



Antidiabetic activity of pentacyclic triterpenes and flavonoids
isolated from stem bark of *Terminalia sericea* Burch. Ex DC

BY

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree of Masters of science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed;

Date:.....

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LIST OF ABBREVIATIONS

AGE:	Advanced glycosylated end products
DM:	Diabetes mellitus
DMSO:	Dimethylsulfoxide
DPPH:	1, 2-diphenyl-2-picrylhydrazil
ERK:	Extracellular signal-regulated kinases
GLUT4:	Glucose transporter
IDDM:	Insulin dependent diabetes mellitus
IRS-1:	Insulin receptor substrate 1
MAPK:	Mitogen-activated protein (MAP) kinases
NIDDM:	Non-insulin dependent diabetes mellitus
NMR:	Nuclear Magnetic Resonance
PEPCK:	Phosphoenopyruvate carboxykinase
TLC:	Thin Layer Chromatography
T1DM:	Type -1 diabetes mellitus
T2DM:	Type- 2 diabetes mellitus
UV:	Ultra violet
WHO:	World Health Organization
XTT:	2, 3-bis-[2-methoxy-4-nitrophenyl]-2H-tetrazolium-5-carboxanilide

Antidiabetic activity of pentacyclic triterpenes and flavonoids isolated from the stem bark of *Terminalia sericea* Burch. Ex DC

Abstract

Diabetes mellitus (DM) represents a series of metabolic conditions associated with hyperglycemia and caused by defects in insulin secretion, and/ insulin action. Exposure to chronic hyperglycemia may result in microvascular complications in the retina, kidney or peripheral nerves. According to the World Health Organization (WHO) global burden of disease, more than 176 million people are diabetic with about two thirds of these living in developing countries. With a long course and serious complications that often result in high incidences of mobility and mortality rate, the treatment of diabetes is often costly. The management of this disease is not without side effects and this is a challenge to the medical system. This has led the researches to seek new antidiabetic agents from plants.

Acetone extract of 8 plants namely *Terminalia sericea* Burch. Ex DC, *Euclea natalensis* A.DC, *Warbugia salutaris* Bertol.f.) Chiov., *Artemisia afra* Jacq.ex Willd., *Aloe ferox* Mill, *Sclerocarya birrea* (A.Richi.) Hochst. subsp. *caffra* , *Spirostachys Africana* Sond and *Psidium guajava* L were evaluated for antidiabetic and antioxidant properties. In addition extracts were tested for cytotoxicity. Different parts of all these plants are traditionally used in South Africa for diabetes treatment. Plants were selected based on ethnobotanical information and phytochemical constituents. For determining inhibitory activity against each enzyme (α -glucosidase and α -amylase), all extracts were tested at concentration that ranged from 2×10^{-5} to 0.2mg/ml for α -glucosidase and 0.025 to 1.25mg/ml for α -amylase and fifty percent inhibition or higher was taken

as significant ($p < 0.05$). The extracts of *A. ferox* and *S. africana* showed no inhibition against α -glucosidase at the highest concentration tested (0.2mg/ml) whereas *A. afra* showed weak inhibition (47.15%). *T. sericea* showed to be a potent inhibitor of α -glucosidase exhibiting 97.44 % inhibition of the enzyme ($p < 0.05$). *W. salutaris*, *S. birrea* and *E. natalensis* also showed good activity on α -glucosidase as they demonstrated 71.84; 97.44 and 92.60 % inhibition respectively ($p < 0.05$). Other plant extracts such as *A. ferox* and *S. africana* did not exhibit any activity on α -glucosidase.

T. sericea and *S. birrea* showed the best inhibitory activity on α -amylase enzyme, exhibiting 91.91 and 94.94 % inhibition respectively at 1.25mg/ml. *A. afra*, *E. natalensis*, *P. guajava* and *W. salutaris* also showed good inhibitory activity on α -amylase enzyme at 1.25mg/ml which was the highest concentration tested ($p < 0.05$).

Low levels of plasma antioxidants is a risk factor associated with diabetes therefore, it has been suggested that plant-based medicines that contain antioxidant properties add an advantage in curbing complications that arise during DM aetiology. The antioxidant activity of plant extracts was carried out using 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay. Six plant extracts which showed good α -glucosidase and α -amylase inhibitory activity were evaluated for antioxidant activity. The radical scavenging activity was measured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance (EC_{50}). The EC_{50} is the amount of antioxidants necessary to decrease initial DPPH absorbance by 50%. All 6 tested plant extracts showed good activity. *W. salutaris* and *T. sericea* demonstrated the highest activity exhibiting EC_{50} values of 5.08 and 5.56 μ g/ml respectively as compared to ascorbic acid/Vitamin C (EC_{50} =2.52 μ g/ml), a well-known potent antioxidant. This was followed by *P. guajava* (EC_{50} =6.97 μ g/ml); *E. natalensis* (EC_{50} =8.46 μ g/ml) and *S. birrea* (EC_{50} =9.41 μ g/ml). *A. ferox* showed EC_{50} value of 48.53 μ g/ml.

It has been suggested that plant extracts and compounds must undergo toxicity test for safety before drug discovery is taken into consideration. Due to the large number of plants screened in this study and limited resources in our laboratory, only the acetone extract of *T. sericea* (which demonstrated good α -glucosidase and α -amylase inhibitory activities) was tested for cytotoxicity. Acetone extract of *T. sericea* demonstrated moderate toxicity against primary vervet monkey kidney cells (VK) cells exhibiting IC_{50} values of 20.94 $\mu\text{g/ml}$ when tested at 400 $\mu\text{g/ml}$. Consequently, the acetone extract of *T. sericea* was selected for the isolation and identification of bioactive compounds. A bio-assay guided fractionation of the acetone extract of *T. sericea* led to the isolation of 4 pure compounds namely β -sitosterol, β -sitosterol-3-acetate, lupeol and 3-one-stigmasterol and two sets of mixtures of isomers (epicatechin-catechin; MI1 and epigallocatechin-gallocatechin; MI2).

Antidiabetic, antioxidant and cytotoxicity activities of isolated compounds were evaluated. β – Sitosterol and lupeol showed best inhibitory activity on α -glucosidase exhibiting 50% inhibitory concentration (IC_{50}) value of 54.50 μM and 66.48 μM respectively ($p < 0.05$). This was followed by the MI2; epigallocatechin-gallocatechin ($IC_{50}=119.34 \mu\text{M}$); β -sitosterol-3-acetate ($IC_{50}=129.34 \mu\text{M}$); 3-one-stigmasterol ($IC_{50}=164.87 \mu\text{M}$) and the MI1; epicatechin-catechin ($IC_{50}=255.76 \mu\text{M}$). During the evaluation of purified compound's inhibitory activity on α -amylase, compounds of interest were lupeol and β -sitosterol which exhibited IC_{50} values of 140.72 μM and 216.02 μM respectively as compared to the positive drug-control acarbose ($IC_{50}=65.25 \mu\text{M}$). Epicatechin-catechin and epigallocatechin-gallocatechin also demonstrated α -amylase inhibitory properties and the IC_{50} values were found to be lower than 100 $\mu\text{g/ml}$. Epigallocatechin-gallocatechin, epicatechin-catechin and lupeol showed good free radical scavenging activity as they inhibited DPPH by 98.19; 96.98 and 70.90 % at 100 $\mu\text{g/ml}$ respectively ($p < 0.05$). The DPPH scavenging activity was very low in case of 3-one-stigmasterol (21.5% inhibition), whilst β -sitosterol and its derivative β -sitosterol-3-acetate did not show any activity.

During cytotoxicity evaluation of pure compounds against monkey kidney cells, all the compounds except β -sitosterol did not inhibit the growth of these cells lines at the highest concentration tested (200 μ g/ml). β -Sitosterol showed moderate toxicity exhibiting IC_{50} values of 197.72 μ M. β -Sitosterol-3-acetate, epicatechin-catechin, lupeol and epigallocatechin-gallocatechin were found to be non-toxic to Vero cells as 100% cell viability was observed when Vero cells were exposed to these samples at 200 μ g/ml.

The compounds isolated and the extract of *T. sericea* demonstrated significant antidiabetic and antioxidant properties as compared to well known drugs acarbose (a known α -glucosidase and α -amylase inhibitor) and Vitamin C (a well known antioxidant). This study is the first to report α -glucosidase, α -amylase and antioxidant properties of epicatechin-catechin, epigallocatechin-gallocatechin, β -sitosterol-3-acetate and stigma-4-ene-3-one isolated from *T. sericea*. In addition, epicatechin-catechin, epigallocatechin-gallocatechin, β -sitosterol-3-acetate and stigma-4-ene-3-one are isolated from *T. sericea* for the first time. Overall all results scientifically validated the traditional use of the bark of *T. sericea* for diabetes in South Africa.

1. Introduction

Diabetes mellitus is a metabolic disorder that is characterized by chronic hyperglycemia (high blood sugar) resulting with carbohydrate, fat and protein metabolism disturbances (Ali *et al.*, 2006). Diabetes mellitus can be classified into (type-1) diabetes also known as insulin-dependent diabetes mellitus (IDDM) and (type-2) diabetes which is known as non-insulin dependent diabetes mellitus (NIDDM) (Oh *et al.*, 2005). In addition, World Health Organization (WHO) also recognizes the third form of diabetes namely gestational diabetes which occurs during pregnancy and has similar signs and symptoms like diabetes (type-2), but with different causes and population distribution (WHO, 1999). Other well characterized forms of diabetes are latent autoimmune diabetes of adults (LADA or type-1.5) (Dineen, 2006).

1.1 History of Diabetes Mellitus

The term diabetes is derived from the Greek word ‘**diabainein**’ which means passing through as extreme urine production is one of the major symptoms of diabetes. In addition, during 1675 Thomas Wills added the name mellitus which means sweet taste in Latin. In 1776, Matthew Dobson proved that the sweet in urine was due to the production of excess kind of sugar in the urine and blood of people with diabetes (Dobson, 1776). Indians in the ancient times also used ants to test for diabetes (which they referred to as sweet urine disease); they used to observe whether ants were attracted to urine or not. Pathogenesis of diabetes was only understood experimentally in 1900. In 1910, Sir Edward Albert Sharpey-Schafer discovered that people who lacked a particular single chemical produced by the pancreas developed diabetes and the single compound was named as insulin (Patlak, 2002).

Insulin is derived from Latin word, ‘insula’ meaning ‘island in reference to insulin-producing islets of Langerhans’ (Patlak, 2002). Sir Frederick Banting, Charles Best and colleagues purified insulin from

bovine at the University of Toronto and this led to the availability of insulin, which was found to be effective treatment for diabetes (Banting, 1922).

1.2 Classification of diabetes mellitus

1.2.1. Type-1 (Insulin dependent diabetes mellitus)

Type-1 diabetes or insulin-dependent diabetes mellitus (IDDM) is an organ specific autoimmune disease which results from damaged β -cells that are situated in pancreas whose primary function is to produce insulin (Jobon *et al.*, 2006). Type-1 is also known as childhood or juvenile diabetes as most people develop it at childhood. The risks associated with this type of diabetes include congenital anomalies such as central nervous system, cardiac and skeletal muscle malformations which usually occur during neonatal stages (Wasserfall and Atkinson, 2006). Type-1 diabetes is the 2nd most common type of diabetes after type-2. It has been shown to be caused as a result of an autoimmune reaction to antigens of β -cells produced by pancreas. Three types of autoimmune exist (Achenbach and Ziegler, 2005; Wasserfall and Atkinson, 2006).

1) Islets cell surface antibodies

These are polyclonal antibodies that react with all the cells of islets i.e. (α , β , and pancreatic polypeptide cells). About 80% of type-1 diabetics have these autoantibodies.

2) Islets cell cytoplasm antibodies

These antibodies are directed against islets cell cytoplasm and about 90% of type-1 diabetics have these antibodies.

3) Specific antigens targets of islets cells

These antibodies are directed to glutamine acid decarboxylase (GAD) and about 80% of type-1 diabetics have these antibodies.

1.2.1.1 Pathogenesis of IDDM (Insulin Dependent Diabetes Mellitus)

Destruction of pancreatic β cells by the autoimmune antibodies leads to a deficiency of insulin secretion. Metabolic disturbances associated with IDDM results from loss of insulin. Type-1 diabetics suffer from excessive production of glucagons, a 29 amino acid polypeptide which is responsible for carbohydrate metabolism. This therefore, results in exaggerated metabolic defects. Patients suffer from ketoacidosis, a formation of ketone bodies. Usually in this case ‘somatostatin’ is administered to suppress glucagon levels in type-1 diabetics. On the other hand, this may have negative effect in patients since it results in impaired ability of the patient to secrete glucagon when there is a decrease in blood glucose levels (hypoglycaemia) (Achenbach and Ziegler, 2005).

One of the major problems associated with type-1 diabetes is that there is a defect in the in the ability of target tissues to respond to insulin (Bailers, 2002). Uncontrolled lipolysis leads to formation of free fatty acids in the plasma and this is due to insulin deficiency. In peripheral tissues such as skeletal muscles, glucose metabolism is suppressed as a result of the formation of free fatty acids. Action of insulin is impaired leading to a decrease in expression of a number genes necessary for target tissues to respond normally for insulin such as glucokinase in liver and glucose transporter 4 (GLU 4) class of glucose transporters in adipose tissues. IDDM leads to an increase in the in hepatic output causing a further damage to glucose and carbohydrate metabolism. Insulin stimulates the storage of food energy just after the meal which is normally stored as glycogen in liver cell as well as skeletal muscles. Insulin

also stimulates liver cells to produce triglycerides and this process is normally disturbed in type-1 diabetics (Bailers, 2002).

1.2.2 Type-2 or Non-insulin dependent diabetes mellitus

Type-2 diabetes (T2DM) is a complex metabolic disorder that is associated with β -cell dysfunction and has varying degrees of insulin resistance (Oh *et al.*, 2005; Deneen, 2006). Type-2 diabetes is usually the product of two distinct abnormalities: β -cell function and decreased insulin sensitivity (Robertson, 2006). It is mostly a genetic disease which is associated with strong familial association and high concordance rates in identical twins (Nelson *et al.*, 1975). Most type-2 diabetics are obese and these people normally have insulin resistance on liver, muscles and adipose tissues which are the major sites of insulin. However, only minority of obese patients develop diabetes, and 20% of type-2 diabetics are not obese, emphasizing that obesity does not cause diabetes rather it contributes to the phenotypic expression of genes that predispose individuals to type-2 diabetes.

1.2.3 Pathogenesis of NIDDM (Non Insulin Dependent Diabetes Mellitus)

The disease results in combination of several genetic determinants that may affect insulin production or insulin sensitivity (Hoppener and Lips, 2006). Any gene mutation or metabolic disturbance leading to defect in insulin secretion, insulin action, glucose transport, or enzyme associated with glucose metabolism can theoretically result in hyperglycemia or clinical diabetes (Tseng, 2004). An example for genetic subtypes of T2DM involves mutations in glucokinase, which phosphorylates glucose to glucose-6-phosphate, leading to impaired glycolysis (Frajans *et al.*, 2001). The impaired glucose transport into skeletal muscle and adipose tissues can result from a variety of mechanisms involving insulin receptor defects. (Le Roith and Zick, 2001). Over expression of tumor necrosis factor

α (TNF α) in muscle cells has been implicated as an inducer of insulin resistance by increasing the serine phosphorylation of insulin receptor substrates (IRS-1) and (IRS-2), resulting in the reduction in the ability of the IRS molecules to dock with receptor and interact with downstream pathway (Le Roith and Zick, 2001). Interleukin-6 (IL-6) has also been found to play an important role in the induction of insulin resistance in adipocytes (Lagathu *et al.*, 2003; Rotter *et al.*, 2003). Chronic treatment with IL-6 to adipocytes can diminish expression of β -subunit receptor, IRS-1 and GLUT4, resulting in reduced glucose transport (Legathu *et al.*, 2003). Insulin-induced activation of β -subunit of insulin receptor, extracellular signal-regulated kinases (ERK-1) and (ERK-2) are also inhibited by IL-6. Although the expression of p38 mitogen activated protein kinases (MAPK) phosphorylation is increased in skeletal muscle in patients with T2DM, the insulin-stimulated p38 MAPK phosphorylation is only noted in non-diabetic subjects, but not in patients with T2DM (Koistinen *et al.*, 2003). Another mechanism leading to hyperglycemia in patients with type-2 diabetes involves the inability to produce hepatic glucose. Enhanced phosphoenopyruvate carboxykinase (PEPCK), an enzyme catalyzing the rate limiting step in gluconeogenesis activity leading increased hepatic glucose production in patients with T2DM (Consoli *et al.*, 1990). Decreased glycogen synthesis has also been reported in patients suffering from T2DM (Le Roith and Zick, 2001). Pancreatic β cell dysfunction has also been demonstrated in patients with type-2 diabetes (Kahn, 2000). Progressive formation of amyloidosis with loss of β cells is always major pathological factor found in patients with T2DM (Marzaban *et al.*, 2003).

1.3 Complications associated with diabetes mellitus

Studies and clinical trials indicate that hyperglycemia is the main cause of complications associated with diabetes mellitus (Lopez-Candales, 2005); however complications are less common and severe in people who control blood glucose levels. One of the major complications of diabetes is the formation of glycosylated end products (AGE) (Vlassara and Palace, 2002). These end products will react with other

proteins to generate free radicals in diabetic patients hence increasing permeability and thickening of blood vessel walls with loss of elasticity (Vlassara and Palace, 2002; Bonnefont-Rousselot, 2000).

1.3.1 Complications relating to diabetes can be divided into two forms

1.3.1.1 Acute complications

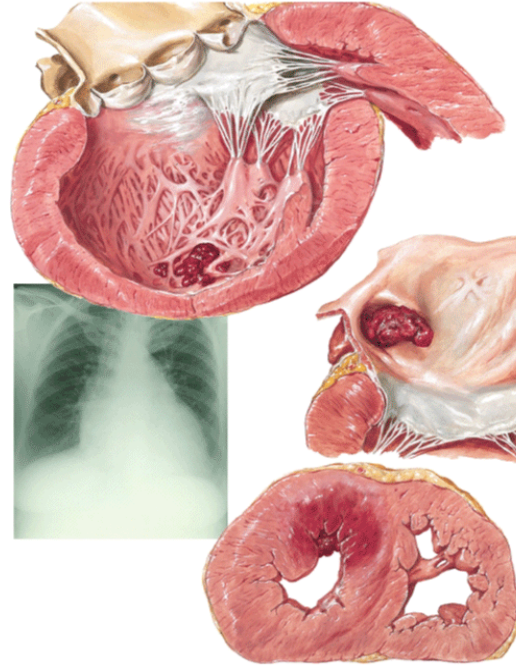
Acute complications include diabetic ketoacidosis, abdominal pain, dehydration, accelerated breath where the patient requires medical emergency. Hypoglycemia or abnormally low sugar that is normally caused by drugs used for diabetes can develop as a result of acute complications. Patients may be agitated, sweaty and may have symptoms of sympathetic activation of Autonomic Nervous System (ANS). Conscious can be lost leading to seizures eventually coma/ brain damage ultimately death may result.

1.3.1.2 Chronic complications

Elevated blood glucose can result in damage of blood vessels. Examples of chronic complications are diabetic retinopathy, coronary artery disease (atherosclerosis, figure 1.1: B), renal failure, limb amputation and eventually premature death (Ortiz-Andrade, 2007). Macrovascular disease leads to cardiovascular complications such as coronary artery disease (angina or myocardial infraction), ischemic stroke and muscle wasting. Diabetic foot (figure 1.1: A) may also result in skin ulcer and infection by gangrene. This is a common cause of adult amputations.



A)



B)

Figure 1.1: Complications that arise as result of diabetic complications, A) diabetic foot that may lead to amputation, B) Atherosclerosis (enlargement of the heart arteries which may lead to cardiac infarction in diabetics)

1.4 Diagnosis of diabetes mellitus

1.4.1 Laboratory diagnosis of Diabetes mellitus

Diagnosis is established exclusively by the demonstration of increased concentration of glucose in the blood. Oral glucose tolerance test has been used for years as a sole diagnostic criterion (Greenberg and Sacks, 2002). There are 3 most recommended criteria of international committee of diabetic experts based on review of epidemiology data, relationship between blood glucose and the impact it has on the microvascular complications. They are:

1. The most evident symptoms of diabetes are thirst, increased urination (polyuria), unexplained loss of weight and increased plasma glucose levels of 200mg/dl (11.1mol/ L).
2. Fasting plasma glucose level of 126mg/dL or (70mmol/L) or higher after overnight (at least 8 hours) fast.
3. Two-hour plasma glucose equals 200mg/dL (11.1mmol/L) or higher during a standard 75g oral glucose tolerance test (Ahmed and Goldstein, 2006). Fasting glucose measurement which is above 126 mg/dL or 7.0mmol/l is considered diagnostic for diabetes mellitus. The diagnostic criteria established by an Expert Committee of the American Diabetes Association (ADA) in 1996 and ratified by WHO is outlined in table 1.1.

Table 1.1: Diagnostic thresholds for diabetes and lesser degrees of impaired glucose regulation (Dineen, 2006)

Category	Fasting plasma glucose	2-hour plasma glucose
Normal	6.1 mmol/litre (110mg/dl)	7.8 mmol/litre (140mg/dl)
IFG	6.1-6.9 mmol/litre (110-125mg /dl)	-
IGT	-	7.8-11.0 mmol/litre (140-199 mg/dl)
Diabetes	≥7.0 mmol/litre (≥ 126mg/dl)	≥11.1 mmol/litre (≥200mg/dl)

IFG: Impaired fasting glucose

IGT: Impaired glucose tolerance

When both tests are performed, IFG or IGT should be diagnosed only when diabetes is not diagnosed by the other tests.

1.5 Epidemiology of Diabetes Mellitus

A global epidemic of diabetes mellitus is predicted for the first quarter of the twenty first century (Greenberg and Sacks, 2002). Diabetes is amongst the five leading causes of deaths these days (WHO, 1994). Table 1.2 illustrates statistics for T2DM.

Table 1.2: Showing non-insulin dependent diabetes statistics worldwide from 1994-2010

	1994		2000	2010
World Region	Population	NIDDM	NIDDM	NIDDM
World	5,638,219	98,868	157,338	215,616
Africa	698,388	4,766	10,881	16,956
Asia	3,879,000	46,864	86,563	126,224
North America	286,041	13,402	15,094	16,787
Latin America	483,862	11,315	14,790	18,179
Europe	510,673	16,044	20,225	24,391
Former USSR	284,654	5,735	8,844	11,946
Oceania	3,346,376	742	941	1,133

***NIDDM (Non-insulin diabetes mellitus)**

1.6 Therapeutic Intervention and limitations associated with α -glucosidase and α -amylase inhibitors

1.6.1 Action of Digestive Glycosidase

In humans digestion of starch occurs in several stages (Breyer and Luo, 1999; Ferey-Roux *et al.*, 1998). Initially, salivary α -amylase breaks down polymeric starch into shorter oligomers. Upon reaching the gut the partially digested starch is then extensively hydrolysed into shorter oligosaccharides by alpha amylase synthesized in the pancreas. This results into formation of mixture of oligosaccharides including maltose, maltotriose and a number of α (1-6) and α (1-4) oligoglucans which then pass through the mucous layer to the brush border membrane (Breyer and Luo, 1999; Ferey-Roux *et al.*, 1998). At this point a number of alpha glucosidases function to further degrade the oligosaccharides to glucose. The glucose is then absorbed and enters the blood stream by means of a specific transport system where it is distributed throughout the body. Glucose then enters into glycolytic pathways where it converted into energy; it can be stored in a form of glycogen for future use (Ryberg, 2000).

1.6.2 Alpha glucosidase and amylase enzymes

One of the therapeutic approaches for reducing postprandial hyperglycemia in patients with diabetes is to prevent absorption of carbohydrates after a meal (Ortiz-Andrade, 2007). Transportation of food into the blood requires smaller molecules (monosaccharides) such as glucose whereas starches, oligosaccharides and disaccharides must be broken down into individual monosaccharides before they can be absorbed in the duodenum and the upper part of the jejunum (Ortiz-Andrade, 2007). This digestion is facilitated by the enteric enzymes such as pancreatic α -amylase (Figure 1.2A) and intestinal α - glucosidase (Figure 1.2B) (Ortiz-Andrade, 2007; deMelo and Gomes, 2006).

Glucosidase and amylase inhibitors have been found to be very effective in reducing postprandial glucose by suppressing the absorption of glucose and these are effective in the treatment and management of diabetes and obesity (deMelo and Gomes, 2006). Current interest in these compounds has been extended to a diverse range of diseases including lysosomal storage disorders and cancer; special attention has been given to those compounds with anti-HIV and diabetic activity (deMelo and Gomes, 2006).

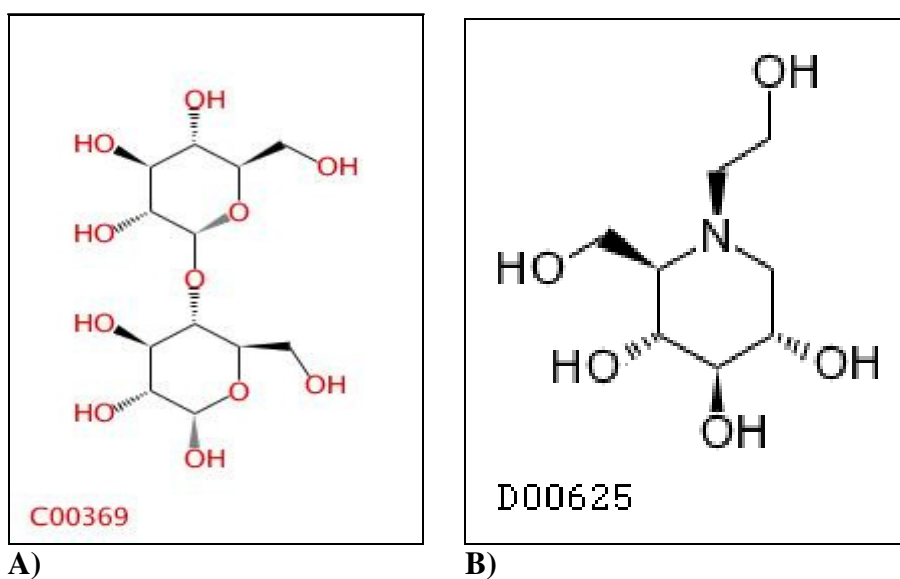


Figure 1.2: Chemical structures of alpha-amylase (A) and alpha glucosidase (B)

1.6.2.1 Alpha glucosidase and alpha amylase inhibitors

There are many pharmaceutical drugs aimed at accomplishing the role of lowering the blood glucose levels hence preventing the onset of complications. A few examples of alpha glucosidase inhibitors are: acarbose (Figure 1.3) and miglitol. These drugs interfere with the action of α -glucosidase that is present in the brush border situated in the small intestine. These drugs function locally in the intestine. They result in abdominal bloating and discomfort, diarrhea and flatulence. Additionally the α -glucosidase

inhibitors result in the reduction in digestion and absorption of glucose into systemic circulation (Skrha, 2007).

