata* increased glucose utilization better than *H. integrifolius* and insulin. Although the mechanism is not well understood, it is likely that the observed enhancement maybe due to compounds present in the plant extracts that have transiently disrupted the cell's mitochondria which in turn activated the AMPK pathway (Eid *et al*., 2010). Activation of AMPK leads to the

phosphorylation and regulation of downstream targets that are involved in various pathways such as acetyl-CoA carboxylase. These pathways increase phosphorylation and activity of mitogen–activitase protein kinase (MAPK) thereby activating insulin-stimulated glucose uptake via Glut4 translocation (Konrad *et al*., 2001). Although *in vitro* studies may not be a direct correlation to studies *in vivo* due to biotransformation of drugs, it is likely that the mechanism of action may be similar to that of metformin, a prominent agent used as an antihyperglycemic agent (Fryer and Carling, 2005). Despite increasing the glucose uptake, there seems to be a release of glucose in the liver cells as the concentration was increased to 500 μg/mℓ. The results suggest that there are compounds in the plant extracts that makes the cells more permeable thereby enhancing the release of glucose out of the cells. Overall more of glucose uptake was observed in the liver cells than muscle cells in the absence of insulin. Metformin used as positive control had little effect than the plant extracts at lower concentrations. However, the effect increased as the concentration was increased to 250 μg/mℓ. It can be assumed that the plant extracts had compounds that mimic insulin activity leading to more glucose absorption and also their entry into the cells or that the signalling cascade they utilize, may play a role (Zygmunt *et al*., 2010). Most plant extracts are reported to mimic insulin through the induction of tyrosine phosphorylation of insulin receptors, IRS1 and PI3K leading to glucose uptake by adipocytes and liver cells (Vijayakumar *et al*., 2005).

Toxicity tests are important and useful to assess the extent of cytotoxicity of various compounds and extracts. There are different *in vitro* assays that can be used to evaluate cytotoxicity such as XTT, MTT etc. Majority of these assays are usually less expensive, quantitative and more reproducible than *in vivo* studies (Saad *et al*., 2006). In this study, only acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* were tested for their toxic effect on the Raw 264.7 cells. This system measures the cellular impedance which depends on the level of the cell confluency after treatment (Urcan *et al*., 2010). The real time experiments are normalised using the media without cell line as the basis of impedance. The value is expressed as Cell Index (CI) which is proportional to the number of viable cells attached to and spreading on the bottom of the microplate wells. An increase in the cell index shows the viability of cells and also indicates that a compound or plant extract is less toxic to the cells. Cell Index of about 0.4 was obtained when the cells were treated at the

lowest concentration of 0.05 mg/mℓ of all plant extracts. At highest concentration of 0.25 mg/mℓ, the Cell Index decreased to less than 0.2. Overall the cells had a comparable Cell Index when treated with *C. abbreviata* and *H. integrifolius* extracts. This shows that the toxicity level of both plant extracts from both species towards the cells is more or less the same. Since *C. abbreviata and H. integrifolius* are shown to have different kinds of compounds in the previous chapters, the observed toxicity effects might be due to some of the compounds they contain. A study conducted by Dharani *et al.* (2008) exhibited that some triterpenes are toxic to humans and other mammals. All cells treated with both plant extracts had a higher Cell Index compared to Actinomycin D which is used to inhibit proliferation of cells.

Both plant species were able to enhance glucose uptake by both muscle and liver cells without insulin. However, further studies should be carried out to understand the molecular mechanism through which these effects are mediated. Also, given that the plant extracts were less toxic in *in vitro* assay, more toxicity tests using *in vivo* assay should be conducted to compare these results. These results are of major importance in providing more information that could lead to development of new treatment for diabetes mellitus.

Chapter 6

Isolation and characterization of active compounds from DCM extracts of *Helinus integrifolius*

6.1. Introduction

Scientific studies show that medicinal activity of plants is attributed to the mixture of different bioactive constituents synthesized by the plants (Rates, 2001). Most of these constituents are present as mixtures with only a small active component. Some of the bioactive compounds are active when combined but others work best individually. According to Rates (2001), the most commonly used method of studying natural products includes the fractionation of a complex mixture, separation and isolation of individual compounds. Separation and determination of active compounds in plants also makes it easier to study the pharmacological, pharmacokinetics and toxicity of the plant (Sun and Sheng, 1998). The presence of other plant constituents such as proteins, fats, sugars and lipids makes it difficult to isolate and determine the active biomolecules. Hence separation techniques with high efficiency and selectivity are required (Wen *et al*., 2004). According to Sasidharan *et al*., (2011) isolation should be guided by bioassays. The bioassays help in identifying the bioactivity of crude extracts and act as a guide for further fractionation and isolation of a bioactive compound. For these purpose, bioassay must be rapid, simple, reliable, reproducible and predictive. One of the ways of obtaining pure active compounds from extracts is through sequential fractionation and qualitative analyses on TLC (Verpoorte, 1989). Thin layer chromatography is a simple and rapid method for analyzing a mixture of plant components. The chemical fingerprint on the TLC plates help in identifying different chemical constituents present in plant extracts (Wagner and Bladt, 1996).

6.2. Objectives

a. To isolate compounds from DCM extracts of *Helinus integrifolius*
b. To test the inhibitory capability of the isolated compound against α-amylase enzyme
c. To compare the polarity of the isolated compound with the crude extract
d. To compare the inhibitory capability of isolated compound with the crude extract

6.3. Materials and methods

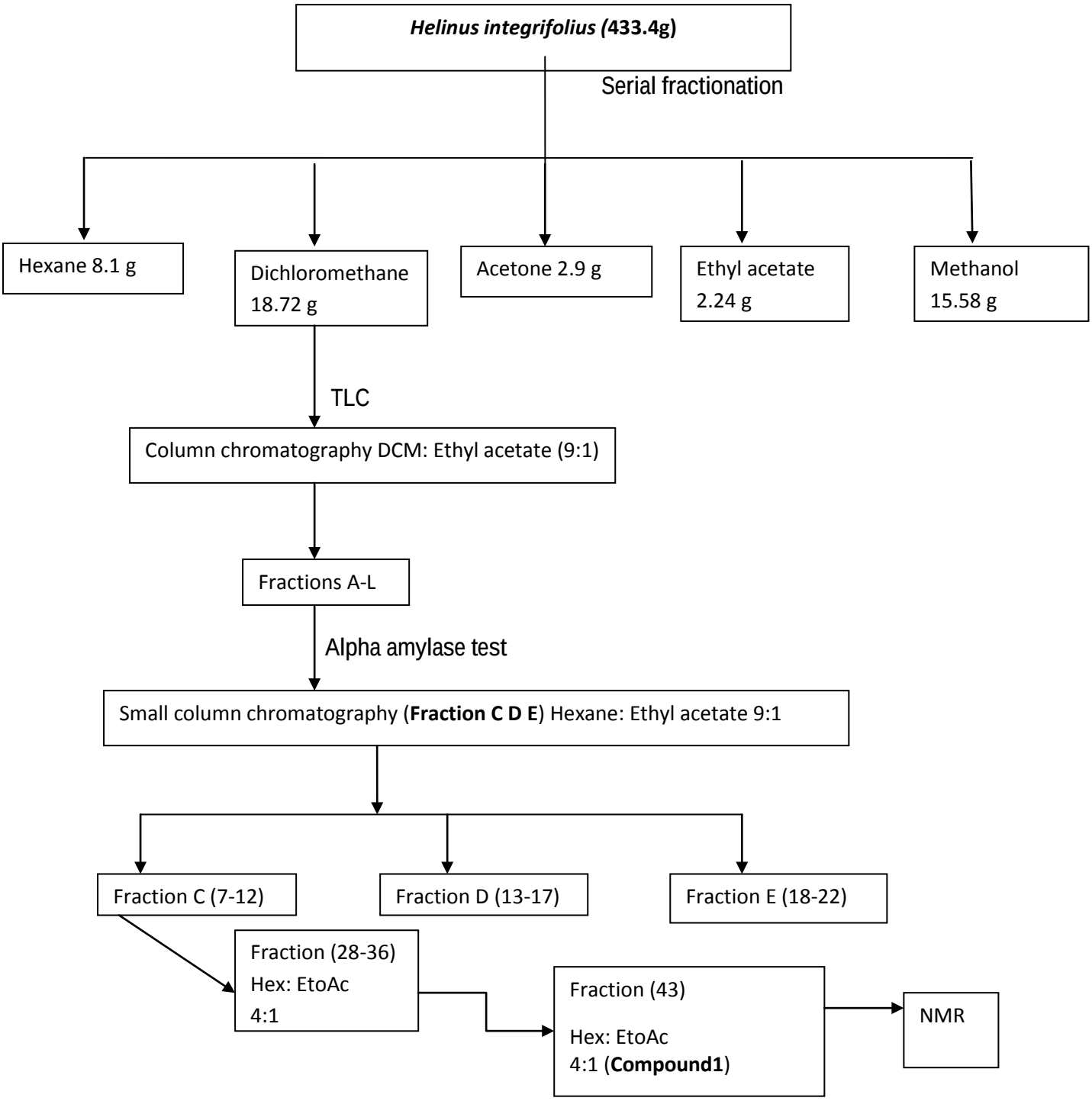


Figure 6.3.1: A schematic representation of the procedure followed when isolating active compounds from DCM extract of *H. integrifolius*.

