UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

Isolation of bioactive compounds and *in vitro* studies on *Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh.) Walp. used in the eastern Free State for the management of Diabetes Mellitus

By

Makhubu, FN

Student number: 2008087963

A dissertation submitted in fulfilment for the award of degree of Master of Science in Botany, Department of Plant Sciences, Faculty of Natural and Agricultural Sciences, University of the Free State, Qwaqwa Campus, Private Bag X13, Phuthaditjhaba, 9866

Supervisor: Dr. Ashafa AOT

Co-Supervisor: Prof. Fouchè G

January, 2017

DECLARATION

I, Makhubu, Fikile Nelly, do hereby declare that the research project submitted for qualification for the Master's Degree in Botany at the University of the Free State represents my own original work and has not been presented for a qualification at another university.

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MAKHUBU, FN

This dissertation has been submitted for examination with our approval as the university supervisor

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Dr. ASHAFA, AOT

DEDICATION

To my beloved mother Malintja Belina Makhubu

ACKNOWLEDGEMENTS

It is a great pleasure to express my deepest thanks to my supervisor Dr. Ashafa Tom Omotayo and also my co-supervisor Prof. Gerda Fouché for their effort, time, support and patience towards a successful completion of this study. It has been a challenging time but their advice and inspiration made the experience worth the while.

I wish to gratefully thank the following people and institution for their contribution towards the success of this project:

The UFS Team: Dr. Mutiu Kazeem, Mr Sabiu Saheed (Phd candidate) and Mr Balogun Fatai (Phd candidate) for their assistance and guidance in the biological screening

The CSIR Team: Mr Jeremiah Senabe for guidance in the laboratory, Dr. Xolani Peter for assisting in isolation of compounds and structure elucidation, Prof. Paul Steenkamp for MS analyses, Dr. Chris Van der Westhuyzen and Dr. Gugulethu Mnguni for their assistance in NMR experiments.

Mme maMokoena for assisting in collection of plant material

Dr. Ojuromi Tessy for proof reading this work

I also extend my gratitude to colleagues and friends, CSIR Natural Product Chemistry group and Phytomedicine and Phytopharmacology Research Group. Moses Mokoena, Sam Leshabane, Rudzani Nthambeleni, Mandisa Mangisa, Vincent Hlatshwayo, Valerie Ramaotsoa, Bongimplilo Mkhize, Martin Moloto, Iviwe Nokalipa, Odwa Gonyela, Valeria Xaba, Seadi Mofutsanyana, Sellwane Moloi, Sellwane Lehasa, Thumeka Tiwani, Nobuhle Mbhele, Kazeem Alayande, Dr. Lanre Akintayo Ogundajo, Anelisa Lingunya, Matshediso Dithlare Mokoena and Kalake Mallane. I am grateful for your advice, help and encouragement at all the times. The moments we shared in the office, lab and outside were the best. Thank you very much for everything.

To my dearest mom (Malintja Belina Makhubu) for her support and giving me the strength and always believing in me, and to the rest of the family, Jabulani, Nkosana, Mamsy, Tshehofatso and my dearest new baby boy, Karabo for their continued encouragement, sacrifices and prayers throughout my educational pursuits and daily life.

National Research Foundation for generously funding my studies and Maluti-A-Phofung Municipality for assisting in outstanding fees, CSIR and the UFS for opportunity to pursue my studies

Most importantly, I would like to thank the Almighty God, this study would not be possible without Him. He gave me strength, good health and always remaining faithful to me. He is indeed magnificent God. I am nothing without Him.

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LIST OF ABBREVIATION

1D	One dimensional
2D	Two dimensional
3D	Three dimensional
^{13}C	Carbon 13
^{1}H	Proton
%	Percentage
µg/l	Microgram per litre
µg/ml	Microgram per mililiter
α	Alpha
°C	Degrees centigrade
ANOVA	Analysis of varience
AcOH	Acetic acid
ATS	African Traditional Systems
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
cm	Centimetre
COSY	Correlation spectroscopy
CSIR	Council for Scientific and Industrial Research
DNS	Dinitrosalycic
DMSO	Dimethyl sulphoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EtOH	Ethanol
EtOAc	Ethyl acetate
ESI	Electrospray ionisation
ESI^-	Electrospray negative mode
ESI^+	Electrospray positive mode
GAE	Galli acid equivalent
g	Grams
h	Hour
HSQC	Heteronuclear Single Quantum Coherence
kg	Kilograms

Km	Michaelis Constant
L	Litre
MeOH	Methanol
MHz	Mega hertz
MS	Mass spectroscopy
Min^{-1}	Per minute
mmol	Millimol
mL	Millilitre
min	Minute (s)
m	Meter
mg	Milligram
m/z	Mass to charge ratio
NBT	Nitroblue Tetrazolium
NMR	Nuclear Magnetic Resonance
ng / ml	Nanograms per mililiter
nm	Nanometers
nM	Nano molar
mM	Millimolar
PMS	Phenazine methosulphate
pTLC	prep Thin Layer Chromatography
pNPG	pNitrophenyl glucopyranoside
QE	Quercetin equivalent
Rf	Retention factor
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
S	Substrate
SEM	Standard error of the mean
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
UV	Ultraviolet
Vmax	Maximal rate
VLC	Vacuum Liquid Chromatography

WHO	World Health Organisation

ABSTRACT

The pravelence of diabetes mellitus is increasing and it is one of the major health problems affecting the world. The challenges with synthetic drugs used in the treatment of hyperglycemia such as acarbose and miglitol include abdominal discomfort, bloating and diarrhoea. The present study isolated and evaluated the active antidiabetic constituents from the roots of *Pentanisia prunelloides* (Rubiaceae) from the eastern Free State Province of South Africa using *in vitro* models. The antidiabetic potential of the water, ethanol, aqueous-ethanol and hexane root extracts of *P. prunelloides* was investigated against the specific activities of α-amylase, α-glucosidase, sucrase and maltase. Furthermore, the antioxidant activity of the extracts was determined using iron chelation, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide anion radical scavenging assays. For the fractions and the isolated compounds, only α-amylase and α-glucosidase assays were used. Fractionation of the ethanol extract was done by vacuum liquid chromatography (VLC), fractions were combined according to thin layer chromatography (TLC) profiles, and further purification of semi-pure compounds was achieved using preparative thin layer chromatography (pTLC) to obtain pure compounds. Isolated compounds were characterised using nuclear magnetic resonance (NMR) (1D and 2D data) and mass spectroscopy (MS). The ethanol extract displayed significantly higher ($p < 0.05$) inhibition of α-amylase (18.51 µg/mL), hexane (18.08 µg/mL) and ethanol (19.73 µg/mL) extract exhibited strongest inhibition of α-glucosidase. Water extract demonstrated strong inhibition of sucrase (3.85 µg/mL), and aqueous-ethanol extract (26.03 µg/mL) on maltase. Kinetic studies showed that the mode of inhibition of α-amylase and α-glucosidase by ethanol extract was mixed non-competitive and non-competitive respectively. Water and ethanol extract displayed higher DPPH (75.42 µg/mL) and (77.06 µg/mL) scavenging abilities than other extracts but not higher than gallic acid. Hexane extract demonstrated significantly higher ($p < 0.05$) superoxide (0.33 µg/mL) and hydroxyl radical (0.51 µg/mL) scavenging abilities while aqueous-ethanol exhibited the strongest iron chelation activity 4.24 µg/mL. Phytochemical analysis of the extract revealed the presence of tannins, terpenoids, alkaloids, saponins, flavonoids and cardiac glycosides. Quantification of phytochemicals revealed total flavonoids of 15.40 mg quercetin equivalent (QE)/g in hexane extract which was not significant ($p > 0.05$) and from water it was 14.70 mg QE/g. The highest tannin concentration of 45.60 mg

gallic acid equivalent (GAE)/g was from aqueous-ethanol which was significantly higher than other extracts ($p < 0.05$). Total phenol from water and aqueous extracts was 0.07 mg GAE/g; alkaloids and saponins were found to be low in the roots of *P. prunelloides*, at 0.6 and 13.9% respectively. Of the 21 fractions obtained, acetylated fraction displayed significantly higher ($p < 0.05$) inhibition of α-amylase 48.06 µg/mL while fraction PP-I-101835BII exhibited strongest inhibition of α-glucosidase (19.53 µg/mL). Three compounds were isolated, two sucrose (acetylated and non-acetylated) and tormentic acid. Tormentic acid inhibited α-amylase and α-glucosidase at 70.45 µg/mL and 28.21 µg/mL respectively. Kinetic analysis revealed that tormentic acid inhibited α-amylase in un-competitive manner and α-glucosidase competitively. The ethanol extract and the isolated tormentic acid exhibited best inhibitory activity on the two enzymes studied, and the presence of phytochemicals in the roots of *P. prunelloides* in this study may be suggested to have contributed greatly to the biological activities of the plant. Tormentic acid appears to be a potential anti-diabetic drug thus supporting usage of the root extract of *P. prunelloides* in the management and treatment of diabetes mellitus in the eastern Free State Province.

Keywords: antioxidants, hyperglycemia, kinetics, phytochemicals, *P. prunelloides*, tormentic acid, α-amylase, α-glucosidase

CHAPTER 1

INTRODUCTION

1.1 General introduction

Most African countries are undergoing a demographic transition and are increasingly coming under the influence of Western lifestyles. South Africa in particular, is one of the developing countries where people have adopted the Western food culture and this has led to increase in consumption of fat, sugar and salt through fast food and others. Increase in population, urbanisation, poverty and lack of exercise could contribute to metabolic disorders such as diabetes mellitus (Deutschländer, 2010).

Diabetes mellitus is a chronic endocrine disorder that affects the metabolism of carbohydrates, fats, proteins, water and electrolytes (Altan, 2003). This disease is considered as one of the non-curable illness but it can be managed through monitoring of the blood sugar over healthy diet, exercise and medication (Diabetes, 2012). Current statistics suggest that about 382 million people live with diabetes worldwide and this number is estimated to increase to 552 million by 2035 (IDF, 2014). The chronic hyperglycemia have risks that lead to long term complications such as dysfunction and failure of organs such as the kidneys, heart and other systemic associated diseases -stroke, feet, nerves, blood vessels and the eyes (Diabetes, 2012).

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Medicines of natural origin offers a great alternative for managing diseases and it is mostly encouraged for chronic diseases (Hemdam and Afifi, 2004). In recent years, research on traditional medicinal plants for the management of diabetes has attracted the attention of scientists. Many plants that are traditionally being used for treating similar symptoms of diabetes have been evaluated and their blood glucose lowering effects have also being confirmed using *in vitro* and *in vivo* models (Day and Bailey, 2006). Plants that have been investigated to have antidiabetic activity include *Urtica dioica, Morinda lucida*, *Allium sativum, Alstonia boonei, Ageratum conyzoides, Bridelia micrantha, Ficus*

exasperate (Gbolade, 2009). Grover *et al*. (2002) reported more than 1,100 plant species have been in use ethno pharmacologically and/ or experimentally for the treatment of diabetes mellitus. The debilitating symptoms, expensive treatment and complications occurring as a result of diabetes mellitus may be prevented or delayed by controlling the amount of glucose in blood (Voulgari *et al*., 2010).

According to Kong *et al*. (2003), series of natural products isolated from plants have been employed as clinical agents and are still in use today. This provides significant information on drug discovery from medicinal plants. Examples of such drugs include quinine from Cinchona bark, which is used for treating of malaria, and modern drug known as Aspirin from *Filipendula ulmaria* used for analgesic and inflammation (Plotkin, 1988; Butler *et al*., 2004). This signifies huge potential that still exists for the discovery of many novel drugs from medicinal plants.

An anti-diabetic agent may work by lowering the blood glucose via stimulating insulin secretion, or improving the insulin sensitivity or inhibiting glucose absorption (Cheng and Funtus, 2005). There are two major primary enzymes, alpha (α) amylase and alpha (α) glucosidase, which play important roles in carbohydrate metabolism.The digestion of starches to glucose requires multiple reactions of α-amylase and α-glucosidase (Bolen *et al*., 2007). Alpha-amylases hydrolyse complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolysed by α-glucosidase to monosaccharides and are then absorbed through the small intestines into the hepatic portal vein (Smith *et al*., 2005). Inhibitors of these enzymes serve as one of the therapeutic approaches for decreasing postprandial hyperglycemia by slowly delaying the digestion of glucose by inhibition of these enzymes in the digestive tract (Deshpande *et al*., 2009).

Hyperglycemia-induced metabolic dysfunction may be caused by reactive oxygen species (ROS) produced in the mitochondrial electron transport chain (Brownlee, 2005). Oxidative stress and free radical induced oxidative damage have long been thought to be the most significant cause of many diseases such as cancer, diabetes, stroke, rheumatoid arthritis, atherosclerosis, arteriosclerosis, neurodegenerative and cardiovascular diseases (Harman, 1992; Babu and Gowri, 2010; Arouma, 2010). It is believed that in the onset

and progression of diabetic complication, free radicals have major roles to play due to their ability to damage lipids, proteins, and Deoxyribonucleic acid (DNA) (Ayepola *et al*., 2014). Therefore, the search for the discovery of antioxidant and antidiabetic agents from plant sources is an important strategy required to combat the widespread nature of this condition.

One of such plant is *Pentanisia prunelloides.*It is a multipurpose plant used traditionally for treating various ailments including tuberculosis, blood impurities, haemorrhoids and pregnancy associated complications (van Wyk *et al*., 2000). The plant was collected in the eastern Free State and evaluated for its antidiabetic activity using *in vitro* assays in attempt to validate its traditional usage. This plant was also evaluated for its antioxidant activity because reports have indicated that diabetes is accompanied by increased production of free radicals (Baynes, 1991). Compounds that could be used to treat diabetes mellitus especially Type II were isolated from the most active extract using chromatographic methods.

1.2 Problem statement

Diabetes mellitus is one of the major health problems that is affecting people throughout the world. Type II diabetes or hyperglycemia is responsible for the development of various complications including impaired fasting glycemia, impaired glucose tolerance as a result of resistance to insulin (Giacco and Brownlee, 2011) and other long term complications due to elevated glucose in the blood (Loghmani, 2005). There are other factors aside hyperglycemia that can lead to the pathogenesis of diabetes such as oxidative stress and hyperlipidemia (Kangralkar *et al*., 2010). The control of hyperglycemia is however one of the most important solutions to retard the progression of the disease.

The use of inhibitors of carbohydrate digesting enzymes (α-amylase and glucosidase) offers a great solution to prevent Type II diabetes. This will control plasma glucose by decreasing the rate of blood sugar from small intestine; thus slowing and interrupting the digestion of starch (Rhabasa-Lhoret and Chiasson, 2003). There are few recognised pharmaceutical drugs that have shown potentials in controlling hyperglycaemia namely acarbose, voglibose and miglitol. These drugs are widely used and are often reported to

cause several side effects, such as cramping, abdominal distention, flatulence and diarrhoea (Bischoff, 1994; Fujisawa *et al*., 2005; Shai *et al*., 2010). This prompted the need for screening and investigation of plants as potential sources of new antidiabetic compounds for primary health care. The medicinal plants that are traditionally used for treating ailments are known to offer good source for finding new, safe and accessible drugs. Therefore, necessitating the search for medicinal plants that will decrease postprandial hyperglycemia and other complications caused by diabetes and will have little or no side effects.

1.3 General aim

The aim of the present study was to evaluate antidiabetic properties and isolatate bioactive compounds of *Pentanisia prunelloides* (Rubiaceae) collected from the eastern Free State Province in South Africa using *in vitro* models.

1.3.1 Specific objectives

The specific objectives are to:

- Extract the roots of *P. prunelloides* using solvents of different polarity and screen the resultant crude extracts for inhibitory activity against carbohydrate metabolizing enzymes (α-amylase and α-glucosidase) using *in vitro* enzyme inhibition bioassays.

- Determine and compare the IC_{50} (inhibitory concentration) of the crude *P. prunelloides* extracts and compare the enzyme inhibitory activity of the extracts with those of known and commercially available enzyme inhibitors.

- Evaluate antioxidants activity of the crude root extracts of *P. prunelloides* root extracts

- Identification of bioactive compounds from *P. prunelloides* active extract using Vacuum Liquid Chromatography and preparative Thin Layer Chromatography fractionation.

- Isolate the active chemical ingredient from the active fraction.

- Elucidate the structure of the isolated compound (s)

- Test the isolated compound (s) for antihyperglycaemic activity

- Validate the traditional usage of *P. prunelloides* as antidiabetic agent by the Basotho tribe from the eastern Free State province

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CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes mellitus

The fastest growing disease, diabetes mellitus is a chronic endocrine disorder that affects the metabolism of carbohydrates, fats, proteins, water and electrolytes (Altan, 2003). It is considered as one of the non-curable illnesses but it can be controlled through monitoring of the blood sugar over healthy diet, exercise and medication (IDF, 2000). Diabetes mellitus is different among individuals and the type an individual has depends on clinical presentation at the time of diagnosis (Diabetes, 2012). The disease has been re- classified by World Health Organization (WHO, 1999) which contains stages that reflect different degrees of the hyperglycemia in individual subjects leading to the diabetes mellitus. According to Harris and Zimmert (1997), the correct measure for organising the epidemiological and the clinical research for the management of diabetes mellitusis through proper classification.

2.2 Types of diabetes mellitus

There are two major types of diabetes recognized in Western countries: insulin dependent diabetes mellitus (IDDM Type I diabetes) and non-insulin dependent diabetes (NIDDM, Type II diabetes). There are other forms in which diabetes mellitus can be classified, including gestational diabetes, secondary diabetes and others (Harris, 2000; WHO, 2002).

2.2.1. Type I diabetes mellitus

As stated by Loghmani (2005), Type I diabetes mellitus is caused by inadequate or absolute absence of insulin. The metabolic imbalance in this type is caused by the autoimmune destruction of pancreatic β-cells which leads to deficiency of insulin secretion. In some patients, the pancreatic α-cells are also abnormal and there is excessive secretion of glucagons. Normally, hyperglycaemia lead to reduced glucogen secretion, but in patients with Type I, glucagon is not suppressed by hyperglycemia (Raju and Raju,

2010). It is referred to as juvenile onset diabetes mellitus as it affects mostly younger individuals (Harris, 2000; Scheen and Lefebvre, 2000).

2.2.1 Type II diabetes mellitus

Type II diabetes is considered as the most common type that affects about 90% of people worldwide (WHO, 2015). Patients with this type are not dependant on exogenous insulin for prevention of ketonuria and are not prone to ketosis. However, they may require insulin for the correction of fasting hyperglycaemia if this cannot be achieved with the use of diet or oral agents. Such patients may develop ketosis under special circumstances such as severe stress precipitated by infections or trauma (Harris and Zimmet, 1997). According to WHO (2009), Type II diabetes is the most prevalent and preventable form when compared to other types, hence the focus of this.

2.2.2 Gestational diabetes mellitus (GDM)

Gestational diabetes is a degree of glucose intolerance with onset or first recognition during pregnancy when the need for insulin increases (Murphy *et al*., 2011). During pregnancy, the need for insulin appears to increase hence gestational diabetes occurs at the late stages of pregnancy (Soumyanath, 2005). This type of diabetes usually disappears once the baby has been delivered but Type II diabetes may develop later in life. (WHO, 2002).

2.2.3 Secondary diabetes

Secondary diabetes develops as a result of other diseases or medication. As reported by Yanase and Nomiyama (2015), these other types of diabetes include diseases such as pancreatic diseases (pancreatitis, cystic fibrosis, surgery). Pancreatic diabetes is one of the most popular secondary diabetes, which causes insulin deficiency following pancreatic diseases, such as pancreatitis and pancreatic cancer; endocrine diseases e.g., cushing's and genetic syndromes which are rare. Davidson (1991) reported that the use of drugs that are not prescribed such as contraceptive pills, steroids and diuretics are the major cause, and contribute to the development of secondary diabetes.

2.2.4 Other forms of diabetes

Other forms of diabetes may arise as a result of complications and are very uncommon (WHO, 2002). As reported by Soumyanath (2005), this type of diabetes results from monogenetic βcell defects in individuals below 25 years of age. Genetic defects in insulin action, disease of the exocrine pancreas, endocrinopathies, drug or chemically induced diabetes, infections and uncommon forms of immune-mediated diabetes are also associated with the diabetes.

2.3 Prevalence of diabetes

The high number of people living with diabetes as viewed by IDF (2015) is expected to reach 205 million by the year 2035. According to WHO (2015), each year 1.5 million deaths are attributed to diabetes with90% linked to Type II diabetes making it the most common type (Figure 2.1). In the Africa region, it was estimated that about 14.2 million adults between 20 and 79 years of age have diabetes (with many undiagnosed cases) and over two thirds (66.7%) are unaware that they have diabetes (IDF, 2015). The high risk of adult having diabetes was reported by (WHO, 2015) which accounts for 9% in the world. In Africa, the estimated number of people with diabetes vary from one country to another such as South Africa (2.3 million), Democratic Republic of Congo (1.8 million), Nigeria (1.6 million) and Ethiopia (1.3 million) (IDF, 2015). It was reported that 17% of deaths aged between 50-79 years in South Africa are due to diabetes and prevalence is higher in men than women (IDF, 2015). Population growth, urbanization, increasing prevalence of obesity and physical inactivity are considered to be the main factors responsible for the increasing prevalence of Type II diabetes mellitus (Wild *et al*., 2004).

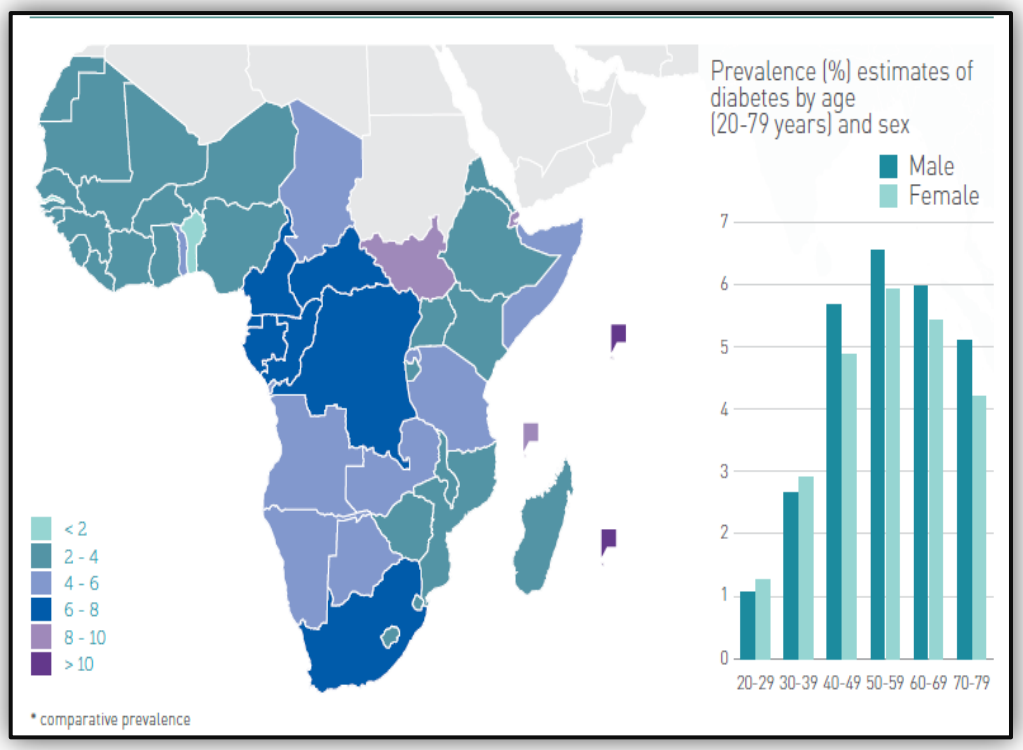


Figure 2.1: Map showing prevalence (%) estimates of diabetes (20-79 years), 2015 (IDF, 2015)

2.4 Mechanism of Type II diabetes

Type II diabetes mellitus can results from an imbalance between insulin sensitivity and / or insulin secretion. The pancreas normally produces insulin, which the body does not utilize correctly (Obimba *et al*., 2014). According to Albright (1997), the imbalance of insulin in this condition are due to peripheral tissue insulin resistance where insulin-receptors or other intermediates in the insulin signalling pathways within body cells are insensitive to insulin and consequently glucose does not readily enter the tissue leading to elevated blood glucose concentrations (hyperglycaemia). This impaired insulin action is often observed in several tissues e.g., skeletal muscle, adipose tissue and the liver. Compensatory hyperinsulinemia maintains glucose level within normal range. However in individuals with high risk of developing diabetes, beta cells function eventually declines and leads to the development of impaired glucose tolerance and eventually overt diabetes mellitus (DeFronzo *et al*., 1992; Stumvoll *et al*., 2005).

2.5 Risk factors of Type II diabetes mellitus

The likelihood of developing Type II diabetes increases with age, lack of activity, obesity, unhealthy diet, smoking, hypertension, low education, women with prior gestational diabetes and family history. Most recently, some specific genes have been documented as risk factors for Type II diabetes (Gadsby, 2002; Alberti *et al*., 2007; Chan *et al*., 2009).

2.5.1. Physical inactivity

Physical activity plays an important role in decreasing the possibility of developing diabetes mellitus. Lack of exercise could lead to several factors such as cholesterol accumulation and hypertension which ultimately can cause diabetes mellitus. An unfavourable blood lipid profile has been reported as a risk factor for Type II diabetes (Jacobsen *et al*., 2002). High cholesterol is linked to Type II diabetes since it affects the levels of different classes' cholesterol. People with diabetes tend to have increased triglycerides, reduced high density lipoprotein (HDL), and sometimes increased low density lipoprotein (LDL). This increases the possibility of developing narrow arteries (Gabriely and Shamoon, 2004). According to Czernichow *et al*. (2002), blood pressure increases with increasing body mass index (BMI); thus explaining the importance of exercising.

Physical activity plays an important role in delaying or prevention of development of Type II diabetes in those at risk directly by improving insulin sensitivity and reducing insulin resistance, and indirectly by beneficial changes in body mass and body composition (Boule *et al*., 2001; Kay and Singh, 2006). This information on the risks suggest that educational attainment promote an interest in own health and acquisition of knowledge that strongly influence people's ability to reduce risk by successfully adopting a healthier life style.

2.5.2. Unhealthy diet

The type of food consumed plays an important role in health. Incorporating good eating habits such as high consumption of fruits and vegetables which are known to be

associated with reduced risk of Type II diabetes mellitus will help in preventing the disease (Montonen *et al*., 2005). Unhealthy diet is one of the major life factor associated with the development of Type II diabetes mellitus (Hu *et al*.,2001). High intake of saturated fat and trans fat adversely also affects glucose metabolism and insulin resistance (Hu *et al*.,2001).

2.5.3. Obesity

According to Nolte and Karan (2001), obesity is a common risk factor for developing Type II diabetes and it normally results in impaired insulin action. In obese people, the adipose tissue releases the highamounts of non-esterified fatty acids, glycerol, hormones and other factors that are involved in the development of insulin resistance. When insulin resistance is accompanied by the dysfunction of the beta cells, insulin secretion normally results in failure to control blood glucose level in Type II diabetes (Hebebrand and Hinney, 2009). Certain genes and environmental factors such as high calorie or fat intake and physical inactivity are known to be associated with diabetes mellitus which can lead to obesity; and insulin resistance follows (Kahn *et al*., 2006; O'Rahilly and Farooqi, 2006).

2.5.4. Smoking

Of all the risk factors that lead to development of diabetes mellitus, smoking is one of the major ones. Facchini *et al*. (1992) reported that smoking leads to insulin resistance and inadequate compensatory insulin secretion response. In addition, Talamini *et al*. (1999) supported the direct negative effect of nicotinic or other components of cigarette on beta cells of the pancreas.

2.6 Complications caused by Type II diabetes

As a consequence of the metabolic derangement in diabetes, various complications develop including macrovascular and microvascular dysfunction (Duckworth, 2001). The consistently high levels of glucose in the blood can lead to serious diseases such as cardiovascular disease, kidney disease, retinopathy and nerve damage (IDF, 2014).

Cardiovascular disease occurs in response to how malfunction in the heart or blood circulation through the body (WHO, 2011). According to WHO (2011), major cardiovascular risk factors such as hypertension and diabetes have been linked to renal diseases. There are several diseases that are associated with heart such as angina, stroke, mycordial infarction (heart attack), peripheral artery disease and congestive heart failure (WHF, 2016). According to WHO (2011), the underlying disease process in the blood vessels results in coronary heart disease and cerebrovascular disease (stroke) known as atherosclerosis which is responsible for large proportion of cardiovascular diseases. Of interest is the report of Touchette (2005) that diabetes medications could cause chemical changes in the blood leading to atherosclerosis.

According to Mestrovic (2016), nephropathy is considered a progressive illness where kidneys become less effective over time and the condition get worse if left untreated. Nephropathy is regarded as a major microvascular complication of diabetes mellitus, and affects approximately one third of all diabetic patients. In affected people, nephrons are unable to filter out impurities in the blood and this accumulates and re-circulates in the blood (IDF, 2013).

Retinopathy is another common complication in diabetes especially in Type II. This complication is insidious and can go unnoticed for years in both Type I and II patients (Vislisel and Oetting, 2010). This disease affects the retina and might lead to permanent loss of vision. In this condition, the blood vessels are normally closed off or may be weaken in the eye, or there is sprouting in the retina which may result in blurry vision and ultimately blindness if not treated (Touchette, 2005).

According to National Diabetes Information Clearinghouse (NDIC, 2009), about 60 to 70 percent of people with diabetes have disorder of neuropathy. As observed by IDF (2013), neuropathy affects the peripheral nerves which might lead to loss of sensation but this condition can only occur for short period of time. This may lead to signal transduction errors which are interpreted aberrantly as pain in hands and feet or loos of sensation. Other serious infections that may occur due to diabetes may be ulceration, and diabetic foot disease resulting in major amputations.

2.7 Role of oxidative stress in diabetes mellitus

Oxidative stress is a conditionwhere there is excessive production of free radicals (Harman, 1992). Free radicals are highly unstable and highly reactive molecules which can react with various organic substrates such as carbohydrates, lipids, proteins and DNA (Zengin and Aktumsek, 2014). Free radicals are formed from molecules through the breakage of a chemical bond such that each fragment keeps one electron by cleavage of a radical to give another radical and also through redox reactions (Halliwell and Gutteridge, 2007; Bahorun *et al*., 2006). Free radicals are continually being produced in the body as the results of normal metabolic processes and they interact with the environmental stimuli (Bisht and Sisodia, 2010).