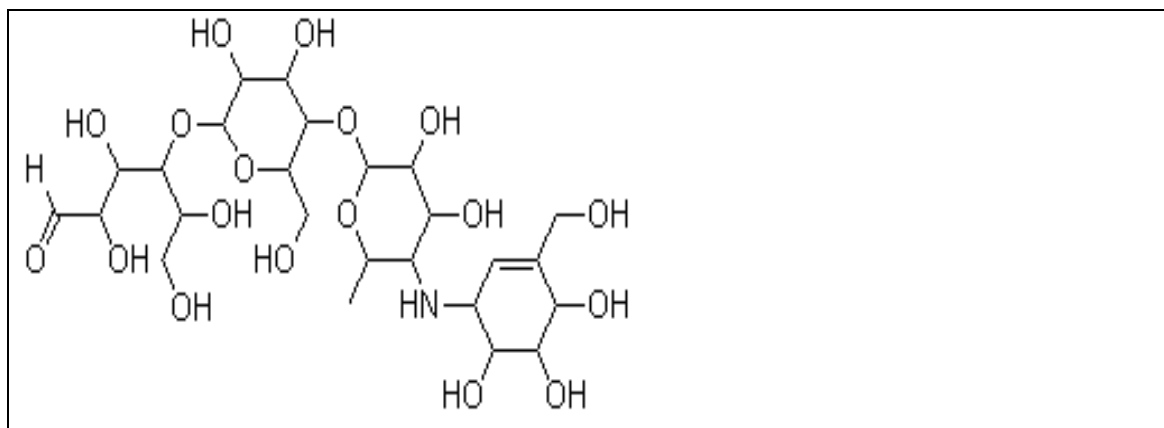


Figure 1.3: Chemical structure of acarbose (Precose)

1.6.3 Sulfonylureas

These drugs are known as endogenous insulin secretagogues since their primary role is to induce the pancreatic release of endogenous insulin and consequently these drugs are effective only when residual pancreatic β cell activity is present (Pleuvry, 2005). These drugs do not have significant effects on circulating triglycerides, cholesterol and lipoproteins that are characteristics in diabetic patients (Skrha, 2007). It is also noted that most sulfonylureas can cross the placenta and cause hypoglycemia in newborns. They can stimulate appetite hence result in weight gain (Pleuvry, 2005).

1.6.4 Biguanides e.g. metformin

In Medieval times, a prescription of a plant known as *Galega officinalis* (goat's rue) was said to relieve symptoms of intense urination that accompanied diabetes mellitus (Witters, 2001). The active constituent that was responsible for lowering of blood glucose levels was discovered to be 'galegine' or 'isoamylene guanidine' (Cusi and DeFronzo, 1998). It was observed that guanidine and certain derivatives were too toxic for the treatment of diabetes mellitus; this resulted in existence of biguanides (two linked guanidine rings) which were useful and three biguanides became available for diabetes therapy in the 1950's.

Phenformin and buformin, with the former becoming quite popular in the 1960's were withdrawn from the pharmacopoeia in the early 1970's due to the appearance of frequent lactic acidosis and increased cardiac mortality (Cusi and Defronzo, 1998). Metformin which is a less lipophilic biguanide became available for the treatment of DM even after 20 years of use in Europe. In 1995, it was approved for use in United States of America (Witters, 2001).

These drugs increase the glucose uptake by the skeletal muscles, thus reducing insulin resistance (Plevry, 2005). This class of drugs functions to lower glucose level by enhancing insulin receptors to increase the absorption of sugars however, these drugs are not ideal to people with liver diseases since the major site the major site of action of metformin is the liver and contraindication in people with liver disease may result (Skrha, 2007).

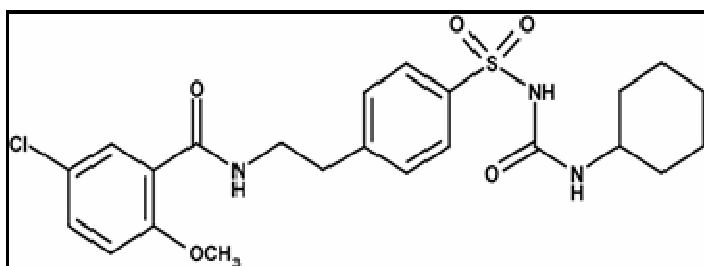


Figure 1.4: Chemical structure of metformin

1.6.5 Meglitinides e.g. prardinic and starlic

Mechanism of action of these drugs involves their binding to a receptor in the pancreatic β -cell that is different from receptors for the sulfonylureas. Additionally the drugs have no effect on circulating levels of plasma lipids (Skrha, 2007). Other drugs that are used for the treatment of diabetes mellitus are illustrated in figure 1.5, table 1.2.

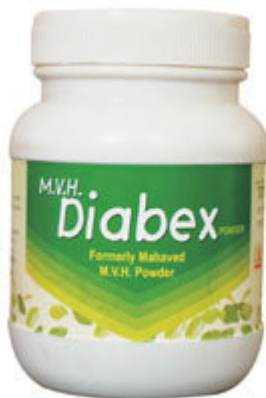


Figure 1.5: Drugs used for the treatment of diabetes mellitus

Table 1.2: Drugs used for the treatment of diabetes mellitus (Li *et al.*, 2004)

Drug group	Example (s)
Insulin	There are many kinds of preparations e.g. Pumps, injections etc
Sulfonyreas (SU)	Tolbutamide (D ₈₆₀ , Orinase), Glibonese (Glyburide, HB419)
Biguanide (BG)	Phenformin (Phenethyldiguanidi Hydrochloridum, Diabenide, DBI), Dimethylbiguanide (FluamineMetformin, Diabex, Mellitin, Metformin
Alpha glucosidase inhibitors (α -GDI)	Glucobay (Acarbose), Voglibose, Miglitol, Emiglitate, Glyset, Precose
Aldose reductase inhibitor (ARI)	Tolrestat, Alredase, Epslstat, Kinedak, Imirestat
Thiazolidinediones (TZD)	Troglitazone, Rosigitazone, Pioglitazone, Englitazone
Carbomoylmethyl benzoic acid (CMBA)	Repaglinide
Insulin-like growth factor (IGF)	IGF-1
Others	Dichloroacetic acid

1.6.6 Insulin

1.6.6.1 Chain of events upon the discovery of insulin

In 1955 ‘Fred Sanger’ was the first to have the amino acids sequence of insulin sequenced (Sanger, 1988), this resulted in him earning Nobel Prize in 1958. It was also the first peptide hormone, circulating

in minute amounts, to be measured using radioimmunoassay (Bersin and Yallow, 1961). In 1967, Don Steiner was the first to determine the pathway behind the biosynthesis of insulin in pancreatic beta cells, specifically as a proinsulin precursor (Steiner and James, 1992). Three-dimensional structure of insulin was discovered by Dorothy Crowfoot Hodgkin and colleagues in 1969 using X-ray crystallographic methods (Adams *et al.*, 1969). In addition, insulin was also the first protein to be synthesized in microorganisms by recombinant DNA technology in the late 1970's. This authenticated the design of insulin in order to optimize the molecule's pharmacodynamic profile for therapeutic purposes.

1.6.6.2 Structure and function

Insulin is the anabolic hormone that is secreted by pancreatic β -cells of the 'Islets of Langerhans' in the pancreas in response to increased circulation levels of glucose and amino acids (Nathanson and Nystrom, 2009). It has two aspects of action, on one aspect it acts as metabolic hormone, while the other aspect is characterized by acting as a growth factor (Mitsuru *et al.*, 2009). The main function of insulin is to maintain normal glucose homeostasis by reducing hepatic glucose production via gluconeogenesis and glycogenolysis. This hormone also aid in promoting glucose uptake primarily by the skeletal muscle and to a lesser extent adipose tissue. This improved glucose uptake involves insulin-stimulated translocation of isoform of the facilitative glucose transporter GLUT-4 to the cell surface, as well as activation of the rate limiting step in glycogen synthesis in muscle cells by insulin (Shulman, 2000).

1.6.6.3 Glucose homeostasis

Homeostasis of glucose is maintained by the highly coordinated interaction of three physiological processes namely, insulin secretion, tissue glucose uptake and hepatic glucose production. The body keeps the supply of glucose to the cells by maintaining a constant concentration of glucose in the blood (Brunner *et al.*, 2009). Normal glucose homeostasis in the body is represented by the balance between

intake (glucose absorption from the gut), tissue utilization (glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, glycogen synthesis) and endogenous production (glycogenolysis and gluconeogenesis) (Meyer *et al.*, 2002). Fatty acids and glucose are considered as the most important fuels. The glucose is mostly used by the brain cells and muscles therefore, to ensure the continuous supply of glucose to the brain and other muscles, metabolic fuels are stored for use in time of need (Brunner *et al.*, 2009). Insulin-an anabolic hormone and some insulin-like growth factors maintain glucose homeostasis (Dunger, 1995). There are several catabolic hormones such as glucagon, catecholamines, cortisol and growth factors that oppose the action of insulin and these hormones are known as anti-insulin or counter-regulatory hormones (Gerich and Campbell, 1988).

1.6.6.4 The role of insulin and glucagon

Insulin is a 51 amino acid protein that is secreted by the β -cells of the pancreatic islets in response to increased blood glucose levels just after a meal. There are several ways by which insulin decreases the plasma glucose levels: 1) the uptake of glucose into tissue, 2) intracellular glucose metabolism, 3) glycogen synthesis (Figure 1.6). The β -cells that are situated in the pancreas's islets of Langerhans secrete insulin (Brunner *et al.*, 2009). There are four main targets of insulin action: the liver, the muscles, adipose tissue and the brain. Upon the binding of insulin to its receptor, a series of reactions which affect glycogen synthesis and glucose transport take place (Kahn, 1988). Glucagon is a small single chain, 29 amino-acid peptide that is secreted by the α -cells of the pancreas. Glucagon mobilizes the fuel reserves for the maintenance of the blood glucose levels after meals by inhibiting glucose-utilization pathways and the storage of metabolic fuels. Its main target is the liver where it stimulates glycogenolysis whilst inhibiting glycogen synthesis, glycolysis and lipogenesis (Brunner *et al.*, 2009). This balance between insulin and glucagon action is a key factor in the control of metabolism. During diabetes mellitus etiology this process is disturbed.

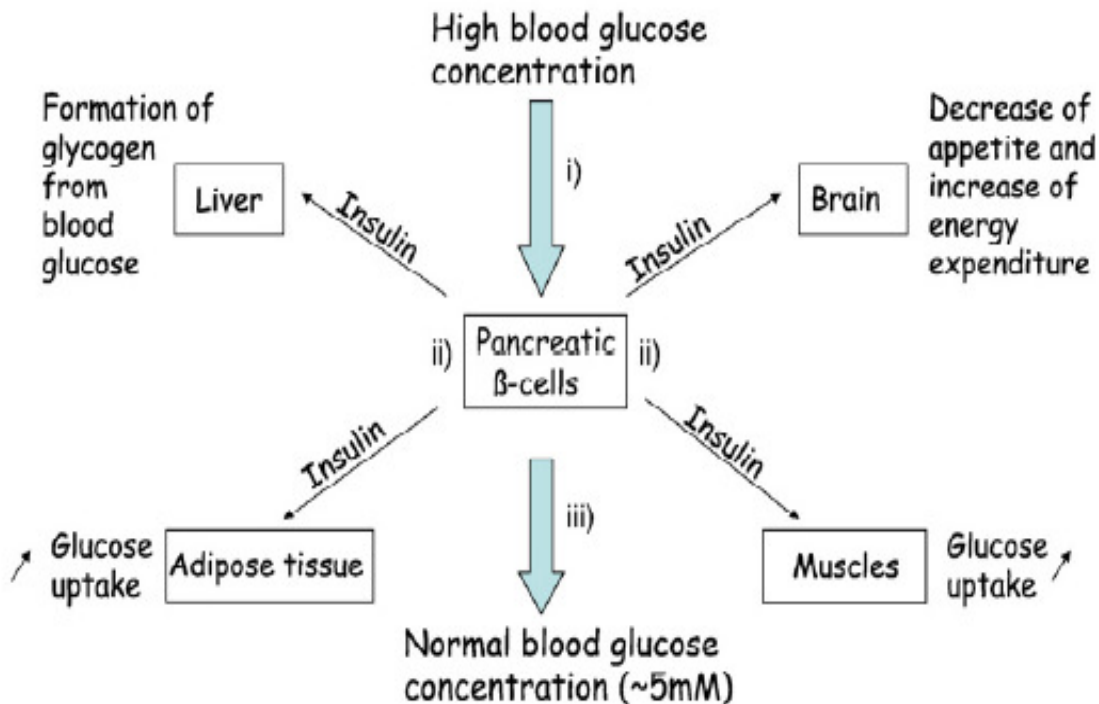


Figure 1.6: Role of insulin for the control of glucose homeostasis. 1) Increase of blood glucose induces the secretion of insulin by the β -cells. 11) The action of insulin in the different insulin targeted tissues allows the return to the normal glucose concentration (Brunner *et al.*, 2009).

1.7 Antioxidants and diabetes mellitus

Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life and our metabolism, since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of Adenosine-5'-triphosphate (ATP). However, problems may arise when the electron flow becomes uncoupled (transfer of unpaired single electrons), generating free radicals. Examples of oxygen-centered free radicals, known as reactive oxygen species (ROS), include superoxide (O_2^-), peroxy (ROO^\cdot), alkoxy hydroxyl, and nitric oxide. The hydroxyl (half-life of 10^{-9} s) and the alkoxy (half-life of seconds) free radicals are very reactive and rapidly attack the molecules in nearby cells. The damage caused by them is

necessary and is dealt with by repair processes. On the other hand, the superoxide anion, lipid hydroperoxides, and nitric oxide are less reactive. In addition to these ROS radicals, in living organisms there are other ROS non radicals, such as the singlet oxygen (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). ROS may be very damaging since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates, and DNA, to induce oxidations, which cause membrane damage, protein modification (including enzymes), and DNA damage. This oxidative damage is considered to play a causative role in aging and several degenerative diseases associated with it, such as heart disease, cataracts, cognitive dysfunction, diabetes and cancer (Pietta, 2000).

Humans have antioxidants systems to protect themselves against free radicals. These systems include some antioxidants produced in the body (endogenous) and those that are obtained from diets (exogenous). Endogenous antioxidants include enzymatic defense, catalase, superoxide dismutase etc. Exogenous antioxidants on the other hand include vitamins A, E, C., carotenoids, and nitrogen containing compounds. Complications that arise in diabetes are as a result of damage that is caused by ROS. Plants offer a wide range of antioxidants that can be beneficial for the management of diabetes (Pietta, 2000).

2. Use of plants against diabetes mellitus

Plants have always provided mankind with all his needs in terms of shelter, clothing, food, flavours and fragrances as not the least, medicines. Plants have formed the basis of sophisticated traditional medicines systems among which are Ayurvedic (Indian), Unani and Chinese and the plants which were used by these medicinal systems are still in use today. Amongst the lesser known medicinal systems is African

partly because African medicine is verbal; some of the precious information of plants with medicinal value is lost and was not recorded.

The search for new molecules nowadays is changing slightly. Previously plants have been used as crude extracts which consisted of numerous active compounds. Some of these compounds may act synergistically, while at times they can have antagonist effects. The focus is now more on ethnobotany and ethnopharmacognosy in which chemists isolate and purify different sources and classes of compounds (Gurub-Fakim, 2006).

Complications associated with diabetes are the major source of morbidity and mortality in patients (Gurub-Fakim, 2006). People are now turning to alternative medicines for chronic illnesses. Plants have always been used for diabetes mellitus. Drugs that treat diabetes are derived directly or indirectly from plants (WHO, 1999). Some plant/ plant products act by lowering blood glucose levels while others work by inhibiting the absorption of glucose from the gut hence prevent the shoot of glucose in the blood just after the meal (Gurub-Fakim). Ethnobotanical information has reported that approximately 800 plants have some anti-diabetic properties, quite a few number of herbs have shown anti-diabetic results when tested using the available technology (Olarcon-Aguilera, 2000). Amongst the plant-derived actives that have shown some anti-diabetic activity include alkaloids, glycosides, polysaccharide, peptidoglycans, steroids, carbohydrates, amino acids and inorganic ions. It is also indicated that screening alpha glucosidase/ amylase inhibitors from plants is increasing (Gurub-Fakim, 2006), however a large number of these plants have not yet being studied in detail for their pharmacological activities. It can be concluded that plants are potential source of anti-diabetic drugs but this fact has not gained enough momentum in the scientific community.

3. Objectives of the study

- To discover and characterize new α -glucosidase and α -amylase inhibitors for the management of diabetes mellitus from natural sources (plants) using bioassay guided fractionation.
- To determine the chemical structure and activities of possible pure compounds isolated by bio-assay guided fractionation.
- To evaluate the efficacy of isolated compounds from plants for diabetes mellitus.
- To investigate oxidant scavenging activity of plant extracts and pure compounds.

4. Structure of the dissertation

Chapter 1:

This chapter gives detailed information on diabetes mellitus. Classification, complication, diagnosis, epidemiology as well as therapeutic intervention of diabetes is discussed in detail.

Chapter 2:

Selected plants for the treatment of diabetes mellitus have been briefly discussed in this chapter. A closer look at the plants chemistry, medicinal value as well as description is further discussed.

Chapter 3:

Inhibitory effect of plant extracts on α -glucosidase and α -amylase has been argued. The antioxidant and radical scavenging capacity of plants extracts have been discussed. In addition, cytotoxicity of selected plant, *T. sericea* against primary vervet monkey kidney cells (VK) is discussed.

Chapter 4:

Isolation, purification and identification of compounds from *T. sericea* using column chromatography and nuclear magnetic resonance and mass spectra have been discussed in this chapter.

Chapter 5:

This chapter displays the inhibitory activities of purified compounds on α -glucosidase and α -amylase. Furthermore, cytotoxicity and antioxidant properties of purified compounds are discussed.

Chapter 6:

This chapter gives a brief overview of all the findings in the study. Recommendations are also looked at in this chapter.

Chapter 7:

Acknowledgements

Chapter 8:

Appendices

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2. Introduction

Traditional medicine derived mainly from plants play a pivotal role in the management of diabetes mellitus (Ahmed *et al.*, 2004; Karunayake and Tennekoon, 1993). World Health Organization (WHO) has recommended the evaluation of the plants used in the treatment of diabetes, as they are considered to be effective, non-toxic, with lesser side effects and are said to be excellent candidates for oral therapy (Day, 1998).

Metformin, a well known antidiabetic drug has been used for the treatment of diabetes mellitus for centuries. Biguanides are a group of plant-derived compounds that are responsible for antidiabetic property in metformin. It is therefore, proposed that plants may contain similar antidiabetic compounds that can be used to lower blood glucose levels. Plants for the present study have been selected based on phytochemistry and ethnobotanical information. Different plant parts (leaves, root bark, and stem bark) were investigated, however, based on literature review these plant parts have not been validated scientifically as yet. The aim of this study therefore, was to investigate antidiabetic and antioxidant property of selected plants. A brief description, distribution, medicinal use and phytochemistry of each plant is discussed. Further details of isolated compounds of plants are illustrated in the appendix B (from dictionary of natural products).

2.1 Selected plants

2.1.1 *Psidium guajava* L

Family: Myrtaceae

Common name: **guava, guajava, kuawa**

2.1.1.1 Description

A small tree to 33 ft (10inch) with spreading branches, the guava is easy to recognize because of its smooth, copper-coloured bark that flakes off showing the greenish layer beneath; and also

because of the attractive “bony” aspect of its trunk which may in time attain a diameter of 10 inch (25cm). Young twigs are quadrangular and downy. The leaves are aromatic when crushed and are evergreen. The fruit gives off a strong, sweet, musky odor when ripe, may be round, ovoid or pear shaped, 2-4 in (5-10 cm) long. When immature before ripening, the fruit is green, hard, gummy within and very astringent (<http://www.hort.purdue.edu/newcrop/morton/>)

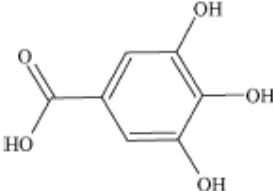
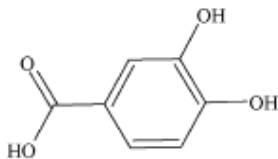
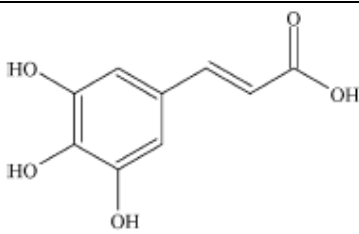
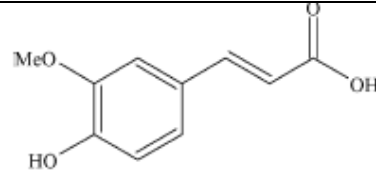
2.1.1.2 Distribution

The guava has been cultivated and distributed by man, birds, animals for so long that its place of origin is uncertain, but it is believed to be an area extending from southern Mexico into or through Central America. It is common throughout all warm areas of tropical America and the West Indies (since 1526), the Bahamas, Bermuda and Southern Florida where it was reportedly introduced in 1847 and was common over more than half the state by 1886. Early Spanish and Portuguese colonizers were quick to carry it from the New World to the East Indies and Guam. It was soon adopted as a crop in Asia and in warm parts of Africa. It occurs throughout the Pacific Islands (<http://www.hort.purdue.edu/newcrop>)

2.1.2.3 Phytochemistry

Guava is rich in tannins, phenols, triterpenes, Flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. The leaves of *P.guajava* are rich in flavonoids, α -pinene, β -pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β -bisabolene, cineol, caryophyllene oxide, β -copanene, farnesene, humulene, selinene, cardinene and curcumene (Zakaria and Mohd, 1994; Li *et al.*, 1999; Rattanachaikunsopon, and Phumkhachorn, 2007).

2.1.2.3.1 Other compounds isolated from *P. guajava***Table 2.1:** Constituents of *P.guajava* isolated from the leaves, roots and bark and their uses (Perez Guti'erez *et al.*, 2008)

Structure	Use
 <p>Gallic acid</p>	Cardioprotective effects against Ischemia-reperfusion. Antioxidant
 <p>Protocatechiuc acid</p>	Antioxidant
 <p>Caffeic acid</p>	Antibacterial and Antioxidant,
 <p>Ferulic acid</p>	Antioxidant, antimutagenic/anticarcinogenic effect and anti-inflammatory

2.1.1.4 Medicinal uses

External use

A decoction of the bark and leaves/ flowers is used topically for wounds, ulcers and skin sores. The roots, bark, leaves and immature fruits because of the astringency, are commonly employed to halt gastroenteritis, diarrhea and dysentery throughout the tropics.

Internal use

It has been used for sore throat, vomiting, stomach pains and also to regulate menstrual periods (Rattanachaikunsopon, and Phumkhachorn, 2007).



Figure 2.1: *Psidium guajava*

<http://images.search.yahoo.com/search/images?p=PSIDIUM+GUAJAVA&toggle>

2.1.2. Terminalia sericea Burch. Ex DC

Family: Combretaceae

Common names: **silver cluster-leaf** (Engl), **vaalboom** (Tswana), **muxonono** (N.Sotho), **mususu** (Tsonga and Venda), **amangwe** (Zulu)

2.1.2.1 Description

Terminalia is a medium sized deciduous or semi deciduous shrub that can grow about 5-8 meters in height (Eldeen *et al.*, 2006). It has an erect trunk and wide spreading crown with bark being grey to pale brown and coarsely fissured (Palmer and Pitman, 1972). Leaves are silver haired and crowned near the branch tips with cream colored flowers that have unpleasant smell. Fruits are about 30mm long with two wide papery wings surrounding the thickening central part.

2.1.2.2 Distribution

The plant is mostly dispersed in sand savanna areas in further North parts of Southern Africa (Van Wyk, 1995).

2.1.2.3 Phytochemistry

Compounds so far isolated from *Terminalia sericea* include a triterpene sericoside resveratrol-3-*O*- β -D-rutinoside, a hydroxystilbene glycoside (Moshi and Mbwambo, 2004).

2.1.2.4 Medicinal uses

Tswana, a South African tribe use root decoction as a remedy for stomach disorders and diarrhea. Decoction and infusion are used for eye lotion and for the treatment of pneumonia. The bark is used for diabetes mellitus and for wound infections (Van Wyk, 1997).



Figure 2.2 : *Terminalia sericea*

<http://www.nparks.gov.sg/nursery/uploadfiles/terminaliasericea03p408.jpg>

2.1.3 Artemisia afra Jacq. Ex Willd

Family: Asteraceae

Common names: **wild/ African wormwood** (Eng); **wilde als** (Afri); **umhlonyane** (Zulu and Xhosa); **lengana** (Tswana)

2.1.3.1 Description

Artemisia afra grows in thick, bushy and slightly disorderly clumps. The stems are thick, woody at the bases but softer and thin towards the top. *A. afra* can grow up to two meters high. Numerous side branches appear from the main stems. Leaves are thinly separated, with the upper surface being dark green while the undersides and the stems are covered with small white hairs, which gives the shrub

the characteristic overall grey color. Flowers bloom in late summer from March to May. The flowers are yellow and small with diameter between 3-4mm (Van Wyk and Gericke, 2000).

2.1.3.2 Distribution

This plant is distributed in Cedeberg Mountains in the Cape, and can spread as far as tropical east Africa, and north of Ethiopia. It prefers to grow at altitudes between 202-440 m on damp slopes, along stream sides and forest margins (Van Wyk *et al.*, 1997).

2.1.3.3 Phytochemistry

Volatile oil which contains largely 1, 8-cineole, α and β thujone, camphor and borneol are largely distributed in *Artemisia* leaves (Graven *et al.*, 1992). ‘Thujone’, has been associated with toxicity during overdose and long use. Terpenoids of eudesmadien and germacratien types as well coumarins and acetylenes are also present in *Artemisia afra* (Dictionary of natural products, 1996).

2.1.3.4 Medicinal uses

Artemisia afra is used for a wide variety of ailments including cough, fever, cold and loss of appetite, colic, headache, earache and diabetes (Van Wyk *et al.*, 1997). *A. afra* has been used to treat inflammatory diseases such as rheumatism, fever and diabetes (Hallowell and Gutteridge, 1989).



