University of Venda

ISOLATION AND CHARACTERIZATION OF ANTIDIABETIC CONSTITUENTS OF *BRIDELIA MICRANTHA*

BY

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DECLARATION

I, Khanyisa Amanda Maluleke, hereby declare that the dissertation for the Master of Science in Chemistry degree at the University of Venda, hereby submitted by me, has not been submitted previously for a degree at this or any other university, that it is my own work in design and in execution, and that all reference material contained therein has been duly acknowledged.

…….…… ……..……

Signature (candidate) Date

DEDICATION

This work is dedicated to the loves of my life:
Mashudu and Floyd, my beloved siblings, who are my reason for being, my dad, who has always been my anchor and my mom (late), who has raised me to become the strong independent woman I am and finally, to my circle of friends.

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ABBREVIATIONS

%	Percent
µg	micro gram
µg/mL	microgram per millilitre
µL	micro litre
^{1}H	Proton
^{13}C	Carbon 13
1D	One dimensional
2D	Two dimensional
ADA	American diabetes association
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
CD26	Cluster of differentiation 26
CD_3OD	Deuterated methanol
CSIR	Council for scientific and industrial research
DEPT	Distortionless enhancement by polarization transfer
DPP	Dipeptidyl peptidase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DM	Diabetes mellitus
DMAA	1,3-dimethylamylamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
ER	Endoplasmic reticulum
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FT-IR	Fourier-Transform Infrared

g	Gram
GAE/g	Gallic acid equivalent per gram
GDM	Gestational diabetes mellitus
GIP	Glucose dependent insulin-tropic polypeptide
GLP	Glucagon-like peptide
GLUT	Glucose transporter
H_2O	Water
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HRMS	High resolution mass spectroscopy
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
IDF	International diabetes federation
INH	Isoniazid
IR	Infrared
LC/MS	Liquid chromatography mass spectroscopy
LFR (1 – 6)	Leaf fractions (1 – 6)
MeOH	Methanol
MES	Maximal electroshock
mg/kg	Milligrams per kilogram
MHz	Mega Hertz
MI	Myocardial infarction
min	Minutes
mL	Millilitres
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
NP-PEG	Natural product – polyethylene glycol
pH	Potential of hydogen

PIC	Picrotoxin
PPAR	Peroxisome proliferator-activated receptor
ppm	Parts per million
PGE	Prostaglandin endoperoxide
pTLC	Preparative thin layer chromatography
PTZ	Pentylenetetrazol
R^2	Correlation coefficient
Rf	Retention factor
RIF	Rifampicin
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Reverse transcriptase
SGLT	Sodium-glucose co-transporter
STM	Streptomycin
STR	Strychnine
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes Mellitus
TLC	Thin layer chromatography
TZD	Thiazolidinedione
UV	Ultraviolet
WHO	World health organisation

ABSTRACT

Bridelia micrantha (Hochst) Baill (*B. micrantha*) is a South African medicinal plant used by traditional healers in the treatment of different human ailments including diabetes, gastrointestinal ailments, joint aches, cough, conjunctivitis, skin problems and malaria. Previous studies have demonstrated the antidiabetic activities of *B. micrantha* crude extracts in *in vivo* studies. However, there are no studies on the compounds responsible for the antidiabetic activity of the plant. The purpose of this study was to isolate and characterize the antidiabetic constituents from *B. micrantha*.

Materials and methods

Crude methanolic extracts of root, stem and leaf were investigated using *in vitro* antidiabetic enzyme assays. Antioxidant activities were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing power assays. The isolation of the antidiabetic constituents was carried out using column chromatography on silica gel. Purification of the active fraction was carried out using preparative thin layer chromatography (pTLC). Structure elucidation of the compound was carried out using Nuclear Magnetic Resonance (NMR) spectroscopy and by comparison with literature.

Results

The results obtained in this study indicated that root, stem and leaf extracts exhibited high inhibition activity against α-glucosidase (98.52, 98.62 and 81.62% respectively). A moderate inhibition against α-amylase enzyme was observed for root (65.62%) and stem (61.86%) extracts. Leaf fraction LFR5 exhibited a high inhibition activity of 96.19% against α-glucosidase. Moreover, the isolated compound showed 96.74% inhibition against α-glucosidase. DPPH results revealed that antioxidant activity of crude extracts was not significantly different and they were concentration-dependent. Reducing power results revealed that stem (119.31 µg/mL) extract had higher activities compared to root (125.17 µg/mL) and leaf (291.88 µg/mL) extracts.

Conclusion

Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside was successfully isolated from *B. micrantha* leaves. Furthermore, quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside demonstrated the ability to inhibit significantly the carbohydrate hydrolysing enzyme α-glucosidase and therefore validate the ethnomedicinal use of *B. micrantha* in the management of diabetes.

1 RATIONALE AND LITERATURE REVIEW

1.1 Diabetes mellitus

Diabetes mellitus (DM) represents a heterogeneous group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or both . Whenever a meal is taken, there is an increase in blood glucose levels that stimulates insulin secretion resulting in an increase in transportation, biotransformation and storage of glucose in muscles and fat tissues (Asmat et al., 2016).

Under normal physiological functions, maintenance of blood glucose concentrations within a narrow range, despite wide fluctuations in supply and demand, relies on a tightly regulated and dynamic interaction between tissue sensitivity and insulin secretion through nutrient availability, hormones and neural inputs (Cerf, 2013; Ozougwu et al., 2013). In the diabetic condition, however, glucose in the blood system cannot efficiently enter the cells, resulting in hyperglycaemia at the extracellular compartment and intracellular hypoglycaemia (Contreras and Gutiérrez-García, 2017). This not only starves all the cells that need glucose for fuel, but also harms certain organs and tissues exposed to the high glucose levels (Siddiqui et al., 2013).

1.2 Demography of diabetes

According to Diabetes Atlas published by International Diabetes Federation (IDF), current estimates are that at least 451 million people worldwide had diabetes in 2017. The prevalence of diabetes is rapidly increasing and it is estimated that by the year 2045, around 693 million people will be suffering from diabetes (Cho et al., 2018). The greatest increase in prevalence is expected in Africa (Figure 1.1).

In Africa, 77.0% of all deaths attributable to diabetes occurred in individuals under 60 years of age in 2017, the highest proportion in the world (Table 1.1). This data emphasises the slow response of healthcare in Africa to the burden of diabetes as the infectious diseases remain the major health priorities. Furthermore, in Africa alone, 3.3 billion USD (6%) was spent on healthcare by people with diabetes in 2017, the lowest in any region despite the region housing 3% of individuals with diabetes (Figure 1.2).

The prevalence of diabetes is rapidly increasing in South Africa. The IDF reported a prevalence of 5.4% for South Africans between the ages of 20 and 79 in 2017 with projections of 6.2% by 2045. Several factors such as population growth, economic transition and urbanization associated with nutrition transition and obesity are thought to be responsible for the increased diabetes prevalence (Pheiffer et al., 2018).

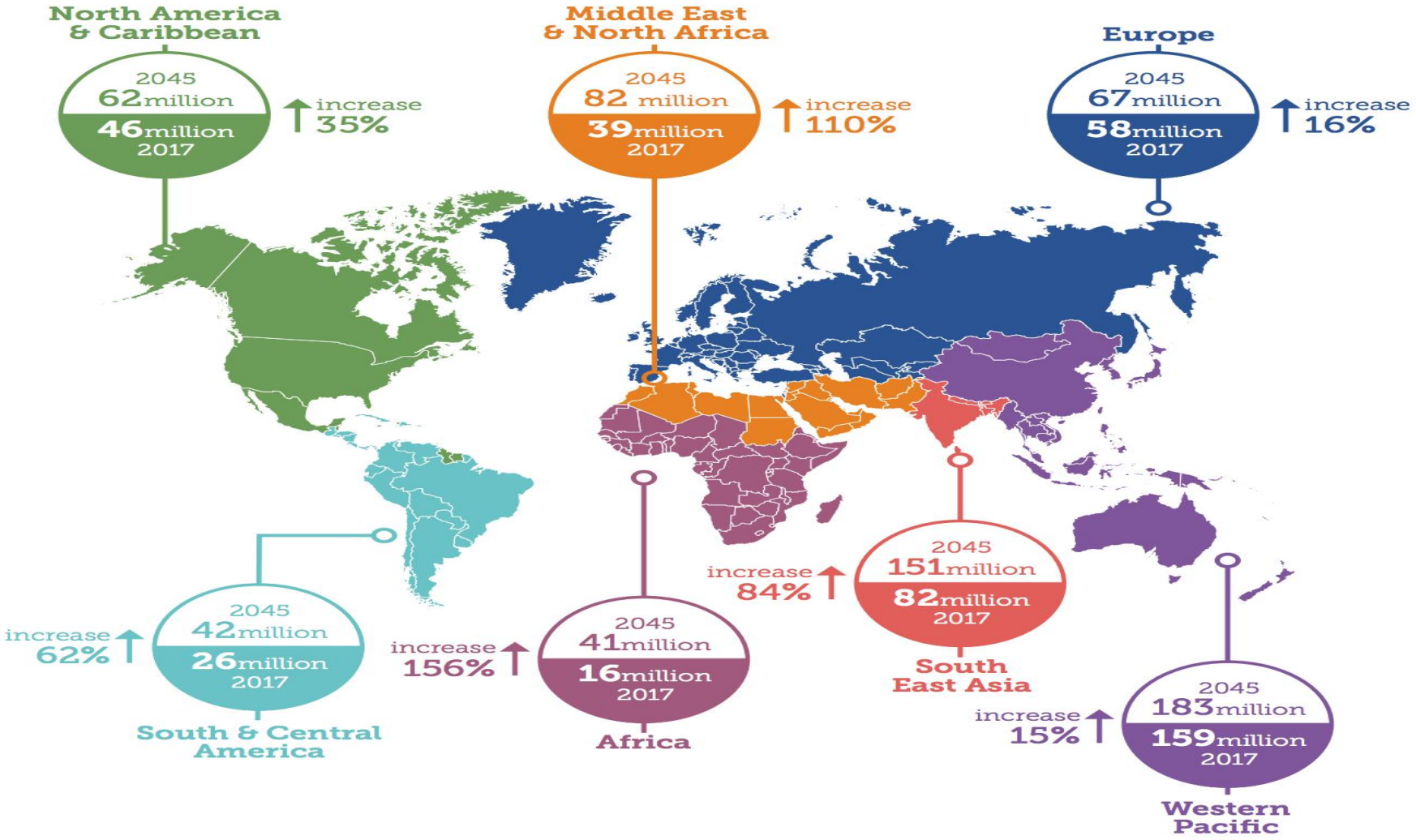
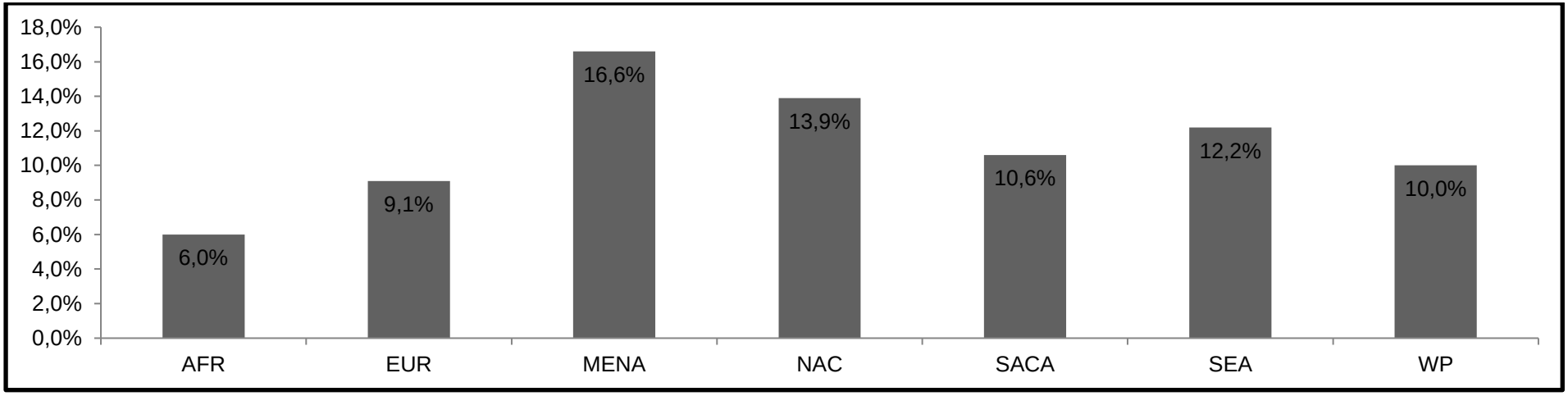


Figure 1.1: Number of people (20-79 years) with diabetes per region in 2017 and 2045 (Atlas, 2017).

Table 1.1: Proportion (%) of people who died from diabetes in 2017 before the age of 60 (Atlas, 2017).

Region	Number of deaths due to diabetes before age 60	Proportion of all deaths due to diabetes occurring before age 60
Africa	0.23 million (0.16-0.39)	77.0%
Europe	0.16 million (0.13-0.22)	32.9%
Middle East and North Africa	0.16 million (0.12-0.21)	51.8%
North America and Caribbean	0.13 million (0.11-0.14)	45.0%
South America and Central America	0.09 million (0.08-0.11)	44.9%
South East Asia	0.58 million (0.47-0.69)	51.5%
Western Pacific	0.48 million (0.43-0.60)	38.0%



AFR-Africa, EUR-Europe, MENA-Middle East and North America, NAC-North America and Caribbean, SACA-South America and Central America, SEA-South East Asia, WP-Western Pacific

Figure 1.2: Percentage of healthcare budget spent on diabetes (20-79 years) by region in 2017 (Atlas, 2017).

1.3 Types of diabetes

There are four major categories of diabetes mellitus: type 1, type 2, gestational and other specific types of diabetes mellitus (Egan and Dinneen, 2019).

1.3.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) diabetes is an autoimmune disease in which β-cells of the pancreas do not produce sufficient insulin (Siddiqui et al., 2013). This is followed by life threatening conditions of hypoglycaemia (low blood sugar) and hyperglycaemia (high blood sugar). When hypoglycaemia develops, cells do not get enough glucose and patients suffer from confusion, loss of consciousness, and can result in a coma (Asmat et al., 2016; Mukherjee et al., 2011)

1.3.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes, accounting for 85-90% of all cases worldwide (de Faria Maraschin, 2013). American Diabetes Association (ADA) defines T2DM as a heterogeneous group of disorders characterized by insulin resistance, a disorder in which the cells in the body do not utilize insulin properly (Association, 2014a). This abnormal regulation of carbohydrate metabolism results in hyperglycaemia. As the disorder progresses, β-cells gradually deteriorate in function and lose the ability to secrete sufficient insulin to overcome insulin resistance, after which insulin therapy will be required (Saisho, 2015; Siddiqui et al., 2013). Factors such as sedentary lifestyles and obesity are prominent leading cause of insulin resistance (Patel et al., 2016).

1.3.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is diabetes that is first diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation (Association, 2018). GDM accounts for 90-95% of all cases of pregnancy and is the most common disorder encountered during pregnancy (Kokic et al., 2018). GDM has been related to substantial adverse health outcome both for the mother and foetus. Possible consequences for the mother include an increased rate of perinatal complications, hypertension during pregnancy and preeclampsia (Kokic et al., 2018; Zhu and Zhang, 2016). Recent studies suggest that women who experience GDM are eight times more likely to develop T2DM as compared to those with normal glucose tolerance in pregnancy (Gupta et al., 2017; Rice et al., 2012).

1.4 Factors associated with pathogenesis of type 2 Diabetes Mellitus

1.4.1 Insulin

Insulin is a small protein that contains 51 amino acids arranged into two chains (A and B) linked by disulfide bonds (Weiss et al., 2014). Pancreatic β-cells initiate synthesis of the insulin precursor, preproinsulin, at the cytosolic side of the endoplasmic reticulum (ER), which is then processed to proinsulin in the luminal side of the ER upon cleavage of its signal sequence by a signal peptidase, forming three evolutionarily conserved disulfide bonds (Figure 1.3). Properly folded proinsulin forms dimers and exits from the ER, trafficking through Golgi complex into immature secretory granules wherein C-peptide is endoproteolytically excised, allowing fully bioactive two-chain insulin to ultimately be stored in mature granules for insulin secretion (Tokarz et al., 2018; Liu et al., 2014).

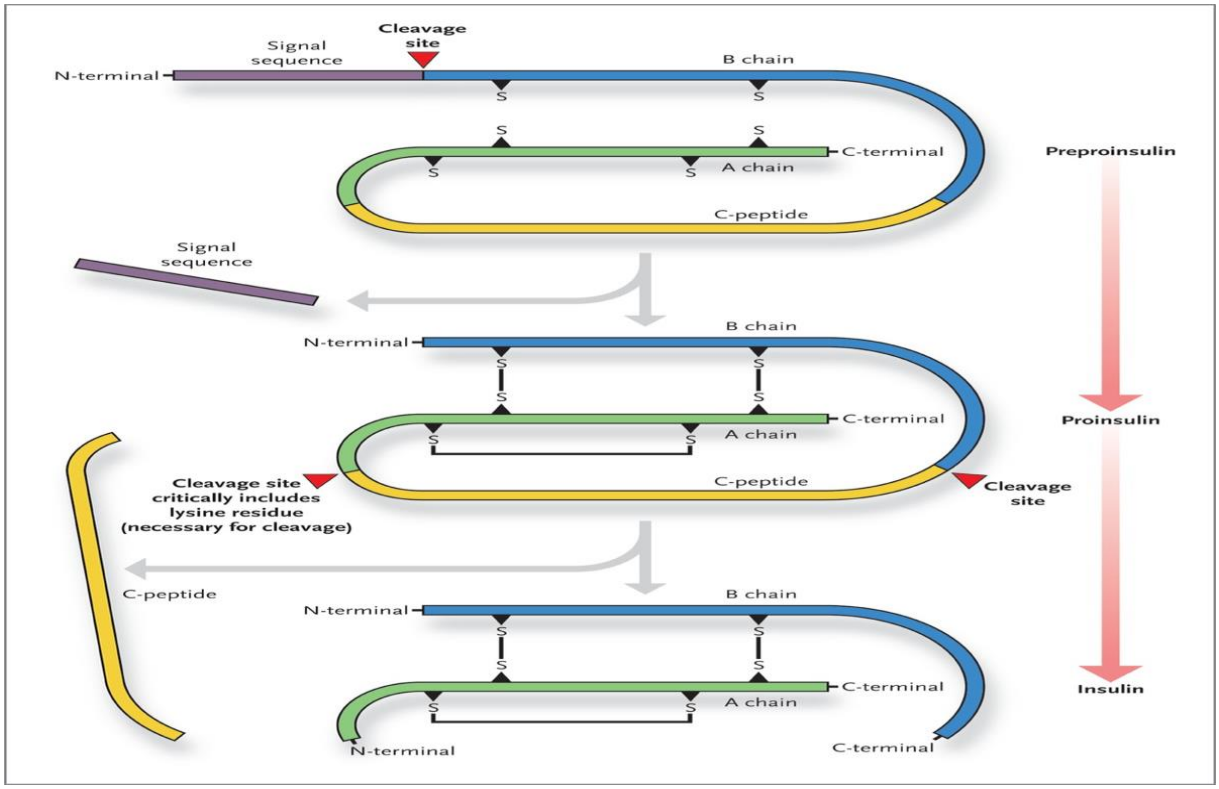


Figure 1.3: Biosynthesis of insulin (https://www.topsimages.com/images/full-cell-diabetes-8d.html).

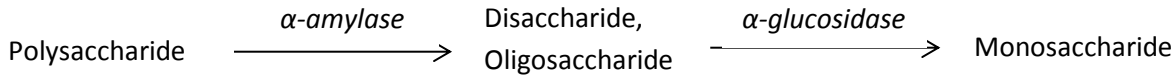
In healthy subjects, insulin secretion is exquisitely exact to meet the metabolic demand. Specifically, β-cells sense changes in plasma glucose concentration and respond by releasing corresponding amounts of insulin (Fu et al., 2013). Secretion of insulin from β -cells is not only an important step in the regulation of glucose homeostasis in healthy individuals, but has also been demonstrated to be impaired (for different reasons) in both

T1DM and T2DM. In fact, in the prediabetic state of T1DM as well as in various forms of T2DM, abnormalities in insulin secretion are an integral component of the pathophysiology (Weiss et al., 2014).

The liver and kidney are the two main organs that predominantly clear insulin from circulation. The liver normally clears approximately 60% of the insulin circulating in the blood, with the kidneys removing 35 – 40% of the endogenous hormone. This ratio is reversed in diabetics receiving insulin replacement therapy (Deutschlander, 2010).

1.4.2 α- Amylase and α-Glucosidase

Sugar in blood originates from the hydrolysis of carbohydrates and is catalysed by digestive enzymes, such as α-glucosidase and α-amylase (Dona et al., 2010). α-Glucosidase is a carbohydrate-hydrolase that liberates α-glucose from the non-reducing terminal of the substrate. It is widely distributed in microorganisms, plants, and animal tissues (Kimura et al., 2004). Similarly, α-amylase is an enzyme that is secreted by the pancreas and salivary glands that can hydrolyse starches and oligosaccharide into simple sugars.