The majority of free radicals that damage biological systems are oxygen-free radicals, known as reactive oxygen species (ROS). Free radical induced oxidative damage has long been thought to be the most significant cause of many diseases such as cancer, diabetes, stroke, rheumatoid arthritis, atherosclerosis, neurodegenerative and cardiovascular diseases (Harman, 1992; Babu and Gowri, 2010; Arouma, 2010). As noted by Ayepola *et al*. (2014), in the onset and progression of the late diabetic complication, the free radicals have a got major role due to their ability to damage the lipids, proteins, and DNA. The elevation of ROS in diabetes may be due to decrease in destruction or the increase in the production by catalase superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). The varying levels of these enzymes in the body makes the tissues susceptible to oxidative stress leading to the development of complications in diabetes (Lipinski, 2001). However, it was documented that ROS can be beneficial in biological systems depending on the environment (Lopaczynski and Zeisel, 2001; Glade, 2003). A beneficial effect of ROS involves the physiological roles in cellular responses to anoxia, such as defense against infectious agents, and in the functioning of cellular signalling systems.

According to Moussa (2008), a component of the utilized oxygen is reduced to water, and the remaining oxygen is transferred to oxygen free radical (O^{-}), an important reactive oxygen species that is converted to other reactive species, such as peroxynitrite ($ONOO^{-}$), hydroxyl radical (OH) and superoxide (H_2O_2). The first step in reduction of oxygen

forming superoxide is endothermic, but subsequent reduction is exothermic (Matough *et al*., 2012).

2.8 Glucose metabolism

2.8.1. Glucose regulation

Glucose is an essential metabolic substrate of all mammalian cells. Glucose and other monosaccharides are transported across the intestinal wall via the hepatic portal vein to liver cells and other tissues (Szablewski, 2011). Humans mobilize carbohydrates from glycogen, free fatty acids from triglycerides and amino acids from proteins during brief or prolonged fasting states to meet energy requirements (Holm and Kasper, 1984).

Glucagon is a hormone secreted from the alpha cells of the pancreas, and is one of the major biomolecules along with insulin that regulate plasma glucose (Shrayyef and Gerich, 2010). Glucagon in the liver stimulates glycogenolysis, which is the breaking down of glycogen, and the export of glucose into the circulatory system (Drucker, 2006). After a carbohydrate meal, there is a rise in blood glucose level sending signals to the pancreas to secrete hormone insulin. Hyperglycemia is a condition characterized by a rapid increase in blood glucose levels. High blood glucose happens when the body has too little insulin or when the body cannot use insulin properly (Deshpande *et al*., 2009). Insulin causes the cells to take glucose out of the blood and store it in almost all tissue in the body, especially the liver (Figure 2.2), musculature and fat tissues, thus keeping the blood glucose from rising too rapidly (Roussel, 1998). Skeletal muscle cells and the liver store the glucose as glycogen, while adipose tissues convert it to lipids. Approximately two hours after meal, blood glucose levels drop causing the pancreas to release glucagon (Szablewski, 2011).

There are two specific types of hyperglycemia. Fasting hyperglycemia, which is defined as a blood sugar level greater than 90-130 mg/dL (5-7.2 mmol/L) without meal for at least 8 h. Postprandial hyperglycemia, defined as a blood sugar level greater than 180 mg/dL (10 mmol/L) after meal (Szablewski, 2011). Postprandial hyperglycemia has been linked to the onset of diabetic complications in Type II diabetic patients due to the generation of

free radicals leading to damage in the retina, renal glomerulus and peripheral nerves (Brownlee, 2005).

Hyperglycemia may be caused by skipping insulin or oral glucose lowering drugs, consumption of foods high in calories and carbohydrates, infection, illness, increased stress, decreased activity or lack of exercise and strenuous physical activity (Szablewski, 2011). Low blood glucose is known as hypoglycaemia, a condition which occurs when blood glucose drops below normal levels. The low blood glucose causes the alpha cells of pancreas to be stimulated, releasing glucagon into the blood, causing the liver cells to convert stored glycogen into glucose and consequently circulated into the blood stream (Figure 2.2). Insulin also cause body cells to take up more glucose leading to decline in blood glucose thus the stimulus for insulin release diminishes (Roussel, 1998). According to Shrayyef and Gerich (2010), hypoglycemia may result from inadequate food intake, over production or administration of insulin. Hypoglycemia can also be caused by infection (higher metabolism and demand for glucose during immune system activity), exercise or situations which increase the body's glucose utilization. This condition is defined as blood glucose level less than 50 mg/dL (2.8 mmol/L) (Szablewski, 2011).

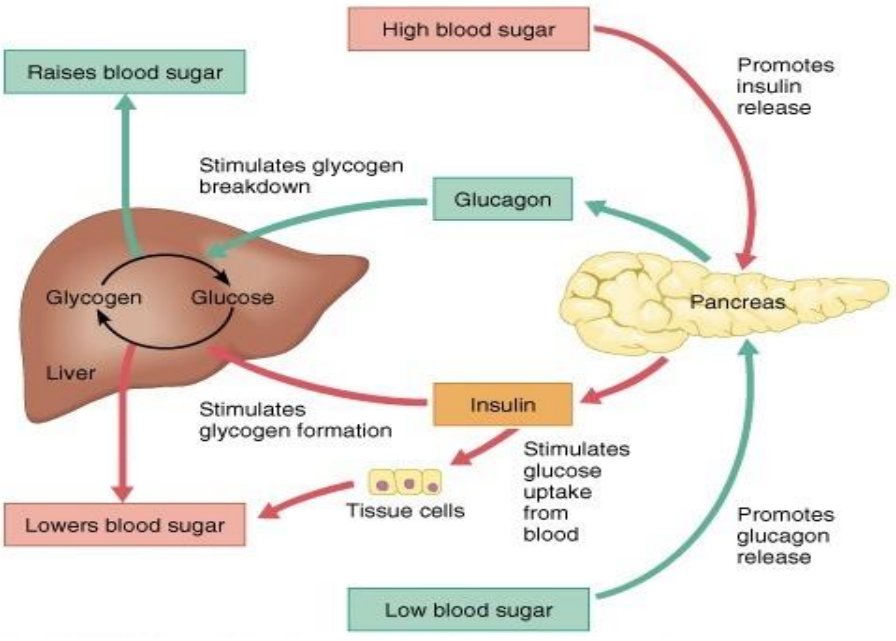


Figure 2.2: The role of glucagon (https:www.atrianceu.com/course-module/3265833-174_diabetes-type-2-module-04)

2.8.2. Digestion and absorption of carbohydrates

The salivary enzyme amylase breaks down food starches into maltose (disaccharide) in the mouth. The disaccharides are broken down into monosaccharides by enzymes maltases, sucrases, and lactases, which are present in the brush border of the small intestinal wall. Maltase breaks down maltose into glucose, while sucrose and lactose are broken down by sucrase and lactase respectively. Sucrase breaks down sucrose into glucose and fructose, and lactase breaks down lactose into glucose and galactose (Saxena and Bhatnagar 1961; Minai-Tehrani *et al*., 2010). Glucose and other monosaccharides (fructose and dextrins) from the digestion of carbohydrates (polysaccharides) are absorbed through the small intestine into the hepatic portal veins, causing elevation of postprandial blood glucose level (Bhat *et al*., 2011) (Figure 2.3).

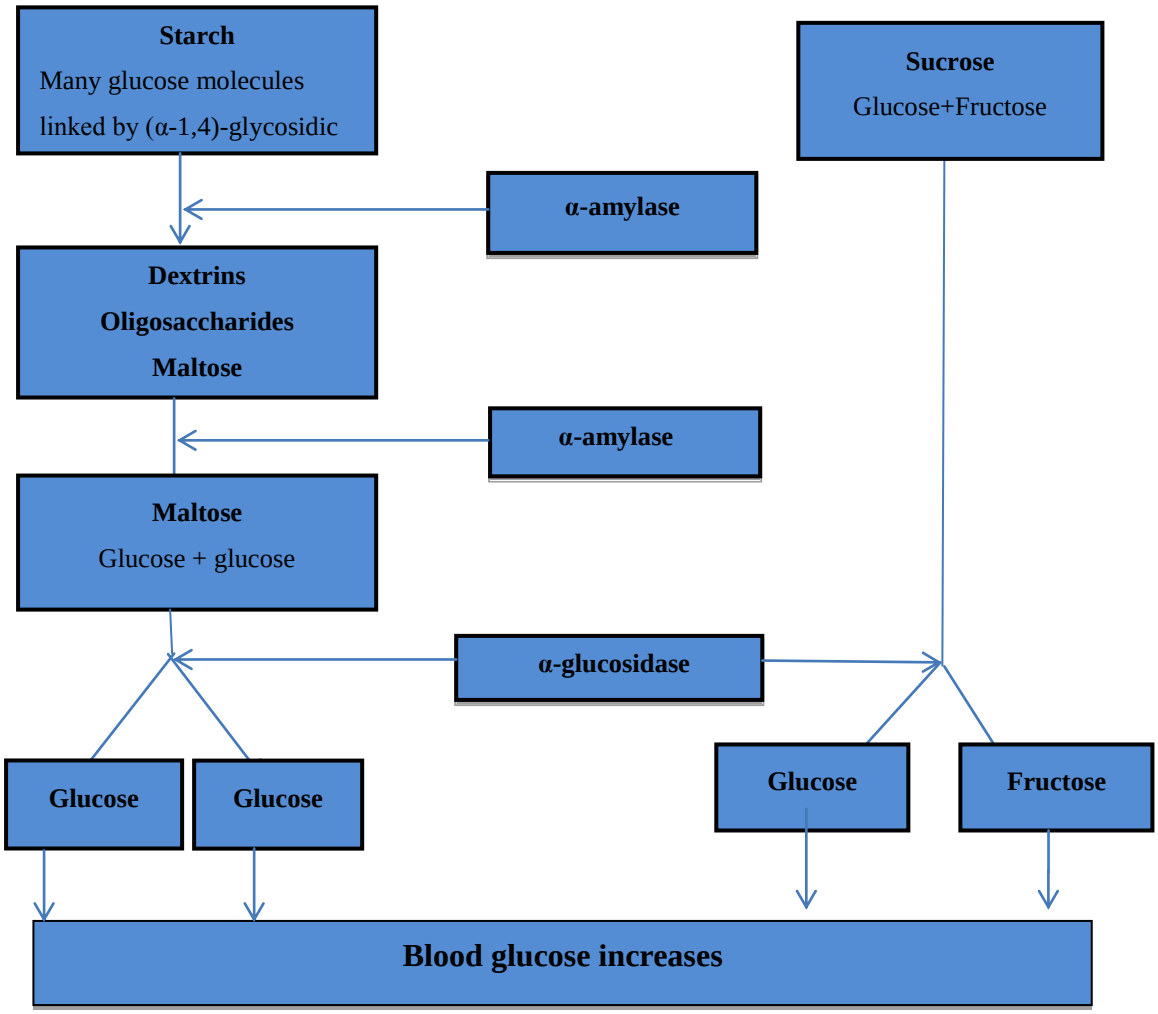


Figure 2.3: Carbohydrate digestion by α-amylase and α-glucosidase (Mkhombo, 2010)

2.9 Treatment for Type II diabetes mellitus

The current available therapy for diabetes is insulin administration which involves various oral hypoglycemic drugs such as sulfonylureas, glucosidase inhibitors, metformin, thiazoldinediones and troglitazone (Rajalakshmi *et al*., 2009). Mamun-or-Rashid *et al*. (2014) reported that the use of antidiabetic medications depend on the nature of the diabetes, age, individual situation and other factors. Injections and oral hypoglycemic agents are used for treating diabetes mellitus. These agents work by improving insulin sensitivity, increasing insulin productivity and decreasing the amount of glucose in blood.

2.9.1. Sulfonylurea

Obimba *et al*. (2014) reported that sulfonylurea decreases fasting and postprandial glucose levels in diabetic patients; by boosting pancreatic insulin secretion. For example, glibenclamide (Figure 2.4) is an oral hypoglycaemic agent that stimulates insulin secretion through the pancreatic β-cells.

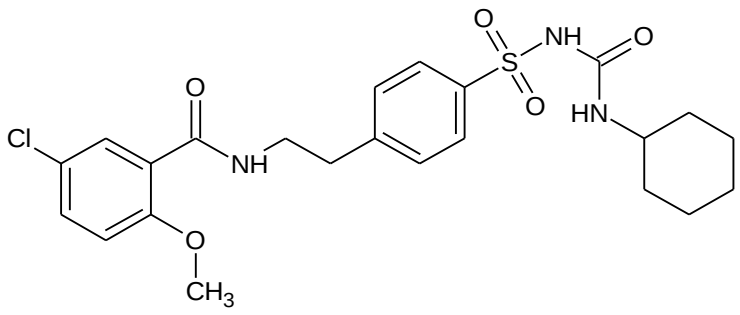


Figure 2.4: Chemical structure of Glibenclamide (Pubmed, 2016)

2.9.2. Metformin

Metformin is a drug that works by stimulating glycolysis in the tissue. It increases the removal of glucose from the blood via glucose to lactate conversion by enterocytes; decreases hepatic glucose production and intestinal absorption of glucose while reducing plasma glucagon (Nolte and Karam, 2001). This drug originates from a perennial herb

known as *Galega officinalis* with the active compound, galegine (Figure 2.5), which is a derivative of guanidine (Nolte and Karam, 2001).

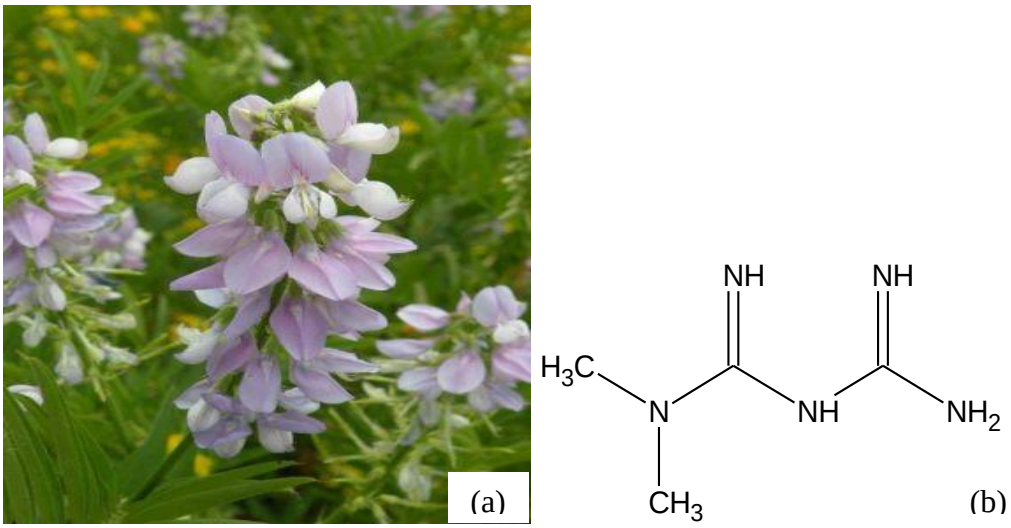


Figure 2.5: (a) *Galega officinalis* L from by Perino, and (b) chemical structure of binguanide metformin from (Parker, 2014)

2.9.3. Acarbose

The report of Arungarinathan *et al*. (2011) emphasised that acarbose (Figure 2.6) is a dissacharide that inhibits the breakdown of other dissacharides in the upper gastrointestinal tract. In addition, acarbose was also reported in another study to inhibit α-glucosidases in competitive and reversible manner (Standl *et al*., 1999). Inhibition of this glucoside hydrolase activity by acarbose delays hydrolysis and digestion of complex carbohydrates in the upper small bowel. This subsequently retards absorption of glucose and reduces postprandial hyperglycemia. It also exerts the same degree of non-reversible blockade on pancreatic α-amylase, which hydrolyzes complex starches to oligosaccharides in the lumen of the small intestine (Arungarinathan *et al*., 2011).

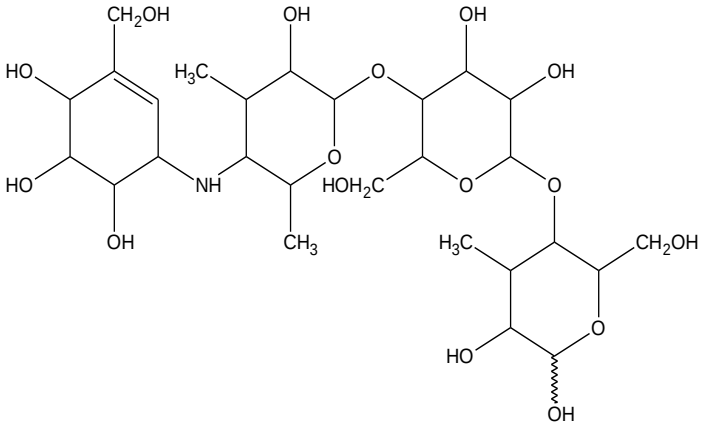


Figure 2.6: Chemical structure of Acarbose (Kalra, 2014)

2.9.4. Thiazoldinediones

Thiazoldinediones is a recently introduced class of oral antidiabetic drug exhibiting the same drug action similar as metformin by enhancing insulin mediated glucose absorption into the cells without raising blood insulin levels (Obimba *et al*., 2014). There are two types of thiazoldinediones that are commercially available namely pioglitazone and rosiglitazone (Figure 2.7). As noted by Yki-Järvinen (2004), thiazoldinediones increase sensitivity by acting on adipose tissues, muscles and liver cells to increase glucose utilization and decrease glucose production.

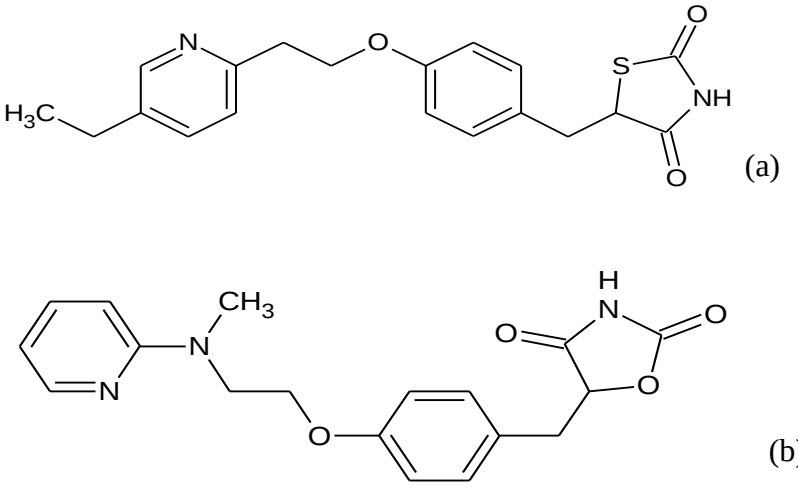


Figure 2.7: Chemical structures of Thiazoldinediones (a) Pioglitazone, (b) Rosiglitazone (Diapedia Collective, 2014).

2.10 Side effects of synthetic antidiabetic drugs

Reduction in weight and increase in body activity usually arise from the use of pharmaceutical drugs in treatment of hyperglycaemia ultimately improve insulin resistance (seldom reaches normality) However, such drugs therapy may be associated with long term underlying and undesirable effects such has hypoglycaemia or weight gain and will ultimately require multiple anti-diabetic agents to maintain adequate glycaemic control (Gerich, 2001; Alberti *et al*., 2007).

Antidiabetic drugs have been found to possess side effects. For instance sulfonylureas reduce blood glucose level for a short term in patients with Type II diabetes. Additionally, they have not been reported to have much benefit for long term complication of the disease since they are associated with the weight gain that might lead to hypertension (Hanefeld, 1998). Metformin has been reported to have a number of undesired side effects such as heart failure, hepatic and renal impairment, and anorexic effect (Hanefeld, 1998). The most frequent toxic effects of metformin are gastrointestinal and risk of lactic acidosis (Nolte and Karam, 2001). The problem associated with undesired side effects posed by drugs had led to several studies using medicinal plants as alternative sources for drug development.

2.11 Traditional medicinal plants and use

Plants form the backbone of all life on earth and they play an important role in the ecosystem. Plants provide food, regulate water cycle, consume carbon dioxide and make oxygen available for other organisms. They also provide habitat for variety of animals and most importantly they are source of medicine (Salisbury and Ross, 1992). Medicinal plants can be defined as any plant that has the ability to prevent, relieve or cure disease or alter the physiological and pathological process; or any plant that is employed as a source of drugs or their precursors (Arias, 1999).

Traditional plants have been in use ever since prehistoric times and are still in use till date worldwide for the treatment, control, and management of a variety of ailments (Philipeon, 2001). Currently in African Traditional Systems (ATS) of medicines, traditional healers and herbalists continue to use herbal remedies for the cure of various ailments even when

there are opposing views from orthodox medical practitioners (Sofowora, 1993; Okigbo and Mmeka, 2006). It is been estimated by WHO (2013) that more than 80% of the world's population is still depending on traditional medicine for primary health care.

In Africa, the use of remedies derived from plants in the treatment of disease by traditional healers is widespread and has been used for years even before the introduction of antibiotics and other modern drugs (Kabir *et al.*, 2005; Rukangira, 2013). According to Schmelzer and Gurib-Fakim (2008), traditional medicine has been used from prehistoric and ancient times and still continues to be used since they are the most affordable and accessible health care systems. Most importantly, they contribute to the rural livelihoods of the people and social equilibrium in Africa. In South Africa, the western and traditional systems of medicines exist; the former dating back only 300 years and the latter possibly to Palaeolithic times (van Wyk *et al*., 1997).

2.11.1 Traditional medicine in South Africa

South Africa has the richest temperate flora and encompasses a rich floristic diversity. There are approximately about 24 000 taxa of 368 families including more than 10% of the world's vascular plant flora on less than 2.5% of the Earth's land surface (Germisthuizen and Meyer, 2003). About 30 000 of flowering plant species have been implicated with a strong history of traditional healing (Louw *et al.*, 2002) and about 3 000 plant species are used as medicine and of these, some 350 species are the most commonly used and traded as medicinal plants in South Africa (van Wyk and Wink, 2004).

In Southern Africa, studies on the use of plants for food and medicinal purposes are widely dispersed due to better understanding of plant diversity by local people (van Wyk *et al*., 1997; WHO 2002). In the report of van Wyk *et al.* (1997), South Africa has a huge diversity of tribes which is reflected in the systems of medicine practised. Traditional healers are commonly known as "inyanga" and "sangoma" Zulu, "ixhwele" and "amagqirha" Xhosa, "ngaka" Sotho, "bossiedokter" and "kruiedokter" in the Western and Northern Cape. Moffet (1997) reported that majority of local Free State communities are dominated by Sesotho speaking tribe, and mainly found in the higher altitude sandy grassland biome of Free State. They mainly use grass, sedge and herbs for cultural and traditional medicinal practices.

The correct scientific documentation of the use of medicinal plants has been poorly recorded since the information was passed on orally among traditional health practitioners for many generations in the African Region (van Wyk *et al*., 1997; WHO, 2013). Collection and documentation of indigenous knowledge is very important and it is required to prevent loss of important information that will be useful for future generations in the use of medicinal plants (van Wyk and Gericke, 2000). Africa is currently lagging behind with validation of some medicinal plants uses and also documentation of their biological activity (WHO, 2002). The documentation of indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources (Muthu *et al.,* 2006).

2.11.2 Medicinal plants with confirmed antidiabetic activities.

Interest in medicinal plant research has increased over the past 10 years or more and over 1 000 plant species have been used for the treatment of Type II diabetes mellitus worldwide (Trojan-Rodrigues *et al*., 2011). Metformin, a drug developed from plant *Galega officinalis* L (Nolte and Karam, 2001), has been used since ancient times in Europe for treating symptoms associated with Type II diabetes mellitus (Whitters, 2001). The potency of this plant is associated with its guanide compound, galegine which is well established due to its hypo-glycaemic and insulin-sensitizing activity (Coman *et al*., 2012).

There are several reports on the importance of medicinal plants in South Africa and their use in treating diabetes mellitus. For instance, *Sutherlandia frutescens* is one of the endemic plants in South Africa and the isolated bioactive chemical compound from the seeds of this plant were found to have hypoglycaemic effects, they are L-canavanine, a non-protein amino acid and pinitol (van Wyk *et al*., 2005*).* Cinnachrome contains the active ingredient cinnulin, which was produced from cinnamon bark and another active compound referred to as methyl-hydroxy-chalcone polymer that regulates blood sugar level (Holford, 2009). *Pteronia divaricata* is used traditionally in South Africa for treating diabetes mellitus and the extract of this plant have been reported to inhibit α-glucosidase and α-amylase enzymes *in-vitro* (Deutschländer *et al*., 2009). Several studies have investigated plants with antidiabetic potential, and active compounds were isolated

from a number of these plants. These include *Urtica dioica*, *Allium sativum*, *Ficus exasperate*, *Aloe vera*, *Cinnamomum tamala*, *Coccinia indica* and *Gymnema sylvestre* (Grover *et al*., 2002; Gbolade, 2009).

According to the study done in Thailand by Wongsa *et al*. (2012), a number of culinary herbs was tested for total phenolic content, antioxidant activity and inhibition assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-amylase and α-glucosidase as potential inhibition activity. The study found out that *Solanum xanthocarpum*, *Ocimum sanctum*, and *Acacia pennata* could be used in the dietary management of Type II diabetes mellitus due to their high contents of polyphenolics, caffeic acid, and p-coumaric acid. *A. pennata* had the strongest effect on α-amylase inhibition. The presence of caffeic acid in *A. pennata* correlated with the strongest effect on α-amylase while α-glucosidase inhibition was linked to the total phenolic content.

The isolated pure compounds from plants are more powerful and easier to prescribe and administer in therapeutics (Saxena *et al*., 2013). The active ingredients from plants may act directly or indirectly in the prevention or treatment of diseases and are used inmaintaining health by stimulating immune system or by preventing diseases (Gurib-Fakim, 2006).

2.12 What are phytochemicals

The medicinal plants are less toxic with minimal side effects and are believed to be safer than the synthetic drugs. Plants produce vast and diverse organic compounds. These naturally occurring chemical compounds have health benefits to humans in managing and preventing diseases. The non-nutrient plant chemical compounds or the bioactive components are normally referred to as phytochemicals (*Phyto* from Greek meaning plant) (Saxena *et al*., 2013). Phytochemicals protects plants from disease and damage either caused by other plants, herbivores or insects or contributes to the plant fragrance, flavour and colour (Saxena *et al*., 2013).

Phytochemicals can be classified into two groups according to their functions and properties, namely primary and secondary metabolites. Primary metabolites play an important role in metabolism and reproduction of cells and they are vital for the survival

of the plant. These metabolites include carbohydrates, lipids, proteins, nucleotides, fatty acids and steroids (Croteau *et al*., 2000; Hanson, 2003). Secondary metabolites are unevenly distributed and their main functions include defence against herbivorus animals, chemical communication, and assistance during interactions and reproduction (Salisbury and Ross, 1992).

2.13 Role and classification of secondary metabolites

Secondary metabolites are organic compounds that are not involved in the normal growth and development of an organism. The production of secondary metabolites normally is from the maximum level during the transition from active growth to stationary phase. The organism that produces this secondary metabolites normally grows in the absence of their synthesis, thus the secondary metabolism in the organism may not be important yet is vital for short survival of the organism (Agostini-costa *et al*., 2012). There are several protective roles that these secondary metabolites provide. For example, they act as antioxidants, free radical scavenging, UV-light absorbing, and anti-proliferation agents. They also protect plants and defend them from harmful pathogens, microorganisms such as bacteria, fungi and viruses (Kennedy and Wightman, 2011). This was supported by the fact that some plants synthesise these chemicals as part of their defence system. For instance, phytoalxenis are produced by plants when they are attacked by bacteria and fungi ence their antibacterial and antifungal properties (Gurib-Fakim, 2006).

There are different classes of secondary metabolites present in plants; they include phenolics, flavonoids, steroids, terpenoids and alkaloids. In different categories in which the secondary metabolites belong to share a distinct structure, the derivatives are made up of structural units or some composed of complex molecules that are compiled by large numbers of simple molecules (Hopkins and Hüner, 2009). The following section briefly discusses some of the classes known and the isolated compounds from this study.

2.13.1 Phenolics

Phenolics are known to be the largest category of phytochemicals and are ubiquitously found across the plant kingdom with about 10 000 structures being identified (Agostini-costa *et al*., 2012). Some of the phenolics are soluble in organic solvents and water which

are known as carboxylic acid and glycosides; while others are large, insoluble polymers (Castillo *et al*., 2012). Phenolics structures vary greatly from simple low molecular weight compounds such as the simple phenols, phenylpropanoids, coumarins, catechols and benzoic derivatives to the most complex higher molecular weight structures such as catechol melanins, lignins, tannins, flavonoids, stilbenes and vitamins (Kennedy and Wightman, 2011; Agostini-costa *et al*., 2012). The phenolic phytochemicals play an important role as defence compounds; exhibit numerous properties that are beneficial to humans. Their antioxidant properties are one of the important properties that determine their roles as protecting agents against free radical mediated disease processes (Saxena *et al*., 2013).

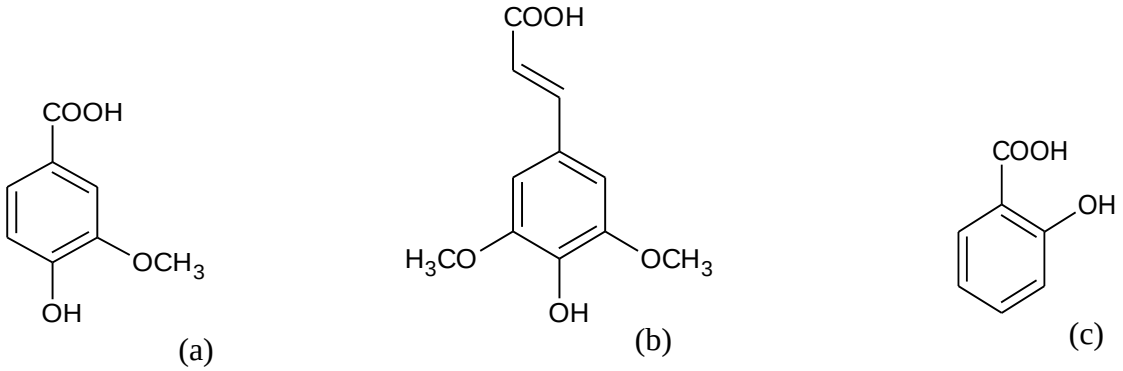


Figure 2.8: Examples of importantly naturally occurring phenolics, (a) phenolic acid, (b) salycylic acid and (c) caffeic acid chemical structures (PubChem, 2016)

2.13.2 Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous among vascular plants. The abundancy of this group has led to more than 4 000 which have been described so far within the part of the plants normally consumed by humans and approximately 650 flavones and 1030 flavonols are known (Harbone and Baxter, 1999). They occur as aglycones, glycosides and methylated derivatives. The six-membered ring condensed with the benzene ring is either -pyrone (flavones and flavonols) or its dihydroderivative (flavonones and flavan-3-ols) (Figure 2.9) (Saxena *et al*., 2013). Flavonoids are responsible for colour of flowers, fruits and sometimes the leaves and play important role in protecting the plants against harmful UV light damaging effects and in pollination and seed dispersal by attracting insects. This class has been subject of considerable scientific and therapeutic interest since they play important role in physiological and dietary

antioxidants, thus by augmenting the body's natural resistance to oxidative damage (Shahidi, 2000).