Figure 2.3 : *Artemisia afra*
<http://www.herbalsmokeshoppe.com/dagga/afra1.gif>

2.1.4 Aloe ferox Mill

Family: Aloaceae

Common names: **Bitter/ Red Aloe** (Engl); **Bitteraalwyn** (Afri); **iNhlaba** (Zulu); **ikhala** (Xhosa)

2.1.4.1 Description

Aloe ferox is a tall single stemmed plant that can grow up to 2-3 meters with leaves arranged in a rosette. After the old leaves have dried, they form a ‘petticoat’ on the stem. The leaves are dull green; sometimes they may be slightly blue with spines present in the upper and lower surfaces. The flowers are carried in a large umbrella-like flower head. The colour of the leaves ranges from yellow-orange to bright red. Flowers emerge between May and August but in colder parts of the country this may be delayed until September (Van Wyk *et al.*, 1997).

2.1.4.2 Distribution

Aloes are distributed ranging over 1000km from the southwestern Cape through the southern Kwazulu Natal. They are found in the southern corner of Free State and Lesotho (Van Wyk *et al.*, 1997).

2.1.4.3 Phytochemistry

The Aloes contain anthrone C-glucoside aloin (barbaloin). Glycoproteins present in Aloes are responsible for wound healing. Aloeresin was recently isolated from Cape Aloes (Manito *et al.*, 2003). Aloeresin A have been reported to demonstrated dose-dependent alpha glucosidase inhibitory activity with IC₅₀ values of 11.94 and 2.16mM against intestinal sucrose and maltase respectively (Jong-Anurakkun *et al.*, 2008).

2.1.4.4 Medicinal Uses

Cape aloes have been used for centuries as laxatives. The leaves and roots are boiled in water and are taken for the treatment of arthritis, eczema, conjunctivitis, hypertension, diabetes and stress. The powdered cape aloe when mixed with Vaseline is applied topically to herpes and shingles (Van Wyk *et al.*, 1997).

2.1.5 *Euclea natalensis* A.DC

Family: Ebeaceae

Common names: **Natal guarri** (Engl); **Natalgwari** (Afri); **Umzimane** (Zulu)

2.1.5.1 Description

The plant grows in coastal forests as a shrub or tree and can reach a maximum height of 10m. At a young age, the bark is white but turns darker and fissured with age. Flowers are small, scented and greenish-white (this is usually noted in May to January) are borne in abundant on branched beads leaves are covered with dense, rust-colored hairs. Attractive fruits to birds are spherical in shape with size between 7-10mm and they usually turn red when ripe (<http://www.up.ac.za/academic/botany/garden/species/1>).

2.1.5.2 Distribution

Euclea natalensis is widely distributed in tropical and subtropical Africa. In Southern Africa it is predominantly found in the East Coast (Port Elizabeth) up to Mozambique extending to Swaziland, Botswana and Zimbabwe.

2.1.5.3 Phytochemistry

Several secondary metabolites have been isolated from *E. natalensis*. Nine of these compounds are naphthoquinones. In addition other compounds that have been isolated from this plant include dihydroxyrsunoic acids (lactone derivatives), triterpenoids and one tetrahydroxyflavone arabonopyranoside (Lall *et al.*, 2006; Van der Kooy *et al.*, 2006). *Euclea natalensis* is rich in pentacyclic terpenoids and triterpenoids (Hutchings *et al.*, 1996). Some triterpenes have been reported to exert antidiabetic activities or potent α -glucosidase inhibitory activities (Luo *et al.*, 2008).

2.1.5.4 Medicinal uses

Zulu people use the roots for bronchitis, pleurisy, chronic asthma and urinary tract infections. Local inhabitants of South Africa are reported to also use the roots for headaches and toothaches (Lall *et al.*, 2006). Roots of *E. natalensis* have shown some antibacterial activity against *Mycobacterium tuberculosis* and some gram-negative bacteria (Lall *et al.*, 2006; Khan *et al.*, 1978).

2.1.6 Warburgia salutaris (Bertol.f.) Chiov.

Family: Canellaceae

Common names: **pepper-bark tree** (Engl.), **peperbasboom** (Afri), **mulanga**, **manaka** (Venda), **shibaha** (Tsonga), **isibhaha** (Zulu)



Figure 2.4 : *Aloe ferox*
http://perso.orange.fr/h.jung/aloes/Aloe_ferox3.jpg

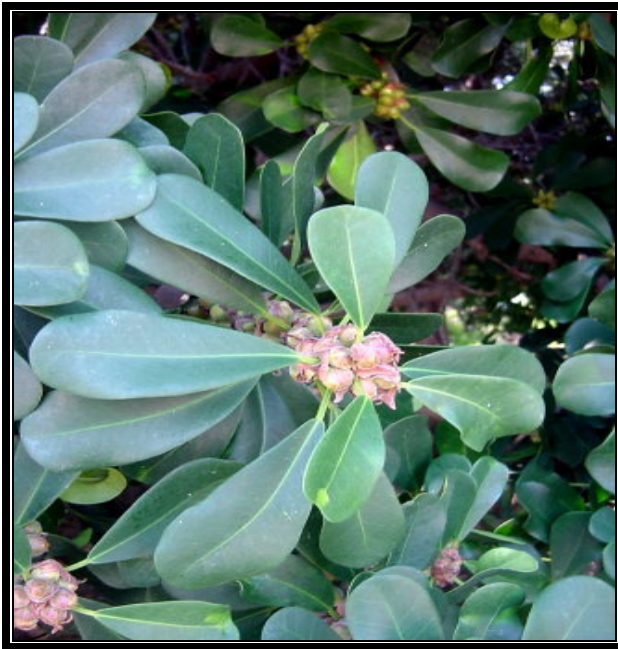


Figure 2.5 : *Euclea natalensis*
http://www.calflora.net/southafrica/images/ficus_natalensis.jpg

2.1.6.1 Description

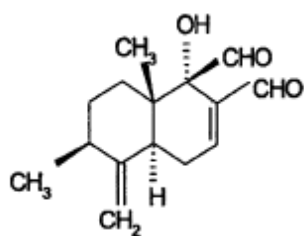
Pepper-bark tree is about 10m in height with rough, molted bark, which is reddish in the inner side. Leaves are rectangle in shape, 60mm long with glossy green on top while they are paler below. The flowers are small, greenish yellow in color with round green fruits (Codd, 1976).

2.1.7.2 Distribution

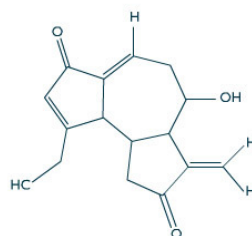
The plant is found in Northeastern parts of South Africa.

2.1.6.3 Phytochemistry

Fractionation of the ethyl acetate extract of the stem bark of *W. salutaris* yielded a known sesquiterpenoid, muzigadial (Rabe and van Staden, 2000). Another sesquiterpene lactone was isolated from *Warbugia salutaris*. The compound demonstrated *in vitro* activity against chloroquine sensitive strain D 10 ($IC_{50} = 0.9\mu\text{g/ml}$) and chloroquine resistant strains (1.2 $\mu\text{g/ml}$) and RSA 11 ($IC_{50} = 0.96\mu\text{g/ml}$) of *Plasmodium falciparum* (Sekhoacha *et al.*, 2007). Other compounds isolated from *W. salutaris* include: warburganal, polygadial, isopolygadial, salutarisolide and mukaadial (Mashimbye *et al.*, 1999).



A)



B)

Figure 2.6: A) Structure of muzigadial; B) Sesquiterpene lactone isolated from *W. salutaris*

2.1.6.4 Medicinal use

Warbugia salutaris has been overexploited by the collectors in the wild for traditional medicinal purposes. The stem bark is most widely sought for the traditional herbal market leading to the species endangered status (Rabe and van Staden, 2000). A bark decoction is taken for colds, influenza, sinus, and other respiratory complaints. Powdered bark on the other hand is applied to sores and used as snuff whilst it is also used for the treatment of malaria (Mander *et al.*, 1995).



Figure 2.7 : *Warbugia salutaris*
<http://www.plantzafrika.com/plantwxyz/warburg.htm>

2.1.7. *Sclerocarya birrea* A.Richi.) Hochst. subsp. caffra

Family: Anacardiaceae

Common names: **marula** (Eng.); **morula** (Northern Sotho); **mufula** (Tshivenda); **ukanyi** (Tsonga)

2.1.7.1. Description

Marula is a medium sized to large deciduous tree with erect trunk and rounded crown. Separate trees bore female and male flowers that produce pollen and fruits for which the tree is famous for. The

fruits are green on the tree but turn yellow after falling-usually from February to June. Compound leaves are mostly crowded at the end of branches (<http://www.plantzafrica.com/plantqrs>).

2.1.7.2. Distribution

The marula is widespread in Africa from Ethiopia in the north to KwaZulu-Natal in the south. In South Africa it is more common in the Baphalaborwa area in Limpopo. It occurs naturally in various types of woodland, on sandy soil or on occasionally sandy loam (<http://www.plantzafrica.com/plants>).

2.1.7.3 Phytochemistry

The bark is known to possess 3.5-20.5% tannin, 10.7% tannin matter and traces of alkaloids (Watt and Breyer-Brandwijk, 1962). The fruit is rich in ascorbic acid and juice extracts yield 33 sesquiterpene hydrocarbons, while kernels yield 54-60% of non-drying oil and 28% protein and iodine (Pretorius *et al.*, 1985; Watt and Breyer-Brandwijk, 1962). The oil-rich seeds contain 64% oleic acid, myristic, stearic and amino acids with prevalence of glutamic acid and arginine. The gum is rich in tannins, whereas the leaves are rich in both tannins and flavonoids (Busson, 1985). A number of investigators have shown that coumarins, flavonoids, terpenoids, and a host of other secondary plant metabolites present in *Sclerocarya birrea*, including arginine and glutamic acid, showed hypoglycemic effects in various experimental animal models (Akah and Okafor, 1992).

2.1.7.4. Medicinal use

Zulu, a South African tribe use bark decoctions as enemas for diarrhea, the bark decoctions are also taken in 300 ml doses for dysentery (Watt and Breyer-Brandwijk, 1962). Bark is also being used for the treatment of proctitis; the Venda people use the bark for treating fevers, stomach ailments and

ulcers (Mabogo, 1990). Roots are used for sore eyes in Zimbabwe whereas in East Africa, roots are ingredient in alcoholic medicine taken to treat internal ailments (Kokwaro, 1976).

2.1.8 Spirostachys africana Sond

Family: Euphorbiaceae (spurge family)

Common names: **Tamboti** (Engl.), **tambotie** (Afri.), **umThombothi** (Zulu), **Modiba** (N.Sotho), **Muonze** (Venda)

2.1.9.1 Description

Spirostachys africana is medium sized, semi deciduous tree that can grow up to 18m. The bark is dark brown to black, thick, rough and neatly cracked into rectangular blocks. It consists of alternative leaves that are up to 70 x 35 mm, these leaves are often visible among the older, green leaves in spring. The flowerheads are 15-30mm long bearing mostly male and female flowers. The flowers of females are attached at the base of each spike. The fruit is characterised by the capsule that is three lobed and opens when ripe (<http://www.plantzafrica.com/plantqrs/spirostachafri.htm>).

2.1.8.2 Distribution

This plant is distributed mostly in KwaZulu-Natal in the South up to Tanzania, it can also be found in all southern African countries except Lesotho (<http://www.plantzafrica.com/plant>).

2.1.8.3 Phytochemistry

Two diterpenoids and nonditerpenoids were isolated from the plant. Other active constituent present in *S. africana* is stachenol (Munkombwe *et al.*, 1997).

2.1.8.4 Medicinal Uses

The leaves of *S. Africana* are used for stomach ulcers, acute gastritis, eye washes, headaches, rashes, emetic, renal ailment, purgatives, blood purification, diarrhoea, and dysentery (Elgarashi *et al.*, 2003).



Figure 2.8 : Image of *Sclerocarya birrea*

<http://www.plantzfrica.com/plantqrs/sclerobirr.htm>



Figure 2.9 : *Spirostachys africana*

http://en.wikipedia.org/wiki/Spirostachys_africana

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<http://www.plantzafrika.com/plantwxyz/warburg.htm>.

<http://www.plantzafrika.com/plantqrs/sclerobirr.htm>.

http://en.wikipedia.org/wiki/Spirostachys_africana.

<http://www.nparks.gov.sg/nursery/uploadfiles/terminaliasericea03p408.jpg>.

<http://www.herbalsmokeshoppe.com/dagga/afra1.gif>.

http://perso.orange.fr/h.jung/aloes/Aloe_ferox3.jpg.

http://www.calflora.net/southafrica/images/ficus_natalensis.jpg.

<http://www.dentistry.leeds.ac.uk/biochem/thcme/diabetes.html>.

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3.1 Introduction

The importance of medicinal plants cannot be ignored. Several medicines for cancer, malaria, tuberculosis, and diabetes mellitus have been developed from plants. An important drug for the treatment of diabetes, ‘metformin’ (a derivative of plant-derived compound), has been used for diabetes for decades; however, the use of this drug is not without drawbacks. These include rigid and multiple dosing regimen. High costs, inaccessibility and untoward effects such as ‘diarrhoea, abdominal pains and flatulence. These factors have contributed to the recent increase in the use of folkloric plant medicines. It is therefore, of vital importance to look for alternative drugs with less side effects. Acetone extracts of plants selected based on ethnobotanical use and phytochemical constituents were tested for their inhibitory activity on α -glucosidase and α -amylase enzymes. Plant extracts were also tested for their antioxidant activity by measuring free radical scavenging activity. In addition, extracts were tested for cytotoxicity on Vero cell lines.

Antioxidant supplements are very important in patients having diabetes mellitus. Low levels of plasma antioxidants is a risk factor associated with diabetes (McCune and Johns, 2002). Complications of diabetes include retinopathy; arterosclerosis, kidney failure, etc which are the leading cause of morbidity and mortality amongst diabetics. These complications arise as a result of low levels of plasma antioxidants which result in oxidative stress (Bayners, 1991). Plants produce biological active compounds. These compounds are known as secondary metabolites or secondary constituents. Amongst these constituents, there are those that act as antioxidants. Examples include tannins, carotenoids, and flavonoids. In addition plants also produce antioxidants such as ascorbic acid and tocopherols (Larson, 1988). It has therefore, been suggested that it will be an added advantage for plant-based medicines for diabetes which will also have antioxidant property.

3.2 Materials and Methods

3.2.1 Plant material

Eight plants used in present study were collected from different locations. *Terminalia sericea* stem bark, *Sclerocarrya birrea* stem bark and *Psidium guajava* leaves were collected from Venda (Limpopo province of South Africa). *Artemisia afra* leaves, *Aloe ferox* leaves, *Warbugia salutaris* leaves, *Euclea natalensis* root bark and *Spirostachys africana* stem bark were collected from the Botanical garden of the University of Pretoria (Table 3.1). The plants were botanically identified by the taxonomist, Prof Van Wyk, and submitted at the H.G.W.J. Schweickerdt Herbarium (PRU) of the University of Pretoria where voucher specimens were deposited.

3.2.2 Preparation of extracts

Plant extracts exist as mixtures of trillions of compounds. It is therefore, of critical importance to find a suitable extractant. For the current study, 50g of dried plant materials were ground to fine powder. Each plant material was soaked in (50 ml) 100% acetone overnight at room temperature. The solvent was then removed and replaced with equal volume of solvent. This procedure was repeated two times. Extracts were filtered and concentrated to dryness using BUCHI rotary evaporator under reduced pressure. Dried extracts were reconstituted in dimethylsulfoxide (DMSO) at 2mg/ml and stored in the dark at 4°C.

3.2.3 Assay for bakers yeast α -Glucosidase inhibitory activity

The assay method was adopted from Collins *et al.*, (1997) and modified accordingly to suite microtiter reading. The enzyme, α -glucosidase (EC 3.2.1.20) and the substrate, p-nitrophenyl α -D-glucopyranoside were purchased from Sigma Chemical Co., (St Louis, MO, USA). The glycohydrolase assay was performed in 96-well microtiter plates.

Table 3.1: Medicinal plants investigated in this study

Species	Family	Part used	Voucher specimen no
<i>Terminalia sericea</i> Burch. Ex DC	Combretaceae	Stem bark	Van Rensburg PRU 38564
<i>Psidium guajava</i> L	Myrtaceae	Leaves	PRU 54544
<i>Sclerocarya birrea</i> (A.Richi.) Hochst. subsp. caffra	Anacardiaceae	Stem bark	PRU 4558/100
<i>Warbugia salutaris</i> Bertol.f.) Chiov.	Canellaceae	Leaves	PRU 094845
<i>Artemisia afra</i> Jacq. ex Willd.	Asteraceae	Leaves	PRU 112085
<i>Aloe ferox</i> Mill	Aloaceae	Leaves	PRU 110308
<i>Euclea natalensis</i> A.DC	Ebenaceae	Roots bark	PRU 095059
<i>Spirostachys africana</i> Sond	Euphorbiaceae	Leaves	PRU 8434

The enzyme was diluted in 50mM Mes-NaOH, pH 6.5. The extracts were allowed to interact with enzyme at room temperature for 5 minutes before the reaction was started by the addition of appropriate substrate. The total reaction volume was 0.2ml. The reaction was allowed to proceed at room temperature for 15 minutes before it was stopped by the addition of 60 μ l 2M glycine-NaOH, pH 10. The assay was performed in triplicates. The final concentration of plant extracts in the wells ranged from 0.02 to 200 μ g/ml. The concentration of positive drug control ‘Acarbose’ ranged from 0.01 to 100 μ g/ml. The highest % of DMSO (1%) did not have any effect on the inhibition of α -

glucosidase enzyme. The coloured product was detected in a Bio-Tek® model microplate reader at 412nm (reference 490nm). Results were analysed using the formula:

$$\text{Percent Inhibition} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

3.2.4 Assay for porcine pancreatic α -Amylase inhibitory activity

3.2.4.1 Digestion of starch with porcine pancreatic α -amylase

Alpha amylase inhibition assay was performed using the chromatogenic method adopted from Sigma-Aldrich which was adapted from Bernfeld (1955). Porcine pancreatic α -amylase was dissolved in ice cold distilled water to give a concentration of 10 units/ml solution. Potato starch (0.5%, w/v) in 20mM phosphate buffer (pH 6.9) containing 6.7mM sodium chloride, was used as a substrate solution. The assay was carried out on glass tubes. Equal amounts (300 μ l) of solution from the tubes were transferred to eppendorfs containing 300 μ l of 3.18M sodium carbonate (NaCO₃). Frozen samples were allowed to thaw and immediately put on ice and vortexed just before use for the Prussian blue assay.

3.2.4.2 Determining reducing sugars (Prussian Blue Assay)

The assay was adapted from Slaughter *et al* (2001) with slight modifications. Except for *Terminalia sericea*, the final concentration of plant extracts in the tubes ranged from 0.025 to 1.25mg/ml. During preliminary tests, *Terminalia sericea* showed activity at higher concentrations hence its concentrations ranged from 0.05 to 1.2mg/ml to determine IC₅₀ values. The concentrations of positive drug control 'Acarbose' ranged from 0.016 to 1mg/ml. The highest % of DMSO did not have any effect on the inhibition of α -glucosidase enzyme. Briefly, equal portions (0.5ml) of sample (diluted with distilled water), solution A (1.6 mM KCN, 0.19 Na₂CO₃); solution B [1.18 mM K₃Fe (CN)₆]; were mixed in glass test tubes capped with aluminium foil. The tubes were placed in vigorously boiling water for exactly 15 minutes and then removed and allowed to cool at room

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temperature for 15 minutes. Once cool, 2.5ml of solution C [3.11mM $\text{NH}_4\text{Fe}(\text{SO}_4)_2$; 0.1 sodium dodecyl sulphate, 0.42% v/v H_2SO_4] was added to the tubes. The tubes were left to stand for 150 minutes for the development of colour. The absorbance was read on a Beckman Coulter Du 720 spectrophotometer at 690nm. Maltose standards covering the range of 0-10 μM in the final assay were assayed by the same procedure. Reducing sugar concentrations are expressed as maltose equivalents. Readings were taken using blanks containing the amounts of inactivated enzyme and starch equivalent to that present in test assays.

3.2.5 DPPH assay

Antioxidant activities of acetone extracts and purified compounds were investigated using the 1,2-diphenyl-2-picrylhydrazil (DPPH) (Sigma-Aldrich, South Africa) antioxidant assay. Following the procedures as described by Toit *et al.*, 2001) for each sample, a dilution series (8 dilutions) was prepared in a 96-well ELISA plate by adding distilled water (100 μl) as a dilution medium. Final concentrations of the acetone plant extracts ranged from 3.125 to 200 $\mu\text{g/ml}$ and of compounds ranged from 0.78 to 100 $\mu\text{g/ml}$. Each concentration was tested in triplicates. Ascorbic acid (AA)/vitamin C was used as a positive control and was tested at the concentrations ranging from 0.52 to 67 $\mu\text{g/ml}$. Ninety microlitres (90 μl) of ethanolic DPPH was added to each well. The plates were covered with aluminium foil and incubated at room temperature for 30 minutes before being read by a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

The radical scavenging capacities of the samples were determined by using a BIO-TEK plate reader to measure the disappearance of DPPH at 550nm. The radical scavenging activity was measured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Toit *et al.*, 2001). The EC_{50} was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in $\mu\text{g/ml}$ for the standard and samples. The EC_{50} is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%. The results are

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expressed as the mg AA equivalents / g dry weight and are calculated as follows: $EC_{50} \text{ AAmg/ml} / EC_{50} \text{ sample (g/ml)} = \text{xmg AA equivalents / g dry weight}$ Zero mg/ml was taken as 100%

3.2.6 Toxicity screening (XTT viability assay)

The cytotoxicity of crude extract of *Terminalia sericea* was investigated by using XTT-based colorimetric assay Cell Proliferation Kit II (Boehringer-Mannheim) following the method as described by Roche (2004). As the extract of *Terminalia sericea* showed good hypoglycemic activity, this extract was further selected for its cytotoxicity evaluation. The final concentration of crude extract tested ranged from 3.125-400 μ g/ml. The final concentration of 'Doxorubicin', a positive control, in the wells ranged from 0.104-12.5 μ g/ml. The final concentration of the extract at which 50% (IC_{50}) of the Vero cells were alive until the 4th day was considered to be the highest concentration which is non-toxic to the cells. These values were calculated using Graph Pad Prism 4 programme.

3.3 Statistical analysis

The final results are expressed as the mean (standard deviation, \pm SE.S). The group means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple range Test was applied to compare the means. Values were determined to be significant when p was less than 0.05 ($p < 0.05$).

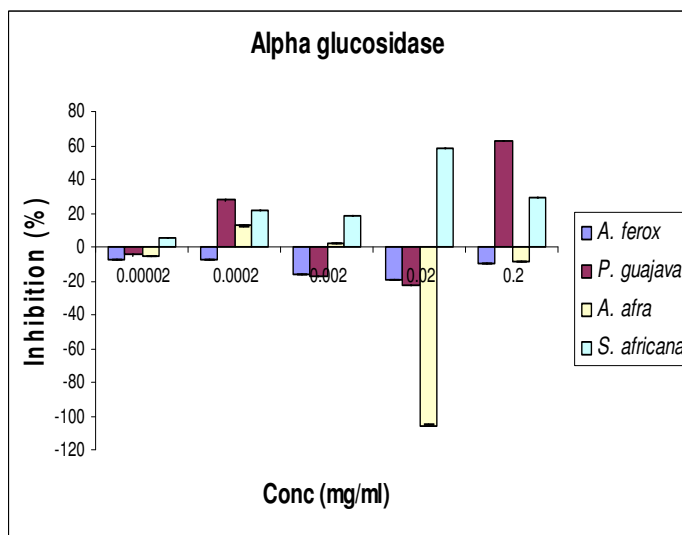
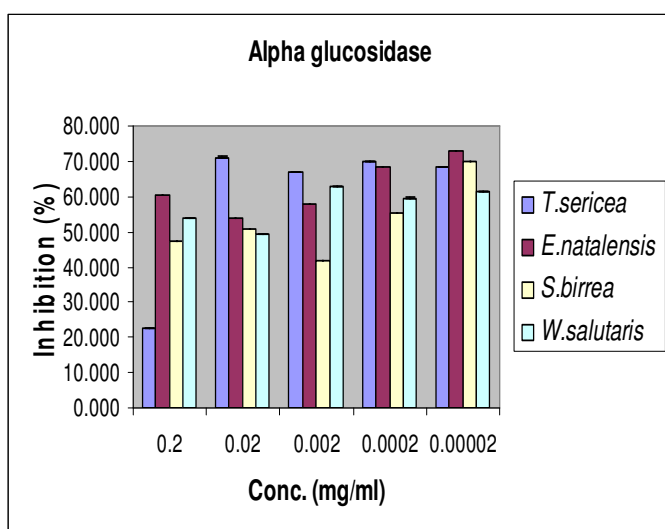
3.3 Results and Discussion

In Africa many plants are traditionally used for the management and control of diabetes mellitus, however few of such have received scientific scrutiny despite the fact that WHO has recommended that medicinal and scientific examination of such plants should be undertaken (WHO Expert Committee of Diabetes Mellitus, 1980). Alpha glucosidase and amylase are enzymes that are

involved in hydrolysis of starch hence they contribute to postprandial hyperglycaemia (Gowri *et al.*, 2007). Inhibitors of these enzyme delay digestion of glucose hence prolonging overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma (Bhandari *et al.*, 2008). Plant or plant products have been investigated with respect to the suppression of glucose production from carbohydrate in the gut.