6.3.1. Serial fractionation

Serial fractionation of the finely grounded powdered leaves of *Helinus integrifolius* weighing 433 g was exhaustively extracted with five different solvents namely; hexane, dichloromethane, acetone, ethyl acetate and methanol respectively as elaborated in section 2.8.1.

6.3.2. Column chromatography

About 10 g of dichloromethane (DCM) extracts was further re-dissolved in 100 mℓ of DCM together with 30 g of silica gel 60 (Merck) for column chromatography (detailed in section 2.8.2).

6.3.3. Isolation of compound from fraction C

Silica gel 60 (50 g) was dissolved in 150 mℓ of hexane to make a slurry and subsequently packed in a glass column (40x4 cm). Fraction C (0.5 g) eluted with 100% DCM was mixed with hexane and 5 g of silica gel 60. The mixture was dried and evenly spread on top of the silica gel in the packed column covered with cotton wool. The column was eluted with 1ℓ of 100% hexane followed by polarity increments with 1ℓ of 10% hexane: ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60 etc) respectively. Fractions were eluted from the column with varying polarities of hexane. Fractions were collected in 20 mℓ test tubes, air dried and further loaded on TLC plates to ascertain the profile of fractions with the same compounds. Fractions with the same compounds were pooled together and tested for alpha amylase inhibition capability. The most active sub-fractions were further run in a small column (20x2 cm) and eluted with increasing polarities of Hex: EtoAc (9:1, 8:2, 7:3 etc respectively). Fractions eluted were loaded on TLC plates, developed in Hex: EtoAc (4:1) systems and sprayed with vanillin/ H_2SO_4 reagent to view the separated compounds. Fractions with single bands on the plates were combined together and purified with hexane. Following purification, a white precipitation was formed. The precipitate was further dried in pre-weighed vials and the compound crystallized out.

To determine which compound was isolated from the crude extract, 0.1 g of white crystals were dissolved in hexane and toluene and loaded on the TLC plate together with DCM crude extract of *H. integrifolius.* The plate was developed in Hex: EtoAc

(4:1) and sprayed with vanillin/sulphuric acid to view the separated compounds. For optimum colour development the plate was further dried in an oven at 110^{o}C for 30 seconds.

The activity of the isolated compound of interest was tested for α-amylase enzyme inhibition using the CNPG3 reagent kit. The compound was dissolved in DMSO to make a concentration of 10 mg/mℓ and further serial diluted. The procedure was followed as in section 2.1.6.1.

6.3.4. Characterization of compounds using nuclear magnetic resonance (NMR)

Nuclear magnetic resonance is a technique used to determine the chemical structure of compounds. The samples were run with a Varian 400 MH_t spectrophotometer and benzene was used as a solvent reference.

6.4. Results

6.4.1. Chemical profiling (Thin layer chromatography)

Phytochemical analysis of extracts from *Helinus integrifolius* obtained after bulk extraction (serial fractionation) was done using TLC method. The plates were developed in three solvent systems (TEA, CEF and EMW) to separate the compounds and further sprayed with vanillin-sulphuric acid reagent to detect separated compounds. All the extracts contained chemical compounds represented by different coloured bands on TLC plates. More compounds were separated better in the TEA mobile system. The methanol extracts had the least number of compounds separated in the TEA system. Only acetone and ethyl acetate extracts had more compounds separated in the EMW and CEF mobile systems.

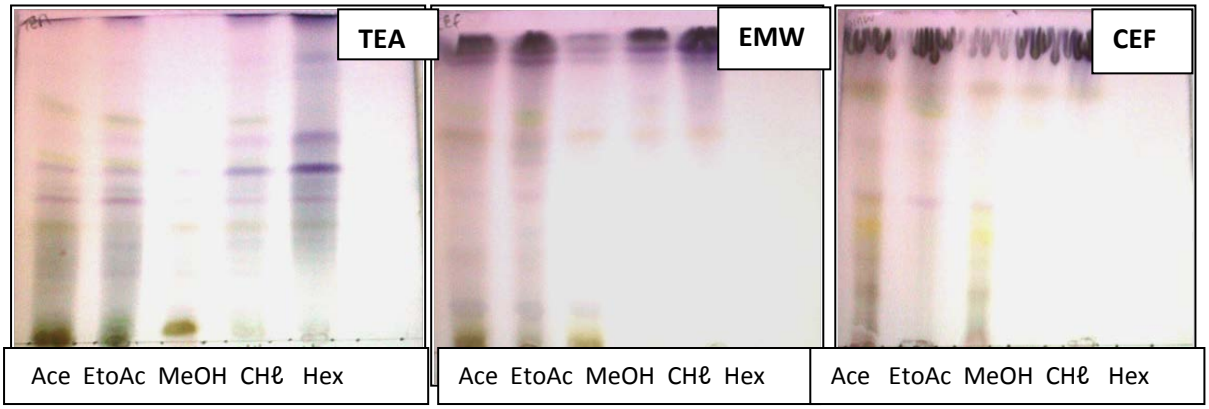


Figure 6.4.1a: Chromatograms of the five extracts of *H. integrifolius* extracted with five different solvents. Ace = Acetone, EtoAc = Ethyl acetate, MeOH = Methanol, CHℓ = Chloroform and Hex = Hexane.

6.4.2. Chromatogram of fractions eluted from the column

Twelve fractions were obtained from the first open column. The plates were developed in a Hex: EtoAc (9:1 and 4:1) mobile phases and sprayed with vanillin-sulphuric acid reagent as depicted in figure 6.4.1a. Different compounds found in DCM fraction of *H. integrifolius* were separated based on their polarity. Most compounds represented by different coloured bands were clearly separated in the 4:1 mobile phase system (B) than 9:1 mobile phase (A).

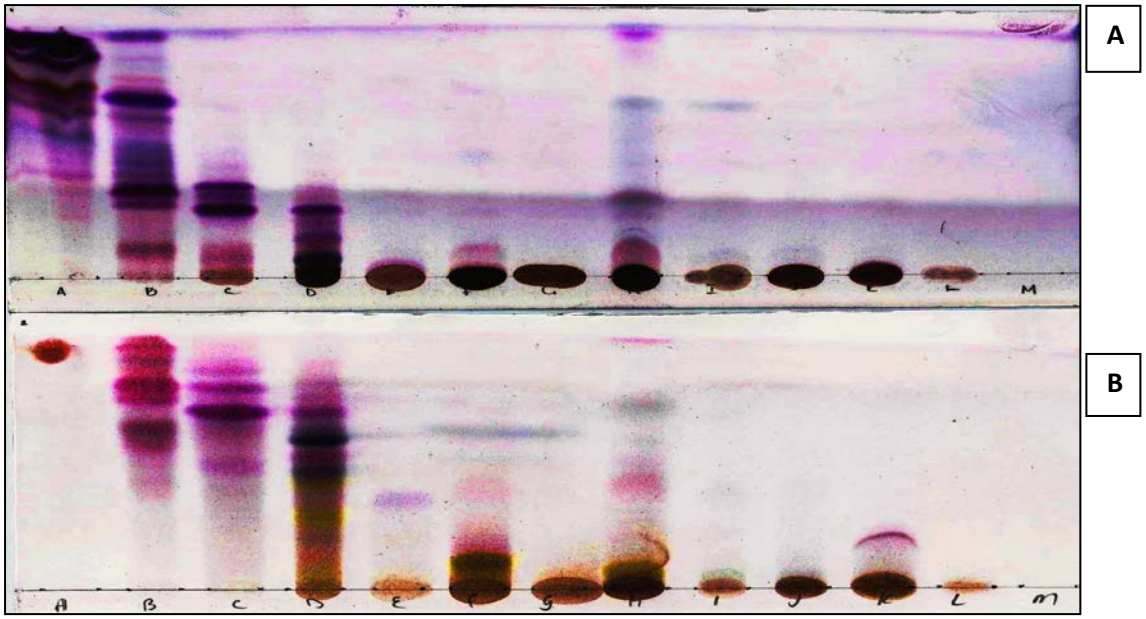


Figure 6.4.2a: Chromatograms of 13 (A-M) fractions eluted from the column, (A-C) with 100% DCM, (D-E) with 90:10 and (F-M) with 50:50 DCM: Ethyl acetate. The plates were developed in hexane: ethyl acetate [9:1(A) and 4:1 (B)] and sprayed with vanillin/ H_2SO_4 reagent.

6.4.3. Fractions tested for α-amylase inhibitory activity

The figures below (6.4.2b-2c) show the inhibitory capability of fractions eluted from the column against α-amylase. All twelve fractions were tested for α-amylase inhibition capability using the CNPG3 reagent kit and acarbose as positive control. Figure 6.4.2b shows inhibition activity of fractions (A-F) and acarbose. Fraction D and acarbose showed a good activity, inhibiting almost 100% of the enzyme at IC_{50} of 1.5 mg/mℓ. Fraction C and F were the second best inhibiting 80% at IC_{50} of 2.5 mg/mℓ. All the fractions inhibited the α-amylase enzyme activity in a concentration dependent manner.