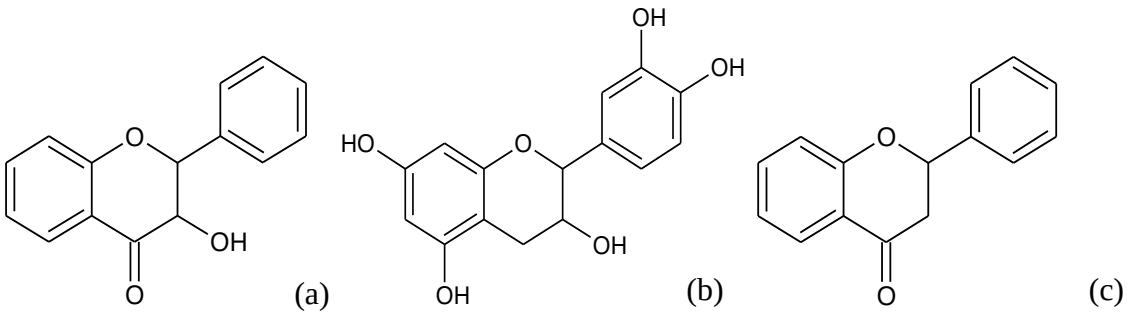


Figure 2.9: Examples of classes of flavonoids. (a) flavanols, (b) flavanols and (c) flavanone structures (PubChem, 2016)

2.13.3 Terpenoids

More than 36 000 terpenoids compounds have been identified, making terpenoids one of the largest classes of plant metabolites. They are a class of natural products which have been derived from five- carbon isoprene units (Bruneton, 1999). They are synthesised and released by plants. Terpenoids comprising one unit are classified as a hemiterpene, those incorporating two isoprene units are monoterpenes, sequiterpenes incorporate three, diterpenes comprise four, sesterpenests includes five, triterpene incorporate six and tetraperpenes eight units (Gurib-Fakim, 2006). The term terpene usually refers to a hydrocarbon molecule while terpenoid refer to a terpene that has been modified such as addition of oxygen. Therefore, the isoprenes are the building blocks of other metabolites such as plant hormones, carotenoids, sterols, rubber, the phytol tail of chlorophyll, and turpentine (Zwenger and Basu, 2008). Most of the terpenoids have cyclic structures. The cyclizations of most of the terpenoid take place in the living systems and they are of an acid catalysed type (Hanson, 2003).

According to Chang *et al*. (2011), more than 80 different triterpenoid structures have been isolated and identified from plants. The triterpenoids are important since they are used as preventive medicines and also are good source of food. Terpernoids compounds that have been isolated from plants and are available for pharmaceuticals application is artemisinin medicine for malaria and also taxol for cancer (Goto *et al*., 2010). Both are useful in prevention of diseases and plays important role in chemotherapy. Terpenoids have been

found to be useful in cancer treatment, have antimicrobial properties such as in antimicrobial, antifungal, antiviral, antiparasitic, antipasmodic, anti-allergic, anti-inflammatory, antihyperglycemic, and immunomodulatory properties (Wagner and Elmadfa, 2003; Shah *et al*., 2009). They are found in vegetables, fruit, and dietary terpenoids may contribute to a decrease in risk of metabolic syndrome.

2.13.4 Alkaloids

Alkaloids are structurally diverse group of over 12 000 cyclic nitrogen-containing compounds that are found in over 20% of plant species (Aniszewski, 2007). They have bitter taste and appear as white; they form water soluble-salt and most of them are well defined crystalline substances which unites with acid to form salts. Nicotine is the only one that has brown liquid (Aniszewski, 2007). They are known as true alkaloids and are highly reactive. Even at low doses, they still possess biological activity. As stated by Woolley (2001), alkaloids have many pharmacological activities and they have often provided lead in the search for new synthetic drugs such as oral hyperglycemic agents (calystegines) (Figure 2.10), antibacterial (berberine), antimalarial (quinine, alstonine) (Figure 2.10), antihypertensive (cevadine, veratrine, reserpine, serpentine, rubijervine) (Figure 2.10) and anticancer actions (camptothecine, demecolcine (desacetylmethylcolchicine), ellipticine, indicine N-oxide, maytansine, pacletaxel, vincristine, vinblastine).

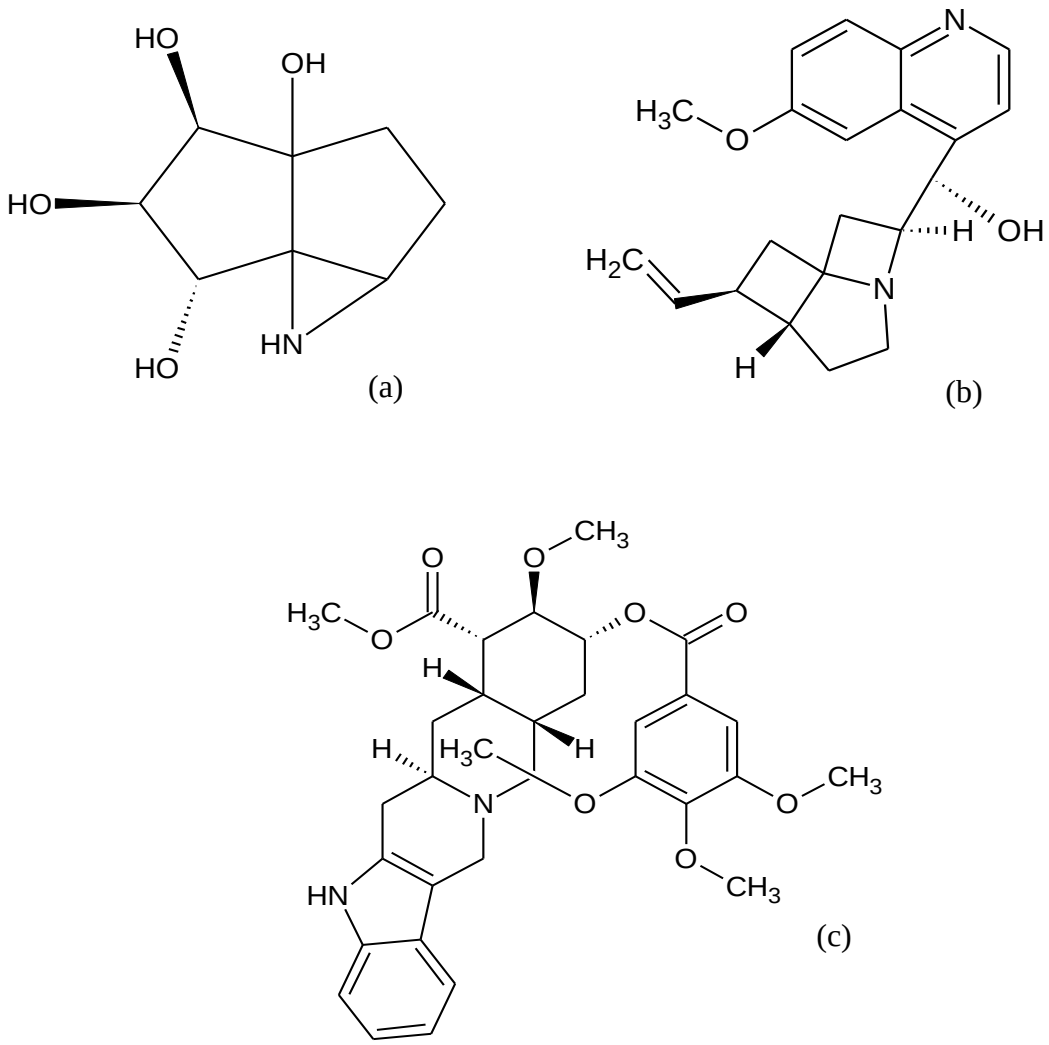


Figure 2.10: Examples of synthetic alkaloid drugs structures (a) calystegines, (b) quinine and (c) reserpine (PubChem, 2016)

2.13.5 Saponins

Saponins are recognized by their ability to produce a soapy lather when shaken with water and they also have the ability of precipitating cholesterol by forming insoluble complexes. They are classified chemically in to two groups, steroidal saponins and triterpenoid saponins on the basis of the chemical structures of their aglycones or saponegins (Shibata, 1977). Steroidal saponins are widely distributed in nature and exhibit various biological activities. They are found to possess properties such as haemolytic activity, toxicity to fish and also form complex formation with cholesterol. They have been found to have antidiabetic, antitumor and antitussive properties (Mimaki

and Sashida, 1996). Triterpenoid saponins (Figure 2.11) are naturally occurring surface active glycosides of triterpenes. They can be divided into two major groups, monodesmosides, in which the aglycone has a singly attached linear or branched chain set of sugars and bisdesmosides in which there are two sets of sugars.

Saponins are one of the great molecules that are structurally diverse. They occur as complex mixtures and have novel bioactivities of relevance to the pharmaceutical industry and agriculture. Their usefulness as ingredients of cosmetics, allelochemicals, food and feeding stuff has generated great interest in the study of these molecules (Guclu-ustundag and Mazza, 2007). Saponins possess a variety of biological activities such as antioxidant, immunostimulant, antihepatotoxic, anticarcinogenic, antidiarrheal, antiulcerogenic, antioxytoxic, hypocholesterolemic, anticoagulant, anti-insect, hepatoprotective, hypoglycemic, neuroprotective, antiinflammatory, haemolytic and inhibition of dental caries and platelet aggregation (Guclu-ustundag and Mazza, 2007; Barbosa, 2014).

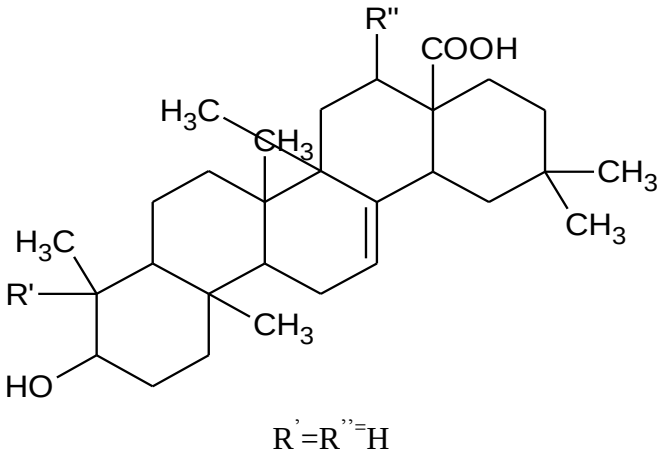


Figure 2.11: Tritepenoid saponin types (PubChem, 2016)

2.14 The Rubiaceae Family

Rubiaceae represent one of the five most rich flowering plant families which rank the fourth in the diversity of the species in Angiosperms (Martins and Nunez, 2015). The family is divided into four subfamilies; the Rubioideae, Chinchonoideae, Antirheoideae and Ixoroideae (Robbrecht, 1988). With recent taxonomy studies, the family is now divided into three subfamilies, which are Rubioideae, Chinchonoidea and Ixoroideae. The

family is known to contain 43 tribes, 637 genera and 13 000 species (Goevarts *et al*., 2006; Martins and Nunez, 2015). They occur on all continents, on the Antarctic continent, tropical or subtropical areas. This family covers the small weedy herbs, trees and shrubs much less perennial to annual herbs of which some are in the tribe Rubieae subfamily, Rubioideae and is broadly distributed in temperate regions (Goevarts *et al*., 2006; Ndlovu, 2007). The flowers in this family are adapted to a wide range of pollinators, the different types of fruits with so many kinds of dispersal mechanism and the plants have different chemical substances that have accumulated in them (Bremer and Eriksson, 2009).

The general morphology of plants in this family includes the leaves which are whorled or opposites with interpetiolar stipules which are sometimes leaf like. The flowers have four or five petals that are united to form a tube. The fruits are usually capsules, drupes and berries (Germshuizen and Clarke, 2003)

2.15 Phytochemicals from some species in Rubiaceae

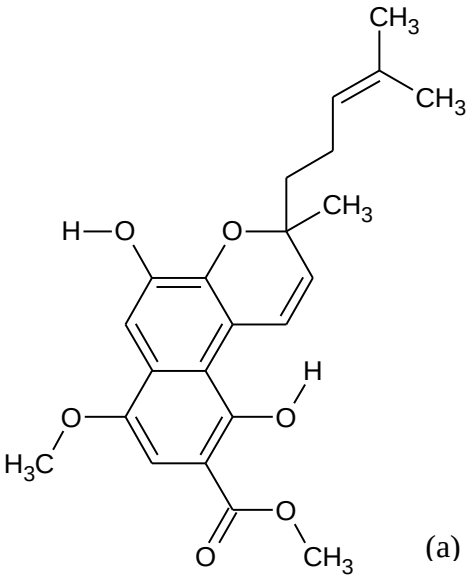
The Rubiaceae family is very large but some plants from this family have not been extensively studied as compared to other families. Recently, the rubiaceae have become the focus of detailed studies (Ndlovu, 2009). However, despite the extensive research on some genus such as *Cinchona*, *Gallium* and *Pentas* not much research has been done on the genus *Pentanisia*, especially the isolation of bioactive compounds for management of chronic disease, agroagriculture, cancer, arthritis and diabetes mellitus.

In the family of Rubiaceae, few alkaloids types have been successfully isolated and found to have pharmacological activity. Genus *Cephaelis* rhizome and *Psychotria* roots were found to contain emetine compound which was responsible for curing amoebic dysentery. Quinine and quinidine were isolated from the *Cinchona* bark and found to possess antimalarial and antiarrhythmia activities respectively (Woolley, 2001).

Verdcourt and Bridson (1991) had stated that some *Pentanisia* are so similar to *Pentas* that only an examination of the ovary will separate them. The genus *Pentas* comprises about 40 species, widely distributed throughout tropical Africa from West Africa and

Somali Republic to Angola and Natal (South Africa), also in tropical Arabia, Madagascar and Comoro Islands (Dessein *et al*., 2000). Phytochemical investigations on members of *Pentas* and *Pentanisia* are sparse. Among so many species of genus *Pentas*, some have been successfully studied and secondary metabolites have been isolated. These include *P. bussei*, *P. longiflora* and *P. lanceolata*.

In Kenya, a decoction of the roots of *Pentas bussei* is taken as a remedy for gonorrhoea, syphilis and dysentery. *P. bussei*, which is commonly used for treatment of malaria in Africa and Asia was found to contain Pentacyclic cyclol-type naphthohydroquinone: eriobrucinol; methyl5,10-dihydroxy-7-nmethoxy-1,1,3α-trimethyl-1a,2,3,3a,10c,10d-hexahydro-1*H*-4-oxacyclobuta[cd] indeno[5,6*a*]naphthalene-9-carboxylate (Bukuru, 2003). Benzochromene was also isolated by Bukuru *et al*. (2002), methyl-5,10-dihydroxy-7-methoxy-3-methyl-3-[4-methyl-3-pentenyl]-3*H*-benzo[*f*]chromene-9-carboxylate (Figure 2.12)



(a)

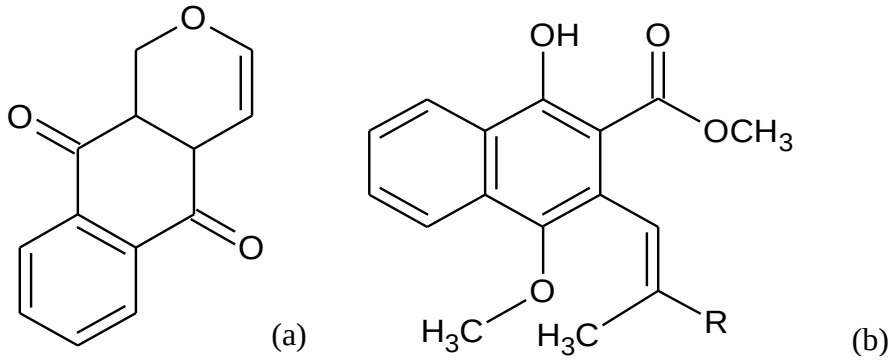
(b)

Figure 2.12: (a) Benzochrome structure (PubChem, 2016) and (b) *Pentas bussei* (http://stewartia.net/engei/engei/Akane_ka/Pentas.html)

According to Endale *et al*. (2012), chromatographic separation of the extract of *Pentas longiflora* (Figure 2.13) led to the isolation of the pyranonaphthoquinones pentalongin (Figure 2.14a) and psychorubrin with IC_{50} values below 1 µg/mL and the naphthalene

derivative mollugin (Figure 2.14b). Pentalongin exhibited antifungal activity against *Pityrosporum ovale*, and algicidal activity against freshwater green algae *Chemydomonas sphagmophilla* var. dysosmos, *Chlorella vulgaris*, and marine algae *Phaeodactylum tricornutum* and *Porphyridium purpureum* (Bukuru, 2003). The compound also showed antibacterial activity against freshwater blue-green bacterium *Anabaena cylindrica* (El-Hady, 1999). A pentalongin hydroquinone diglycoside, harounoside (Harouna *et al*., 1995), has been isolated from *Mitracarpus scaber* Zucc (Rubiaceae), a plant used in African traditional medicine (Niger) for antifungal and antiparasitic activity (Adjanohoun *et al*., 1985).

Figure 2.13: *Pentas longiflora* (https://www.flickr.com/photos/svkett/8470380901/sizes/l)



R- H; 3-Hydroxymollugin; 3-Methoxymollugin

Figure 2.14: Chemical structures (a) Pentalongin (b) and Mollugin (PubChem, 2016)

Bukuru (2003) investigated the roots of *P. lanceolate* (Figure 2.15) and isolated the following compounds stigmasterol and the anthraquinones damnacanthol, rubiadin-1-

methyl ether, rubiadin, 1,3-dihydroxy-2-methoxymethyl- 9,10-anthraquinone (lucidin-*ω*-methyl ether), damnacanthol- 3-*O*-methyl ether (Figure 2.16b), and rubiadin-1-methyl ether-3-*O*-primeveroside. Schripsema *et al*. (2007) isolated a series of iridoid glucosides from the roots of *P. lanceolate.* Seven of these iridoid glucosides were identified besides asperuloside (Figure 2.16) and asperulosidic acid five new iridoids were isolated, tudoside, 13*R*-*epi*-gaertneroside, 13*R*-*epi*epoxygaertneroside, and a mixture of *E*-uenfoside and *Z*-uenfoside.

Figure 2.15: *Pentas lanceolate* (https://www.flickr.com/photos/svkett/8470380901/sizes/l)

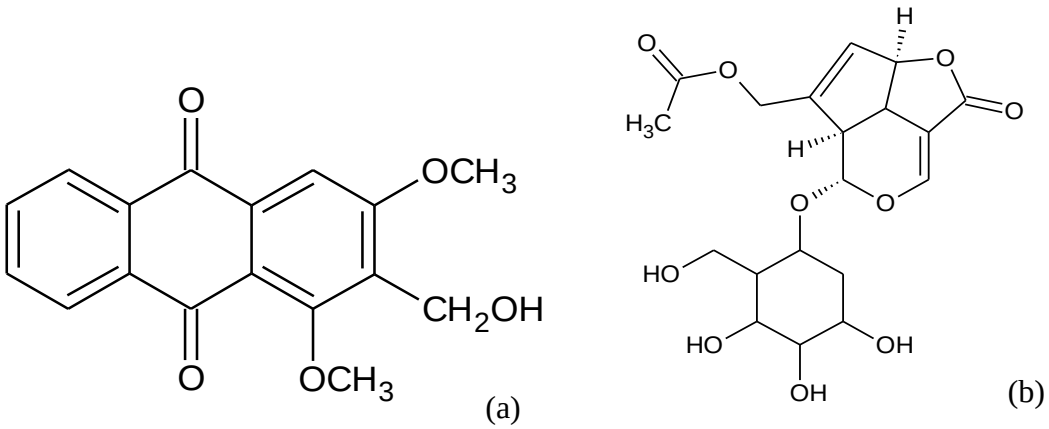


Figure 2.16: Chemical structures of isolated compounds (a) Damnacanthol-3-O-methyl ether (b) and speruloside. (PubChem,2016)

The plant under investigation is *Pentanisia prunelloides* and it belongs to family Rubiaceae

2.16 *Pentanisia prunelloides*

The genus *Pentanisia* is made up of 20 species worldwide which are found in tropical Africa and with three occurring in Southern Africa (The Plant List, 2013). This species include *P. arenaria* (Hiern) Verdc, *P. annua* K. Schu, *P. monticola* (K. Krause), *P. veronicoides* (Baker) K.Schum, *P. foetida* Verdc, *P. rubricaulis* (K. Schum.) Karehed & B. Bremer, *P. sykesii* Hutch, *P. logipendunculata* Verdc., *P. gossweiler* (Verdc.) Karehed & Bremer, *P. calcicola* Verdc, *P. confertifolia* (Baker) Verdc, *P. longituba* (Franch.) Oliv, *P. microphylla* (Franch.) Chiov, *P. procumbens* R.D.Good, *P*. renifolia Verdc, *P. ouranogyne* S. Moore, *P. prunelloides* subsp. latifolia (Hochst.) Verdc. The three species that are found in South Africa are *P. prunelloides* (Klotzsch) Walp, *P. schweinfurthii* Hiern and *P. anguistifolia* (Hochst.) Hochst (The Plant List, 2013). *P. prunelloides* and *P. angustifolia* are clonal perennials, which inhabit grasslands in South Africa and southern Mozambique (Launert, 1989).

Pentanisia prunelloides is a perennial herb belonging to Rubiaceae commonly called wild verbena (English), *setimamollo* (Sotho) translated as fire extinguisher, *icimamlilo* (Zulu) putting out the fire and *sooibrandbossie* (Afrikaans) little heartburn bush (van Wyk *et al*, 2009; Moteete and van Wyk, 2011).

2.16.1 Plant description

Pentanisia prunelloides is an erect perennial plant that grows to about 30 cm height. It is characterised by its stout hairy stems (Figure 2.17) emerging from red tuberous root and have hairy leaves which are oblong shaped and borne in pairs. The plant produces flowers that are pale purple in colour during summer and are densely grouped in ends of the branch (van Wyk *et al.*, 2005).

Figure 2.17: General morphology of *P. prunelloides* showing roots, flowers and leaves

2.16.2 Plant distribution

The species is widely distributed in the eastern parts of South Africa and its area of distribution accords well in the union as would be expected from the pyrophytic habit of the plant (Verdcourt, 1958) (Figure 2.18).

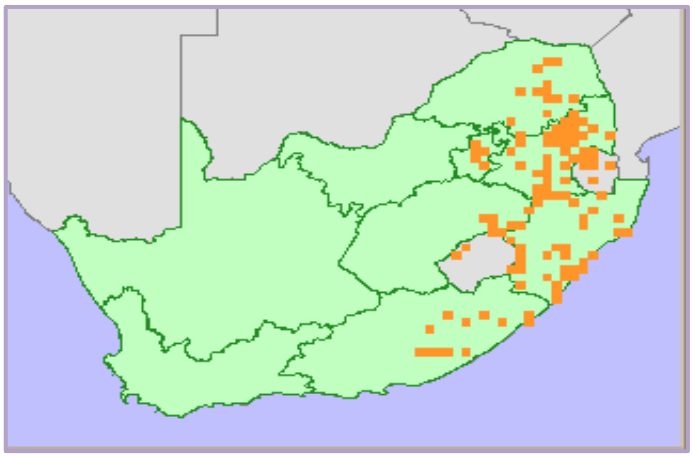


Figure 2.18: Map indicating distribution of *P. prunelloides* in the eastern part of South Africa (Google map, 2016)

2.16.3 Medicinal uses of *Pentanisia prunelloides*

The roots and leaves of this plant are mostly used for treating variety of ailments such as heartburn, fever, tuberculosis, blood impurities, haemorrhoids, toothache, chest pains, sore joints, swellings and snake bites (Hutchings *et al*., 1996, Neuwinger, 2000). The plant has been reported to be effective in pregnancy, for easy child birth and the leaf for retained placenta (van Wyk *et al*., 2000), leaves may also be used for treating diarrhoea (Madikizela *et al*., 2012). Several studies have established that boiled grated dried bulb taken orally stops vomiting (Bisi-Johnson *et al*., 2009), the roots are used for managing gynaecological complaint such dysmenorrhoea (Steenkamp, 2003); for swelling of the stomach the decoction of bruised and boiled root are mixed with sour milk and taken orally (Smith, 1985) and the root extract for treating aches and pains (Kaidoo *et al*., 1997).

2.16.4 Pharmacological activities attributed to *P. prunelloides* extracts

Several studies have been conducted to evaluate the pharmacological effect of *P. prunelloides*. Extracts from these plants have shown great antibacterial activity against *Bacillius subtilis*, *Staphylococcus aureus*, *Escherichia coli*, K*lebsiella pneumoniae* and this has led to isolation of major non polar antibacterial compound, palmitic acid (Figure 2.19) (Yff *et al*., 2002). Madikizela *et al* (2013) also reported the antibacterial activity of some *P. prunelloids* extracts, against *Klebsiella pneumoniae, Mycobacterium aurum* A+ and *Staphylococcus aureus*. The plant was also found to possess anti-inflammatory activity, antiviral and antioxidant activity by inhibiting cyclooxygenase -1 (COX-1), inhibiting viral replication of the Influenza A virus (Yff *et al*., 2002; Muleya *et al.*, 2015) and scavenging the DPPH radicals through hydrogen transferring reactions which provides the strong evidence of their ability to intercept reactive oxygen species (ROS) (Mpofu *et al*., 2014).

2.16.5 Reported phytochemistry on *P. prunelloides*

Palmitic acid (Figure 2.19) is one of the most abundant saturated fatty acid within plants, humans, animals, microorganisms which constitutes about 16 to 45% of the lipid profile (Porto, 2014). According to Mancini *et al*. (2015), palmitic acid can be synthesized

endogenously through *de-novo* lipogenesis, the metabolic pathway stimulated by increased intake of carbohydrates and alcohol. It is considered that flowing palmitic can stimulate insulin resistance by decreasing phosphorylation of the insulin receptor and insulin receptor substrate-1. In muscle cells, it decrease oxidation of fatty acids and glucose which elevates fatty acid and glucose levels in tissues and blood, and decrease adiponectin production, which may both promote insulin resistance (Porto, 2014). As demonstrated by Yang *et al*. (2013), *in vitro* studies revealed that palmitic acid directly impairs insulin signaling in cultured rat hepatocytes and myotubes as well as pancreatic cells vitality and insulin secretion.

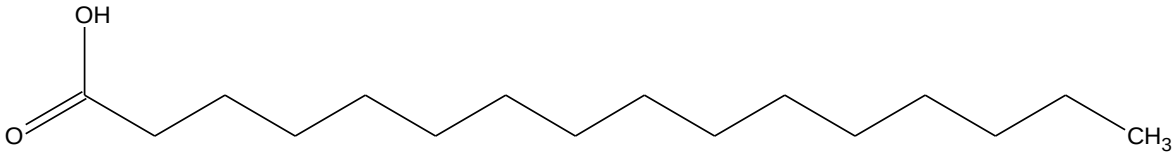


Figure 2.19: Structure of Palmitic acid (PubChem, 2016).

Mpofu *et al.* (2014) conducted the phytochemical studies on the rhizome of *P. prunelloides* and find the plant to contain alkaloids, saponins, anthraquinones, tannins, flavonoids and anthocyanidins when doing quantitative screening and it was the first report to detect and isolate diosgenin (Figure 2.20a) (steroidal saponin) and oleanolic acid (Figure 2.20b). Diosgenin provide several health benefits to human, as anti-cancer where the effects of diosgenin *in vitro* is through different mechanisms (Raju and Mehta, 2009), anti-HIV (Wang *et al*., 2011) and have antidiabetic properties (McAnuff *et al*., 2005). The antidiabetic effect of diosgenin in experimental models has shown to decrease plasma glucose in streptozotocin-induced diabetic rats by comparison to the diabetic controls suggesting its anti-diabetic properties (McAnuff *et al*., 2005).

Oleanolic acid is a triterpenoid compound that exists widely in food, medicinal herbs and other plants. This compound exists widely in natural plants in the form of free acid or aglycones for triterpenoid saponins (Wang and Jiang, 1992). According to the review conducted by Liu (1995), there are several studies conducted on this compound and it was found to be non-toxic and has been used in cosmetics and health products. Also the compound was found to provide good pharmacological properties such as hepatoprotection, anti-inflammation, antitumor and antihyperlipidemia activities.

The roots of *P. prunelloides* were reported (Ndlovu, 2007) to contain high levels of alcohol precipitate solids (0.7-7.0%), and these include polysaccharides and glycoproteins, amino acids (α-aminobutyric acid, valine, allo-isoleucine, serine, aspartic acid, asparagine and alanine. Several unidentified terpenes of medium and low polarity together with surcose and (-)-epicatechin were also isolated.

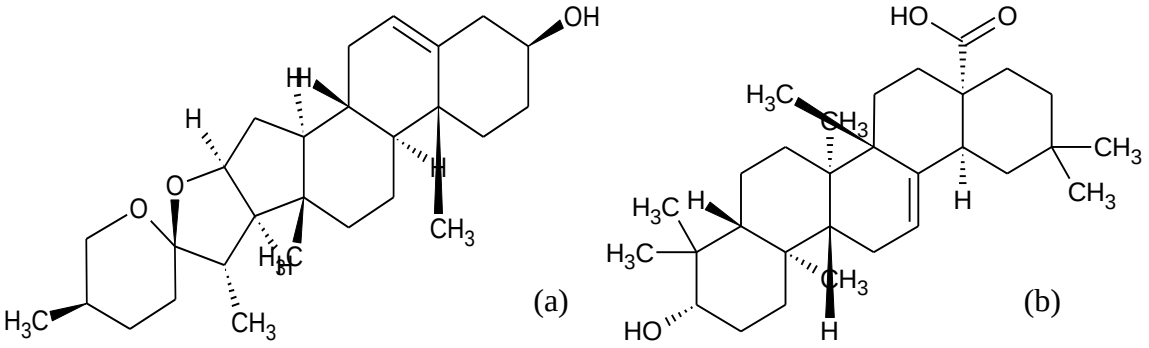


Figure 2.20: Chemical structures (a) Diosgenin, and (b) Oleanolic acid (PubChem, 2016)

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CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

Drug discovery from plants still continues to grow as a more valuable source of new drug leads (Spainhour, 2005). Louw *et al*. (2002) recorded about 30 000 flowering plants that have been implicated with a strong history of traditional healing in South Africa. About 3000 of these plant species are used as medicines and from these only 350 species are the most commonly used (van Wyk and Wink, 2004). This vast diversity poses a problem financially in that resources are wasted when trying to prepare assays of each individual plant or the isolation of bioactive components from the leaves, roots, flowers, twig and fruits whereas conducting an assay from a single part of the plant can be pursued (Colegate and Molyneux, 1993). According to Cordell *et al*. (1993), there are five systematic approaches for the selection of plants that might contain new biological agents; ethnopharmacology, chemotaxonomy, random, taxonomy and the information-managed approach.