Inhibition of these enzymes can retard carbohydrate digestion, thus causing a reduction in the rate of glucose absorption into the blood. Therefore, inhibition of these enzyme activities in digestive organs is considered to be a therapeutic approach for managing diabetes (Kalita et al., 2018).

1.4.3 Oxidative stress

Several studies have indicated that oxidative stress, mediated mainly by hyperglycaemia-induced overproduction of free radicals, contributes to the pathogenesis and progression of diabetes and related complications (Newsholme et al., 2016). There are multiple sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways (Bajaj and Khan, 2012). Non-enzymatic sources of oxidative stress originate from the autoxidation of glucose to generate $^{\bullet}OH$ radicals (Mauricio, 2015).

1.5 Complications of Diabetes

There are two categories in which diabetes complications are classified: microvascular and macrovascular complications. Microvascular complications are due to damage of small blood vessels whereas macrovascular complications are caused by damage to larger blood vessels (Organization, 2013). Microvascular complications include damage to the kidneys

(nephropathy), leading to renal failure; eyes (retinopathy), leading to blindness; and the nerves (neuropathy), resulting in impotence and diabetic foot disorders that can result in infections and amputations. Macrovascular complications include cardiovascular diseases, such as strokes, heart attacks, and blood flow insufficiency to the legs (Organization, 2013).

1.5.1 Microvascular complications

1.5.1.1 Diabetic retinopathy

Diabetic retinopathy is the most frequent cause of vision loss among adults aged 20-74 in developing countries (Solomon et al., 2017). It is generally classified as either non-proliferative or proliferative. Non-proliferative retinopathy occurs when small blood vessels become partly occluded, leading to microaneurysms and leaking of capillary fluid (Stitt et al., 2016). Microaneurysms are small vascular dilatations that occur in the retina, often as the first sign of retinopathy vision is affected if the macula is involved (Blair, 2016). Proliferative retinopathy is characterized by neovascularization (i.e. growth of abnormal blood vessels) originating from the retina and optic disc as a severe complication of diabetes mellitus (Duh et al., 2017; de Carlo et al., 2016). These new vessel growths occur along the vitreoretinal interface, leading to severe vision loss, preretinal fibrosis, bleeding into the vitreous cavity and traction retinal detachment (Roy et al., 2016; Alghadyan, 2011).

1.5.1.2 Diabetic nephropathy

Diabetic nephropathy is due to damage to small vessels in the glomeruli of the kidneys and affects up to 40% of individuals with diabetes (Thomas and Kodack, 2011). Diabetic nephropathy is commonly defined by abnormally high albumin levels in urine in a patient without known renal disease (Gross et al., 2005). The earliest indicator is microalbuminuria, defined as albumin excretion of less than 30 mg/day or a urinary albumin/creatinine ratio of greater than 3.0 mg/mmol (Bennett and Aditya, 2015). It is the leading cause of end stage renal disease and is more common among individuals with diabetes who smoke, and who have uncontrolled hypertension and chronically high blood glucose (Bennett and Aditya, 2015; Ghaderian et al., 2015; Williams et al., 2012). Despite the use of therapy to protect the kidneys of individuals with diabetes, end stage renal disease is increasing in the diabetic population at the rate of about 9% a year (Krolewski, 2015).

1.5.1.3 Diabetic neuropathy

Diabetic neuropathy is recognized by the ADA as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes (Association, 2014b) . As with other microvascular complications, risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycemia (Girisha and

Viswanathan, 2017), and some individuals may possess genetic facets that affect their predisposition to developing such complications (Chawla et al., 2016).

Diabetic neuropathy affects nearly two-thirds of all individuals with diabetes, and is probably the result of chronic high blood glucose that damages the nerves and blood vessels. Other risk factors include increasing age, obesity, and having concurrent peripheral vascular disease (Sharp and Clark, 2011).

1.5.2 Macrovascular Complications

Among macrovascular diabetes complications, coronary heart disease has been associated with diabetes in numerous studies beginning with the Framingham study (Fowler, 2011). More recent studies have shown that the risk of myocardial infarction (MI) in diabetic patients is equivalent to the risk in nondiabetic patients with a history of previous MI. Diabetes is also a strong independent predictor of risk of ischemic stroke of antherothrombotic cause and cerebrovascular disease, as in coronary artery disease (Arboix, 2015). Risk of cognitive impairment and stroke-related dementia is elevated in patients with diabetes (Saedi et al., 2016).

1.6 Treatment of T2DM

Progress in understanding the metabolic staging of diabetes over the past few years has led to significant advances in regimen for treatment of this devastating disease (Martins et al., 2008). The present treatment of diabetes is focused on controlling and lowering blood glucose to normal level (Asif, 2014). The mechanisms of both western medicine and traditional medicines to lower blood glucose are: to simulate β-cell of pancreatic islet to release insulin; to resist hormones which raise high blood glucose; to increase the leading out of glycogen; to enhance the use of glucose in the tissues and organ; to clear away free radicals, resist lipid peroxidation and protein; to improve microcirculation in the body (Tu et al., 2013; Hui et al., 2009). In recent diabetic treatments, α-amylase and α-glucosidase inhibitors are most warranted because they decrease post-prandial hyperglycaemic conditions (Patel and Zaidi, 2012; Kumar, 2011). However, these agents have been found to possess side effects (Figure 1.11).

1.6.1 Oral antidiabetic drugs

There are seven classes of oral antidiabetic drugs currently available, which can be classified into insulin secretagogues (sulfonylureas, meglitinides), insulin sensitizer (biguanides, thiazolidinediones), decrease glucose flux (α-glucosidase inhibitors), incretin mimetic agents (DPP-4 inhibitor), and glycosuric agent (SGLT-2 inhibitor) (Baiga et al., 2018; Tsang, 2012).

1.6.1.1 Biguanides

Metformin is the only biguanide available in clinical practice, biguanides such as phenformin and buformin have been withdrawn from the market due to high incidence of lactic acidosis (Sogame et al., 2013). Metformin activates adenosine monophosphate-activated protein kinase (AMPK) in the liver, causing hepatic uptake of glucose and inhibiting gluconeogenesis through complex effects on the mitochondrial enzymes (Chaudhury et al., 2017). Metformin improves insulin sensitivity, leading to reduction in insulin resistance by activating insulin receptor expression and enhancing tyrosine kinase activity (Viollet et al., 2012). It is widely recognised that metformin improves glycaemic control with a good safety profile, lack of associated hypoglycaemia and reduced cardiovascular mortality. However, 25% of patients suffer gastrointestinal side effects such as nausea, vomiting and diarrhoea (McCreight et al., 2016; Bouchoucha et al., 2011).

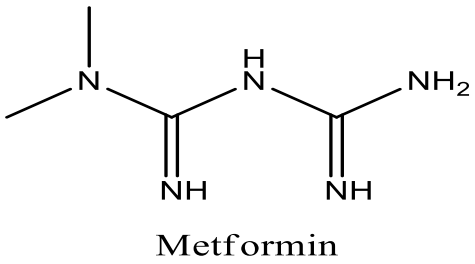


Figure 1.4: *Galega officinalis* (http://www.freenatureimages.eu/) and chemical structure of biguanide (metformin)

1.6.1.2 Sulfonylureas

Sulfonylureas have long been established in the treatment of T2DM, since the 1950s, and were the first oral antidiabetic medications to be introduced into clinical practice. They are still widely used and are the second-line recommended choice of oral hypoglycaemic treatment after metformin (He et al., 2015). Sulfonylureas work by lowering the blood glucose level by increasing insulin secretion in the pancreas by blocking the K_{ATP} channels in the β-cells of Langerhans islets (Scheen et al., 2015).

Sulfonylureas are classified into two groups; first generation, which includes chloropropamide and tolbutamide and second-generation, which includes glipizide, glimperide and glyburide. Currently in clinical practice, second-generation sulfonylureas are prescribed and more preferred over first generation agents because they have greater potency, enabling treatment at lower doses (Tahrani et al., 2016).

Hypoglycaemia is the major side effect of all sulfonylurea, while minor side effects such as headache, dizziness, nausea, hypersensitivity reactions and weight gain are also common (Chaudhury et al., 2017).

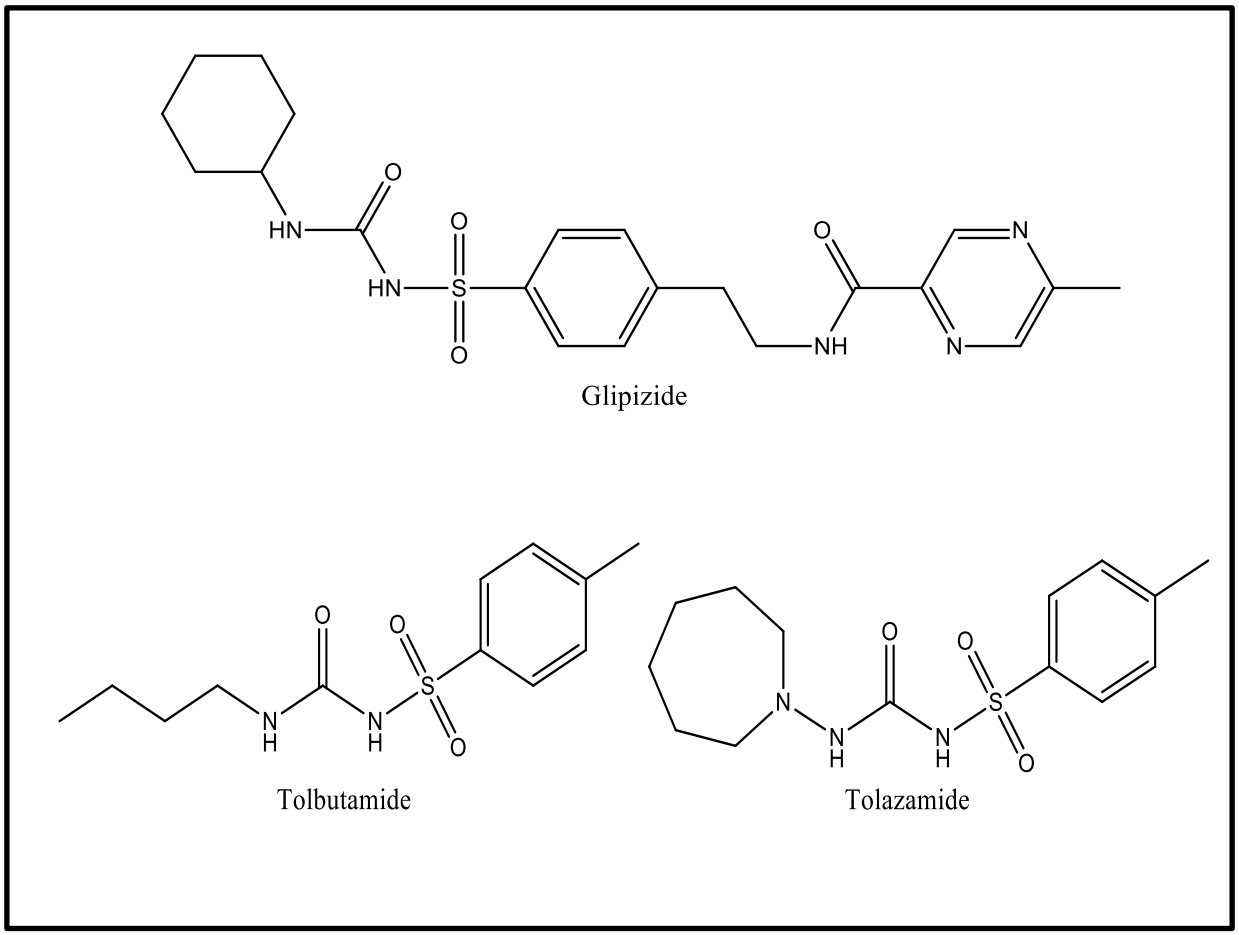


Figure 1.5: Chemical structures of the first generation sulfonylurea (tolbutamide and tolazamide) and second generation sulfonylurea (glipizide).

1.6.1.3 Meglitinides

Meglitinides (nataglinides and repaglinides) are non-sulfonylurea secretagogues, which were approved as treatment for T2DM in 1997. Meglitinides bind to a different part of the sulfonylurea receptor in β-cells of the pancreas, the interaction with the receptor is weaker than that of sulfonylurea meaning that a shorter duration of action and higher blood sugar level is required before it can stimulate β-cells' insulin secretion (Grant and Graven, 2016). In general, the efficacy of repagalinide is comparable to other sulfonylureas but less with netaglinides. Side effects are similar to sulfonylurea with less weight gain (Tsang, 2012).

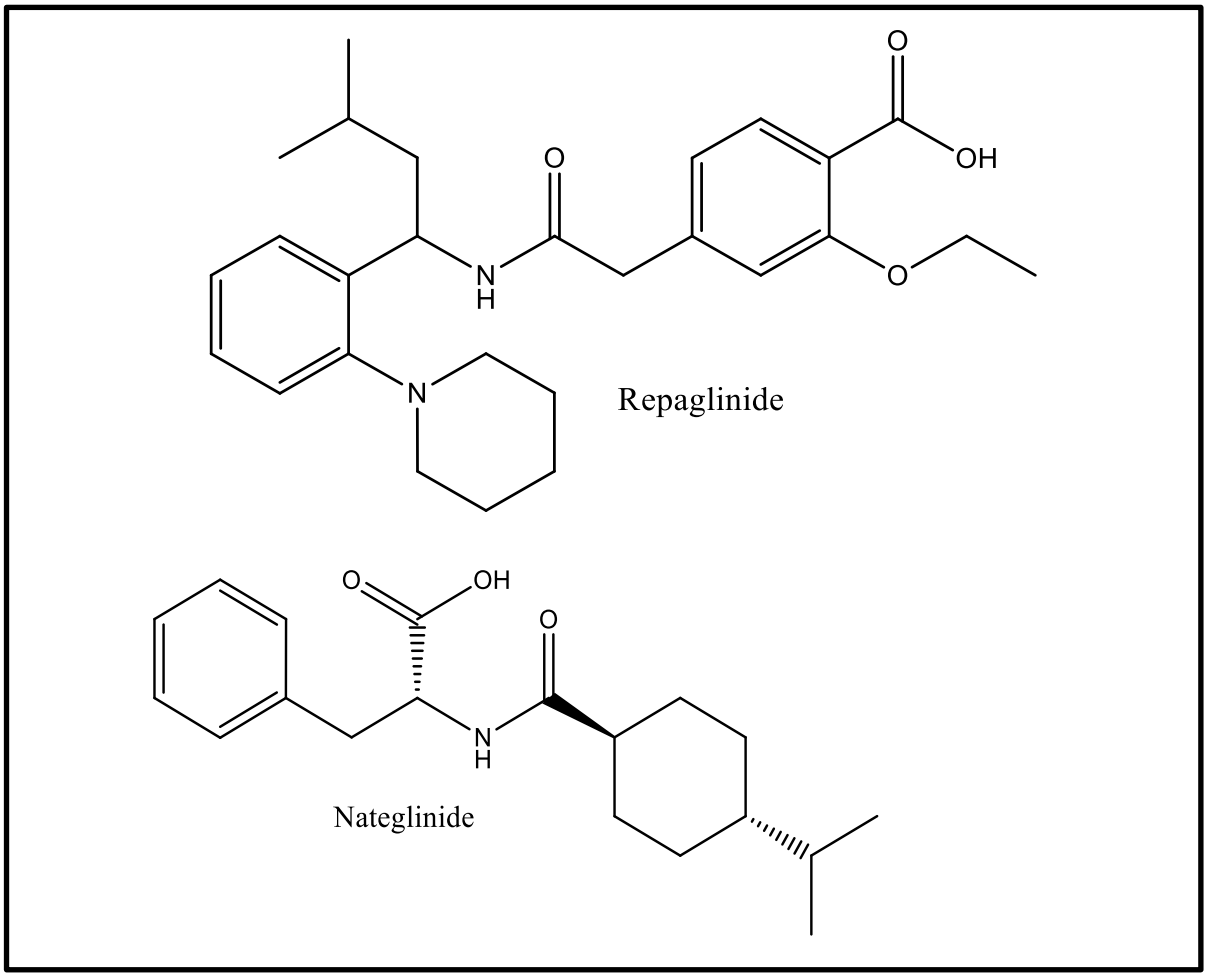


Figure 1.6: Chemical structures of meglitinides (repaglinide and nateglinide).

1.6.1.4 Thiazolidinediones

Like biguanides, thiazolidinediones (TZDs) improve insulin action. Rosiglitazone and pioglitazone are representative agents. TZDs act as peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, lowering pasma glucose, triglyceride and fatty acid levels in patients with type 2 diabetes (He et al., 2015). The major actions of TZDs are to improve insulin sensitivity, increase adipogenesis, prevent β-cell function and modulate hepatic gluconeogenesis (Tsang et al., 2012). However, there are high concerns of risks overcoming the benefits. Namely, combined insulin-TZD therapy causes heart failure. Thus TZDs are not preferred as first-line or even step-up therapy (Chaudhury et al., 2017).

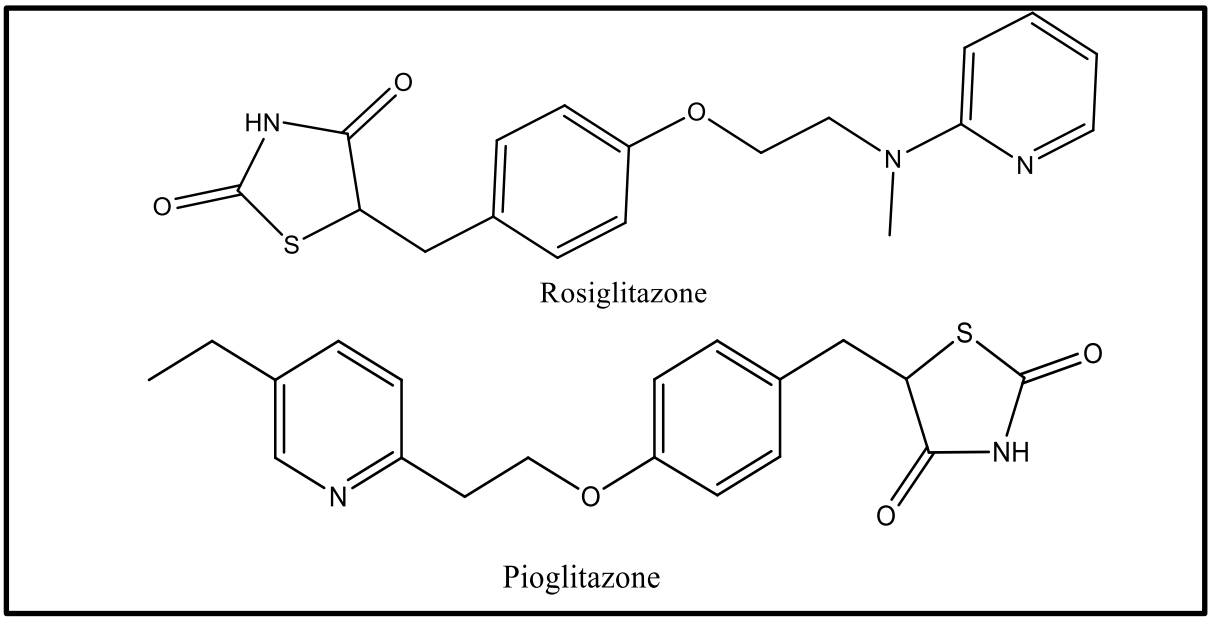


Figure 1.7: Chemical structures of thiazolidinediones (Pioglitazone and Rosiglitazone)

1.6.1.5 α-Glucosidase inhibitors

α-Glucosidase inhibitors of the pancreatic α-amylase and membrane bound intestinal α-glucosidase enzymes are competitive reversible inhibitors. The mechanisms of all α-glucosidase inhibitors are similar (Tsang, 2012). α-Glucosidase inhibitors are the most effective antidiabetic agents primarily targeting postprandial hyperglycaemia (He et al., 2015). They competitively bind to the oligosaccharide binding site of the α-glucosidase enzymes, thereby preventing enzymic hydrolysis. These inhibitors provide an alternative means to reduce post-prandial hyperglycaemia by retarding the rate of digestion and adsorption of starch and other oligosaccharides and disaccharides (Kokil et al., 2010).

Acarbose, the first α-glucosidase inhibitor to be commercially available and the most widely used, was introduced in the early 1990s. Acarbose is most effective against glucoamylase, followed by sucrase, maltase and dextranase. It also inhibits α-amylase but has no effect on β-glucosidase such as lactase (Derosa and Maffioli, 2012). Other drugs in this family include miglitol and voglibose. α-Glucosidase inhibitors can lower post-prandial glucose and improve hyperglycaemic control without causing weight gain or hyperglycaemia. Their good safety record is a further advantage, but limited gastrointestinal tolerability has limited their use. The relatively high cost of these drugs is another limiting factor. Side effects of α-glucosidase inhibitors include diarrhoea, abdominal pain, bloating and flatulence (Kokil et al., 2010; Krentz and Bailey, 2005).