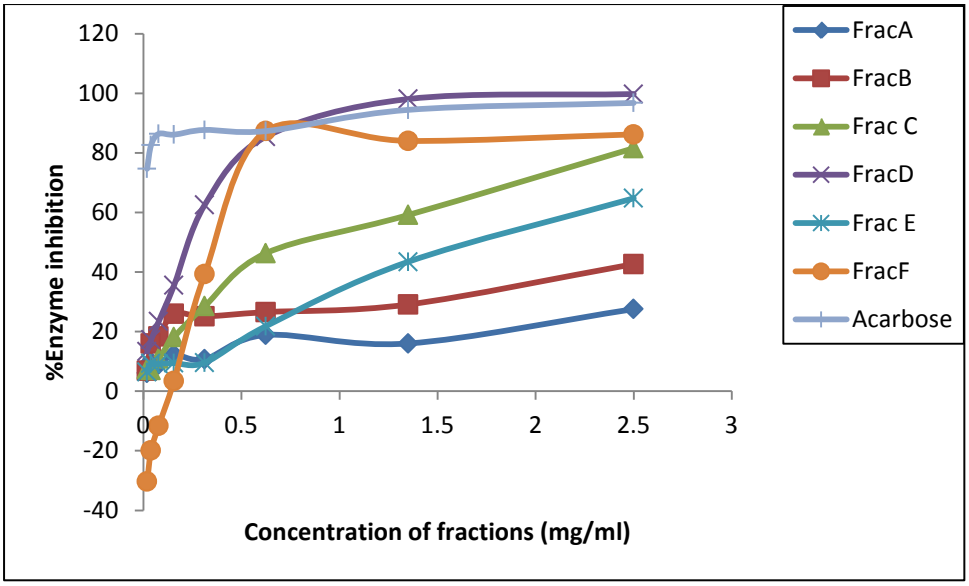


Figure 6.4.2b: Alpha amylase inhibition by fractions (A-F) eluted from the column and dissolved in DMSO to make up 10 mg/mℓ. Acarbose was used as a positive control.

The figure below shows the inhibitory activity of fractions (G-L). All the fractions inhibited α-amylase enzyme in a concentration dependent manner. Only fractions J and K inhibited more than 50% of the enzyme activity. Fraction I was the least active fraction inhibiting less than 10% of the enzyme activity. Acarbose inhibited almost 100% of the enzyme activity.

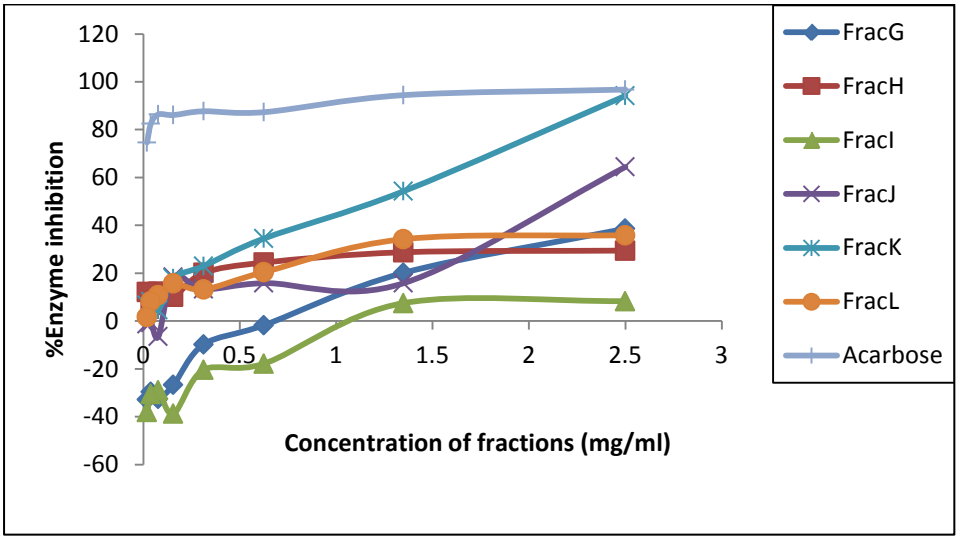


Figure 6.4.2c: Alpha amylase inhibition by fractions (G-L) eluted from the column and dissolved in DMSO to make up 10 mg/mℓ. Acarbose was used as a positive control.

6.4.4. Purification of fraction C

Fraction C was further run in an open small column. The sub-fractions were eluted with increasing polarity of DCM: EtoAc (90:10) and visualized on TLC plates as shown in figure 6.4.2d. Fractions with a single band (28-36) were combined together and re-eluted in a small column and also visualized on TLC plates as depicted in figure 6.4.2e. Different coloured bands on TLC plates symbolize compounds obtained from each fraction.

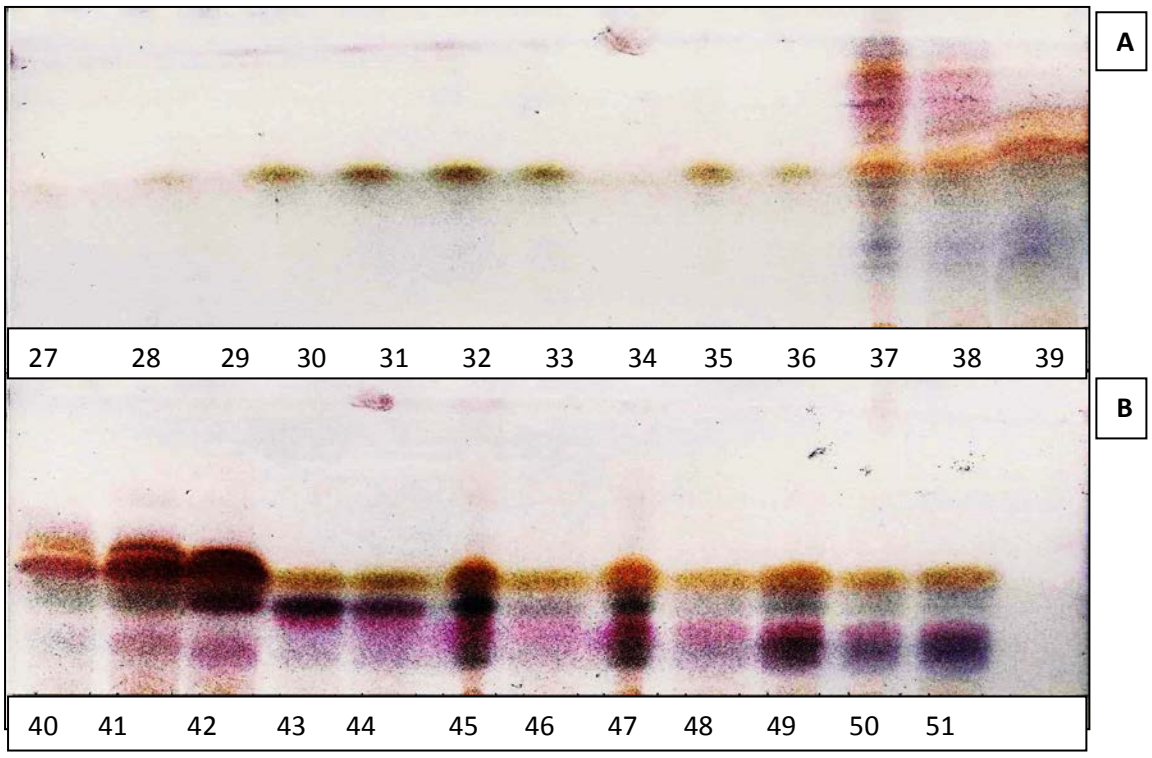


Figure 6.4.2d: Chromatograms obtained from sub-fraction C. The plates were developed in Hex: EtoAc (4:1) and sprayed with vanillin/ H_2SO_4 reagent. A= (fraction 27-39) and B = (fraction 40-60).

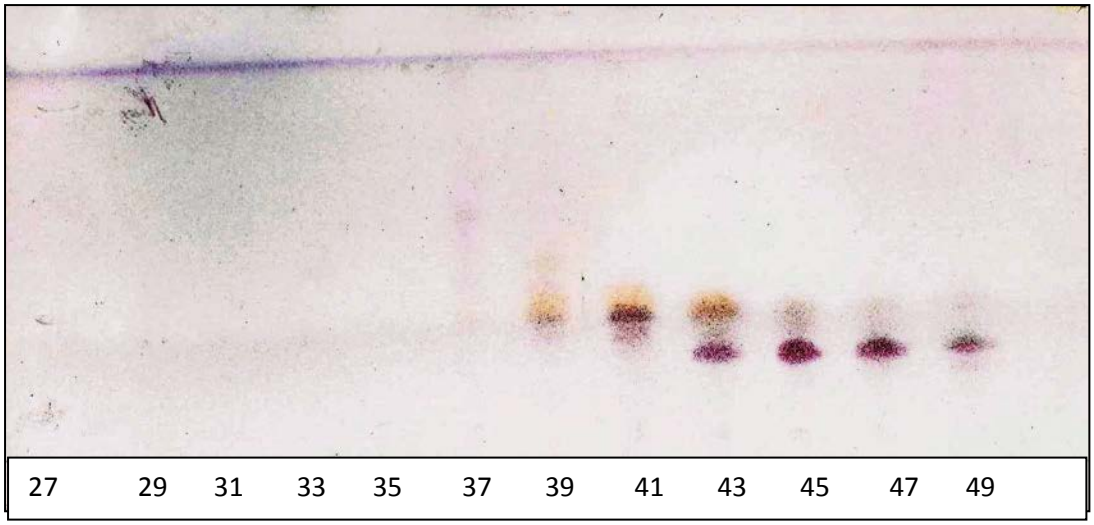


Figure 6.4.2e: Chromatogram obtained of sub-fractions (28-36) from sub-fraction C eluted with Hex: EtoAc (90:20) and developed in Hex: EtoAc (4:1). Plates were sprayed with vanillin/ H_2SO_4 reagent to view the compounds.