Ethnopharmacology is the use of local materials for medicinal purposes by ethnic groups usually designated as those indigenous to a geographical area (Houghton, 1999). This approach uses the information obtained either orally or written which provides record for evaluation on the use of medicinal plants (Colegate and Molyneux, 1993). Chemotaxonomic approach is only restricted for a certain class of secondary metabolites that have been identified as possessing activity. For example, Amaryllidaceae alkaloids may be considered as being biologically active therefore plants thought likely to contain related compounds are collected (Colegate and Molyneux, 1993; Farnsworth, 1994). In random approach, all species are collected irrespective of the previous knowledge on the species for evaluation (Colegate and Molyneux, 1993). In taxonomic approach, plants of

families or genera considered to be of interest are sought from diverse locations (Colegate and Molyneux, 1993). Lastly, the information-managed approach involves the target collection of species which are selected by database surveillance as plants that are proven to have biologic activity. These are collected with the anticipation of discovering new chemicals entities (Colegate and Molyneux, 1993).

3.2 Systematic approach for plant selection

In this study, the ethnopharmacological approach was chosen because the local people had a better understanding of the use of plant diversity to sustain their living, by exploiting plant natural products for food and medicine purposes (van Wyk *et al*., 1997). The majority of local communities in the eastern Free State are dominated by Sesotho speaking tribes who are mostly found in the higher sandy grassland biome of the province. The use of medicinal plants for therapeutic purposes is still being practiced for primary health care. For example, *Artemisia afra* for treatments of cold (Moffett, 2010). The knowledge of the use of medicinal plants was obtained through consultations with the traditional healers who claimed it is being passed through ancestral calling.

For the purpose of this study, diabetes mellitus was investigated and found to be one of the common metabolic disorders that rural people in the Free State suffer from and have found a way of managing it using local medicinal plants.

3.3 Selection of candidate plant

The plant used for this study was selected based on the report of Tshabalala (2012) in area of Maluti-A-Phofung as advised by traditional healers. Herbalists were consulted to assist in providing information and collection of selected species of *Pentanisia prunelloides*. Through literature and assistance of the traditional healers, the part of the plant to be used was also defined.

3.4 Plant collection and identification

Pentanisia prunelloides was collected naturally from a multi population in the eastern part of Free State near Golden Gate at Beste Farm in October 2015. The location was

found at a mountainous area with the following coordinates: latitude 28º 23’ 55.60”S and longitude 28º 47’ 18.83” E (Figure 3.1). The plant was further authenticated by Dr. E. Sieben at the University of the Free State herbarium, in Qwaqwa campus. The voucher specimen (MakMed 03/ 2015/ QHB) was prepared and deposited at the university as well.

3.5 Extraction procedures

3.5.1 Preparation of plant extracts

The fresh root materials were separately rinsed under running water to remove debris and then dried in an Ecotherm oven (Laboratory Consumables Pty, RSA) at a temperature of 45 °C until constant weight was reached. The dried root material was ground using an electric blender (Nanning Mainline Food Machinery Company Ltd, China) to powdered form and weighed. Exactly 20 g of the powdered root material wasseparately extracted in four solvents of different polarities (200 mL) namely hexane, ethanol, aqueous-ethanol (1:1) and distilled water. The set up was placed on a Labcorn Platform shaker (Laboratory consumables, PTY, Durban, South Africa) at 115 rpm revolutions for 24 h. The extraction mixtures were filtered using a Whatman No.1 (Whatman, UK) filter paper to obtain a homogenous mixture. The filtrates of the hexane, ethanol and aqueous-ethanol solvents was concentrated using an oven at 37 °C until the solvent had totally evaporated. Water filtrates were placed in water bath at 45 °C until dried. The dried crude extracts were stored in a fridge at 4 ºC until use. Percentage yield of the extract is defined as the total dry mass after evaporation of the solvent and was calculated using the equation:

$$\%\, Yield = \frac{mass\ of\ dry\ extract\ (g)}{total\ mass\ of\ dry\ powdered\ sample\ (g)}\ X\ 100\%$$

3.5.2 Bulk extraction

Freshly dried roots of *P. prunelloides* (1000g) were extracted six times with absolute ethanol (1000 mL of 99% EtOH) for 48 h with occasional stirring using stirrer. The suspension was then filtered through cheese cloth and then concentrated using rotary evaporator (Cole-Parmer, South Africa). This was stored in an airtight container in the cold room at 4 °C until processing.

3.6 Collection site

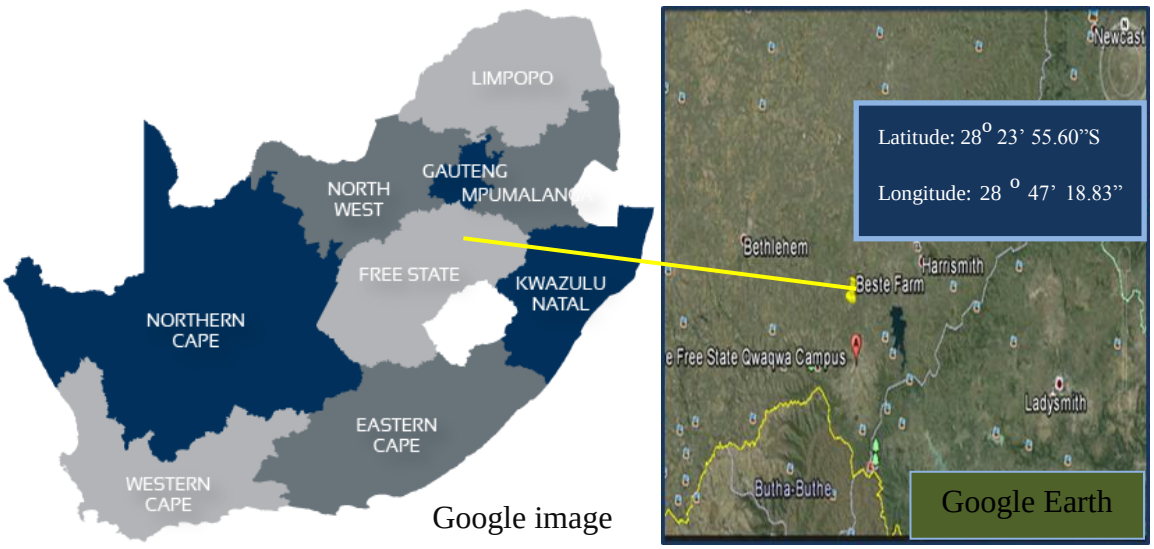

Figure 3.1: Map showing the collection site of *P. prunelloides* in the eastern Free State Province

Hyperglycemia, condition that is characterised by the rapid increase of glucose in blood is linked to the onset of diabetic complications in diabetic patients. Two enzymes, the α-amylase and α-glucosidase are found to be implicated by this pathological effect (Brownlee, 2005). The use of inhibitors of these digesting enzymes (α-amylase and α-glucosidase) offers a great solution to prevent Type II diabetes. Tihis will help to control plasma glucose by decreasing the supply of blood sugar from small intestine thus slowing and interrupting the digestion of the starch (Rabasa-Lhoret and Chiasson, 2004). There are synthetic drugs used for treatment of diabetes mellitus, but these drugs are reported to cause several side effects, such as cramping, abdominal distention, flatulence and diarrhoea (Fujisawa *et al*., 2005). Therefore, finding the natural inhibitors of these enzymes is the key strategy for controlling diabetes mellitus.

3.7 Antidiabetic Assays

The activity of crude extracts and standard (acarbose) was performed at various concentrations (6.25-100 µg/mL). The activity was evaluated using percentage inhibition and was also analysed using the IC_{50} values (concentration required for 50% inhibition of enzymatic activity) for each extract and acarbose to determine potency.

3.7.1 Alpha-glucosidase inhibitory activity

The effect of the plant extract on α-glucosidase activity was determined according to the method described by Adisakwattana *et al*. (2009) with modifications. The substrate solution *p*-nitrophenyl glucopyranoside (pNPG), sucrose and maltose were prepared in 0.02 M phosphate buffer (pH 6.9), 100 µL of glucosidase (E.C.2) was pre-incubated with 50 µL of the different concentrations (6.25-100 µg/mL) of the extracts (ethanol, aqueous-ethanol, hexane and water) for 10 min. Then 50 µL of 5 mM (pNPG), 25 mM maltose or 50 mM sucrose in in 0.02 M phosphate buffer (pH 6.9) to start the reaction. The reaction mixture was incubated at 37 ˚C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The enzyme activities were determined by measuring the yellow coloured para-nitrophenol released from pNPG absorbance at 405 nm (α-glucosidase) or 540 nm (maltase and sucrase) using micro plate titre reader (Model 680, BIO-RAD). The control was prepared using the same procedure replacing the extract with distilled water while activity of standard by replacing the extract with acarbose (6.25-100 µg/mL). Percentage inhibition was calculated, thus;

$$\%\ Inhibition = \left[\frac{(\Delta Acontrol - \Delta Asample\ test)}{\Delta Acontrol}\right] X\ 100$$

Where ΔAcontrol = Acontrol –Ablank and ΔAsample test = Asample test – Ablank
Sample test is either extract or compound. Concentrations of extracts and compounds resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically

3.7.2 Mode of α-glucosidase inhibition

The mode of inhibition of α-glucosidase by the extract and compound were determined using the extract and compound with the lowest IC_{50} according to the modified method described by Ali *et al*. (2006). Ethanol extract and tormentic acid were selected because they possessed the lowest IC_{50} for the inhibition of α-glucosidase as compared to the other extracts and compounds, i.e. the lower the IC_{50} values for the inhibition of an extract and compound towards an enzyme, the stronger the inhibition of the enzyme. Briefly, 50 µL of the (5 mg/mL) ethanol extract and tormentic acid was pre-incubated

with 100 µL of α-glucosidase solution for 10 min at 25 ˚C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50 µL of phosphate buffer (pH 6.9). 50 µL of PNPG at increasing concentrations (0.20 – 1.0 µg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25˚C and 500 µL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a para-nitrophenol standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition.

3.7.3 Alpha-amylase inhibitory activity

The assay was carried out using the modified method procedure of McCue and Shetty (2004). Briefly, a total of 50 µL extract was placed in a tube and 50 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was pre-incubated at 25 ˚C for 10 min, after which 50 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then further incubated at 25 ˚C for 10 min. The reaction was terminated by adding 200 µL of dinitrosalicylic acid (DNS) reagent. The tubes were then incubated in boiling water for 10 min and cooled at room temperature. The reaction mixture was diluted with 300 µL of distilled water and the absorbance was measured at 540 nm using micro plate titre reader (Model 680, BIO-RAD). A control was prepared using the same procedure replacing the sample test with distilled water. The α-amylase inhibitory activity was calculated as percentage inhibition, thus;

$$\%\, Inhibition = \left[\frac{(\Delta Acontrol - \Delta Asample\ test)}{\Delta Acontrol}\right] X\ 100$$

Where ΔAcontrol = Acontrol –Ablank and ΔAsample test = Asample test – Ablank
Sample test is either extract or compound. Concentrations of extracts and compounds resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically

3.7.4 Mode of α-amylase inhibition

The mode of inhibition of α-amylase by the plant extracts and compounds was conducted using the most potent extract (ethanol) and tormentic acid according to the modified method described by Ali *et al*., (2006). Ethanol extract was selected because although its IC_{50} for the inhibition of α-amylase was greater than that of acarbose it would be less likely to cause the side effects associated with the excessive inhibition of this enzyme, while tormentic acid was selected since its IC_{50} even though it was lower than acarbose but the inhibition was mild which is likely to cause side effects. Briefly, 250 µL of the (5 mg/mL) ethanol extract was pre-incubated with 250 µL of α-amylase solution for 10 min at 25˚C in one set of tubes. In another set of tubes, α-amylase was pre-incubated with 250 µL of phosphate buffer (pH 6.9). 250 µL of starch solution (1%) at increasing concentrations (0.20 – 1.0 µg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25˚C, and then boiled for 5 min after addition of 500 µL of DNS to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition.

3.8 Antioxidant assays

Antioxidants are substances when present in food or in the body at low concentrations compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substance (Halliwell and Gutteridge, 1981). Humans have evolved highly complex defense antioxidant strategy (enzymic and nonenzymic) which works synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage (Rahman, 2007). The antioxidants can either be endogenous (internally synthesized) or can be obtained exogenously (consumed) for example, can be part of a diet or as dietary supplements (Rahman, 2007; Asmat *et al*., 2015). Endogenous antioxidants play important role in maintaining cellular functions and complete well-being of an individual. However, under conditions which leads to production of oxidative stress, the endogenous antioxidants may not be enough and dietary antioxidants may be essential to maintain the optimal cellular functions (Rahman, 2007).

There are two components of endogenous antioxidant defense system, that is, antioxidant enzymes and low molecular weight antioxidants. The low molecular antioxidants includes the vitamins A, C and E, ascorbate, tocopherol, glutathione, ubiquinone and thioredoxin, and proteins, such as transferrin, ferritin, ceruloplasmin, lipoic acid, hemopexin, hepatoglobin and albumin that are capable of chelating metal ions (Acworth *et al*., 1997; Grune *et al*., 2005). Vitamins are very important part of the biological systems as they play an important part in the different biochemical processes. They act as antioxidants by detoxifying the free radicals.

Reactive Oxidative Species (ROS) such as the superoxide anion radical (O_2-) and hydroxyl radicals (OH^-) are physiological metabolites, their excessive levels in human body have been linked to the onset of diseases such as cancer, stroke and diabetes (Niedowicz and Daleke, 2005). A natural or dietary component that can either scavenge ROS to stop radical chain reaction or that can inhibit the oxidants from being formed in the first place is considered as the most active which will help in combating the widespread nature of diabetes mellitus.

In this study, ascorbic acid and gallic acid were used as standard antioxidants. While ascorbic acid was used for the DPPH radical scavenging assay, the gallic acid was used for the other assays (superoxide anion radical, hydroxyl radical scavenging and metal ion chelation).

3.8.1 DPPH free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated by a modified method of Saha *et al*. (2008). Different concentrations (6.25-100 µg/mL) of the extracts (150 µL) were mixed with 150 µL of 0.4 mmol/L methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability of each extract was subsequently calculated with respect to the reference (which contains all the reagents without the test sample). DPPH free radical scavenging ability of a standard antioxidant was also tested by replacing the extract with ascorbic acid (6.25-100 µg/mL). The capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ Scavenging\ effect\ (\%) = [(\frac{Ao-As}{Ao}) * 100]$$

Where Ao and As is the absorbance of control and sample respectively

3.8.2 Superoxide anion radical scavenging ability

Measurement of superoxide anion scavenging activity of the various extracts was based on the method described by Liu *et al*. (1997). Superoxide radicals were generated in 50 µL of Tris-HCl buffer (16 mM, pH 8.0) containing 50 µL of NBT (50 mM) solution, 50 µL NADH (78 mM) solution and different concentrations (6.25-100 µg/mL) of *P .prunelloides* extracts (100 µL). The reaction started by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture. The reaction mixture was incubated at 25˚C for 5 min, and the absorbance measured at 560 nm. Superoxide anion radical scavenging ability of a standard antioxidant was also tested by replacing the extract with gallic acid (6.25-100 µg/mL). The capability to scavenge the superoxide anion radical was calculated using the following equation:

$$Superoxide\ Scavenging\ effect\ (\%) = [(\frac{Ao-As}{Ao}) * 100]$$

Where Ao and As is the absorbance of control and sample respectively

3.8.3 Hydroxyl radical scavenging ability

The ability of the root extracts of *P. prunelloides* to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the modified method described by Oboh and Rocha (2006). Briefly, 40 µL freshly prepared extracts (6.25-100 µg/mL) was added to a reaction mixture containing 20 µL 20 mM deoxyribose, 80 µL 0.1 M phosphate buffer, 10 µL 20 mM hydrogen peroxide and 10 µL 500 mM FeSO4, and the volume was made up to 200 µL with distilled water. The reaction mixture was incubated at 37 ˚C for 30 min, and the reaction was stopped by the addition of 50 µL of 2.8% TCA (trichloroacetic acid), this was followed by the addition of 50 µL of 0.6% TBA solution. The mixtures were subsequently incubated for 20 min and the absorbance was measured at 532 nm in a microplate reader (Model 680, BIO-RAD). Hydroxyl radical scavenging

ability of a standard antioxidant was also tested by replacing the extract with gallic acid (6.25-100 µg/mL). The capability to scavenge the hydroxyl radical was calculated using the following equation:

$$Hydroxyl\ Scavenging\ effect\ (\%) = [(\frac{Ao-As}{Ao}) * 100]$$

Where Ao and As is the absorbance of control and sample respectively

3.8.4 Iron Chelation assay

The chelation of ferrous ions by the root extracts of *P. prunelloides* was determined by the modified method of Dorman *et al*. (2003). Briefly described, 200 µL of 0.2 mM FeCl2 was added to 40 µL aliquots of extracts (6.25-100 mg/mL). The reaction was initiated by the addition of 5 mM ferrozine (80 µL) and the mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance was then measured at 562 nm in a microplate reader (Model 680, BIO-RAD, USA). Iron chelating potential of a standard antioxidant was also tested by replacing the extract with gallic acid (6.25–100 µg/mL). The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as:

$$\text{ferrozine}-\text{Fe2} + \text{complex formation}\ (\%) = [(\frac{Ao-As}{Ao}) * 100]$$

Where Ao and As is the absorbance of control and sample respectively

3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 statistical package (GraphPad Software, USA). The data were analysed by one way analysis of variance (ANOVA) followed by Bonferroni test. All the results were expressed as mean ± SEM for triplicate determinations.

3.10 Qualitative analysis of secondary metabolites

Phytochemical composition of the plant extracts were determined by the method described by Trease and Evans, 1996; Sofowora, 2006

3.10.1 Test for tannins: To 0.5 mL of the extract solution, 1 mL of distilled water and one to two drops of ferric chloride solution were added and observed for brownish green or a blue colouration

3.10.2 Test for terpenoids: A 5 mL of extract was mixed with 2 mL of chloroform in a test tube. 3 mL of concentrated H_2SO_4 was carefully was added to the mixture to form a layer. An interface reddish brown colouration was formed for the presence of terpenoids

3.10.3 Test for Alkaloids: A 1 mL of 1% HCl was added to 3 mL of extract in a test tube and was treated with few drops of Meyer's reagent. A creamy white precipitate indicated the presence of alkaloids

3.10.4 Test for saponins: A 5 mL of extract was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with three drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins.

3.10.5 Test for flavonoids: A few drops of 1% NH3 solution was added to the extract in a test tube. A yellow colouration was observed for the presence of flavonoids

3.10.6 Test for cardiac glycosides: A 5 mL of extract was mixed with glacial acetic acid containing one drop of ferric chloride. The above mixture was carefully added to the 1 mL of concentrated H_2SO_4. The presence of cardiac glycosides was detected by the formation of brown ring.

3.10.7 Test for phlobotannins: About 10 mL of extract was boiled with 1% HCl in boiling tube. Deposition of red precipitate indicated the presence of phlobatannins.

3.11 Quantitative analysis of secondary metabolites

3.11.1 Determination of total alkaloids

To a 250 mL beaker, 5 g of sample was mixed with 200 mL of 10% acetic acid in ethanol and the mixture was allowed to stand for 4 h. The filtrates were collected and concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise until precipitation was complete. The solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue, alkaloid, were dried through evaporation of solvent and weighed (Harborne, 1973). Percentage yield of the extract is defined as the total dry mass after evaporation and was calculated using equation:

$$\%\, Yield = \frac{mass\ of\ dry\ weight\ (g)}{total\ mass\ of\ dry\ powdered\ sample\ (g)}\ X\ 100\%$$

3.11.2 Determination of total saponins

Ten grams of powder material was extracted in 100 mL of 20% aqueous-ethanol. The mixture was heated over a hot water bath for 4 h with continuous stirring at about 55 °C, this was followed by filtration. The residue was re-extracted with 100 mL 20% ethanol. The combined extracts were reduced to 40 mL over water bath at about 90 °C. The concentrate was transferred into a 250 mL separation funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. This purification process was repeated two times. 60 mL of *n*-butanol was then added to the extract. The *n*-butanol extracts was washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution after washing was heated in a water bath (Obadoni and Ochuko, 2001). After evaporation the samples were dried in an oven to a constant weight and the saponin content was then calculated using equation:

$$\%\, Yield = \frac{mass\ of\ dry\ weighed\ (g)}{total\ mass\ of\ dry\ powdered\ sample\ (g)}\ X\ 100\%$$

3.11.3 Determination of total tannins

The total tannins of the roots extracts of *P. prunelloides* were determined by Folin - Ciocalteu method as described by Miean and Mohamed (2001). In 10 mL volumetric flask, 0.1 mL of extract was mixed with 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu phenol reagent, 1 mL of 35% Na_2CO_3 solution and was further diluted to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100 μg/mL) were prepared. Absorbance for test and standard solutions were measured against the blank at 725 nm with Biowave II (Biochrom, UK) spectrophotometer. The tannin content was expressed in terms of mg of gallic acid equivalent (GAE) /g of extract.

A standard curve of absorbance against gallic acid concentration was prepared and results were expressed as percentage w/w i.e. tannin content (% w/w) = $GAE \times V \times D \times 10^{-3} \times 100$
GAE - gallic acid equivalent (μg/mL), V - total volume of sample (mL), D - dilution factor

3.11.4 Determination of total flavonoids

Ten milligram of quercetin was dissolved in 100 mL of methanol (100 μg/mL) and then further diluted to 10, 20, 40, 80 or 160 μg/mL (Lin and Tang, 2007). The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of methanol, 0.1 mL of aluminium ($AlCl_3$) chloride (10%), 0.1 mL of 1 M potassium acetate and 2.8 mL of diluted water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with Biowave II (Biochrom, UK) spectrophotometer. The amount of $AlCl_3$ (10%) was substituted by the same amount of distilled water in blank. Quantification was done on the basis of a standard curve of quercetin.

A standard curve of absorbance against quercetin concentration was prepared and results were expressed as percentage w/w i.e. flavonoids content (% w/w) = $QE \times V \times D \times 10^{-3} \times 100$
QE - quercetin equivalent (μg/mL), V - total volume of sample (mL), D - dilution factor

3.11.5 Determination of total phenolic content

Total phenol contents in a plant extract were determined by the method of Singleton *et al*. (1999) with modification of Folin-Ciocalteu by Wolfe *et al*. (2003). 50 µL of aliquot extract (1 mg/mL) was mixed with 50 µL of Folin-Ciocalteu reagent (10%) and 50 µL of water. 150 µL of 7.5% of Na_2CO_3 was added to neutralize the reaction. The tubes were mixed thoroughly and incubated for 40 min at 45 °C. Absorbance was then measured at 765 nm using a Biowave II (Biochrom, UK) spectrophotometer. The total phenolic content was expressed as mg/mL gallic acid using the equation obtained from calibration curve (using 0.05 mg/mL - 0.5 mg/mL of gallic acid)

A standard curve of absorbance against gallic acid concentration was prepared and results were expressed as percentage w/w i.e. phenolic content (% w/w) = $GAE \times V \times D \times 10^{-3} \times 100$
GAE – gallic acid equivalent (µg/mL), V - total volume of sample (mL), D - dilution factor

3.12 Experimental Techniques for Isolation

Four techniques were used to screen and isolate chemical components of *Pentanisia prunelloides.* The chromatographic techniques used were Vacuum Liquid Chromatography (VLC), Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC). Nuclear Magnetic Resonance (NMR) was used for characterisation of the isolated compounds.

3.12.1 Vacuum Liquid Chromatography (VLC)

Vacuum Liquid Chromatography is one of the simplest, cost reducing and time saving method used for the effective fractionation of a large quantity of a material by gradient elution and the solvent is maintained by the vacuum that is for solvent to move, the vacuum must be in operation (Pelletier *et al*., 1986). The column is prepared in a sintered glass funnel (with dry silica gel) where the arm of the volumetric flask for collection is connected to the vacuum. To have uniform packing, the silica is poured slowly and evenly and it is left to settle before application of the sample. The sample is applied uniformly at the top of the support and then cotton wool is embedded on top of the

sample so that when the solvent is introduced in the column, it will move evenly and not cause skew movement. Step-gradient elution is used and the column is allowed to be dry after each collection to allow all the compounds of the chosen solvent is in one collector.

3.12.2 Thin Layer Chromatography (TLC)

Thin Layer chromatography is widely used for isolation of active compounds from fractions or the crude extracts. This technique gives an overall picture of the type and number of secondary metabolites present in a plant. As compared to other chromatographic methods, it is the simplest and cheapest method for detecting plant constituents. It requires little sample of nanolitres to microlitres that is not too diluted, can spot many samples in one plate, needs small volume of solvent for developing the plate and results can be obtained in short period of time (Sherma, 2000).

The sample mixture was evenly spotted on a TLC plate (either aluminium or glass plate), along a straight line and the plate was developed by placing it in an upright position in a closed TLC development chamber with the chosen mobile phase. The capillary forces will draw the solvent up the plate and fractions will separate at the different rates depending on their polarity and retention affinity by the stationary phase. Different spots are equivalent to the different analytes characterised by retention factor (Rf) values. This is defined as the measure of the fraction of the distance moved by the solvent, calculation is as follows:

$$Rf = \frac{distance\ moved\ by\ the\ analyte}{distance\ moved\ by\ solvent\ front}$$

3.12.3 Preparative Thin Layer Chromatography (pTLC)

Preparative TLC is used to separate and isolate large amounts of compounds than normally separated by analytical TLC. This is because of the thickness of layers of silica gel which ranges between 1 and 2 mm that allows the large application of volume of sample.

After development, the plates are visualised under UV light (254 nm and 366 nm), if some compounds require spray, the plate is covered with aluminium foil and only the edge is left and sprayed with 1% vanillin-sulphuric reagent, use heat from hairdryer to develop the colours. Then the band of interest is matched and marked then scraped off using spatula (for glass backed silica gel TLC plates) or were cut off (for aluminium TLC plates) then concentrated and filtered using methanol and chloroform.

pTLC was carried out using glass backed silica gel F_{254} and aluminium. The collected analytes were dissolved in methanol and ethyl acetate, depending on whether the compound will dissolve or not, and then loaded into the TLC plates within the two lines which were 2.5 cm from the bottom across the plate with a ruler. The samples were then loaded using a thin tipped pipette and the plate was then dried with flowing air. Then visualisation was followed as described above.

3.12.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectroscopy gives information on the environment in which the nuclei of the atoms are found in molecules and compounds. It is the study of the interaction of radio frequency (RF) of the electromagnetic radiation (EMR) with unpaired nuclear spins in an external magnetic field to extract structural information about the given sample (James, 1998).

The structures of the isolated compounds were interpreted using various 1D and 2D NMR experiments (such as proton - ^{1}H, carbon - ^{13}C, Heteronuclear Multiple Bond Correlation - HMBC, Heteronuclear Single Quantum Coherence - HSQC, Correlation SpectroscopY - COSY) to determine the proton to carbon relation and the chemical environment they are in for structure elucidation (Silverstein *et al.*, 2005).

Two Varian NMR instruments allowing analysis at 600 MHz and 400 MHz were used. Deuterated Chloroform ($CDCl_3$) and methanol (CD_3OD) were used for dissolving of non-polar and polar compounds respectively. The duration of the experiments that were run, depended on the purity and quantity of the samples. For accurate mass detections of

the compounds, analysis by the Mass spectroscopy (UPLC-QTOF-MS) was used. All samples were run at a set temperature of 25 °C.

3.13 Acetylation

The acetylation of compounds reduces the polarity of polar compounds that are normally difficult when separating using columns. A decrease in polarity often results in better resolution. When doing structure elucidation in NMR, it is often challenging because some hydroxyl containing carbons have tendency of being difficult to differentiate them and hydrogen peaks of those carbons sometimes they overlap.

The derivatives are usually less polar than the original substances and with the replacement of the active hydrogen in the groups, the tendency to form hydrogen bonds decreases substantially and their volatility increases so that non-volatile unstable compounds can be analysed by GC (Drozd, 1981)

3.14 Isolation of compound PP-I-101840B

Ethanol extract (10 g) was separated on silica gel vacuum liquid chromatography (VLC) of particle size (35-70 µM) using trial stepwise gradient mixture of approximately 500 mL (v/v) 6:4 Acetone:$CHCl_3$; 5:5 MeOH:$CHCl_3$ and 98% MeOH as eluent to give 5 fractions. Fraction 5 (PP-I-101803D) was further separated using VLC using trial stepwise mixture containing mixture of 5:2 Acetone:MeOH; 98% $CHCl_3$; 5:4:1 MeOH:Acetone:$CHCl_3$; 98% MeOH; 98% $CHCl_3$ as eluent to give 5 fractions. Fraction 1 (PP-I-101805A 0.83g) was chromatographed using preparative thin layer chromatography (prep-TLC) employing $CHCl_3$:MeOH:AcOH (9:1:1) as eluent to give fraction PP-I-101828 and pTLC was done on this fraction employing pTLC 8:2:1 $CHCl_3$:MeOH:AcOH to give compound PP-I-101840B (1.2 mg) Figure 3.2.