3.3.1 α -Glucosidase and Amylase inhibitory activity

Eight plant extracts were tested for alpha glucosidase and amylase inhibition activity (Table 3.2, figures 3.3-3.5). The inhibition percentage of extracts ranged from 47.15 ± 0.02 to 97.57 ± 0.01 at 0.2mg/ml. The extracts of *A. ferox* and *S. africana* showed no inhibition against α -glucosidase at the highest concentration tested (0.2mg/ml) whereas *A. afra* showed weak inhibition (47.15%). *T. sericea* showed to be a potent inhibitor of α -glucosidase exhibiting 97.44 % inhibition of the enzyme ($p < 0.05$). *W. salutaris*, *S birrea* and *E. natalensis* also showed good activity on α -glucosidase as they demonstrated 71.84; 97.44 and 92.60 % inhibition respectively ($p < 0.05$). Other plant extracts such as *A. ferox* and *S. africana* did not exhibit any activity on α -glucosidase.(Table 3.2, figure 3.1 and 3.3)



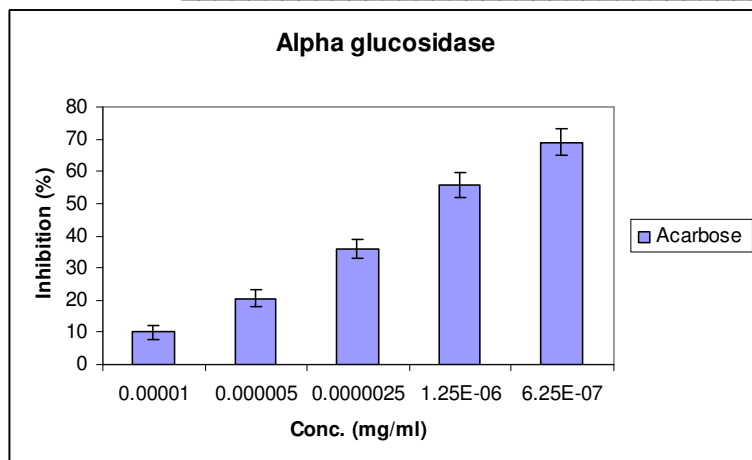


Figure 3.1: Inhibition of α -glucosidase using p-nitrophenyl α -D-glucopyranoside as a substrate, by the extracts and positive drug-control; Acarbose

T. sericea and *S. birrea* showed the best inhibitory activity on α -amylase enzyme, exhibiting 91.91 and 94.94 % inhibition respectively at 1.25mg/ml. *A. afra*, *E. natalensis*, *P. guajava* and *W. salutaris* also showed good inhibitory activity on α -amylase enzyme at 1.25mg/ml which was the highest concentration tested ($p < 0.05$). (Table 3.2, figure 3.2 and 3.3). For determining the inhibitory activity against each enzyme, all extracts were tested at 1.25mg/ml for alpha amylase and 0.2mg/ml for alpha glucosidase and 50% inhibition or higher was taken as significant ($p < 0.05$).

T. sericea stem bark demonstrated good activity on both enzymes. *T. sericea* has been used in the folk medicine for the treatment of many ailments including diabetes. Several compounds have been isolated from the plant (Fyhrquist *et al.*, 2002). In a study done by Tshikalange *et al.*, (2008), it was found that *T. sericea* inhibited α -glucosidase by $90.00 \pm 0.01\%$. In a similar study, *Terminalia superba* and its constituents, gallic and methyl gallate showed significant α -glucosidase inhibitory activity (Wansi *et al.*, 2007). On the other hand Gao *et al.*, 2007 indicated that *T. chebula* also showed good inhibitory effect against rat intestinal maltase activity.

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W. salutaris, *S. birrea*, *E. natalensis* and *P. guajava* also demonstrated good α -glucosidase and amylase inhibitory activities. *S. birrea* dichloromethane: methanol (1:1) extract has been found to decrease blood glucose levels while increasing plasma insulin levels in STZ rats (Dimo *et al.*, 2007). A considerable improvement was seen in glucose tolerance during an oral glucose tolerance test in diabetic rats treated with the extract and this led to the speculation that this improvement could be associated with stimulation of insulin. In a study done by Venter and colleagues (2008), organic extract (methanol) of *S. birrea* caused a noted increase in glucose utilization in Chang liver cells. Quite a few studies have reported hypoglycemic activity of *P. guajava* extracts. A study done recently has revealed that the *P. guajava* methanol extract has shown significant inhibition of alpha-glucosidase activity in the small intestine of diabetic mice (Wang *et al.*, 2007). In another study, aqueous leaf extracts of *P. guajava* were tested in STZ-induced (Ojewule, 2005) and alloxan-induced diabetic rats (Mukhtar *et al.*, 2004) an ethanol extract was tested on alloxan-induced hyperglycemic rats (Mukhtar *et al.*, 2006), a butanol-soluble fraction of the leaves was tested Lepr^{db}/Lepr^{db} mice and has shown to significantly decrease the blood glucose levels in rats (Oh *et al.*, 2005). Other plant extracts such as *A. ferox* and *S. africana* showed weak inhibition on alpha glucosidase (inhibition less than 50%). In a study done by Jong-Anurakkun *et al.*, 2008; Aloeresin A isolated from *A. ferox* have been reported to demonstrated dose-dependent α - glucosidase inhibitory activity with IC₅₀ values of 11.94 and 2.16mM against intestinal sucrose and maltase respectively. In the present study the extract of *A. ferox* did not exhibit any α -glucosidase activity. *E. natalensis* also demonstrated good α -glucosidase and α -amylase inhibitory activities.

There are no reported data on the activity of *E. natalensis* on both enzymes; however, it is rich in pentacyclic triterpenoids. Triterpenoids have been reported to possess both potent α - glucosidase and α -amylase inhibitory activities (Luo *et al.*, 2008). *W. salutaris* which also exhibited α -glucosidase and α -amylase inhibitory activities is rich in sesquiterpenoids. In a study done by Choudhary *et al.*,

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(2001), sesquiterpenoids isolated from the air-dried roots of *Ferula mangolica* exhibited significant α -glucosidase inhibitory activity (IC₅₀ value =79.87 μ M).

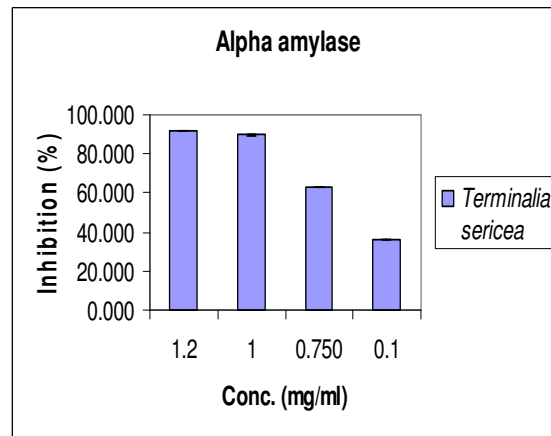
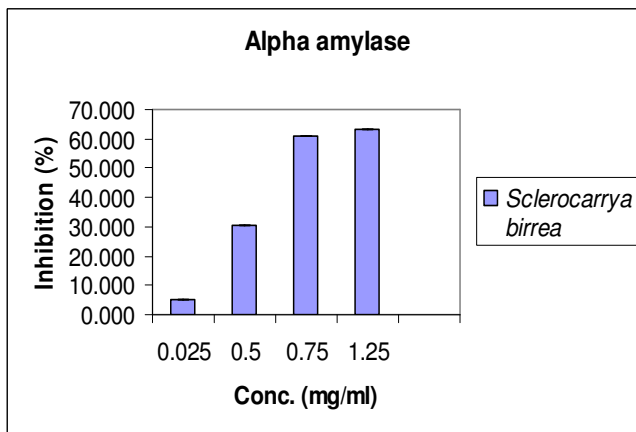
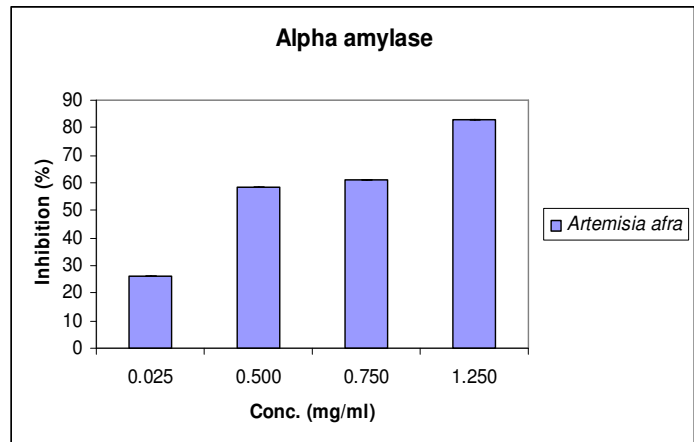
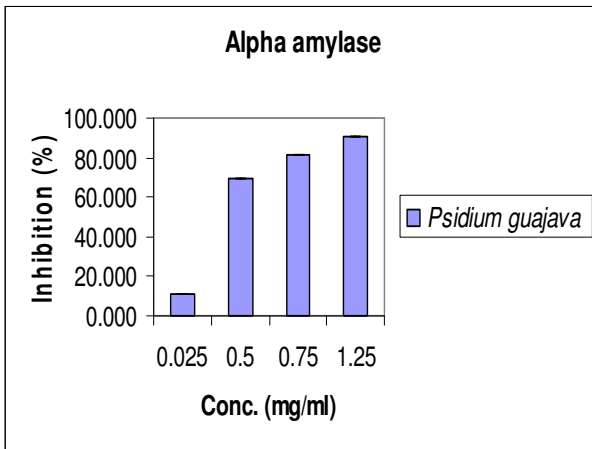
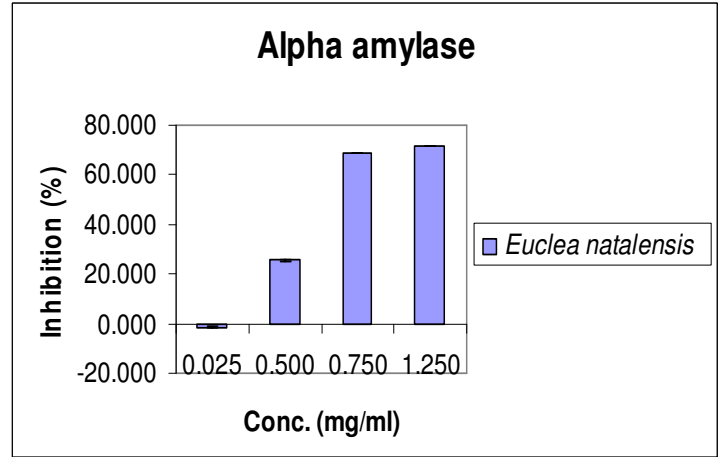
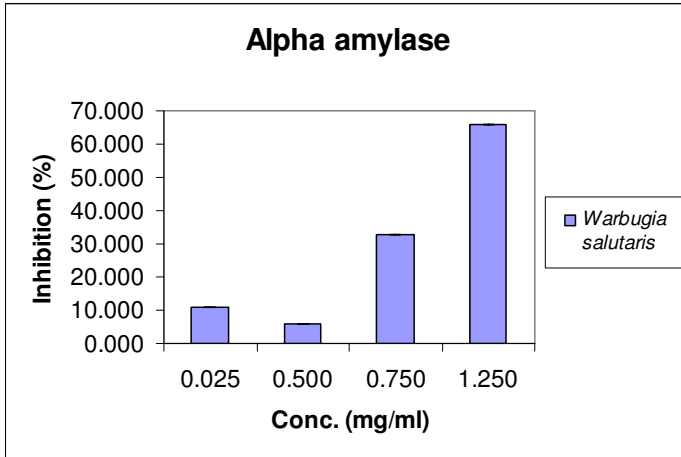
Table 3.2: Effect of plant extracts on the inhibition of α -glucosidase and α -amylase enzymes

Plant	α-glucosidase Inhibition % (SD) at the concentration of 0.2mg/ml	α-amylase Inhibition % (SD) at the concentration of 1.25mg/ml
<i>Terminalia sericea</i>	97.44 \pm 0.03	91.91 \pm 0.10
<i>Sclerocarya birrea</i>	97.44 \pm 0.04	94.94 \pm 0.01
<i>Artemisia afra</i>	47.15 \pm 0.02	74.00 \pm 0.01
<i>Euclea natalensis</i>	92.60 \pm 0.04	74.54 \pm 0.04
<i>Psidium gaujaya</i>	62.74 \pm 0.19	89.14 \pm 0.01
<i>Warbugia salutaris</i>	71.84 \pm 0.27	89.21 \pm 0.06
<i>Aloe ferox</i>	ni ^a	ni ^b
<i>Spirostachys africana</i>	ni ^a	ni ^b
Acarbose	80.63 \pm 0.03	73.40 \pm 0.03

^a: no inhibition of α -glucosidase enzyme at the highest concentration tested (0.2mg/ml)

^b: no inhibition of α -amylase enzyme at the highest concentration tested (1.25mg/ml)

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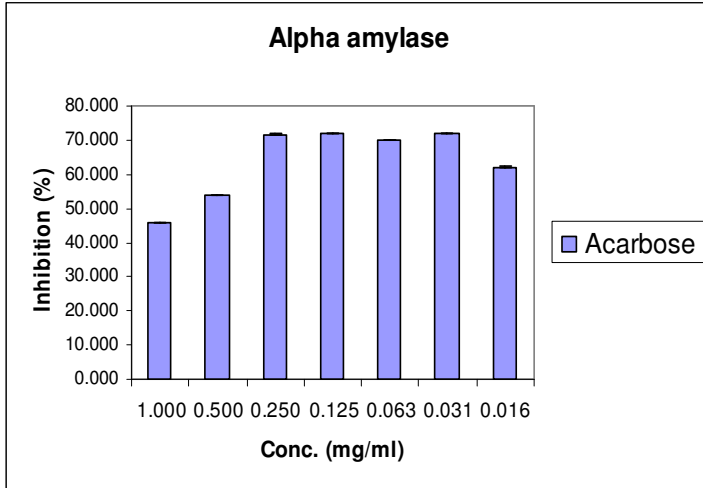


Figure 3.2: Inhibition of α -amylase using soluble potato starch as a substrate, by different concentrations of the extracts and acarbose (standard control).

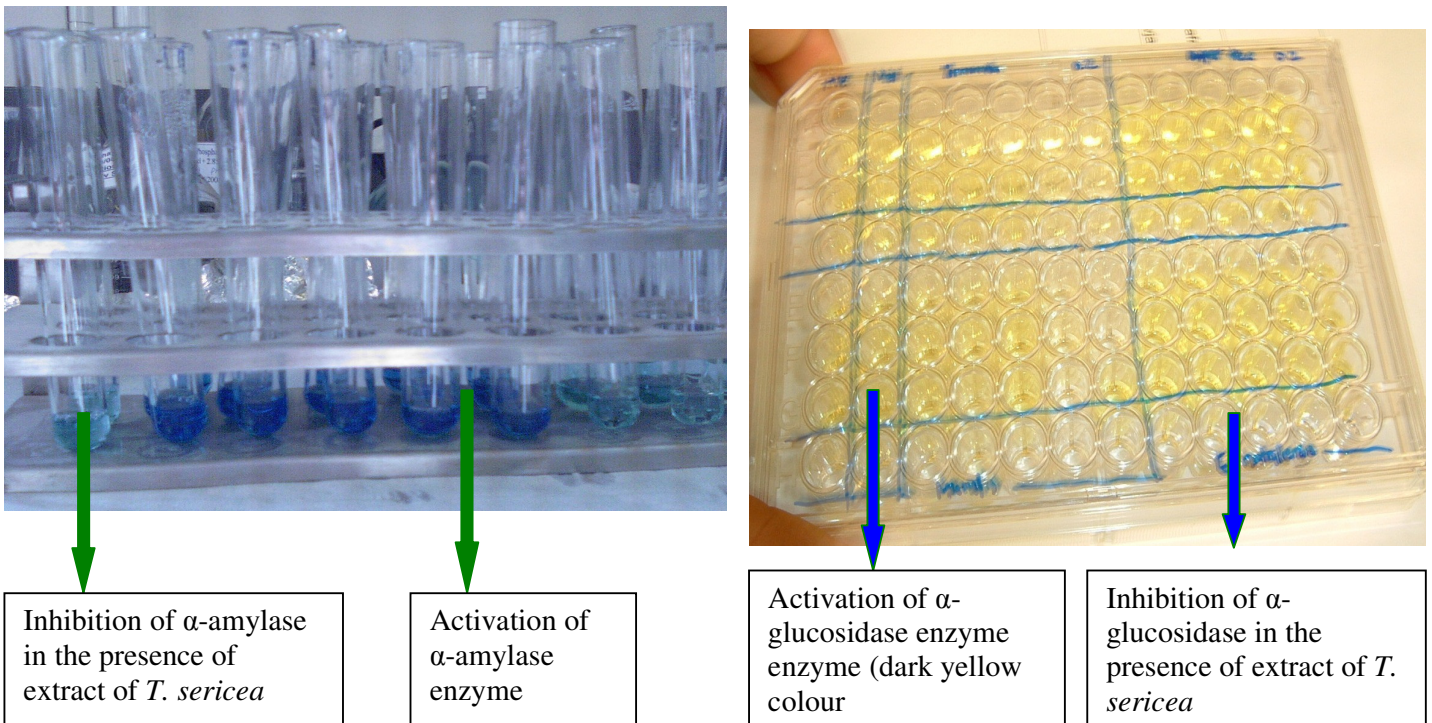


Figure 3.3: A) α - Amylase in glass tubes: activation of α -amylase enzyme results in the formation of dark blue colour, when this activity is inhibited; a light blue colour results

B) Inhibition of α -Glucosidase enzyme by the plant extracts. Activation of the enzyme results in formation of yellow colour on 96-well plates, inhibition is indicated by light-yellow colour as compared to the wells where enzyme is activated.

3.3.2 Antioxidant activity

Cellular damage/ oxidative injury arises from the formation of free radicals which appears to be the fundamental mechanism underlying a number of human neurodegenerative disorders such as cancers, diabetes and inflammation (Perez Guitierrez *et al.*, 2008). Free radicals are natural by products of human metabolism which attack cells, proteins and enzymes present in cells (Masoko and Eloff, 2007). They are produced as a result of body's use of oxygen such as respiration and some cell-mediated immune response, however, environmental factors such as cigarette smoke; automobile exhaust, radiation and air pollution contribute to the formation of free radicals (Li and Trush, 1994). The body does produce antioxidants that scavenge/quench these free radicals; however, the antioxidant defence systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level.

Plants contain a variety of free radical scavenging molecules. These may include phenolic compounds (flavonoids, phenolic acids, quinones, tannins); nitrogen containing compounds (alkaloids) Terpenes (carotenoids) (Zeng and Wang, 2001). The antioxidant activity of plant extracts was carried out using DPPH assay. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. As shown in table 3.3, figure 3.4 and 3.5 all six tested plant extracts showed good activity. *P. guajava* and *T. sericea* exhibited the highest activity with the IC_{50} values of $6.97 \pm 0.03 \mu\text{g/ml}$ and $5.56 \pm 0.01 \mu\text{g/ml}$ respectively when compared to ascorbic acid ($IC_{50}=2.52 \pm 0.01 \mu\text{g/ml}$), a well-known potent antioxidant. These plant extracts demonstrated significant results ($p < 0.05$). This was followed by *W. salutaris* ($IC_{50}=5.08 \pm 0.01 \mu\text{g/ml}$); *E.*

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natalensis (IC_{50} = 8.46 \pm 0.01 μ g/ml) and *S birrea* (IC_{50} = 9.41 \pm 0.01 μ g/ml). *A. ferox* showed IC_{50} value of 48.53 \pm 0.01 μ g/ml (Table 3.3).

The antioxidant activity of these traditional medicinal plants may come in part from the antioxidant vitamins; phenolic compounds most particularly flavonoids or tannins. *P.guajava* contains high levels of phenolic compounds, which may attribute to its high antioxidant activity (Qian and Nihoimbere, 2004). It has been previously shown to possess antioxidant properties (Gutierrez *et al*, 2008); which are associated with its phenolic compounds such as protocatechin acid, ferulic acid, quercetin and guavin B (Thoipong *et al.*, 2005). In qualitative analysis of antioxidant activity using DPPH on a thin layer chromatography (TLC) plate developed with acetone and methanol extracts of *T. sericea*, antioxidant compounds in extracts were clearly indicated as clear spots (Masoko and Eloff, 2007). Several flavonoids that demonstrated antioxidant properties have been isolated from *T. fagifolia*, a species belonging to *Terminalia* genus (Garcez *et al.*, 2006).

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Table 3.3: A summary of fifty percent inhibitory concentration (IC₅₀ values) of extracts on alpha (α) - glucosidase, α -amylase and DPPH

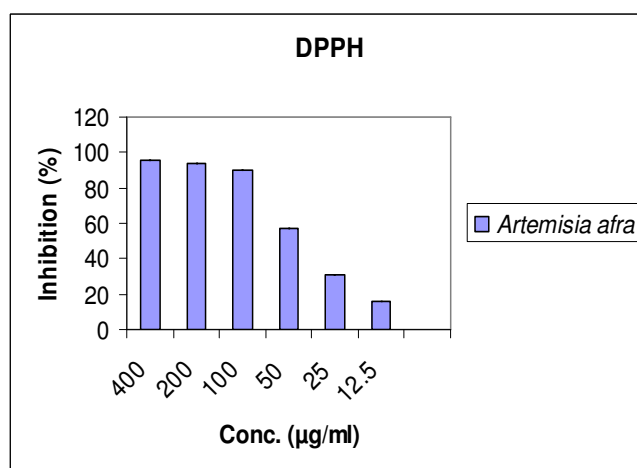
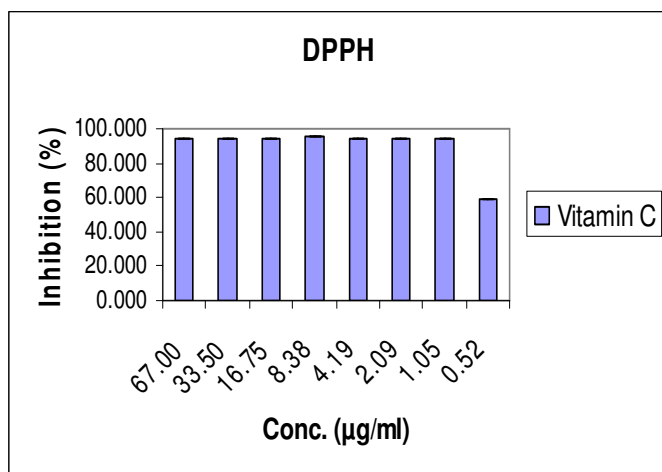
Plant	IC ₅₀ α -Glucosidase (μ g/ml)	IC ₅₀ α -Amylase (μ g/ml)	IC ₅₀ DPPH (μ g/ml)
<i>Terminalia sericea</i>	83.41± 0.05	55 ± 0.04	5.56 ± 0.05
<i>Sclerocarya birrea</i>	100± 0.03	100 ± 0.01	9.41 ± 0.03
<i>Artemisia afra</i>	N/A	150 ± 0.01	48.53± 0.01
<i>Euclea natalensis</i>	95.55 ± 0.08	60.50± 0.05	8.46 ± 0.01
<i>Psidium gaujva</i>	96.67 ± 0.15	85.36 ± 0.06	6.97 ± 0.03
<i>Warbugia salutaris</i>	188± 0.01	71.23±0.09	7.50 ± 0.01
<i>Aloe ferox</i>	N/A	N/A	N/T
<i>Spirostachys africana</i>	N/A	N/A	N/T
Acarbose ^a	60 ± 0.09	42 ± 0.49	
Vitamin C ^b	-	-	2.52 ± 0.89

N/A: not active at the highest concentration tested

N/T: not tested (these plants did not show activity on α -glucosidase and α -amylase hence they were not tested for DPPH)

a: positive drug control for α -glucosidase and α -amylase inhibition assay

b: positive drug control for antioxidant bioassay



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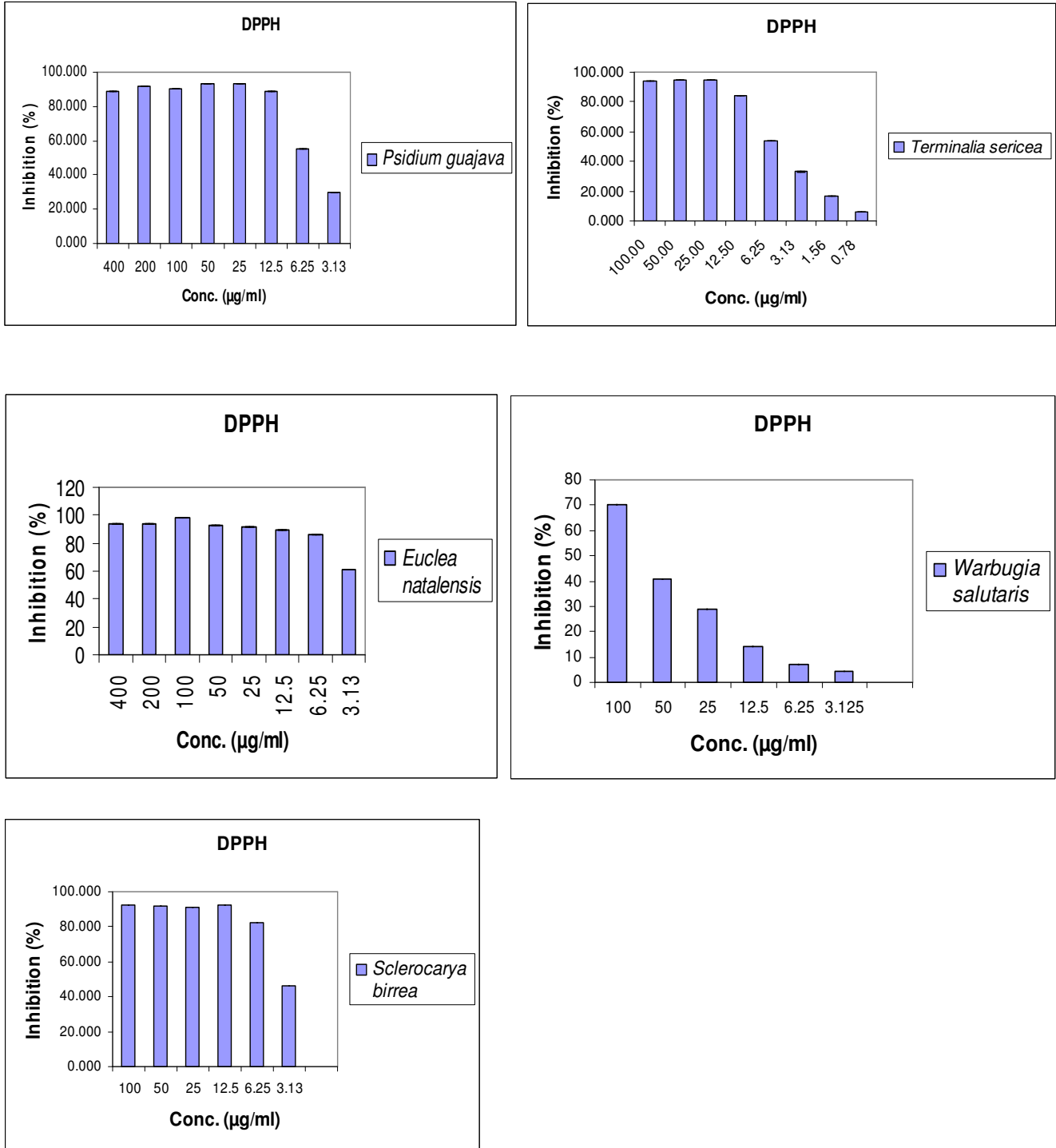


Figure 3.4: The percentage inhibition of DPPH by the acetone extracts of selected plants and Vitamin C (standard control)

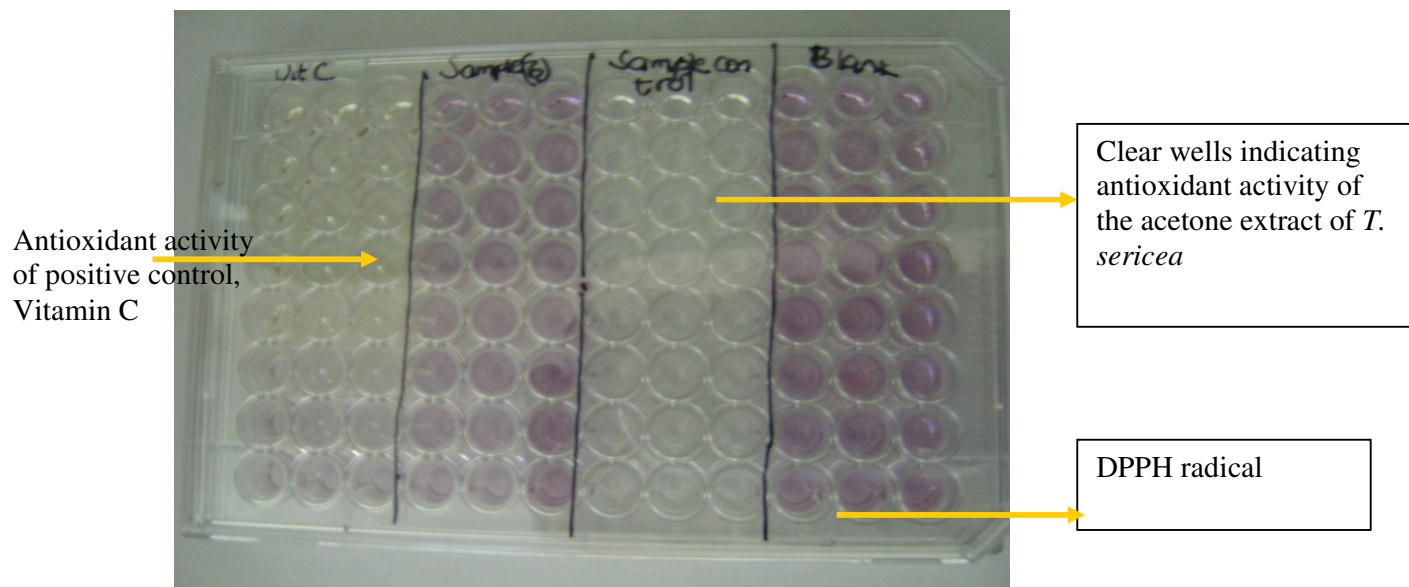


Figure 3.5: Antioxidant activity of *Terminalia sericea*.