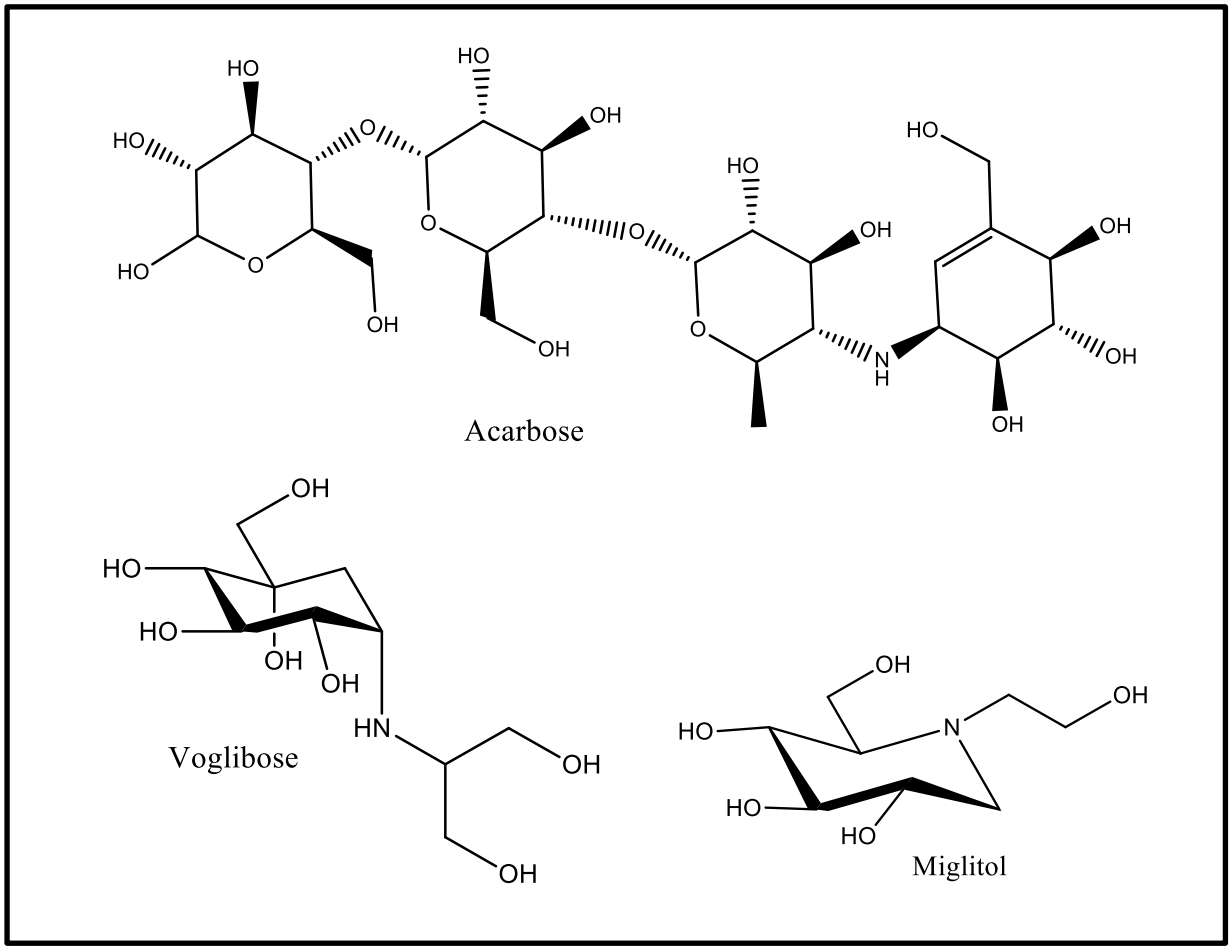


Figure 1.8: Chemical structures of α-glucosidase inhibitors (acarbose, voglibose and miglitol).

1.6.1.6 Dipeptidyl peptidase-4 (DPP-4) inhibitors

Dipeptidyl peptidase-4 (also known as adenosine deaminase complexing protein 2 or CD26) is a ubiquitous atypical serine protease that degrades two incretin hormones, glucose-dependent insulin-tropic polypeptide (GIP) and glucagon-like polypeptide 1 (GLP-1). These incretin hormones contribute more than 70% to insulin response to blood glucose changes (Mascolo et al., 2016). The highly selective DPP-4 inhibitors, sitagliptin, saxagliptin, vildagliptin, and linagliptin, prevent normal rapid degradation of endogenous glucagon-like-peptide-1 (GLP-1) (Tsang, 2012). Neither weight loss nor nausea occurs with DPP-4 inhibitors. The most commonly reported adverse events have been mild infections such as nasopharyngitis, upper respiratory tract infection, and headaches (Tsang, 2012).

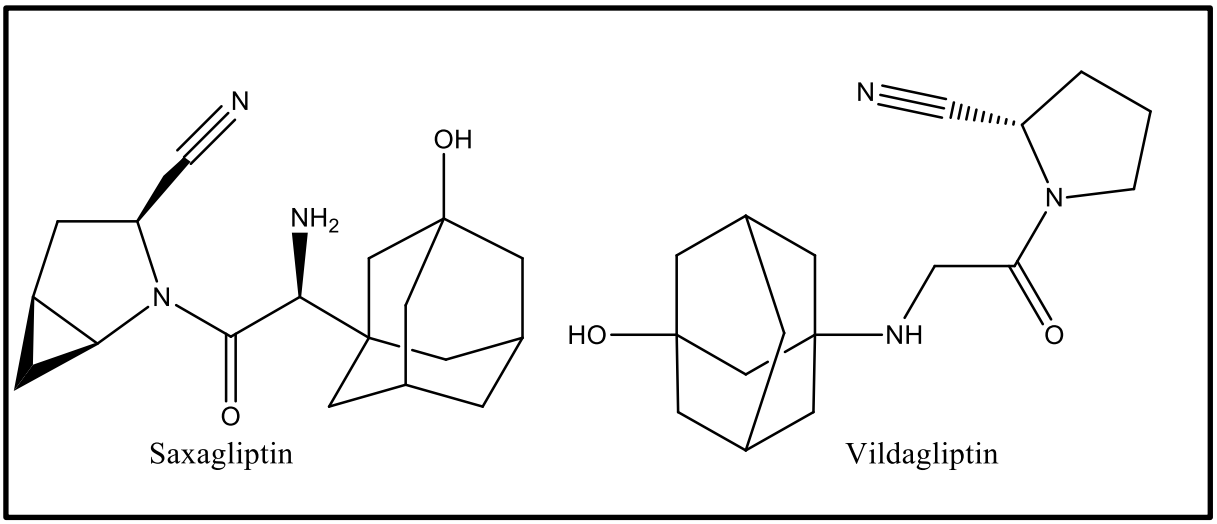


Figure 1.9: Chemical structures of Dipeptidyl peptidase-4 (DPP-4) inhibitors (saxagliptin and vildagliptin)

1.6.1.7 SGLT-2 inhibitors

The Sodium–glucose cotransporters (SGLTs) are responsible for glucose reabsorption in the kidneys. SGLT-2 inhibitors, function by lowering the tubular resorption of glucose (Monami et al., 2018). Inhibition of SGLT-2 results in normalization of blood glucose levels and amelioration of insulin resistance by augmenting insulin signalling and increasing glucose transporter GLUT-4 and glycogen synthase activity in muscle. Furthermore, correction of the hyperglycaemia improves *β*-cell function (He et al., 2015). Potential problems with SGLT-2 inhibitor are risk of urinary tract infection and diuretic effect of glycosuria (Tsang, 2012).

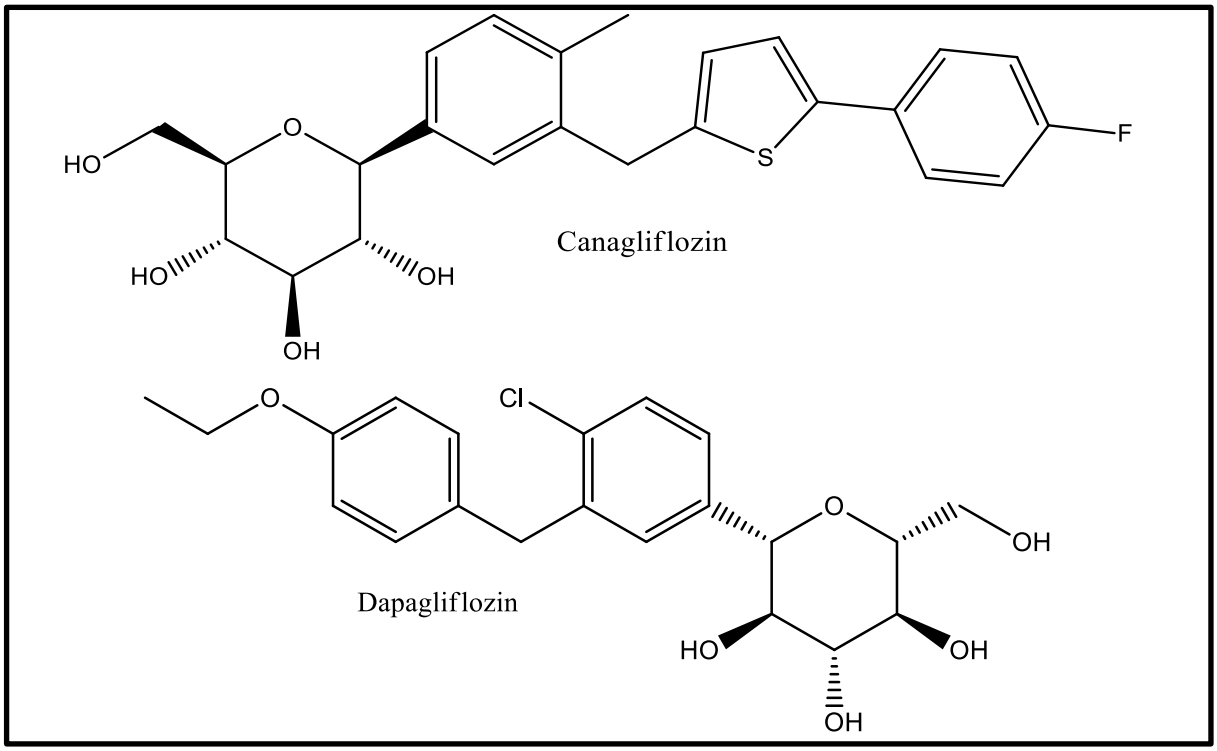


Figure 1.10: Chemical structures of SGLT-2 inhibitors (dapagliflozin and canagliflozin)

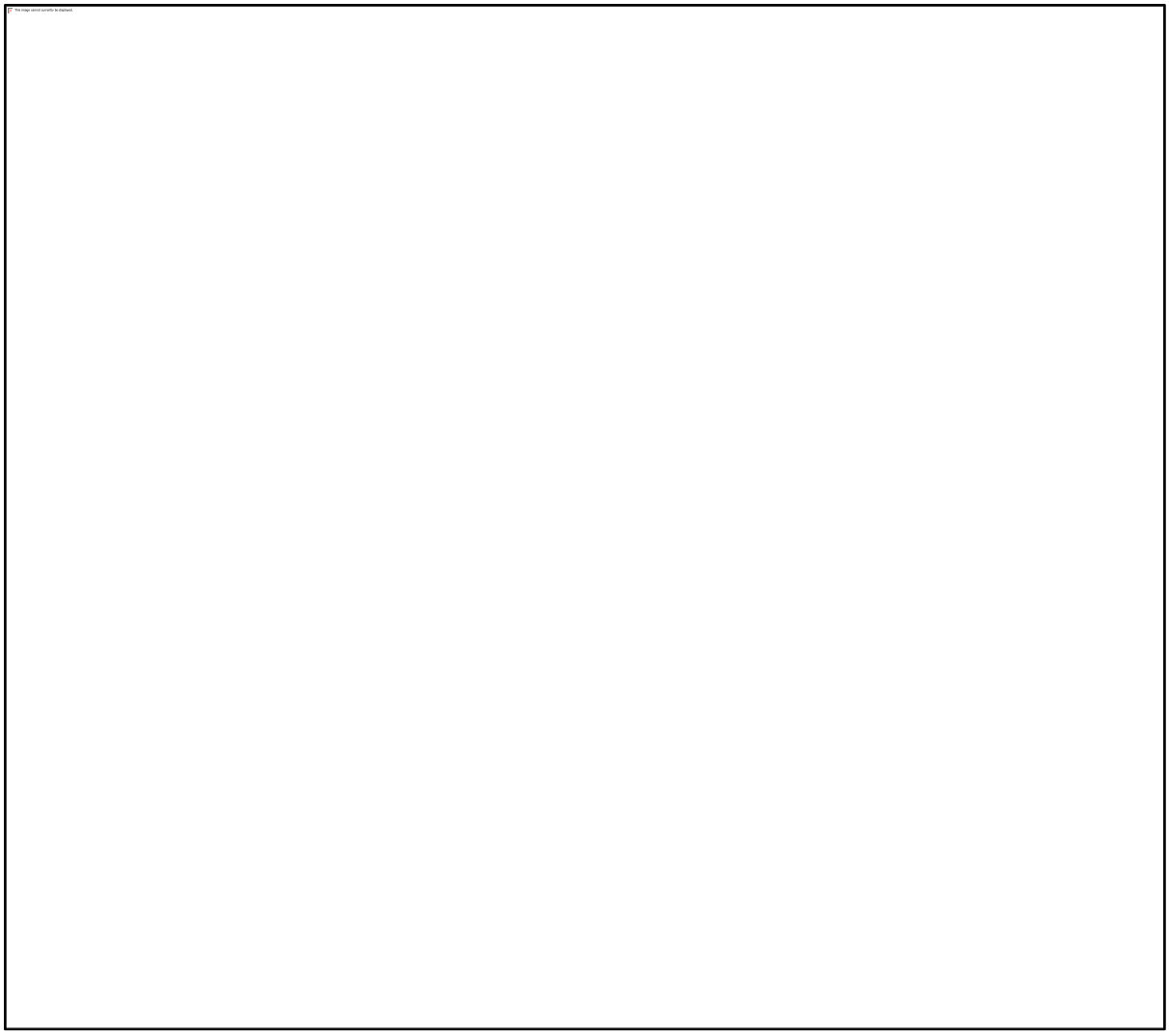


Figure 1.11: The side effects of current antidiabetic agents and their mechanisms of lowering blood glucose in diabetes mellitus (Adapted from Vinayagam et al. (2016)).

1.7 Plants for diabetes therapy

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them (Ahmad et al., 2008). Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically as antidiabetic and antihyperlipidemic remedies. Despite the presence of known antidiabetic medicine in the pharmaceutical market, diabetes and the related complications continue to be a major medical problem (Malviya et al., 2010). Many conventional drugs have been derived from prototypic molecules in medicinal plants. Even the discovery of widely used hypoglycaemic drug, metformin came from the traditional approach of using *Galega officinalis* (Joseph and Jini, 2011).

Table 1.2: Herbal products available on the South African market for the management of blood glucose levels.

PRODUCT	NAPPI CODE	PLANT	PHARMACOLOGICAL ACTION	REFERENCE
Cinnachrome	708102-001	Cinnamon bark	Assist in regulating blood sugar levels	(Ibrahim, 2014)
Diabecinn	704686-001	Cinnamon bark	Increases insulin sensitivity	(Oldani, 2011)
Diavite	707941-001	Propolis grandulosa	Glucose stabiling	(George et al., 2011)
Manna DFM43	705846-001	Propolis grandulosa	Retard the absorption of glucose in the blood and reduces glycaemic index value of food	(Huisamen et al., 2013)
Probetix	711050-001	Sutherlandia frutescens	Reverse insulin resistance and decrease intestinal glucose uptake	(Chadwick et al., 2007)

1.8 Phytoconstituents with antidiabetic activities

Synthetic antidiabetic drugs are well known and currently in use for the management of diabetes, however, the associated costs and side effects highlight the relative shortcomings of this treatment strategy. Based on the WHO recommendations, hypoglycaemic agents of plant origin used in traditional medicine are imperative as they are effective, non-toxic, with less or no side effects and are considered to be excellent aspirants for oral therapy (Gaikwad et al., 2014). The recognised antihyperglycaemic activity of most plants is achieved either by reinstating the function of pancreatic tissues by causing an increase in insulin output or by inhibition of glucose absorption in the gut (Noor et al., 2013; Malviya et al., 2010). Moreover, during the past few years many phytochemicals responsible for antidiabetic effects have been isolated from the plants. Several phytoconstituents such as alkaloids, glycosides, flavonoids, saponins, dietary fibres, polysaccharides, glycolipids, peptidoglycans, amino acids and others obtained from various plant sources that have been reported to be potent hypoglycaemic agents (Patel et al., 2012).

1.8.1 Polysaccharides

Polysaccharides are the most abundant natural products produced by plants and are well recognized for their importance in our food (Lovegrove et al., 2017; Simpson, 2016). In recent years, polysaccharides have attracted various researchers' attention as they are the main active fraction of various antidiabetic plants (Sun et al., 2018; Wang et al., 2016). Details of some polysaccharides exhibiting antidiabetic activity are listed in table 1.3.

1.8.2 Glycosides

Glycosides are plant secondary metabolites which are made up of two components including glycone (a carbohydrate component) and aglycone (a non-carbohydrate component). The former component usually consists of one or more glucose units and the latter component may be any one of the plant secondary metabolites from alkaloids, phenolics or terpenoids (Lin et al., 2016). The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, glucosinolates, coumarin glycosides and anthraquinone glycosides. Table 1.3 shows a number of glycosides that possess antidiabetic activity.

1.8.3 Phenolics

Another class of plant secondary metabolites are phenolics which are widely distributed. They contain benzene rings, with one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Lin et al., 2016). Phenolic compounds are ubiquitous in edible vegetables, fruits and nuts. Some examples of common simple phenolics with antidiabetic properties include ellagic acid, caffeic acid and gallic acid (Hanhineva et al., 2010).

1.8.4 Flavonoids

Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known. Flavonoids are characterized by two substituted benzene rings A and B linked by a heterocyclic pyran ring C. They can be widely classified into different categories like anthocyanins, catechins, flavonols, flavones, flavanones etc (Gaikwad et al., 2014). (Figure 1.12) and usually occur as aglycones, glycosides and/or methylated derivatives. Various flavonoids have been reported to exhibit antidiabetic activities. Some flavonoids with antidiabetic activity are listed in Table 1.3.

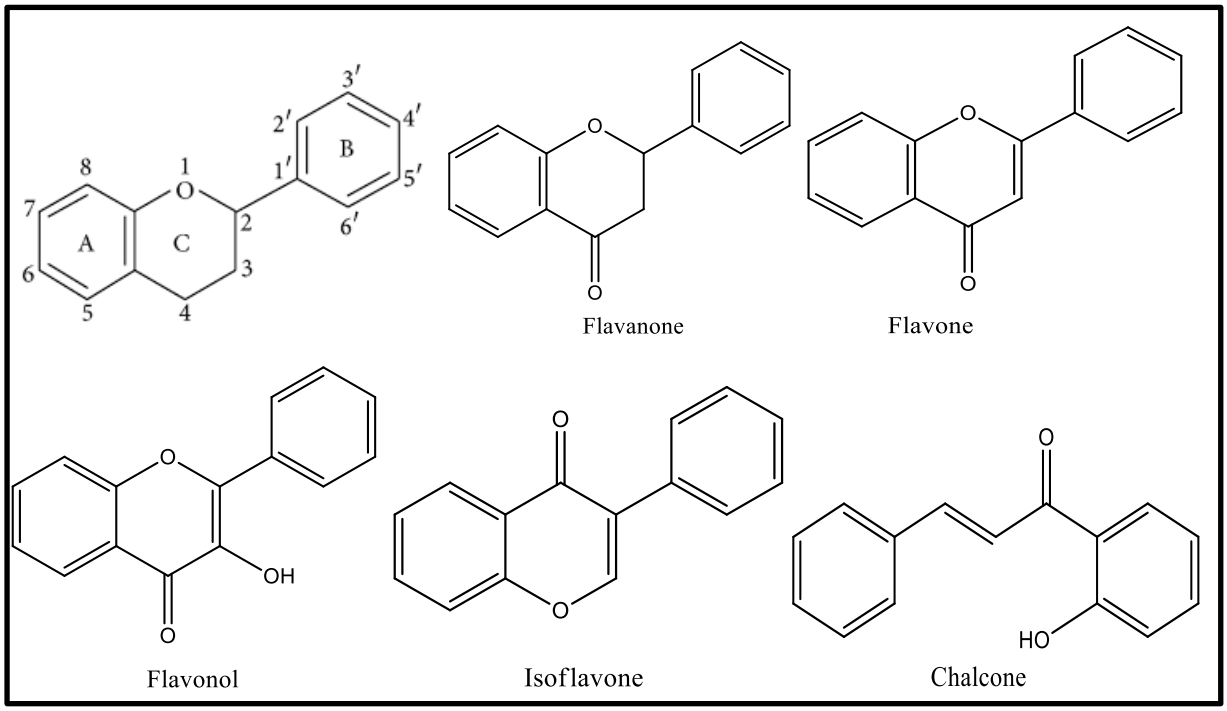


Figure 1.12: Chemical structures of flavonoid classes

1.8.5 Alkaloids

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds derived mostly from amino acids. These secondary metabolites are produced by about 20% of plant species (Kaushal, 2018). Those compounds characterized by nitrogen atoms in heterocyclic rings are classified as true alkaloids. In contrast, the, protoalkaloids, do not have nitrogen atom(s) in heterocyclic rings, while the pseudoalkaloids are not derived from amino acids, but may have nitrogen atoms in heterocyclic rings. Various alkaloids have been isolated from numerous medicinal plants, and investigated for possible hypoglycaemic activity. Table 1.3 shows some of the known antidiabetic alkaloids.