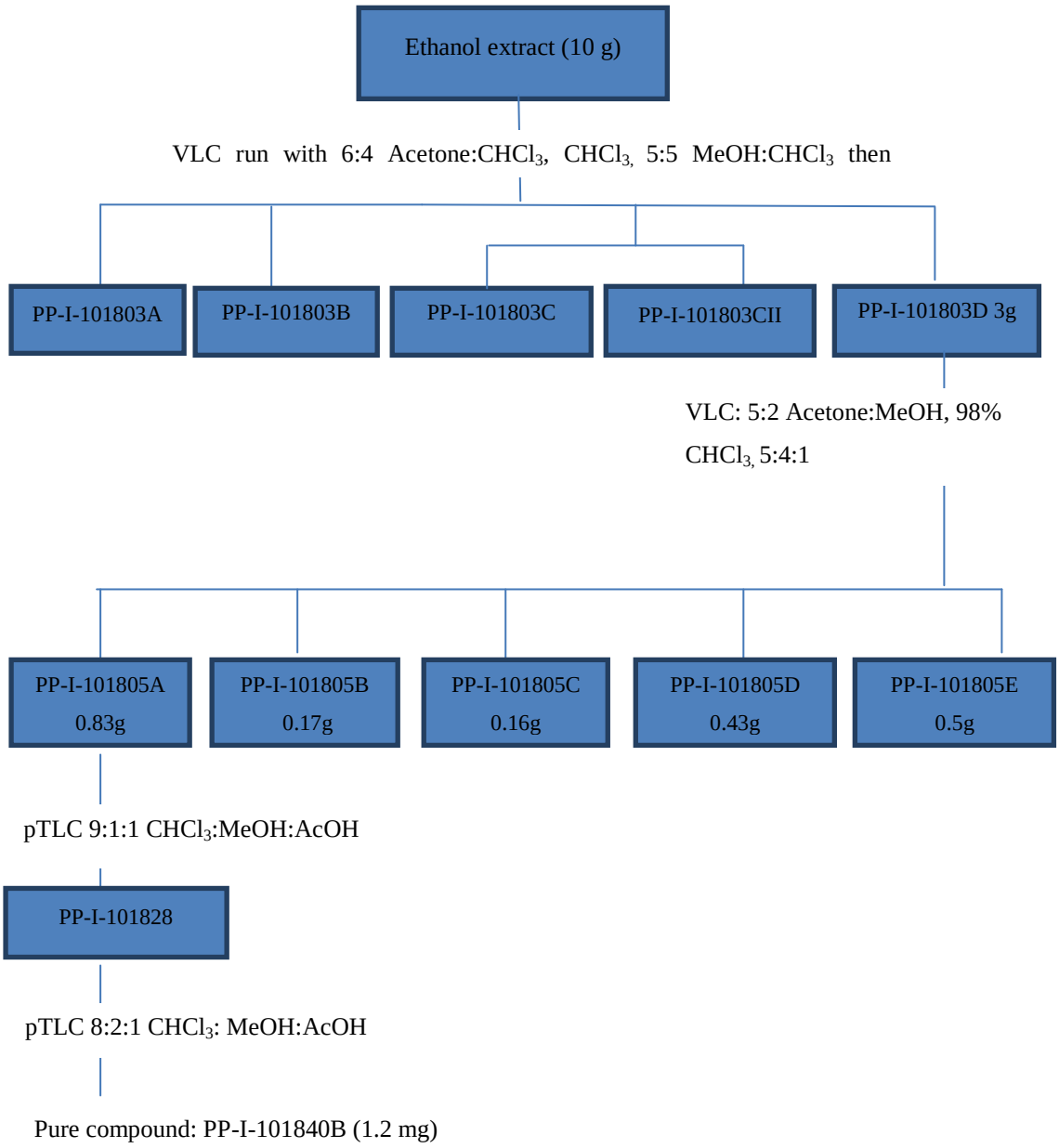

Figure 3.2: Stepwise method for isolation of compound PP-I-101840B

Ethanol extract (3 g) was separated on VLC of particle size (35-70 µM). The elution was started with hexane:EtOAc (6:4) followed by stepwise gradient mixture of $CHCl_3$:MeOH in ratio (9:1, 8:2 and 7:3) and lastly methanol was used to wash all the remaining compounds. Each collection was made up to 200 mL. Total of twenty one fractions were obtained and dried in a fume hood with flowing air.

3.15 Isolation of compound PP-I-101841A and PP-I-101841D

Acetylation of methanol fraction (2 g) was achieved by dissolving the fraction in a mixture of pyridine-acetic anhydride (1:1) in a round-bottomed flask. The reaction mixture was stirred for 48 h at room temperature and then 3 mL ethanol was added to react with the excess acetic anhydride. The resultant mixture was poured into an ice-chloroform mixture and the acetylated product was extracted three times using liquid-liquid partitioning in a separating funnel. The organic phases were combined and the solvent removed under vacuum. Acetylated fraction was chromatographed using pTLC employing EtOAc:Hexane:AcOH (5.5:4.5:0.5 v/v) as eluent to give two compounds PP-I-1018741A (10.1 mg) and PP-I-1011841D (6.9 mg), where compound PP-I-1011841D appeared to be poorly acetylated making it non-acetylated.

All the obtained fractions were screened for the chemical compounds that they contained on aluminium backed TLC plates (10 x 7 cm). The solvent front was marked and the plates were allowed to dry before visualisation under UV 254 nm and UV 366 nm. The plates were sprayed with the 1% vanillin-sulphuric acid solution for visualisation. A number of solvent systems were tried until the optimal solvent system was obtained for each of the fractions collected. The same procedure for identifying single compounds was followed. For documentation, pictures were taken since some of the compounds have tendency of fading away after spraying.

The isolated compounds were further analysed using Nuclear Magnetic Resonance (1D and 2D experiments) and mass spectroscopy.

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CHAPTER 4

RESULTS

4.1 Crude extracts percentage yield of *P. prunelloides*

The percentage yields of water, ethanol, aqueous-ethanol (1:1) and hexane extracts are presented in Table 4.1

Table 4.1: Percentage yield of root extracts of *P. prunelloides*.

Solvent	Mass extracted (g)	% yield
Water	3.05	15.25
Ethanol	0.73	3.65
Aqueous-ethanol (1:1)	5.81	29.05
Hexane	0.34	1.7

The aqueous-ethanol (29.05%) had the highest percentage yield, followed by water (15.25%), then ethanol (3.65%) and hexane (1.7%).

4.2 Alpha amylase inhibition

The *in vitro* α-amylase inhibitory studies revealed that all the crude extracts of *P. prunelloides* inhibited the specific activity of porcine pancreatic α-amylase (Figure 4.1). The hexane extract demonstrated the strongest inhibitory activity against the α-amylase at all the concentrations which was significantly higher ($p < 0.0.5$) than other extracts and standard (acarbose). Water, ethanol, aqueous-ethanol extracts and acarbose inhibited this enzyme similarly at all the concentrations, except that water extract had the highest inhibition at 50.0 µg/mL.

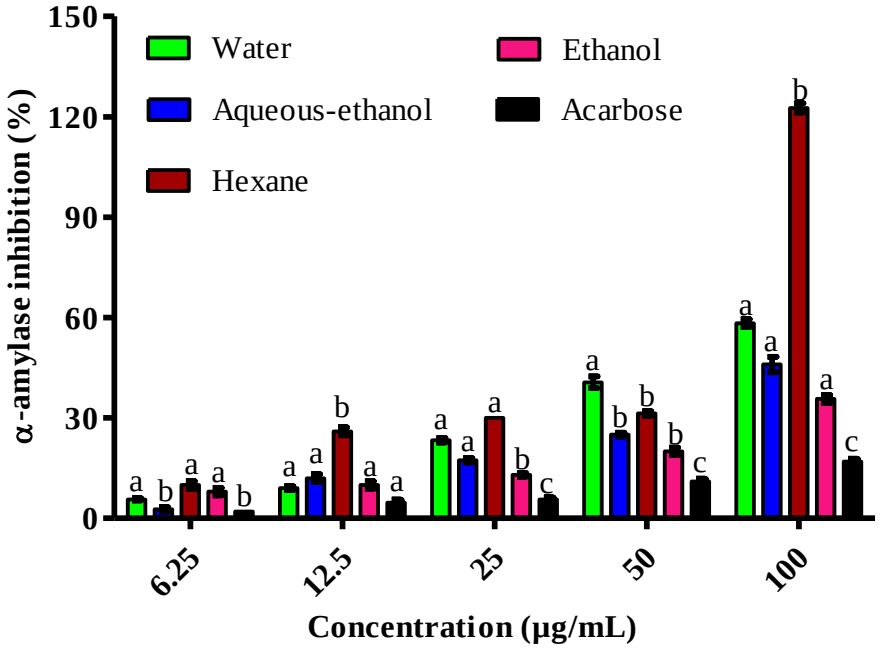

Figure 4.1: Inhibitory effects of extracts of *P. prunelloides* on the activities of α-amylase. Values are expressed as mean ± SEM of triplicate determinations. Bars not sharing a common letter at the same concentration are significantly different ($p < 0.05$) .

The IC_{50} (concentration of inhibitor producing 50% inhibition) values of *P. prunelloides* root extracts against α-amylase revealed that out of all the extracts, hexane extract had the lowest IC_{50} value of 0.48 µg/mL. Water, aqueous-ethanol extracts and acarbose were not significantly different ($p > 0.05$) from one another. The ethanol extract had the highest IC_{50} value of 18.51 µg/mL (Table 4.2).

Table 4.2: IC_{50} values of α-amylase inhibition by acarbose and root extracts of *P. prunelloides.*

Extracts	IC_{50} (µg/mL)
Water	8.82 ± 0.53^{a}
Aqueous-ethanol	9.03 ± 2.08^{a}
Hexane	0.48 ± 0.35^{b}
Ethanol	18.51 ± 1.78^{c}
Acarbose	9.87 ± 1.61^{a}

The values are expressed as mean ± SEM of triplicate determinations (n=3). Means not sharing a common superscript are significantly different ($p < 0.05$).

A further analysis on the mode of inhibition of the most potent of the extracts (ethanol-extract) suggests a characteristic mixed non-competitive inhibition (Figure 4.2). From this, the exerted mixed inhibition pattern on α-amylase which altered the Km and Vmax values are presented in (Table 4.3)

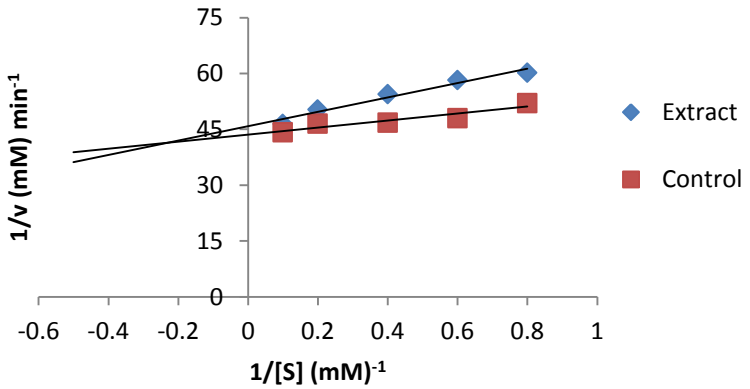

Figure 4.2: Mode of inhibition of α-amylase by *P. prunelloides* ethanol root extract.

Table 4.3: Effect of ethanol extract of *P. prunelloides* on kinetic parameters of α-amylase

Kinetic parameters	α-amylase	
	Control	Extract
Km (mM)	0.002	0.001
Vmax (mM)min^{-1}	0.023	0.022

4.3 Alpha glucosidase inhibition

The *in vitro* α-glucosidase inhibitory studies revealed that all the crude extracts of *P. prunelloides* inhibited the specific activity of α-glucosidase, maltase and sucrase (Figure 4.3). Hexane and ethanol extracts demonstrated the highest percentage inhibition against α-glucosidase followed by water extract (Figure 4.3a). The high inhibitions of these extracts were observed in all the concentrations, where at lower concentration of 6.25 µg/mL, they have demonstrated strong inhibitions of 44 % (hexane) and 32 % (ethanol) and at high concentrations inhibitory activity was not significant different ($p > 0.05$)

The ethanol, aqueous-ethanol and hexane extracts inhibitory activities on sucrase were not significant different ($p > 0.05$) from one another. Water (35%), ethanol (30%) and acarbose (29%) had the highest inhibition of sucrase at 6.25 µg/mL (Figure 4.3b).

The inhibition by aqueous-ethanol extract and acarbose on maltase were not significantly different from one another at 6.25 µg/mL ($p > 0.05$) and this similar inhibition was also observed in ethanol and hexane extract at the same concentration. In all the concentration tested except 100 µg/mL, aqueous-ethanol extract exhibited the strongest inhibition of the maltase while hexane extract displayed more than 100% at 100 µg/mL (Figure 4.3c).

The IC_{50} values of *P. prunelloides* root extracts against α-glucosidase revealed that ethanol and hexane extracts had the lowest IC_{50} values of 19.73 µg/mL and 18.08 µg/mL respectively and were lower than all the screened extracts and standard. Water extract had the lowest IC_{50} value of 3.85 µg/mL against sucrase and was significantly lower ($p < 0.05$) than other extracts and standard. Aqueous-ethanol extract had the lowest IC_{50} value of 26.03 µg/mL against maltase and was lower than all other extracts and standard (Table 4.4).

Further analyses on the mode of inhibition of ethanol extract (more potent against α-glucosidase) suggest a characteristic of non-competitive inhibition (Figure 4.4). The exerted non-competitive inhibition pattern on α-glucosidase witch altered the Km and Vmax values presented in (Table 4.5)

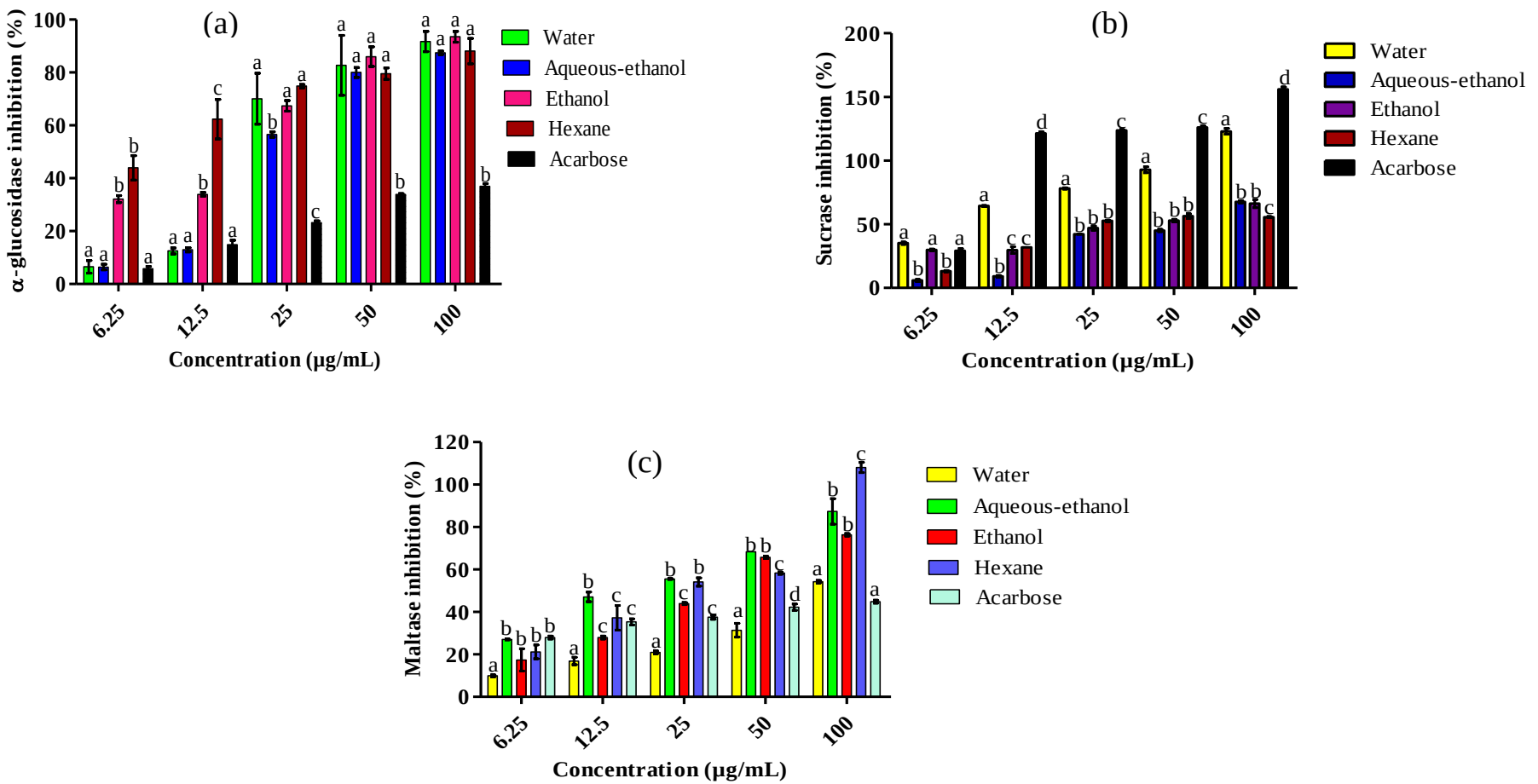

Figure 4.3: Inhibitory effects of different extracts of *P. prunelloides* on the activities of (a) α-glucosidase, (b) sucrase, (c) and maltase. Values are expressed as mean ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration are significantly different ($p < 0.05$) .

Table 4.4: IC_{50} values of *P. prunelloides* root extracts and acarbose against α-glucosidase

Samples	IC_{50} (µg/mL)		
	α-glucosidase	**Sucrase**	**Maltase**
Water	36.01 ± 6.21[a]	3.85 ± 2.11[a]	90.49 ± 2.90[a]
Ethanol	19.73 ± 1.53[b]	51.48 ± 5.31[b]	44.84 ± 1.88[c]
Aqueous-ethanol	40.33 ± 0.49[a]	64.36 ± 0.84[c]	26.03 ± 0.33[b]
Hexane	18.08 ± 0.03[b]	61.66 ± 1.43[c]	31.59 ± 2.81[b]
Acarbose	129.4 ± 5.11[c]	30.65 ± 1.13[d]	122.1 ± 4.64[d]

The values are expressed as mean ± SEM of triplicate determinations (n=3). Means not sharing a common superscript for each enzyme are significantly different ($p < 0.05$).

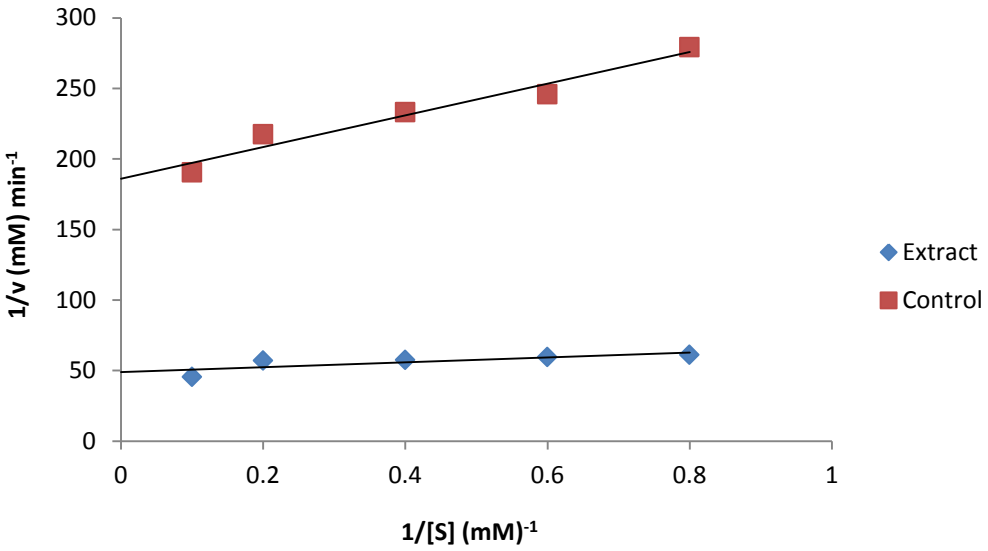

Figure 4.4: Mode of inhibition of α-glucosidase by ethanol extract

Table 4.5: Effect of ethanol extract of *P. prunelloides* on kinetic parameters of α-glucosidase

Kinetic parameters	α-glucosidase	
	Control	Extract
Km (mM)	$4.79x^{-5}$	0.001
Vmax (mM)Min^{-1}	0.005	0.020

4.4 *In vitro* antioxidant effects of root extracts of *P. prunelloides*

The *in vitro* antioxidative capacities for the extracts under investigation were studied using different models of antioxidant activities. They were determined using1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anion radical, hydroxyl radical and metal ion chelation. In the DPPH assay (Figure 4.5a), the percentage inhibition by ascorbic acid (standard) was significantly higher ($p < 0.05$) than all the tested extracts except at the lowest concentration, 6.25 μg/mL, where the ethanol and aqueous-ethanol extracts had the highest inhibition. Ethanol extract was significantly higher than aqueous-ethanol extract at all the concentrations except at 12.5 μg/mL where inhibition was similar.

For the superoxide anion scavenging assay (Figure 4.5b), there was no significant difference in all the tested concentrations except with ethanol, where its inhibitory percentage was low at 6.25, 12.50 and 25.0 μg/mL. Gallic acid was significantly higher than all the tested extracts at 100 μg/mL. For the hydroxyl radical scavenging abilities of the extracts and the standard (Figure. 4.6a), there was no significant difference ($p > 0.05$) between ethanol extract and gallic acid at all the concentrations except at 6.25 μg/mL where gallic acid was higher than that of ethanol extract. The ability of the extracts to chelate ions (Figure 4.6b) was determined and the values for the water extract were significantly higher at all concentrations except at 6.25 μg/mL, where the inhibition of aqueous-ethanol extract was high. The metal chelating potentials of all the extracts were higher than that of gallic acid in all the concentrations tested.

Table 4.6: IC_{50} values for the free radical scavenging abilities of different extracts of *Pentanisia prunelloides*

Samples	IC_{50} (µg/mL)			
	DPPH	**Superoxide**	**Hydroxyl**	**Iron Chelation**
Ethanol	77.06 ± 0.22^{a}	92.98 ± 1.20^{a}	42.99 ± 1.50^{a}	64.86 ± 3.20^{a}
Aqueous-ethanol	142.7 ± 0.47^{b}	74.72 ± 1.94^{b}	24.78 ± 0.72^{b}	4.24 ± 0.03^{b}
Water	75.42 ± 2.28^{a}	118.7 ± 0.66^{c}	12.28 ± 0.51^{c}	20.30 ± 1.56^{c}
Hexane	88.67 ± 1.57^{c}	0.33 ± 0.02^{d}	0.51 ± 0.43^{d}	37.47 ± 0.19^{d}
Gallic Acid	34.03 ± 1.56^{d}	55.50 ± 0.12^{e}	65.70 ± 1.48^{e}	101.7 ± 2.26^{e}

The values are expressed as mean ± SEM of triplicate determinations (n=3). Means not sharing a common superscript are significantly different ($p < 0.05$).

The IC_{50} value for the free radical scavenging abilities of the *P. prunelloides* extracts are presented in Table 4.6. Ascorbic acid had the lowest IC_{50} for DPPH (34.03 µg/mL), which was significantly lower ($p < 0.05$) than other extracts. Ethanol and water extracts had minimal inhibition of IC_{50} value of 77.06 and 75.42 µg/mL respectively. Hexane extract displayed the lowest IC_{50} (0.33 µg/mL) for the inhibition of superoxide anion radicals which was significantly lower than other extracts and standard (gallic acid). Hexane extract also showed good activity in inhibiting hydroxyl radicals with IC_{50} value of 0.51 µg/mL and was significantly lower than all the extracts and standard (galli acid) followed by water extract 12.28 µg/mL. In the metal chelation assay, aqueous-ethanol extract had the lowest IC_{50} of 4.24 µg/mL, and it was lower than other extracts and standard.

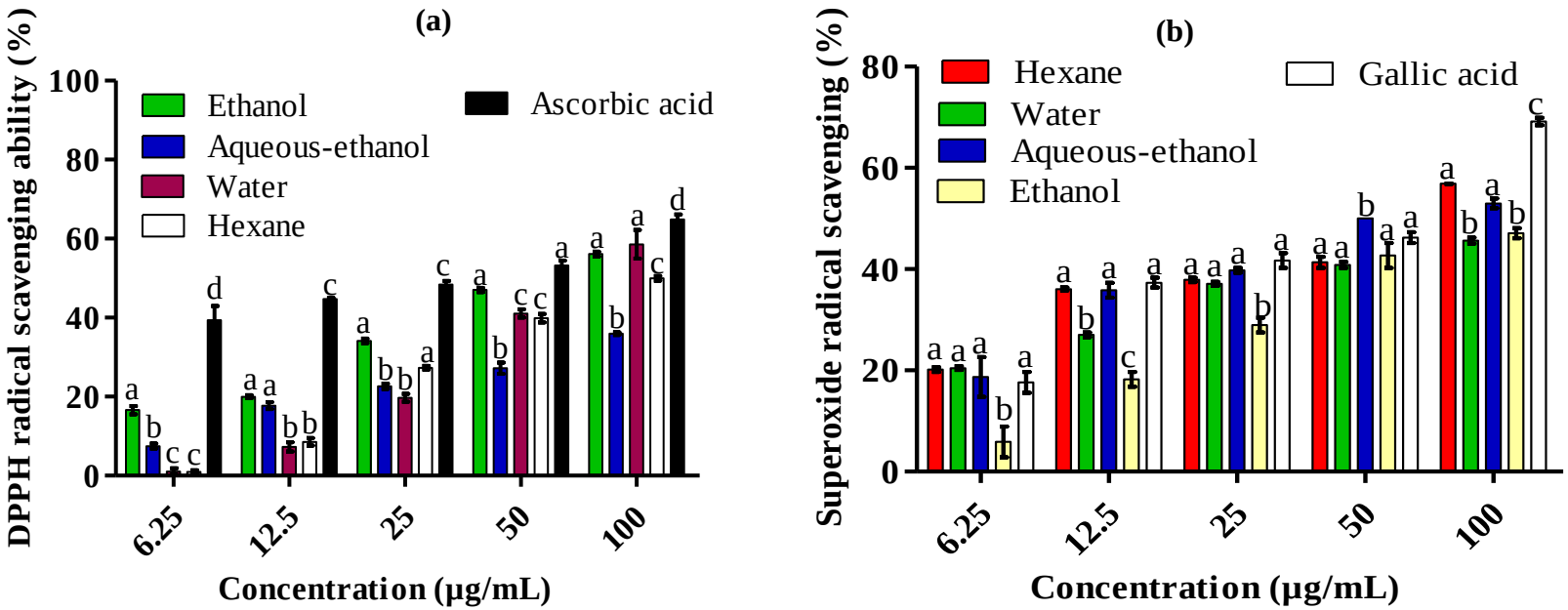

Figure 4.5: Scavenging abilities of different extracts of *Pentanisia prunelloides* against (a) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, (b) and superoxide anion radical. Values are expressed as mean ± SEM of triplicate determinations. Bars not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

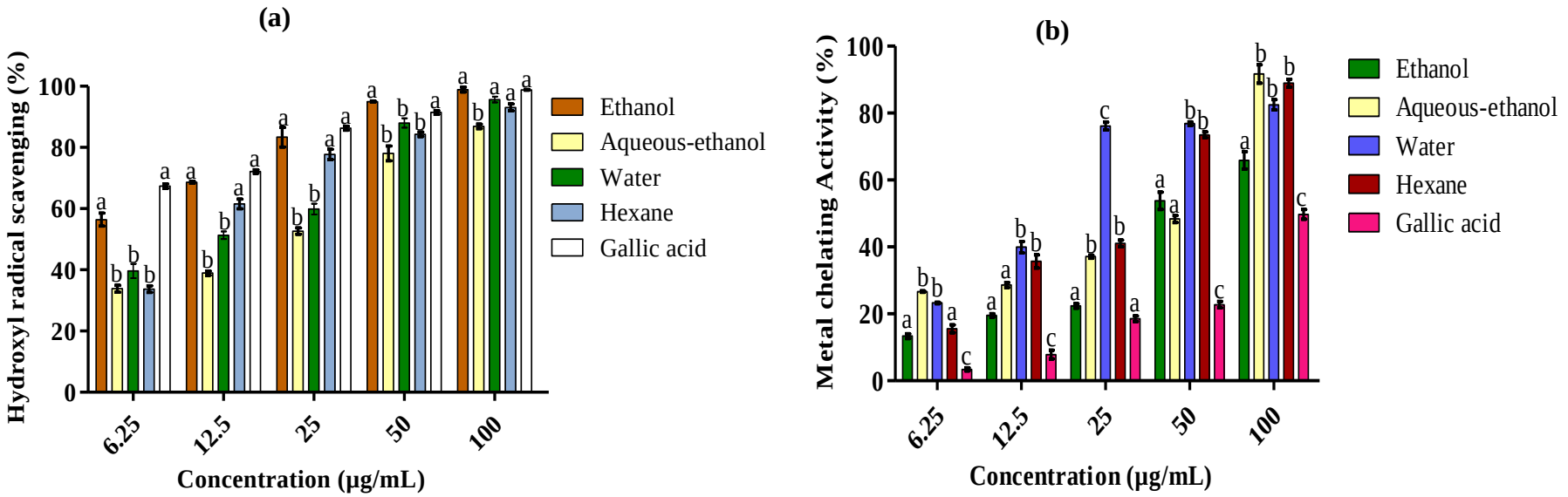

Figure 4.6: Scavenging abilities of different extracts of *Pentanisia prunelloides* against (a) hydroxyl radical, and (b) metal chelation. Values are expressed as mean ± SEM of triplicate determinations. Bars not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

4.5 Phytochemical screening analyses

The results of the chemical group screening for the presence of tannins, saponins, alkaloids, terpenoids, flavonoids, phlobatannins and cardiac glycosides are presented in Table 4.7. Tannins, terpenoids and saponins were present in all the root extracts. Alkaloids were only detected in water extracts, flavonoids in ethanol extract and cardiac glycosides in aqueous-ethanol extract.

Table 4.7: The phytochemical constituents of t extracts of *P. prunelloides* roots

Phytochemical type	Water	Ethanol	Hexane	Aqueous-ethanol
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Alkaloids	+	-	-	-
Saponins	+	+	+	+
Flavonoids	-	+	-	-
Cardiac glycosides	-	-	-	+
Phlobatannins	-	-	-	-

(+) detected, (-) not detected

The results of the quantity of saponins and alkaloids of the root of the plant are presented in Table 4.8, while those of the total flavonoids, tannins and phenolics are shown in Table 4.9. The roots of *P. prunelloides* was found to contain low quantity of alkaloids with percentage less than ten that is 0.6%, while saponins were also found to be less but with percentage of 13.9%. Total flavonoids of different extracts of *P. prunelloides* were determined. Hexane extract and water extracts were found to contain more flavonoids other than other extracts with values of 15.40 and 14.70 mg quercetin/g respectively, and these results are not significantly different from each other ($p > 0.05$). Also, ethanol and aqueous-ethanol extracts had quantified flavonoids similarly even though at lower concentration than above mentioned extracts, quantity among ethanol and aqueous-ethanol extracts were not significant different. The total tannins contents of the root extracts were evaluated using concentrations from 20-100 µg/mL, the contents of tannins from the extracts varied from 27.54 to 45.60 mg gallic acid/g. The aqueous-ethanol

extract has been found to be rich in tannins, with total tannin of 45.50 mg gallic acid/g, and it was significantly higher than other extracts. Total phenolics contents from different extracts ranged between 0.04 to 0.07 mg gallic acid/g. Water and aqueous-ethanol extracts had the highest total phenolic content of 0.07 mg gallic acid/g and these results were similar.

Table 4.8: The quantitative analysis of phytochemicals of extracts of *P. prunelloides* roots

Phytochemical type	Mass extracted (g)	% yield
Alkaloids	0.03	0.6
Saponins	1.39	13.9

Table 4.9: Total flavonoids, total tannins and total phenols of the root extracts of *P. prunelloides*

Extracts	Total flavonoids (expressed in mg/g quercetin)	Tannins (expressed in mg/g gallic acid)	Total phenols (expressed in mg/g gallic acid)
Water	14.70 ± 0.06^{a}	27.54 ± 0.03^{a}	0.07 ± 0.002^{a}
Ethanol	13.21 ± 0.14^{b}	34.87 ± 0.04^{a}	0.04 ± 0.003^{b}
Aqueous-ethanol	13.74 ± 0.01^{b}	45.60 ± 0.05^{b}	0.07 ± 0.002^{a}
Hexane	15.40 ± 0.17^{a}	30.21 ± 0.08^{a}	0.04 ± 0.000^{b}

Values are presented as mean ± SEM (n=3)

Values with different superscript in the same column for each parameter are significantly different ($p < 0.05$) to each other.