The expression of the antioxidant activity of plant extracts in mg vitamin C equivalent has the benefits that the antioxidant is quantified and different plant extracts are compared. The IC_{50} values (the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%) were calculated (IC_{50} VIT C mg/ml/ IC_{50} sample (g/ml) = x mg vitamin C equivalents/g dry weight) and are listed in Table 3.4.

Table 3.4: EC_{50} values of acetone extracts of selected plants and number of cups equivalent in RSA (Radical scavenging capacity) to a single 200mg vitamin C capsule.

Extracts	IC_{50} (μ g/ml)	Mg vitamin C equivalents/g dry weight (IC_{50} value)
<i>Terminalia sericea</i>	5.56	453.24
<i>Sclerocarya birrea</i>	9.41	260.09
<i>Artemisia afra</i>	48.53	51.93
<i>Euclea natalensis</i>	8.46	297.87
<i>Psidium guajava</i>	6.97	361.54
<i>Warbugia salutaris</i>	7.50	336.00
Vitamin C	2.52	336.00

Standard deviation values (present as error bars on graphs) were all \leq a 3.1 % confidence interval

3.3.3 Cytotoxicity of acetone extract of *T. sericea* on Vero cell lines

Cytotoxicity is a mechanism of cell-killing property that is carried by either a chemical compound, or a mediator cell called T. cell (Roche, 2004). Various methods for the determination of in vitro cytotoxicity exist; examples include brine shrimp, lactate dehydrogenase (LDH) and colorimetric assays. Lall and Meyer have reported that plant extracts and isolated compounds must undergo toxicity tests before drug discovery is taken into consideration. In this study, cytotoxicity of compounds isolated from *Terminalia sericea* has been investigated on Vero cell lines based on the tetrazolium reagent: sodium-2-3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT). This method has been described as straightforward, reliable, efficient and inexpensive means of determining the toxicity of extracts and isolated compounds (Williams *et al.*, 2003).

The IC₅₀ (dose that inhibits 50% cell growth after incubation period) of the plant extract is reported in table 3.5, figure 3.6. *Terminalia sericea* acetone extract showed significant toxicity against VK cells exhibiting IC₅₀ value of 20.94 ± 1.32µg/ml respectively. These findings correlate with those of Tshikalange *et al.*, (2005) where it was found that methanol extract of *T.sericea* exhibited similar results (IC₅₀ value was found to be 24.00µg/ml). In a study done by Fyhrquist *et al.*, 2006; root extract of *T. sericea* was found to be more cytotoxic against T- 24 cancer cell lines (IC₅₀=25.35 µg/ml).

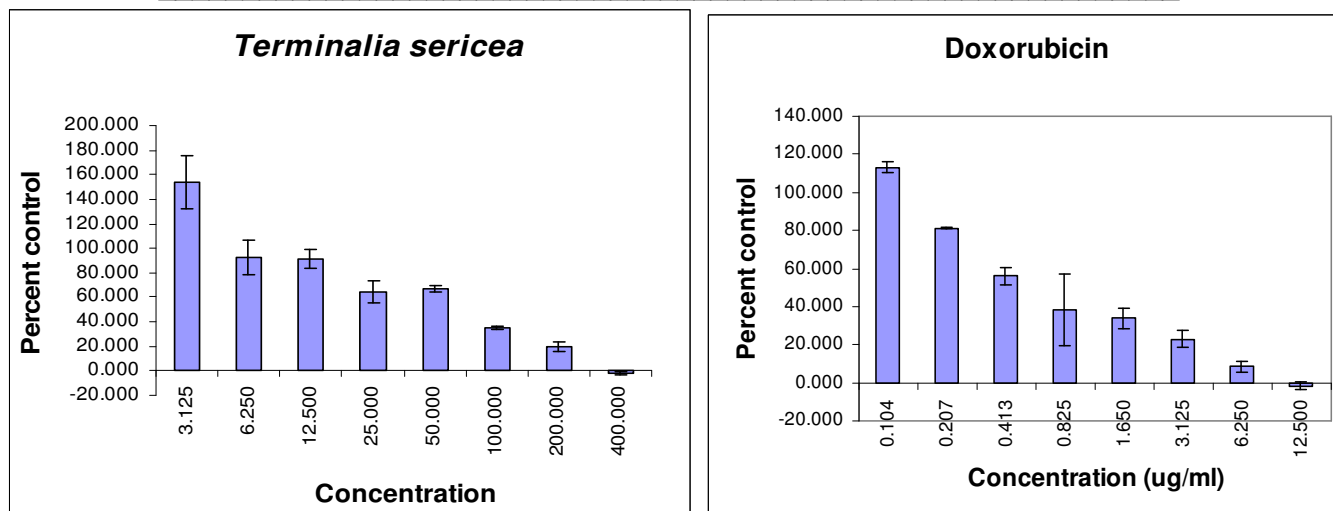


Figure 3.6: Effect of *T.sericea* crude extract and (Doxorubicin) on normal Vero cells

Table 3.5: IC₅₀ values of crude extract of *T. sericea* after 4 days on Vero cells

Plant extract/ compound	Vero Cell lines IC ₅₀ (μ g/ml) \pm SD
Doxorubicin	0.2449 \pm 0.120
<i>Terminalia sericea</i>	20.94 \pm 1.32

3.4 Conclusion

This study investigated the antidiabetic activity of eight selected plants, focusing on inhibitory effects of alpha glucosidase and alpha amylase. *Psidium guajava* leaves, *Terminalia sericea* bark, *Sclerocarya birrea* bark and *Euclea natalensis* roots showed good α -glucosidase and α -amylase inhibitory activities. Several factors have been considered for choosing a plant for the isolation and identification of compounds. Factors included:

- The availability of the plant as isolation of pure compounds requires a large amount of material (2kg).

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-
- It is feasible to collect the leaves and the bark of the plant as harvesting of the roots (as in the case of *P. guajava*) of the plant could destroy the plant.
 - The plant that has been traditionally used for the treatment of diabetes and which has no scientific evidence of its hypoglycemic activity.

Due to the above mentioned factors, *T. sericea* was chosen for the isolation and identification of pure compounds. This study is the first to report α -glucosidase inhibitory activities of all the plants except *T. sericea* which has been previously reported to possess α -glucosidase inhibitory activities. In addition, all the plants except *A. ferox* have never been reported for α -amylase activity.

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4. 1 Introduction

Recently medicinal plants have become the focus of intense study in terms of conservation and to whether their traditional uses are supported by pharmacological effects (Masoko *et al.*, 2005) The acetone extract of the stem bark of *T. sericea* showed good hypoglycaemic activity and hence was selected for the identification of bioactive principle(s). *Terminalia* belongs to the family Combretaceae which consists of 20 genera and 300 species (Tan *et al.*, 2002). The largest genera include *Combretum*, *Terminalia* and *Quisqualis*. About 32 *Terminalia* species occur in tropical Africa (North of the tropic of Capricorn) and about 7 occurring only in subtropical Africa.

4.1. 1 Medicinal uses and biological activity of *T. sericea*

There are many reports on the medicinal uses of *Terminalia sericea* for different ailments (Tshikalange *et al.*, 2008; Moshi and Mbwambo, 2005; Fyhrquist *et al.*, 2002). It has been selected as one of the fifty most important medicinal plants in Africa by the Association for African Medicinal Plant Standards (<http://www.aamps.net>). Dried fruits are traditionally used for the treatment of tuberculosis (TB). Roots decoctions are used for stomach troubles, wounds, diarrhoea, inflammation and sexual transmitted diseases (Eldeen *et al.*, 2006). Aqueous and organic extracts made from leaves, roots and bark have been reported to have antimicrobial activity (Bruneton, 1995). In Tanzania, the bark of *T. sericea* is used for the treatment of diabetes, diarrhoea and gonorrhoea (Moshi and Mbwambo, 2005)

Figure 4.1

It has been reported that biological activities of *Terminalia sericea* were mainly attributed to triterpenoids, saponins and tannins (Bombardelli *et al.*, 1974). Several pentacyclic triterpenoids have been isolated from *Terminalia* species. The triterpenoids sericoside, arjunglucoside and an aglycon of sericoside have been found in the roots and stem bark of *Terminalia sericea* (Fyhrquist *et al.*, 2002; Bombardelli *et al.*, 1974)



A)

B)



C)

Figure 4.1: Different plant parts belonging of *Terminalia sericea* (Combretaceae)
A=bark, B=fruit, C=leaves and twigs

(http://calphotos.berkeley.edu/cgi/img_query?statTerminalia+sericea&title_tag=Terminalia+sericea)

Anolignan B (a polyphenol) was isolated from the roots of *T. sericea* (Eldeen, 2006). It was reported that anolignan B with its constituent, anolignan A inhibited the enzyme HIV-reverse transcriptase. In a study done by Rimando *et al.*, 1994, anolignan B was reported to have *in vitro* cytotoxic effects against fibrosarcoma cell line. Other medicinal activities that have been reported on anolignan B include: antibacterial activity, anti-inflammatory, inhibition of COX-1 and COX-2 (Eldeen *et al.*, 2006).

Other compound that has been isolated from *T. sericea* includes resveratrol-3-O- β -D-rutinoside. It has been reported that *Terminalia* species possesses imino sugars [polyhydroxyalkaloids (PHA) or aza sugars]. These compounds are of significance because it has been reported earlier that the compounds inhibited glycohydrate enzymes thus are useful in carbohydrate-mediated disorders such as diabetes, HIV and various cancers (Durant *et al.*, 2007) (Appendix A).

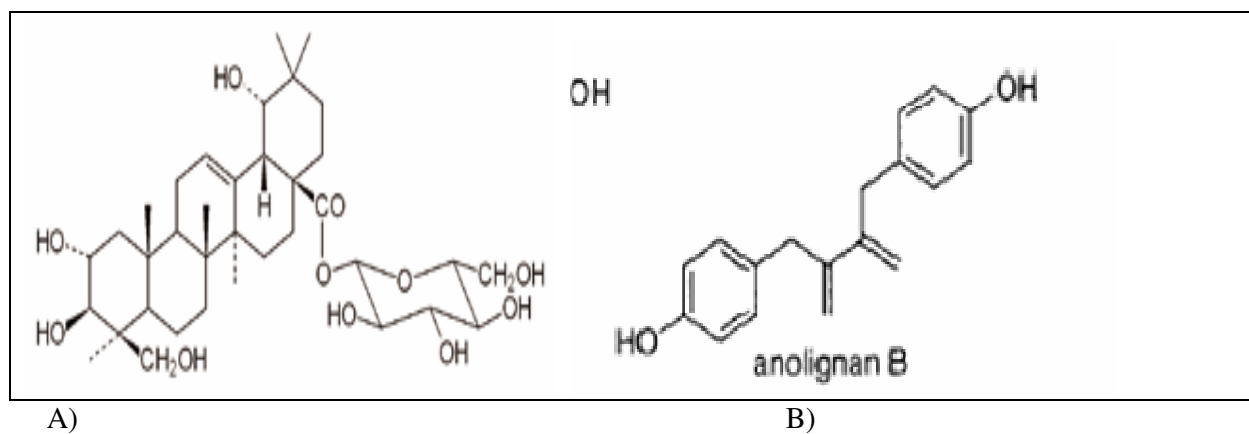


Figure 4.2: Chemical structures of sericoside (A) and Anolignan B (B) isolated from *T. sericea*

4.2 Materials and methods

4.2.1 Extraction and isolation of pure compounds

Air-dried powdered stem bark of *Terminalia sericea* (1.8kg) was extracted with 100% acetone for 48 hours. The acetone extract was filtered and evaporated under reduced pressure. The total concentrated extract (80g) was subjected to silica gel column chromatography (CC, size 10cm x 20cm, figure 4.3) using hexane/ethyl acetate (EtOH) mixtures of increasing polarity (0-100%) followed by 100% methanol (MeOH), a total 30 sub-fractions (250ml) were collected (Figure 4.4). The fractions were combined on the basis of thin layer chromatography (TLC) leading to 13 main fractions (Figure 4.4). TLC of 13 pooled fractions was developed with Methanol: Dichloromethane: 95:5. TLC plates were then examined under (254 and 366nm) after development and also dipped in vanillin (15g vanillin, 500ml ethanol and 10ml concentrated sulphuric acid) and heated to detect compounds which do not absorb UV. The 13 main fractions were tested on alpha glucosidase enzyme to evaluate their inhibitory activity. Results are shown in table 4.2. Fraction 1, 3 and 6 showed good inhibitory activity on α -glucosidase exhibiting IC_{50} values of 22.50, 40.85 and 50.01 μ g/ml respectively. Fraction 1 was subjected to silica gel CC and eluted using hexane/ ethyl acetate of increasing polarity which yielded compound **1** and 5 sub-fractions. Sub-fraction 1 of the main fraction 1 was rechromatographed on silica gel column which was eluted with ethyl acetate-hexane to give compounds **2** and **3**. Fraction 3 was subjected to sephadex CC and eluted using 100% ethanol which yielded compound **4**. Fraction 6 was further separated on silica gel CC using hexane/ethyl acetate mixtures of increasing polarity followed by 100% chloroform to give compound **5** and **6** (Figure 4.5). The structural elucidation of isolated compounds were identified by their physical (mp, $[\alpha]_D$) and spectroscopic (1H and ^{13}C NMR) data. All the NMR data of all isolated compounds are illustrated in appendix A.

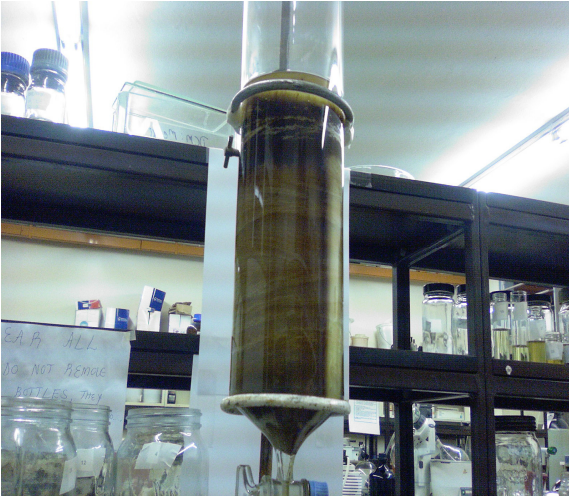
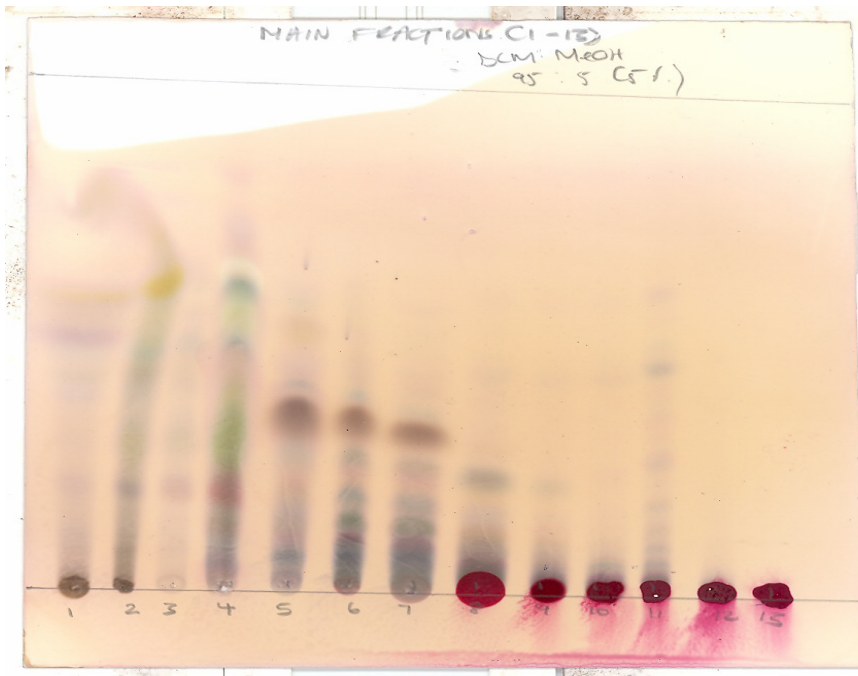
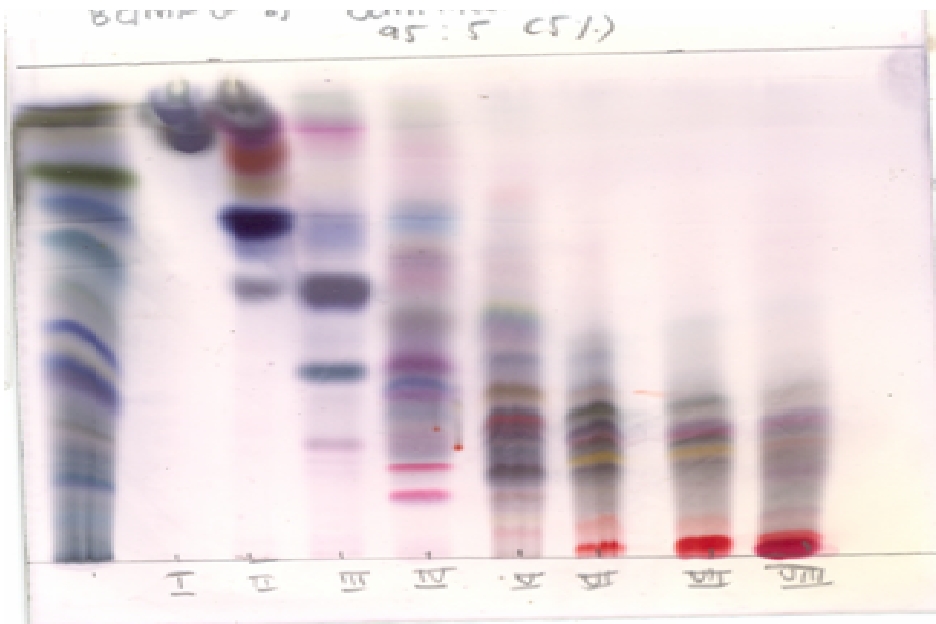


Figure 4.3: Silica gel column chromatographic purification of acetone extract of *T. sericea*



A)



B)

Figure 4.4: TLC plates of fractions obtained from chromatographic separation of acetone extract of *T. sericea*

Solvent system: A) and B) DCM: MeOH (95:5)

Detection: Vanillin in H₂SO₄

Terminalia sericea stem bark powder

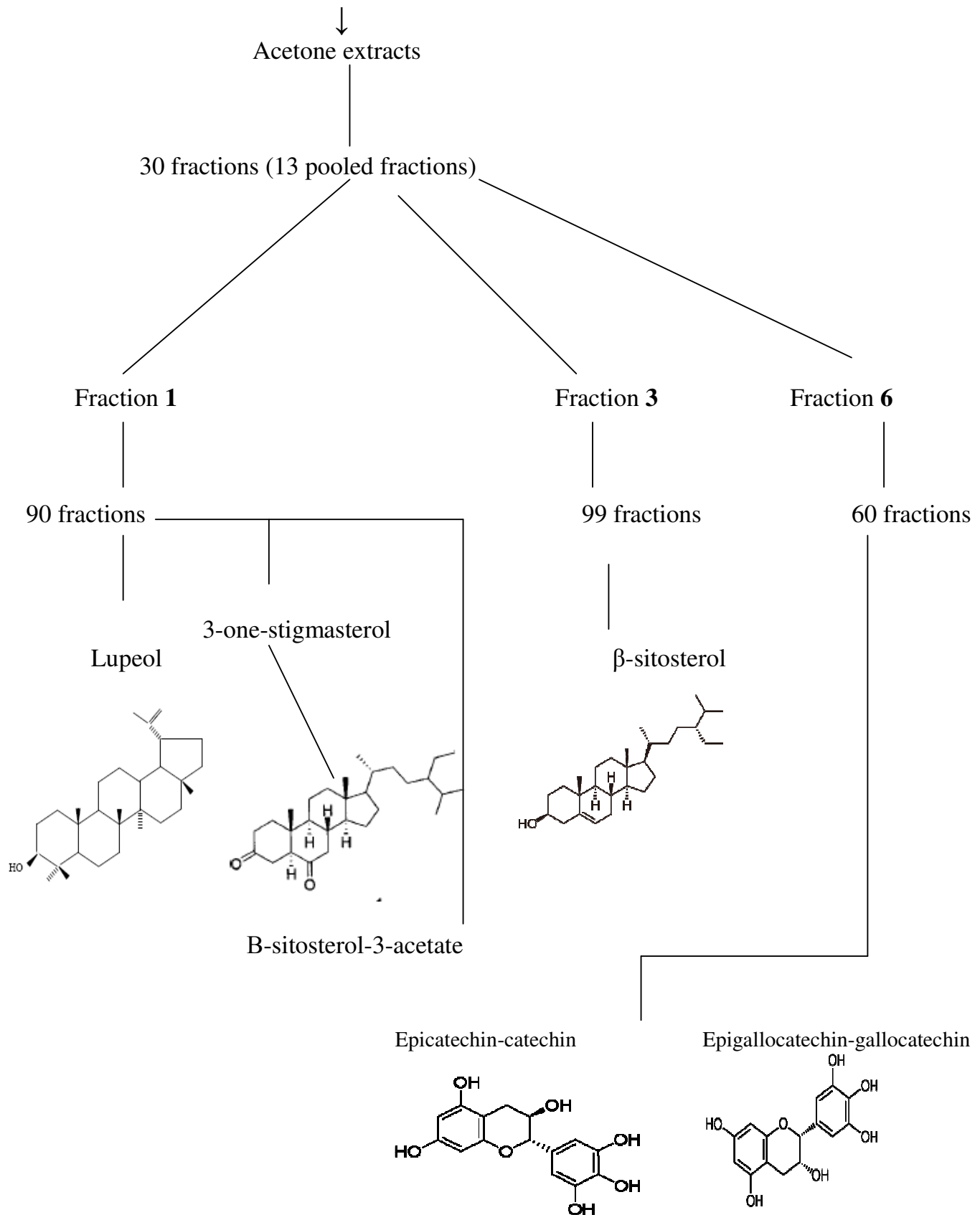


Figure 4.5: Isolation of compounds from the acetone extract of *Terminalia sericea*

4.2.2 Determining alpha glucosidase inhibition by the fractions

Alpha glucosidase activity of 13 main fractions of acetone extracts of *Terminalia sericea* was determined as described in chapter 3 (section 3.2.4). The fractions were tested at the final concentration of 0.1mg/ml.

4.2.3 Statistical analysis

The final results are expressed as the mean (standard deviation, \pm SE.S). The group means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple range Test was applied to compare the means. Values were determined to be significant when p was less than 0.05 ($p < 0.05$).

4.3 Results and Discussion

4.3.1 Alpha glucosidase activity

Alpha glucosidase inhibition by the fractions was conducted to determine the most active fraction. Fractions 1, 3 and 6 showed the most significant alpha glucosidase inhibitory activity exhibiting 95.56 ± 0.25 ; 81.33 ± 1.25 and $89.50 \pm 0.85\%$ inhibition respectively ($p < 0.05$) (figure 4.3)

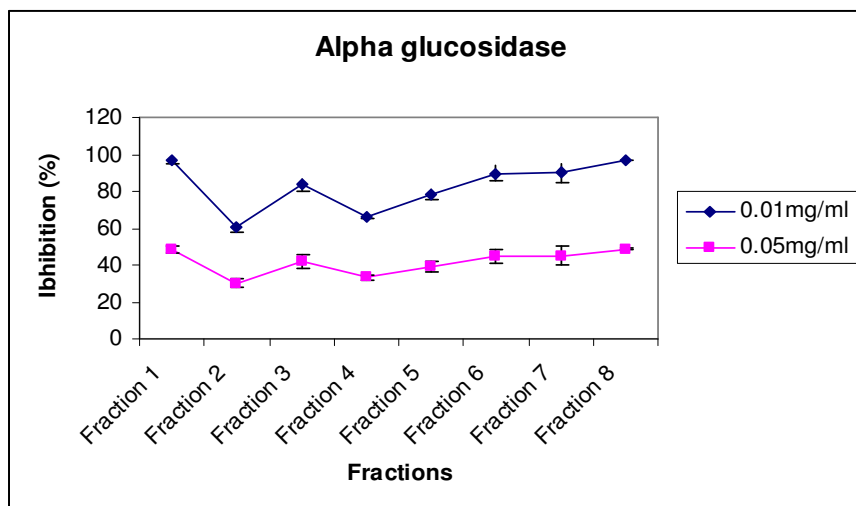


Figure 4.6: Dose dependent inhibition of alpha glucosidase enzyme by fractions using p-nitrophenyl α -D-glucopyranoside as a substrate.