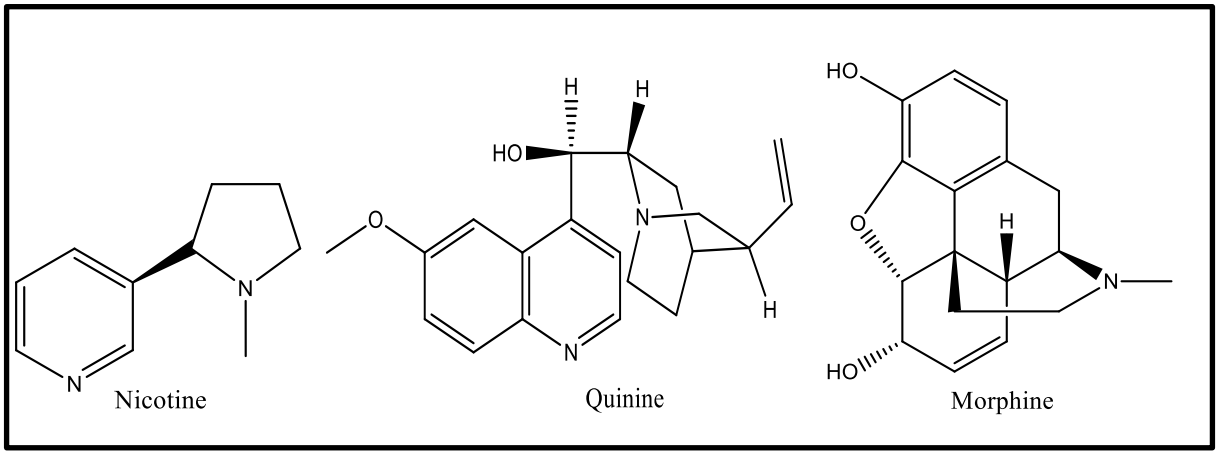


Figure 1.13: Chemical structures of some common alkaloids

1.8.6 Terpenoids and steroids

Terpenoids, also known as isoprenoids, are a large family of compounds including carotenoids, tocopherol, phytol, sterols and hormones (Pattanaik and Lindberg, 2015). They are synthesized from isoprenoid units, and share metabolic pathways with fatty acids. Terpenoids are classified according to the number of isoprene units into hemiterpenoids (C5) monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), triterpenoids (C30) and tetraterpenoids (C40) (Yazaki et al., 2017).

Triterpenoids and steroidal glycosides, referred to collectively as saponins, are bioactive compounds that exist naturally in most plants and are reported to possess potent hypoglycaemic activity. Examples of terpenoids and steroids with antidiabetic activities are listed in Table 1.3.

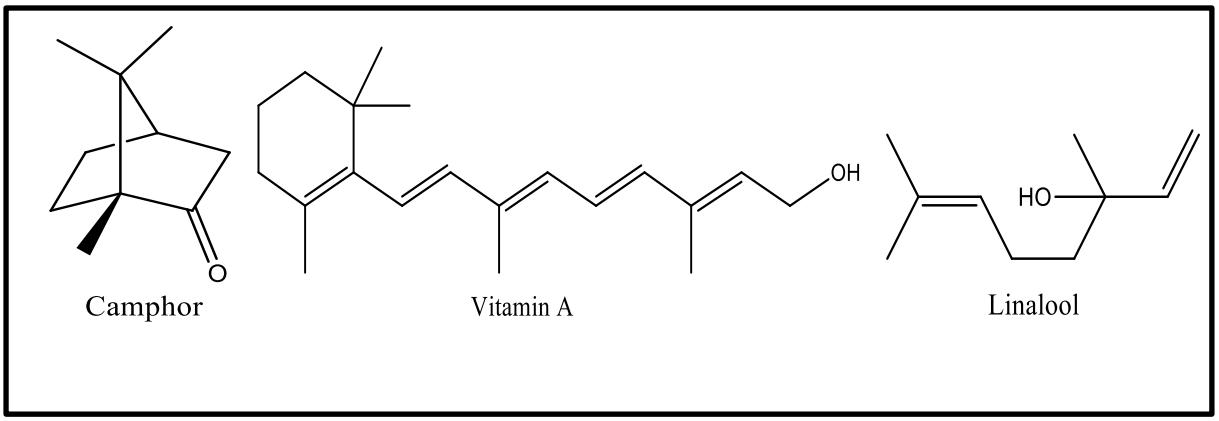


Figure 1.14: Chemical structures of some common terpenoids.

Table 1.3: Important phytoconstituents used to treat diabetes mellitus (Adapted and modified from Gaikwad et al. (2014)).

COMPOUND	PLANT NAME	PARTS USED
Carbohydrates (Polyssaccharides)		
Aconitans A-D	*Aconitum carmichaeli*	Roots
Atractans A	*Atractylodes japonica*	Rhizomes
Ganoderans A and B.	*Ganoderma lucidum*	Fruit bodies
Galactomannan gum	*Cyamopsis tetragonolobus* *Amorphophallus konjac*	Seeds Tubers
Glycosides		
Kalopanax	*Kalopanax pictus*	Stem
Jamboline or antimellin	*Syzygium cumini*	Seeds
Myrciacitrins I and II	*Myrcia multiflora*	Leaves

Myrciaphenones A and B		
Neomyrtillin	*Vaccinium myrtillus*	Leaves
Perlargonidin 3-o-α- l rhamnoside	*Ficus bengalensis*	Bark
Pseudoprototinosaponin AIII Prototinosaponin AIII	*Anemarrhena asphodeloides*	Rhizome
Vitexin, isovitexin and Isorhamnetin 3-O-β-D-rutinoside	*Microcos paniculata*	Leaves
Flavonoids		
Bengalenoside	*Ficus benghalensis*	Stem
Cyanidin-3-galactoside Epigallocatechin gallate	*Camellia sinensis*	Leaves
(-)-3-O-galloylepicatechin (-)-3- O-galloylcatechin	*Bergenia ciliata*	
Genistein	*Glycine* spp.	Soya beans
Hesperidin, naringin	*Citrus* spp.	
Prunin	*Amygdalus davidiana* var. *davidiana*	Stems
Kaempferitrin	*Bauhinia forficate*	Leaves
Kaempferol	*Jindai soybean*	Leaves
Kolaviron	*Garcinia kola*	
Mangiferin	*Anemarrhena asphodeloides*	Rhizomes
Marsupsin, pterostilbene	*Pterocarpus marsupium*	Heartwood
Quercetin	*Chamaecostus cuspidatus*	
Rutin		
Shamimin	*Bombax ceiba*	Leaves
Alkaloids		
Berberine	*Berberis* spp. *Tinospora cordifolia*	Roots, stem-bark
Casuarine 6-o-α-glucoside	*Syzygium malaccense*	Bark
Catharanthine, vindoline and vindolinine	*Catharanthus roseus*	Leaves, stems
Calystegine B2	*Nicandra physalodes*	Fruits
Cryptolepine	*Cryptolepis sanguinolenta*	
Harmane , norharmane,	*Tribulus terrestris*	
Jambosine	*Syzygium cumini*	Seeds, fruits, bark
Jatrorrhizine, magnoflorine, palmatine	*Tinospora cordifolia*	
Javaberine A, javaberine A	*Talinum paniculatum*	Roots

hexaacetate, javaberine B hexaacetate		
Lepidine and semilepidine	*Lepidium sativum*	Seeds
Lupanine	*Lupinus perennis*	
Mahanimbine	*Murraya koenigii*	Leaves
Piperumbellactam A	*Piper umbellatum*	Branches
Radicamines A and B	*Lobelia chinensis*	
Swerchirin	*Swertia chirayita*	
Tecomine	*Tecoma stans*	
Trigonelline	*Trigonella foenum-graecum*	Seeds
1-deoxynojirimycin	*Morus alba*	Leaves, bark
Terpenoids and Steroids		
α-amyrin acetate Fruits	*Ficus racemosa*	Fruits
Andrographolide	*Andrographis paniculata*	Leaves
3 β -acetoxy-16 β -hydroxybetulinic acid	*Zanthoxylum gilletii*	Stem
Bassic acid	*Bumelia sartorum*	Root bark
Charantin	*Momordica charantia*	Seeds, fruits
Christinin A	*Zizyphus spina-christi*	Leaves
Colosolic acid, maslinic acid	*Lagerstroemia speciosa*	Leaves
Corosolic acid	*Vitex* spp.	Leaves
Elatosides E	*Aralia elata*	Root cortex
Escins-IIA and IIB	*Aesculus hippocastanum*	Seeds
Forskolin	*Coleus forskohlii*	
Ginsenosides	*Panax species*	Rhizomes
Gymnemic acid IV	*Gymnema sylvestre*	Leaves
Momordinic	*Kochia scoparia*	Fruit
β-sitosterol	*Azadirachta indica*	
Senegin derivatives	*Polygala senega*	
Miscellaneous		
Ferulic acid	*Curcuma longa*	Leaves seeds
Ginseng polypeptides	*Panax ginseng*	Roots
4-hydroxyisoleucine	*Trigonella foenum-graecum*	Seeds
Kotalanol	*Salacia reticulate*	
Masoprocol	*Larrea tridentate*	
Paeoniflorin, 8-debenzoylpaeoniflorin	*Paeonia lactiflora*	Root

1.9 Plant under study: *Bridelia micrantha*

1.9.1 Scientific classification

Bridelia micrantha (Hochst.) Baill. is a small to medium sized tree belonging to the family Phyllanthaceae. It is commonly known in English as coastal golden leaf. In South Africa, *B. micrantha* is commonly known as bruin stinkhout (Afrikaans); munzere (Tshivhenda) motsere (Sotho) and ndzerhe (Tsonga). *B. micrantha* can be classified as Kingdom: Plantae – Plants; Phylum: Tracheophyta – Vascular plants; Class: Magnoliopsida – Dicotyledons; Order: Malpighiales – Flowering plants; Family: Phyllanthaceae; Genus: *Bridelia*; Species: *B. micrantha* (Rivers et al., 2017).

1.9.2 Botanical description

B. micrantha is a semi-deciduous to deciduous tree up to 20 m tall with a dense, rounded crown and tall, bare stem. The bark on young branches are grey-brown and smooth, on older branches and stems dark brown and rough, cracking into squares. The branches are often thorny, slash thin, fibrous, and brown to dark red (Mbahin et al., 2007). Leaves are simple, entire, distichous, often alternate, glabrous to slightly hairy, elliptical to oblong in shape. Flowers occur in clusters in leaf axils, yellow in colour, unisexual with triangular sepals and small petals. Male flowers have stamens and filaments that are fused into a column at the base, but free and spreading above with rudimentary ovary. Female flowers are nearly sessile with ovary and styles fused at the base. The fruit is a globose, fleshy drupe, black in colour when ripe with brownish seeds (Maroyi, 2017).

Figure 1.15: Characteristic features of *B. micrantha*

1.9.3 Distribution

B. micrantha has been recorded in several countries throughout tropical Africa (Figure 1.16). *B. micrantha* is found in a variety of habitats, ranging from savanna and woodland to seasonally flooded grassland, riverine forest, swamp forest and the margins of mangrove swamps, often from sea-level in West Africa to around 2500 m altitude in East Africa. *B.*

micrantha is a pioneer species that tolerates a wide diversity of soils, different rainfall regimes and can withstand moderate frost (Maroyi, 2017).

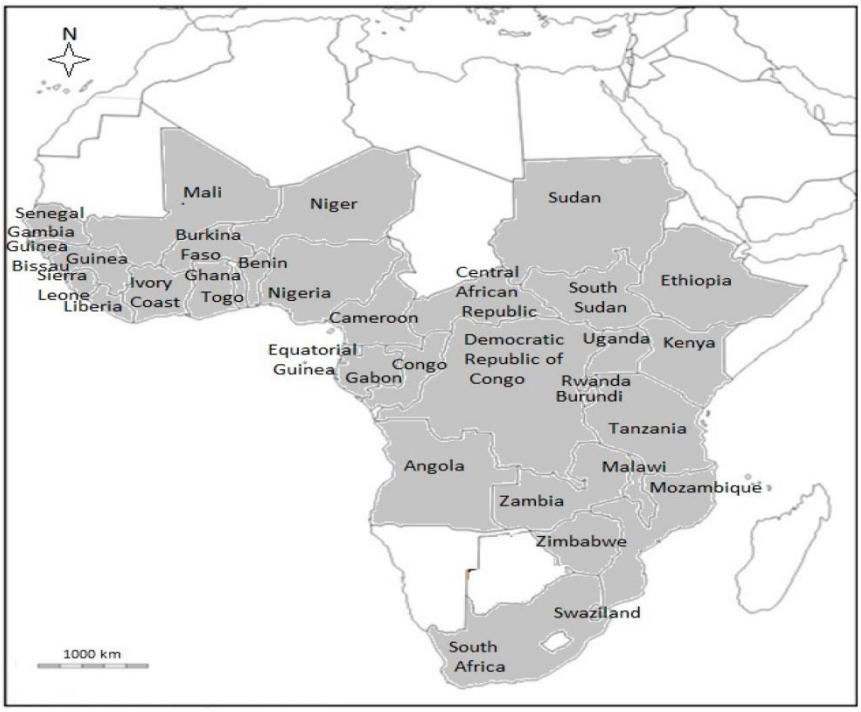


Figure 1.16: Distribution of *B. micrantha* on the mainland tropical Africa (Adapted from Maroyi (2017)).

1.9.4 Ethnomedicinal uses

B. micrantha has been identified as one of the few plant species that should be integrated in the domestication process in farming systems in sub-Saharan Africa to support medicinal, nutritional and income security of local communities through household use and marketing of its fresh or dried fruits. Due to its popularity as an herbal medicine, *B. micrantha* is sold as such in the herbal medicine or “muthi” markets in Cameroon, Malawi, Nigeria and South Africa (Maroyi, 2017). In South Africa, *B. micrantha* is used in traditional medicine for diabetes, gastrointestinal ailments, paralysis and painful joints (Deutschländer et al., 2009; Lin et al., 2002), as an emetic for poisons (Orwa et al., 2009), toothaches, cough, conjunctivitis, skin problems such as ulcers, boils and rashes and as an antimalarial (Ajaiyeoba et al., 2006).

1.9.5 Phytochemistry

Pegel and Rogers (1968) reported the presence of friedelin, epi-friedelin, anthocyanidin, taraxerol, taraxerone, caffeic acid and phenolic derivatives such as gallic and ellagic acid from the leaves and stem of *B. micrantha*. Phytic acid, trans-triacontyl-4-hydroxy-3-

methoxyxinnamte, betulinic acid and catechin were isolated from the bark of *B. micrantha* (Munayi, 2016; Akinyeye and Olatunya, 2014). Cycloartenol, cycloartenol acetate, ergosterol, stigmast-8(14)-en-3-ol, and 5,6-epoxy-7-bromocholestan-3-one from fruits and acacic acid lactone, quercetin, quercetin-3-O-glucoside, oleanolic acid from the stem and leaves of *B. micrantha* (Shelembe et al., 2016; Shelembe, 2014). Some of these compounds, particularly alkaloids, essential oils, flavonoids, phenolics and tannins could be responsible for some of the ethnomedicinal uses of *B. micrantha.*

1.9.6 Pharmacological activity

1.9.6.1 Antibacterial

The antibacterial activities of *B. micrantha* methanol, acetone and hexane (bark, roots and seeds) extracts were tested using the disc diffusion and the micro-dilution methods against *Aeromonas hydrophila, Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Enterobacter cloacae, Enterococcus fecalis, Escherichia coli, Klebsiella pneumoniae, Pantoea agglomerans, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella cholerae-suis, Serratia marcescens, Staphylococcus aureus* and *Shigella flexneri*. The extracts of *B. micrantha* showed activity against all pathogenic organisms except *K. pneumoniae* and *S. cholerae-suis* (Adeyemi et al., 2008; Samie et al., 2005). Stem, leaf extract and fractions of *B. micrantha* showed activity against *H. pylori* with zone of inhibition ranging from 0 - 25 mm (Adefuye and Ndip, 2013; Okeleye et al., 2011; Adeyemi et al., 2008). However, detailed assessment to identify the active compounds has not been carried out.

1.9.6.2 Antifungal

Mabeku et al. (2011) evaluated the antifungal activities of methanol, ethanol, hexane, ethyl acetate, aqueous, mixture of methanol and water, and mixture of ethanol and water stem extracts of *B. micrantha* against *Candida albicans* and *Candida glabrata* using the disc diffusion assay and broth micro-dilution methods. The ethanol extract of stem of *B. micrantha* gave a diameter zone of inhibition of 10 mm against *C. glabrata*. The phytoconstituents responsible for the antifungal activity of *B. micrantha* have not been identified.

1.9.6.3 Antidiarrheal

The methanol bark extract of *B. micrantha* was evaluated against different experimental models of diarrhoea in rats as well as bacteria that cause diarrhoea such as *E. coli, Plesiomonas shigelloides, Salmonella virchow and Shigella dysenteriae* and *S. flexneri*. The methanolic bark extract of *B. micrantha* showed weak inhibitory activities against *P. shigelloides and S. flexneri*. Based on the results in experimental rat models, there were significant reductions in faecal output and frequency of droppings when plant extracts were

administered compared with castor-oil treated rats. All plant extracts also significantly retarded the propulsion of charcoal meal and significantly inhibited the prostaglandin E2 (PGE2)-induced enteropooling (Lin et al., 2002). The phytoconstituents responsible for this activity have not been identified.

1.9.6.4 Anthelmintic

The aqueous and organic bark extract of *B. micrantha* was evaluated for anthelmintic activities using a standard motility assay against a levamisole resistant strain of the nematode *Caenorhabditis elegans*. The extent of activity of the extracts was presented as average percentage of worm death and statistically compared to a negative control. *B. micrantha* aqueous and organic bark extracts showed 89.4% and 80.7% dead worms respectively, higher than the negative control (Waterman et al., 2010). The compounds responsible for this activity have not been identified.

1.9.6.5 Antimycobacterial

Green et al. (2011) evaluated the antimycobacterial activities of the n-hexane sub-fraction of ethyl acetate fractions from acetone extracts of *B. micrantha* stem using the resazurin microplate assay against *M. tuberculosis*. The n-hexane fraction showed 20% inhibition of *M. tuberculosis* H37Ra and almost 35% inhibition of *M. tuberculosis* isolate resistant to all first-line drugs at 10 µg/mL. The primary ethyl acetate fraction showed minimum inhibitory concentration (MIC) value of 8.25 µg/mL against H37Ra *M. tuberculosis* strain. The fraction also inhibited the growth of *M. tuberculosis* isolate resistant to isoniazid (INH), ethambutol (EMB), streptomycin (STM) and rifampicin (RIF) at a concentration of 50 µg/mL. N (b)-benzyl-14-(carboxymethyl), benzene, 1,3-bis (3-phenoxyphenoxy), 2-pinen-4-one were the major compounds of the fraction.

1.9.6.6 Antioxidant

Onoja et al. (2014) evaluated the antioxidant activities of the hydromethanolic extract of *B. micrantha* stem using 2,2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay. *B. micrantha* extract produced concentration-dependent increase in percentage antioxidant activity in DPPH photometric assay. The *B. micrantha* extract demonstrated a potent antioxidant activity with 50% inhibitory concentration (IC_{50}) of <25 µg/mL concentration in DPPH photometric assay. Shelembe et al. (2016) identified the antioxidant compounds of the stem of *B. micrantha* as Quercetin and Quercetin-3-O-glucoside.

1.9.6.7 Antidiabetic

Crude extracts of *B. micrantha* were found effective in reducing fasting blood glucose levels in diabetic mice *in vivo*. Even in crude form, the effects were comparable to that of the standard glibenclamide, an oral sulfonylurea with proven antidiabetic activity (Omeh et al.,

2014; Nwaehujor et al., 2015; Adika et al., 2012). In another study, Eton and Abo (2008) evaluated the antidiabetic activity of extracts and fractions from leaves of *B. micrantha* in alloxan diabetic rats. The MeOH/H_2O fraction exhibited significant ($p < 0.05$) reduction of blood glucose of 34.2% when compared to control rats. The antidiabetic activity of *B. micrantha* stem was evaluated *in vitro* using α-glucosidase, β-glucosidase and maltase glucoamylase assays. The methanolic extract was found to be the most potent inhibitor of α-glucosidase and maltase glucoamylase with IC_{50} values of 1.06 ± 0.10 µg/mL and 1.12 ± 0.10 µg/mL respectively. The extracts were not potent inhibitors of β-glucosidase (Ezekiel et al., 2018). Ajao et al. (2018) investigated the preventive effect of *B. micrantha* leaf extract on insulin resistance using high salt diet rats. Fasting blood glucose level and fasting blood insulin level were measured. At doses of 50 and 200 mg/kg/day, *B. micrantha* lowered the above parameters significantly. The compounds responsible for these effects have not been identified.