4.6 Bulk extraction and fractionation results

Based on the high activity of the ethanol extract in most of the conducted assays, in particularly the anti-diabetic assays, this extract was selected for further isolation of active compounds. For this, exactly 1000 g of the powdered root sample of *P. prunelloides* was exhaustively extracted with ethanol. The resultant reddish brown residue of 15.1 g was obtained.

Fractions were obtained using vacuum liquid chromatography (VLC). Ethanol extract (10 g) was fractionated and total of eleven fractions were obtained. Fraction PP-I-101805A yielded 0.83 g which was further purified using preparative thin layer chromatography (pTLC) and thin layer chromatography (TLC) to obtain a pure compound. Three gram of ethanol was fractionated and has resulted in twenty one fractions. Fraction PP-I-101835E (methanol) yielded 2.3 g out of which 2 g was acetylated to give two pure identified compounds and other three compounds from acetylation were not elucidated due to time.

4.7 *In vitro* inhibitory effects of *P. prunelloides* fractions obtained from VLC on α-amylase and α-glucosidase activities

Fractions were obtained in VLC. The obtained fractions were further determined for their α-glucosidase and α-amylase inhibitory activities. The activity of the fractions and acarbose were performed at various concentrations (6.25-100 µg/mL). To determine potency, the activity was evaluated using percentage inhibition and was also analysed using the IC_{50} values.

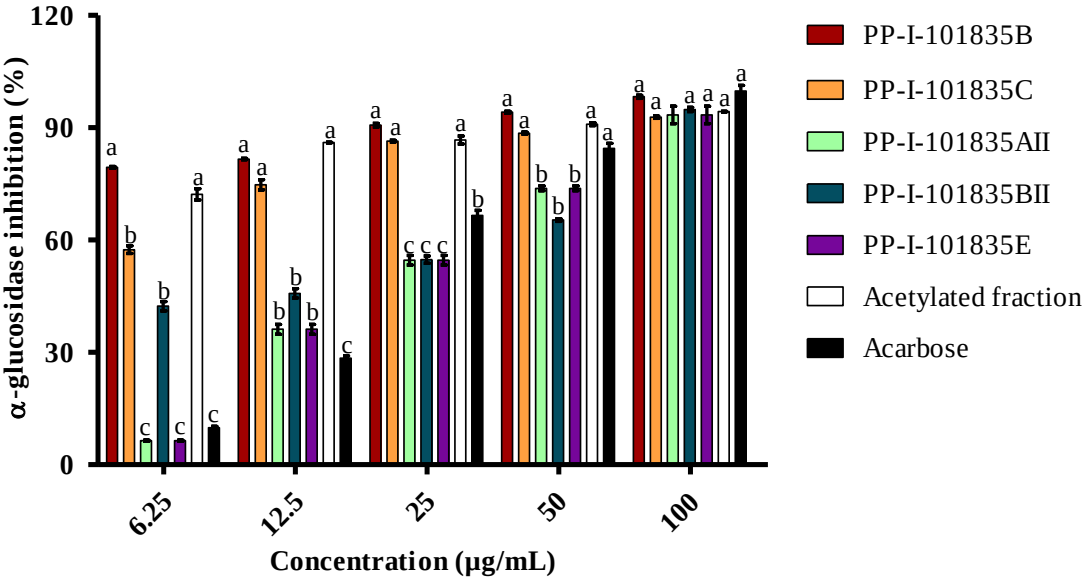

Figure 4.7: Inhibitory effects of different fractions of *P. prunelloides* on the activities of α-glucosidase Values are expressed as mean ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

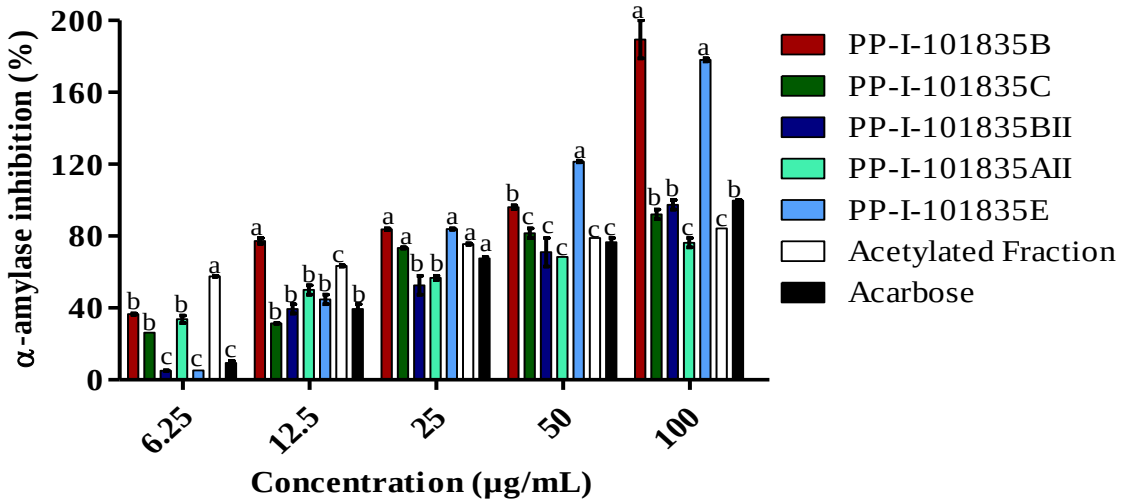

Figure 4.8: Inhibitory effects of different fractions of *P. prunelloides* on the activities of α-amylase. Values are expressed as mean ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

Acetylated fraction and fraction PP-I-101835BII had inhibited α-glucosidase (Figure 4.7) similarly at all the concentrations. At high concentration of 100 µg/mL, all the fractions and standard were not significantly different from each other ($p > 0.05$).

Fraction PP-I-101835B had inhibited α-amylase higher than other fractions and standard, at concentration 12.5, 25.0 and 100 µg/mL. Acetylated fraction had highest inhibition at 6.25 µg/mL while PP-I-101835E was higher at concentration 50.0 µg/mL. Other fractions had inhibited this enzyme similarly at all the concentrations (Figure 4.8)

The results from Table 4.10 had revealed that PP-I-101835BII had the lowest IC_{50} value of 19.53 µg/mL, which was significantly lower ($p < 0.05$) than other fractions and standard, and this fraction had mild inhibition against α-amylase with the IC_{50} value of 34.98 µg/mL. The IC_{50} values of PP-I-101835AII and PP-I-101835E were not significantly different from each other, and had IC_{50} less than 50%, while the standard (acarbose) had lower IC_{50} value these two fractions.

Table 4.10: IC_{50} values of *P. prunelloides* fractions and acarbose against α-glucosidase and α-amylase

	IC_{50} (µg/mL)	
Fractions	**α-glucosidase**	**α-amylase**
PP-I-101835AII	34.97±0.63[a]	20.03±4.27[a]
PP-I-101835B	165.6±7.34[b]	15.06±0.15[b]
PP-I-101835BII	19.53±0.39[c]	34.98±0.65[c]
PP-I-101835C	66.08±0.51[d]	22.29±0.62[a]
PP-I-101835E	44.33±0.93[a]	16.94±0.54[b]
Acetylated Fraction	171.3±8.20[b]	48.06±0.57[d]
Acarbose	29.77±0.22[a]	28.17±0.43[c]

Values are expressed as mean ± SEM of triplicate determinations (n=3). Means not sharing a common letter are significantly different ($p < 0.05$).

4.8 TLC plates for the isolated compounds

Total of six compounds were isolated, three were identified. The TLC plates together with Rf values are presented in Figure 4.9. The TLC plate of hexane: EtOAc (6:4) shows the occurrence of purple and clear colour of spots. About three major compounds are observed, but our main compound appears at Rf of 0.52 (Figure 4.9 (a)). This purple compound could only be detected after spraying with vanillin. In the second TLC plate of hexane:EtOAc (5:5), two compounds of interest were observed after spraying with purple colour which faded quickly after removal from the oven. Their Rf values were 0.75 for one compound and named PP-I-101836C and the other one was at Rf 0.60 named PP-I-101836E (Figure 4.9 (b)). The compound PP-I-101836E was not pure since one spot was visible after viewing under UV light while the compound of interest was clear after spraying. In Figure 4.9 (c), two major compounds were observed in PP-I-101840, from $CHCl_3$:MeOH:AcOH, the compound of interest appears to be at Rf of 0.78 (PP-I-101840B), which was observed under UV light. The other two compounds of interest were found in Figure 4.9 (d), these compounds are from acetylation of methanol fraction (PP-I-101835E). They were detected after spraying and are brown in colour. The Rf values were 0.74 for compound PP-I-101841A and 0.35 for compound PP-I-101841D. The solvent used to obtain these two compounds is EtOAc:Hexane:AcOH (5:5: 4.5: 0.5).

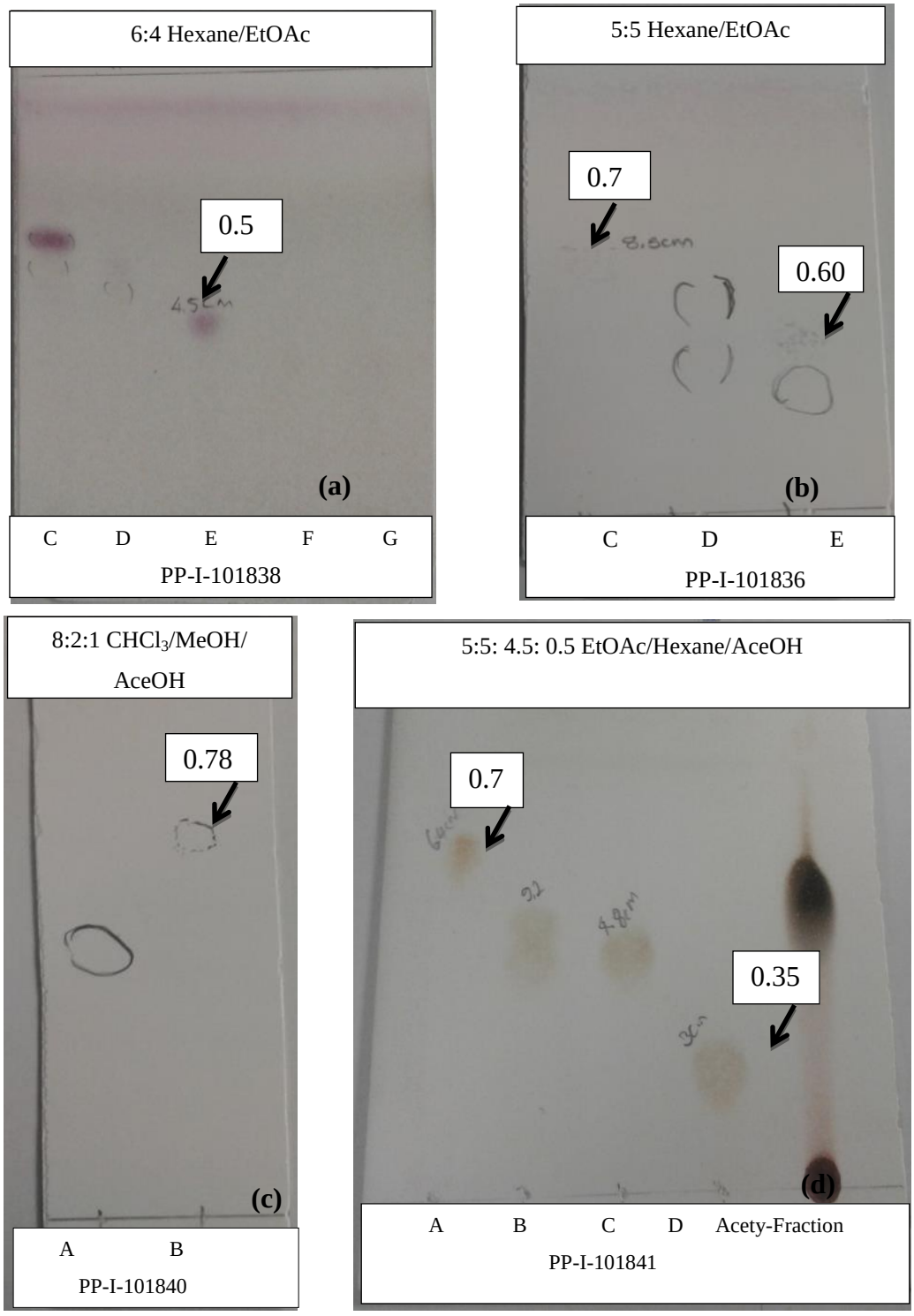

Figure 4.9: TLC plates for the isolated compounds (a) PP-I-101838 compound, (b) PP-I-101836 compounds, (c) PP-I-101840 compounds, and (d) PP-I-101841 acetylated compounds

4.9 In *vitro* inhibitory effects of isolated compounds on α-glucosidase and α-amylase activities

Four compounds (PP-I-101836C, PP-I-101836E, PP-I-101838E and PP-I-101840B) were isolated from different fractions and were tested against specific activities of α-glucosidase and α-amylase. PP-I-101836E inhibited α-glucosidase higher than other compounds and standard, except at 50.0 and 100 µg/mL, where standard had the highest inhibition. Compound PP-I-101836C and PP-I-101840B had similar inhibition of α-glucosidase at all concentrations (Figure 4.10).

Compound PP-I-101838E had the highest inhibition against α-amylase (Figure 4.11) at all the concentrations, though at some concentrations the inhibitions by compounds were denoted as not significance. This compound had higher percentage than other compounds and acarbose. At high concentration of 100 µg/mL, PP-I-101838E and PP-I-101836C were not significantly different from each other; they had inhibited this enzyme similarly.

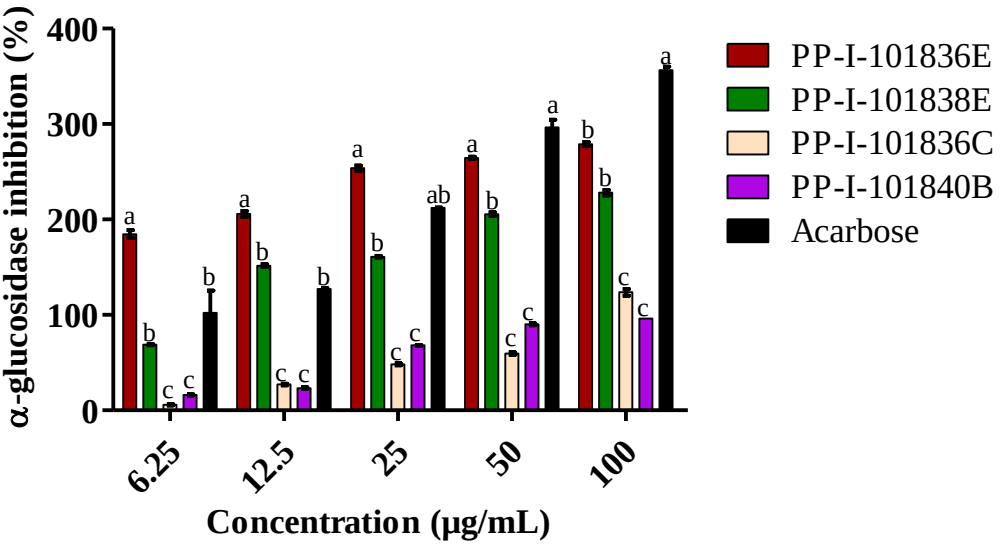

Figure 4.10: Inhibitory effects of isolated compounds from *P. prunelloides* on the activities of α-glucosidase. Values are expressed as mean ± SEM of triplicate determinations. Bars not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

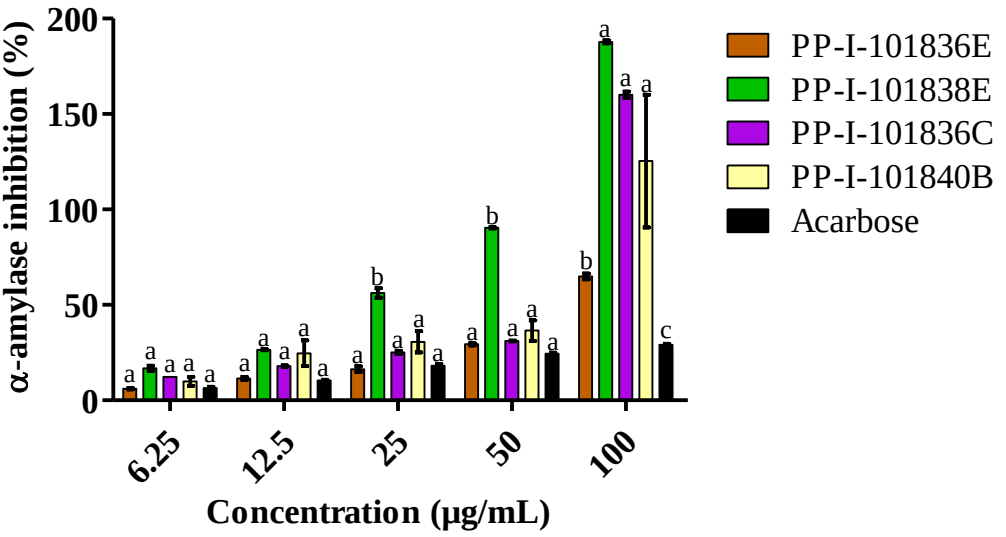

Figure 4.11: Inhibitory effects of isolated compounds from *P. prunelloides* on the activities of α-amylase. Values are expressed as mean ± SEM of triplicate determinations. Bars not sharing a common letter at the same concentration are significantly different ($p < 0.05$).

Table 4.11: IC_{50} values of isolated compounds and acarbose against α-glucosidase and α-amylase

	IC_{50} (µg/mL)	
Compounds	**α-glucosidase**	**α-amylase**
PP-I-101836E	163.0 ± 4.56^{a}	78.42 ± 1.81^{a}
PP-I-101838E	45.02 ± 2.07^{b}	24.61 ± 0.21^{b}
PP-I-101836C	36.33 ± 0.64^{b}	39.23 ± 0.33^{c}
PP-I-101840B	28.21 ± 0.33^{c}	$70. 45 \pm 1.58^{a}$
Acarbose	19.04 ± 0.10^{d}	180.2 ± 3.99^{d}

Values are expressed as mean ± SEM of triplicate determinations. Means not sharing a common letter are significantly different ($p < 0.05$).

The IC_{50} values of the isolated compounds against α-glucosidase and α-amylase are presented in Table 4.11. Compound PP-I-101840B had the lowest IC_{50} value of 28.21 µg/mL against α-glucosidase and it was significantly lower ($p < 0.05$) than other compounds but not standard (19.04 µg/mL). PP-I-101838E demonstrated the lowest IC_{50} with value of 24. 61 µg/mL against α-amylase. The IC_{50} values for compound PP-I-101836E and PP-I-101840B were not significantly different ($p > 0.05$) from one other, while acarbose showed the highest IC_{50} value of 180.2 µg/mL.

The analysis on the mode of inhibition of the most potent compound (PP-I-101840B) against α-glucosidase suggests a characteristic competitive inhibition (Figure 4.12).

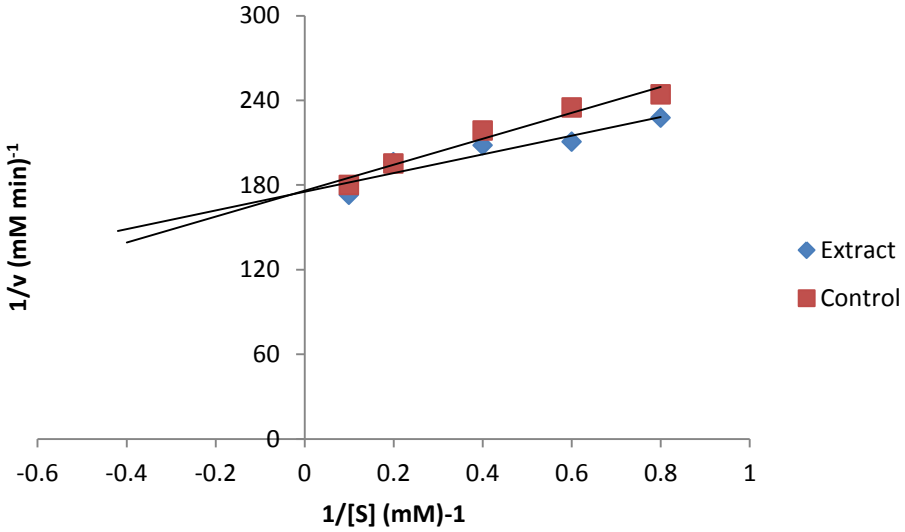

Figure 4.12: Mode of inhibition of α-glucosidase by PP-I-101840B

Mode of inhibition of α-amylase by PP-I-101840B is presented in Figure 4.13 and the compound has exerted uncompetitive type of inhibition.

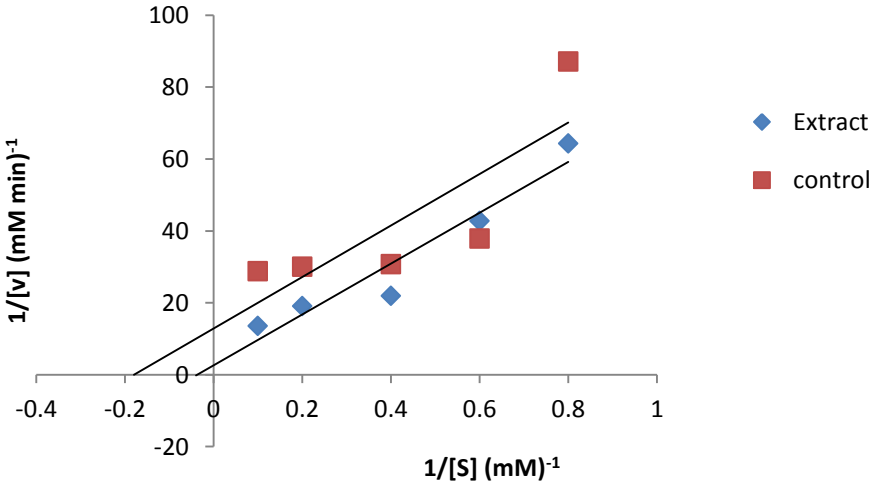

Figure 4.13: Mode of inhibition of α-amylase by PP-I-101840B

4.10 Structure elucidation

Three compounds were elucidated, two sucrose (PP-I-101841D and PP-I-101841A) and tritepenoid saponin tormentic acid (PP-I-101840B). Compound PP-I-101841D is a

sucrose which did not acetylate properly while compound PP-I-101841A is an acetylated sucrose. Apart from these, other compounds were isolated which could not be identified. Compound PP-I-101836C was found to be tritepernoid but structure elucidation could not be done and was also tested for anti-diabetic assays

4.10.1 Structural elucidation for compound PP-I-101840B (Tormentic acid)

Tormentic acid (Figure 4.14) was isolated as single spot which was colourless (1.2 mg) using $CHCl_3$:MeOH:AcOH (8:2:1) mobile phase. The NMR data obtained was found to be similar to that reported in the literature (Sun *et al*., 1992; Tanaka *et al*., 2003) and the accurate mass obtained from the HRTOFMS spectra confirmed the structure. From the mass spectra data obtained the ESI^- (negative mode) of the spectra showed a pseudo molecular ion at m/z 487.34 and the ESI^+ (positive mode) showed a pseudo molecular ion at m/z 471.35. The observed molecular ion is in agreement with the proposed molecular formula of $C_{30}H_{47}O_5$. Supplementary information is given in Appendix 1.

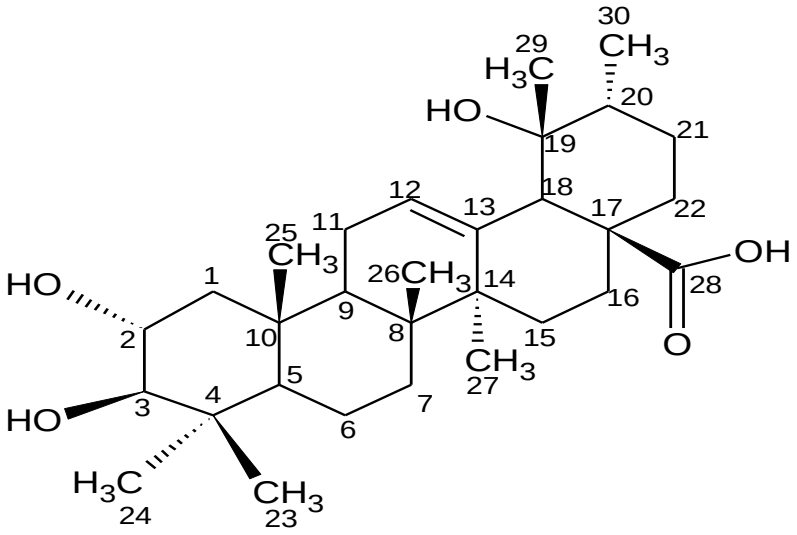

Figure 4.14: Structure of compound PP-I-101840B (Tormentic acid)

Table 4.12: ^{1}H- and ^{13}C-NMR data of compound PP-I-101840B (Tormentic acid) in CD_3OD

	Isolated compound		Literature	
Position	**δC (ppm)**	**δH (ppm)**	**δC[#1] (ppm)**	**δH[#2] (ppm)**
1	42.67		43.3	
2	67.35	3.92	68	3.62
3	80.29	2.49	80.9	2.91
4	41.42		42.1	
5	49.79		50.1	
6	19.45		20.1	
7	34.28		34.9	
8	39.55		40.2	
9	48.43		49	
10	39.6		40.2	
11	24.87		25.5	
12	129.31	5.27	130.2	5.28
13	140.5		140.8	
14	42.92		43.5	
15	27.58		28.1	
16	26.93		27.4	
17	49.36		49.8	
18	55.44	2.52	55.9	2.50
19	73.98		74.7	
20	43.22		43.9	
21	29.85		30.4	
22	39.32		39.8	
23	29.37		30	
24	25.09		25.07	
25	16.78		17.4	
26	17.86		17.7	
27	27.33		27.9	
28	181.46		183.1	
29	22.4		23.3	
30	17.02		17.7	

*Data obtained CD_3OD, ^{13}C and ^{1}H (600 and 400 MHz)
#1 Data obtained in CD_3OD, ^{13}C (JEOL GX-400)
#2 Data obtained in CD_3OD (400 MHz)

4.10.2 Structural elucidation for compound PP-I-101841D (Sucrose)

Sucrose (Figure 4.15) was isolated as a single brown spot (10.1 mg) using EtOAc:Hexane:AcOH (5:5: 4.5: 0.5). This compound did not undergo complete acetylation judging from the absence of acetyl groups after acetylation. The NMR data issimilar to that reported in the literature (Yan *et al*., 2014). The accurate mass obtained from the HRTOFMS spectra showed a pseudo molecular ion at m/z 341.10 in the ESI^- (negative mode) and the ESI^+ (positive mode) showed a pseudo molecular ion at m/z 365.15. Supplementary information is given in Appendix 1 Supplementary information is given in Appendix 2.

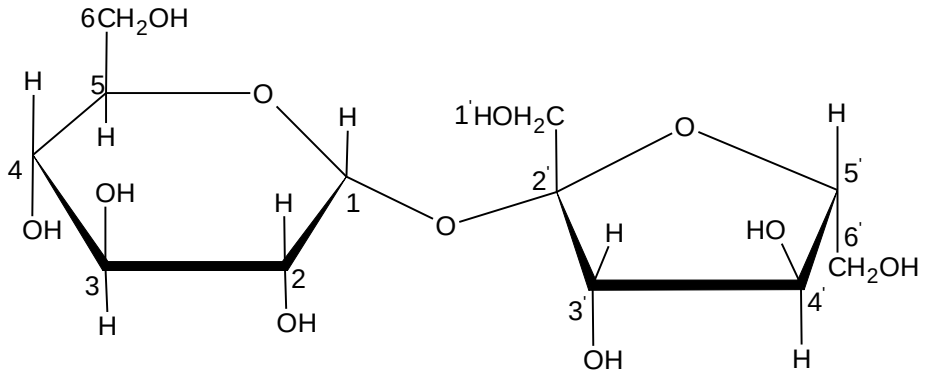

Figure 4.15: Structure of compound PP-I-101841D (Sucrose)

Table 4.13: ^{1}H- and ^{13}C-NMR data of compound PP-I-101841D (Sucrose) in CD_3OD

	Isolated compound[#]		Literature[*]	
Position	**δ_C(ppm)**	**δH(ppm)**	**δ_C(ppm)**	**δH(ppm)**
1	64.27	3.61	65.2	3.59
2	105.53	3.67	105.0	3.68
3	79.79	5.41	79.7	5.49
4	74.58	4.09	74.0	4.37
5	83.91	3.85	84.2	3.91
6	63.59	3.77	63.2	3.77
1'	93.89	4.83	93.4	5.5
2'	71.62	3.61	71.5	3.61
3'	76.95	5.4	77.2	5.22
4'	67.07	3.45	69.3	3.58
5'	74.75	3.85	74.6	3.94
6'	62.45	3.8	62.0	3.77

* Data obtained in CD_3OD, ^{13}C and ^{1}H (600 and 150 MHz, J in Hz)

Data obtained CD_3OD, ^{13}C and ^{1}H (600 and 400 MHz)

4.10.3 Structural elucidation for compound PP-I-101841A (Acyl sucrose)

Acyl sucrose was isolated as a single brown spot (6.9 mg) using EtOAc:Hexane:AcOH (5:5:4.5:0.5). This is an acetylated compound and theNMR data is similar to that reported in the literature (Min *et al*., 2014). The accurate mass obtained from the HRTOFMS spectra showed showed a pseudo molecular ion at m/z 639.10 in the ESI^- negative mode and the ESI^+ positive mode showed a pseudo molecular ion at m/z 612.27^+. Supplementary information is given in Appendix 3.