6.3.5. Further purification of fraction C

Fractions 41 – 49 were purified with hexane until a pure white precipitates was obtained. About 10 µℓ of the precipitate of each fraction was loaded on TLC plate (figure 6.3f). The plates were developed in Hex: EtoAc (4:1), sprayed with vanillin/ H_2SO_4 and heated in an oven at 110^{o}C until optimum colour development. Purple coloured bands represent compounds of interest.

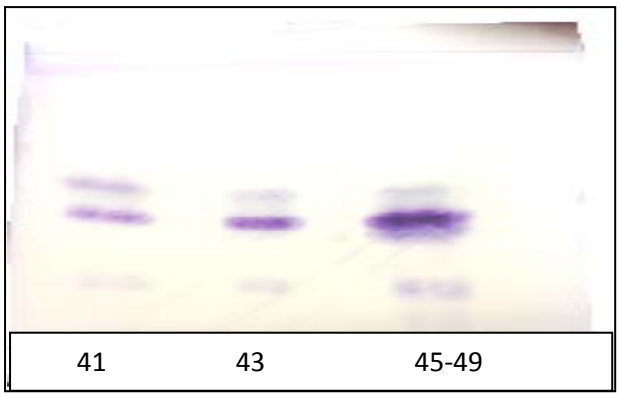


Figure 6.4.2f: Chromatogram showing the target compounds from pooled fractions. The chromatogram was developed in Hex: EtoAc (4:1) and sprayed with vanillin/H_2SO_4 for visualization of the compounds.

6.4.6. Comparing the polarity of the target compound with those of crude extract

The precipitates were allowed to dry, weighed and stored. Fraction 34 was re-dissolved in hexane and toluene. About 10 µℓ of each was loaded on TLC plates together with DCM crude of *H. integrifolius* (figure 6.4g). The plate was developed in Hex: EtoAc (4:1) and sprayed with vanillin/H_2SO_4, heated in an oven at 110^oC until optimum colour development. Purple coloured bands symbolize compounds eluted from each fraction.

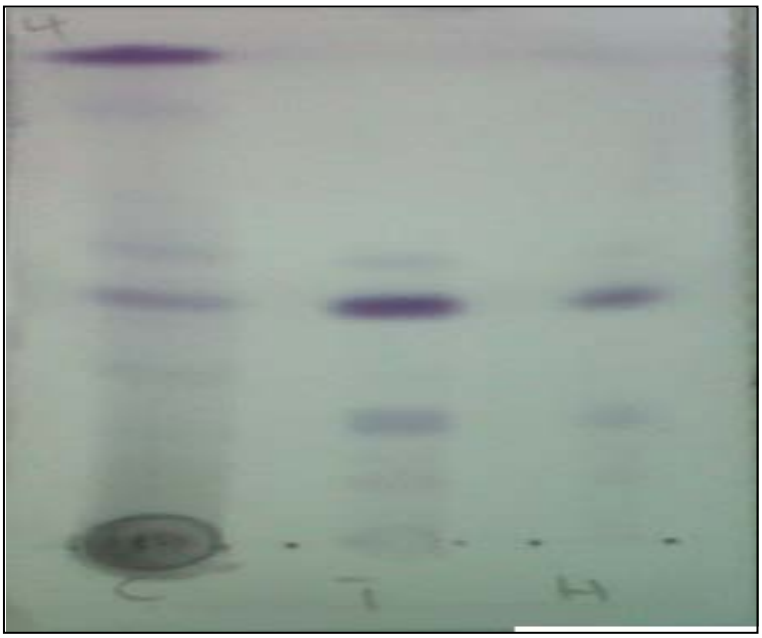


Figure 6.4.2g. Chromatogram comparing DCM crude extract *of H. Integrifolius* with the target compound from fractions 43 dissolved in toluene and hexane. The chromatogram was developed in Hex: EtoAc (4:1) and sprayed with vanillin/H_2SO_4 for visualization of the compounds. C- Crude, T- toluene and H- hexane.

6.4.7. Determining the inhibitory activity of the target compound

To evaluate the activity of the compound isolated, the precipitate was re-dissolved in DMSO to make a final concentration of 10 mg/mℓ and tested for α-amylase inhibitory capability using the reagent kit assay. The activity of the compound was compared with DCM crude extract and acarbose after serial dilution. The crude inhibited 50% of the enzyme at 1.2 mg/mℓ whilst fraction 43 didn't inhibit more than 50% of the

activity of the enzyme. Acarbose (<2.5 mg/mℓ) used as positive control, inhibited about 90% of the activity of the enzyme.

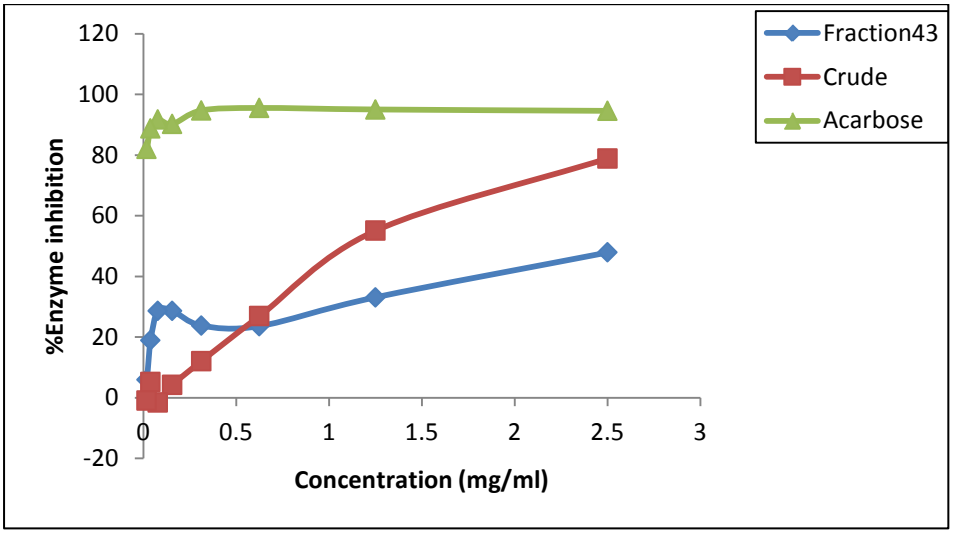


Figure 6.4.2h: Alpha amylase enzyme inhibition by crude and compound isolated from fraction 43 of the DCM extract of *H. integrifolius*.

6.4.8 Structures of amyrin

After testing the activity of the isolated compound, NMR revealed that the compound is a mixture of α and β amyrin. Both the compounds elute at the same RF because of the same polarity. The only difference is the methyl positioning on the E- ring.

Alpha Beta

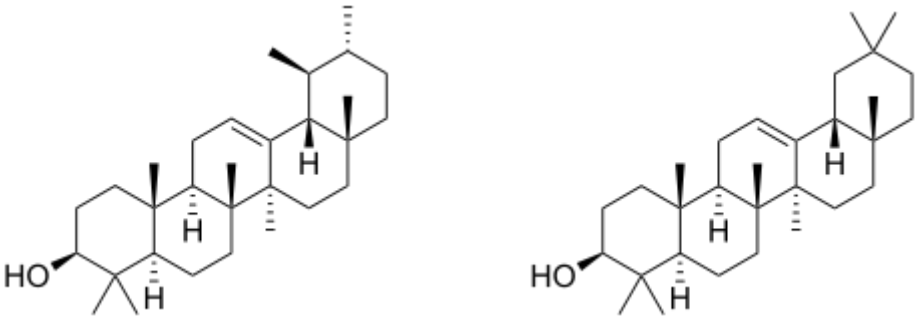


Figure 6.4.2i-j: Structures of alpha and beta amyrin

6.4 Discussion and conclusion

People have always relied on natural products as a source of drugs. Countries such as Egypt, China and India have been using plant-derived drugs for many years as a form of traditional medicine (Balandrin *et al*., 1993). Plant based drugs play an integral role in the health care system. According to WHO, 80% of the world's population rely on traditional medicine for their primary health care (Gurib-Akim, 2006). Plants used as traditional medicine contain a wide variety of phytochemicals that can be used to treat both chronic and infectious diseases. They also provide an opportunity in the discovering of new drugs due to their chemical diversity (Cosa *et al*., 2006). It has become clear that the medicinal value of plants lies within the bioactive phytochemical present in the plant. Therefore identification, isolation, purification and characterization of bioactive compounds in plants extracts is of paramount importance.

In the present study, a compound was isolated from DCM extract of *H. Integrifolius* leaves. Although not so many studies have been conducted with this plant, it is reported to be rich in saponins. Its leaves are mainly used in Africa to treat malaria, hysteria, arthritis, paralysis and backache (Nanyingi *et al*., 2008). A study conducted by Goodson (1920) shows that the leaves of *H. Integrifolius* contains acotonic acid, quercitin, saponins and scyllitol. More compounds such as lupane, ziziberylanic acid, boswellic acid etc has been isolated from the leaves of related species in the same family of Rhamnaceae (Palejkar *et al*., 2012; Segismundo *et al*., 2008).