1.9.6.8 Antiviral

The leaf methanol extracts of *B. micrantha* were evaluated against human immunodeficiency type 1 reverse transcriptase by assessing inhibition of the ribonucleic acid (RNA) dependent deoxyribonucleic acid (DNA) polymerase activity by measuring the degree of incorporation of methyl-3H thymidine triphosphate using polyadenylic acid. The methanol extract of the leaves of *B. micrantha* inhibited the polymerase with IC_{50} value of 23.5 µg/mL and the ribonuclease H with IC_{50} value of 18.9 µg/mL (Bessong et al., 2004). In another study, Bessong et al. (2006) investigated the antiviral activities of root methanol extracts of *B. micrantha* against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and integrase (IN). The n-butanol fraction obtained from the crude methanol extracts of *B. micrantha* inhibited the RNA-dependent-DNA polymerization (RDDP) activity of HIV-1 RT with an IC_{50} of 7.3 µg/mL. However, the chemical constituents responsible for the antiviral activity of *B. micrantha* have not been identified.

1.9.6.9 Anticonvulsant and Sedative

The anticonvulsant effects of stem crude extracts of *B. micrantha* were investigated using mice model maximal electroshock (MES), strychnine (STR), pentylenetetrazol (PTZ), picrotoxin (PIC), isonicotinic hydrazide acid (INH)-induced convulsions and diazepam-induced sleep in assessing the sedative effects. Results obtained revealed that *B. micrantha* stem extracts at the doses of 34 and 67 mg/kg protected 100%, 80%, 80% and 80% of mice from PIC, STR, PTZ and MES-induced seizures, respectively. *B. micrantha* also delayed the onset to seizures in the INH test (Bum et al., 2012). The responsible phytochemicals have not been identified.

1.9.6.10 Antinociceptive

The antinociceptive effects of *B. micrantha* stem extracts at the doses of 50, 100 and 200 mg/kg were investigated using male Wistar rats via acetic acid-induced writhing reflex and tail flick methods and the effects of *B. micrantha* on thiopentone-induced narcosis was also investigated. *B. micrantha* extracts exhibited a significant dose-dependent decrease in the mean number of abdominal constriction in the acetic acid-induced writhing reflex when compared to the negative control. Both the extract (200 mg/kg) and paracetamol (400 mg/kg) produced 61.85% and 73.08% inhibition of writhing reflex, respectively (Onoja et al., 2014). The chemical constituents responsible for this activity have not been identified.

1.9.6.11 Antischistosomal

The antischistosomal activities of hexane, methanol and water extracts (bark) of *B. micrantha were tested* on Swiss white mice infected with *Schistosoma mansoni* with praziquantel as control. There were no significant difference between worm reduction percentages of praziquantel (75.2%) compared to 48.7% and 63.4% demonstrated by hexane and water extracts of *B. micrantha* respectively (Waiganjo et al., 2016). Chemical constituents responsible for this activity have not been identified.

1.9.6.12 β-Lactamase Inhibitory

The anti- *β* -lactamase activities of methanolic stem extracts of *B. micrantha* were evaluated by assessing the inhibition activities (over 90%) against four classes of lactamases, viz. TEM-1, OXA-10, IMP-1 and P99. *B. micrantha* extracts had strong inhibition activities of 99.2% and 92.0% against OXA-10 and P99 respectively. After elimination of tannins, the extracts were further tested for anti-*β*-lactamase activities with OXA-10 demonstrating potent inhibitory activity with 50% inhibitory concentration (IC_{50}) value of 0.02 mg/mL (Gangoue-Pieboji et al., 2009). Further research aimed at isolating and elucidating the chemical structure of the active constituents of *B. micrantha* has not been done.

1.9.6.13 Insecticidal

The leaf extracts of *B. micrantha* were investigated for insecticidal activities against *Podagrica uniforma* (Jacoby) and *Nisotra dilecta* (Jacoby) insect pests of *Abelmoschus esculentus* (okra) with synthetic insecticide cypermethrin as a control. Results obtained revealed that *B. micrantha* extracts were effective in reducing the insect population and improved *A. esculentus* fruit yield compared to cypermethrin (Adesina et al., 2016). No study has been done to determine the phytochemical/s responsible for the activity.

1.9.6.14 Toxicity and Cytotoxicity

Steenkamp et al. (2009) investigated the cytotoxicity of *B. micrantha* using human adenocarcinoma cells of the cervix (HeLa), human breast cells (MCF-12A), lymphocytes

(both resting and stimulated) and primary porcine hepatocytes. Toxicity was found to be concentration dependent when HeLa and MCF-12A cells were exposed to *B. micrantha* extracts. Munayi (2016) found that the crude extract of *B. micrantha* showed a cell viability of 31.5% at the tested concentration (10 µg/ml) with an IC_{50} value of 9.43µg/ml and thus showed good activity towards the drug sensitive leukemia cell lines. The compound, trans-triacontyl-4-hydroxy-3-methoxycinnamate showed an interesting cell viability of 31.13% at 1 µg/mL.

1.10 Problem statement

Bridelia micrantha is commonly used traditionally for the treatment of diabetes, gastrointestinal ailments, paralysis and painful joints amongst others. Pharmacological studies have revealed that various parts of the plant possess antidiabetic, antiviral, antidiarrheal, antibacterial, antioxidant, anticonvulsant, insecticidal and cytotoxicity activities. Phytochemical studies have also reported the presence of friedelin, epi-friedelin, flavonoids, tannins, gallic acid, ellargic acid, sterol, saponin, anthocyanidin, delphinidin, caffeic acid, taraxerone and taraxerol. However, there are limited studies identifying the compounds responsible or contributing to the pharmacological activities. Despite the use of *B. micrantha* traditionally for the treatment of diabetes, there are limited studies validating the traditional use of the plant. This project was therefore designed to evaluate the antidiabetic activities and to isolate and characterize the compound responsible.

1.11 Aim

The aim of this study was to isolate and characterize the compounds responsible for the antidiabetic activity of *Bridelia micrantha* plant.

1.12 Objectives

- Isolate and purify compounds using chromatographic techniques
- Determine the structure of compounds using spectroscopic techniques
- Evaluation of the antidiabetic activities using α-amylase and α-glucosidase assays
- Evaluation of antioxidant activities using DPPH radical scavenging and reducing power assays

2 MATERIALS AND METHODS

2.1 Sample collection

The leaf, stem and root of *B. micrantha* were collected during the month of May 2017 at the University of Venda campus. Plant identification was confirmed by Prof MP Tshisikhawe, in the Department of Botany, University of Venda. The voucher specimen was deposited at the University of Venda Herbarium and assigned a voucher number MKUV0001. The plant materials were air-dried at room temperature for 3 weeks. The materials were then ground to coarse powder using an industrial grinding mill (Dietz-Moteren KG, Deltingen unter Teck, Germany).

2.2 Extraction

Initially, 50 grams (g) of leaves; stem and root material were each soaked with 200 millilitres (mL) of methanol for 24 hours (h) with occasional shaking. Each extract was filtered using Whatman no1 filter papers (Whatman, UK) to obtain a homogeneous mixture and concentrated using a rotary evaporator (BUCHI, Switzerland) at 50°C.

To determine the most suitable solvent for extraction, each 50 g of ground leaf material was separately extracted with seven solvents of different polarities (i.e. Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol) with occasional shaking for 24 h. Each extract was filtered and concentrated using a rotary evaporator at 50°C.

The percentage yield of extracts was calculated using the following equation:

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

- **Bulk extraction**

877.174 g of the leaf material was extracted successively with hexane, dichloromethane and methanol for 72 h each as shown in figure 2.1 below. All the extracts were filtered out using filter papers (Whatman no 1) and concentrated in a rotary evaporator. The extracts were then transferred to appropriately labelled vials and stored at 4°C until further use.

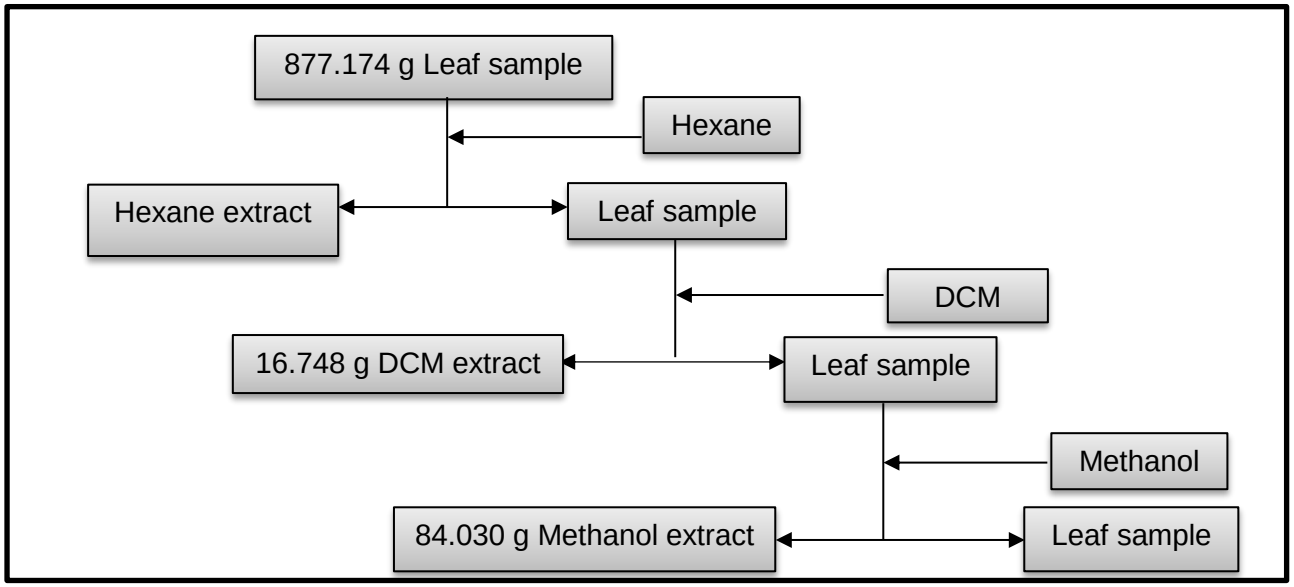


Figure 2.1: Schematic diagram showing successive extraction of leaf material.

2.3 Phytochemical screening

2.3.1 Total phenolic content

The total phenolic content of the crude extract and fractions was determined according to a method by Anokwuru et al. (2017). A working concentration of 1 mg/mL of the crude extract and fractions was prepared, 20 µL of samples were placed into a 96 well plate containing 80 µL of deionized water and 20 µL of 10% folin ciocalteu (Sigma-Aldrich, USA) was added to the diluted sample and was allowed to stand for 1 min before 60 µL of 7% Na_2CO_3 (MINEMA, RSA) was added to the mixture to stop the reaction. 120 µL of deionized water was added to the mixture for further dilution and the mixture was allowed to stand for 30 min. The absorbance was measured using a tunable microplate reader spectrophotometer (VersaMax™, China) at 760 nm and results were expressed as milligrams of gallic acid equivalents per gram of plant extract in dry weight (mg/g), as calculated by the following equation:

$$Gallic\ acid\ equivalents\ (GAE) = c\ \times v/m$$

Where, c = concentration of gallic acid obtained from the calibration curve (mg/mL);
v = volume of extract (mL) and m = mass of extract (g). The activity of the test samples ranged from 336.97 – 348.76 µg/mL, while Gallic and Ascorbic acid recorded 10.69 and 85.95 µg/mL, respectively

2.3.2 Total flavonoid content

The total flavonoid content of the crude extract and fractions was determined according to a modified method reported by Olajuyigbe and Afolayan (2011). A working concentration of 1

mg/mL of the crude extract and fractions was prepared and 100 µL of 2% $AlCl_3$ (MINEMA, RSA) was added to 100 µL of the crude extract and fractions in a 96 well and allowed to stand at room temperature for 60 min. Absorbance was recorded at 420 nm using a tunable microplate reader spectrophotometer (VersaMax™, China). Total flavonoid content was expressed as quercetin equivalent per gram of the fractions as well as the crude extract.

$$Quercetin\ equivalents\ (QE)\ = c\ \times v/m$$

Where, c = concentration of quercetin obtained from the calibration curve (mg/mL);
v = volume of extract (mL) and m = mass of extract (g).

2.3.3 Thin Layer Chromatography (TLC)

TLC is primarily used as an inexpensive method for separation, qualitative identification or for the semi quantitative visual analysis of samples. TLC chromatographic technique gives a clue as to how many components are in an extract. Advantages of TLC include rapid analysis time because many samples can be analysed simultaneously, low solvent usage on a per-sample basis and a high degree of accuracy and precision (Tuzimski and Sherma, 2015).

The dilute sample was spotted along a straight line at the bottom the TLC plate (about 0.5 cm from the base). Commercially prepared TLC aluminium sheets of 20 x 20 cm Silica gel Alugram xtra SIL G/UV 254 was used. The plate was cut to size of 5 x 5 cm. The plate was then placed in a developing tank containing the following chosen solvent systems:

i. Ethyl acetate/methanol/water (81:11:8)
ii. Ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:26)
iii. Chloroform/glacial acetic acid/methanol/water (64: 32:12:8).

For visualization of chemical compounds, two separate spray reagents (Appendix 1) were used for each TLC plate i.e.

i. Natural product reagent, for detection of flavonoids and;
ii. Sulphuric acid spray then heating, for detection of terpenoids.

Development occurs as the mobile phase moves through the layer, and the components of the sample move at different rates according to polarity and retention affinity to create the separation. The different spots are equivalent to the different compounds characterized by retention factor (Rf) values, calculated as follows:

$$Rf = \frac{distance\ traveled\ by\ the\ compound}{distance\ traveled\ by\ the\ solvent\ front}$$

2.3.4 Fractionation – Column Chromatography

The methanol extract was re-dissolved in methanol, adsorbed with silica gel then air-dried until it was in powder form. The powdered material was then subjected to column chromatography prepared with silica gel and hexane slurry. The eluting solvent initially was hexane/ethyl acetate (70: 30) followed by hexane/ethyl acetate (30: 70). 100% ethyl acetate was then used and the polarity was gradually increased from ethyl acetate/methanol (90:10) to ethyl acetate/methanol (30: 70). About 960 mL of each elution mixture was collected. The fractions collected were monitored by TLC as described above. The fractions collected with the same elution solvents were combined together since they showed similar spots on the TLC. A total of 6 fractions (LFR) were obtained. The fractions were then concentrated using a rotary evaporator, transferred to appropriately labelled vials and allowed to stand in the fume hood to evaporate residual solvents.

2.3.5 Purification - Preparative Thin Layer Chromatography (pTLC)

Preparative thin layer chromatography (pTLC) is used to separate compounds of quantities larger than are normal for analytical TLC by utilizing a thick layer of adsorbent (0.5-5 mm). LFR5 (selected based on the amount obtained) was re-dissolved in methanol then applied as a long streak using a thin tipped pipette within two lines which were 1.5 cm from the bottom of the plate and dried with flowing air. Development was done using Ethyl acetate/ Methanol/ Water (81:11:8) solvent system. The plates were visualized under UV light (254 nm), the regions of interest were marked and scraped off. The separated materials were filtered from the sorbent using methanol. This afforded three compounds (Figure 2.2).

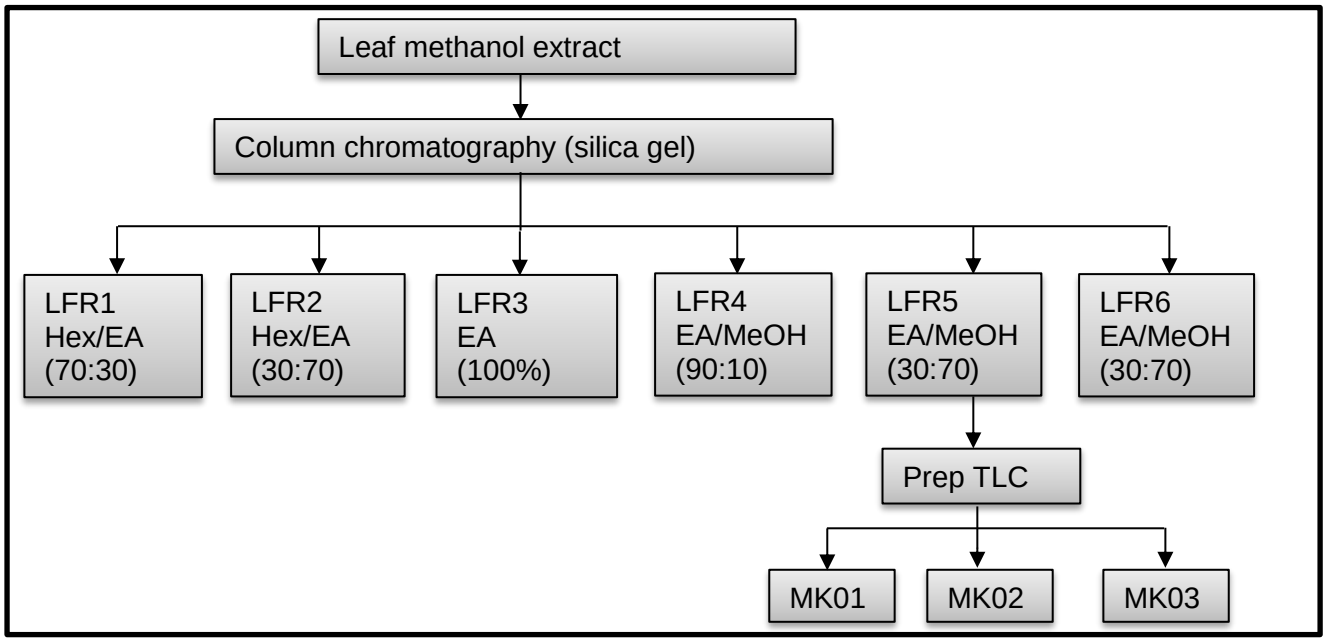


Figure 2.2: Schematic diagram showing purification of compounds

2.3.6 Structural elucidation

Fourier Transform – Infrared (FT-IR) spectra were recorded on a Bruker Alpha Fourier Transform IR spectrometer, with absorption maxima reported in terms of wavenumbers (cm^{-1}).

High resolution mass spectra (HRMS) were measured on a Waters Synapt G2 Quadrapole time-of-flight mass spectrometer connected to a Waters Acquity ultra-performance liquid chromatography (UPLC). The samples were dissolved in a mixture of acetone and dichloromethane and injected at a volume of 1 µL at a mode of 10:1 at a temperature of 280°C. The source temperature was set at 300°C. Helium gas was used as the carrier gas. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 704).

Nuclear magnetic resonance (NMR) is a spectroscopic technique involving a magnetic field in which a sample is placed. The sample is then subjected to radiofrequency radiation at the appropriate frequency, allowing for the absorption of energy depending on the type of nucleus, whether, for example, it is a 1H or 13C (Stuart, 2012).

A Bruker Avance NMR instrument allowing analysis at 400 MHz was used in this study. Deuterated methanol (CD_3OD) was used to prepare the NMR samples. Both 1D and 2D NMR experiments were conducted on the isolated compound for structural elucidation, with the former providing information on the carbon skeleton as well as the specifics of the hydrogen atoms and the latter providing information on the proton to carbon relation and the chemical environment they are in.

2.3.6.1 Spectroscopic data

Yellow amorphous powder, Negative HRESIMS m/z: 613.0872. ^{1}H-NMR (400 MHz, CD_3OD): δ_H 7.73 (1H,s, H-2'), 7.62 (1H, d, *J* = 8.4 Hz, H-6'), 6.90 (1H, d, *J* = 8.8, H-5), 6.42 (1H, s, H-8), 6.23 (1H, s, H-6), 5.33 (1H, s, H-1'''), 5.27 (1H, *J* = 7.6, H-1''), 0.98 (3H, d, *J* = 6 Hz, H-6'''), 3.34 - 3.96 (9H, m, glc: 2'' - 6'', rha: 2''' - 5''')

^{13}C-NMR (100 MHz, CD_3OD): δ_c 178.08 (C-4), 164.71 (C-7), 161.71 (C-5), 157.65 (C-9), 157.06 (C-2), 148.46 (C-4'), 145.37 (C-3'), 134.21 (C-3), 121.81 (C-6'), 121.64 (C-1'), 116.15 (C-2'), 114.63 (C-5'), 104.26 (C-10), 102.90 (C-1''), 102.20 (C-1'''), 98.55 (C-6), 76.96 (C-3''), 76.67 (C-5''), 74.29 (C-2''), 71.93 (C-5'''), 70.70 (C-4'''), 70.64 (C-2'''), 70.48 (C-3'''), 69.79 (C-4''), 61.11 (C-6'') and 16.26 (C-6''') ppm.

2.4 Antioxidant assays

2.4.1 DPPH radical scavenging method

The DPPH radical scavenging activity was measured according to a modified method by Anokwuru et al. (2017). A 125 mM of DPPH/ ethanol solution was prepared by dissolving 10 mg of DPPH (Sigma-Aldrich, USA) into 200 mL of ethanol. A 100 µL of distilled water was added into each well of a 96 well plate. Concentrations of 1 mg/mL of the crude extract and fractions were prepared. A volume of 100 µL of the samples was added into the first three wells. Serial dilutions were done using multi-channel micro pipette to get concentrations of 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL. A 200 µL of DPPH/ethanol solution was added to each well containing the mixtures. The plate was kept in the dark for 30 min and the absorbance was measured using a tunable microplate reader spectrophotometer (VersaMax™, China) at 517 nm. The ability of the samples to scavenge DPPH was calculated using the equation:

$$\%\ Inhibition\ = \frac{Abs_{DPPH}\ -\ Abs_{sample}}{Abs_{DPPH}} \times 100$$

The antiradical properties of the methanol extracts (root, stem and leaves) of *B. micrantha* were evaluated as the DPPH IC_{50} which is the concentration of antioxidant required to reduce the DPPH free radical by 50%. Basically, a high DPPH radical scavenging activity is associated with a low IC_{50} value. Gallic acid and Ascorbic acid were used as positive controls.