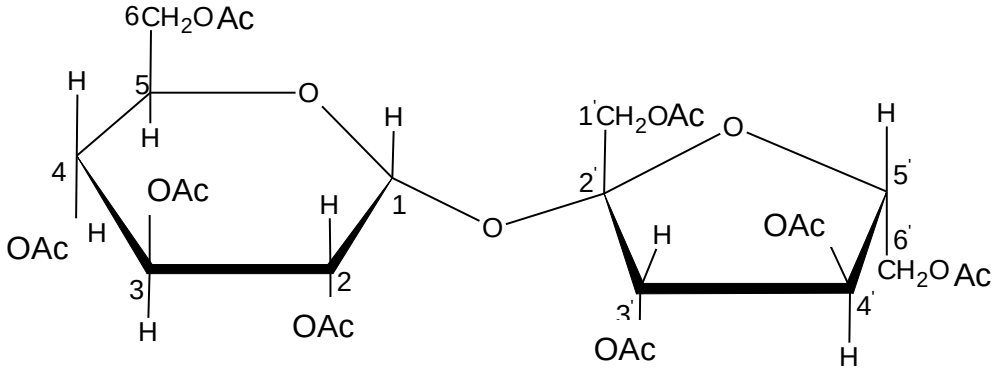

Figure 4.16: Structure of compound PP-I-101841A (Acyl sucrose)

Table 4.14: ^{1}H- and ^{13}C-NMR data of compound PP-I-101841A (Acyl sucrose) in CD_3OD

	Isolated compound #		Literature *	
Position	**δ_C(ppm)**	**δ_H(ppm)**	**δ_C(ppm)**	**δ_H(ppm)**
	20.63 (Acetyl CH_3)		20.6 (Acetyl CH_3)	
1	91.45	5.61	91.08	5.61
2	70.08	4.82	70.4	4.87
3	77.45	5.44	75.87	5.44
4	71.13	5.02	68.4	5.08
5	71.91	4.29	68.62	4.3
6	63.42	4.17	63	4.18
1'	64.99	4.16	61.86	4.16
2'	105.42		104.09	
3'	69.96	5.45	69.76	5.45
4'	76.59	5.37	75.16	5.36
5'	80.58	4.22	79.23	4.22
6'	63.78		63.71	
	171.89-172.64 (Acetyl C=O)			

* Data obtained in $CDCl_3$, ^{1}H and ^{13}C (400 and 100 MHz)

Data obtained CD_3OD, ^{13}C and ^{1}H (600 and 400 MHz)

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CHAPTER 5

DISCUSSION

5.1 *In vitro* inhibitory effects of *P. prunelloides* root extracts on α-amylase and α-glucosidase activities

One of the important strategies for the management of diabetes mellitus is to maintain near normal blood glucose levels in fasting and postprandial (Bailey, 2000; Ortiz-Andrade *et al*., 2007). Management of diabetes without side effects is still posing a challenge in the health sector. Diet rich in carbohydrates causes a sharp increase in the level of blood glucose as the complex carbohydrate in food is rapidly absorbed in the intestine (Kazeem *et al*., 2013). According to Rhabasa-Lhoret and Chiasson (2004), inhibition of the activity of α-amylase and α-glucosidase delay the degradation of carbohydrate, which in turn cause a decrease in the absorption of glucose, and as a result the reduction of postprandial blood glucose level. Acarbose and miglitol are α-glucosidase inhibitors which act competitively and modulate the postprandial digestion and absorption of carbohydrates (Nolte and Karam, 2001; Kim *et al*., 2005). These two compounds have different binding affinities; acarbose and miglitol both target α-glucosidases: sucrase, maltase, glycoamylase and dextranase. α-amylase can only be targeted by acarbose while isomaltase and β-glucosidase are targeted by miglitol (Nolte and Karam, 2001). These inhibitors are known to causes gastrointestinal discomfort such as flatulence and diarrhoea (Shai *et al*., 2010).

In this study, the effects of *P. prunelloides* on the activities of α-amylase and α-glucosidase were investigated *in vitro.* The potency of an enzyme inhibitor is usually determined and reported in terms of an IC_{50} value (inhibitor concentration corresponding to 50% inhibition). Low IC_{50} values suggest a higher affinity of the enzyme for the inhibitor. All the extracts (water, ethanol, aqueous-ethanol and hexane) investigated in this study demonstrated significant potency in inhibiting the activities of α-amylase and α-glucosidase. The hexane extract had the strongest inhibitory effect against the α-amylase with appreciable activity of more than 70 %, which was supported

by the lowest IC_{50} (0.48 µg/mL). This result is in agreement with previous reports which indicated that excessive inhibition of pancreatic *α*-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Apostolidis *et al*., 2007; Cheplick *et al.*, 2010). Though α-amylase inhibitory activity had positive effects on prevention of hyperglycemia linked to Type II diabetes mellitus, mild inhibitory activity is desirable. Hence the ethanol extract was found to be the most suitable of the extracts showing mild/moderate activity in inhibiting this enzyme. Ethanol extract had the highest IC_{50} value of 18.51 µg/mL and the reference drug, acarbose was found to be weak inhibitor against α-amylase in this study. This is consistent with other reports that either acarbose is described as a very weak inhibitor with an IC_{50} value of about 1 mg/mL or no inhibition of α-amylase (Subramanian *et al*., 2008).

To characterise the effectiveness of an extract or drug, mode of inhibition (that is inhibition type) is often applied (Smith *et al*., 2005). Inhibitors of enzymes obeying Michaelis-Menten kinetics are often identified and differentiated from one another by means of their effect on the kinetic constants, Km (the Michaelis-Menten constant) and Vmax (maximum velocity of the enzyme) using Lineweaver-Burk plots (Mathews and van Holde, 2004). The mode of inhibition of ethanol extract done on Lineweaver-Burk plot presented mixed non-competitive type of inhibition. This type of inhibition explains that the active components in the extract did not compete with the substrate. This suggests that inhibitors bind to a separate site on the enzyme to retard the conversion of disaccharides to monosaccharides (Ogunwande *et al*., 2007).

Similarly, the inhibitory activities of the roots extracts of *P. prunelloides* were investigated using α-glucosidase together with maltase and sucrase. Maltase and sucrase are forms of α-glucosidase which catalyse the hydrolysis of maltose and sucrose to their constituent monosaccharides respectively (Toda *et al*., 2000). In this study, hexane and ethanol extracts exhibited the strongest activity against α-glucosidase. The IC_{50} values of these extracts on α-glucosidase were lower than those of other extracts and the standard (acarbose). The activity of hexane and ethanol extracts on α-glucosidase could be attributed to the presence of the phytochemicals present in the extracts, thus α-glucosidase can be inhibited by both polar and no-polar compounds.

For the sucrase assay, water extract demonstrated the lowest IC_{50} which was significantly lower than all the other extracts and the standard. This result implied that the active components in the water (both polar and non-polar) were responsible for the activity. The high activity shown by water extract agrees to the traditional preparation of the plant thus these results validate the use of *P. prunelloides* roots by traditional healers in the management of diabetes mellitus. Aqueous-ethanol demonstrated the strongest inhibition on maltase, making it the most active extract than other tested extracts and the standard. The strong inhibition shown by the aqueous-ethanol extract may suggest that the active components are largely polar in nature.

The strong inhibition of α-glucosidase, sucrase and maltase by these extracts (ethanol, hexane, water and aqueous-ethanol, respectively) agrees with the reports Kwon *et al*. (2007) that natural α-glucosidase inhibitors from plants have shown strong inhibitory activity against α-glucosidase and therefore can be potentially used as an effective therapy for the management of postprandial hyperglycemia with minimal side effects.

Ethanol extract showed the best activity on both α-amylase and α-glucosidase activity hence the mode of inhibition was studied. Lineweaver-Burk plot for ethanol extract showed that this extract inhibit α-glucosidase non-competitively. This suggests that the active component of the extract binds to a site other than the active site of the enzyme and combine with either free enzyme or enzyme substrate complex possibly interfering with the action of both (Mayur *et al*., 2010).

5.2 *In vitro* antioxidant potential of *P. prunelloides* root extracts

The overloading of free radicals in human body also known as oxidative stress plays an important part in the development of chronic and degenerative illness such cancer, diabetes mellitus, autoimmune disorders, cardiovascular and neurodegenerative disease (Pham-Huy *et al*., 2008). Antioxidants fight against free radicals and as such protecting the body from various diseases *(*Khatoon *et al*., 2013). Oxidative damage can be overcome by many synthetic drugs available but these drugs are associated with adverse side effects (Suwalsky and Avello, 2014). The antioxidant activities of plants may act by preventing the production of free radicals or by neutralizing/scavenging free radicals

produced in the body or chelating the transition metal composition (Amic *et al*., 2003). Hence continuous search and study on the anti-oxidative properties of medicinal plants is imperative and should be given dedicated attention. These plant-derived antioxidants include ascorbic acid, carotenoids and phenolic compounds (Ghani, 2003). Ascorbic acid (vitamin C) is a water-soluble micronutrient required for multiple biological functions (Duarte and Lunec, 2005). Gallic acid is an antioxidant which is used as a remote astringent in cases of internal haemorrhage as well as in the treatment of albuminuria and diabetes (Deutschländer, 2009).

The radicals investigated in this study have been implicated in the pathogenesis of Type II diabetes and its associated complications (Maritim *et al*., 2003). The use of different models for this anti-oxidative studies were supported since a single method cannot give a full evaluation of the anti-oxidative capabilities due to the involvement of multiple mechanisms in the induction of oxidative stress (Ceriello, 2006).

The antioxidant activity of the extract was determined using the stable DPPH radical. DPPH is a free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule (Auwal and Islam, 2014). The DPPH radical scavenging ability of the extract was based on its ability to decolourize the deep purple colour that is measured from the changes in absorbance (Wettasinghe and Shahidi, 2000). Using this model, the ethanol extract exhibited remarkable activity when compared with other extracts, but less active than the standard (ascorbic acid), which showed the lowest IC_{50} value. This result is expected since ascorbic acid (reference drug) is an established anti-oxidative drug for DPPH radicals (Susanti *et al*., 2007). The ability of the ethanol extract to scavenge this radical higher than other extracts have shown potency since it was able to reduce the stable free radical of DPPH to the yellow coloured diphenylpicrylhydrazine (Wettasinghe and Shahidi, 2000).

Muleya *et al*. (2015) reported high activity of *P. prunelloides* acetone root extract in DPPH radical scavenging assay. Results obtained from the current study revealed that water extract had the highest activity in scavenging DPPH radical. Thus, the active components in the water extract are capable of donating hydrogen to a free radical in order to remove electron which is responsible for radical's reactivity (Auwal and Islam,

2014). The potency demonstrated by water extract shows the ability to inhibit oxidative stress which consequently might ameliorate Type II diabetes and its associated complications.

Superoxide radical anion (O_2^-) originates from the one-electron reduction of free molecular oxygen by nicotinamide adenine dinucleotide phosphate oxidase, which is a membrane-bound enzyme (Babior, 2000). The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of vessels (large and small) and in the myocardium (Giacco and Brownlee, 2011). This radical is known to be very harmful to cellular components that contributes to tissue damage and other diseases, since it is known to be a precursor of most reactive oxygen species (Khan *et al*., 2012). In this study, all the extracts (ethanol, aqueous-ethanol, water and hexane) scavenged superoxide uniformly at all concentrations, except at high concentration where the standard (gallic acid) had strong inhibition. The ability of the root extracts of *P. prunelloides* to inhibit this radical agrees to the findings of Giacco and Brownlee (2011) and Khan *et al*. (2012) suggesting that the plant might assist in the reduction of production of superoxide and it will also reduce the damaging effects of the cellular components that contributes to tissue damage and other diseases such as diabetes mellitus.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage. They cause reduction of disulfide bonds in proteins specifically fibrinogen resulting in unfolding and scrambled refolding into abnormal spatial configurations (Lipinski, 2011). The ethanol extract and the standard (gallic acid) were found to be more effective in hydroxyl radical inhibition than other extracts. As noted by Kazeem and Ashafa (2015), hydroxyl radicals are highly reactive in causing enormous biological damage to any living cell, but this untoward effect may be mitigated by the presence of ethanol extract of *P. prunelloides*.

Humans are unable to eliminate iron released from the breakdown of transfused red blood cells and thus the excess of this ion is deposited in the liver, spleen and endocrine organs. The accumulation of the toxic metal ion causes tissue damage and leads to various complications such as heart failure, endocrine abnormalities like diabetes, liver failure hypothyroidism and ultimately early death (Loukopoulos, 2005; Taher *et al*., 2006). In

this study, the aqueous-ethanol extract was found to be the most active extract which effectively interfered with the formation of ferrous and ferrozine complex, thus suggesting that it has chelating activities and captures ferrous before ferrozine formation (Enein *et al*., 2003). The ability of aqueous-ethanol extract to successfully interfere with the formation of ferrous and ferrozine complex as noted by Enein *et al*. (2003) can assist in removal of toxic ions formed that causes damages and other complications which leads to diabetes mellitus (Taher *et al*., 2006).

Antioxidant activity of the extracts is strongly dependent on the types of solvent used because compounds with different polarity exhibit different degrees of antioxidants potential (Kumaran, 2007; Caunii *et al*., 2012). It is obvious from the results of this study that the extracts with non-polar and polar compounds showed radical scavenging activities. Our assertions are in agreement with the report of Caunii *et al*. (2012) and it may be logically inferred that *P. prunelloides* is endowed with promising antioxidant constituents that protect against a wide range of free radical-induced diseases including diabetes mellitus.

5.3 Phytochemicals and biological screening

The presence of secondary metabolites contributes to the medicinal value of a plant (Edeoga *et al*., 2005). The root extracts of *P. prunelloides* indicated the presence of saponins, tannins, flavonoids, terpenoids, alkaloids and cardiac glycosides. The differences in the percentage composition of each phytochemicals in the screened extracts in this study could be due to the varying extraction solvent used, which is probably due to differences in polarity.

Saponins provide several health benefits such as reducing cholesterol levels in the intestinal tract, therefore, it helps in mitigating obesity and antimutagenicity thus preventing cancer cells from growing (Mpofu *et al*., 2014). van Wyk and Wink (2004) have reported that saponins have antidiabetic, lipid and cholesterol lowering activities. The non-sugar parts of saponins have a direct antioxidant activity; hence, reducing the risk of cardiovascular disorders (Mpofu *et al*., 2014). Diosgenin (type of saponin) was isolated from the roots of *P. prunelloides* and has been implicated in reducing glucose levels in the blood (McAnuff *et al*., 2005) and antioxidant activity (Son *et al*., 2007;

Jayachandra *et al*., 2009). Mpofu *et al*. (2014) reported the presence of saponin in the n-hexane extract and fractions of *P. prunelloides* rhizome. It is obvious from the results of this study that all extracts have saponins (13.9 %) indicating the ability of this plant to manage diabetes (McAnuff *et al*., 2005).

Studies have shown that tannins possess multiple biological activities including antioxidant, anticancer and antimicrobial activities (Gin *et al*., 1999; Amarowicz, 2007), and have antidiarrhoeal properties (Bruneton, 1995). Tannins have been reported to have health benefits in Alzheimer and diabetes (Ono *et al*., 2004), and effective in protecting the kidneys (Bajaj, 1988). Mpofu *et al*. (2014) reported the presence of tannins in the rhizomes of *P. prunelloides*. In this study, tannins were present in all the extracts but the concentration differs. The highest concentration of tannins was found in the aqueous-ethanol extract with concentration of 45.60 mg gallic acid/g. The high concentration of tannins in aqueous-ethanol extracts may be attributed to the polar compounds in the extract. The roots of *P. prunelloides* are used traditionally for treating diarrhoea, the presence of tannins in all the extracts agrees with the work of Bruneton (1995) that tannins have anti-diarrhoeal properties. These results are in agreement with the report of Ono *et al*. (2004), that the presences of tannins have health benefits in diabetes. Thus *P. prunelloides* root extracts have potential in managing diabetes mellitus.

Flavonoids and phenolic acids are the largest classes of plant phenolics and are known to have good antioxidant activity both *in vitro* and *in vivo* (Kasote *et al*., 2015). The presence of flavonoids in ethanol root extract of *P. prunelloides* was supported by Robak and Gryglewski (1988), which stated that flavonoids are most effective antioxidants mainly because they can easily scavenge superoxide anions (Cook and Samman, 1996). Mpofu *et al*. (2014) had reported the presence of flavonoids in the n-hexane extract and fractions of *P. prunelloides* rhizomes.

Although the presence of flavonoids was very low in all the extracts, Song *et al*. (2009) reported that flavonoids may preserve beta-cell function by reducing oxidative stress-induced tissue damage. Therefore the compound protects against the progression of insulin resistance to Type II diabetes, hence the presence of flavonoids in this plant may be a good potential for managing diabetes mellitus.

Plants that contain high levels of phenolics are considered to be good sources of antioxidants and therefore it is important to quantify the total phenolics in plants (Gorinstein *et al*., 2004). According to Li *et al*. (2010), phenolic compounds possess multiple biological properties such as antitumor, antimutagenic and antibacterial properties. In this study, the total phenolics in the root extracts of *P. prunelloides*was generally low although water and aqueous-ethanol extracts had the higher concentration (0.07 mg gallic acid/g) when compared to the other extracts. As reported by Mayur *et al*. (2010), flavonoids are phenolic compounds with antioxidant and antidiabetic potentials due to the presence of hydroxyl groups that confer scavenging ability on them. The low quantity of phenolics and flavonoids detected in the extracts of *P. prunelloides* suggest that the antioxidant and antidiabetic effects may be due to the presence of other compounds.

Terpernoids are generally soluble in common organic solvents. However, low molecular weight terpenoids such as essential oils, are sparingly soluble in water and usually extracted with non- polar solvents (Bruneton, 1999). Terpenoids have been reported to have various health benefits such as anti-inflammatory and antimicrobial activities (Shimon, 2009) and known to poses antioxidants (Graßmann, 2005). Muleya *et al*. (2015) reported the presence of terpenoids in the roots of *P. prunelloides* and showed remarkable antimicrobial activity. Terpenoids were found to be present in all the extracts of *P. prunelloides* and their availability agrees with the report of Graßmann (2005). The antioxidant activity of the extracts may be attributed to the presence of terpenoids thus, the plant can be used for managing diabetes.

The presence of alkaloids in plants do not feature strongly in herbal medicine since they are known to be extremely toxic. However, they have always been important in allopathic systems where the dose is controlled and in homoepathy, where dose-rate is so low as to be harmless (Trease and Evans, 2005). Alkaloids have been reported to show effect on the nervous system, digestive system, blood circulatory system and they can act as anticancer and anti-inflammatory agents (Jacobsen and Salguero, 2003). Various alkaloids such as catharanthine, leurosine and vindoline have been reported to have hypoglycemic effect (Deutschländer, 2009). Mpofu *et al*. (2014) reported the presence of trace amounts of alkaloids in n-hexane crude extract and fractions of *P. prunelloides* rhizomes. In this

study, alkaloids were only found in water extract and quantity was low making up to 0.6 %. The low content of alkaloids in the roots of *P. prunelloides* is significant since it supports the findings of Trease and Evans (2005) that plant parts containing high alkaloids content are not recommended in herbal medicinal practices because such regions are extremely toxic when consumed in large quantity.

In small doses, cardiac glycosides are used medicinally for controlling congestive heart failure (Harborne and Baxter, 1993). Generally, concentrations of cardiac glycosides in plants are very low, lower than 1% (Bruneton, 1995). In this study, cardiac glycosides were only present in aqueous-ethanol extract. The results are in agreement of the report of Harborne and Baxter (1993), thus the plant can be used for controlling congestive heart failure without any fatality. It is important, however, that the concentration of the cardiac glycoside is known for proper treatment.

Three compounds were isolated and two identified as sucrose and triterpenoid saponin (Tormentic acid). The sucrose obtained from the study was not tested while tormentic acid was tested for α-glucosidase and α-amylase inhibitory activities. Sucrose is a widely known and available disaccharide composed of glucose and fructose monomers and with a moderate glycemic index (Foster-Powell *et al*., 2002). The uses of sugar in the food industry as studied by Cotton *et al*. (1955) include as sweetner and flavour, antioxidant effect and gel formation. Taddio *et al*. (2009) reported that sucrose has analgesic and calming effects on newborns. The obtained sucrose in this study from the roots of *P. prunelloides* relates with the findings of Ndlovu (2009). Triterpenoids have been reported to have antidiabetic effect (Chen *et al*., 2006) and antioxidant properties (Somova *et al*., 2003). Ivorra *et al*. (1989) also reported hypoglycemic effect of triterpenoids while Wu *et al*. (2014) reported that the compound lowers hyperglycemia and hypertriglycemia in high fat-fed mice. Tormentic acid have also been reported to possess anti-inflammatory effects (Jung *et al*., 2005; Chang *et al*., 2011; An *et al*., 2011).

5.3.1 *In vitro* inhibitory effects of the active compounds isolated from *P. prunelloides* on α-amylase and α-glucosidase activities

The activity of the fractions and compounds were determined *in vitro* on α-amylase and α-glucosidase. Fraction PP-I-101835BII was found to be the best fraction to inhibit both

enzymes. This fraction was obtained from chloroform: methanol (9:1), compounds from this fraction are non-polar. Therefore, activity of this fraction may be attributed to the presence of non-polar compounds which might have worked in synergy. Compound PP-I-101836C was obtained from fraction PP-I-101835BII and this compound was found to be the second best active compound which had similar inhibition on α-glucosidase with compound PP-I101840B. Compound PP-I-101836C was developed from non-polar solvent and the structure elucidation for this compound was not done.

Tormentic acid inhibited α-glucosidase and α-amylase in competitive and uncompetitive manner respectively, which was different from the mixed non-competitive and non-competitive patterns observed in α-amylase and α-glucosidase of the ethanol crude extracts. The competitive inhibition of α-glucosidase by tormentic acid suggests that the inhibitory component of this compound binds reversibly to the active site of the enzyme and occupies it in a mutually exclusive manner with the substrate (Copeland, 2000). These results are in agreement with Kim *et al*. (2005), that acarbose and miglitol are competitive inhibitors of α-glucosidase. Thus tormentic acid act competitively on this enzyme while modulating the postprandial digestion and absorption of starch and disaccharides. This is because any plant or drug which is a strong inhibitor of α-glucosidase could serve as effective therapy for postprandial hyperglycemia with minimal side effects (Kwon *et al*., 2006). The uncompetitive inhibition of α-amylase by tormentic acid suggests that it binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate inhibitor complex (Bachhawat *et al*., 2011).

The high activity shown by the compounds obtained in this study suggests that non-polar compounds belonging to class of triterpenoids in the roots of *P. prunelloides* are responsible for the elicited anti-diabetic properties. The inhibitory activities and mode of inhibition exhibited by tormentic acid on the specific activities of α-glucosidase and α-amylase is reported for the first time in this study. Overall, the results from the study add to the existing information that the investigated compound is a good candidate for the management and treatment of diabetes mellitus.

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CHAPTER 6

CONCLUSION

General conclusions

Aqueous-ethanol extract gave the highest average percentage yield when compared to water, ethanol and hexane extracts with the latter giving the lowest yield. This suggests that the amounts of non-polar compounds in the roots are very small. Ethanol being a solvent of high polarity, it was interesting to find that the active compound was obtained from non-polar fraction. The activity of the ethanol extract was higher than the isolated compound which suggests that other phytochemical components of the extracts contribute to the inhibition of the tested enzymes which are believed to be working in synergy.

The presence of phytochemicals in the root extracts of *P. prunelloides* has effects on the scavenging activity of free radicals and the modulatory properties of the tested enzymes. The ethanol extract had the best antidiabetic effect judging by its inhibitory role on both α-amylase and α-glucosidase. The water, hexane and aqueous-ethanol extracts showed significant antioxidant potentials in inhibiting/ scavenging DPPH, superoxide, hydroxyl and metal ion radicals. The isolated tormentic acid from the roots of *P. prunelloides* might be a potential drug for diabetes mellitus.

Ethanol and hexane extracts showed the best activity on α-glucosidase, water extract was best in sucrase while aqueous-ethanol was found to be best in maltase. The best activity on α-amylase was found to be only the ethanol extract. On the basis of the current study, it can be concluded that ethanol extract possess anti-diabetic action through inhibition of α-amylase and α-glucosidase over mixed non-competitive and non-competitive type of inhibition pattern respectively.

The high radical scavenging activity shown by water, hexane and aqueous-ethanol extracts on DPPH, superoxide, hydroxyl radical scavenging and metal ion chelating propose the use of the plant as a promising anti-oxidant which can protect against a wide

range of free radical-induced diseases including diabetes mellitus. A total of eight compounds were isolated from the roots of *P. prunelloides*, however only three were identified namely saponin triterpenoid (tormentic acid) and two sucrose compounds (acetylated and non-acetylated).

Tormentic acid and the other isolated compound (PP-I-101836C) were found to be active in inhibiting α-amylase and α-glucosidase enzymes. This observation indicates that tritepernoid class are responsible for the inhibitory activity of α-amylase and α-glucosidase in this plant. However, tormentic acid was found to have high inhibitory activity against both α-amylase and α-glucosidase enzymes; and mode of inhibition by this compound on α-glucosidase appeared to be competitive while on α-amylase was uncompetitive. Tormentic acid appears to be to a potential anti-diabetic drug due to its good activity which might be good for standardisation and commercialisation.

Native people in most developing countries including South Africa still rely on traditional medicines for the management of diabetes mellitus. The findings from the current study support the traditional use of *Pentanisia prunelloides* in the management of diabetes mellitus and further document the use of this plant in eastern Free State Province of South Africa.

Recommendations

Additional studies are needed in order to investigate other possible mechanisms *in vitro* for hypoglycaemic activity of the root extracts of *P. prunelloides* and tormentic acid

The isolated compounds are needed to be tested in synergy since the extract was better in terms of α-amylase and α-glucosidase activity than the isolated compound

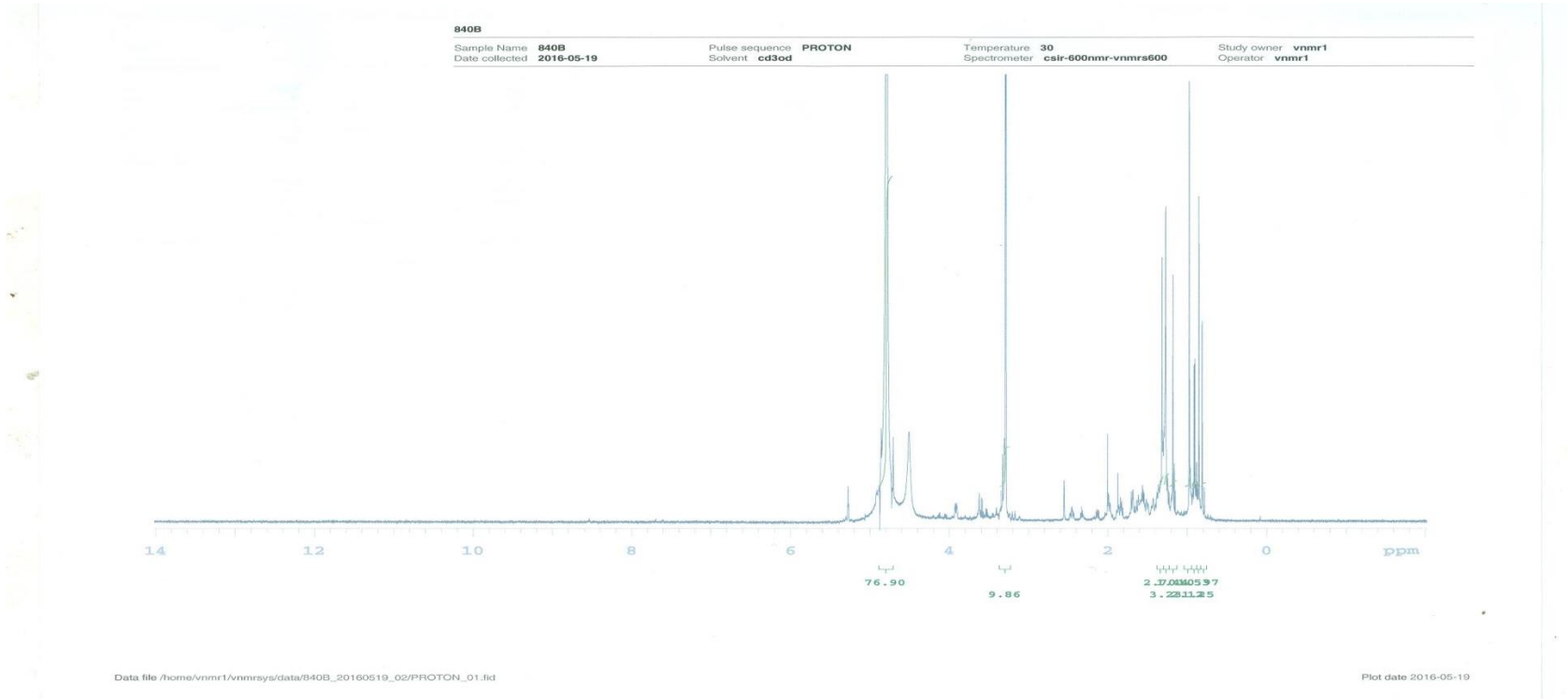
The leaves of *P. prunelloides* should be tested for their biological activities in comparison with the roots because the leaves are sometimes used. An attempt to isolate bioactive compounds from the leaves is also necessary

Traditionally, *P. prunelloides* is used in conjunction with other plants. Biological tests may be necessary to prove possible synergy between the different plants that are normally co-administered with *P. prunelloides.*