The preliminary isolation of active compounds from DCM extract using silica gel column chromatography resulted in 12 fractions. The fractions were loaded on TLC plates to view the separated compounds. The analysis made it easier to view similar fractions and combine them together. As expected, different compounds with different polarities were observed on the TLC plates. Most of the compounds were separated better in the Hexane: Ethyl acetate (4:1) than in 9:1 mobile phase. From the obtained fractions, the majority of the compounds were purple in colour when sprayed with vanillin/sulphuric reagent indicating the presence of non-polar terpenoids (Wettasinshe *et al*., 2001). This is mainly due to the saponins present in the plant hence the name soap plant. To ensure the isolation of antidiabetic compounds, the fractions were further tested for α-amylase inhibition capability at

different concentrations using the CNPG3 reagent kit. The fractions inhibited the enzyme in a concentration dependent manner. Fractions (C, D, E, F, J and K) had better inhibitory capability with fraction (D) even more potent than the positive control acarbose.

The compound of interest which formed white crystals after purification from fraction C yielded only 0.25 g after drying. Previous studies conducted reported that plant extracts contain low concentration of active compounds and a large number of promising compounds. The quality of bioactive compounds differs from plant to plant. As such, some can easily be isolated. Some bioactive compounds are lost during extraction and isolation process especially when using silica gel chromatography. The activity of the isolated compound was compared with the activity of crude extracts. The isolated compound had a low inhibitory activity against α-amylase as compared to the crude extract (figure 6.4.2h). This is not surprising because very often isolated compounds are less active than crude extracts (Nwodo *et al*., 2010). The loss of activity might be due to the attempts of purifying the compound and loss of synergism with other compounds.

It is very vital for administration of medicinal plants with the knowledge of the chemical constituents they contain. From the findings it shows that different bioactive compounds with different polarities can be isolated from crude extracts of *Helinus Integrifolius*. It also shows that it is better to use crude extracts for better activity of the plant. More studies should be conducted on *H. Integrifolius* since it is shown to contain very important phytochemicals with good activity. The NMR data of the isolated compound determined are presented in the appendix.

Chapter 7

General discussion and conclusion

Diabetes mellitus is one of the leading causes of death and have already reached an epidemic proportion globally. So far it can only be managed with oral agents, healthy diet and exercises. Despite the progress in current treatment, it is still vital to search for new drugs since the available commercialized drugs have limitations (Kavishankar *et al*., 2011). According to Kashani *et al*., (2012), medicinal plants contribute significantly to providing new leads upon which new drugs can be synthesized. The search for new treatment from medicinal plants is steadily growing. There are currently 800 medicinal plants with antidiabetic activity have been reported. Their antidiabetic activity is ascribed to different bioactive compounds they synthesize. Most of the efficacy of these plants has been demonstrated in animal model studies (Martineau *et al*., 2010). In the present study, ten medicinal plants used to treat various diseases were tested for their *in vitro* antidiabetic activity. They were investigated for their inhibitory capability against α–glucosidase enzyme from mammalian rat and α–amylase enzyme from hog pancreas. Inhibition of carbohydrate digesting enzymes is one of the ways in which postprandial hyperglycemia can be prevented and lowered in diabetic patients.

In 1999, Bruneton reported that the pharmacological and medicinal activity of plants is dependent on the phytochemicals they posses. The phytochemical analyses of the plants under investigation were determined qualitatively and quantitatively. Different mass of plant extracts from each plant species were obtained after extracting with different solvents. Hexane extracted the least amount of material. The highest material extracted with chloroform indicate that the solvent was able to penetrate cell membrane of plants thus extraction high amount of endocellular components, suggesting that most of the plant species had intermediate to less polar compounds. This was also confirmed by the qualitative TLC fingerprint whereby the compounds were separated clearly in the non-polar TEA mobile system. A broad spectrum of compounds separated according to their polarities was viewed on the TLC fingerprint represented by different coloured bands. Majority of the bands turned purple/dark bluish in colour after spraying vanillin sulphuric acid reagent indicating the presence of terpenoids (Wettasinghe *et al*., 2001). Different qualitative

tests were also carried out to check the presence of different metabolites. All plant extracts tested positive for phenols and flavonoids but negative for starch. This was observed when the colour of extracts did not change to blue/black after adding iodine. Few of the acetone and ethyl acetate extracts tested positive for glycosides and steroids. Quantitative analysis of phytochemicals showed that plant extracts contain different amounts of bioactive compounds. Most plant extracts had more of total phenolics than flavonoids and condensed tannins.

Different plant parts of different species have different pharmacological activities such as antimicrobial, anticancerous, antioxidant, antimicrobial etc. In the present study, almost all plant extracts exhibited antioxidant activity at various concentrations. The plant extracts had radical scavenging ability and were able to donate atoms to DPPH in turn reducing its colour to yellow. Just like previously conducted studies, antioxidant activity of the plant extracts increased as the concentration of the extracts was increased. Most scientific studies show that antioxidant activity of plants is ascribed to their phenolic content. However in this study not all the plants exhibited that correlation as presumed. For example *A. venosum,* acetone extracts had 22 GAE/mg of phenolics but its antioxidant activity was very high at low concentration of 0.019 mg/mℓ as compared to the ethyl acetate extracts with 72 GAE/mg. This shows that there are other compounds present in the plants apart from phenolics which are responsible for antioxidant activity of the plants. Antioxidants are compounds that prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Most antioxidants are abundant and widespread in the plant kingdom, in fruits, vegetable and some beverages. They form part of the daily human diet (Dai and Mumper, 2010) and also play a vital role in human health. According to Hollman and Katan (1999), antioxidants reduce the risk of oxidative stress related diseases such as cancer, cardiovascular diseases and diabetes. A decrease in the levels of plasma antioxidants is a risk factor associated with diabetes making the treatment with antioxidants, essential. Most plants with antidiabetic activity have been found to possess antioxidant activity (Raphel *et al*., 2002). For example, a study conducted by Matsumato *et al*. (1993) show that tea phenolics rich in antioxidant activity had antidiabetic activity and was also able to inhibit α-amylase and α-glucosidase enzyme activity. This was also observed in the present study whereby plant extracts

with antioxidant activity had better inhibitory ability against the carbohydrate digesting enzymes

Carbohydrate digesting enzymes (α-amylase and α-glucosidase) are the main enzymes responsible for digestion of dietary carbohydrates. Inhibition of these enzymes delays the absorption of glucose in the small intestines consequently reducing postprandial hyperglycemia. In this study, intermediate polar extracts of *C. abbreviata* and *H. integrifolius* were better inhibitors of both carbohydrate digesting enzymes. They were even more potent than acarbose which was used as the positive control and is currently employed in type 2 diabetic patients. The results obtained for *C. abbreviata* are in support with studies conducted by Shai *et al*., (2010) and also its traditional use by people in Botswana to treat diabetes. Since *C. abbreviata* had more tannins than the rest of the other plant extracts it can be deduced that its activity is ascribed to the tannins present. Tannins form complexes with enzymes and consequently retarding the activity of hydrolyzing polysaccharides. Hanhinneva *et al*. (2010) reported that tannins such as catechins are able to inhibit alpha glucosidase enzyme and also delay the absorption of glucose in the small intestines. Antidiabetic activity of *H. integrifolius* has never been reported before, however it is reported that is it used to treat paralysis, infertility and arthritis in some part of Africa. All the plant extracts inhibited α-glucosidase better than α-amylase enzyme which is consistent with previous studies conducted. They inhibited both the enzymes in a concentration-dependent manner and non-competitively. This was observed when the value of K_m remained the same while the value of V_{max} changed. The results shows that the plant extracts did not compete to bind to the active site of the enzyme with the substrate. Rather they bound on a separate site on the enzyme.

Another important factor to control diabetes is through the enhancement of glucose uptake by the cells. Currently diabetic patients use Biguanides and Thiazolidinediones to increase the sensitivity of peripheral and hepatic cells to insulin. Peripheral and hepatic cells are the major disposals of glucose in the body. As such, glucose uptake by these cells results in decrease in plasma glucose. There are certain polyphenols obtained from diet and medicinal plants which are able to enhance peripheral glucose uptake in insulin or non-insulin sensitive tissues. For instance phenolics such as tannins and flavonoids are able to induce hepatic

glucokinase activity, suppress gluconeogenetic enzyme expression and also activate hepatic AMPK (Wolfram *et al*., 2006; Lin *et al*., 2007). Adenosine monophosphate activated protein kinase (AMPK) is reported to be one of the important therapeutic targets for diabetes. Its activation assists in restoring energy balance by decreasing energy consuming processes. Many plant products are known to activate AMPK by disrupting the mitochondrial through metabolic stress (Winder and Thomson, 2007). The obtained data also confirmed earlier report by Wolfram *et al*., (2006) and Lin *et al*., (2007), since both the acetone and ethyl acetate extracts of *C. abbreviata* and *H. integrifolius* were able to increase glucose uptake by the muscle and liver cells without insulin. Although the mode of action is not known, the plant extracts increased glucose uptake in a concentration dependant manner. There was an appreciable increase in uptake when the cells were treated with *C. abbreviata.* However there was an unexpected decrease when the liver cells were treated with *C. abbreviata* at high concentration of 500 μg/mℓ. This might be due to the presence of some compounds in *C. abbreviata* that makes the cells more permeable and aids in release of glucose.