Table 4.1: Fifty percent inhibitory concentration (IC_{50} values) of fractions on alpha (α) - glucosidase

<i>Fraction(s)</i>	<i>IC_{50} α-Glucosidase (μg/ml)</i>
<i>Fraction 1</i>	22.50
<i>Fraction 3</i>	40.85
<i>Fraction 6</i>	50.01

4.3.2 Characterization of compound (1+2)

Mixture (1+2), Catechin and epicatechin

The compounds were isolated as inseparable mixture, the NMR data from the spectra obtained (1H and ^{13}C) in CD_3OD are listed in Table 4.2 and show excellent agreement with the spectra obtained from authentic catechin and epicatechin, respectively, in the same solvent. The vicinal J_{HH} couplings for protons at positions 2–4 confirmed the relative stereochemistry at these centers (Khallouki *et al.*, 2007; Jesus *et al.*, 2007). The mixture was identified as catechin-epicatechin (Figure 4.7).

4.3.3 Characterization of compound (3+4)

Mixture (3+4) Gallo catechin and galloepicatechin

The mixture gave the same NMR profile of ^1H and ^{13}C (Table 4.2) of the previous mixture except for the ring B which showed a single peak (at 6.22 or 6.33) with the corresponding carbon signal at 115 (3) or 106 (4). The relative percentage of both mixtures was determined from their peak intensities either in ^1H or ^{13}C NMR signals. The mixture was identified as gallo catechin-galloepicatechin (Figure 4.7).

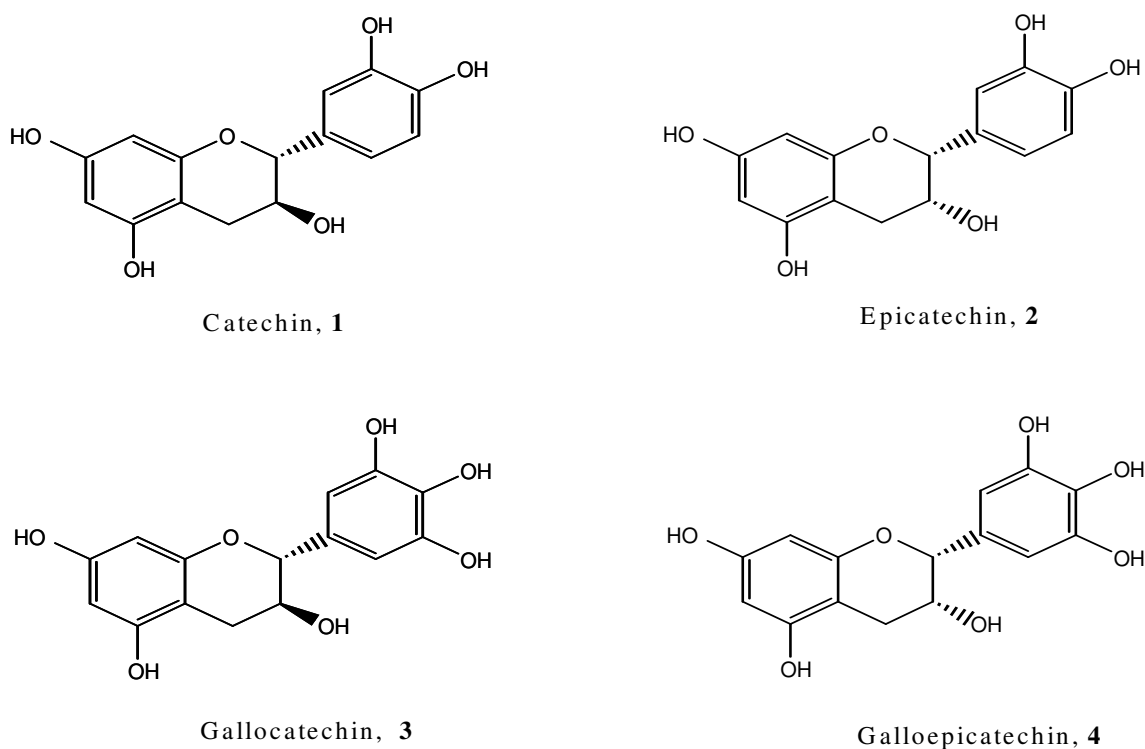


Figure 4.7: Chemical structure of catechin, epicatechin, gallo catechin and galloepicatechin isolated from the bark of *T. sericea*

Table 4.2: ^1H NMR and ^{13}C NMR data of catechin-epicatechin and gallo catechin-epigallocatechin

	Mixture 1 + 2				Mixture 3+4			
	Catechin (~ 59.0%)		Epicatechin (~41.0%)		Galocatechin (~32.0%)		Epigallocatechin (~68.0 %)	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
2	4.56, d, 7.5	82.6	4.81 m	79.3	4.40 d, 9.7	81.0	4.84 m	78.1
3ax	3.97, m	68.2		66.8	3.74, m	66.3		65.0
3eq		68.8	4.19 m	66.8		66.3	3.95, m	65.0
4ax	2.52, dd, 15.8, 8.0	29.0	2.91 dd,16.5, 5.5	28.7	2.33 m	27.4	2.33, m	28.1
4eq	2.79, dd, 12.8 5.4	29.0	2.74, m	28.7	2.68, m	27.4	2.68, m	28.1
5		157. 1		156. 8		155.3		155.8
6	5.88, d,2.2	96.0	5.91, d, 2.2	95.6	5.85, s	95.0	5.85,s	95.0
7		157. 6		157. 5		156.2		156.2
8	6.01, d, 2.2	95.4	6.01, d,2.2	96.0	5.66, br s	93.8	5.68, br. s.	94.1
9		157. 8		157. 4		156.5		156.4
10		100.. 6		99.5		99.0		98.6
1`		132.		132.		132.5		132.1



		1		2				
2`	6.89, d, 1.8	115. 2	7.04, d, 1.8	115. 2	6.22, s	106.0	6.35, s	106.0
3`		145. 6		145. 5		145.6		145.4
4`		145. 2		129. 3		129.7		129.7
5`	6.74 (d, <i>J</i> 8.0 Hz	115. 6	6.7, d, 8.2	145. 3		145.6		145.4
6`	6.70, dd, 8.0, 1.8	120. 0	6.84, dd, 8.2, 1.8	115. 4	6.22, s	106.0	6.35, s	106.0

4.3.4 Characterization of compound 5 (1)

¹H-NMR (CDCl₃, 400 MHz): δ 4.69 and 4.56 (each 1H,m, H-29), 3.18 (1H, dd, H-3), 2.39 and 1.93 (each 1H, m,H-19, 21A), 1.71 (1H, t, H-15A), 1.69 (3H, s, H-30), 1.68 (2H, d, H-12A, 1A), 1.67 (1H, t, H-13), 1.61 (1H, d, H-2A), 1.54 (1H, q, H2B), 1.54, 1.49 and 1.42 (each 1H, d,H-64, 16A, 11A), 1.42 (1H, m, H-22A), 1.41 (2H, m, H-7), 1.39 (1H, q, H-6B), 1.38 (1H, t, H-16A), 1.37 (1H, t,H-18), 1.33 (1H, m, H-21B), 1.28 (1H, d, H-9), 1.29 (1H,q, H-11B), 1.20 (1H, m, H-22B), 1.07 (1H, q, H-12A),1.04 (3H, s, H-23), 1.01 (1H, d, H-15A), 0.98 (3H, s, H-23), 0.97 (3H, s, H-27), 0.91 (1H, t, H-18), 0.27,0.84, 0.79 (each 3H, s, H-25, 28, 24) and 0.69 (1H, d, H-5).

¹³CNMR (CDCl₃, 100 MHz): δ 150.8 (C-20), 109.3 (C-29), 78.9 (C-3), 55.2 (C-5), 50.3 (C-9), 48.2 (C-18), 47.9(C-19), 42.9 (C-17), 42.7 (C-14), 40.7 (C-8), 39.9 (C-22),38.8 (C-4), 38.6 (C-1), 38.0 (C-13), 37.1 (C-10), 35.5 (C-16), 34.2 (C-7), 29.8 (C-21), 27.9 (C-23), 27.4 (C-15), 27.3 (C-2), 25.0 (C-12), 20.9 (C-11), 19.2 (C-30), 18.2(C-6), 17.9 (C-28), 16.1 (C-25), 15.9 (C-26), 15.3 (C-24) and 14.5 (C-27).

The compound was isolated from the non-polar fractions through different silica gel columns. The compound obtained as a white powder. The identification of the compound was completed using NMR spectra (^1H and ^{13}C) and TLC comparison with authentic sample exist in our lab. The presence of seven methyl singlets and an olefinic function in the ^1H -NMR spectrum revealed that compound **1** may be pentacyclic tri-terpenoidal type in nature. The comparison of ^1H -NMR chemical shifts with that of the reported data similar type of compounds and lupeol has led to the conclusion that compound **1** is lupeol (Figure 4.8). This is a very common chemical compound found in higher plants (Imam *et al.*, 2007). This compound has been previously isolated from bark of *Heitiera utilis* and *Euphorbia laterifolia* (Blair *et al.*, 1970; Lavie *et al.*, 1968).

4.3.5 Characterization of compound 6 (4)

The isolated pure compound was characterized and identified by spectral analysis. Compound 4 was obtained as colourless crystals, m.p. 137. in ^1H NMR: it showed signals at 3.52 (1H, s, H-3), 5.35 (1H, s, 6-H), in addition to cluster of methyl signals in the aliphatic region, this compound was effectively identified as β -sitosterol (Figure 4.8) based on comparison of NMR data obtained with those in literature and comparison with the authentic exist in our lab. (Chen *et al.*, 2008).

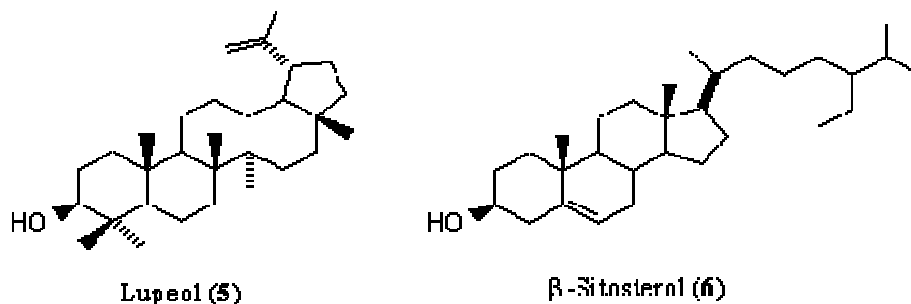


Figure 4.8: Chemical structure of lupeol and β -sitosterol isolated from the bark of *T. sericea*

4.3.6 Characterization of compound 7 (6)

IR: (KBr) cm^{-1} : 3429, 3373 (AOH), 2959, 2866, 1463, 1367(C@C); **MS** (m/z): 414 (M⁺), 400, 387 329, 303, 213, 99, 85, 71, 57, 43, 29;

^1H NMR: (CDCl_3 , 500 MHz): 3.52 (1H, s, AOH), 5.35 (1H, s, 7-H);

¹³C NMR: (CDCl₃, 125 MHz): 140.7 (C-5), 121.7 (C-7), 71.7(C-1), 56.7 (C-17), 56.0 (C-14), 50.1 (C-21), 45.8(C-10), 42.3 (C-13), 19.8 (C-29), 19.4 (C-28), 19.0 (C-25), 18.7 (C-26), 11.9 (C-27), 11.8 (C-4).

The compound was isolated as colorless powder and showed similar pattern of NMR like the previous compound except the downshift of proton H-3 at 4.50 and appearance of acetate signal at 2.04, which indicated the acetylation of the 3-OH, and the compound finally identified as β -sitosterol-3-*O*-acetate (Figure 4.9). The identification was confirmed by comparison of the isolated compound with the authentic acetate derivative of β -sitosterol that exists in the lab.

4.3.7 Characterization of compound 8 (7)

The isolated pure compound was characterized and identified by spectral analysis. It was isolated as white needles. The isolated compound showed a typical ¹HNMR spectra like the previous two compound except the downfield shift of the olefinic proton to 5.72 and the disappearance of H-3 signal. The compound was identified as stigma-4-ene-3-one from comparison of the NMR data with those published in literature (Figure 4.9) (Seca *et al.*, 2000).

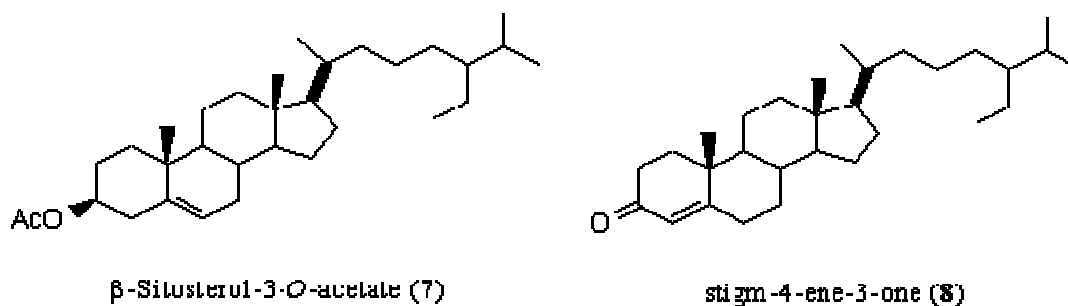


Figure 4.9: Chemical structure of β -sitosterol-3-acetate and stigma-4-ene-one isolated from the bark of *T. sericea*

4.4 Conclusion

Isolation of triterpenes

Triterpenes represent a diverse class of natural products. There have been reports of thousands of triterpenes structures as well as hundreds of new derivatives that are discovered each year. Pentacyclic triterpenes have 30-carbon skeleton that comprise five, six membered rings (ursanes and lanostanes) of four, six-membered rings and one, five-membered ring (lupanes and hopanes) (Chaturvedi *et al.*, 2008). The production of pentacyclic triterpenes is based on the arrangement of squalene epoxide molecules. These molecules are predominantly present in fruits, vegetables and many medicinal plants. At least 4000 known triterpenes have been reported to most of which occur freely while others occur as glycosides (saponins) or in special combined forms (Jiri, 2003). Pentacyclic triterpenes have a wide continuum of biological activities that can be useful in medicine. Among this class of compounds, lupeol [lup-20 (29)-en-3b-ol] occurs across a multitude of taxonomically diverse genera.

The isolation of all the compounds (1-6) was achieved by means of various physical (solvent extraction, chromatography) and spectral techniques. Compound 3 was the first ever compound to be isolated from *T. sericea*.

In conclusion, lupeol and β -sitosterol have previously been isolated from *Terminalia sericea* and *T. glautens*. In addition, epicatechin and catechin have been isolated from *Terminalia catappa*, a same genus as *T. sericea*. Stigma-4-ene-3-one has previously been isolated from *Hibiscus cannabinus*. A flavan-3-ol, galliccatechin, was first isolated from the leaves and twigs of *T. arjuna*. (Lin *et al.*, 2000). This study is the first to report the isolation of β -sitosterol-3-acetate, stigma-4-ene-3-one, mixture of epicatechin-catechin and epigallocatechin-galliccatechin from *Terminalia sericea*.

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isolated from *Terminalia sericea*

5.1 Introduction

A bioassay guided fractionation led to the isolation of 4 pure compounds namely β -sitosterol, β -sitosterol-3-acetate, lupeol and 3-one-stigmasterol and two sets of mixtures of isomers (epicatechin-catechin and epigallocatechin-gallocatechin. Lupeol and β -sitosterol have been isolated from the roots of *Terminalia sericea* before. Epicatechin and catechin have been isolated from *Terminalia catappa*, a same genus as *T. sericea*. Stigma-4-ene-3-one has previously been isolated from *Hibiscus cannabinus*. A flavan-3-ol, gallocatechin, was first isolated from the leaves and twigs of *T. arjuna*. β -Sitosterol-3-acetate, stigma-4-ene-3-one, Epicatechin-catechin and epigallocatechin-gallocatecin from *Terminalia sericea* are reported for the first time. It was decided to evaluate the α -glucosidase, α -amylase, antioxidant and cytotoxicity activities of the isolated compounds.

5.2 Materials and Methods

The materials and methods for all the assays are described in chapter 3.

5.3 Statistical analysis

The final results are expressed as the mean (standard deviation, \pm SE.S). The group means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple range Test was applied to compare the means. Values were determined to be significant when p was less than 0.05 ($p < 0.05$).

isolated from *Terminalia sericea*

5.4 Results and Discussion

There are more than 200 pure compounds from plant sources that have been reported to show blood glucose lowering activity (Marles and Farnworth, 1994). The wide variety of chemical compounds contributes to the different mechanisms of lowering blood glucose levels they are responsible for (Ali *et al.*, 2006). In addition, it has long been recognized that many naturally occurring substances have inhibitory effect of α -glucosidase and amylase in plant materials such as fruits, leaves, seeds etc (Shim *et al.*, 2003). Studying those promising bioactive constituents open doors to new diabetic drugs discovery.

5.3.1 α -Glucosidase and Amylase inhibitory activity

The results of alpha glucosidase and alpha amylase inhibitory activities of compounds isolated from *Terminalia sericea* are shown in table 5.1, figure 5.1. This study reports that, from the six isolated compounds, β -Sitosterol and lupeol showed best inhibitory activity on α -glucosidase exhibiting 50% inhibitory concentration (IC_{50}) value of $54.49 \pm 0.01 \mu M$ and $66.48 \pm 0.02 \mu M$ respectively ($p < 0.05$). This was followed by epigallocatechin-gallocatechin ($IC_{50} = 119.34 \pm 0.01 \mu M$); β -sitosterol-3-acetate ($IC_{50} = 129.36 \pm 0.01 \mu M$); stigma-4-ene-3-one ($IC_{50} = 184.87 \pm 0.01 \mu M$) and epicatechin-catechin ($IC_{50} = 255.80 \pm 0.02 \mu M$).

During the evaluation of purified compound's inhibitory activity on α -amylase, compounds of interest were lupeol and β -sitosterol which exhibited IC_{50} values of $140.72 \mu M$ and $216.02 \mu M$ respectively as compared to the positive drug-control acarbose ($IC_{50} = 65.25 \mu M$). Epicatechin-catechin and epigallocatechin-gallocatechin also demonstrated α -amylase inhibitory properties and the IC_{50} values were found to be lower than $100 \mu g/ml$. In a study done by Mai *et al.*, (2007) it was

isolated from *Terminalia sericea*

found that catechins possess α -glucosidase inhibitory activities (93% inhibition) at the final concentration of 0.8mg, suggesting that these compounds might be possible new sources of α -glucosidase inhibition.

Lupeol (Lup-20(29)-en-3-ol) is a naturally occurring triterpene that is abundant in various fruits, has been isolated from many medicinal plants including *Hieracium pilosella*, *Tamaindus indica*, *Crataeva nurvala*, *Arbutus unedo* (Gawronska-Grzywacz and Krzaczek, 2007; Imam *et al.*, 2007). In a study done by Ali *et al.*, 2006 it was found that lupeol inhibited alpha amylase enzyme by 60% and these findings are similar of the present study where lupeol inhibited α -amylase enzyme by 70% at the highest concentration tested. In a recent study lupeol, β -sitosterol and stigmaterol obtained from a methanolic extract of seeds of *Cinchorium intybus* demonstrated good α -amylase inhibitory activities (IC_{50} values=250 μ M, 300 μ M and 500 μ M) respectively (Rahman *et al.*, 2008).

Table 5.1: Fifty percent Inhibitory concentration (IC_{50}) values of compounds on alpha (α)-glucosidase and α -amylase enzymes

<i>Compounds</i>	<i>IC₅₀ α-Glucosidas (μM)</i>	<i>IC₅₀ α-Amylase (μM)</i>
<i>Acarbose (positive drug Control)</i>	93.22	60.25
<i>β-sitosterol</i>	54.49	215.95
<i>β-sitosterol-acetate</i>	129.36	N/A
<i>stigma-4-ene-3-one</i>	184.87	N/A
<i>Epigallocatechin & Gallocatechin</i>	119.34	328.06
<i>Epicatechin & Catechin</i>	255.796	304.89
<i>Lupeol</i>	66.48	140.72

N/A: NOT ACTIVE at the highest concentration tested

isolated from *Terminalia sericea*

In a recent study by Shabana *et al.*, (2009) it was found that the millet seed coat inhibited both alpha glucosidase and pancreatic amylase in a dose dependent manner. Mass spectra of the finger millet extract showed the presence of naringenin, kaempferol, luteolin glycoside (+)-catechin/ (-)-epicatechin etc (Shabana *et al.*, 2009). It has been reported that plant phenolic compounds modulate the enzymatic breakdown of carbohydrates by inhibiting amylases and glucosidases (McDougall *et al.*, 2005). Furthermore, flavonoids, like antioxidants may prevent the destruction of pancreatic β -cells function due to oxidative stress thus reducing the incidence of type-2 diabetes (Song *et al.*, 2005). Sabu *et al.*, (2002) observed the antidiabetic and free radical scavenging activities of tea polyphenols such as gallic acid (GC), epigallocatechin (EGC), epicatechin (EC), epicatechin gallate (EGCG) which correlates to the findings of the present study of isolated compounds from *T. sericea*.

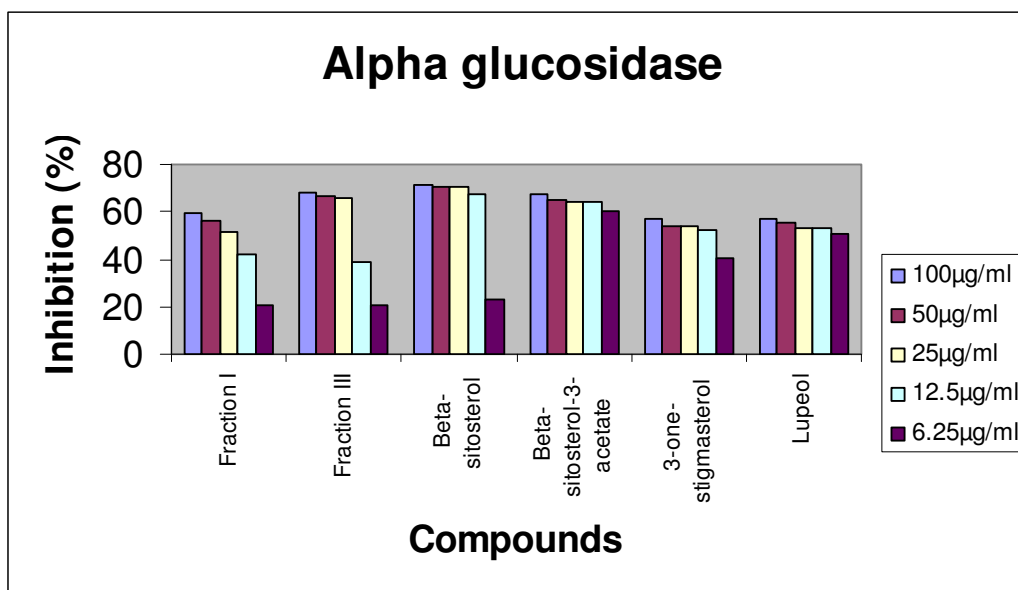


Figure 5.1: Inhibitory activity of compounds isolated *T. sericea* on α -glucosidase

isolated from *Terminalia sericea*

5.3.2 Antioxidant activity

DPPH is a stable radical that has the maximum absorption of 517nm which can readily undergo scavenging in the presence of an antioxidant (Lu and Yeap, 2001). The advantages of using DPPH assay for the determination presence of antioxidant includes: This bioassay can accommodate multiple samples period, it is sensitive enough to detect active ingredients at low concentrations, as a result DPPH has been used to evaluate the antiradical activity of various samples (Pioa *et al.*, 2004; Yu *et al.*, 2002). Table 5.2 depicts the DPPH scavenging activity of the compounds isolated from *Terminalia sericea*. As established, epigallocatechin-gallocatechin, epicatechin-catechin and lupeol showed high radical scavenging activity as they inhibited DPPH by 98.19; 96.98 and 70.90 % at 100 μ g/ml respectively ($p < 0.05$). The two isolated isomers namely epigallocatechin-gallocatechin, epicatechin-catechin are polyphenolic plant antioxidants. They belong to the family of flavan-3-ols (Li *et al.*, 2007). Epigallocatechin-gallocatechin and epicatechin-catechin inhibited DPPH by more than 95% similar to our findings where it was found that similar compounds caused more than 95% inhibition on DPPH at 100 μ g/ml suggesting they had scavenged the whole amount of DPPH (Han *et al.*, 2008). On the other hand, the activity of scavenging DPPH was very low in case of β -sitosterol (21.5% inhibition). β -Sitosterol-3 and its derivative, β -sitosterol-acetate-3-acetate did not show any activity, (table 5.2).