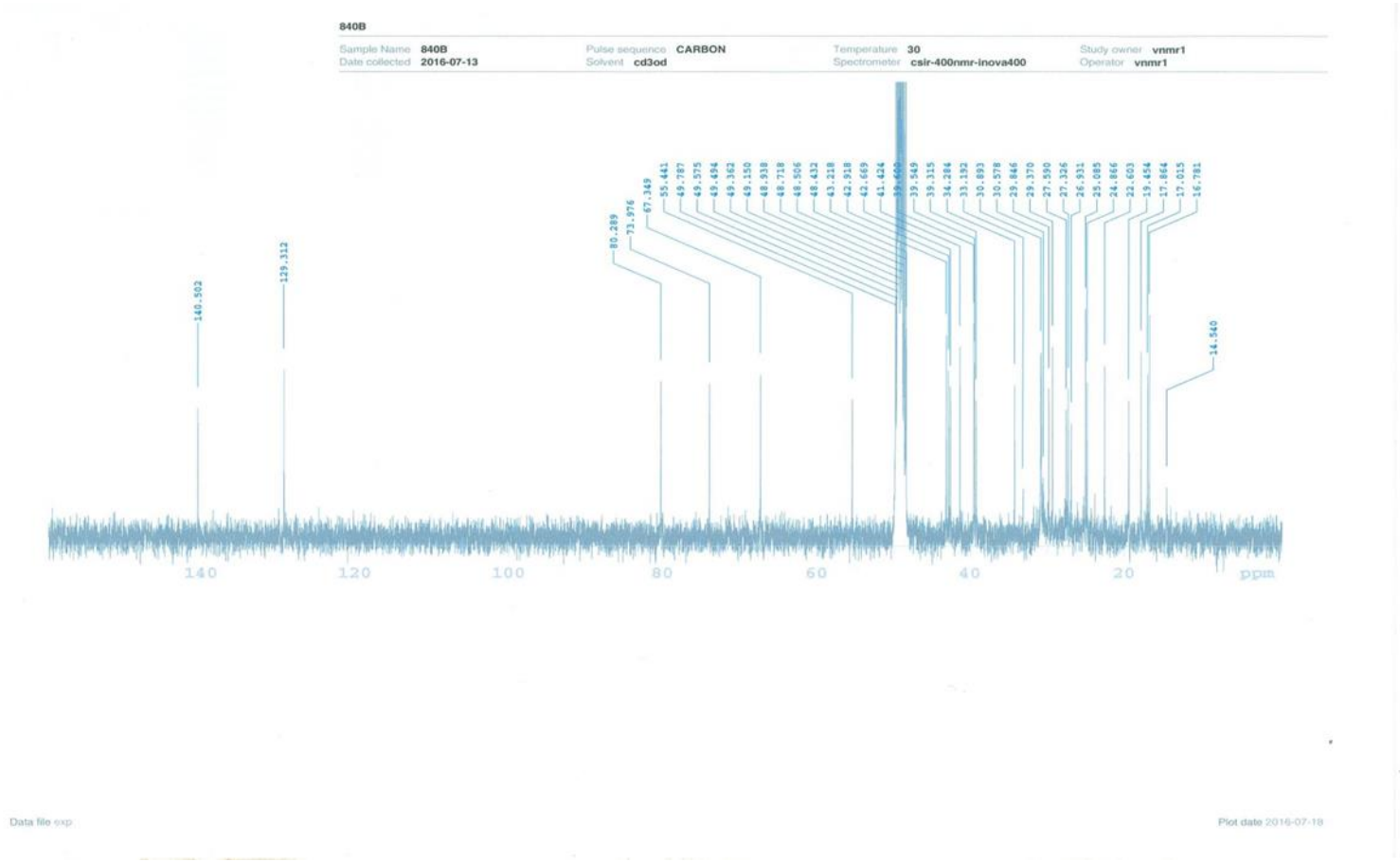
APPENDICES

Appendix 1

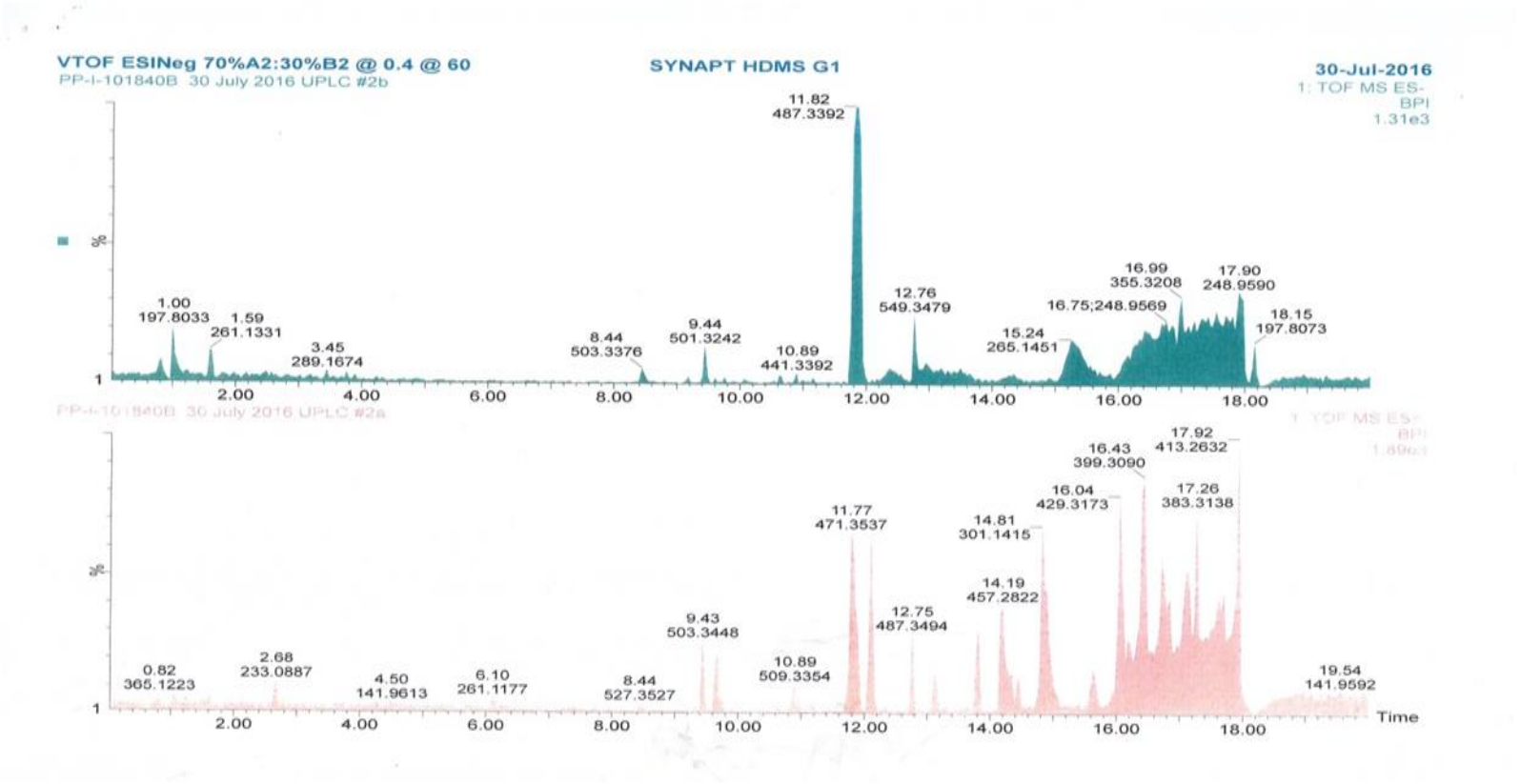
1. Compound PP-I-101840B (Tormentic acid) ^{1}H-NMR



2. Compound PP-I-101840B (Tormentic acid) ^{13}C-NMR



3. Compound PP-I-101840B (Tormentic acid) Mass analysis



Elemental Composition Report

Single Mass Analysis

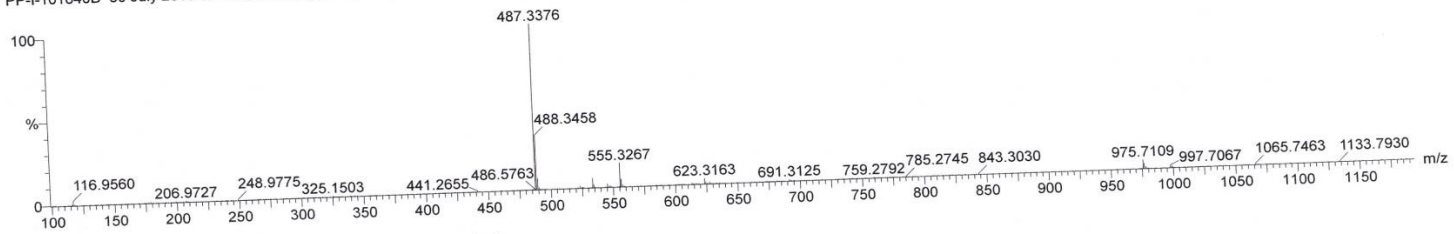
Tolerance = 10.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
98 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 10-40 H: 1-100 O: 0-20

VTOF ESINeg 70%A2:30%B2 @ 0.4 @ 60
PP-I-101840B 30 July 2016 UPLC #2b 305 (11.852) Cm (303:306)

SYNAPT HDMS G1

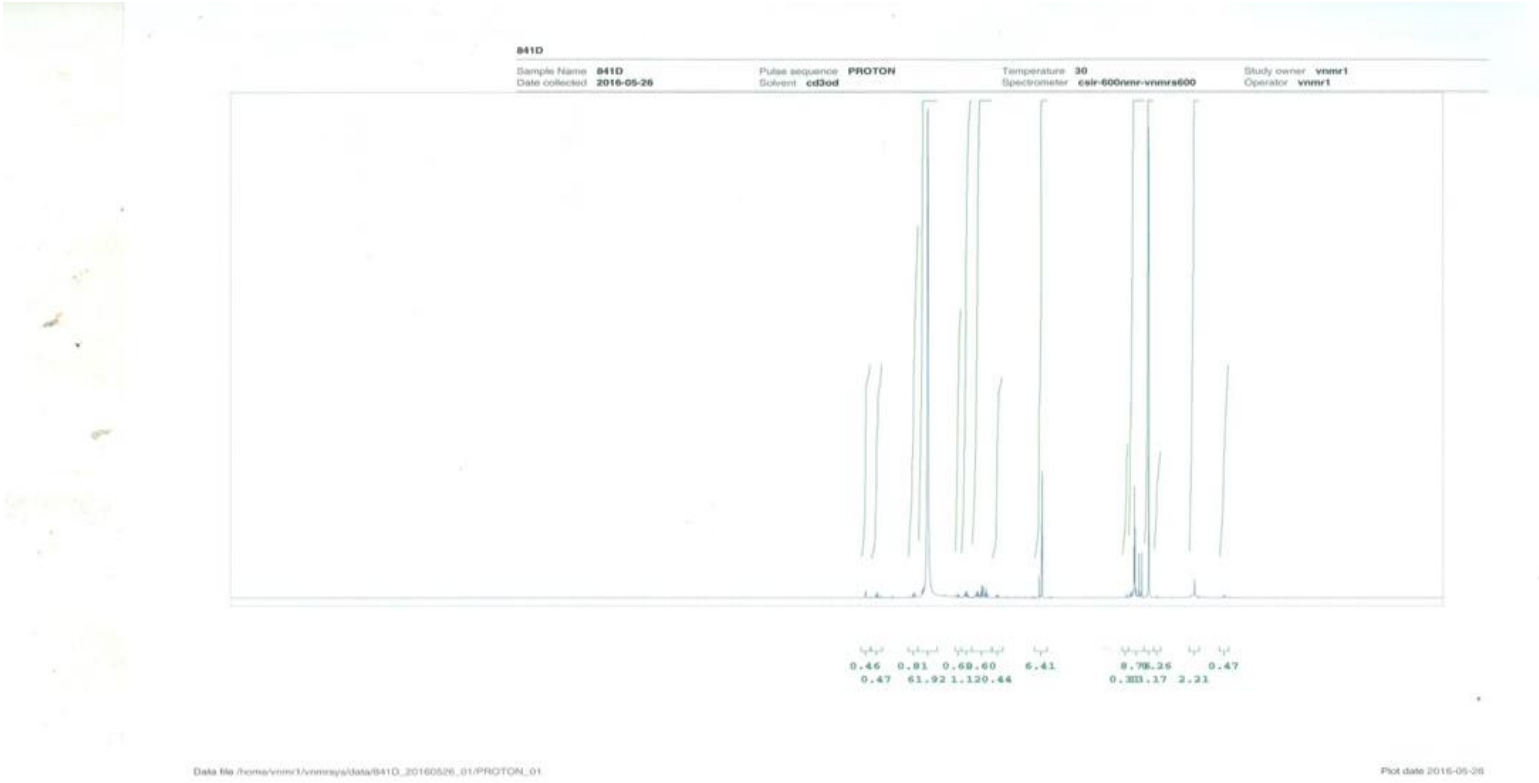
30-Jul-2016
1: TOF MS ES-
4.99e+003



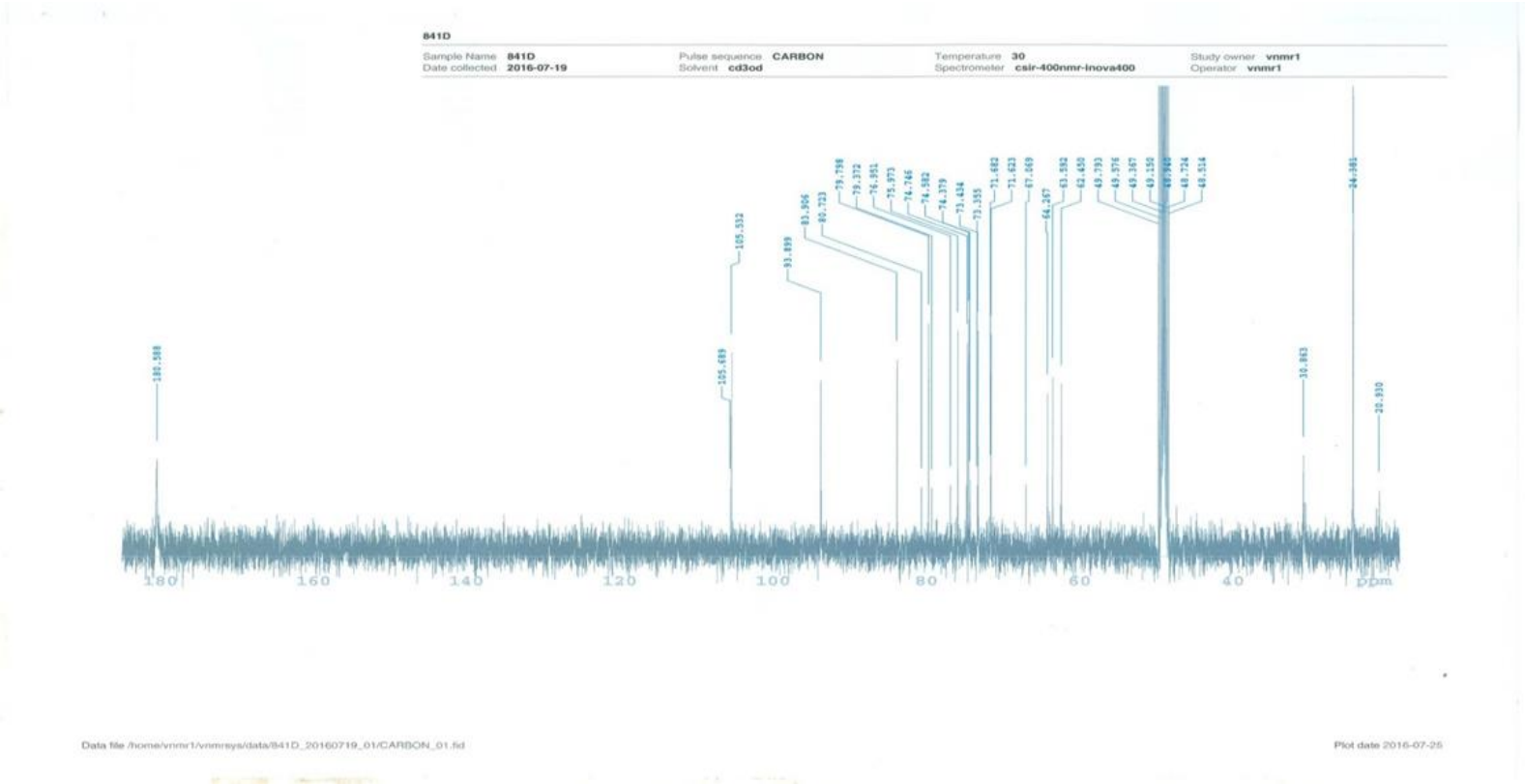
Minimum:		10.0	10.0	-1.5			
Maximum:				100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
487.3376	487.3424	-4.8	-9.8	7.5	15.2	0.0	C30 H47 O5
	487.3365	1.1	2.3	16.5	21.1	5.9	C37 H43

Appendix 2

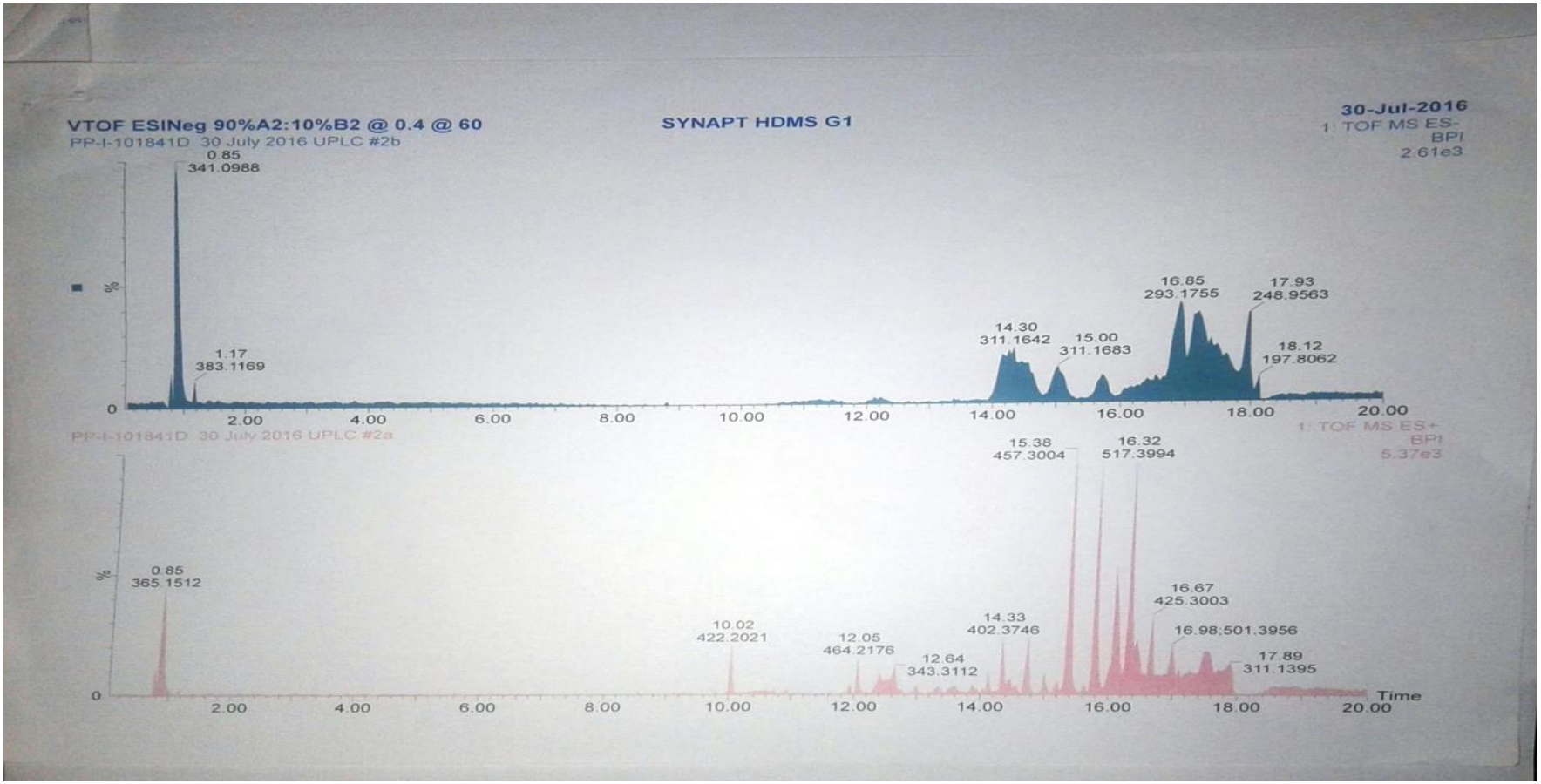
4. Compound PP-I-101841D (Sucrose) ^{1}H-NMR

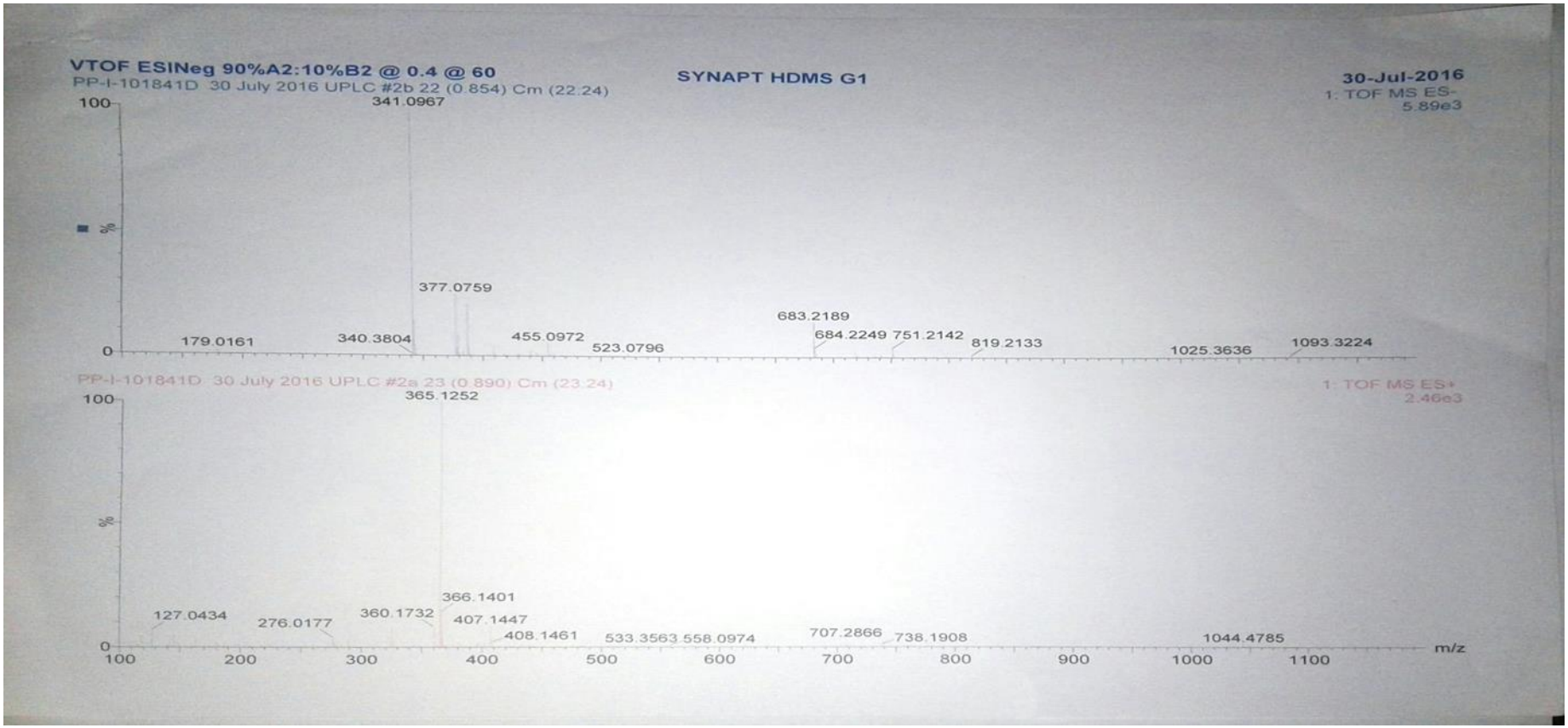


5. Compound PP-I-101841D (Sucrose) ^{13}C-NMR



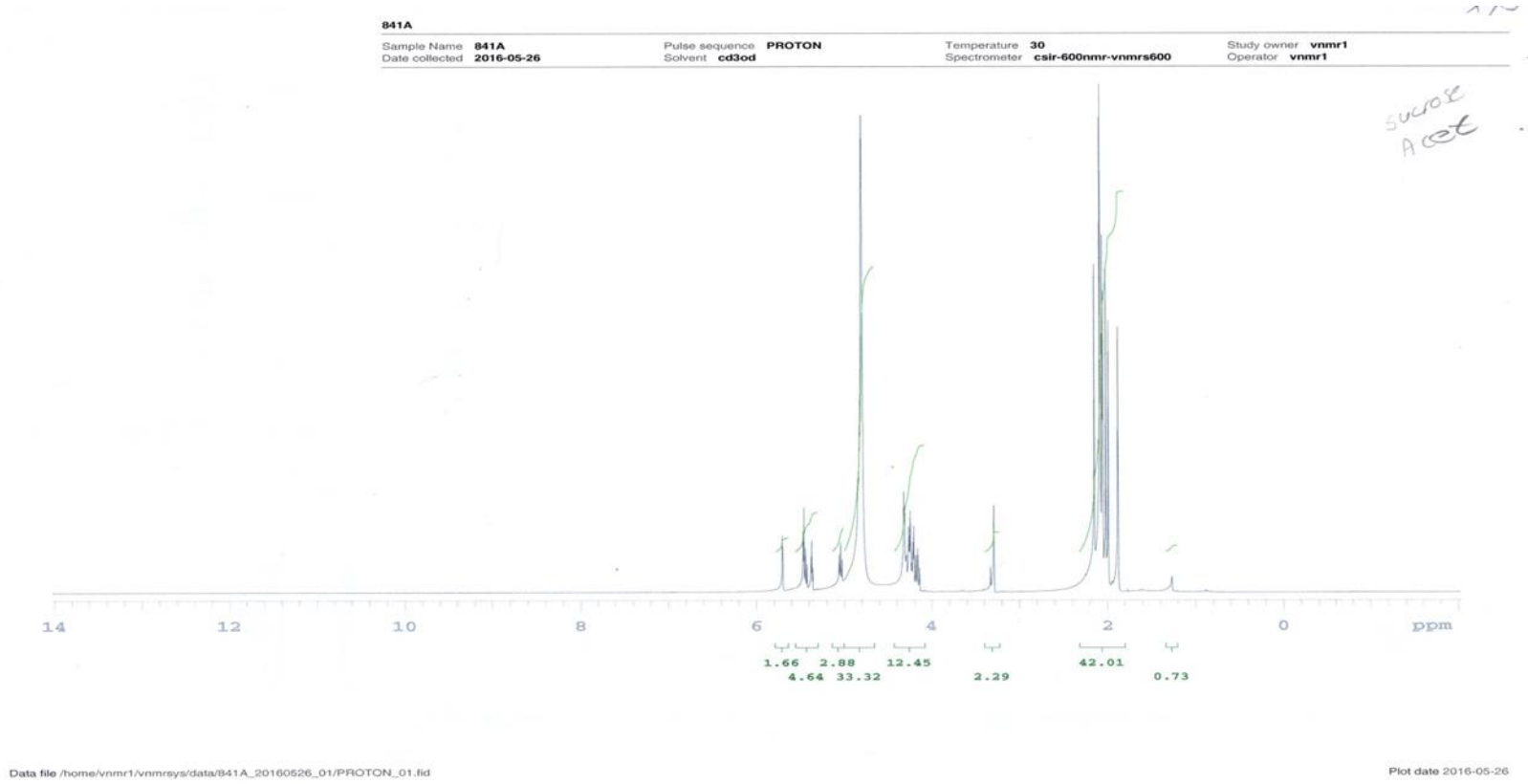
6. Compound PP-I-101841D (Sucrose) Mass analysis



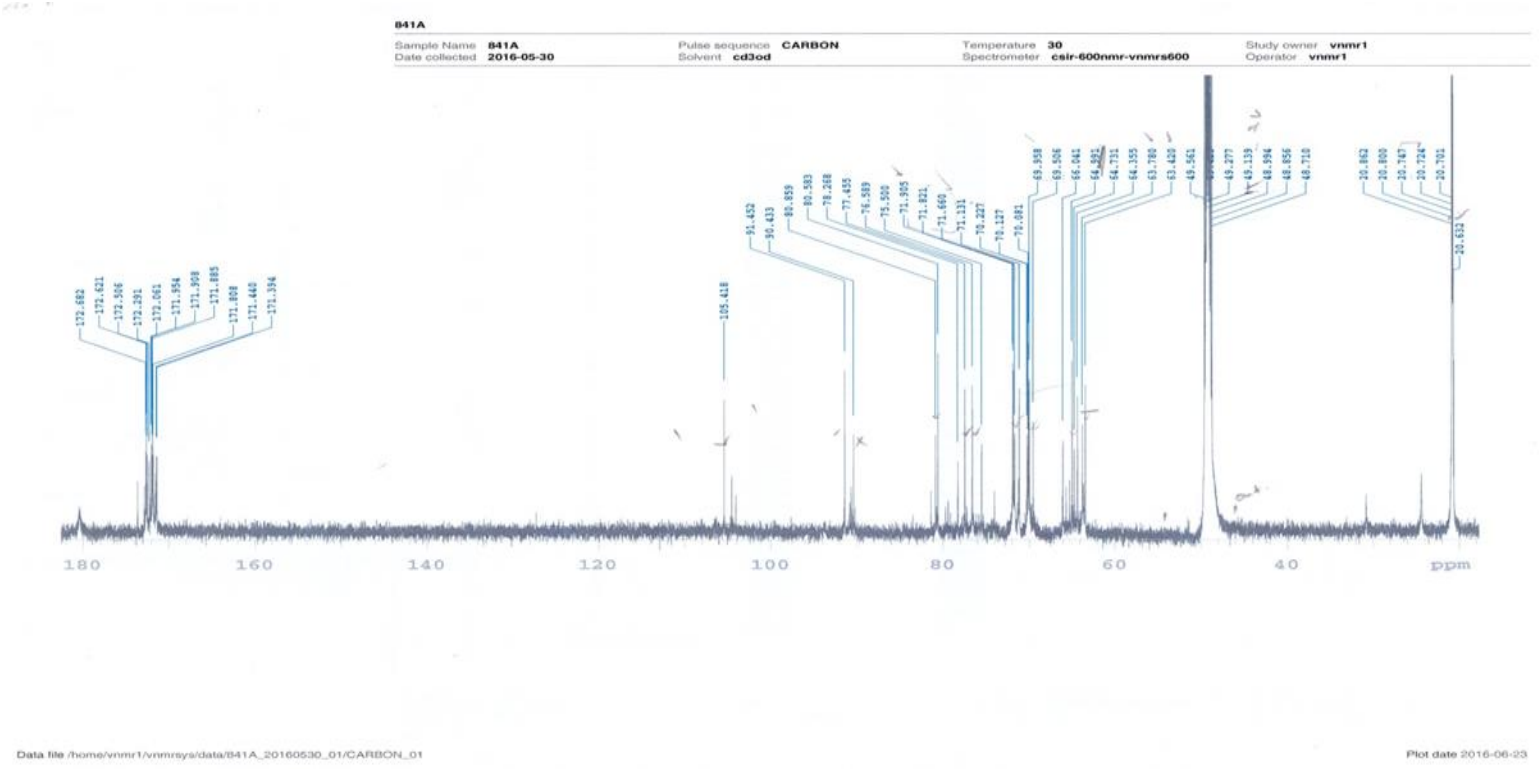

VTOF ESINeg 90%A2:10%B2 @ 0.4 @ 60
PP-I-101841D 30 July 2016 UPLC #2b 22 (0.854) Cm (22.24)
SYNAPT HDMS G1
30-Jul-2016
1: TOF MS ES-
5.89e3
100
%
0
341.0967
377.0759
683.2189
179.0161
340.3804
455.0972
523.0796
684.2249
751.2142
819.2133
1025.3636
1093.3224
PP-I-101841D 30 July 2016 UPLC #2a 23 (0.890) Cm (23.24)
1: TOF MS ES+
2.46e3
100
%
0
365.1252
366.1401
127.0434
276.0177
360.1732
407.1447
408.1461
533.3563
558.0974
707.2866
738.1908
1044.4785
100
200
300
400
500
600
700
800
900
1000
1100
m/z

Appendix 3

7. Compound PP-I-101841A (Acyl sucrose) ^{1}H-NMR



8. Compound PP-I-101841A (Acyl sucrose) ^{13}C- NMR



9. Compound PP-I-101841A (Acyl sucrose) Mass analysis