Both *C. abbreviata* and *H. integrifolius* extracts were able to increase glucose uptake better than metformin used as positive control. Metformin is a drug used by type 2 diabetic patients who are insulin resistance. It increases the muscle insulin sensitivity and decrease conversion of glycogen to glucose by the liver. Although the mechanism of action is not known, seeing that the extracts were able to increase glucose uptake in the absence of insulin is suggestive that the plant extracts mediated glucose uptake in an insulin independent mode, just like metformin. Very few studies have been conducted on *H. integrifolius* and few compounds have been isolated from the plant. Since the plant is rich in saponins, most of the compounds viewed on the TLC plates were triterpenes. The isolated compound from the DCM extract of *H. integrifolius* was found to be a mixture of α and β amyrin which is a triterpene in nature. The compound has been isolated and identified using NMR. This technique has made it possible to elucidate the structure of the compound. α, β-Amyrin is a pentacyclic triterpene which belongs to a group of ursane and oleanane. Different pharmacological activities such as anti-inflammatory, antinociceptive, antioxidant, gastroprotective etc of this compound have been reported in numerous studies. An *in vivo* study conducted by Santos *et al*., (2012)

demonstrates that α, β-Amyrin have antihyperglycemic activity in diabetes induced rats models. The present study also confirmed the antihyperglycemic effects of this compound since it was able to inhibit α-amylase enzyme from hog pancreas. Numerous studies also reported that pentacyclic terpenoids can interact with hydrophobic chains of many enzymes and consequently inhibit their action in a non specific mode (James and Dubery, 2009). Although the compound inhibited α-amylase enzyme, its activity was lower than the crude extract, supporting the traditional use of the whole plant extracts to treat diseases.

Even though plant extracts show potential in treating different diseases, there is less awareness about their toxicity. Toxicity tests are very important since they help people to become aware of the toxic effects that the plant might have. Toxicity tests can be done using different *in vitro* or *in vivo* assays. Cytotoxicity tests conducted in this study were performed *in vitro* using the xCelligence system on Raw 264.7 cells. Only the acetone and ethyl acetate extracts of *C. abbreviata* and *H. Integrifolius* were tested for cytotoxicity. The toxicity of the plant extracts was measured by measuring the cell index (CI) after 10 hrs of treatment with both extracts. The Cell Index decreased as the concentration of the extracts was increased, showing that high concentrations of the plant extracts had toxic effect on the cells. The CI of the cells was comparable after treatment with both plant extracts.

The interest of finding new treatment for diabetes mellitus from medicinal plants is growing because of the negative side effects associated with the current treatment. This study revealed the presence of various secondary metabolites that have the ability to inhibit carbohydrate digesting enzymes from plants investigated. This information could lead to the discovery of new treatment to manage diabetes from the plants investigated and other natural products. Therefore research on these natural products should be encouraged to help find treatment for diabetes and other life-threatening chronic diseases. Further studies needs to be conducted such as *in vivo* studies, clinical trials and the isolation of compounds from the active extracts.

Chapter 8

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Appendix

Nuclear magnetic resonance structures of isolated compound.

The structure of the isolated compound in chapter6 was elucidated using NMR Varian 400 MHz instrument. This technique is used to determine physical, chemical, electronic and structure of molecules. These characteristics are obtained due to the chemical shift present on the resonant frequencies of the nuclei available in the sample. This technique was used to analyse the ^{13}C and ^{1}H of the compound isolated.

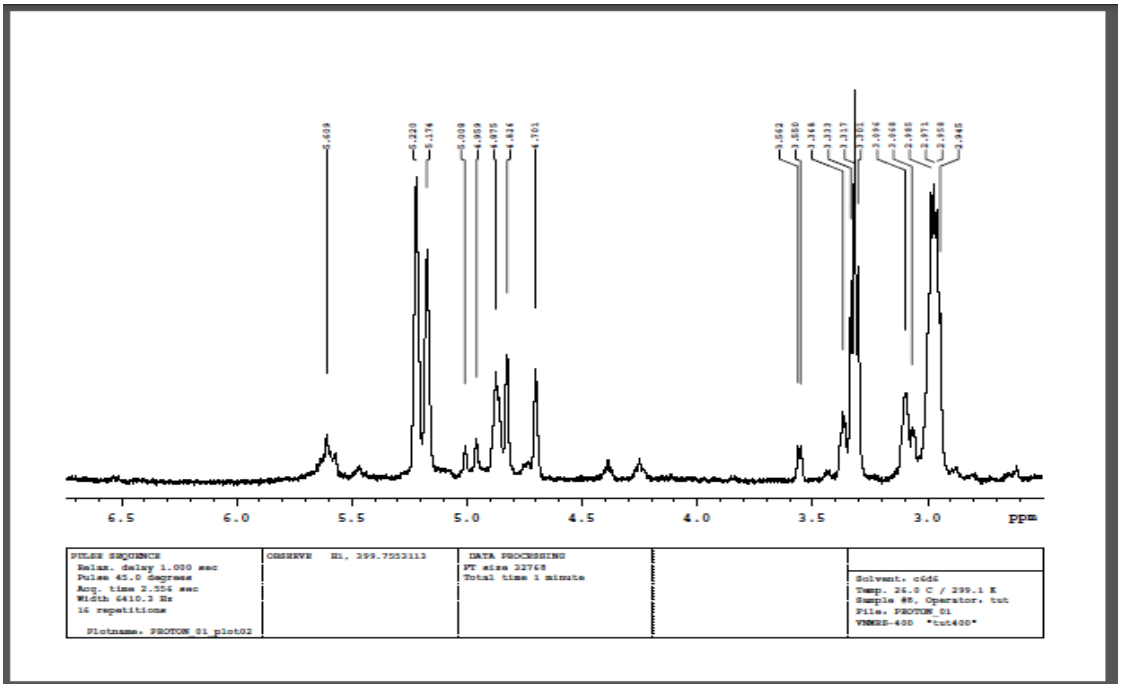

Figure1: The ^{1}H-NMR spectrum of compound isolated from DCM extracts of *Helinus integrifolius.*

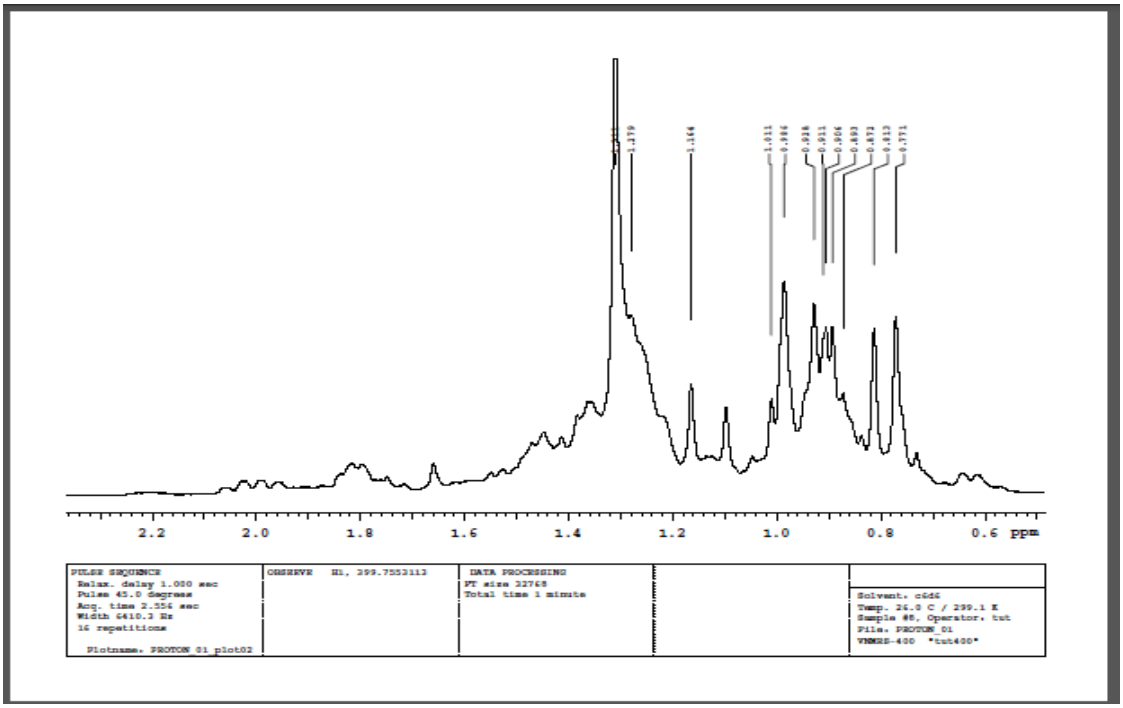

Figure2: The ^{1}H-NMR spectrum of a compound isolated from DCM extract of *Helinus integrifolius.*