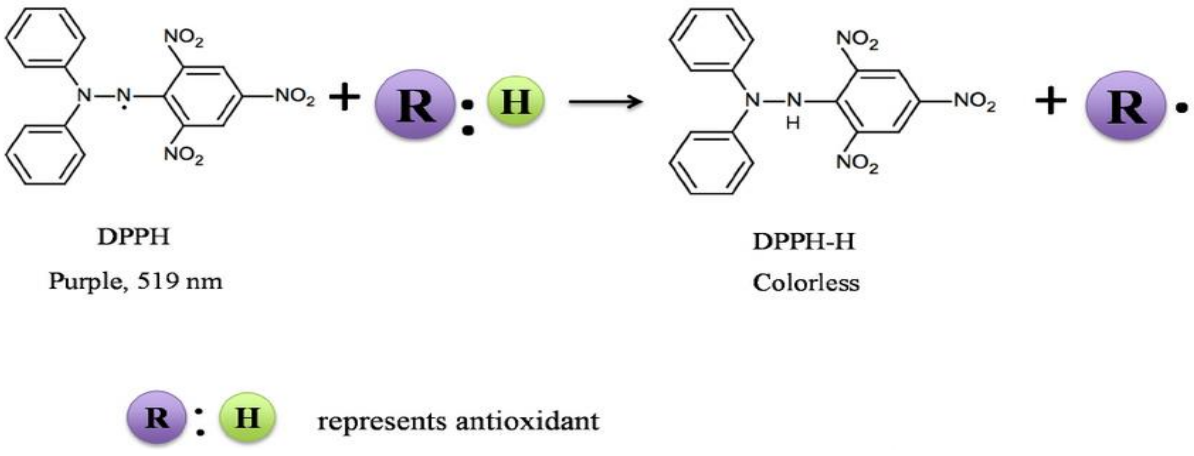


Figure 2.4: Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant (Liang and Kitts, 2014).

2.4.2 Ferric reducing power assay

The ferric reducing power was determined by a modified method cited by Anokwuru et al. (2017). Concentrations of 0.5 mg/mL of the crude extract and fractions were prepared. A volume of 50 µL of the sodium phosphate buffer (0.2 M, 6.6 pH) and 50 µL of the samples and standards, gallic acid (Sigma-Aldrich, USA) and ascorbic acid were added into each well of a 96 well plate and serial dilution was done using a multi-channel micro pipette. A 50 µL volume of potassium hexacynate (1%) was added to each well. The plate was placed in an incubator for 30 min at 50 °C. After incubation, 50 µL of trichloroacetic acid (10%) was added. A volume of 80 µL of each mixture was transferred to another well plate and 80 µL of distilled water was added then 15 µL of ferric chloride (0.1 % w/v) (Associated Chemical Enterprise, RSA) was added. Reading was done by tunable microplate reader (VersaMax™, China) at 700 nm.

2.5 Antidiabetic assays

2.5.1 α- Amylase Inhibition assay

The assay was carried out with 1% soluble starch as the substrate (Sigma, S2004), human salivary α-amylase (1 mg/mL)(Sigma, E8140), 20 mM phosphate buffer (pH 6.9)(Sigma, S0751), standard inhibitors, acarbose (1 mg/mL)(Sigma, A8980) and DNS reagent (1% 3,5-dinitrosalicylic acid)(Sigma, D0550) and 12% potassium sodium tartrate (Sigma, S6170) in 0.4 M NaOH.

In 2 mL control tubes, 40 µL phosphate buffer and 20 µL α-amylase were added. For the blank, 60 µL of the phosphate buffer without the enzyme was added. Aliquots of 10 µL phosphate buffer, 20 µL α-amylase and 30 µL acarbose were added to the inhibitor tubes. To the solvent control tubes, aliquots of 10 µL phosphate buffer, 20 µL α-amylase and 30 µL solvent (10% Dimethyl sulfoxide DMSO) were added to solvent control tubes. For the test samples, 10 µL phosphate buffer, 20 µL α-amylase and 30 µL test samples were added.

The tubes were incubated in a water bath at 37°C for 30 min. To all the test tubes, 90 µL of 1% starch was added. The tubes were further incubated for 20 min in a water bath at 37°C. 50 µL of DNS reagent was added and the tubes were placed in boiling water for 5 min. The tubes were then left to cool down and then 500 µL of water was added to each tube. All the samples were done in triplicate at a final concentration of 200 µg/mL.

Test samples that showed α-amylase inhibition were recognized by the formation of a pale brown product while the blank and the inhibitors showed no colour change. The contents in the tubes were then transferred into the respective wells in a 48 well plate (Corning, USA).

The absorbance was measured at 540 nm on a Tecan-Infinite 500 multi-well spectrophotometer (Tecan group LTD, Switzerland).

$$\%\ Inhibition\ = \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}} \times 100$$

Where $\Delta Abs_{control}$ = $Abs_{control}\ -\ Abs_{blank}$ and $\Delta Abs_{sample}\ = Abs_{sample}\ -\ Abs_{blank}$
Sample is either extract or compound. This assay was carried out at CSIR.

2.5.2 α- Glucosidase Inhibition assay

In a 96 well plate (Corning, USA), 30 µL phosphate buffer (20 mM, pH 6.9) and 10 µL α-glucosidase (100 µg/mL) (from *Saccharomyces cerevisiae*) were added to the control wells. For the blank wells, 40 µL phosphate buffer was added. To the inhibitor wells, 20 µL of dimethylacrylic acid (DMAA) (1 mg/mL), 10 µL phosphate buffer and 10 µL α-glucosidase were added. For the test samples, 20 µL sample (1 mg/mL), 10 µL phosphate buffer and 10 µL α-glucosidase were added. Since extracts have pigments which might interfere with the assay, sample controls were prepared. Sample controls contained 80 µL phosphate buffer and 20 µL sample. The plate was incubated at 37°C for 30 min. 60 µL substrate was added to each well and the plate was further incubated for 15 min. All samples were done in triplicate at a final concentration of 200 µg/mL. The absorbance was read on a Tecan micro-plate reader.

$$\%\ Inhibition\ = \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}} \times 100$$

Where $\Delta Abs_{control}$ = $Abs_{control}\ -\ Abs_{blank}$ and $\Delta Abs_{sample}\ = Abs_{sample}\ -\ Abs_{blank}$
Sample is either extract or compound. This assay was carried out at CSIR.

2.6 Statistical analyses

Statistical analyses were carried out with the STATISTICA (StaSoft Inc., Tulsa, OK, USA) package. Data was presented as mean ± SD. Mean differences were assessed by one-way ANOVA with the Fisher LSD test.

3 RESULTS

3.1 Extraction

The different parts (leaves, stem and roots) of *B. micrantha* were extracted with methanol. The yields obtained from the different plant parts extracts are shown in Table 3.1. The highest yield was observed for the stem extract, followed by the root extract and then leaf extract.

Table 3.1: The yields of extracts obtained from different part of *B. micrantha*.

Plant part	Mass extracted (g)	% Yield
Root	10.09	20.18
Stem	11.76	23.51
Leaf	3.37	6.74

Different solvents were assessed for their effectiveness in extracting constituents of the leaf components of *B. micrantha* (Figure 3.1). Methanol proved to be the best extracting solvent for *B. micrantha*. The least extraction yield was obtained for hexane.

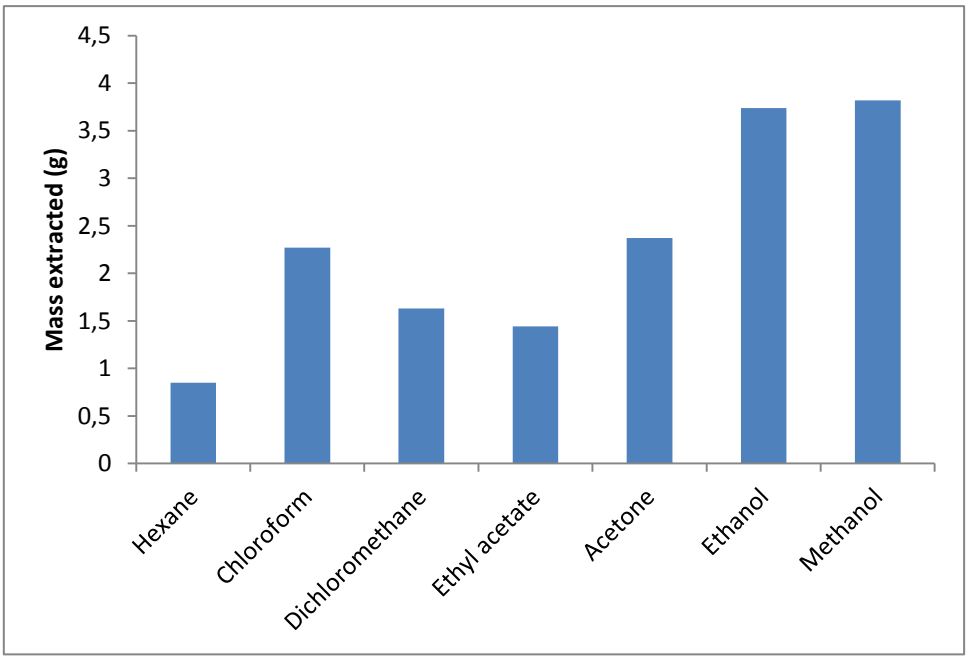


Figure 3.1: Effect of solvents on extraction yield of *B. micrantha* leaf

3.2 Bulk extraction and fractionation results

About 800 g of ground *B. micrantha* leaf material was first defatted with hexane then successively extracted using dichloromethane and methanol to yield extracts of 15.849 g

and 84.030 g respectively. The methanol extract was further selected for fractionation based on the amount obtained. The extract was subjected to column chromatography using silica gel as the stationary phase. A gradient mixture of solvents was used as the mobile phase. The fraction yield and eluting solvent mixture are shown in Table 3.2.

Table 3.2: Fraction yields and solvent mixture ratio

Fractions	Elution solvent	Mass (g)
LFR1	Hex/EA (70:30)	2.511
LFR2	Hex/EA (30:70)	5.290
LFR3	100% EA	0.364
LFR4	EA/MeOH (90:10)	1.244
LFR5	EA/MeOH (70:30)	13.447
LFR6	EA/MeOH (30:70)	7.368

LFR: Leaf fraction; Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol.

3.3 Isolation of compounds

Further isolation of compounds from LFR5 was selected based on the high mass obtained, three compounds were isolated from the fraction using preparative TLC (pTLC). The TLC plates together with the Rf values of the compounds are presented in figure 3.2 below. Development with E.M.W (81:11:8) showed the occurrence of three orange spots which were detected after staining the plate with natural product polyethylene glycol (NP-PEG) reagent revealing that fraction LFR5 is a pure fraction of flavonoids. The spots observed at Rf values 0.19, 0.32 and 0.52 were named MK01, MK02 and MK03 respectively.

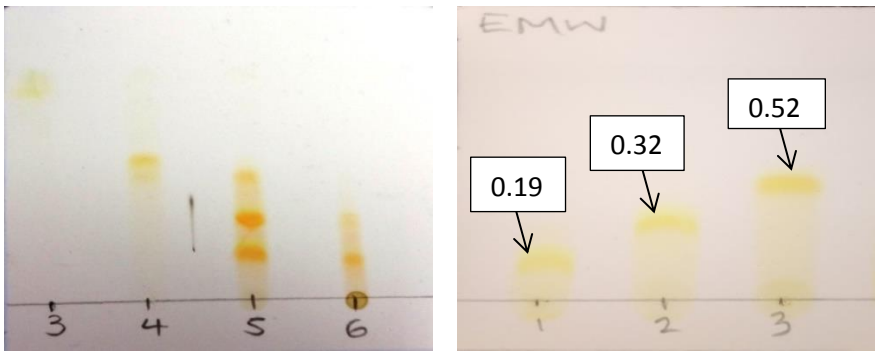


Figure 3.2: TLC plates stained with NP-PEG reagent showing fractions (left) and isolated compounds (right).

3.4 Phytochemical screening

3.4.1 Total phenolic content

The calibration curve (Y = 0.0034x + 0.4375, R^2 = 0.9955) obtained from gallic acid was used to quantify the total phenolic content in the crude extracts. The absorbance values obtained were replaced in this equation to determine the total phenolic contents presented in Figure 3.3. The total phenolic contents in the examined crude extracts were found to be highest in the stem extract (9.57 ± 4.59 mg GAE/g) followed by root (7.34 ± 3.17 mg GAE/g). The lowest phenolic content was observed for the leaf extract (4.76 ± 1.10 mg GAE/g).

3.4.2 Total flavonoid content

The calibration curve (Y = 0.0211x + 0.0555, R^2 = 0.999) obtained from quercetin was used to quantify the total flavonoid content in the crude extracts. The total flavonoid content presented in Figure 3.3 was found to be highest in leaf (1.86 ± 0.35 mg QE/g) followed by root (0.57 ± 0.13 mg QE/g) and the stem extract (0.43 ± 0.05 mg QE/g).

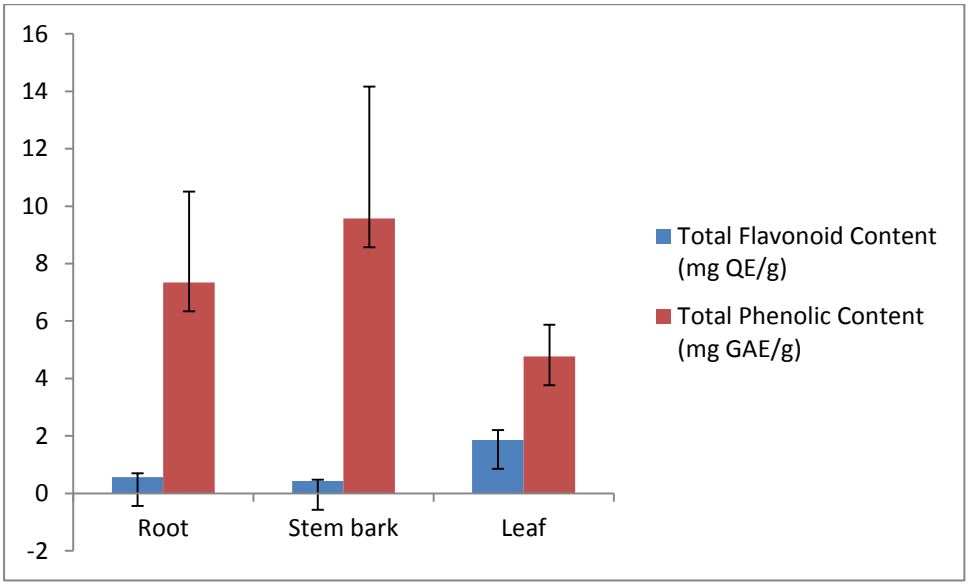


Figure 3.3: Total flavonoid (mg QE/g) and total phenolic content (mg GAE/g) of different plant parts.

3.5 Antioxidant activities

The results of the antioxidant activities of the crude extract, fractions and pure compound are presented in Table 3.3.

3.5.1 DPPH scavenging method

There was no significant difference between the DPPH free radical scavenging ability of the crude extracts and LFR5. The standards (gallic acid and ascorbic acid) displayed

significantly ($p \leq 0.01$) higher activity compared to the extracts. Nevertheless, there was no significant difference among the IC_{50} values of the crude extracts and the fractions.

3.5.2 Reducing Power assay

The root and stem extracts displayed significantly ($p < 0.05$) higher reducing power activity compared to the leaf extract and fraction LFR5. Gallic acid displayed significantly higher reducing power activity compared to ascorbic acid and the extracts.

Table 3.3: Inhibition of DPPH (IC_{50}) and reducing power ($IC_{0.5}$) represented as average of triplicates ± standard deviation of the mean. Values with dissimilar letters in a column are significantly different ($p \leq 0.01$).

Test sample	DPPH radical IC_{50} values (µg/mL)	Reducing Power $IC_{0.5}$values (µg/mL)
Roots	348.76 ± 12.82^{b}	125.17 ± 10.46^{b}
Stem	345.56 ± 44.84^{b}	119.31 ± 8.22^{b}
Leaves	336.97 ± 86.93^{b}	291.88 ± 74.34^{c}
LFR5	298.22 ± 19.39^{b}	226.92 ± 49.80^{c}
Gallic acid	10.69 ± 7.05^{a}	12.28 ± 2.01^{a}
Ascorbic acid	85.95 ± 27.95^{a}	78.45 ± 8.55^{b}

3.6 Percentage inhibition on α-glucosidase and α-amylase

The results of the percentage inhibition of crude extracts and isolated compound (200 µg/mL) on α-glucosidase and α-amylase are presented in Table 3.4. The crude extracts displayed high inhibition of α-glucosidase at the tested concentration. The fraction obtained from leaf (LFR5) displayed higher activity compared to leaf crude extract. The compounds MK01 and MK02 were not active. However, compound MK03 displayed similar activity as LFR5 and comparable to positive control (DMAA). The percentage inhibition of α-amylase by the crude extracts was lower than the activity against α-glucosidase. Only the stem and root crude extracts displayed percentage inhibition >50 %. None of the isolated compounds displayed any activity against α-amylase.

Table 3.4: Percentage Inhibition of test samples on α-glucosidase and α-amylase

Test sample	% Inhibition ± SD α-glucosidase assay	% Inhibition ± SD α-amylase assay
Root	98.52 ± 0.38	65.62 ± 1.71
Stem	98.62 ± 0.02	61.86 ± 0.39
Leaf	81.62 ± 0.62	27.79 ± 0.21
LFR5	96.19 ± 0.20	26.93 ± 2.25
MK01	NI	NI
MK02	41.62 ± 1.43	NI
MK03	96.74 ± 0.39	NI
Acarbose	NI	80.18 ± 1.18
DMAA*	99.85 ± 0.09	-

NI = No inhibition

*The acarbose was used as a positive control in the α-amylase assay but it did not inhibit α-glucosidase. Therefore, after screening different compounds and drugs against α-glucosidase, it was found that DMAA inhibits α-glucosidase hence it was used as a positive control in this assay.

3.7 Antidiabetic activity of leaf extracts of various solvents

The α-amylase and α-glucosidase activity of the leaf extracts obtained with various solvents were evaluated. All the extracts exhibited weak activity against α-amylase. IC_{50} values for α-glucosidase activity are depicted in table 3.5. The methanol and ethanol extracts were significantly ($p < 0.05$) higher than the other extracts and control.

Table 3.5: α-Glucosidase (IC_{50}) represented as average of triplicates ± standard deviation of the mean. Values with dissimilar letters in are significantly different ($p<0.05$).

Extracts	α-glucosidase IC_{50} values (µg/mL)
Acetone	23.80 ± 0.47^{b}
Chloroform	34.60 ± 0.36^{b}
Dichloromethane	$>112 \pm 0.36^{b}$
Ethyl acetate	$>112 \pm 0.40^{b}$
Ethanol	3.75 ± 1.00^{a}
Hexane	110.00 ± 0.57^{b}
Methanol	4.58 ± 1.51^{a}
DMAA	91.30 ± 0.74^{b}

4 DISCUSSION

4.1 Effect of solvent on extraction yield

The extraction of plant constituents is the first step in isolation of bioactive compounds present in medicinal plants. Solvent extraction is the most frequently used technique due to efficiency, ease of use and wide applicability. Generally, the extract yield depends on the nature of the extracting solvent, composition of sample, extraction time as well as temperature (Do et al., 2014). The solubility of polyphenols is governed by the chemical nature of the plant sample as well as the polarity of solvents used. Furthermore, polyphenols may be associated with other plant constituents such as carbohydrates and proteins. Therefore, there is no universal extraction procedure suitable for the extraction of all plant polyphenols. Depending on the solvent system employed during extraction, a mixture of polyphenols soluble in the solvent will be extracted from the plant matrix. It may also contain some non-phenolic substances such as sugar, organic acids and fats. As a result, additional steps may be required to remove those unwanted components (Dai et al., 2010). The most suitable solvents are aqueous mixtures containing methanol, ethanol, acetone and ethyl acetate (Dorta et al., 2012).

In this study, the effect of seven different polarity solvents on extraction yield of *B. micrantha* leaves was evaluated. Methanol had the highest yield followed by ethanol. The lowest yield was observed for hexane. In one study (Adefuye et al., 2011); the extraction efficiency of different solvents on the stem bark of *B. micrantha* was evaluated. The results obtained for the different solvents were as follows: Methanol (4.58 g) > ethanol (3.19 g) > acetone (2.95 g) > ethyl acetate (0.35 g). These results corroborate with the findings of our study and therefore confirm that methanol is the best solvent for extraction of *B. micrantha* constituents. The results also corroborate with findings of other authors investigating the effect of solvent systems on extraction yields of medicinal plants (Złotek et al., 2016; Do et al., 2014).