Catechin and epicatechin are epimers with (-)-epicatechin and (+) and they are common isomers that are abundant in nature. On the other hand, epigallocatechin and gallocatechin contain an additional phenolic hydroxyl group when compared to the former (Li *et al.*, 2007). Flavonoids have been reported as being potential therapeutic agents for type 1 diabetes (Yazdanparast *et al.*,

isolated from *Terminalia sericea*

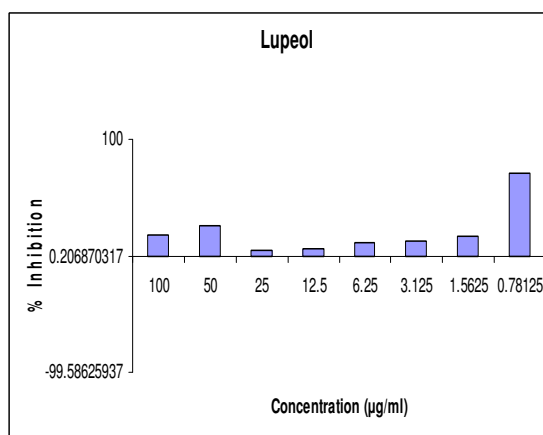
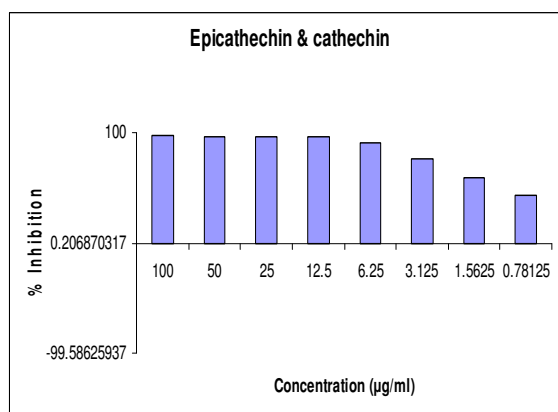
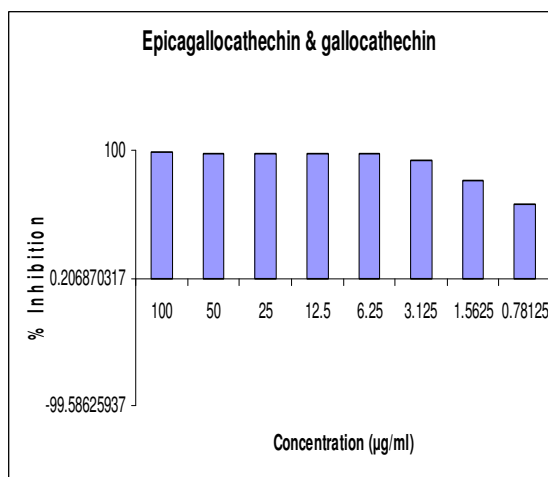
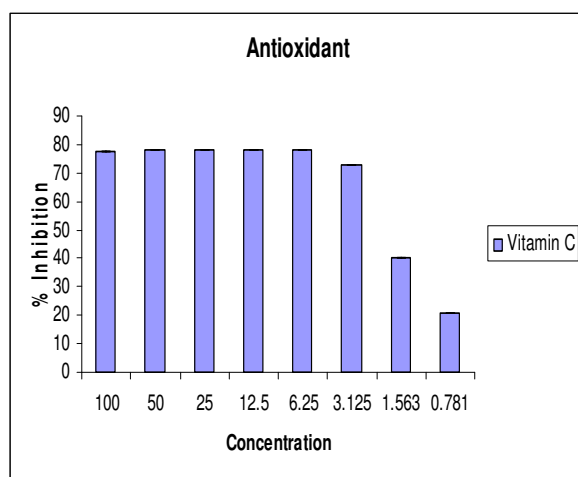
2007). Therefore, currently there is intensive focus on polyphenolic phytochemicals such as flavonoids (Coskun *et al.*, 2005). Narvaez-Mastache *et al.*, (2008) previously reported that catechin and epicatechin that were isolated from *Eysenhardtia subcoriacea* demonstrated strong radical scavenging properties against diphenylpicrylhydrazil (DPPH). Our results are in agreement with the findings of Yu *et al.*, (2007), where it was found that epicatechin, isolated from *Garcinia mangostona* exhibited significant antioxidant activity when DPPH was used. Epicatechin-a flavan-3-ol has previously been isolated from *Hibiscus esculentus*. This plant demonstrated good *in vitro* antioxidant potential and the major antioxidant molecule was identified to be epigallocatechin (Shui and Peng, 2004). It has been reported that several derivatives of stigmasterol such as stigmasterol, stigmastadienol and stigmastadiene which were isolated from *Hibiscus tiliaceus* have demonstrated *in vitro* antioxidant effects using *Saccharomyces cerevista* defective in antioxidant defense and exposed to oxidative stress induced by hydrogen peroxide and tert-butylhydroperoxide (Rosa *et al.*, 2006; Wang *et al.*,2000). Contrary to these finding, stigma-4-ene-3-one (a derivative of stigmasterol) which was isolated from *T. sericea* in our findings did not demonstrate antioxidant activity. This difference could be due to the different assays used and the difference in the chemical structure of these compounds. β - Sitosterol on the other hand did not show any DPPH scavenging effects and our study correlates with the study done by Han and colleagues (2008), where they did not find antioxidant effect of β -sitosterol.

isolated from *Terminalia sericea*

Table 5.2: Inhibition of DPPH (percent) by the compounds at the concentration of 100 μ g/ml

Compounds	DPPH (%) activity)
Vitamin C	2.5
β -sitosterol	21.504
β -sitosterol-3-acetate	N/A
Stigma-4-ene-3-one	N/A
Epigallocatechin - Galocatechin	98.19
Epicatechin -Catechin	96.98
Lupeol	70.9

N/A=not active at the highest concentration tested



isolated from *Terminalia sericea*

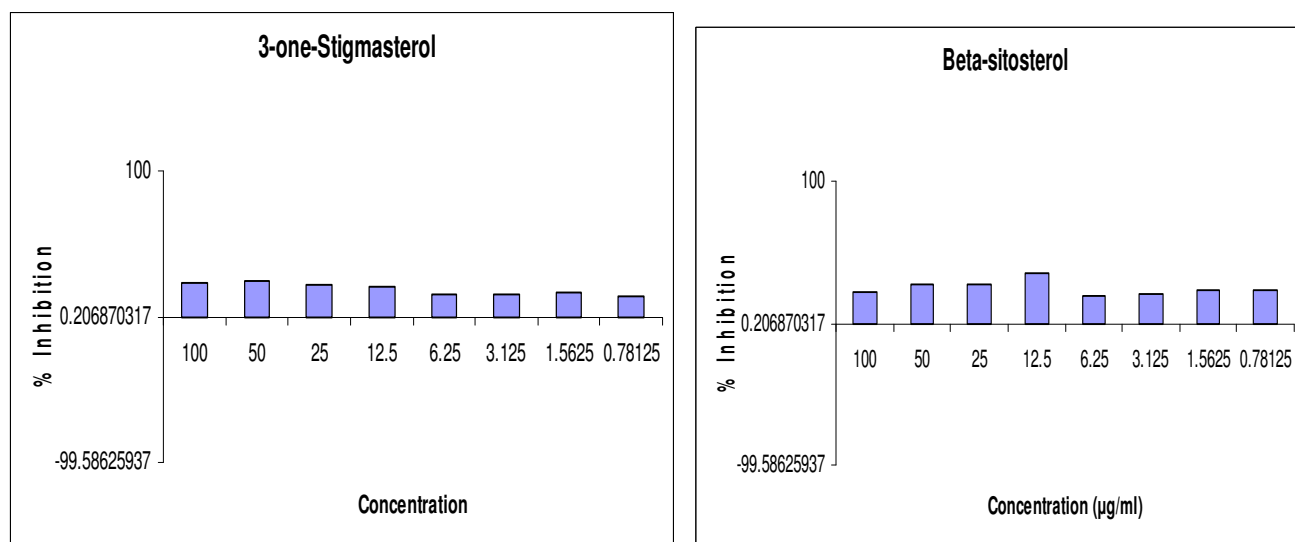


Figure 5.2: Antioxidant activity of isolated compounds from *T. sericea*

5.3.3 Cytotoxicity of isolated compounds on Vero cell lines

Compounds isolated from *Terminalia sericea* were evaluated for their *in vitro* activity against the growth of Vero cell lines. All the compounds except β -sitosterol did not inhibit the growth of these cells lines at the highest concentration tested (200 μ g/ml). β -Sitosterol showed moderate toxicity exhibiting IC_{50} values of $192.72 \pm 2.8 \mu$ M. β -Sitosterol-3-acetate, epicatechin-catechin, lupeol and epigallocatechin-gallocatechin were found to be non-toxic to Vero cells as 100% cell viability was observed when Vero cells were exposed to these samples (table 5.4). β -Sitosterol did not demonstrate cytotoxicity on Vero cells, however, Moon *et al.*, (2007) suggested that the same compound induced apoptosis in MCA-102 fibroblasts.

isolated from *Terminalia sericea*
Table 5.4: IC₅₀ values of isolated compounds from *T. sericea* after 4 days on Vero cells

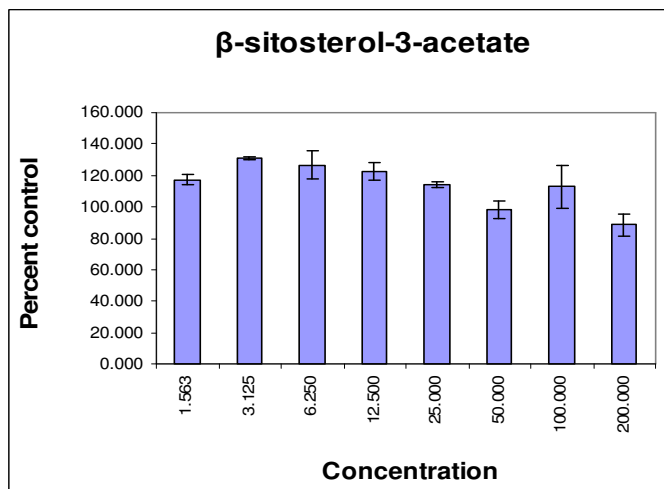
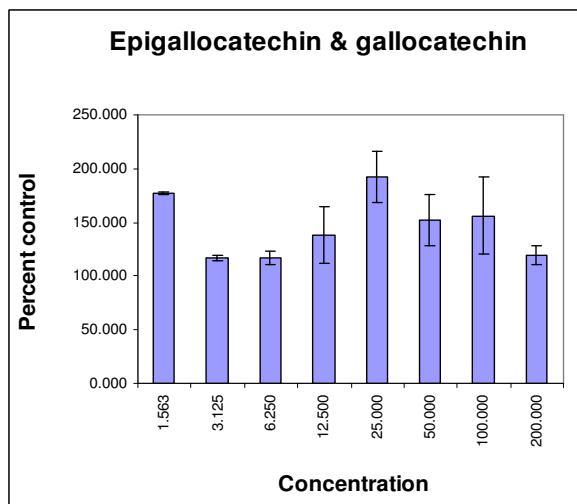
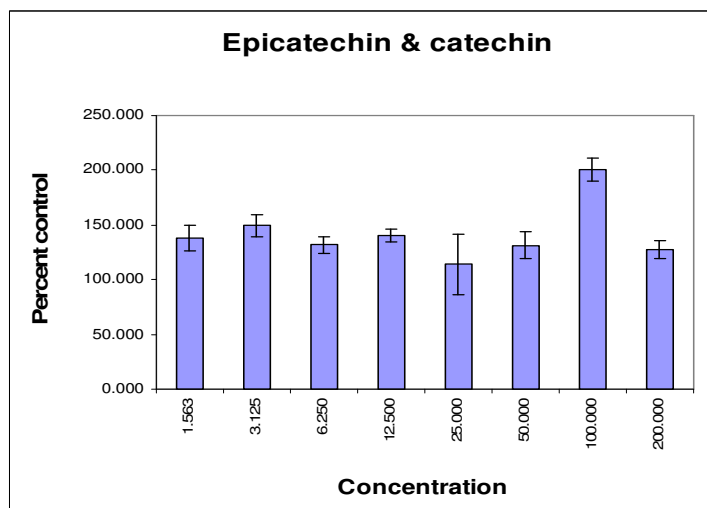
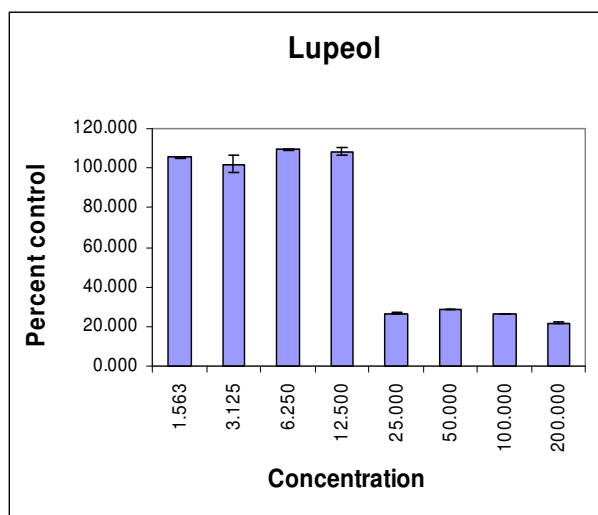
Plant extract/ compound	Vero Cell lines IC₅₀ (μg/ml) \pm SD	Vero Cell lines IC₅₀ (μM) \pm SD
Doxorubicin	0.2449 \pm 0.120	0.41 \pm 0.12
Lupeol	>300.9 \pm 2.43	705.14 \pm 0.12
β -sitosterol-3-acetate	>200.00 \pm 0.659	482.25 \pm 0.659
Epigallocatechin – galocatechin	>200.00 \pm 0.265	653.02 \pm 0.27
Epicatechin - catechin	>200.00 \pm 4.93	689.00 \pm 4.93
β -sitosterol	82.0 \pm 2.8	197.72 \pm 2.80

SD: Standard deviation

Lupeol isolated from *Spirostachys africana* had shown no toxicity on Vero cell lines with the IC₅₀ value of 300.09 μ g/ml (Mathabe *et al.*, 2008). You *et al.*, (2003) have reported that lupeol did not inhibit the growth of tumor cell lines such as SK-MEL-2 and B16-F10 melanoma. On the other hand lupeol exhibited weak cytotoxicity (IC₅₀= >100 μ g/ml) when tested against melanoma B16 cells and human cancer cell lines (Chaturvedula *et al.*, 2002; Liu *et al.*, 2004).

isolated from *Terminalia sericea*

Catechin derivatives: epicatechin-catechin and epigallocatechin-gallocatechin did not demonstrate any toxicity on Vero cell lines in the present study. This confirms the findings by Pragon *et al.*, (2008) where *Erythroxylum cuneatum* extract was tested on Vero cells, demonstrated no toxicity (IC₅₀ value of 366 μ g/ml). The active compound isolated from the plant was (+)-catechin (Pragon *et al.*, 2008). This might explain non-toxicity properties observed from all catechin-derived compounds isolated.



isolated from *Terminalia sericea*

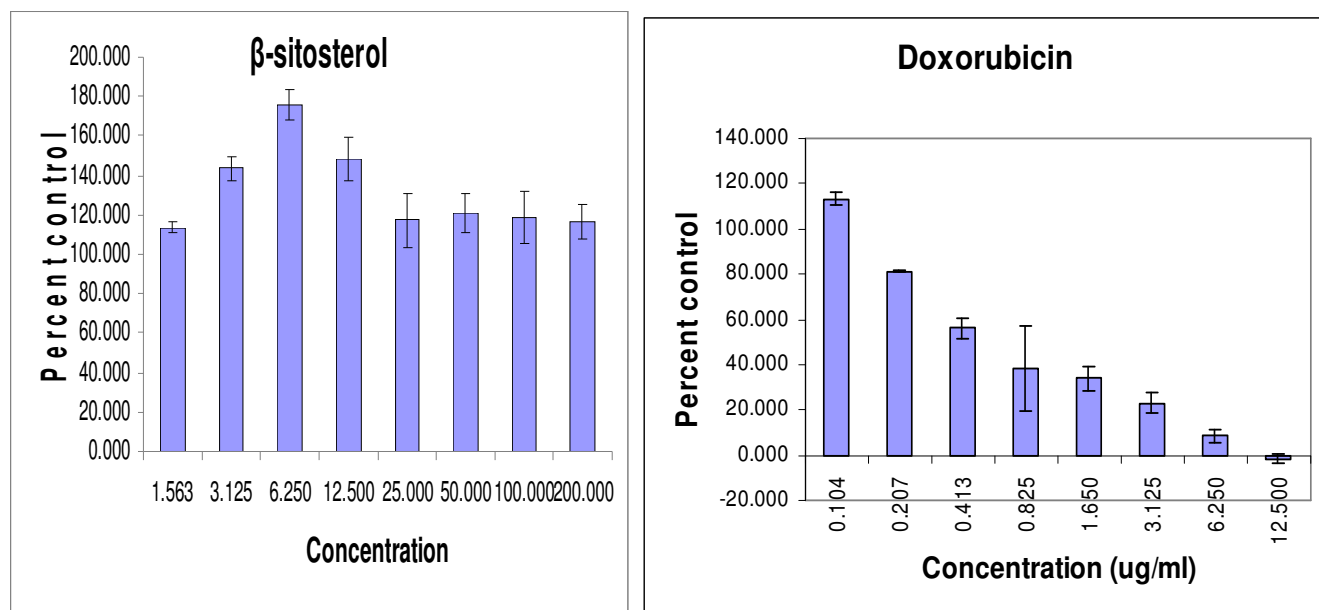


Figure 5.3: Effect of isolated compounds on the viability of Vero cells

5.4 Conclusion

Compounds belonging to triterpenes and flavonoids that were isolated from the stem bark of *Terminalia sericea* were tested on alpha glucosidase, amylase and DPPH assays for their antidiabetic and antioxidant properties. Compounds were also tested for cytotoxicity on Vero cell lines. This study is the first to report α -glucosidase, α -amylase and antioxidant properties of epicatechin-catechin, epigallocatechin-gallocatechin, β -sitosterol-3-acetate and stigma-4-ene-3-one isolated from *T. sericea*. In addition, epicatechin-catechin, epigallocatechin-gallocatechin, β -sitosterol-3-acetate and stigma-4-ene-3-one are isolated from *T. sericea* for the first time.

T. sericea is moderately toxic to Vero cells. This could be due to the solvent used. Ideally water extracts (which are less toxic) are used traditionally however due to their low activity other organic solvents are recommended for *in vitro* studies. Compounds have demonstrated good antioxidant

isolated from *Terminalia sericea*

and hypoglycemic activities. As these compounds can be synthesized in the labs in large quantities, this will be an added advantage and will open doors for drug discovery.

5.5 References

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Chapter 8: Appendix A-¹HNMR and ¹³CNMR of isolated compounds from *Terminalia sericea*

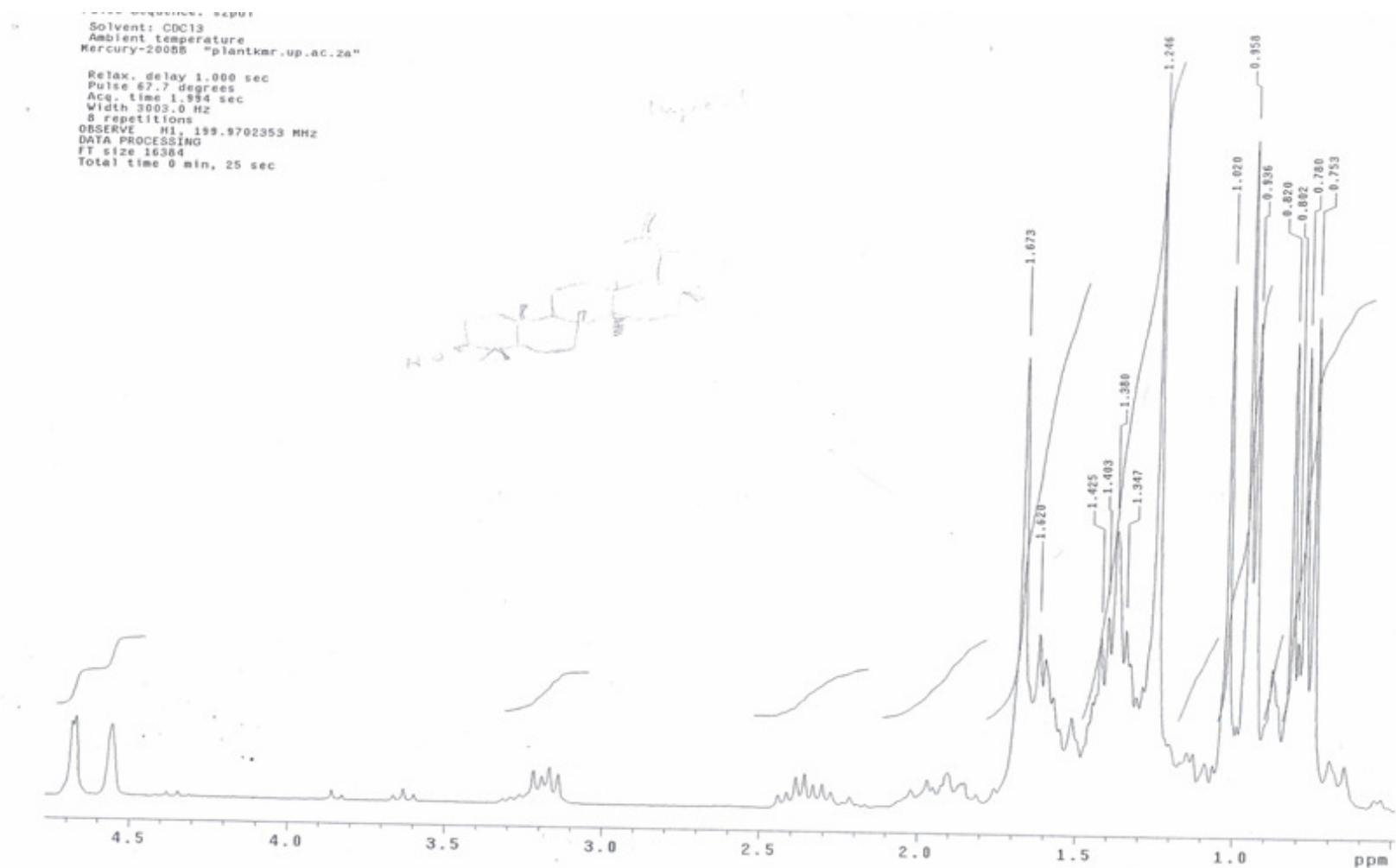


Figure 8.1: ¹HNMR spectrum of 'lupeol' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A-¹HNMR and ¹³CNMR of isolated compounds from *Terminalia sericea*

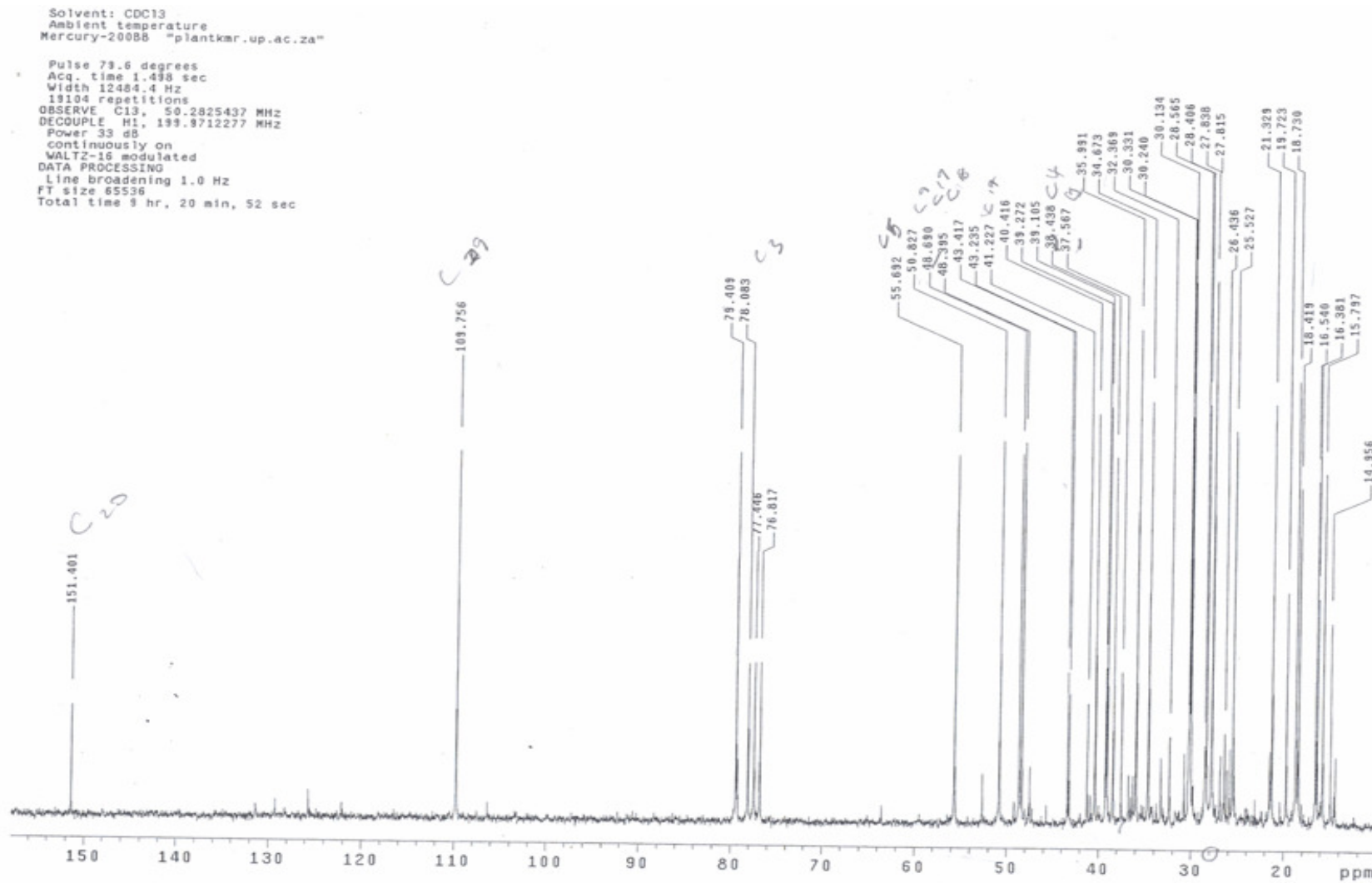


Figure 8.2: ¹³CNMR of 'lupeol' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A-¹HNMR and ¹³CNMR of isolated compounds from *Terminalia sericea*