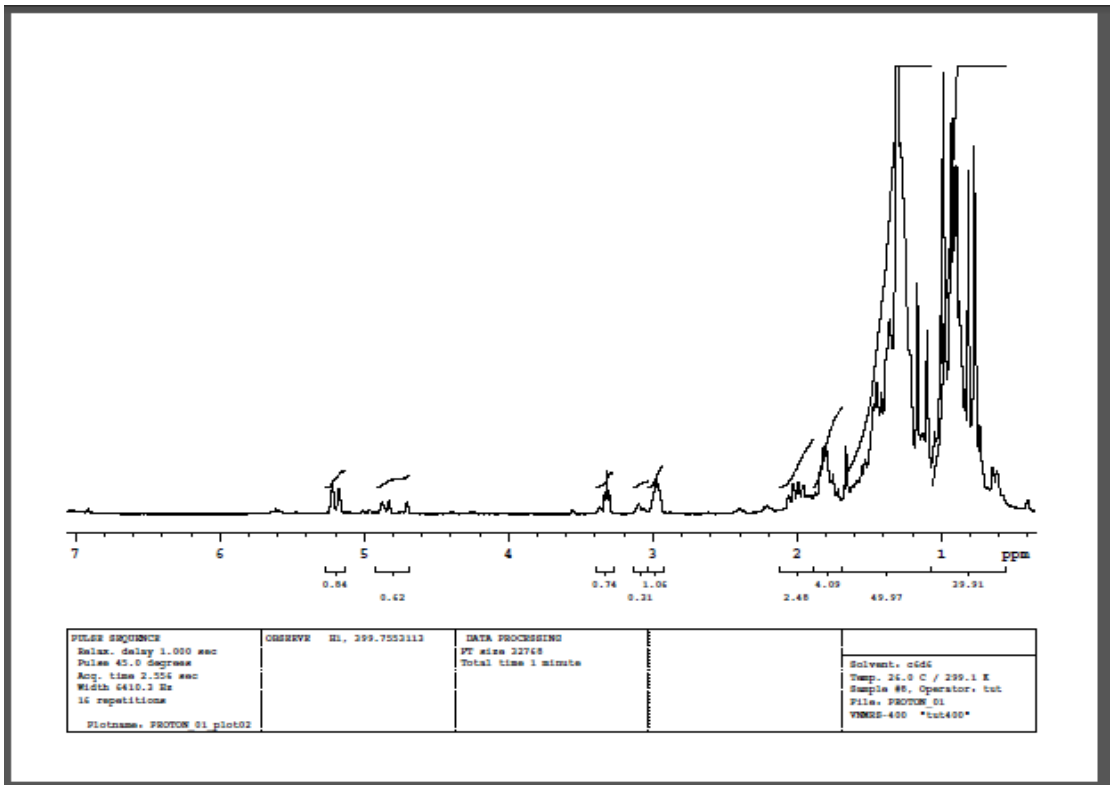

Figure3: The ^{1}H-NMR spectrum of a compound isolated from DCM extract of *Helinus integrifolius.*

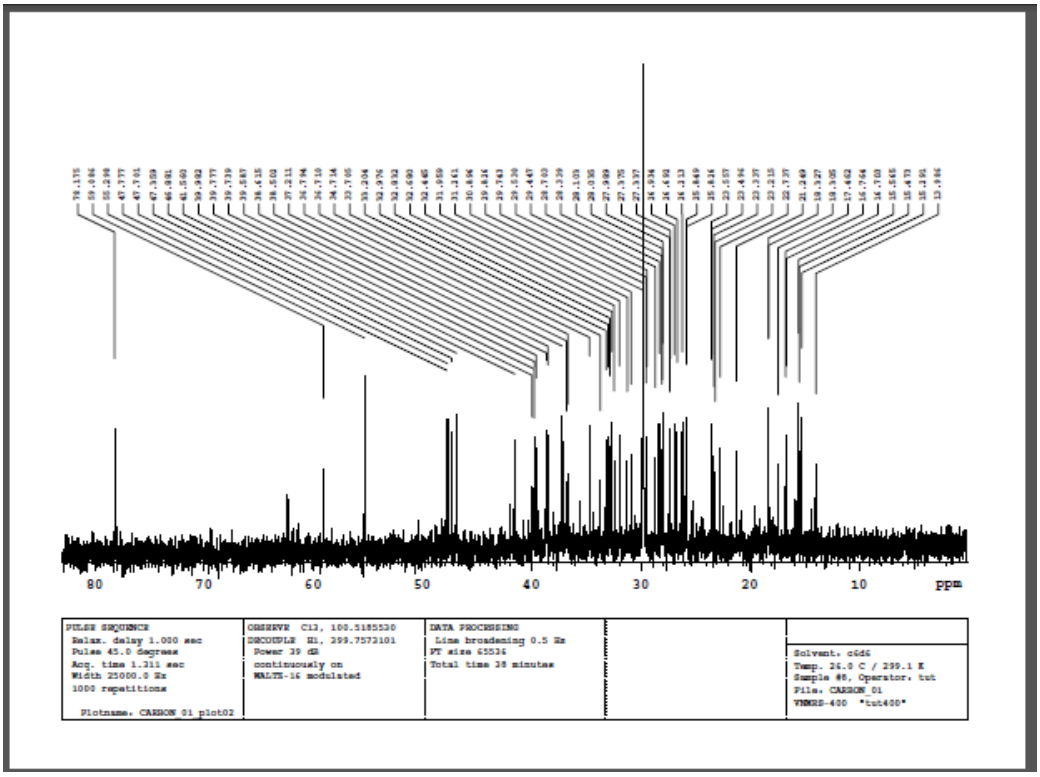

Figure4: The ^{13}C-NMR spectrum of a compound isolated from DCM extracts *of Helinus integrifolius.*

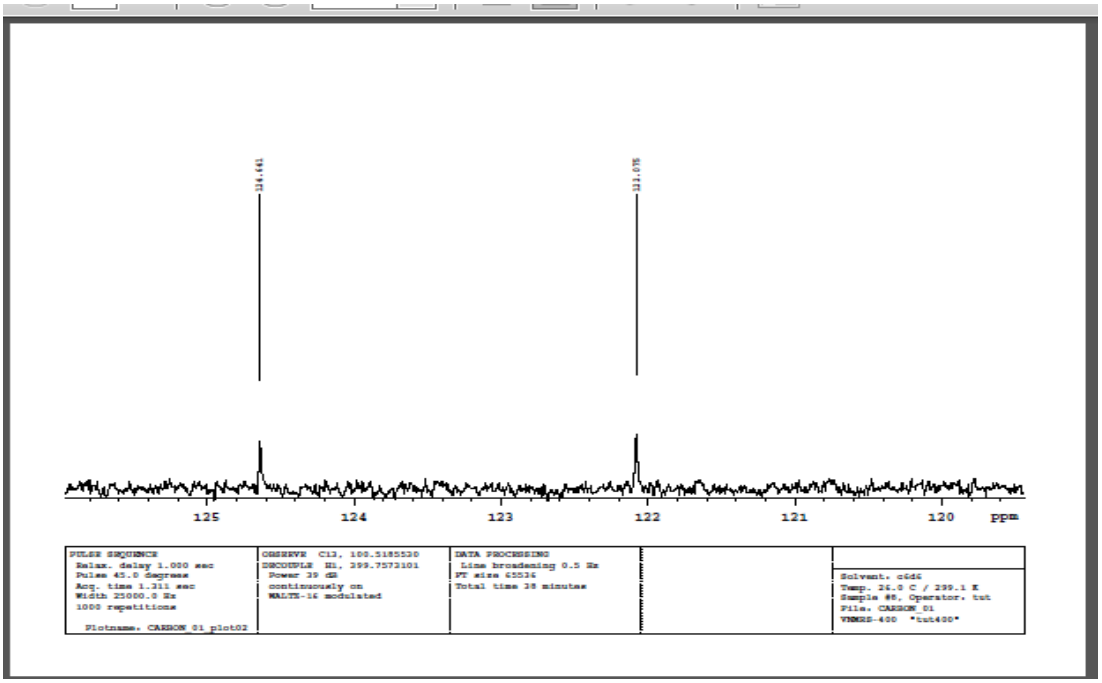

Figure5: The ^{13}C-NMR spectrum a compound isolated from DCM extract of *Helinus integrifolius.*