4.2 Structural elucidation

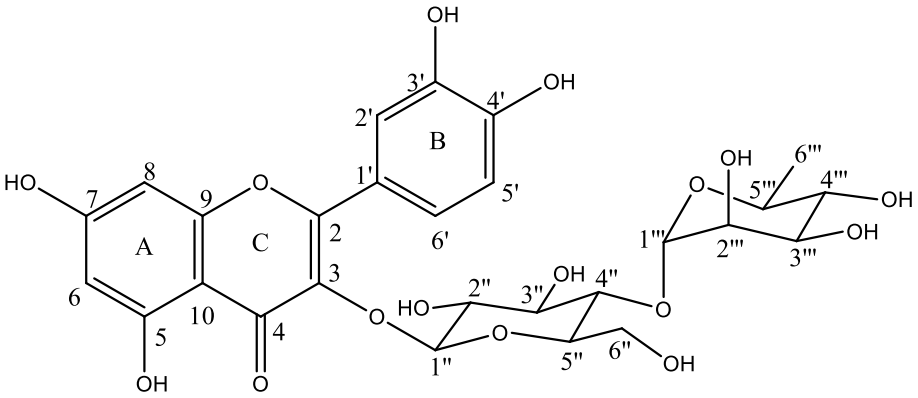


Figure 4.1: Structure of compound MK03 (quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside)

Compound MK03 was obtained as a yellow powder (20.2 mg). The TLC developed in Ethyl acetate: Methanol: Water (3:1:1) exhibited an Rf value of 0.52 and the spot appeared bright yellow under UV indicating the presence of quercetin. IR spectra at 3273.47 cm^{-1} (OH stretching vibration of phenol), at 2918.52 cm^{-1} (methylene's stretching vibration of the glycosyl) and at 1651.00 cm^{-1} (C = O, flavone ring) suggesting the presence of a quercetin glycoside. Its molecular formula, $C_{27}H_{30}O_{16}$ was confirmed by HRMS, m/z: 613.0872.

The ^{1}HNMR spectra of compound MK03 exhibited characteristic resonances of a flavonol. The aromatic region displayed the ABX system with proton signals at δ_H 7.73 ppm (s, H-2'), δ_H 7.62 ppm (d, J = 8.4, H-6') and δ_H 6.90 ppm (d, J = 8.8, H-5') due to 3, 4-dihydroxylated pattern for ring B. A 5, 7-dihydroxylated pattern for ring A exhibited protons resonating at δ_H 6.42 ppm (s, H-8) and δ_H 6.23 ppm (s, H-6) assigning quercetin as the aglycone (Sambandam et al., 2016). The NMR could not recognize the hydroxyl groups due to their high rates of proton exchange resulting from the electronegativity of the oxygen atom. The signals displayed at 3.35 – 3.74 ppm in association with the presence of a doublet at δ_H 5.27 ppm (J = 7.6 Hz) assigned for the anomeric proton H-1'' due to diaxial position with H-2'' suggested the presence of a β-linked glucose unit. Furthermore, a distinct broad singlet displayed at δ_H 5.33 ppm (s, H-1''') assigned the anomeric diequitorial proton which was in association with a doublet at δ_H 0.98 (d, J=6 Hz, H-6''') revealed the presence of a α-linked rhamnose unit.

The ^{13}C-NMR and DEPT 135 spectra revealed the disappearance of 10 quaternary carbons at δ_C 178.08 (C-4), 164.71 (C-7), 161.60 (C-5), 157.65 (C-9), 148.46 (C-4'), 157.06 (C-2), 144.51 (C-3'), 134.21 (C-3), 121.64 (C-1') and 104.26 ppm (C-10). The 5 methine carbons of the aromatic region were observed at δ_C 121.81 (C-6'), 116.15 (C-2'), 114.63 (C-5'), 98.55

(C-6) and 93.36 ppm (C-8). The presence of glucoside and rhamnose moieties were further confirmed by chemical shifts of oxymethine carbons at δ_C 76.96 – 61.11 (C-2'' – C-6"), and especially the carbons at δ_C 102.90 (C-1''), 102.20 (C-1'''), 61.11 (C-6'') (observed below the plane on the DEPT 135 spectrum), and 16.26 (C-6''').

For the protons displayed in the aromatic region, the HMBC spectrum was used to confirm correlations between carbons and protons separated by two or three bonds. For protons on the catechol ring B, H-2' (δ_H 7.73) showed correlation with C-6'(δ_C 121.64), C-4 (δ_C 148.46) and C-2 (δ_C 145.37); The proton H-6' (δ_H 7.62) correlates with C-2' (δ_C 116.15), C-4'(δ_C 148.46) and C-2 (δ_C 145.37) whereas H-5' (δ_H 6.88) correlates with C-1'(δ_C 121.81) and C-3'(δ_C 144.51). The protons on the benzopyrone ring AC displayed correlations of H-6 (δ_C 7.62) with C-8 (δ_C 93.36) and C-10 (δ_C 104.26); and H-8 (δ_H 6.42) with C-6 (δ_C 98.55) and C-10 (104.26). The HMBC spectrum also revealed important correlations between δ_H 5.27 ppm (d, J = 7.6, H-1'') and C-3 (134.21) which showed that the glucose is linked to the aglycone at C-3. A cross peak between δ_C 69.78 (C-4'') and δ_H 5.33 ppm (s, H-1''') established the linkage point of rhamnose to the glucose moiety.

The HSQC spectrum was used to confirmed correlations on the catechol ring B of H-2' (δ_H 7.73) with C-2' (δ_C 116.15); H-6' (δ_H 7.62) with C-6' (δ_C 121.64); H-5' (δ_H 6.88) with C-5' (δ_C 114.63) and the correlations on the benzopyrone ring AC of H-8 (δ_H 6.42) with C-8 (δ_C 93.36); H-6 (δ_C 7.62) with C-6 (δ_C 98.55) and H-1'' (δ_H 5.27) with C-1'' (δ_C 102.20).

4.3 Phenolic content and Antioxidant activity of various plant parts extracts

Increase in levels of oxidative stress has been linked with lipid peroxidation, non-enzymatic glycation of proteins and oxidation of glucose which contributes towards diabetes and its complications. Antioxidants have already shown to be prospective agents in the treatment of diabetes.

The current study investigated the antioxidant potential of extracts from different parts of *B. micrantha* using DPPH radical scavenging and reducing power assays as a prelude to find compounds that could be used to ameliorate T2DM and associated complications. The DPPH results in the current study revealed that the antioxidant activity of the *B. micrantha* extracts increased with increasing concentration. This observation is in agreement with the results obtained by various authors (Shelembe et al., 2016; Onoja et al., 2014).

In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{3+}) in ferric chloride to ferrous (Fe^{2+}). In this assay, the test solution changes to various shades of green depending on the reducing power of the antioxidant samples. The

reducing power assay showed weak antioxidant activities for all extracts. The crude extracts produced IC_{50} values ranging from 119.31 – 291.88 µg/mL. The stem extract showed higher activity in comparison to root and leaf extracts.

Antioxidants have the capability to reverse the effects of alloxan (2,4,5,6-tetraoxypyrimidine), a strong oxide agent capable of producing free superoxide radicals which, as it is well known, shows selective toxicity to β-cells and create conditions conducive to the development of diabetes (Kikumoto et al., 2010). Alloxan also causes a decrease in the activity of superoxide dismutase, the enzyme responsible for protecting β-cells from injury (Song et al., 2013). Therefore, this study reveals that *B. micrantha* possess antioxidant activities which could slow down or eliminate the production of ROS thereby, reversing diabetic conditions.

4.4 Antidiabetic activities of various plant part extracts

Postprandial hyperglycaemia is the main risk factor in the development of T2DM. α-Glucosidase and α-amylase inhibitors that reduce postprandial hyperglycaemia have a key role in the treatment of T2DM. Due to side effects of many synthetic inhibitors, many studies have focused on searching for natural inhibitors of α-glucosidase and α-amylase from plants. In this study, the effect of various plant part extracts of *B. micrantha* on the activities of α-glucosidase and α-amylase were investigated *in vitro*.

All the extracts in this study were found to be active against α-glucosidase with activity ranging from 81.62% - 98.62%. The root and stem extracts showed the strongest inhibitory effect against α-glucosidase of 98.52% and 98.62%, respectively. These results were comparable to the result obtained for DMAA (99.85%) which was used as a control in this study. The strong inhibition of the stem methanolic extract of *B. micrantha* is in agreement with a report by Ezekiel et al. (2018) which found the extract to be a potent α-glucosidase inhibitor with IC_{50} of 1.06 ± 0.1 µg/mL.

The stem and root extracts produced higher activity compared to the leaf extract in the current study. However, the leaf extract was selected for further purification due to conservation purpose. The fractionation of the leaf (LFR5) produced results comparable to that of the stem and root extracts. This fraction was obtained from ethyl acetate: methanol (70:30), compounds from this fraction are polar. Inhibition of α-glucosidase by fraction LFR5 of *B. micrantha* agrees with a report by Kwon et al (2007) that phenolic rich extracts have a strong inhibitory effect against α-glucosidase.

In a study conducted by Omeh et al. (2014), the methanolic leaf extract of *B. micrantha* was tested *in vivo* for subacute antidiabetic effects in alloxan-induced hyperglycaemic rats. The subacute treatment of the extract (125, 250 and 500 mg/kg) produced 75, 68 and 63% reduction in fasting blood sugar level respectively, on day 14 of treatment. In a similar study by Adika et al. (2012), oral administration of the methanol leaf extract of *B. micrantha* on alloxan-induced diabetic mice produced maximum reduction (77.7%) in blood glucose level at 500 mg/kg at the sixth hour.

The root and stem extracts also demonstrated moderate activity (Table 3.4) against α-amylase (65.62% and 61.86% respectively). Previous reports indicate that excessive inhibition of pancreatic α-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Cheplick et al., 2010; Apostolidis et al., 2007). Though α-amylase inhibitory activity had positive effects on prevention of hyperglycemia linked to T2DM, mild inhibitory activity is desirable.

Amongst the wide array of enzymes, glucosidase is presumed to be an effective therapeutic target, which catalyse the cleavage of glycosidic bonds to liberate glucose from the non-reducing end of oligosaccharide and polysaccharide chains (de Melo et al., 2006). Furthermore, α-glucosidase inhibitors inhibit maltase and sucrase in intestine, consequently delaying absorption of sugars from the gastrointestinal tract and hence decrease post prandial hyperglycaemia in T2DM patients (Moelands et al., 2018).

The findings of the current study demonstrate that extracts of *B. micrantha* have antidiabetic properties and further support its use in South Africa for the management of diabetes mellitus.

4.5 Effect of extraction solvent on antidiabetic activity

The choice of solvent is a major factor in extraction of bioactive compounds. In this study, different solvents (hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol) were investigated for their effect on *in vitro* inhibition of carbohydrate hydrolysing key enzymes.

All the extracts demonstrated inhibition activity against α-glucosidase. These findings suggest that α-glucosidase enzyme is inhibited by both polar and nonpolar constituents of *B. micrantha*. This study corroborates with other studies that evaluated the α-glucosidase inhibition activities of polar and nonpolar solvent extracts (Laoufi et al., 2017). The potency of methanol and ethanol extracts in inhibiting α-glucosidase was significantly ($p < 0.05$) higher than DMAA which was used as a positive control.

B. micrantha exhibited weak activity against α-amylase. As already mentioned above, excessive inhibition of α-amylase results in undesirable effects. Therefore, in the present study, since methanol and ethanol showed no significant differences, methanol was considered to be the best solvent for extracting antidiabetic constituents of *B. micrantha* as the methanol extract possess strong inhibition activity against α-glucosidase and also gave the best extraction yield.

4.6 Antidiabetic activity of isolated compound

Previous studies investigated numerous key structural features of flavonoids to inhibit α-glucosidase and α-amylase enzymes *in vitro*. The present study is in agreement with previous reports that have indicated that glycosylation of flavonoids, in this case quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside was ineffective in inhibiting α-amylase. This observation is likely due to increased polarity and steric hindrance which possibly weakens the binding interactions between quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside and the catalytic site of the α-amylase enzyme.

Acarbose was used as a positive control in this study. Acarbose is a pseudotetrasaccharide containing a non-hydrolysable nitrogen-linked bond that suppresses α-amylase activity. Regarding the antidiabetic effect of acarbose, the associated side effects caused by excessive inhibition of pancreatic α-amylase results in bacterial fermentation of undigested carbohydrates in the large intestine (Bishoff, 1985). It is therefore supported that any bioactive compound exhibiting α-glucosidase inhibition activity instead, may be an effective therapeutic agent for the management of post prandial hyperglycaemia than acarbose (Kwon et al., 2008).

The results of the present study revealed that quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside strongly inhibits α-glucosidase activity. The findings of the current study is in agreement with studies suggesting that 5,7-dihydroxyflavone structure of flavonoids has been shown to be crucial for potent α-glucosidase inhibition (Sheliya et al., 2015; Gao et al., 2007; Tadera et al., 2006). Several studies have demonstrated that polyglycosylated flavonoids such as rutin showed higher α-glucosidase activity than the quercetin aglycone (Oboh et al., 2015). The current study reports for the first time the anti-hyperglycaemic activity of quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside isolated for the first time in *B. micrantha*.

5 GENERAL CONCLUSIONS

The aim of this study was to isolate and characterize the antidiabetic constituents of *B. micrantha*. A total of 3 compounds were isolated from the leaf methanolic extract of *B. micrantha*, however, only one compound exhibited antidiabetic activity and was therefore identified as quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside. Due to its good inhibition activity against α-glucosidase, quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside is a potential antidiabetic agent. The findings from the current study support the traditional use of *B. micrantha* in the treatment of diabetes mellitus.

Future work on the plant includes evaluation of toxicity and hypoglycaemic activity of isolated bioactive compound, quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside *in vivo* as the glycoside moieties are hydrolysed *in vivo* and might produce different results. It is also recommended that UPLC-PDA studies be carried out to evaluate whether the bioactive compound isolated from *B. micrantha* leaf is the same compound that is responsible for the inhibition activities observed in root and stem extracts.

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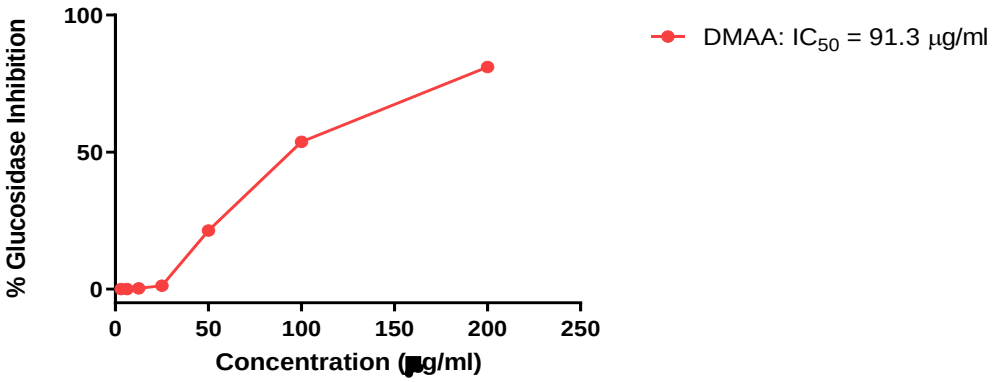
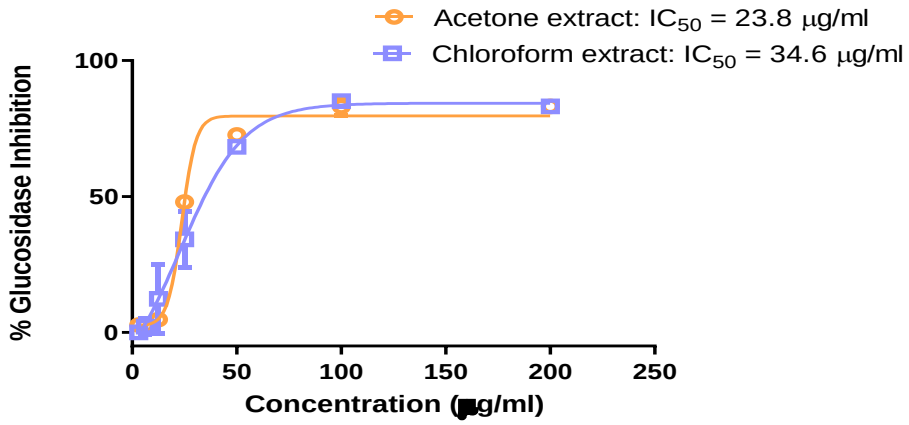
APPENDICES

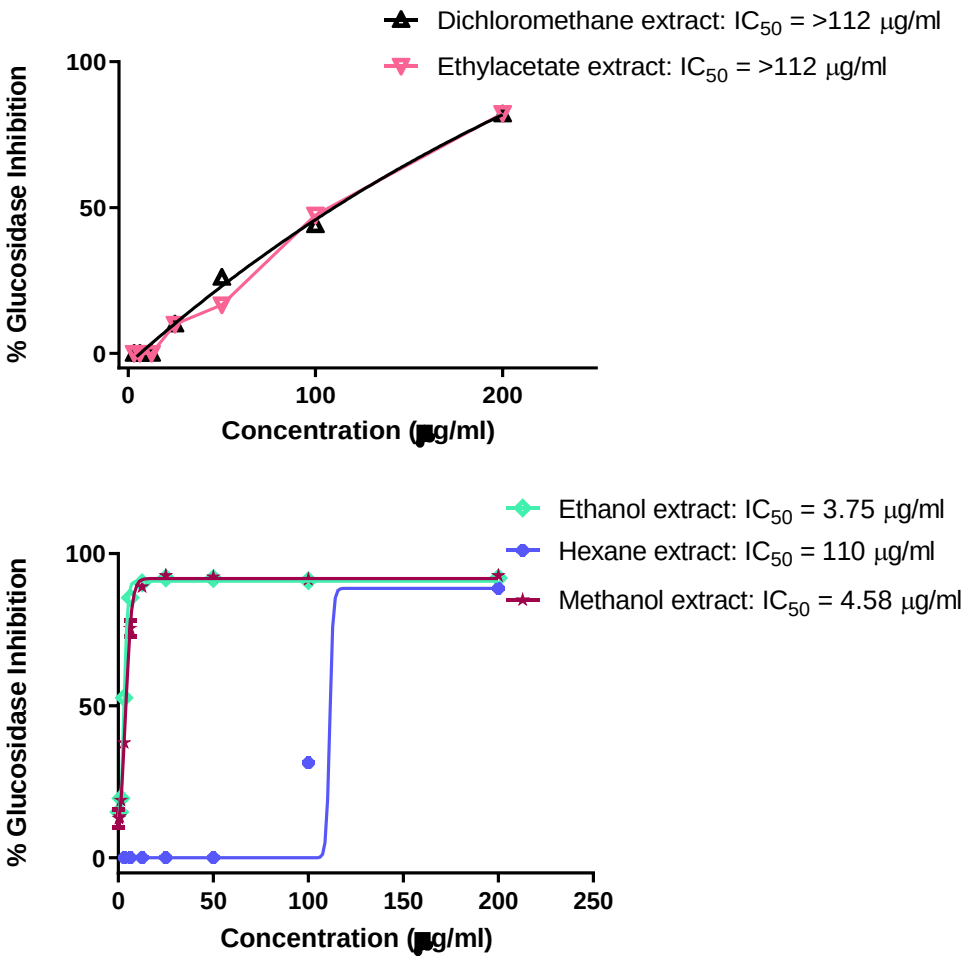
Appendix 1: Stains used for developing TLC plates

	Preparation of TLC Stains
NP-PEG reagent	1% Methanolic diphenylboric acid + 5% ethanolic polyethylene glycol (PEG)-4000
H_2SO_4	20% Sulphuric acid in ethanol

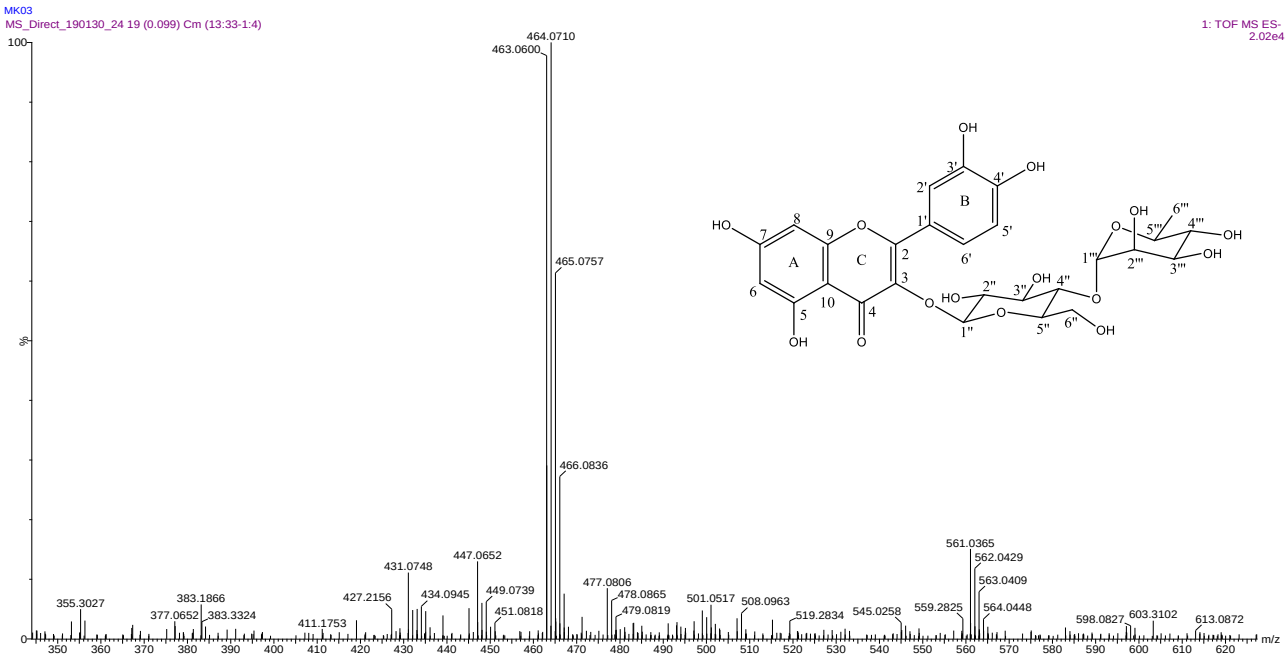
Appendix 2: IC_{50} of various solvent extracts against α-glucosidase