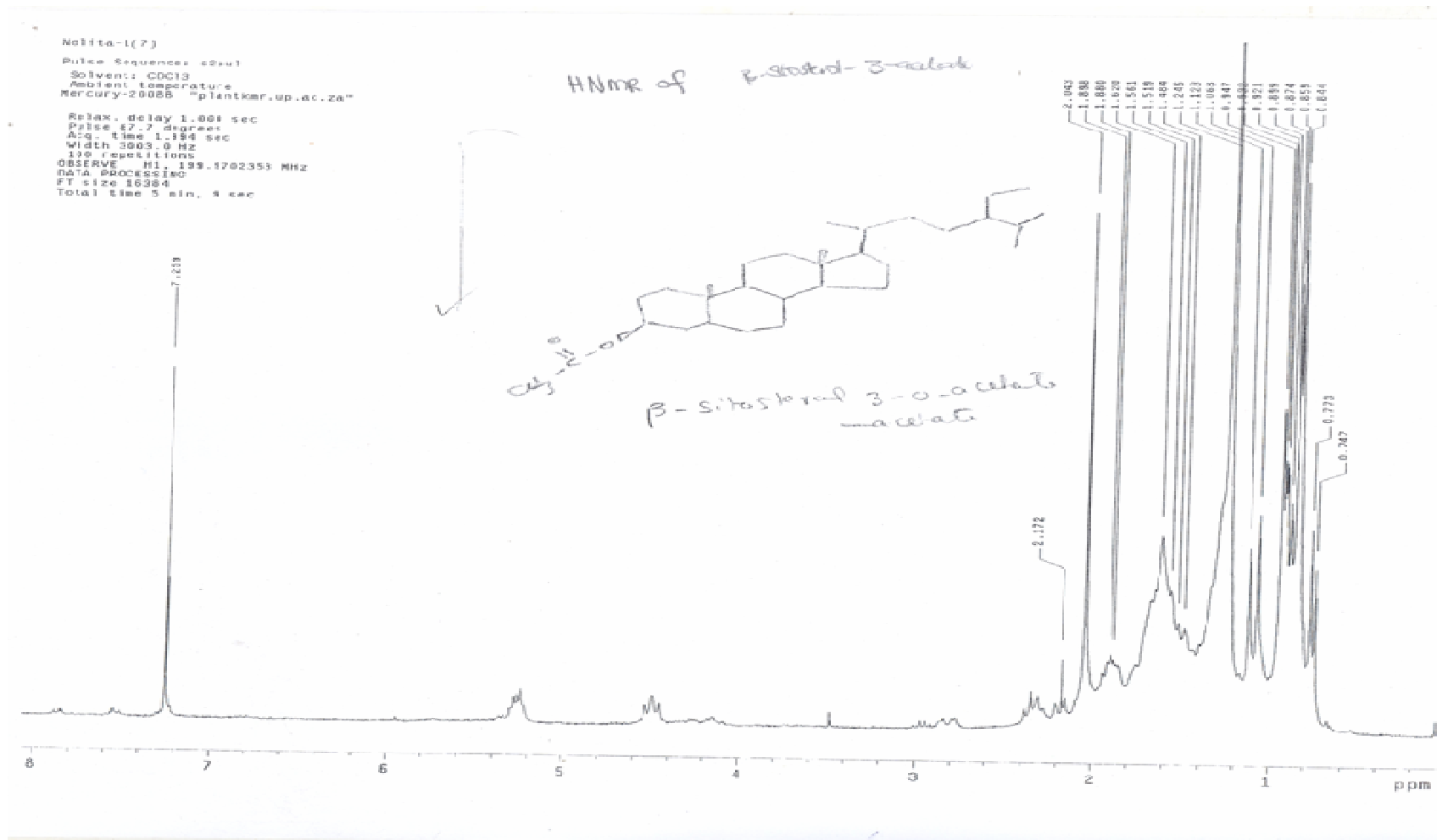


Figure 8.3: ¹HNMR spectrum of ' β -sitosterol-3-acetate' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A- ^1H NMR and ^{13}C NMR of isolated compounds from *Terminalia sericea*

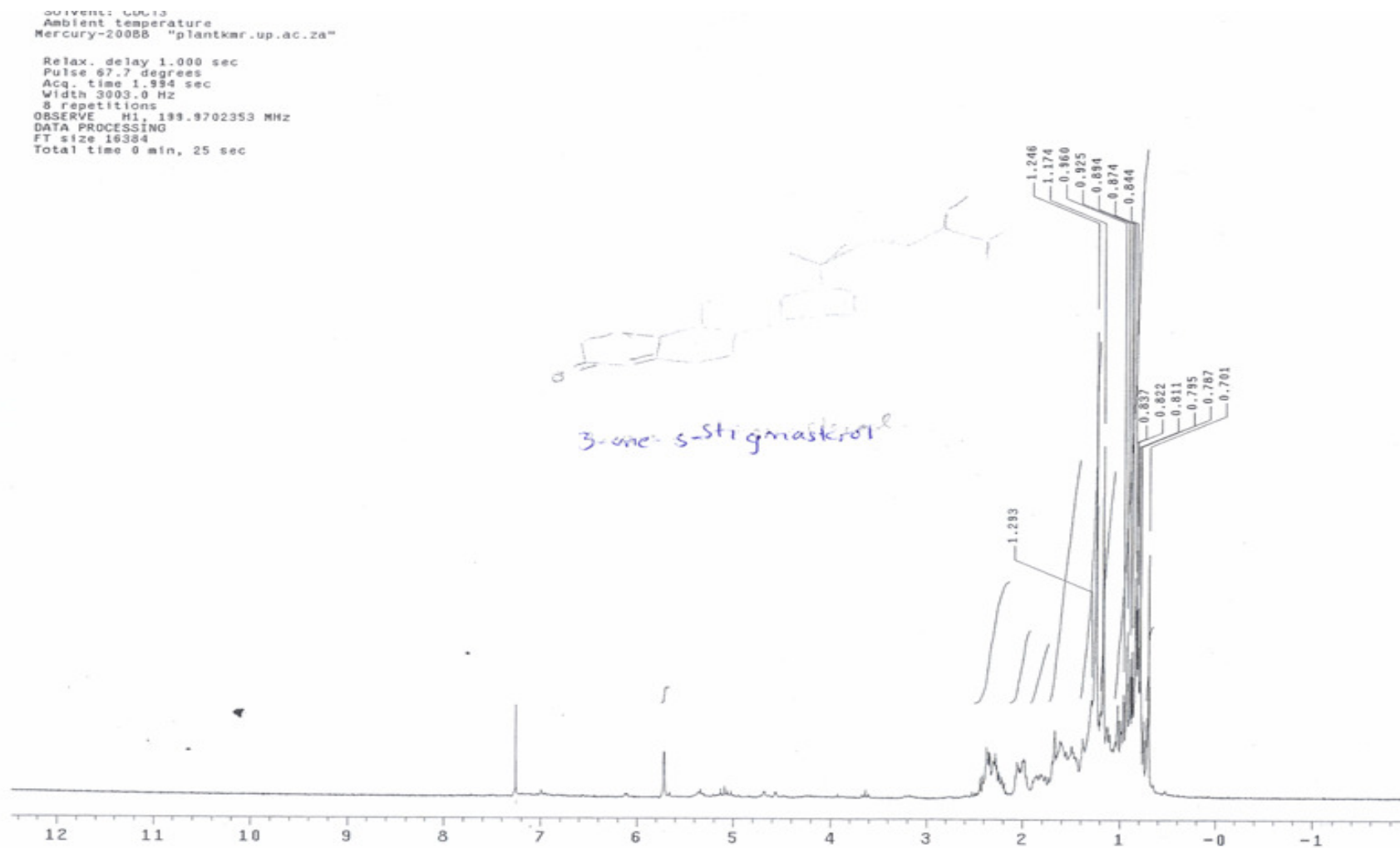


Figure 8.4: ^1H NMR spectrum of '3-one-stigmasterol' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A- ^1H NMR and ^{13}C NMR of isolated compounds from *Terminalia sericea*

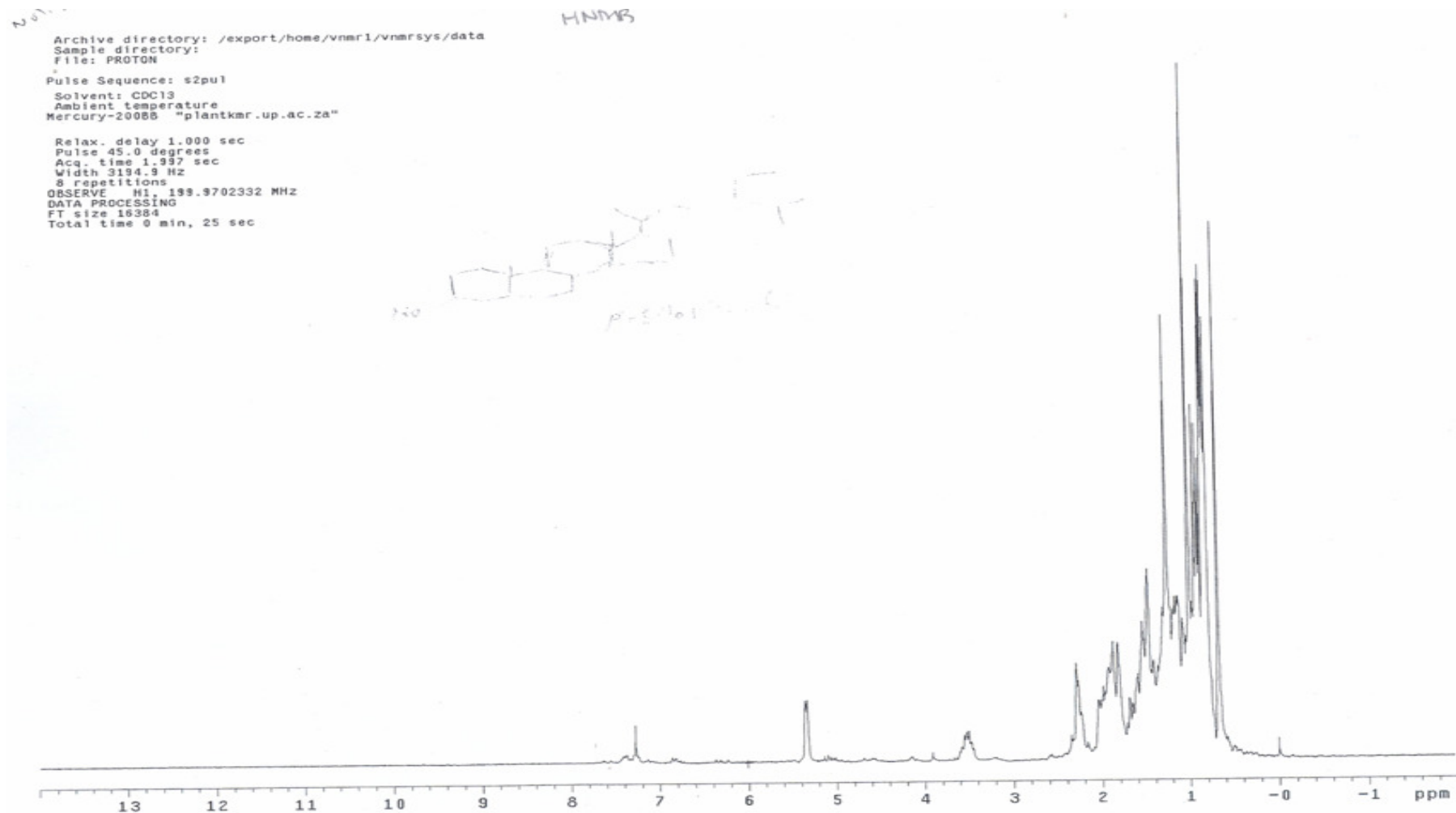


Figure 8.5: ^1H NMR spectrum of ' β -sitosterol' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A- ^1H NMR and ^{13}C NMR of isolated compounds from *Terminalia sericea*

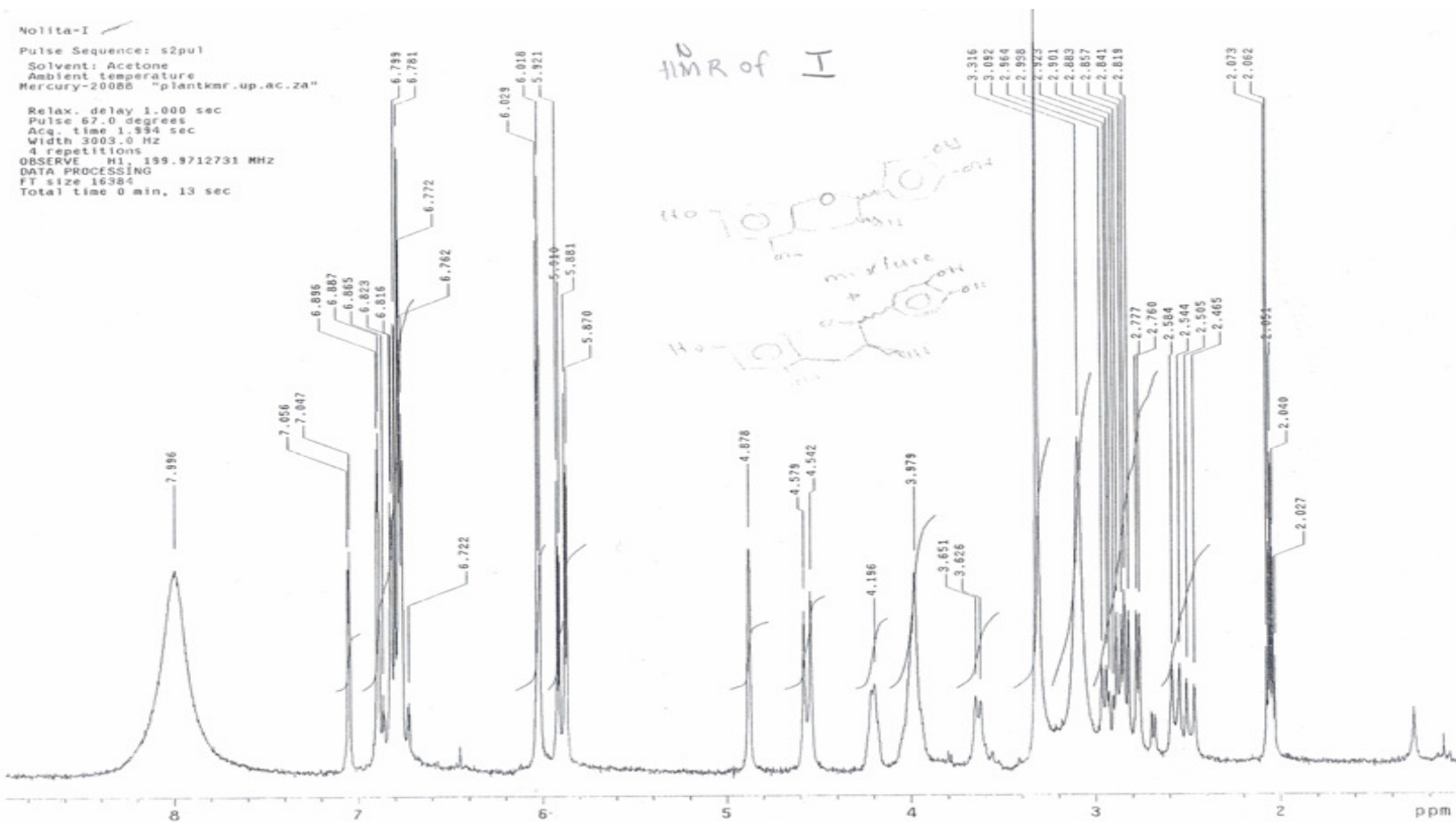


Figure 8.6: ^1H NMR spectrum of 'catechin-epicatechin' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A-¹HNMR and ¹³CNMR of isolated compounds from *Terminalia sericea*

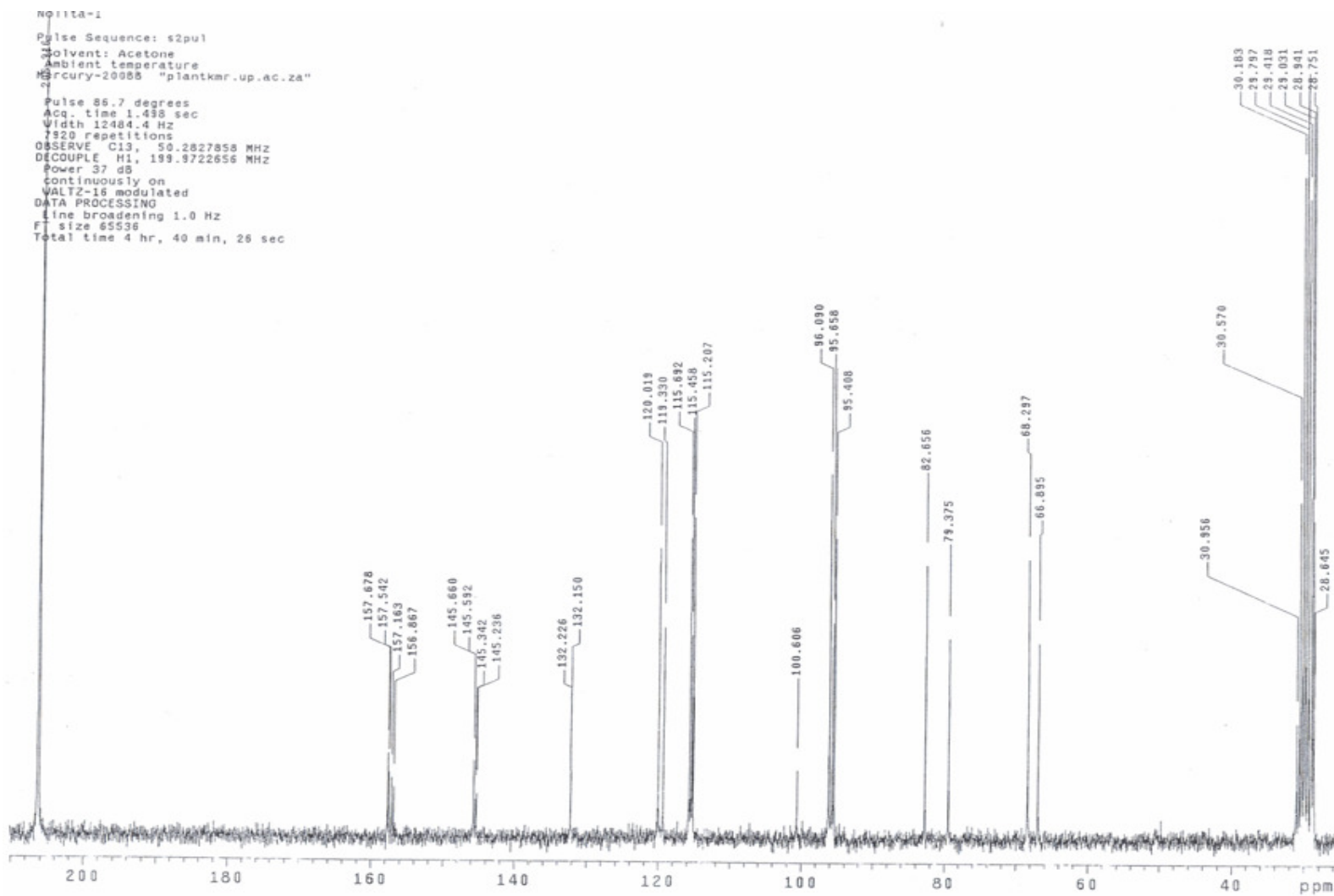


Figure 8.7: ¹³CNMR of ‘catechin-epicatechin’ isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A- ^1H NMR and ^{13}C NMR of isolated compounds from *Terminalia sericea*

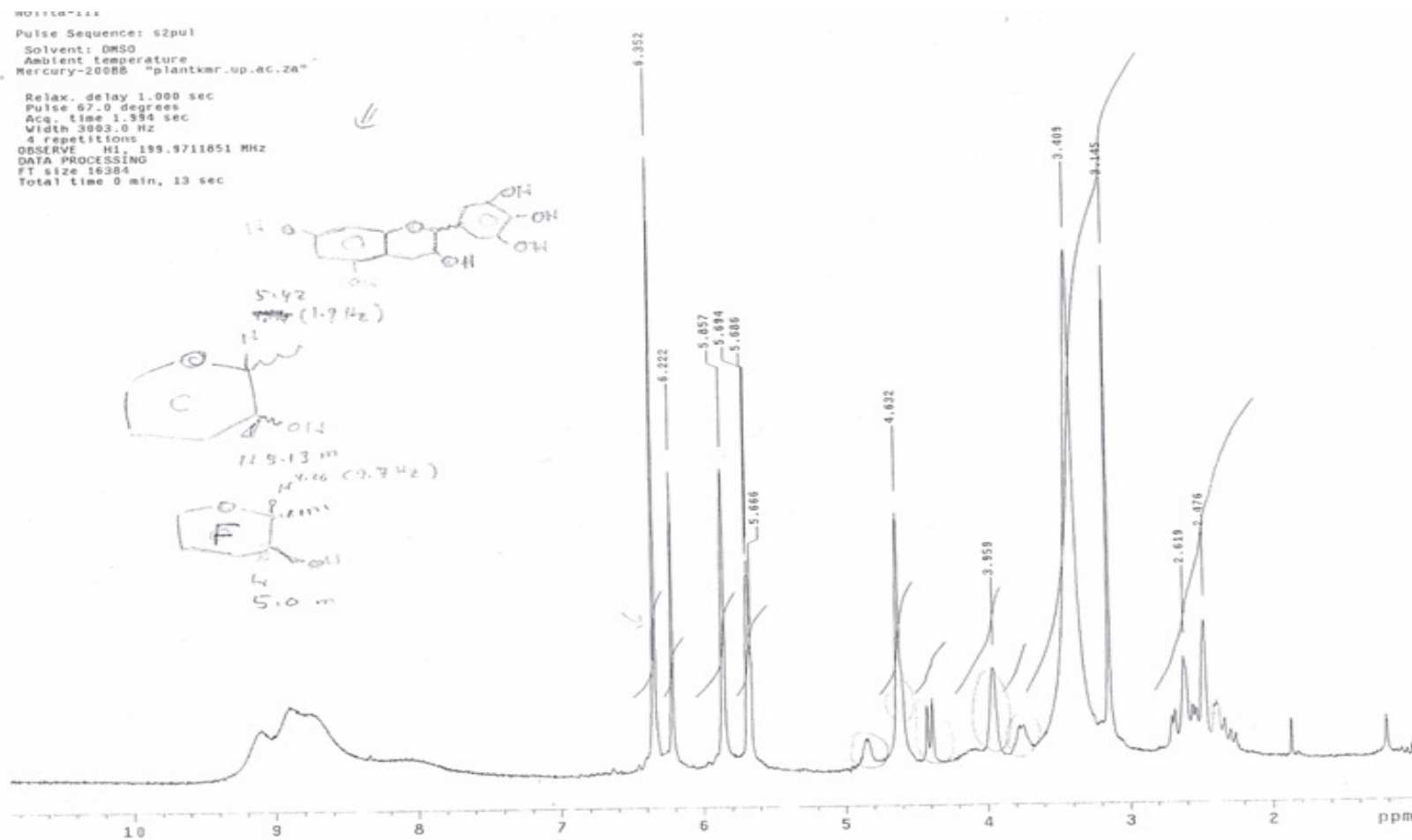


Figure 8.8: ^1H NMR spectrum of 'gallocatechin-epigallocatechin' isolated from the acetone extract of *Terminalia sericea*

Chapter 8: Appendix A-¹HNMR and ¹³CNMR of isolated compounds from *Terminalia sericea*

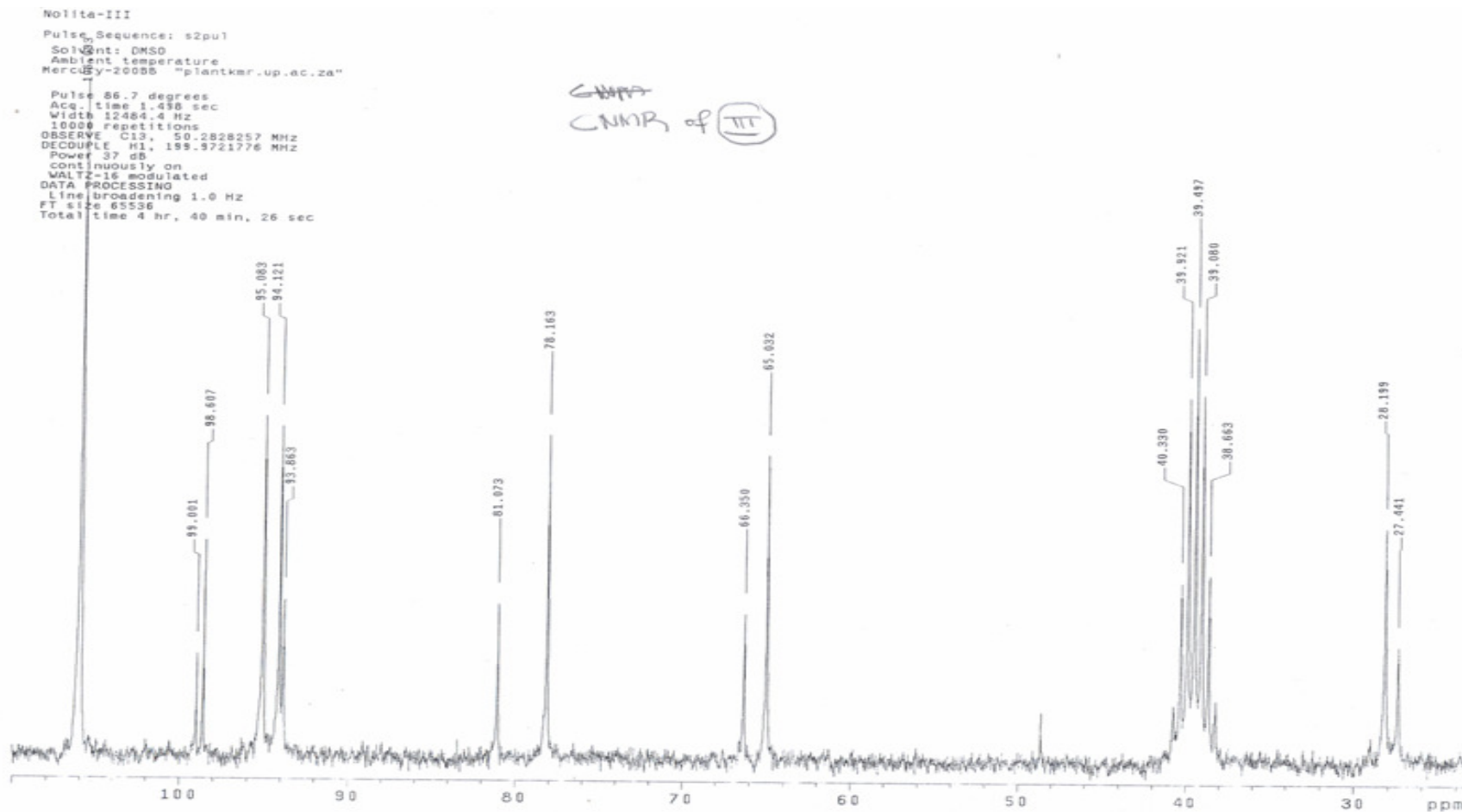


Figure 8.9: ¹³CNMR of 'gallocatechin-epigallocatechin' isolated from the acetone extract of *Terminalia sericea*

8.3 Publications and conference presentations resulting from this thesis

8.3.1 Publications

Article in preparation

Antidiabetic activity of pentacyclic triterpenes and flavonoids isolated from stem bark of *Terminalia sericea* Burch.Ex DC, N Nkobole, N Lall, PJ Houghton and AA Hussein

8.3.2 Conference presentations

National

Post-graduate Symposium (2008) University of Pretoria (oral presentation), N Nkobole, N Lall, PJ Houghton and AA Hussein. HYPOGLYCAEMIC COMPOUNDS FROM *TERMINALIA SERICEA* BURCH. EX DC. (COMBRETACEAE)

SAAB conference (2009) University of Stellenbosch (oral presentation), N Nkobole, N Lall, PJ Houghton and AA Hussein. HYPOGLYCAEMIC COMPOUNDS FROM *TERMINALIA SERICEA* BURCH. EX DC. (COMBRETACEAE)