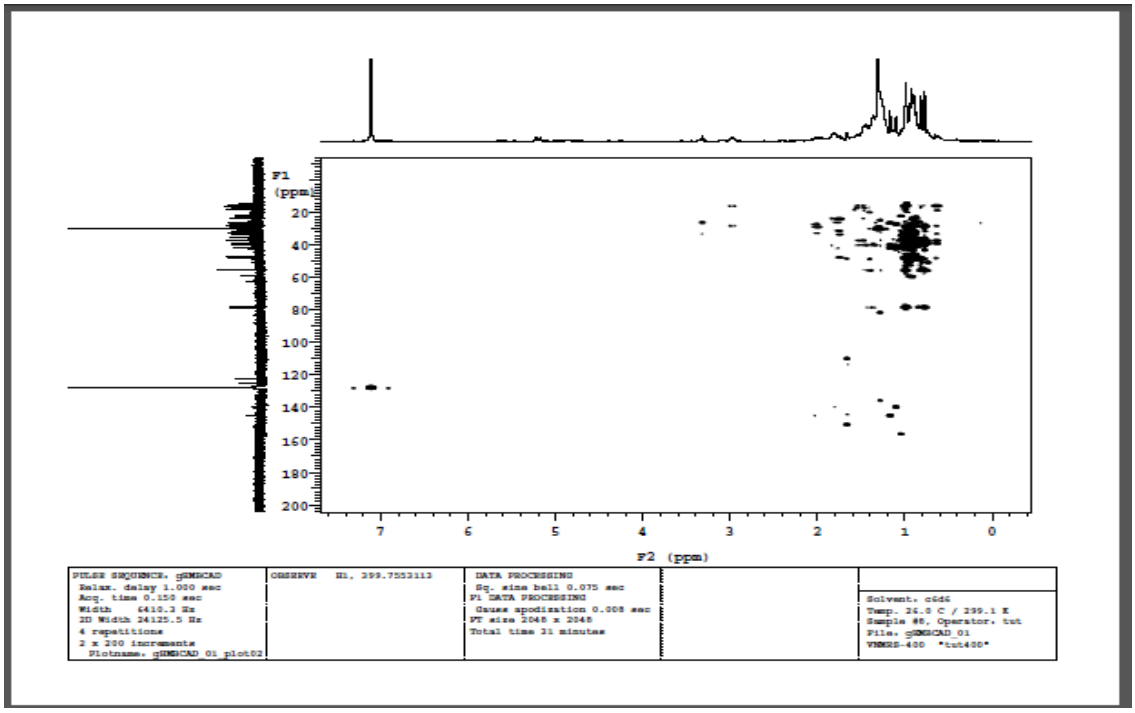

Figure6: The HMBCAD-NMR spectrum a compound isolated from DCM extract of *Helinus integrifolius.*

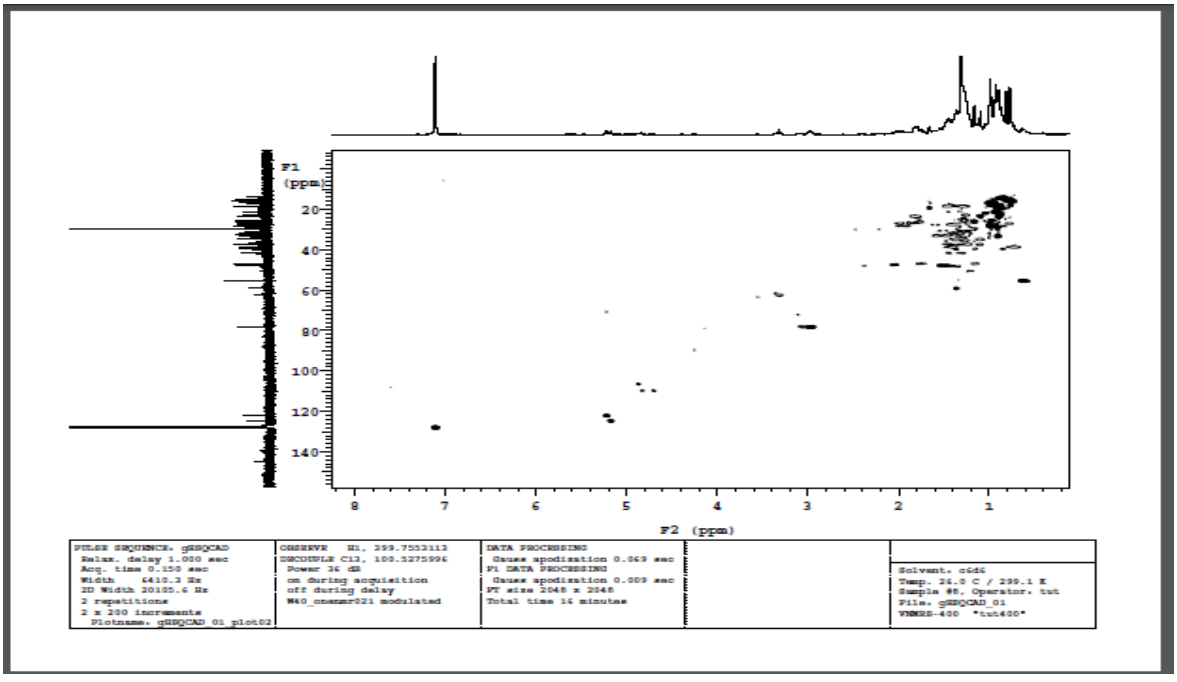

Figure7: The HSQCAD-NMR spectrum a compound isolated from DCM extract of *Helinus integrifoliu*

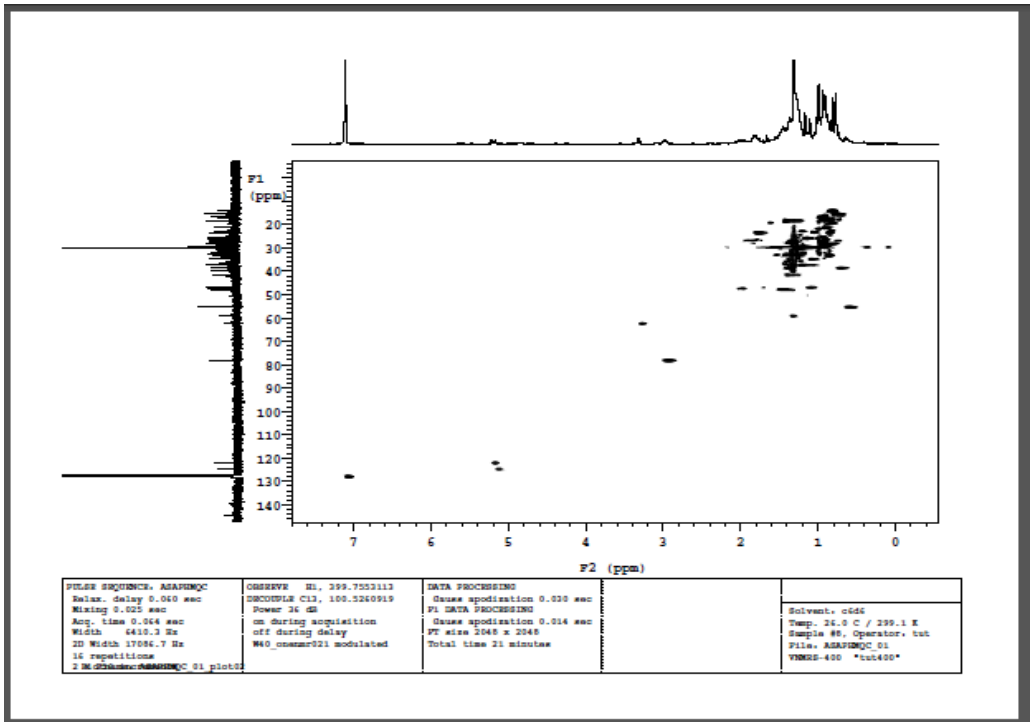

Figure8: The HMQC-NMR spectrum a compound isolated from DCM extract of *Helinus integrifolius.*

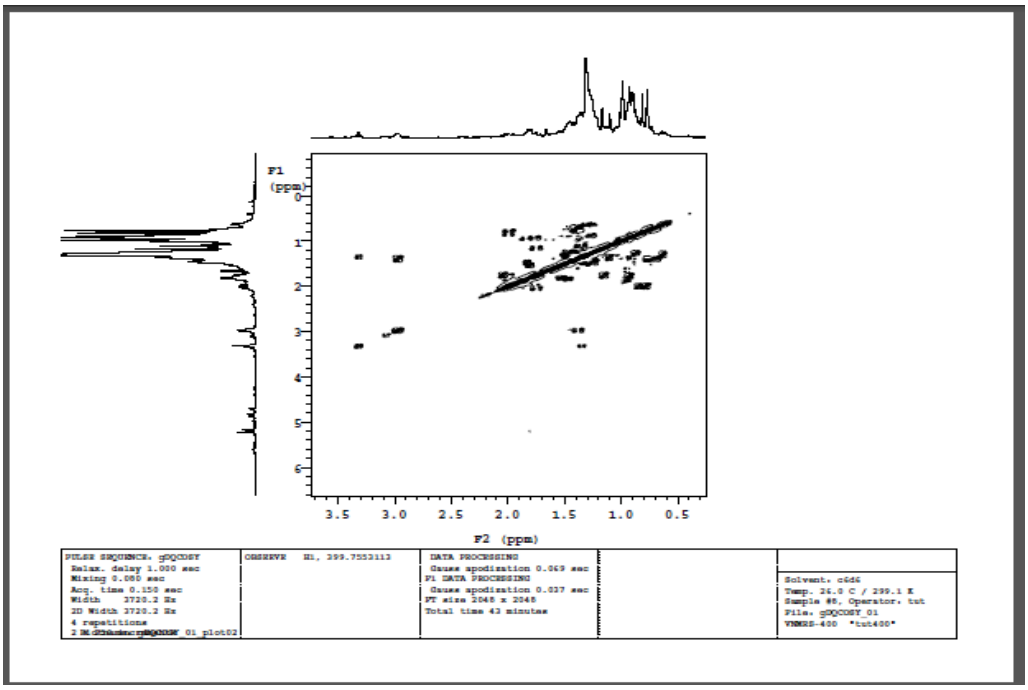

Figure9: The COSY-NMR spectrum a compound isolated from DCM extract of *Helinus integrifolius.*