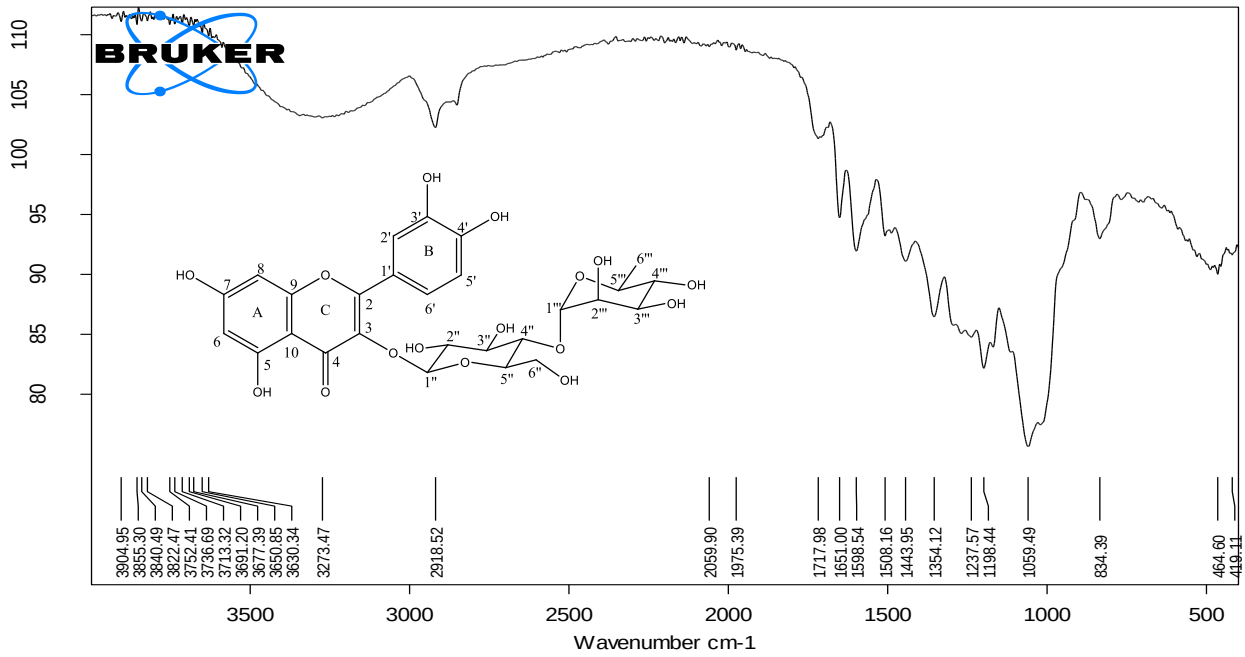




Appendix 3: Mass spectrum of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



Appendix 4: IR spectrum of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside

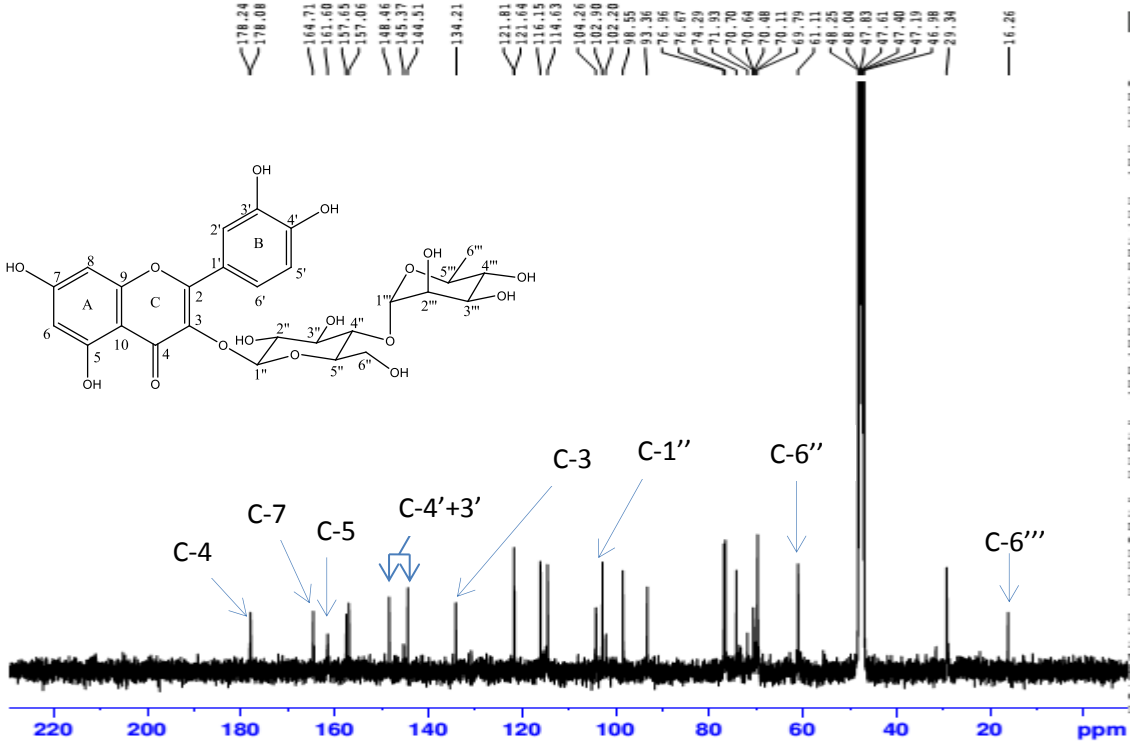


Appendix 5: 1D and 2D NMR data of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside

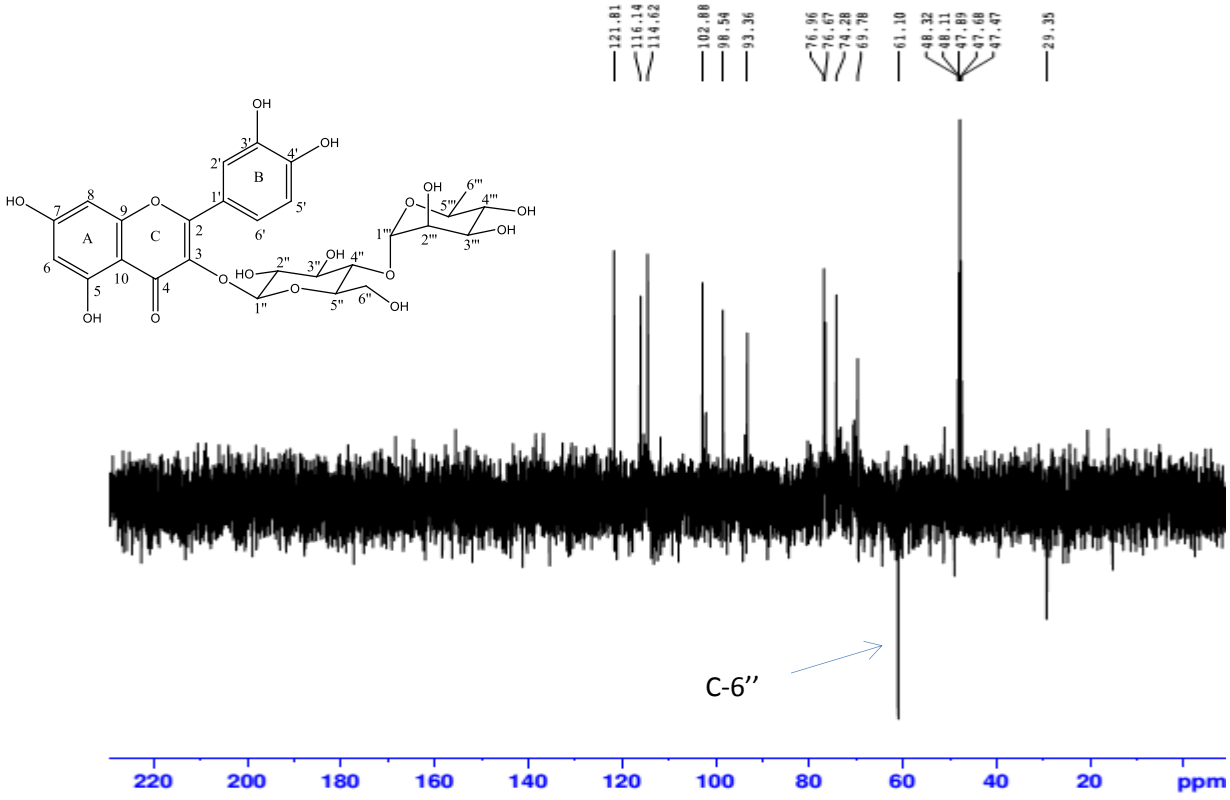
Position	^{1}H NMR δ multiplicity, J (ppm)	^{13}C NMR δ (ppm)	HMBC (H→C)	HSQC (H→C)
2	-	157.65	-	-
3	-	134.21	-	-
4	-	178.08	-	-
5	-	161.60	-	-
6	6.22 (1H,s)	98.55	C-5, C-7,C-8, C-10	H-6 & C-6
7	-	164.71	-	-
8	6.42 (1H,s)	93.36	C-6, C-7, C-9, C-10	H-8 & C-8
9	-	157.06	-	-
10	-	104.26	-	-
1’	-	121.81	-	-
2’	7.73 (1H,s)	116.15	C-1’,C-3’, C-4’,C-6’, C-2	H-2’ & C2’
3’	-	144.51	-	-
4’	-	148.46	-	-
5’	6.88-6.90 (1H,d,J=8.8 Hz)	114.63	C-3’, C-4’, C-6’	H-5’& C-5’
6’	7.60-7.62 (1H,d,J=8.4Hz)	121.64	C-2’, C-4’, C-2	H-6’& C-6’
1’’	5.25-5.27 (1H,d,J=7.6Hz)	102.90	C-3	H-1’’ & C-1’’
2’’	3.56-3.59 (1H,m)	74.29	-	-
3’’	3.22-3.26 (1H,m)	76.96	-	-
4’’	3.60-3.62 (1H,m)	69.79	-	-
5’’	3.43-3.49 (1H,m)	76.67	-	-
6’’	3.95-3.96 (1H,d,J=5.2Hz)	61.11	-	-
1’’’	5.33 (1H,s)	102.20	C-4’’	H-1’’’& C-1’’’
2’’’	3.95-3.96 (1H,m)	70.64	-	-
3’’’	3.70-3.74 (1H,m)	70.48	-	-
4’’’	3.43-3.47 (1H,m)	70.70	-	-
5’’’	3.95-3.96 (1H,m)	71.93	-	-
6’’’	0.97-0.98 (3H,d,J=6Hz)	16.26	-	H-6’’’& C-6’’’

S= singlet, d= doublet, m= multiplet

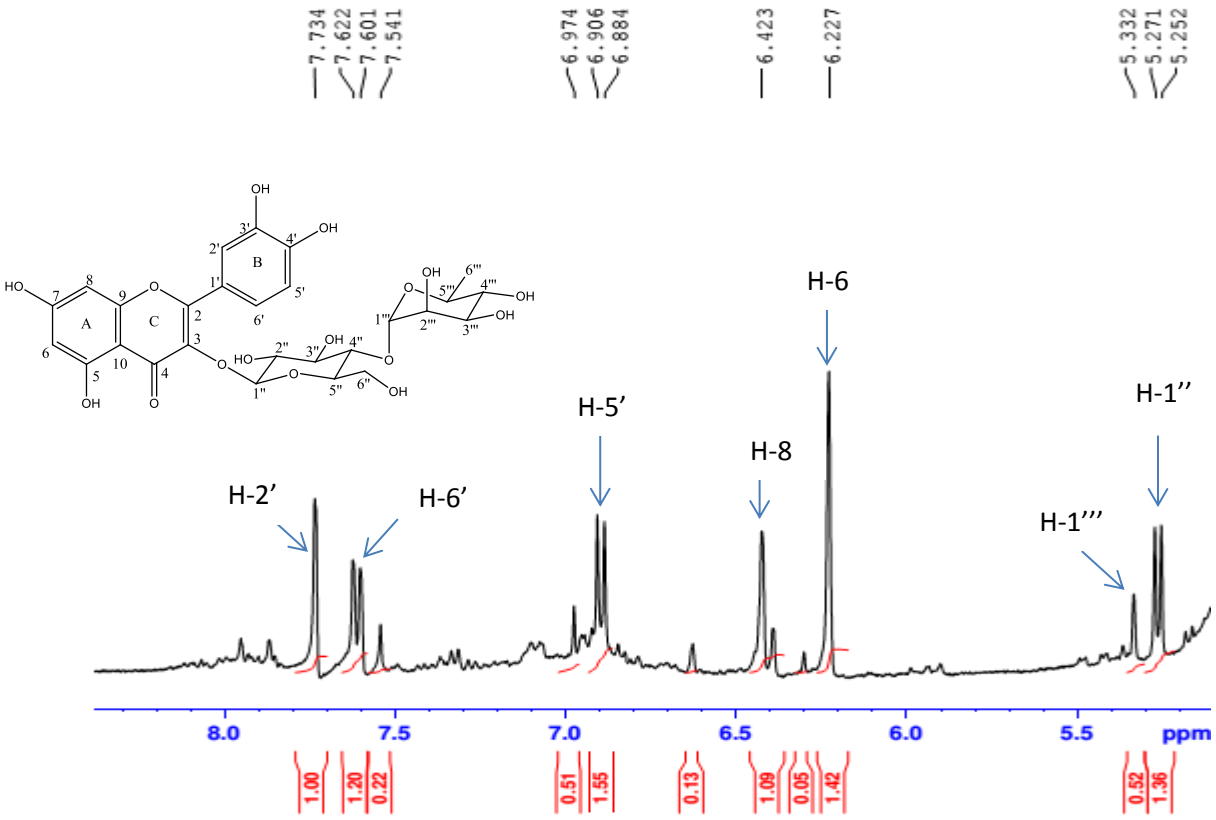
Appendix 6: ^{13}C-NMR of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



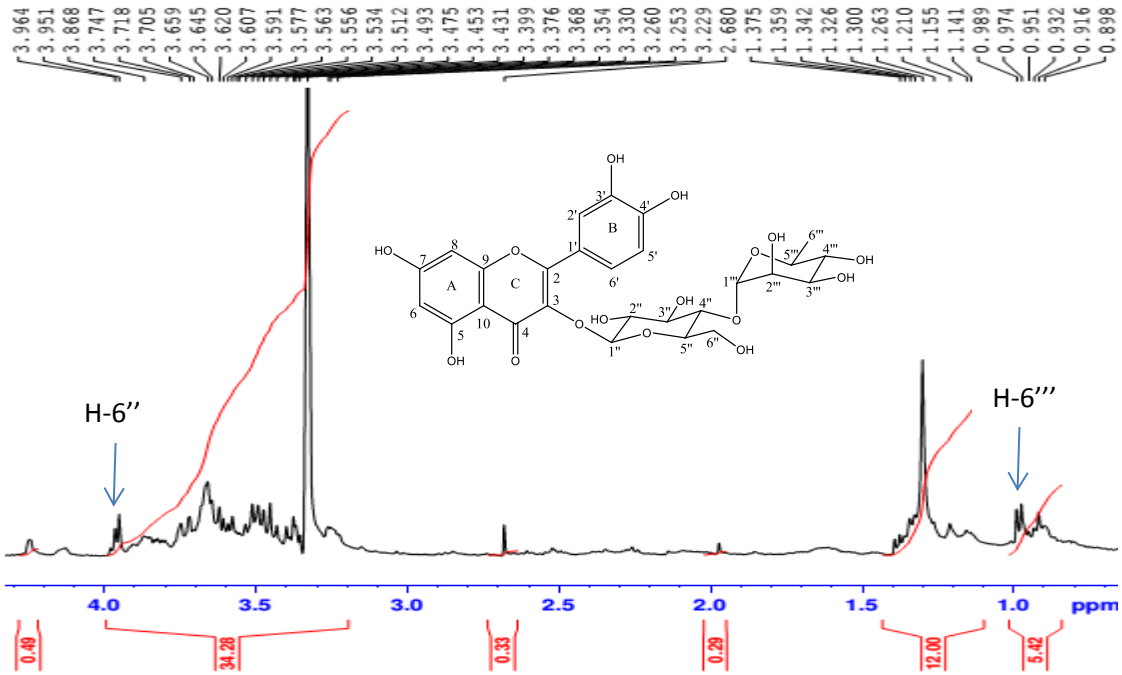
Appendix 7: DEPT 135 NMR of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



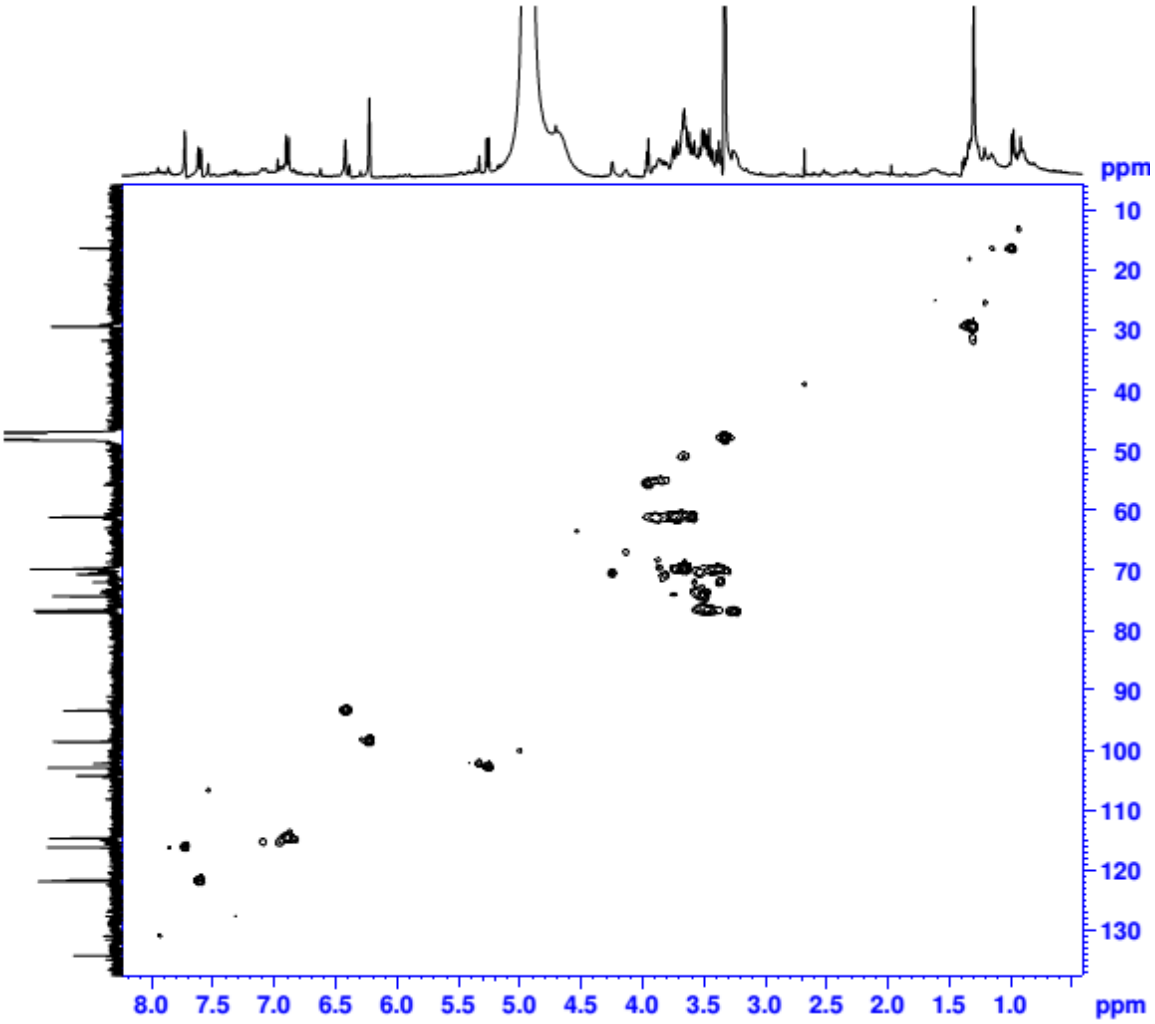
Appendix 8: ^{1}H-NMR expansion of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



Appendix 9: ^{1}H-NMR expansion of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



Appendix 10: HSQC of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside



Appendix 11: HMBC of Quercetin-3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnoside