Bidens pilosa extract and sub-fractions induce adipogenesis and exert glucose uptake in 3T3-L1 adipocytes

BY

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DECLARATION

I Mantwampe Motse Tolo declare that the dissertation under the title *Bidens pilosa* extract and sub-fractions induce adipogenesis and exert glucose uptake in 3T3-L1 adipocytes is my own work in design and execution, that it has not been submitted for any degree or examination in any other University and that all the sources I have quoted or used have been duly acknowledged.

Signature:

Date   : 01/12/2020

DEDICATION

I would like to dedicate this study to my family for their endless support, more especially to my late aunt Enicah Mantsho Rankwe who passed on after suffering from diabetes and lastly to everyone out there battling with diabetes.

ACKNOWLEDGEMENTS

"The secret of getting ahead is getting started" ~ Mark Twain

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LIST OF ABREVIATIONS

α	Alpha
β	Beta
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
ACE	Angiotensin-converting enzyme
ADA	American diabetes association
ADM	Adipocyte differentiation medium
AGEs	Advanced glycation end-products
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CC_{50}	Cytotoxic concentration exhibiting 50% activity
CEF	Chloroform: ethyl acetate: formic acid
CO_2	Carbon dioxide
DEX	Dexamethasone
DM	Diabetes mellitus
DMEM	Dulbecco's minimum-eagle medium
DMSO	Dimethylsulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Concentration exhibiting 50% activity
EMW	Ethyl acetate methanol water
FBS	Foetal bovine serum

FPG	Fasting plasma glucose
FRP	Ferric reducing power
g	Gram
GAE	Gallic acid equivalence
GDM	Gestational diabetes mellitus
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
Glut4	Glucose transporter4
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
IL6	Interleukin-6
IR	Insulin receptors
l	litre
Mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
MTT	3-(4,5-dimethylthiozyl-2-yl)-2,5-diphenyl tetrazolium bromide
NaOH	Sodium hydroxide
NIDDM	Non-insulin dependent insulin diabetes mellitus
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
QE	Quercetin equivalence
ROS	Reactive oxygen species
RS	Receptive species

TCA	Trichloroacetic acid
TEA	Toluene: ethyl acetate: ammonia hydroxide
TIDM	Type I diabetes mellitus
TLC	Thin layer chromatography
TNFα	Tumour necrotic factor alpha
T2DM	Type II diabetes mellitus
TZDs	Thiazolidinediones
USA	United States of America
UV	Ultraviolet
v/v	Volume to volume
W/V	Weight per volume
WAT	White adipose tissue
WHO	World Health Organization

CONFERENCES AND PRESENTATIONS

Poster presentations

Tolo, M.M., Bagla, V.P., Poopedi, K.W., Mampuru, L.J. and Mokgotho, M.P. (2018). Antiglycation and α-amylase inhibition potential and cytotoxicity studies of *Bidens pilosa* crude extract and sub-fractions extract and sub-fractions using 3T3-L1 adipocytes. The South African society of Biochemistry and Molecular Biology conference (SASBMB) (Potchefstroom University).

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Oral presentations

Tolo, M.M., Bagla, V.P., Poopedi, K.W., Mampuru, L.J. and Mokgotho, M.P. (2018). Phytochemical constituent profiles and antioxidant activity of six different extraction formulations for analysis of *Bidens pilosa* crude extract and its sub- fractions. South African Medical Research Council (SAMRC) (Cape Town).

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ABSTRACT

Diabetes mellitus has become a global epidemic, particularly type 2 diabetes. Obesity is one of the causes of type 2 diabetes mellitus due to its link with induced insulin resistance. There is no cure for diabetes mellitus and, as such, it is managed by using standard drugs which have side effects, and can be toxic, expensive and unavailable. People have resorted to the use of medicinal plants to treat diabetes and its complications. The aim of this study was to test the anti-obesity and anti-diabetic properties of *Bidens pilosa* crude extract and its sub-fractions using C2C12 myoblasts and 3T3-L1 adipocytes. The crude extract and the most active sub-fractions were selected for further analysis because of their ability to stimulate glucose uptake and induction of adipogenesis.

Bidens pilosa leaves were selected for this current study. They were firstly extracted using absolute methanol and further subjected to solvent-solvent fractionation to obtain the *n*-butanol, ethyl acetate, water, hexane, chloroform and 35% water in methanol sub-fractions. Qualitative phytochemical analysis was performed using thin layer chromatography (TLC) and standard chemical tests. Total phenolic and flavonoid content were determined quantitatively using a calorimetric method with Folin-Ciocalteu's reagent. For their antidiabetic potential, the extracts were evaluated chromogenically and calorimetrically for antiglycation and α-amylase inhibitory activity. The cytotoxicity of the extracts on 3T3-L1 preadipocytes and C2C12 myotubes were determined using the MTT assay. The adipogenesis inducing effect of the extract was tested using the adipogenesis kit.

More compounds were found on chromatograms eluted in EMW mobile phase (Ethyl acetate: methanol: water). The extracts were shown to contain a variety of secondary metabolites, and high phenolic and flavonoids contents. Crude, chloroform, *n*-butanol and water sub-fractions had high antioxidant activity. Alpha amylase activity was highly inhibited in the crude extract and all sub-fractions, with the highest inhibitory activity observed in the crude extract and the chloroform, *n*-butanol and water Sub-fractions (IC_{50} 1.25 ± 2.5 mg/ml). The cytotoxic profiles indicated that all extracts are non-cytotoxic at concentrations of 15.63 µg/ml. Extracts at a concentration of 31.25 µg/ml were shown to stimulate the accumulation of triglycerides using 3T3-L1 adipocytes. The extracts also exhibited significant

(P < 0.05) glucose uptake activity. In conclusion, *Bidens pilosa* contains constituents that inhibit α-amylase, antiglycation formation and modulates uptake of glucose in 3T3-L1 adipocytes. The use of *B. pilosa* in combination with insulin revealed the synergistic effects in facilitating glucose uptake in both C2C12 myotubes and 3T3-L1 adipocytes. This suggests that there might be some binding compounds found in the plant extracts that are responsible for the stimulation of expression of several genes that encode for proteins involved in the metabolism of glucose. However, the use of *B. pilosa,* in combination with metformin, results in a decreased glucose uptake. *Bidens pilosa* have the fast-acting insulin mimetic properties. Furthermore, the plant was shown to stimulate the accumulation of triglycerides in 3T3-L1 adipocytes, signifying the plant can induce adipogenesis at 30μg/ml.

Chapter 1: Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a metabolic condition characterised by an increase in the level of glucose due to the deficiency in insulin secretion and/or resistance associated with this hormone. Diabetes mellitus is partially caused by the excessive presence of carbohydrates in the diet, and abnormal protein and fat metabolism (American Diabetes Association, 2010). If blood glucose levels remain high, over a long period of time, this can result in long-term damage of organs such as the kidneys, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death (Ziegler *et al.*, 2009).

There are many pathologic processes involved in the development of diabetes. One such process involves the destruction of pancreatic β-cells, which are responsible for the production of insulin, thus leading to deficiency or abnormalities in insulin and resistance to insulin action. The deficiency in insulin action occurs as a result of improper insulin secretion and or destroyed responses of tissues to insulin at one or more points in the complex pathways of hormone action (Ozougwu *et al.*, 2013).

1.2 Prevalence of diabetes mellitus

Diabetes mellitus is one of the largest metabolic epidemics the world has faced, in both the developed and developing nations. In 2019, a total of 463 million people are estimated to be living with diabetes, representing 9.3% of the global adult population (20–79 years). This number is expected to increase to 578 million (10.2%) in 2030 and 700 million (10.9%) in 2045 (Saeedi *et al.*, 2019). The prevalence of diabetes in women in 2019 is estimated to be 9.0%, and 9.6% in men. The increase of diabetes with age leads to a prevalence of 19.9% (111.2 million) in people aged 65–79 years. In 2045, diabetes prevalence is projected to reach 11.9%, 11.8% and 4.7% in high-, middle- and low-income countries, respectively (Ajlouni *et al.*, 2019). Of all people living with diabetes, 67.0% are living in urban areas, prevalence being higher in urban than in rural areas (10.8% vs. 7.2%) (Saeedi *et al.*, 2019).

1.3 Classification of diabetes mellitus

Diabetes mellitus is classified into three groups, according to their primary causes, namely: type I, type II and gestational diabetes mellitus (Kenneth, 2006). A huge factor underlying these different types of diabetes is the deficiency in insulin, defective insulin or lack of tissue responsiveness to insulin.

1.3.1 Type I diabetes mellitus

Type I diabetes mellitus (TIDM) is known as a chronic autoimmune disease which is characterised by elevated blood glucose levels, known as hyperglycaemia, due to insulin deficiency that occurs as a consequence of the loss of pancreatic islet β-cells (Search study group, 2004; Eisenbarth 1986; Atkinson *et al.*, 2014). Type I diabetes mellitus is a common endocrine and metabolic condition occurring in children. In the majority of patients (70-90%), the destruction of β-cells is a result of TIDM-related autoimmunity (concomitant with the formation of TIDM-associated autoantibodies). There are factors contributing to type I diabetes mellitus which are heterogenous: environmental factors, such as diet and chemical agents among individuals genetically predisposed, are major factors contributing to the autoimmune activity of the pancreas (Marshall *et al.*, 2013).

1.3.2 Type II diabetes mellitus

Type II diabetes mellitus (T2DM) is defined as a disease caused by dysregulation of carbohydrate, lipid and protein metabolism, and results from impaired insulin secretion, insulin resistance or a combination of both. T2DM is the most common type as it accounts for more than 90% of all cases. It is caused mainly by continuously impaired insulin secretion by pancreatic β-cells, usually upon a background of pre-existing insulin resistance in skeletal muscle, liver and adipose tissue (DeFronzo, 2009). Type II diabetes mellitus is an on growing global health problem, closely related to the epidemic of obesity. Individuals affected with type II diabetes have a higher chance of microvascular and macrovascular complications. The development or causes of diabetes mellitus are not fully understood, nonetheless, environmental factors such as unhealthy diet, lack of physical activities and obesity and genetic disorders have been shown to contribute to the development and pathophysiological

disturbances that are responsible for impaired glucose homeostasis in T2DM (DeFronzo *et al.*, 2015).

1.3.3 Gestational diabetes mellitus

Gestational diabetes is a type of diabetes which occurs during pregnancy. In previous years, any form of hyperglycaemia which occurred during pregnancy was referred to as gestational diabetes mellitus (GDM), regardless of whether the condition occurred before the pregnancy or continued after the pregnancy. Recently, GDM is diagnosed in the second or, more commonly, third trimester and differs from type I and type II diabetes (American Diabetes Association, 2016). Women with GDM are mostly asymptomatic, so screening is important for detection. In a normal pregnancy, insulin resistance develops in the second trimester and continues until birth. The mode of action is not fully understood but is known to be associated with the production of hormones, cytokines or adipokines by the placenta. Insulin secretion also increases, resulting in standardised glucose concentrations. Gestational diabetes normally develops because of pre-existing elevated insulin resistance and reduced insulin secretion. The imbalance between insulin resistance and secretion that occurs during pregnancy may lead to hyperglycaemia. Gestational diabetes is associated with maternal and foetal complications (Whalen and Taylor, 2017).

1.4 Carbohydrate metabolism

Carbohydrate metabolism is the biochemical process accountable for the metabolic production, breakdown and interconversion of carbohydrates in the body (Maughan, 2009). Humans consume a lot of carbohydrates, and digestion breaks down complex carbohydrates into a few simple monomers (monosaccharides) for metabolism: glucose, fructose, and galactose (Hall, 2015).

1.4.1 Carbohydrates digestion enzymes

Alpha amylase and alpha glucosidase enzymes play a role in the digestion of carbohydrates into glucose molecules. Alpha amylase is responsible for breaking down long chain carbohydrates into oligosaccharides and disaccharides, and alpha glucosidase responds to the end product by breaking down starch and disaccharides to glucose molecules (Bhosale and Hallale, 2011). Improvement in meal tolerance and diabetic control leads to a decrease in the rate of carbohydrate intake and post prandial blood glucose level. Alpha amylase and glucosidase inhibitors are the potential targets in the development of lead compounds for the treatment of diabetes (Anderson and Chen, 1979).

1.5 Glucose metabolism and regulation

The rate at which glucose enters the blood circulation is called plasma glucose concentration. It is well balanced by the measure of glucose removal from the circulation (glucose disappearance). Circulating glucose is derived from three sources namely: intestinal absorption during the fed state, glycogenolysis and gluconeogenesis. Other sources of circulating glucose originate directly from hepatic processes: glycogenolysis, the breakdown of glycogen, the polymerized storage form of glucose; and gluconeogenesis, the formation of glucose primarily from lactate and amino acids during the fasting period (Wallum *et al.*, 1992). Glucagon is a hormone which plays a huge role in glycogenolysis and gluconeogenesis during the fasting period. Glycogenolysis is the major procedure by which glucose is produced during the first 8– 12 hours of fasting. Glucagon promotes this process and thus enables glucose appearance in the circulation. Over longer periods of fasting, glucose, produced by gluconeogenesis, is released from the liver.

Glucoregulatory hormones, such as insulin, glucagon, amylin, GLP-1, glucose-dependent insulinotropic peptide (GIP), epinephrine, cortisol and growth hormone, are responsible for maintaining circulating glucose concentrations in a relatively narrow range (Aronoff *et al.*, 2004). Amongst these, insulin and amylin originate from the β-cells and glucagon from the α-cells of the pancreas, and GLP-1 and GIP from the L-cells of the intestine. The glucoregulatory hormones of the body are structured to keep circulating the concentration of glucose in a moderately small range. During the fasting process, glucose leaves the circulation at a steady rate. To keep pace with the removal of glucose, the endogenous glucose formation is fundamental. Renal gluconeogenesis contributes considerably to the foundational glucose pool only during times of serious starvation. After reaching a post-meal peak, blood glucose gradually decreases during the next few hours, eventually returning to the fasting state. In the prompt post-feeding process, elimination of glucose into skeletal muscle and adipose tissue is driven mainly by insulin (Gerich *et al.*, 2001).

1.6 β-cell hormones

Insulin, a small protein composed of two polypeptide chains containing 51 amino acids, is a key anabolic hormone secreted in response to increased blood glucose and amino acids, following ingestion of a meal. Insulin applies its mechanism of action through binding to specific receptors found on a lot of cells of the body, including fat, liver and muscle cells. The major function of insulin is to stimulate glucose disappearance. Insulin helps control postprandial glucose in three ways: Initially, insulin signals the cells of insulin-sensitive peripheral tissues, and primarily skeletal muscle, to increase their uptake of glucose (Gerich *et al.*, 1974). Secondly, insulin acts on the liver to stimulate glycogenesis. Lastly, insulin simultaneously inhibits glucagon secretion from pancreatic β-cells, thus signalling the liver to stop producing glucose via glycogenolysis and gluconeogenesis. Another mode of action of insulin involves the activation of fat synthesis, development of triglyceride accumulation in fat cells, formation of synthesis of protein in the liver and muscle and proliferation of cell growth (Cryer *et al.*,1993). Insulin action is attentively regulated in response to circulating glucose concentrations. Insulin is not stimulated when the blood glucose concentration is ≤ 3.3 mmol/l but is stimulated as the glucose concentration increases over this threshold. Postprandially, the production of insulin appears in two stages: The first rapid release of preformed insulin, followed by

elevated insulin synthesis and release in response to blood glucose. Long-term stimulation of insulin occurs when there is high concentration of glucose (D'Alessio *et al.*, 2004).

1.7 Glucose uptake and transport in skeletal muscle and adipose tissue

The transport of glucose in the skeletal muscle is the first step and categorised under physiological conditions, such as glucose metabolism, as the rate limiting step (Zeil *et al.*, 1988). As such, transportation of glucose in cells and the possibilities of disturbance in glucose homeostasis is of major regulatory significance. For decades, it has been established that the uptake, utilisation and disposal of glucose in skeletal muscles and adipose tissues are enhanced by insulin. The pattern of molecular events directing the insulin-mediated glucose transport is initiated at the muscle cell membrane where insulin binds to the receptors of the plasma membrane (Khan *et al.*, 1976).
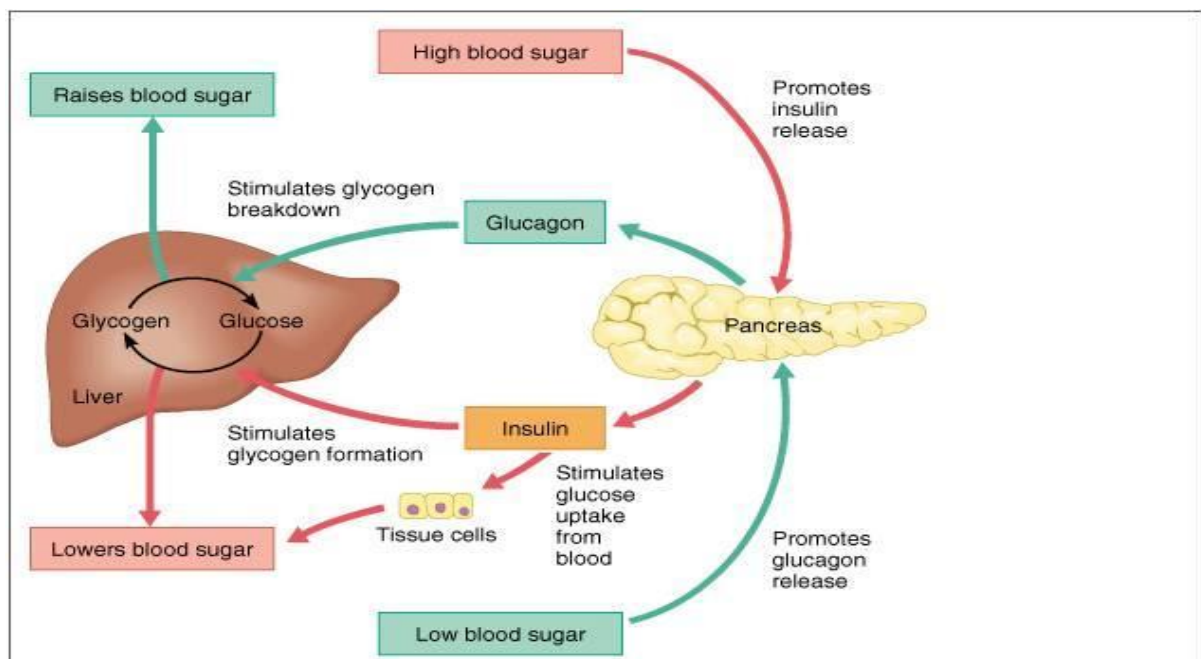
Figure 1.1: Metabolism of glucose and regulation of blood glucose levels are controlled by a sophisticated hormonal system (Cummings, 2001).

1.8 Complications of diabetes mellitus

The complications of diabetes mellitus are much less in people who have well controlled blood sugar levels. These complications include hypoglycaemia, hyperglycaemia, diabetic coma and nonketotic hyperosmolar coma. Chronic complications occur because of a mix of microangiopathy, macrovascular disease and immune dysfunction in the form of autoimmune disease or poor immune response, most of which are difficult to manage. Macrovascular problems can lead to cardiovascular disease and erectile dysfunction. Female infertility may be due to endocrine dysfunction with impaired signalling on a molecular level (Nathan, 1993).

Other health problems compound the chronic complications of diabetes such as smoking, obesity, high blood pressure, elevated cholesterol levels and lack of regular exercise which are accessible to management as they are modifiable. Non-modifiable risk factors of diabetic complications are the type of diabetes, age of onset, and genetic factors (Moore *et al.*, 2009).

1.8.1 Microvascular disease complications of diabetes.

1.8.1.2 Diabetic retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes, classified by a lesion that arises on the retina of the eye. This means that there are alterations in vascular permeability, capillary micro aneurysms, capillary degeneration and excessive formation of new blood vessels (neovascularization). It contributes to ~ 10,000 new cases of blindness every year (Fong *et al.*, 2004). The chances of developing diabetic retinopathy, or other microvascular complications of diabetes, relies upon both the span and the severity of hyperglycaemia. Development of diabetic retinopathy in patients with type II diabetes was found to identify with both the severity of hyperglycaemia and the presence of hypertension. Most patients with type I diabetes are shown to have signs and symptoms of retinopathy after 20 years of diagnosis (Keenan *et al.*, 2007). This is opposite to type II diabetes as development of retinopathy may begin as early as 7 years before the diagnosis of diabetes. There are a few proposed pathological pathways by which diabetes may cause the development of retinopathy. Aldose reductase may be a major contributor in the

development of diabetes complications. Aldose reductase is the initial enzyme in the intracellular polyol pathway. This pathway converts glucose into glucose alcohol (sorbitol). High glucose levels give rise to the flux of sugar molecules through the polyol pathway, which leads to sorbitol accumulation in cells. Osmotic pressure from sorbitol accumulation has been hypothesised as a basic component in the improvement of diabetic microvascular inconveniences, including diabetic retinopathy (Gabby, 1975).

Proliferative stages of diabetic retinopathy occur in the development of new blood vessels, blindness and visual impairment (Tarr *et al.*, 2013). Frequent increase of glucose in the blood stream, in combination with an increase in blood pressure and high cholesterol, are the major causes of retinopathy. Regular eye check-ups and managing glucose levels at or close to normal concentrations is required (Chistiakov, 2011).

1.8.1.2 Diabetic nephropathy

Diabetic nephropathy is the leading cause of kidney failure in diabetes. It is classified by the presence of proteinuria with microalbuminuria. Diabetic patients with microalbuminuria normally develop proteinuria and overt diabetic nephropathy. This progression occurs in both type I and II diabetes. The initial treatment of diabetic nephropathy, as for other complications of diabetes, is prevention. Like other microvascular complications of diabetes, there is a strong link between glucose control (as estimated by hemoglobin A1c [HbA1c]) and the risk of developing diabetic nephropathy. Treatment to maintain the minimal safe glucose level is required to prevent or manage diabetic nephropathy (Fowler, 2008). Treatment with angiotensin-converting enzymes (ACE) inhibitors have not appeared to improve microalbuminuria in patients with type I diabetes but has been shown to lower the risk of having nephropathy and cardiovascular occasions in patients with type II diabetes (Ruggenenti and Remuzzi, 1998).

1.8.2 Protein glycation and its implications in diabetic complications

Glycation is known to be a key molecular feature of diabetes complications resulting from chronic hyperglycaemia. Glycation is characterised by the carbonyl group of reducing sugars reacting non-enzymatically with the amino group of proteins, nucleic acids and other molecules in order to initiate glycation, also known as Amadori products or fructose products (Singh *et al.*, 2011). Subsequently, Amadori products go through a sequence of irreversible reactions forming highly reactive carbonyl species (RCS), such as glyoxal, methylglyoxal and 3-deoxy-glucosone (Negre-Salvayre *et al.*, 2008). At the end, these reactive carbonyls react with the amino, sulfhydryl and guanidine functional groups of intracellular and extracellular proteins to form the stable advanced glycation end products (AGEs). The reactive carbonyl species can also be produced from sugar glycoxidation contributing to the AGE formation (Muthenna *et al.*, 2012). Accumulation of AGEs has been implicated as a major pathogenic connection between hyperglycaemia and long complications of diabetes (Matsuura *et al.*, 2002)

1.9 The relationship between obesity and diabetes

Obesity is defined as state of inflammation. The plasma concentration of inflammatory mediators, such as tumour necrosis factor-a (TNF-α) and interleukin-6 (IL-6), are raised in the insulin resistant status of obesity and type II diabetes (Hotamisligil *et al.*,1993). This brings about the mechanisms involved in obesity which lead to the development of type II diabetes mellitus. There are two mechanisms which may be involved in the pathogenesis of inflammation. Firstly, glucose and macronutrient intake lead to oxidative stress and inflammatory alterations. Chronic conditions such as excessive food intake (obesity) might thus be a proinflammatory state with oxidative stress. Secondly, the increased concentrations of TNF-α and IL-6, linked with obesity and type II diabetes, might inhibit insulin action by blocking insulin signal transduction. This might prevent the anti-inflammatory effect of insulin, which in turn might result in the development of inflammation (Yudkin *et al.*, 1999).

1.10 Macronutrient intake and inflammation

An increase in the concentration of fibrinogen has an insightful value in the formation of obesity itself. Glucose intake stimulates acute oxidative stress and inflammation at the cellular and molecular level for a period of three hours (Esposito *et al.*, 2002), and a mixed fast-food meal also influences the same responses for the upcoming four hours. However, restriction of a diet in the obese for a certain time period causes a significant decrease in oxidative stress, which is noticeably enhanced in the obese (Dandona *et al.*, 2001). Fasting for a period of 48 hours might cause 50% decrease in reactive oxygen species (ROS) generation by leucocytes and a reduction in the expression of NADPH oxidase, the enzyme that converts molecular oxygen to the superoxide radical. The superoxide radical is responsible for the activation of the redox sensitive proinflammatory transcription factor, NF-kB, which activates the transcription of most proinflammatory genes. Hence, the pro-oxidant and proinflammatory effects of a lot of macronutrient intakes in normal subjects are the same as those found in the obese in their basal fasting state. There are high probabilities that the proinflammatory state of the obese is linked to chronic excessive macronutrient intake. Therefore, increased concentrations of inflammation sensitive proteins, fibrinogen, ceruloplasmin, orosomucoid and x-antitrypsin are predictive of future weight gain. In type II diabetes, the presence of hyperglycaemia further exacerbates the proinflammatory state (Niess and Simon, 2007).

1.11 Anti-inflammatory effect of insulin

The state of insulin resistance influences inflammation since insulin exerts an anti-inflammatory effect at the cellular and molecular level *in vitro* and *in vivo*. A small dose infusion of insulin (2.5 IU h21) reduces ROS generation by mononuclear cells, diminishes NADPH oxidase expression and intranuclear NF-kB binding, stimulates IkB expression and represses plasma intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) concentrations (Aljada *et al.*, 2002). Mental health contributes to the rise in plasma IL-6 concentrations. Mental stress might stimulate an inflammatory state (Padgett and Glaser, 2003). However, the mode of action is not fully understood as to how stress could result in worsening

ofglucose homeostasis in known diabetics, or whether stress can cause diabetes on its own (Surwit *et al.*, 1992).
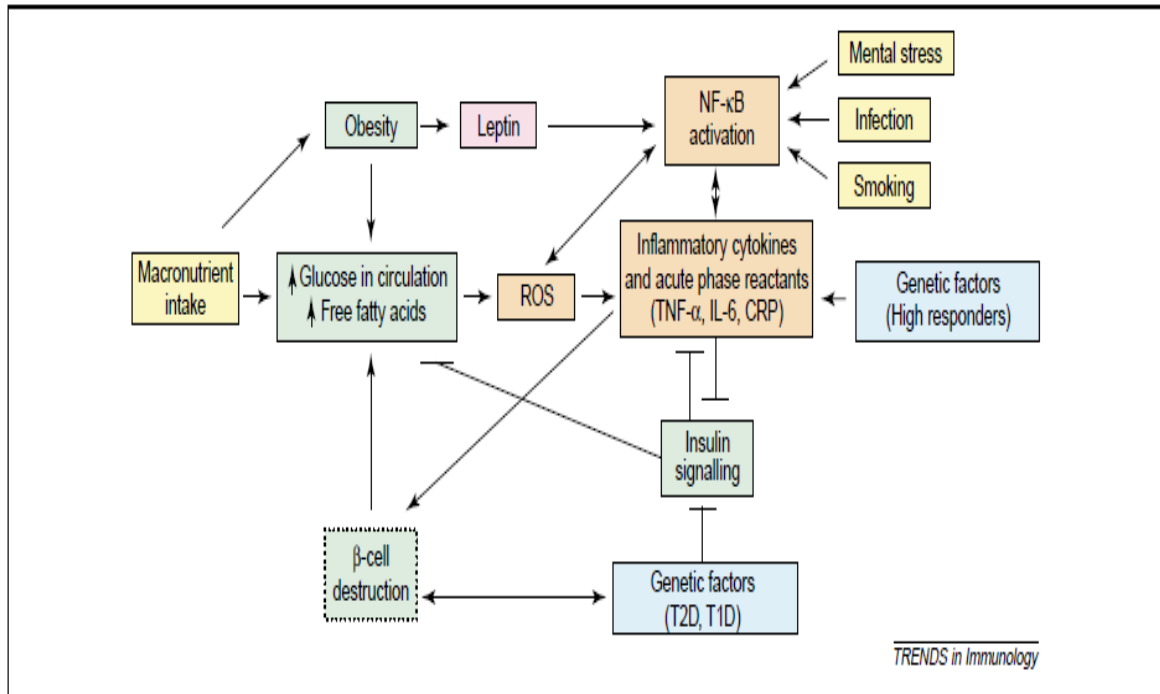
Figure 1.2: The induction of reactive oxygen species (ROS) generation and inflammation (NF-kB activation) by macronutrient intake, obesity, free fatty acids, leptin, infection, smoking, mental stress and genetic factors. Interference with insulin signalling (insulin resistance) leads to hyperglycaemia and proinflammatory changes. Abbreviations: CRP, C-reactive protein; T1D, type I diabetes; T2D, type II diabetes (Dandona *et al* 2004).

1.12 Diagnosis of diabetes mellitus

Diagnosis of diabetes mellitus remain a challenge due to the fact that it is a slow progressive pathogenesis disease. The early detection of the disease is difficult because it is asymptomatic at this stage. Diabetes may be diagnosed based on plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT) (Welsh *et al.*, 2016).

1.12.1 Fasting and 2-Hour Plasma Glucose

The fasting plasma glucose (FPG) measures the fasting glucose level, an FPG with greater or equal to 7 mmol/L or 126 mg/dL is taken to be diabetic. Another method to diagnose a diabetic patient is to measure the plasma glucose concentration. A plasma glucose concentration greater or equal to 11.1 mmol/L or 200 mg/dL, measured after 2 hours administration of 75 g glucose, is considered to be diabetic. However, factors such as age, race, ethnicity, hemoglobinopathies, family background and concomitant, plays a vital role in reliability of the diagnosis and therapy (Diabetes Care, 2018).

1.13 Treatment and management of diabetes mellitus

Diabetes mellitus is a complex disease which requires serious management, continuous medical care and multifactorial risk-reduction strategies beyond glycaemic control. Continuous self-care management education by the patients and support are important to prevent complications and to minimize the risk of long-term complications (American Diabetes Association, 2018).

Ninety years on, the management of type I and type II diabetes aims on improving glycaemic control, by ways of changing lifestyle and pharmacological treatment, with the aim of decreasing microvascular and macrovascular complications. Technology plays a major role in monitoring and delivery of treatment in diabetes and communication with healthcare professionals, while new therapies for lowering glucose are used to target key pathophysiological deficiencies in the development of diabetes (Rösen *et al.*, 2001). Besides major improvements in care, there is still no

cure for diabetes mellitus. Nonetheless, considerable research is being carried on in these fields. It has been discovered that obesity is the major factor contributing to the development of diabetes mellitus. Therefore, what is needed is to cut down or decrease the amount of food normally consumed; carbohydrate must be specially restricted, and the patient should concentrate more on protein and fat. Organised instruction programs are viewed as fundamental to enhance patient inspiration and strengthening of self-administration skills (American Diabetes Association, 2018).

1.13.1 Non-pharmacological management of diabetes mellitus

1.13.1.1 Physical activity therapy

There are various advantages to physical activity in patients with diabetes, and these extend to the elderly. The advantages incorporate weight loss or maintenance, lower blood pressure, improved cardiovascular fitness, improved lipid profile and an increased sense of well-being (Wheeler, 1999). Exercise increases glucose uptake into active muscles maintained by the production of hepatic glucose, with an increased dependence on carbohydrate to fuel muscle activity as exercise intensifies. A decrease in blood glucose levels has been reported with both aerobic and resistance exercise up to 72 hours post exercise. Although exercise improves blood glucose levels and insulin action in the short term, the risk for hypoglycaemia among patients who do not take insulin or insulin secretagogues is minimal (Powers *et al.*, 2017).

1.13.1.2 Nutrition therapy

Nutrition is regarded as the "cornerstone of diabetes care". It is a complex, controversial and evolving area. Dietary administration of diabetes has the goal of improving or maintaining quality of life, in addition to the physiological health and nutritional status of patients. Up to 10% of the daily energy needed can take the form of added sugars, such as table sugar and/or sugar containing products, without impairing diabetes control in most Type II patients (Wolever *et al.*, 1999). The amount of nutritional dietary intake plays a role in the post-prandial blood glucose concentration. Therefore, a proper planned meal prescribed by a qualified dietician

can help in the management and regulation of blood glucose level (Morris and wylie-Rosett, 2010).

1.13.2 Pharmacological therapies

1.13.2.1 Insulin therapy

Insulin is a hormone produced in the pancreas by the islets of Langerhan, which regulates the amount of glucose in the blood stream. Insulin therapy is the mainstay of hyperglycaemia treatment in diabetic patients (White *et al.*, 2003). Endogenous basal insulin maintains glucose homeostasis by regulating hepatic glucose production. The ideal strategy for managing insulin therapy in diabetic patients involves a basal-bolus approach, using a combination of a long-acting basal insulin and bolus doses of a rapid- or short-acting insulin prior to meals (Umpierrez *et al.,* 2012). Administering roughly half of the total daily insulin requirement as a basal insulin, once or twice daily, maintains consistent blood glucose concentrations at night and between meals. Giving the other half of the everyday insulin requirement in divided bolus doses before meals prevents postprandial hyperglycaemia. Supplemental amendment portions of a fast or short-acting insulin might be utilised to address excursions in blood glucose, with dosing guided by blood glucose estimations and insulin affectability, which relies on patient-explicit variables such as capacity to eat dinners, age and renal hindrance (Teuscher, 2007).

1.13.2.2 Oral therapies

Since the 1950s, metformin and sulfonylurea have been used for the treatment of type II diabetes and still stand to be the first- and second-line choices following diagnosis. Current studies have shown that metformin specifically prevents the development of the mitochondrial isoform of glycerophosphate dehydrogenase, decreases cytosolic dihydroxyacetone phosphate and elevates the cytosolic NADH-NAD ratio. This results in decreased plasma glucose and lactate levels, and reduced gluconeogenesis, hepatic glucose secretion and endogenous glucose production. Metformin can be used with all other diabetes therapies, including, insulin and is also used normally for managing gestational diabetes and polycystic ovary syndrome.

Metformin is related to improved cardiovascular outcomes in patients with diabetes (Kahn *et al.*, 2014).

Insulin sensitizers like sulfonylurea and meglitinides induce insulin secretion from the β cells of the pancreas and are thus related to hypoglycaemia and gaining of weight. They are productive at decreasing HbA1c and rapidly decrease glucose levels, which is helpful in highly symptomatic patients following diagnosis. Older generation sulfonylurea, such as tolbutamide and glibenclamide, have been superseded by new versions such as glimepiride, which are short acting, reducing the risk of hypoglycaemia and are more effective, although more expensive (American Diabetes Association, 2009).

Thiazolidinediones (TZD, PPARγ agonists) have been used for over 20 years. The mode of action involves a decreased free fatty acid accumulation, reduction in inflammatory cytokines, elevation in adiponectin levels and preservation of β cell structure and function, which leads to improvement in insulin resistance and β cell failure. The incretin treatments incorporate the subcutaneously injectable GLP-1 receptor agonists, exenatide, liraglutide, lixisenatide, dulaglutide and albiglutide, and the oral DPP-IV inhibitors sitagliptin, saxagliptin, vildagliptin, linagliptin and alogliptin. GLP-1 receptor agonists initiate GLP-1 receptors in the little digestive system and DPP-IV inhibitors inactivate the catalyst DPP-IV, which ordinarily separates GLP-1. These drugs have been shown to increase insulin discharge by means of β cells and decrease glucagon emission, hepatic glucose generation and glucose take-up from the stomach, also advancing satiety. All these modes of actions result in improved glycaemic control (HbA1c decrease up to 1.6%) with less risk of hypoglycaemia and frequently significant weight reduction (up to 8 kg) with GLP-1 agonists, and weight neutrality with DPP-IV inhibitors (Achakzai *et al.*, 2009).

1.14 Current antidiabetic drugs and their shortcomings

1.14.1 Biguanides

The most commonly used biguanides is metformin, it is normally used to monitor overweight or obese patients. Metformin activates adenosine monophosphate-activated protein kinase in the liver, causing hepatic uptake of glucose and inhibiting gluconeogenesis through complex effects on the mitochondrial enzymes. Although

the drug is effective it has been associated with possible gastrointestinal disturbances (Lorenzati *et al.*, 2010)

1.14.2 Sulfornylureas

Sulfonylureas decreases blood glucose level by increasing insulin secretion in the pancreas by blocking the KATP channels. They also lower gluconeogenesis in the liver. Sulfonylureas decrease breakdown of lipids to fatty acids and reduce clearance of insulin in the liver. Hypoglycemia is the major side effect of all sulfonylureas, while minor side effects such as headache, dizziness, nausea, hypersensitivity reactions, and weight gain are also common (Ronacher *et al.*, 2015).

1.14.3 Thiazolidinedione

Thiazolidinedione decrease plasma glucose and insulin level and improve some of the abnormalities of lipid metabolism. Mechanisms of action include diminution of free fatty acid accumulation, reduction in inflammatory cytokines, rising adiponectin levels, and preservation of β-cell integrity and function, all leading to improvement of insulin resistance and β-cell exhaustion. However, there are a lot of 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. There is high risk of bone fractures predominately in women (Inzucchi *et al.*, 2015)

1.14.4 Alpha- Glucosidase inhibitors

Alpha-glucosidase inhibitors such as acarbose, miglitol and voglibose are used for tratement of type II diabetes. They work by preventing the digestion of carbohydrates such as starch and table sugar. These agents are commonly effective for postprandial hypoglycaemia but have side effects such as flatulence and diarrhoea (Olokoba *et al.*, 2012).

Chapter 2: Literature review

2.1 Traditional herbal remedies for primary health care

Traditional medicinal plants, also referred to as herbal medicines, botanical medicines or phytomedicines, are attributed to the medicinal products of plant roots, stems, leaves, bark, seeds and foods grown from the ground that can be utilized to advance general wellbeing and treat ailments. The various product of plants may be utilised straightforwardly in a prescription formula or processed into different ready-to-use products. Traditional systems of medicines, such as herbal medicines, have been practised for hundreds of years for health care by people in countries of the South-East Asia region, as well as in other parts of the world. Traditional medicine is still a significant and a valuable source of remedies that has been practiced by millions of people around the world to secure their health. It has been developed from experimental encounters and from perceptions made by people who use them. It encapsulates age-old wisdom and information obtained over thousands of years and forms an integral part of the social and cultural heritage of people and countries. The system has been acquired from one generation to the next by custom and tradition (WHO, 2010).

Traditional medicine is increasingly popular as a cost-effective alternative to standard drugs. The World Health Organization (WHO) states that 80% of the population in some Asian and African nations rely upon traditional medication for essential medicinal health needs. Traditional medicinal products constitute multi-billion-dollar industries worldwide. Traditional and folklore medication, obtained from age to age, is rich in domestic formulas and collective practice. Incorporating ideas and techniques for the protection and reclamation of wellbeing, traditional medicine has produced a fount elective drug, new pharmaceuticals and healthcare products. The utilization of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1997). Furthermore, an increasing dependence on the utilisation of medicinal plants in the developed countries has allowed for the extraction and improvement of a few medications and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1997).

2.2 Medicinal plants as antioxidant

Several epidemiological studies have discovered that there is a link between the intake of food rich in polyphenols (for example, fruits, vegetables and grains) and the risk of age-related diseases in humans. This association is mostly assigned to the capability of the antioxidant activities of flavonoids and different polyphenols, as established in vitro, to scavenge a wide range of oxygen, nitrogen and chlorine species (Ketsawatsakul *et al.*, 2000, Pannala *et al.*, 1997 and Silva *et al.*, 2002). An antioxidant is characterised as "any substance that delays, prevents or expels oxidative harm to a target molecule (Halliwell and Gutteridge, 2015). Therefore, antioxidants may serve to manage the level of free radicals and other "receptive species" (RS) to limit oxidative damage. There is a lot of anecdotal proof on protective effects of medicinal plants. Some of these effects include antidiabetic, antimicrobial, antiviral, anti-inflammatory, antiallergic, immunosuppressive, immunostimulatory and cancer chemoprevention effects (Huseini *et al.*, 2006). These beneficial effects are mostly assumed to be antioxidant mechanisms because medicinal plants are rich sources of polyphenols.

2.3 Bioactive compounds in plants

There are two types of compounds found in plants, primary metabolites and secondary metabolites. Primary metabolites are chemical substances which are involved in direct growth, reproduction and development of plants. These includes respiration, carbon fixation and photosynthesis (Achakzai *et al.*, 2009). Secondary metabolites are the chemical substances that are not directly involved in the growth and development of plants. They lead to the biosynthesis of simple to complex metabolites, utilising the intermediates from the primary metabolites through specific pathways. The function of secondary metabolites in defence may include deterrence, anti-feedant activity, toxicity or acting as precursors to physical defence systems. Some of the important secondary metabolites include phenolics, alkaloids, terpenoids, vitamins etc. Most secondary metabolites take a role in defensive mechanism against biotic factors like protection from attack of pathogens, herbivores and allelopathy, etc., (Athanasiadou and Kyriazakis, 2004, Khan and Singh, 2008).

2.3.1 Phenolic compounds

Phenolic compounds are secondary metabolites that are derived from pentose phosphate, shikimate and phenylpropanoid pathways involved in plants (Randhir *et al.*, 2004). Phenolic compounds are one of the most extensively appearing group of phytochemicals, which are of major physiological and morphological importance in plants. These includes properties such as anti-allergenic, antioxidant, anti-microbial, anti-inflammatory, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia *et al.*, 1997). Phenolic compounds have been associated with the health benefits obtained from high intake of fruits and vegetables (Hertog *et al.*,1993). The compounds could be a dominant determining factor of the antioxidant potential of food. They are classified into different groups namely (i) phenolic acids (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolics. Phenolics essentially serve as a host of natural antioxidants, which are used as nutraceuticals, found in apples, green tea and red wine, with the ability to combat cancer, and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents.
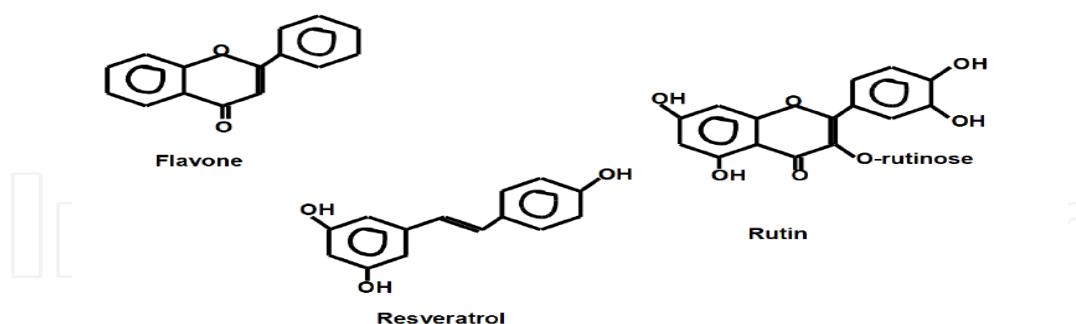
Figure 2.1: Basic structures of some pharmacologically important plant derived phenolics (Doughari, 2012).

2.3.2 Flavonoids

Flavonoids are an important group of polyphenols mostly distributed among the plant flora. Structurally, they are made of more than one benzene ring (a range of C15 aromatic compounds) and several studies support their use as antioxidants or free

radical scavengers (Kar, 2007). The compounds are formed from parent compounds known as flavans. There are more than four thousand flavonoids and some are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in about 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, calchones and catechin and leucoanthocyanidins.

FLAVONE (FLAVONOL)

ANTHOCYANIDIN (ANTHOCYANIN)

DIHYDROFLAVONE (DIHYDROFLAVONOL)

FLAVAN (FLAVANOL)
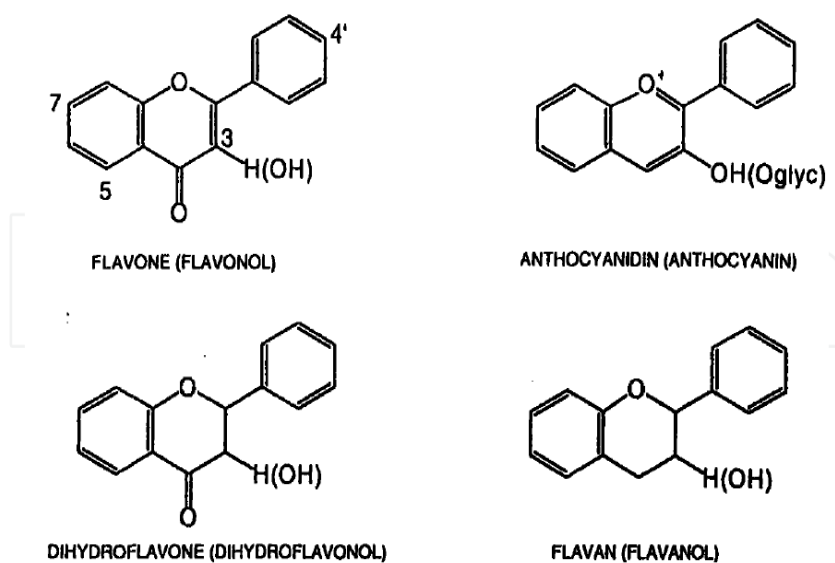
Figure 2.2: Basic structures of some pharmacologically important plant derived flavonoids (Doughari, 2012).

2.3.3 Glycosides

Glycosides are a group of chemical compounds known as condensation products of sugars, such as polysaccharides, with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemiacetal entity of the carbohydrate must essentially take part in the condensation. Glycosides are classified as colourless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulphur) water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides have carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Kar, 2007; Firn, 2010). Alcohol, glycerol or phenol represents aglycones. Glycosides are neutral in reaction and may be readily hydrolysed into its components with ferments or mineral acids. Glycosides are characterised based on the type of sugar component, chemical nature of aglycone or pharmacological action. The rather older or trivial names of glycosides usually has a suffix 'in' and the names essentially included the source of the glycoside.

α-Terpineol Cinnamyl acetate Eugenol Taxifolin-7-O- β-glucosid
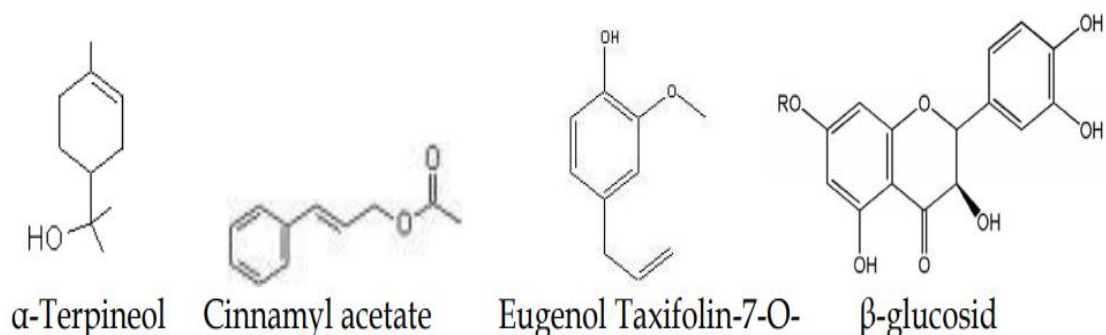
Figure 2.3: Basic structures of some pharmacologically important plant derived glycosides (Doughari, 2012).

2.3.4 Saponins

The word saponins is obtained from *Saponaria vaccaria* (*Quillaja saponaria*), a plant which abounds in saponins and was once used as soap. Therefore, saponins carry out 'soap like' behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal. Usually, the sugar is attached at C-3 in saponins, because in most

sapogenins there is a hydroxyl group at C-3. *Quillaja saponaria* is known to contain toxic glycosides, quillajic acid and the sapogenin senegin. Quillajic acid is strenutatory and senegin is toxic.

2.3.5 Tannins

Tannins are a large group of phenolic compounds which have high molecular weight and are mostly found in plant flora. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a similar property to tan i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or a carboxylic group (Kar, 2007). Tannins interact with proteins, carbohydrates, gelatin and alkaloids. They are grouped into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and, according to the type of acid they produce, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as an antiseptic and this activity is due to the presence of the phenolic group. Common examples of hydrolysable tannins include the aflavins (from tea), daidezein, genistein and glycitein.
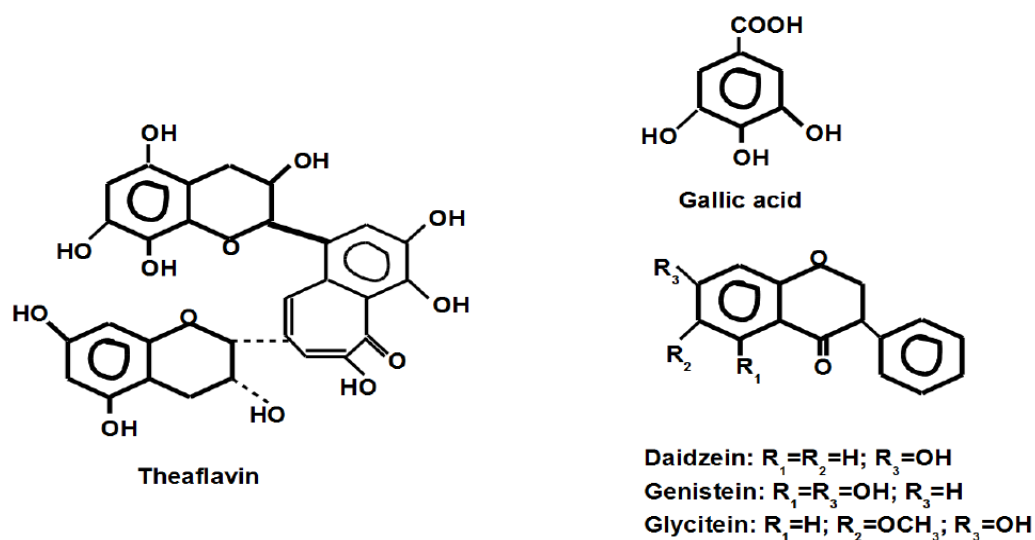
Figure 2.4: Basic structures of some pharmacologically important plant derived Tannins (Doughari, 2012).

2.3.6 Terpenes

Terpenes are among the largest outspread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, found in liquid form, mostly in essential oils, resins or oleoresins (Firn, 2010). Terpenoids constitute hydrocarbons of plant origin of general formula $(C_5H_8)_n$ and are classified as mono-, di-, tri- and sesquiterpenoids based on the number of carbon atoms. Examples of most known terpenes are terpinen-4-ol, thujone, camphor, eugenol and menthol. *Diterpenes (C20)* such as resins and taxol, the anticancer agent, are common example. Examples of *triterpenes (C30)* are steroids, sterols and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. Common triterpenes: amyrins, ursolic acid and oleanic acid. *Sesquiterpene (C15)*, like monoterpenes, are important parts of many essential oils (Martinez *et al.,* 2008). The sesquiterpene behaves as an irritant when tested externally and. when consumed internally. their action resembles that of a gastrointestinal tract irritant. Several sesquiterpene lactones have been screened and generally they have antimicrobial (particularly antiprotozoal) and neurotoxic action.

β-caryophyllene   Terpenolen   α-Cubebene
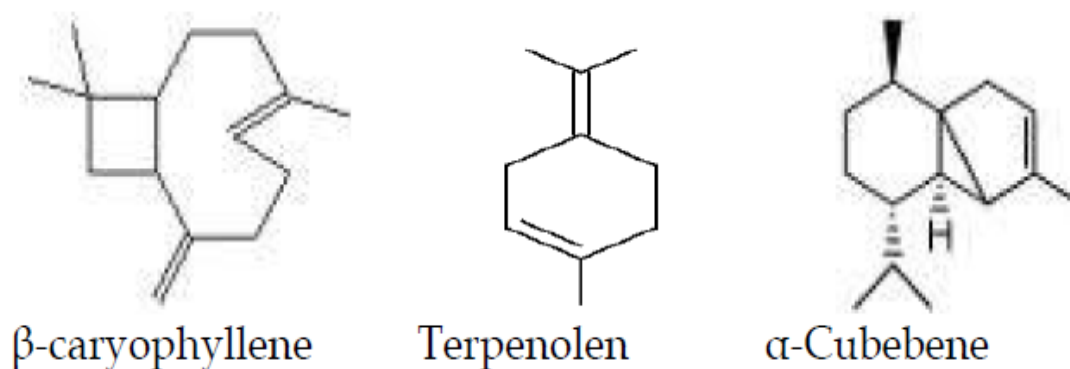
Figure 2.5: Basic structures of some pharmacologically important plant derived tarpenes (Doughari, 2012).

2.4 Anti-diabetic properties of medicinal plants

Free radicals have been involved in the cause of age-related diseases, such as diabetes mellitus. Compounds that can scavenge free radicals have great potential

in ameliorating these disease processes (Wilson, 1988). Therefore, antioxidants have a huge impact in protecting the human body against damage by reactive oxygen species (Lollinger, 1981). Elevated oxidative stress has been postulated in the diabetic state. Oxygen free radical activity has the ability to start peroxidation of lipids, which in turn may lead to glycation of protein, inactivation of enzymes and changes in the structure and function of collagen, basement and other membranes, and participate in the long-term complications of diabetes (Boynes, 1991). Oxidative stress in diabetes coexists with a reduction in the antioxidant status (Collier *et al.*, 1990), which can increase the deleterious effects of free radicals. Antioxidants, such as vitamins C and E, have been shown to decrease the oxidative stress in experimental studies carried out in diabetes (Garg and Bansal, 2000). Supplementation of the antioxidant vitamin C has been indicated to reduce glycosylated haemoglobin in diabetic patient (Davie *et al.*, 1992). A lot of plant extracts and plant products have indicated to have significant antioxidant activity (Doughari, 2012 and Sylvie *et al.*, 2014).

2.5 Potential of medicinal plants in inhibiting protein glycation

Many plant species constituents contain polyphenols, which possess the ability to remove free radicals developed following glycation and its end products from the system (Kazeem *et al.*, 2012). The oxidation process is believed to play a huge part in the development of advance glycation end products (AGEs). Therefore, medicinal plants with an antioxidant agent or metal-chelating property may reduce the process of AGEs formation by inhibition of further oxidation of Amadori product and metal-catalyzed glucose oxidation. According to the world health organization, about 80% of the world's population practice the use of medicinal plants as a remedy to prevent diabetes complications such as protein glycation enhanced by free radical formation (Yamaguchi *et al.*, 2000). Several animal studies have indicated that inhibitors of glycation reaction, aminoguanidines, can decrease or inhibit the development of different diabetic vascular complications. However, a standard agent, used as glycation inhibitors, has been reported to have some undesirable side effects such as being too toxic to human cells. This has led to an increase in interest of medicinal plants as a way to inhibit glycation reaction.

2.5 The plant under study: *Bidens pilosa*

Bidens pilosa is a plant that is easy to maintain and grow and is found all over the world. It is known to be a rich source of food and medicine for humans and animals (Bremer, 1994 and Pozharitskaya *et al.*, 2010). There is expanding interest worldwide for the utilization of *B. pilosa. Bidens pilosa* has been described by its Botany, traditional usage, phytochemistry, pharmacology, and toxicology.

2.5.1 Botany

There are 230 to 240 species of *B. pilosa* found worldwide. *B. pilosa* has several varieties such as *B. pilosa* var. *radiata*, var.*minor*, var. *pilosa,* and var. *bisetosa*. *Biden pilosa* is an erect, perennial herb generally distributed across temperate and tropical locales. *Bidens pilosa* is either glabrous or bushy, with green inverse leaves that are serrate, lobed or dismembered. It has white or yellow flowers, and long narrow ribbed black achenes (seeds). It develops to an average height of 60 cm and a maximum of 150 cm in favourable environments (Alcaraz, 1988). Preferably *B. pilosa* develops well in conditions and environment where there is adequate sunlight with moderately dry soil. Nonetheless, it can develop and survive in arid and barren land from low to high elevations. *B. pilosa* is known to have originated in South America and subsequently spread throughout the world (Ge,1990). *Bidens* species and their varieties bear different names based on their attributes. For instance, Bidens species are known by such names as Spanish needles, beggar's ticks, devil's needles, cobbler's pegs, broom sticks, pitchforks and farmers' friends in English and some other languages because of their sticky achenes (Alcaraz, 1988).

2.5.2 Traditional uses

Traditionally *B. pilosa* is utilised as an herb and ingredient in teas or herbal medication. Its shoots and leaves, dried or fresh, are utilised in sauces and teas (Chiang *et al.*, 2003). All parts of *B. pilosa* plant, the aerial parts such as leaves, flowers, seeds and stems and the roots, fresh or dried, are used as ingredients in folk medicines. It is mostly prepared in a form of dry powder, decoction, maceration or tincture (Redl *et al.*, 1994). Normally, *B. pilosa* is applied as dry powder or tincture when used externally, and as a powder, maceration, or decoction when

used as an internal remedy (Rybalchenko *et al*., 2010). Different parts of the plant are used to treat most disorders, such as inflammation, immunological disorders, digestive disorders, infectious disorders, cancer, wounds and metabolic disorders such as diabetes (Dimo *et al*., 2011). *Bidens pilosa* is an extraordinary source of phytochemicals and is rich in flavonoids and polyynes. Plants which have flavonoids are known to have anti-cancer and anti-inflammatory properties and contain antioxidants. However, the bioactivities of only seven of the 60 flavonoids present in *B. pilosa* have been studied.

2.5.3 Pharmacological properties

Traditionally *B. pilosa* is utilised to treat a lot of ailments, such as a stomach ulcer, diabetes, malaria and cancer. All parts of this plant are used in different preparations for treatment of various diseases. *Bidens pilosa* possesses anti-inflammatory (Pereira *et al*., 1999), antidiabetic and antihyperglycemic (Hsu *et al*., 2009), antioxidant immunomodulatory, antimalarial, antibacterial properties, and have dilatory activities (Dimo and Dongo, 2000).

Figure 2.6: A representative image of *Bidens pilosa (*Bartolome *et al*., 2013).

2.6 Toxicity and safety concern of the medicinal plants

Medicinal plant species which are utilised for the management of various ailments, on a big scale, are reported to have adverse side effects. Most standard drugs are generated from biologically active plant chemicals and their medicinal mode of action are attributed to different active chemicals found in plants. Synthetic drugs normally contain a single chemical, whereas medicinal plants constitute a diverse mixture of 400 or more chemicals (George, 2011). It's comparatively simple to identify the activity and side effects of a single chemical, but there is huge challenge for scientists to identify all the active complex interactions and synergies that are involved between all the different chemicals in the plant, or crude plant extract. consisting of all these chemicals which is used traditionally (Boullata and Nace, 2000).

Plants which are mostly used in traditional medicine are assumed to be safe. The assumption on safety is based on their lifetime span usage in the treatment of diseases through the knowledge acquired over centuries. However, current scientific research has proven that most plants, taken as food or in traditional medicine, are potentially toxic, mutagenic and carcinogenic (Schimmer *et al.*, 1988, Higashimoto *et al.*, 1993; Kassie *et al.*, 1996; Fernandes De Sã Ferrira and Ferrão Vargas, 1999). Commonly used traditional medicines were investigated for their toxicity and safety in relation to their long-term effects. It was discovered that most plant species, when utilised at very high concentrations, result in serious side effects, such as DNA damage, chromosomal aberrations and/or non-disjunction or chromosome lagging (Taylor *et al.*, 2003). The safety of patients utilising traditional medicine is compromised because they are increasingly dependent on sellers who are inadequately trained to supply proper plant medicine. Highly equipped traditional healers are reliable sources of medicine, but traders are seldom qualified (Cunningham, 1988). An increasing number of healers do not show formal training or adequate knowledge, skill and experience to practice successfully (Bodenstein, 1973). Besides, inadvertent misidentification and the misadministration of traditional plant medicines is mostly the result of purposeful adulteration because the motivation to market medicinal plants is mostly not to give a required service, but to make money (Manana and Eloff, 2001).

2.7 Background and study rationale

Obesity has become a huge health problem globally (Finkelstein *et al.,* 2012). The world is thus in the midst of a worldwide obesity epidemic. The complications of obesity are taking a major public health toll, which will only worsen in future years. Among the most devastating of these complications is type II diabetes (T2D). Most patients with T2D are obese or overweight. Currently, there is no cure for diabetes mellitus; it is managed through standard drugs, which have undesirable side effects such as toxicity. The current diabetes drugs are available and cheaper, however the most effective ones are expensive and inaccessible to the majority of the rural poor. People in rural areas are at higher risk of being obese because rural healthcare facilities are less likely to have nutritionists, dietitians or weight management experts available. These areas may equally lack exercise facilities and infrastructure to encourage physical activity (Haughton *et al.*, 2012). The prevalence of obesity and the undesirable effects of current antidiabetic drugs, coupled with their unavailability and economic impact of the diseases, remains a great challenge to the society at large.

Medicinal plants play a big role in the treatment of human diseases, especially in the developing world. The World Health Organisation came to a realisation that an effective health agenda for developing countries can not only depend on western medicine and have emphasized the need for the utilization of medicinal plant resources in order to achieve the objectives of primary health care (Dahlberg *et al.*, 2009). Medicinal properties of plant remedies have been attributed to the presence of secondary metabolites, such as polyphenols, steroids, alkaloids, terpenoids and glycosides (Yao *et al.*, 2009). A growing number of the world's population have resorted to the use of plant derived remedies and medicines for many reasons, which include their low cost, easy accessibility and less side effects (Payyappallimana *et al.*, 2010).

In that regard, this current study aimed to evaluate the anti-diabetic potential of the crude extract of *B. pilosa* and its sub-fractions in both 3T3-L1 and C2C12 cell models. Plants are known to contain a variety of secondary metabolites. These secondary metabolites can be beneficial in that they could elicit anti-obesity and anti-diabetic properties based on their ability to regulate various metabolic pathways. More

importantly, they could be capable of exhibiting different pharmacological activities, such as hypoglycaemic activity, cytotoxicity, anti-glycation and potentiation of glucose uptake. Despite these facts, a large majority of medicinal plants have not been evaluated, in different test systems, for the identification of active principles contained in the treatment for a variety of diseases.

2.8 Aim of the study

The aim of the study was to evaluate the anti-diabetic and anti-obesity properties of *B. pilosa* crude extract and sub-fractions on C2C12 muscle cells and 3T3-L1 adipocytes in *vitro* models.

2.9 Objectives

The objectives of the study were**:**

i. To analyse the phytochemical constituents of the *B. pilosa* methanol crude extract and its fractions using thin layer chromatography and standard chemical tests.

ii. To determine the antioxidant capacity of the methanol leaf extract of *B. pilosa* and sub-fractions using the DPPH and ferric ion reducing assays.

iii. To determine the antiglycation potential of the methanol leaf extract of *B. pilosa* and sub-fractions.

iv. To determine the cytotoxic effect of the crude extract of *B. pilosa* and sub-fractions on 3T3-L1 pre-adipocytes and C2C12 muscle cells using the MTT assay.

v. To evaluate the ability of *B. pilosa* crude extract and sub-fractions in stimulating glucose uptake in 3T3-L1 adipocytes and C2C12 myotubes.

vi. To evaluate the effect *B. pilosa* crude extract and sub-fractions on adipogenesis in 3T3-L1 adipocytes.

Chapter 3: Methods and Materials

3.1 Plant collection, extraction and sub-fractionation

The plant was harvested at Soekmekaar Village in Limpopo province and identification confirmed by experts at Larry Leach Herbarium, University of Limpopo. The selection of the plant was based on literature survey of reports of their anti-diabetic properties by traditional healers and elders in the villages in Limpopo province. The leaves of *B. pilosa* (30 g) were extracted using methanol (300 ml). The supernatant of the plant material was filtered using a Whatman no.1 filter paper into pre-weighed glass vials. The filtrate was left under a stream of air until it was completely dried, after which the total mass was determined. The methanol crude extract was then subjected to solvent-solvent fractionation to yield six semi-purified extracts (*n*-butanol, chloroform, ethyl-acetate, hexane, 35% water in methanol and water) according to the method of Suffness and Douros (1979).
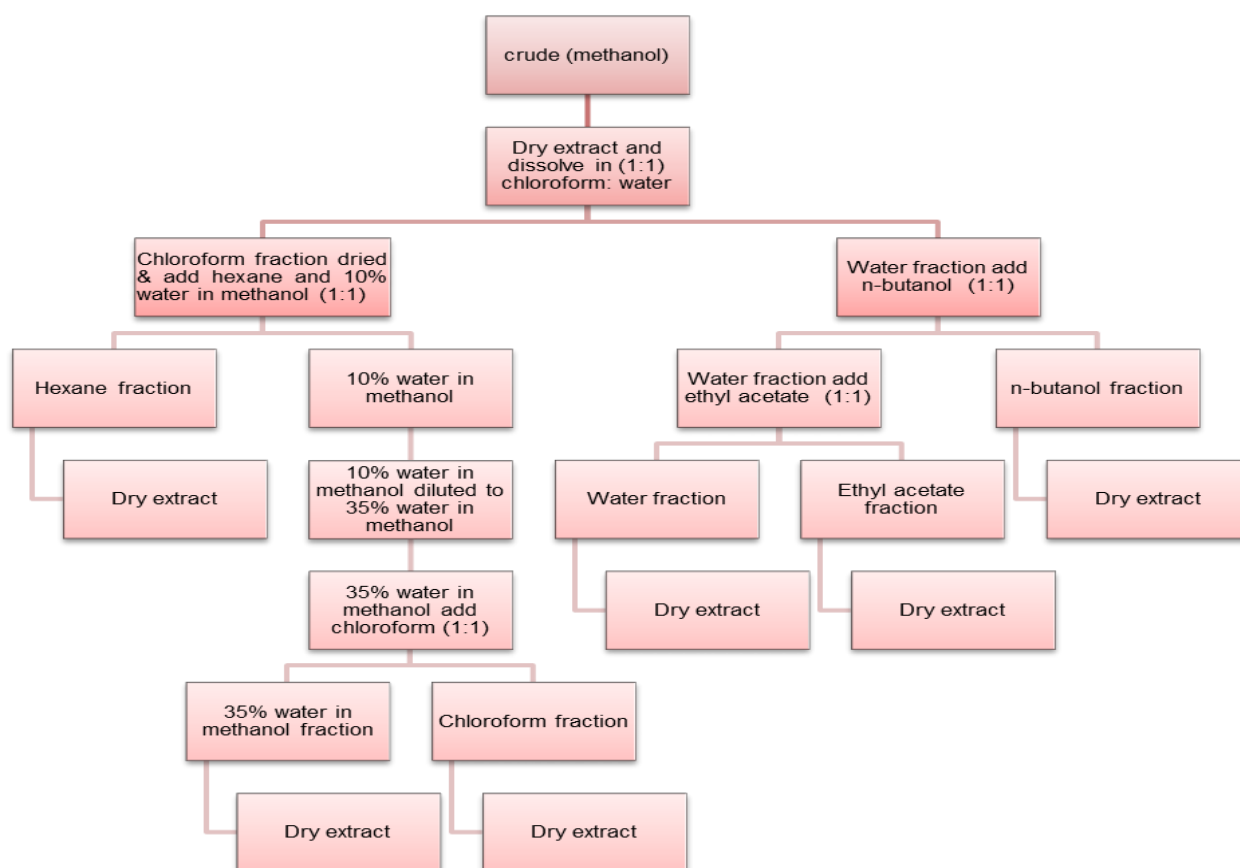
Figure 3.1: solvent -solvent fractionation diagram (suffness and Duoros 1979).

3.2 Qualitative phytochemical analysis

3.2.1 Thin layer chromatography Profiling

Fingerprinting of compounds was done using Thin Layer chromatography (TLC), according to the method of Kotze and Eloff (2002), where TLC plates where loaded with 10 µg/ml of the crude extract and sub-fractions. The TLC plates were eluted in different mobile phases, primarily polar Ethyl acetate: Methanol: Water (40:5:4:5) [EMW], Chloroform: Ethyl acetate: Formic acid (5:4:1) [CEF] (intermediate polarity) and Toulene: Ethanol: Ammonium hydroxide (18:2,0:2) [TEA] (non-polar). The separated compounds were detected by spraying with different sprays: DPPH, vanillin- sulphuric acid and p-Anisaldehyde heated at 110 °C for optimal colour development.

3.2.2 Chemical tests for various secondary metabolites

All chemical tests for secondary metabolites were performed according to the methods of Sofowara (1993) and Harborne (1973).

3.2.2.1 Test for phenols

An amount of 2 ml of *B. pilosa* crude extract and sub-fractions were added to water and warmed at 45-50 °C. Thereafter 2 ml of 3% ferric chloride ($FeCl_3$) was added. Formation of green or blue colour will indicate the presence of phenols.

3.2.2.2 Test for tannins

Approximately 1 ml of *B. pilosa* crude extract and sub-fractions were added to 1 ml of 3% $FeCl_3$. A greenish black precipitate signifies the presence of tannins.

3.2.2.3 Test for terpenoids

An amount of 5 ml of *B. pilosa* crude extract and sub-fractions were mixed in 2 ml chloroform. To carry out the reaction, 3 ml of concentrated sulphuric acid was carefully added to observe a reddish-brown coloration between the upper and lower layer.

3.2.2.4 Test for saponins

The presence or absence of saponins was determined by adding 0.2 ml of *B. pilosa* crude extract and sub-fractions and mixed with 5 ml of distilled water. The extracts were shaken vigorously for 5 minutes. Persistence of foam was the indicator for saponins.

3.2.2.5 Test for flavonoids

Approximately 1 ml of *B. pilosa* crude extract and sub-fractions were added to 10% $FeCl_3$ ferric chloride. The mixture was shaken vigorously for 5 minutes. A woolly brownish precipitate indicated the presence of flavonoids.

3.3 Quantitative phytochemical analysis

3.3.1 Total phenolic content

The quantity of phenols present in the methanol crude extract and sub-fractions was determined by Folin-Ciocalteu's phenol reagent, following the method obtained from Humadi and Istudor (2009). A stock solution of 100 mg/ml of the crude methanol extract and sub-fractions were prepared. Folin-Ciocalteu reagent (50 µl) and distilled water (450 µl) were added to the crude extract and sub-fractions (100 µl) and left for 5 minutes in the dark at room temperature. The reaction continued with the addition of 7% of sodium carbonate solution (500 µl). Distilled water was added to make a final volume of 500 µl. Thereafter, the mixture was incubated in the dark for 90 minutes at room temperature. Absorbance was read at a wavelength of 750 nm using a spectrophotometer. The total phenolic content was determined by linear regression from a tannic acid calibration standard curve.

3.3.2 Total Flavonoid Content

Total flavonoid content was determined using the aluminium chloride colorimetric method obtained from (Chang *et al*., 2002). A stock solution (10 mg/ml) of the crude extract and sub-fractions were prepared. Each of the extracts (25 µl) was mixed with 10% aluminium chloride (25 µl), 1 M potassium acetate (25 µl) and distilled water

(700 μl). The mixture was left to stand at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm in triplicate using a spectrophotometer (Beckman Coulter-DU730). The total flavonoid content was determined by linear regression from a quercetin calibration curve standard.

3.4 Quantitative antioxidant activity

3.4.1 Free radical scavenging activity

The amount of antioxidants present in the methanol crude extract and sub-fractions was determined by its free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) following a method of Deby and Magotteaux (1970). Each plant extract was serially diluted with distilled water in a 96 well plate. The final volume of each plant extract's concentration was 1 mg/ml - 0.0625 mg/ml. L-ascorbic acid (vitamin C) was used as a positive control, and dimethylsulfoxide (DMSO) as the negative control. The decrease in absorbance was measured at 490 nm using a microtiter-plate multimode detector (Promega-Glomax Multidetection System). The degree of discolouration indicates the scavenging potential of the extracts in terms of hydrogen donating ability. The absorbance values obtained were converted to percentage scavenging activity using the following formula:

$$\% \text{ inhibition} = \frac{((A490 \text{ nm of blank} - A490 \text{ nm of sample}) \times 100)}{(A490 \text{ nm of blank})}$$

3.4.2 Ferric ion reducing power

The ferric ion reducing power of the different methanol crude extract and sub-fractions were determined according to the method of Benzie and Strain (1996). Various concentrations (1 mg/l - 0.0625 mg/ml) of the extracts in deionised water (100 μl) were prepared. A blank was prepared without extract, while ascorbic acid was used as the reference standard. These were then mixed with 0.2M phosphate buffer (250 μl) (pH 7.4) together with potassium ferricyanide (250 μl) and incubated at 50°C for 20 minutes. After incubation, aliquots of trichloroacetic acid (250 μl) were added to the mixture. The mixture was transferred to a new plate (250 μl) and mixed with distilled water (125 μl) and freshly prepared ferric chloride solution (25 μl). The

absorbance of the samples was measured at 700 nm using a microtiter-plate multimode detector (Promega-Glomax Multi detection System). Percentage reducing power was calculated according to the following percentage formula:

Percentage inhibition = $\underline{(A700nm \ of \ sample - 1) \times 100)}$

$\qquad\qquad\qquad (A700nm \ of \ blank)$

Experiments were done in triplicate in three independent trials.

3.5 Anti-glycation activity

The antiglycation assay was performed according to the methods reported by Matsuura and colleagues with slight modification (Matsuura *et al.*, 2002). In all experiments, the final reaction volume was 1 ml and was performed in 48 well plates. Albumin (1 mg/ml final concentration) was incubated with glucose (500 mM final concentration) in the presence of arbutin or PBS, as control buffer, at different concentrations (10 mg/ml - 0,625 mg/ml). The reaction was allowed to proceed at 60°C for 24 hours and thereafter, stopped by adding 10 µl of 100% (w/v) trichloroacetic acid (TCA). The TCA-added mixture was kept at 4°C for 10 minutes before centrifugation at 10000 g. The precipitate was re-dissolved with alkaline PBS (pH 10), and immediately quantitated for the relative amount of glycated BSA based on fluorescence intensity by a spectrofluorometer. The excitation and emission wavelengths used were at 370 nm and 440 nm, respectively. Five concentrations of crude were analysed in triplicate. Percentage inhibition was calculated using the formula provided below and the sample concentration required for 50% inhibition of BSA glycation was calculated:

% inhibition= $\underline{OD \ blank - (OD \ sample - OD \ sample \ negative)}$ x 100

$\qquad\qquad\qquad\qquad OD \ blank$

3.6 Partial α-amylase enzyme inhibition assay

The partial inhibition of pancreatic α-amylase was determined using the chromogenic method adapted by Sigma-Aldrich with few modifications (Bernfeld, 1955). *Bidens pilosa* crude extract and sub-fractions were reconstituted in DMSO and serially

diluted (40 µl plant and 160 µl distilled water), pre-incubated with 200 µl (4 U/ml) of pancreatic α-amylase (Sigma-Aldrich) and dissolved in ice-cold distilled water for 5 minutes at room temperature. The reaction was initiated by addition of 400 µl of 0.5% potato starch solution prepared in 20 mM phosphate buffer pH 6.9, containing 6.7 mM sodium chloride, and incubated at 37 °C for 5 minutes. Final concentrations in each reaction mixture were 1 mg to 0.5 mg/ml of each plant extract, 1 U/ml of α-amylase and 0.25% (w/v) potato starch. To stop the reaction,100 µl of DNS reagent (96 mM 3,5-dinitrosalicyclic acid, 5.31 M sodium potassium tartrate in 2 M sodium hydroxide) was added and heated for 15 minutes at 85 °C in a water bath. After heating, 900 µl of distilled water was mixed with 100 µl aspirate from each sample mixture and absorbance read at 540 nm. The control mixtures were performed in the same way, with the plant extract replaced by 40 µl of DMSO or acarbose (serial dilution) and with the blank, the enzyme solution was replaced by distilled water. Background readings were eliminated by subtracting the absorbance of the extract without substrate and enzyme from the absorbance of the extract and substrate mixture. The results obtained were expressed as percentage inhibition of alpha amylase using this formula:

% inhibition= $\underline{\text{OD control} - \text{OD sample extract}}$ x 100

                          OD control

3.7 Cell culture maintenance

The C2C12 muscle and 3T3-L1 pre-adipocyte cells were kindly provided by the South African Medical Research Council (SAMRC). The cell lines were maintained in Dulbecco's minimum-eagles medium (DMEM) (Hyclone, Thermo Scientific) supplemented with 10% (v/v) foetal bovine serum (FBS) and 100 µg/ml ciprofloxacin and incubated at 37 °C and 5% CO_2 in a humidified incubator (Heracell 150i CO_2 Incubator, Thermo Scientific). The culture medium was replaced with fresh medium every 2–3 days.

3.7.1 C2C12 muscle cell differentiation

The C2C12 muscle cell differentiation was induced by refreshing the growth medium (DMEM supplemented with 10% FBS and 100 µg/ml ciprofloxacin) with differentiation

medium (DMEM supplemented with 2% FBS and 100 µg/ml ciprofloxacin) for 6 days. The medium was changed after every 2 days with the monitoring of the morphological change of C2C12 to that of myotubes within this period (Jing, 2012).

3.7.2 3T3-L1 pre-adipocyte cell differentiation

The 3T3-L1 pre-adipocytes were differentiated using a method of Mazibuko *et al*. (2015). The growth medium (DMEM supplemented with 10% FBS and 2% ciprofloxacin) was replaced with differentiation medium (0.5 mM IBMX, 1 µM dexamethasone, 5 µg/ml insulin, DMEM, 10% FBS and 100 µg/ml ciprofloxacin) and incubated for 6 days in a humidified incubator at 37°C and 5% CO_2. On the fourth day, differentiation medium was replaced with post differentiation medium (DMEM, insulin, 10% FBS and 100 µg/ml ciprofloxacin) until the eighth day. By the eighth day, the 3T3-L1 pre-adipocytes were fully differentiated. The change in morphology of the differentiated cells was observed after every two days with the change of media. Cells were stained with Oil-red-O to visualize the amount of accumulated fats droplet using 40X magnification.

3.8 Cytotoxicity assay

3.8.1 MTT assay

Toxic effects of each plant extract were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, SA) assay according to the method of Mossman (1983). The C2C12 muscle and 3T3-L1 cells were seeded at a density of 5×10^3 cells/well in 96 well plates and incubated at 37 °C and 5% CO_2 overnight. Both cell lines were treated with various concentrations of *B. pilosa* crude extract and sub-fractions for 24 hours. After 24 hours of treatment, the treatment medium was aspirated carefully, replaced with 100 µl of MTT (1 mg/ml) in each well and further incubated for 3 hours at 37 °C in the incubator. The untreated cells served as the experimental control. Curcumin (Sigma Aldrich, SA) and DMSO served as positive and negative controls, respectively. The formazan product was solubilized in 100 µl of DMSO the absorbance reading at 560 nm recorded using the Glomax microtiter plate reader (Promega, U.S.A). Untreated cells served as an experimental

control from which the toxic effects of the extracts were determined. Results obtained were expressed as percentage viability against concentrations.

% inhibition=OD blank − <u>(OD sample − OD sample negative)</u> x100

OD blank

3.9 Glucose uptake assay

Glucose concentration in the medium was determined after the incubation period by the glucose oxidase method using a commercial kit [KAT Laboratories and Medicals (PTY) LTD]. Differentiated C2C12 and 3T3-L1 cells were treated with non-cytotoxic concentrations of the extracts for 3 hours and 6 hours in a humidified incubator at 37°C. After 3 hours and 6 hours of incubation, 100 µl of each sample treatment was mixed with 100 µl of glucose oxidase substrate and further incubated at 37°C for 30 min. The absorbance of the free glucose remaining in the samples was read at 540 nm using the Glomax microtiter plate reader (Promega, U.S.A). Untreated cells were used as a standard control and insulin (1 µg/ml) and metformin (1 µg/ml) were both used as positive controls and in combination with the plant extracts. The results obtained were expressed as % glucose uptake by the cells.

3.10 Adipogenesis assay

The adipogenesis assay was performed using the kit from Sigma Aldrich. Differentiated cells were treated with non-cytotoxic concentrations of *B. pilosa* extract and active sub-fractions and incubated for 48 hours. Adipocyte differentiation medium (ADM) was used as a positive control and untreated cells as negative control. After 48 hours treatment, the medium was removed and cells were washed once with PBS. One hundred microliters of lipid extraction buffer was added in each well, the plate was sealed and incubated for 30 minutes at 90°C. The plate was cooled and agitated for 1 minute to homogenise the solution. Fifty microliters of each treatment was transferred to a new 96 well plate and 2 μl of lipase solution (Sigma-Aldrich, Germany) was added to each well and incubated for 10 minutes at room temperature. Fifty microliters of adipogenesis master mix was added to each well and further incubated for 30 minutes at room temperature. The plate was protected from light during the

incubation period. The amount of accumulated triglycerides was measured at an absorbance of 560 nm using Glomax Microtiter Plate Reader (Promega, USA).

3.11 Statistical analysis

The results obtained were analysed using Graphpad Instat Statistical Software, version 3.0; the results were expressed as ± SEM of the mean, from triplicates of three to four independent experiments. Statistical evaluation of the results was determined using the one way analysis of variance (ANOVA), employing the Dunnett multiple comparisons test and P values of <0.05, <0.001 and 0.001 were considered significant.

Chapter 4: Results

4.1 Plant extraction and yield quantification

The percentage yields of *B. pilosa* sub-fractions were obtained after solvent-solvent fractionation obtained from crude methanol extract. The yield quantifications were calculated by taking the dry masses extracted by each of the sub-fractions as a percentage of the initial dry mass of crude methanol extract. Water had the highest percentage yield of 19.5% while ethyl acetate had the lowest percentage yield of 1.3% (fig. 4.1).
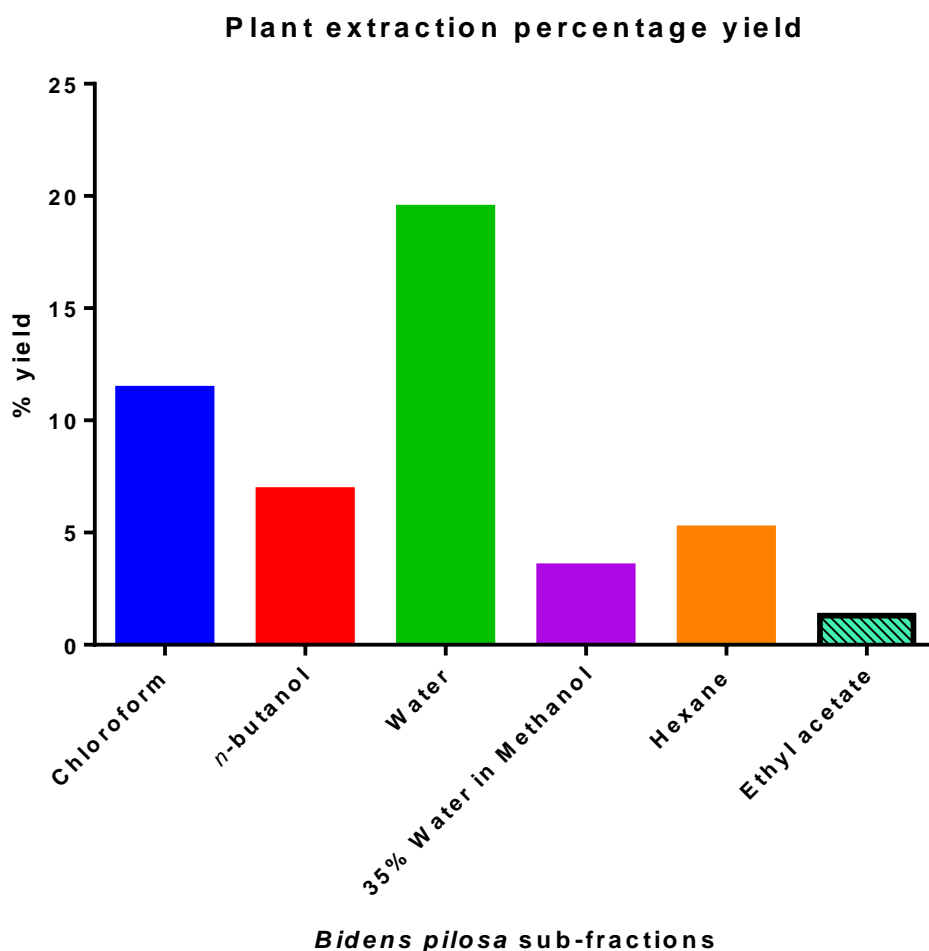

Figure 4.1: The percentage yields of *B. pilosa* sub-fractions obtained after solvent- solvent sub-fractions of crude methanol extract.

4.1 Phytochemical analysis

4.1.1 The qualitative phytochemical analysis and UV active compound profile on thin layer chromatography plates

Thin layer chromatography (TLC) was used to separate the chemical constituents of *B. pilosa* crude extract and sub-fractions to establish their phytochemical fingerprints. Different compounds migrate to the top of the plates according to their polarities in each of the mobile phases. The bands on top of the plates are the most polar while those at the bottom of the plates are less polar. Under visible light different bands were observed on the TLC plates. These different bands are an indication of different compounds present in the extracts. Some compounds were not visible under normal light and could only fluoresce under ultraviolet light at a wavelength of 360 nm and 254 nm. When observed under the UV light more bands were visible with different colours, an indication of the presence of different compounds. More compounds were shown to fluoresce on chromatograms eluted in EMW followed by CEF. They also had more compounds that fluoresce under the UV light as compared to visible light. The intensities of the colour of bands is an indication of the concentration of the compound within the extract. The TLC plates were then sprayed with vanillin sulphuric acid reagent spray to further visualise compounds that were not seen under visible and UV light (fig 4.2). To visualise the presence of phenolic compounds, the plates were sprayed with p- Anisaldehyde (fig 4.3). Chloroform, 35% water in methanol and *n*-butanol had the highest phenolic compounds respectively, whereas hexane and water resulted in the least phenolic compounds. In comparison with the crude extract and sub-fractions in different mobile phases, chloroform had more compounds followed by 35% water in methanol, *n*-butanol, crude, hexane and water sub-fractions. The mobile phase EMW best separated the chemical compounds present in *B. pilosa* crude extract and sub-fractions.
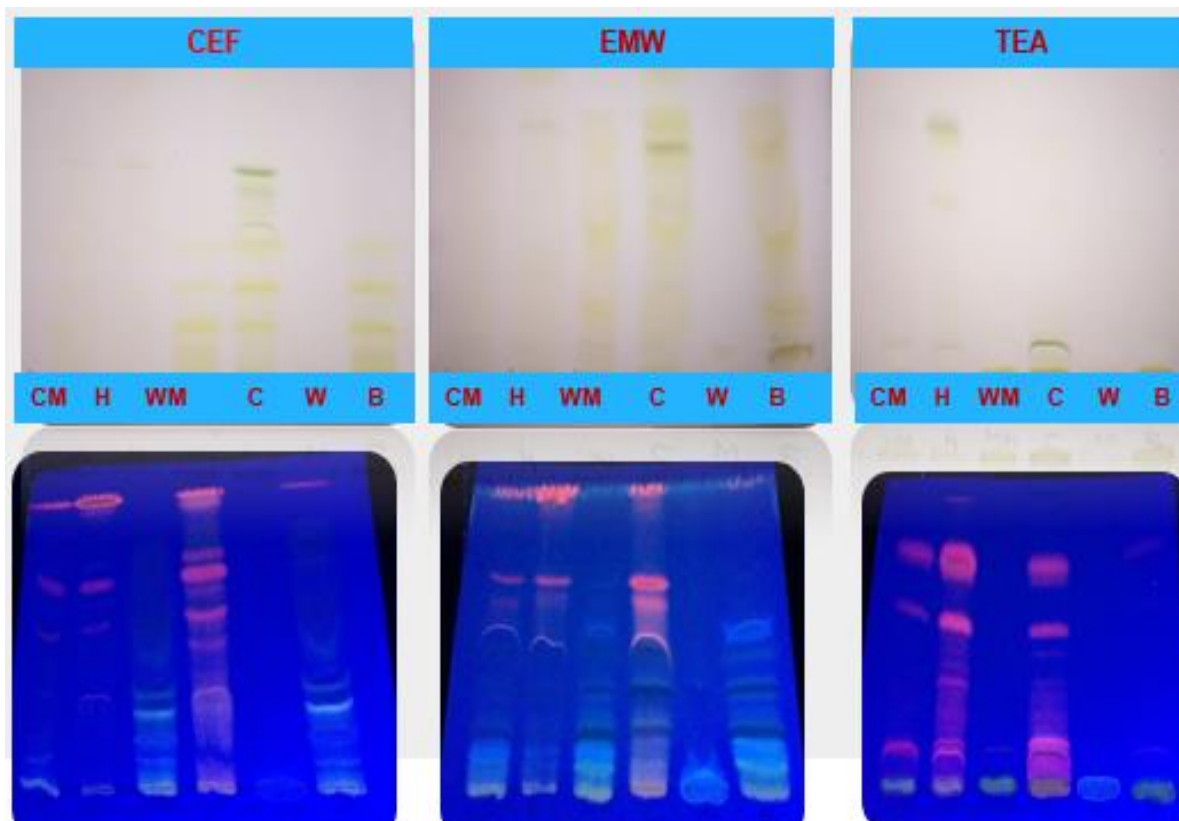
Figure 4.2: TLC fingerprint profile and UV active compounds of *B. pilosa* crude methanol and sub-fractions under UV light (365 nm). The plates were developed using TEA [Toluene: Ethanol: Ammonium hydroxide, (72: 8: 8)], EMW [Ethyl acetate: Methanol: Water (40: 5.48: 4)] and CEF [Chloroform: Ethyl acetate: formic acid, (10: 8: 2)]. CM=Crude Methanol, H=Hexane, WM=35% Water in Methanol, C=Chloroform, W=Water, B=*n*-Butanol.
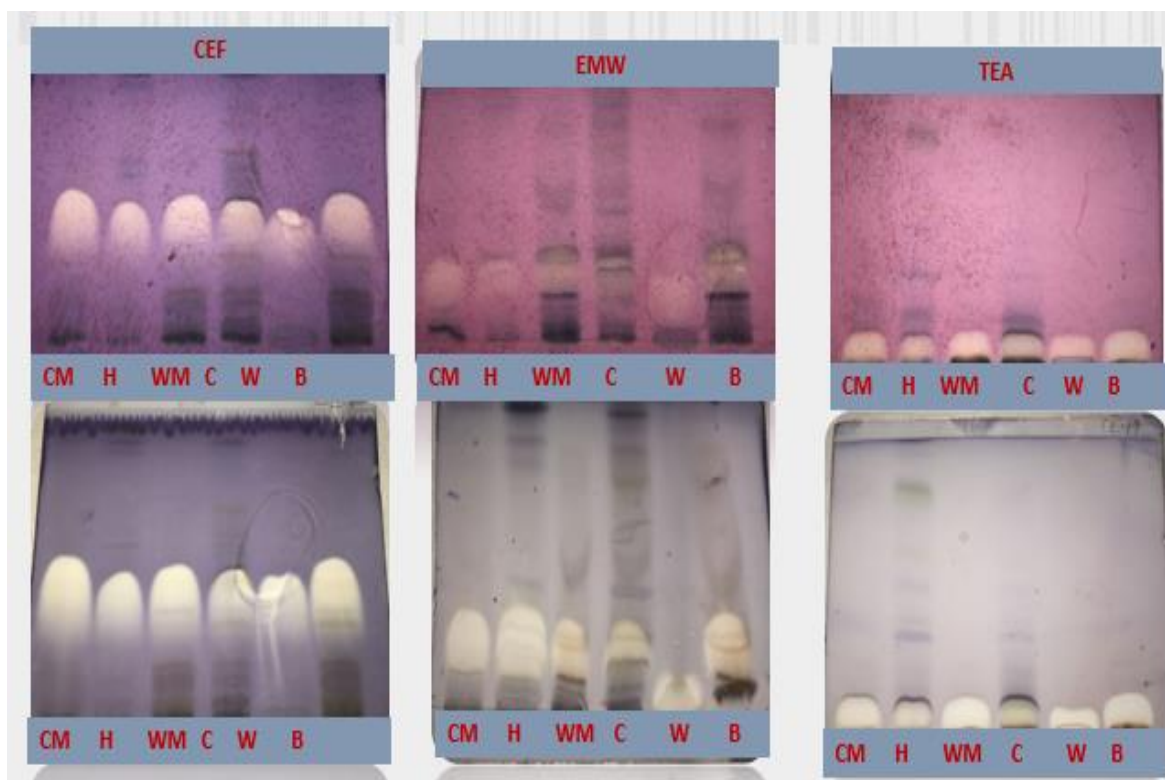
Figure 4.3: TLC fingerprint plates sprayed with p-Anisaldehyde and acidic vanillin. The plates were developed using TEA [Toluene: Ethanol: Ammonium hydroxide, (72: 8: 8)], EMW [Ethyl acetate: Methanol: Water (40: 5.48: 4)] and CEF [Chloroform: Ethyl acetate: formic acid, (10: 8: 2)]. CM=Crude Methanol, H=Hexane, WM=35% Water in Methanol, C=Chloroform, W=Water, B=*n*-Butanol.

4.1.2 Chemical tests for secondary metabolites

Bidens pilosa crude extract and sub-fractions were tested for the presence of various secondary metabolites, namely tannins, phenols, flavonoids, steroid and terpenoids. The positive sign is an indication of the presence of the phytochemical while the negative sign indicates its non-presence or detection. Tests for these various secondary metabolites were performed using standardised calorimetric tests. Tannins and phenols were found to be present in the crude extract and all the sub-fractions, except 35% water in methanol, whereas terpenoids were found present in crude extract and sub-fractions, except for *n*-butanol sub-fraction. All the various secondary metabolites tested were found to be present in the chloroform sub-fraction. There was a slight difference in the presence/ no presence of steroids and flavonoids in the crude extract and sub-fractions. Nonetheless, *B. pilosa* crude extract and sub-fractions where shown to have most of the secondary metabolites evaluated (table 4.1).

Table 4.1: The presence and absence of various secondary metabolites in *B. pilosa* crude extract and sub-fractions.

Phytochemicals	Crude Methanol	Chloroform	*n*-Butanol	Water	35% Water in methanol	Hexane
Phenols	+	+	+	+	-	+
Terpenes	+	-	+	+	+	-
Flavonoids	+	+	+	-	+	-
Saponins	+	+	+	-	+	+
Steroids	+	-	+	-	-	-
Tannins	+	+	+	+	+	+
Glycosides	+	+	-	-	+	+

Key: Present (+) and Absent (-)

4.1.3 Qualitative antioxidant

The TLC plates were sprayed with DPPH solution for determination of antioxidant activity. The yellow bands that appear on the purple background are an indication of the presence of antioxidant activity. Different solvent systems were used to separate the antioxidant compounds according to their polarities, TEA, CEF and EMW mobile phases. The TLC profiles indicate that more antioxidant compounds were found in the CEF mobile phase. This reveals that the nature of antioxidant compounds found in the *B. pilosa* are mostly intermediate polar. More antioxidant compounds were found in the EMW mobile phase and few antioxidant compounds were present in the TEA mobile phase, as indicated by only one band for crude extract and sub-fractions. *n*-Butanol and 35% water in methanol had more antioxidant compounds in EMW and CEF mobile phases. This is indicated by the presence of yellow bands. No bands were visible for hexane sub-fraction, an indication of absence of antioxidant activity (fig. 4.5).
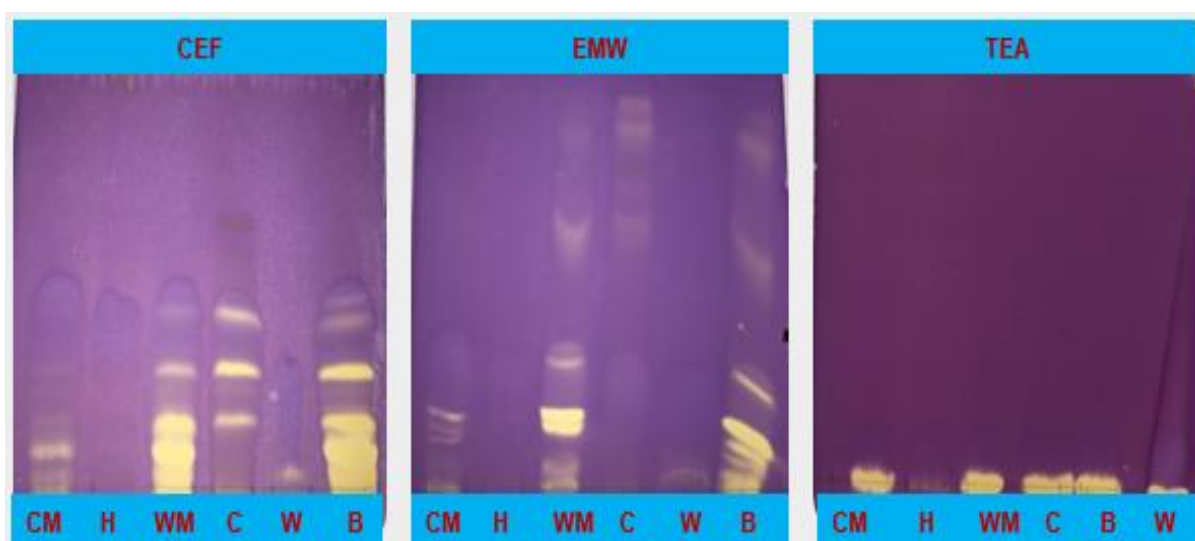
Figure 4.4: TLC plates sprayed with DPPH for visualisation of antioxidants compounds. The plates were developed using TEA [Toluene: Ethanol: Ammonium hydroxide, (72: 8: 8)], EMW [Ethyl acetate: Methanol: Water (40: 5.48: 4)] and CEF [Chloroform: Ethyl acetate: formic acid, (10: 8: 2)]. CM=Crude Methanol, H=Hexane, WM=35% Water in Methanol, C=Chloroform, W=Water, B=*n*-Butanol.

4.1.3 Quantitative phytochemical analysis

4.1.3.1 Total phenolic content

The total phenolic content of the crude extract and sub-fractions were determined by a linear regression formula obtained from a standard curve and expressed as tannic acid equivalence per milligram (TAE/mg). Water had the highest phenolic content followed by *n*-butanol and chloroform sub-fractions respectively, whereas hexane had the lowest phenolic content followed by 35% water in methanol (fig. 4.6).
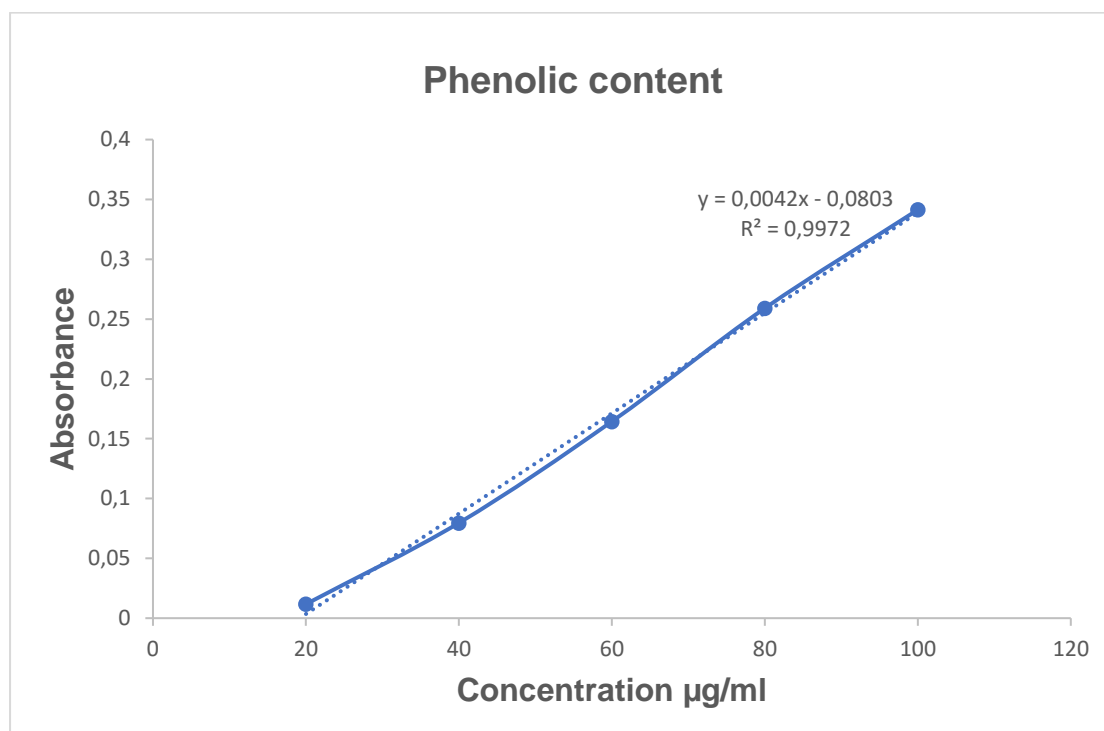
Figure 4.5: standard curve of phenolic content constructed using tannic acid.
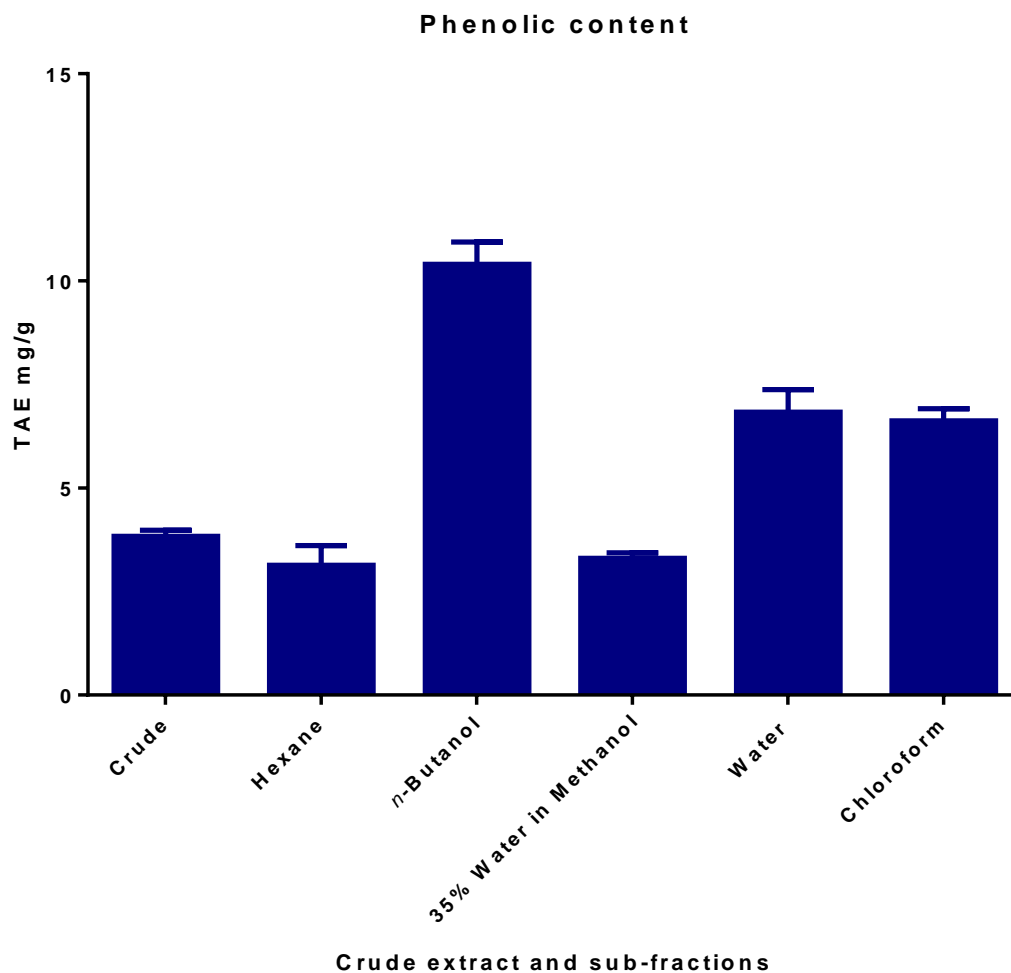
Phenolic content

Figure 4.6: Total phenolic content of *B. pilosa* crude extract and sub-fractions represented as tannic acid equivalents (TAE mg/g). The results are expressed as ± SD from duplicates of three independent experiments.

3.1.3.2 Total flavonoid content

The total flavonoid content of the crude extract and sub-fractions was determined by a linear regression formula obtained from a standard curve and expressed as quercetin equivalence per milligram (QE/mg). Chloroform had the highest flavonoid content, followed by *n*-butanol, whereas water had the lowest flavonoid content. This indicates that the presence and concentrations of various phytochemicals differ with each sub-fractions and solvent used (fig. 4.8). `
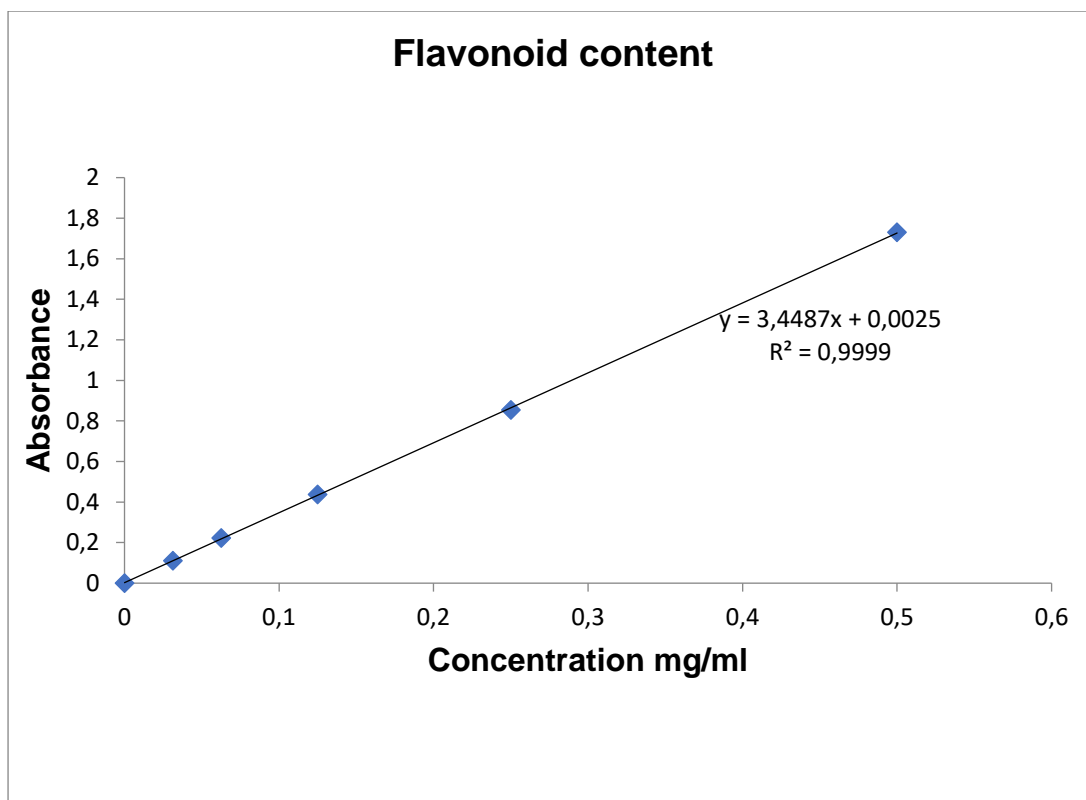
Figure 4.7: The quercetin standard curve used for quantification of flavonoids compounds
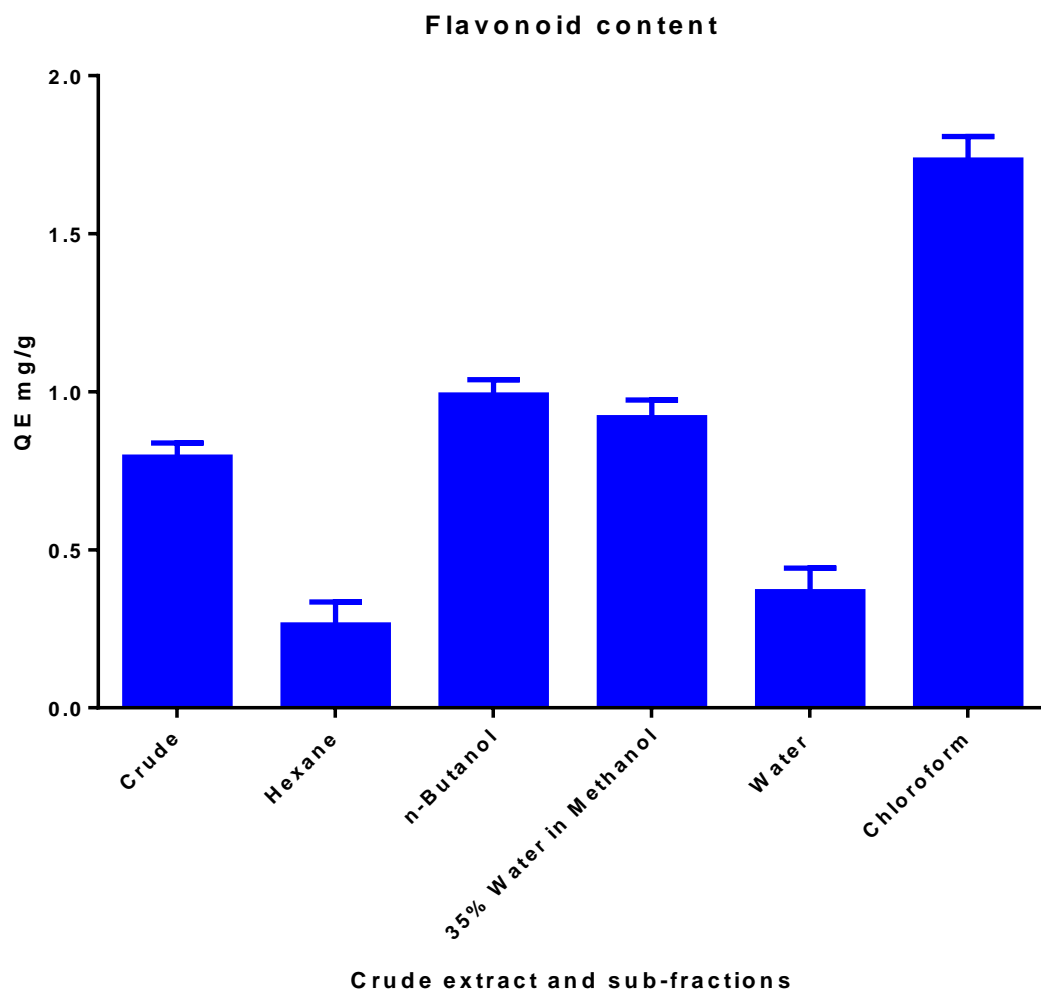
Figure 4.8: Total flavonoids content of *B. pilosa* crude extract and sub-fractions represented as quercetin equivalents (QE mg/g). The results are expressed as ± SD from duplicates of three independent experiments.

4.1.3.2 Quantitative antioxidant analysis

The DPPH radical scavenging activity assay measures the ability of compounds with antioxidant activity to turn DPPH from its unstable state, known as hydrazyl, to its stable state known as hydrazine. Therefore, in the event of antioxidant activity, DPPH will change from its original colour (purple) to different intensities of yellow according to different concentrations. This is based on the ability to donate a hydrogen atom, which stabilizes the DPPH free radical. The results show an increase in concentration leads to an increase in percentage scavenging activity of the *B. pilosa* crude extract and sub fraction. Chloroform sub-fraction had the highest percentage scavenging activity followed by water, *n*-butanol, methanol crude extract and 35% water in methanol sub-fractions respectively. Hexane sub-fraction showed poor percentage scavenging activity (figs. 4.9 & 4.10).
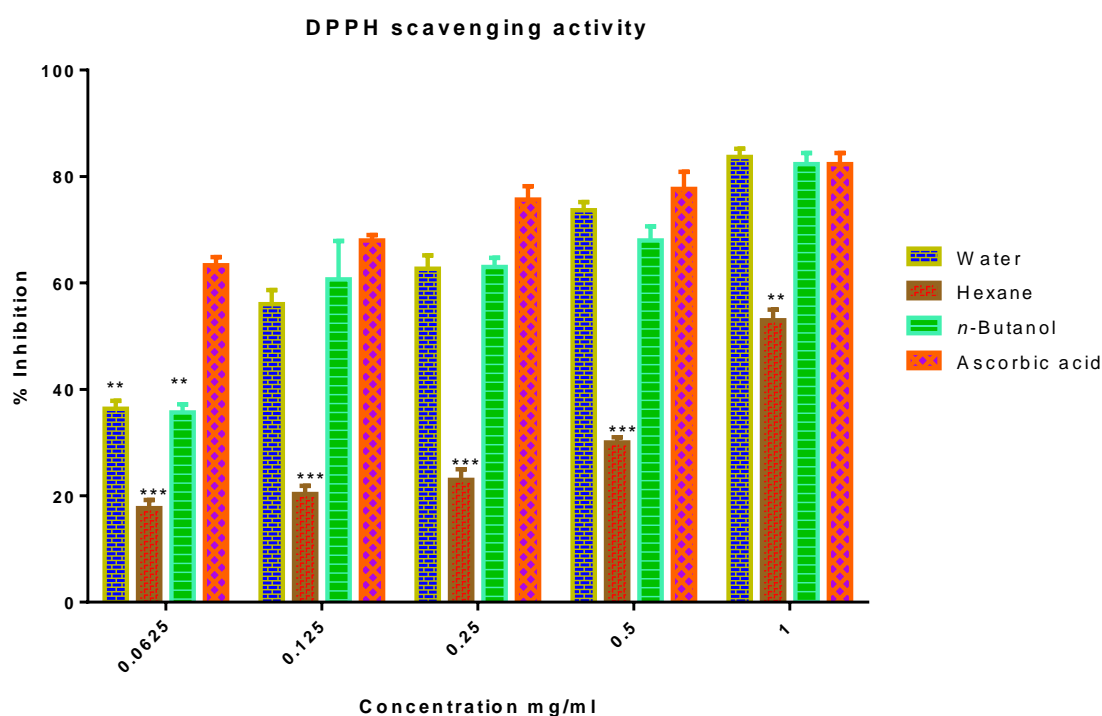

Figure 4.9: The percentage of the DPPH free radical scavenged by different concentration of *B. pilosa* crude extract and sub-fractions. Ascorbic acid was used at the positive control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

D P P H s c a v e n g i n g a c t i v i t y
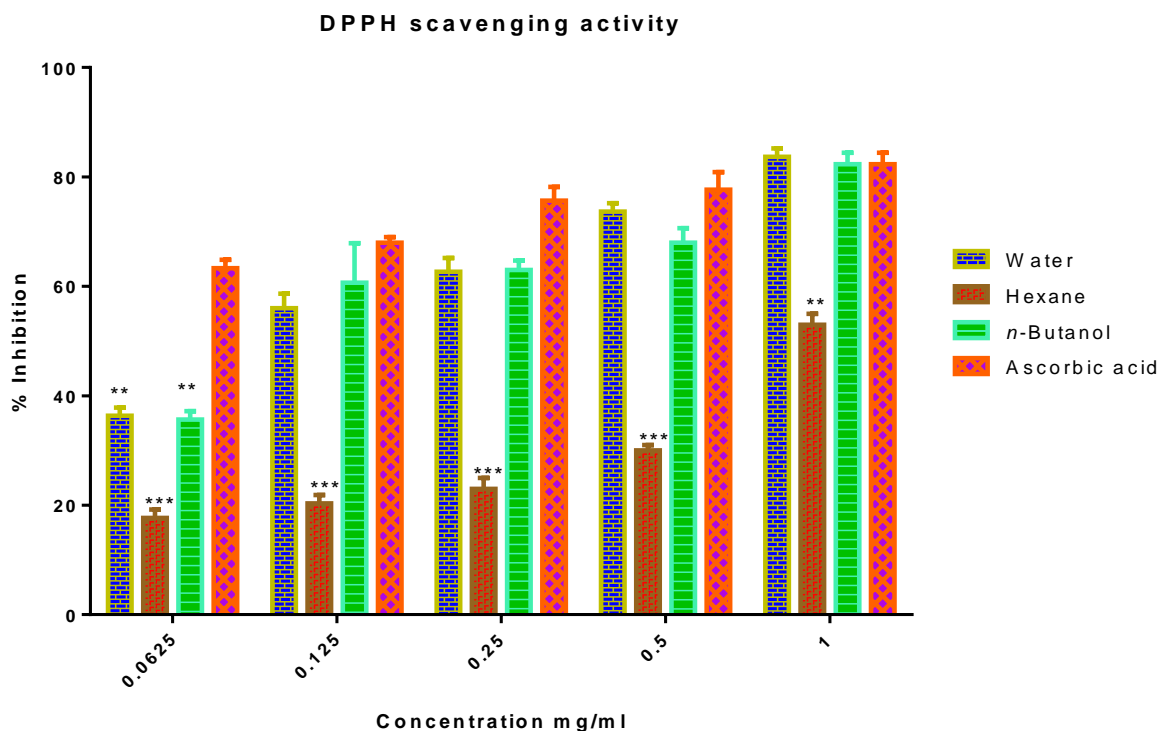
Figure 4.10: The percentage of the DPPH free radical scavenged by different concentration of *B. pilosa* sub-fractions. Ascorbic acid was used at the positive control. The data represent the mean $\pm$ SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

4.1.3.3 Ferric ion reducing power (FRP)

Ferric reducing power (FRP) measures the formation of Pearl's Prussian blue complex as a result of the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidant compounds. FRP assay is concentration dependent, an increase in concentration shows an increase absorbance. Formation of the blue colour, which is an indication of Pearl's Prussian blue complex, indicates the presence of antioxidant. Water sub-fraction is shown to have a higher ferric reducing power with an increase in concentrations, followed by crude extract, chloroform and *n*-butanol sub-fractions, whereas 35% water in methanol and hexane sub-fractions had the least ferric

reducing power. This correlates with the DPPH assay results, thus demonstrating that the mode of antioxidant activity of *B. pilosa* is different as they can donate both protons (DPPH assay) and electrons (FRP assay). Furthermore, these results demonstrate that crude extract and sub-fractions can have different strengths pertaining to various mechanisms used to prevent oxidative damage (fig. 4.11).
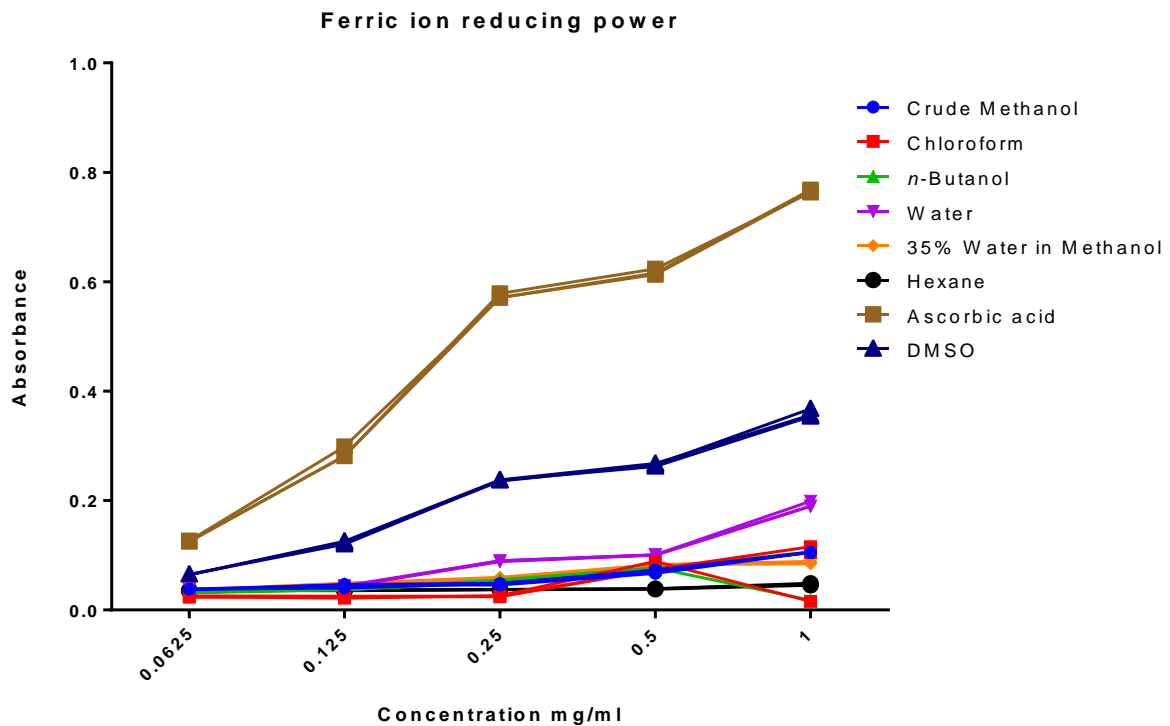
Figure 4.11: The absorbance ferric ion reduced by different by different concentrations of *B. pilosa* crude extract and sub-fractions. Ascorbic acid is used at the positive control. The data represent the mean ± SEM of three independent experiments

4.2 Antiglycation activity

Antiglycation activity of *B. pilosa* crude extract and sub-fractions was tested for the ability to inhibit the formation of glycated BSA on different concentrations. Arbutin was used as a positive control since it's a known inhibitor of glycation reaction. *Bidens pilosa* crude extract and sub-fractions resulted in a concentration dependant antiglycation activity, expressed as percentage inhibition of glycation. Arbutin had the highest antiglycation activity at all concentrations compared to the plant extracts. At lower concentrations, 35% water in methanol sub-fractions had higher anti-glycation activity. A concentration of 2.5 µg/ml crude extract showed a higher antiglycation activity followed by water and *n*-butanol subfractions, whereas hexane sub-fractions indicated a poor antiglycation activity at lower concentrations. Overall *B. pilosa* crude extract and sub-fractions were shown to have a good potential as glycation inhibitors (fig 4.12).
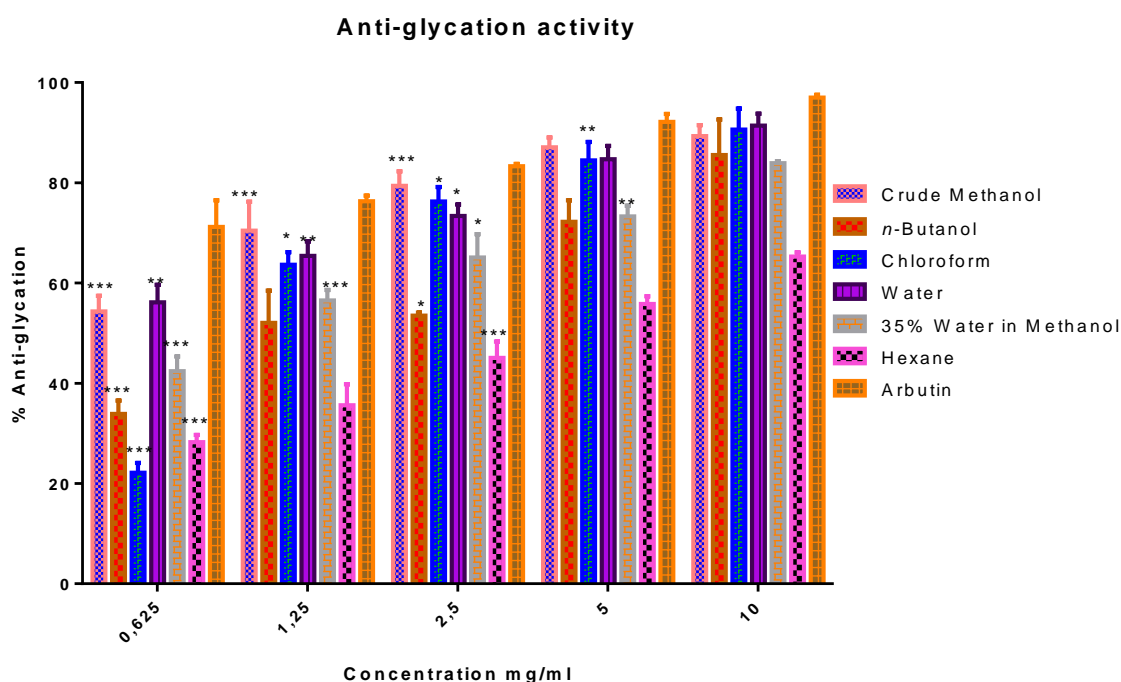
Figure 4.12: The percentage of antiglycation potential in different concentration of *B. pilosa* crude extract and sub-fractions. Arbutin was used as a positive control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered

significant at an sterisk(*) for p < 0.05, two asterisks(**) for p < 0.01 and three asterisks(***) for p < 0.001.

## 4.3 Partial α-amylase inhibitory activity

The ability of *B. pilosa* crude extract and sub-fractions, to partially inhibit the pancreatic α-amylase enzyme, was tested and the IC_{50} determined. An increase in concentration of the plant extract and sub-fractions resulted in an increase in the inhibition of pancreatic α-amylase. Acarbose was used as a positive control and resulted in higher percentage inhibition of the α-amylase enzyme compared to crude extract and sub-fractions. The order of the most active extracts was as follows: chloroform sub-fraction>water sub-fraction> n-butanol sub-fraction> crude extract. An IC_{50} of the crude extract and sub-fractions was found to be at a concentration of 0.125 mg/ml except for 35% water in methanol sub-fraction (fig 4.13).

α-amylase inhibition
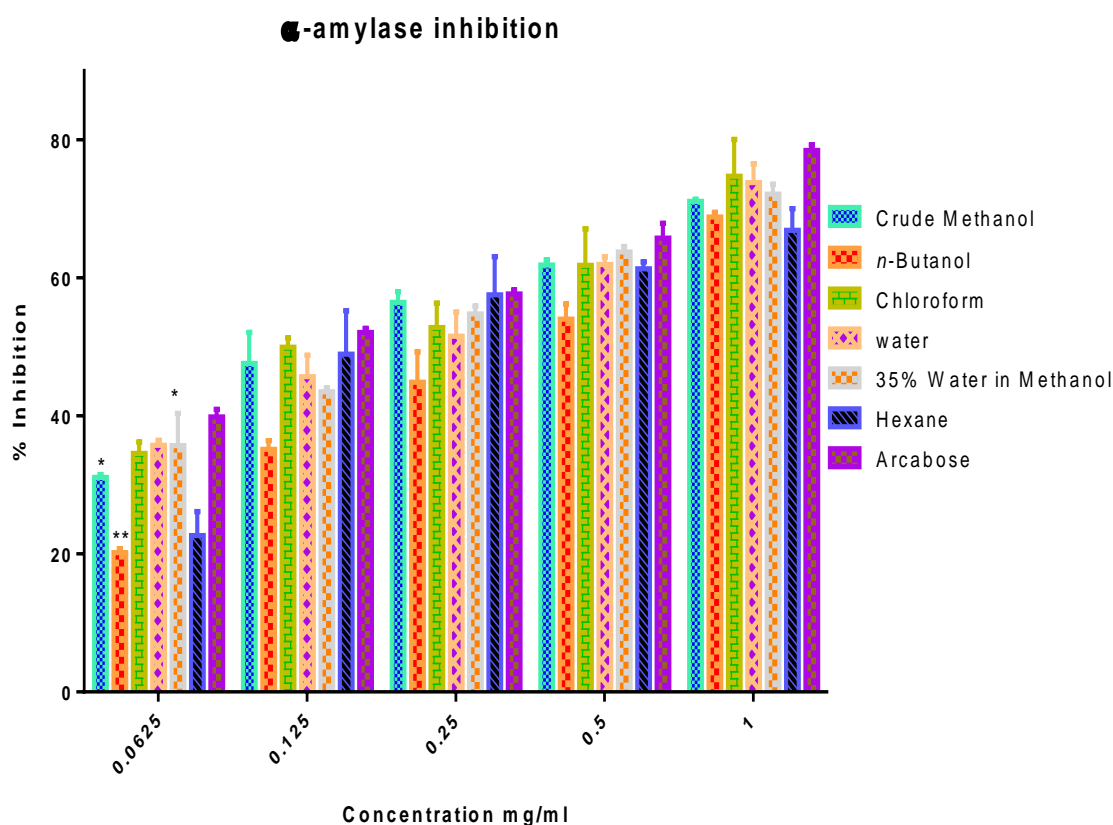
Figure 4.13: Percentage inhibition of α-amylase enzyme using different concentrations of *B. pilosa* crude extract and sub-fractions. Acarbose was used as

a positive control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

4.4 Cytotoxicity assay

It was observed that the viability of the 3T3-L1 pre-adipocytes and C2C12 myoblasts decreased in a concentration dependent manner. This indicates that when the highest concentration (500 µg/ml) of crude extract and sub-fractions was applied to cultures, the number of viable cells decreased. The results also show that the effective concentration of extracts was 15.63 µg/ml in both cell lines. The concentration of 15.63 µg/ml crude extract and sub-fractions resulted in maximum cell growth with approximately 80% of viability, after treatment for 24 hours, as indicated in Figures 4.14 - 4.17. The C2C12 cells were more sensitive to treatment with the n-hexane and 35% water in methanol sub-fractions as viability percentages of lower than 70% were observed. The 3T3-L1 cells, on the other hand, were more sensitive to the n-hexane sub-fraction as indicated by viability percentages of less than 60%. The 3T3-L1 cells seemed to be more resistant to the treatment compared to the C2C12 cells. Thus, the MTT assay showed that the cells well tolerated the extracts at concentration of 15.63 µg/ml. This maximum tolerated concentration was chosen for this study.
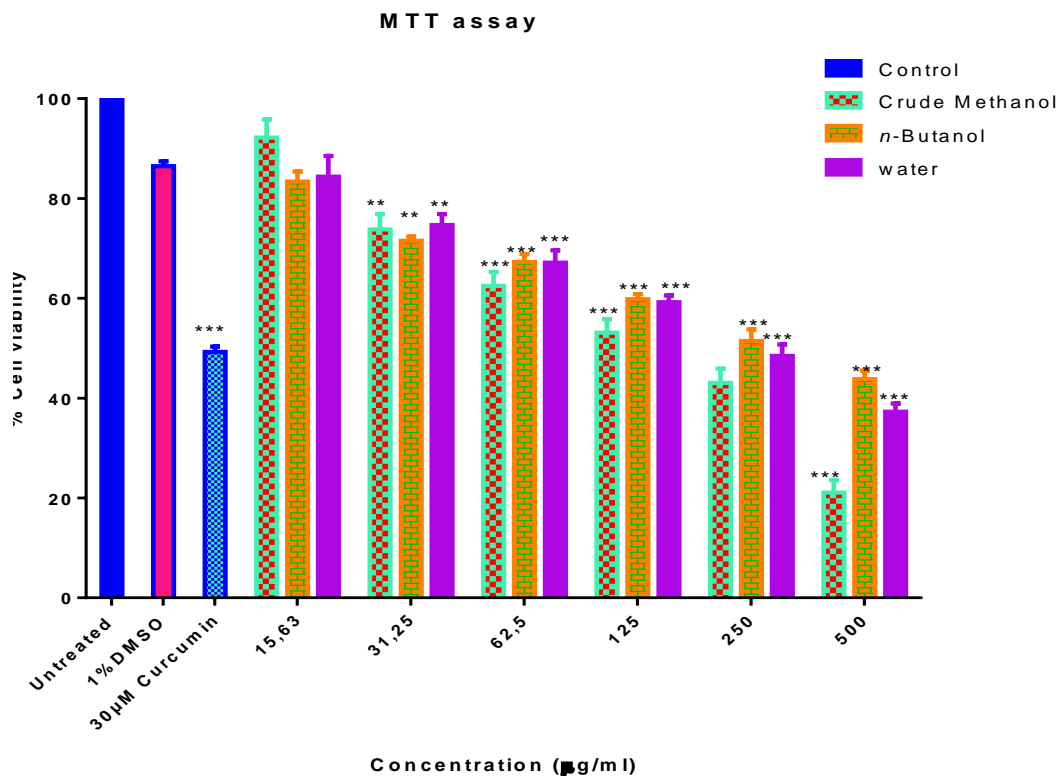
Figure 4.14: The cytotoxic effects of *B. pilosa* crude extract and sub-fractions (water and *n*-butanol) using different concentration on C2C12 muscle cells treated with different concentrations (15.63 µg/ml -500 µg/ml). The experiment was carried out for 24 hours using MTT assay. Untreated cells and Curcumin were used as experimental and positive controls respectively. DMSO was used as a negative control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
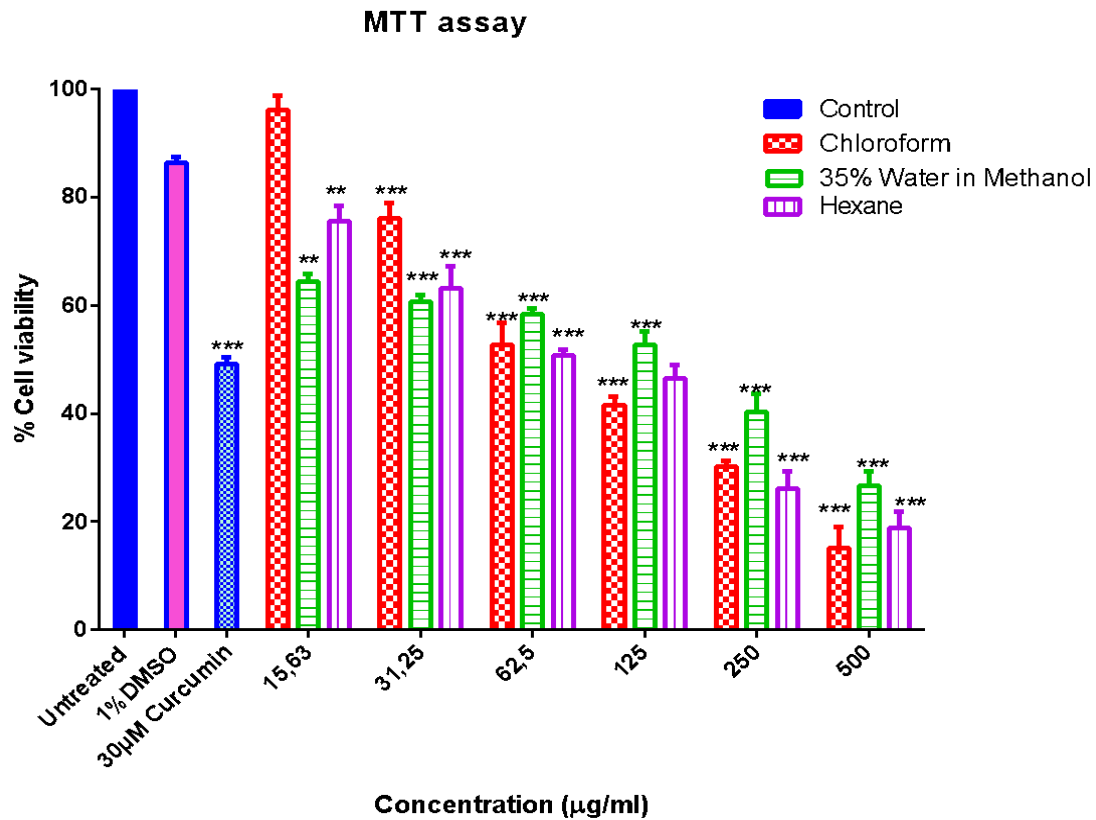
Figure 4.15: The effect of *B. pilosa* sub-fractions (Chloroform, 35% water in methanol and hexane) on C2C12 muscle cells treated at different concentrations (15.63µg/ml -500µg/ml). The experiment was carried out for 24 hours using MTT assay. Untreated cells and Curcumin were used as experimental and positive controls respectively. DMSO was used as a negative. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
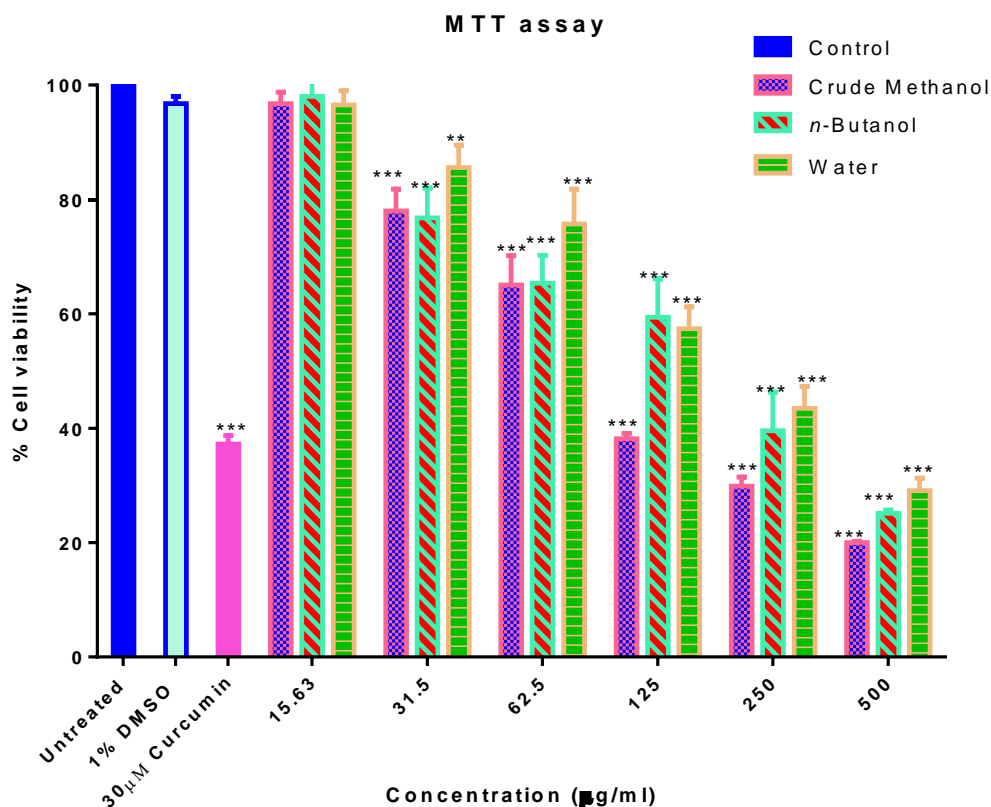
Figure 4.16: The effect of *B. pilosa* crude methanol and sub-fractions (*n*-butanol and water) on 3T3-L1 pre-adipocytes treated at different concentrations (15.63 µg/ml - 500 µg/ml). The experiment was carried out for 24 hours using MTT assay. Untreated cells and Curcumin were used as experimental and positive controls respectively. DMSO was used as a negative control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Figure 4.17: The effect of *B. pilosa* sub-fractions (Chloroform, 35% water in methanol and hexane) on 3T3-L1 pre-adipocytes treated at different concentrations (15.63 µg/ml -500 µg/ml). The experiment was carried out for 24 hours using MTT assay. Untreated cells and Curcumin were used as experimental and positive controls respectively. DMSO was used as a negative control. The data represent the mean ± SEM of three independent experiments. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Differentiation of C2C12 cells from murine myoblast cells to fibroblast cells

Cells have to go through a differentiation process in order for the glucose uptake to take place. The murine myoblasts were differentiated to fibroblasts, as demonstrated by the pictures in figure 4.18. The C2C12 cells were differentiated using decreased serum conditions (2% foetal bovine serum) for 6 days. Morphological changes were observed from day 0 to day 6, where murine myoblast cells were fully differentiated to fibroblast cells. At day 0, the cells were not differentiated and still fully attaching to the plate, appearing like multipolar like cells. At day 4, there was an observed morphological change. Cells displayed an elongated polynucleated characteristic. At day 6, the cells were fully confluent and had undergone differentiation. At this stage, cells appeared as contractile muscle-like as compared to their somewhat round shape un-differentiated appearance.
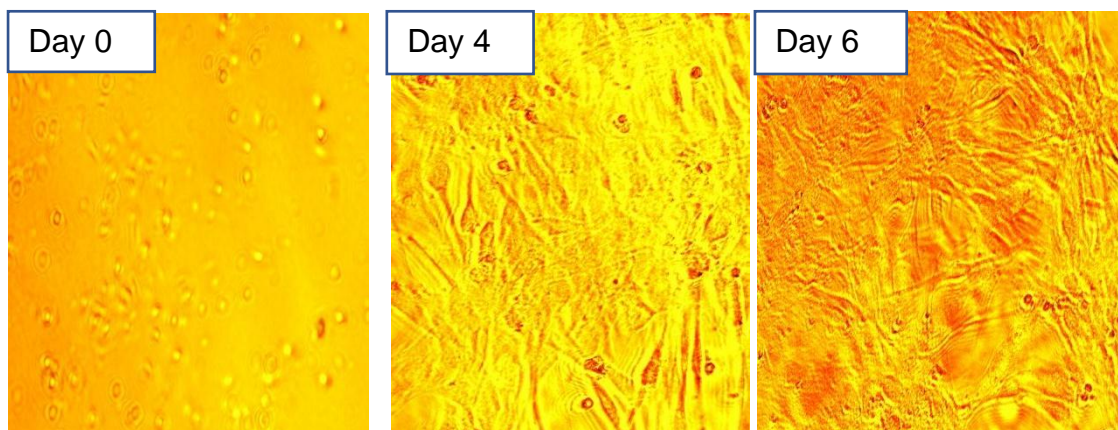
Figure 4.18: The changes in morphology as an indication of differentiation of murine myoblast cells to myo-tubules from day 0 to day 6 in differentiation media. The differentiation of C2C12 murine myoblasts to myo-tubules was characterised by an observed changes form round fusiform shape to elongated confluent tubular like shape. The images were captured using 40X magnification.

Differentiation of 3T3-L1 pre-adipocytes into 3T3-L1 adipocytes

The 3T3-L1 pre-adipocytes were differentiated as illustrated in the figure 4.19. Cells were differentiated using the adipocytes differentiation media containing 10% foetal bovine serum, 1 µM dexamethasone (DEX), 0,5 mM 3-isobutyl-1-methylxanthine and 1 µg/ml insulin. On day 0, the cells were not differentiated, as they have elongated fibroblast like morphology. There was an observed change in morphology on day 4, as a result of growing the cells in adipocyte differentiation media. The cells appeared more adipocyte-like in morphology. On day 8, cells had fully differentiated into adipocytes.
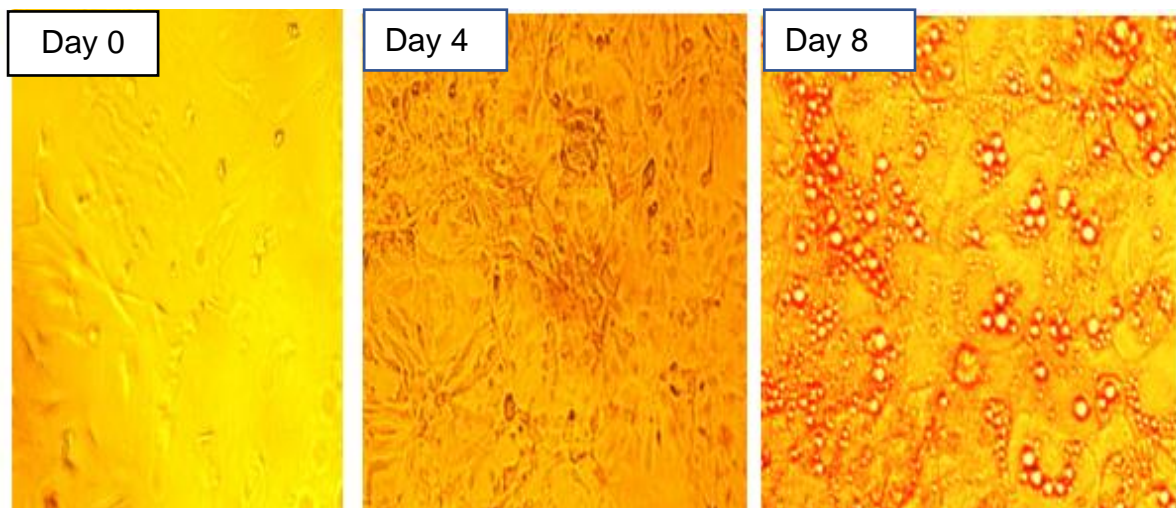
Figure 4.19: The transition of 3T3-L1 pre-adipocytes in to mature adipocytes from day 0 to day 8. Differentiation of 3T3-L1 is characterised by morphological changes from elongated fibroblast into adipocytes morphology. The cells were stained using Oil-red O to visualise the accumulated fats droplets using 40X magnification.

4.5 Glucose uptake assay

The ability of *B. pilosa* crude extract and sub-fractions to stimulate glucose uptake was determined by measuring the decrease in the concentration of glucose in the supernatant medium in the differentiated C2C12 and 3T3-L1 cells. Insulin and metformin were used as positive controls and in combination with the plant extracts. The cells were treated with plant extracts in the presence and absence of both insulin and metformin. The treatment was for 6 hours and experimental samples were taken at 3-hour intervals. The results obtained indicated that untreated cells and cells treated with DMSO resulted in no glucose uptake by both cell lines, whereas cells treated with insulin and metformin showed high amount of glucose utilisation. Treatment of cells with insulin and a combination of plant extracts led to a significant increase in the stimulation of glucose in the differentiated cells. However, cells treated with metformin in combination with plant extracts resulted in decrease in glucose taken up by the cells (figs. 4.20 - 4.27).
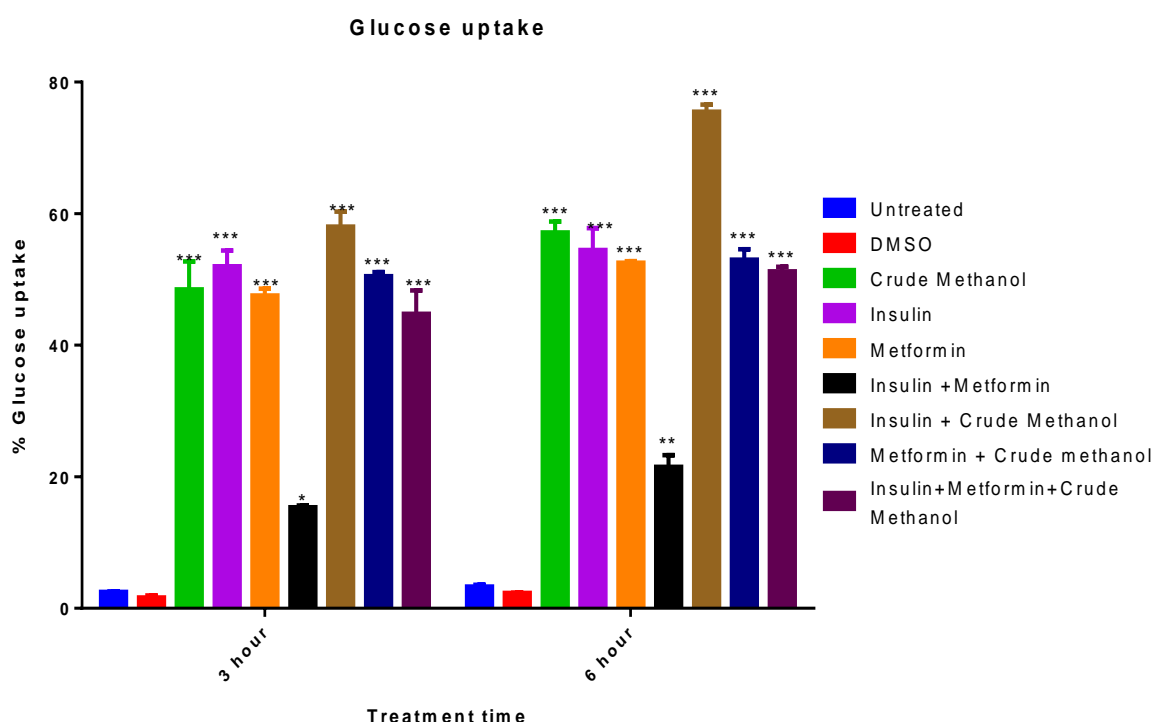
Figure 4.20: The effects of *B. pilosa* crude extract in the presence, absence and combination of insulin and metformin at a concentration of 15 µg/ml in stimulating glucose uptake in differentiated C2C12 cells. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and

metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
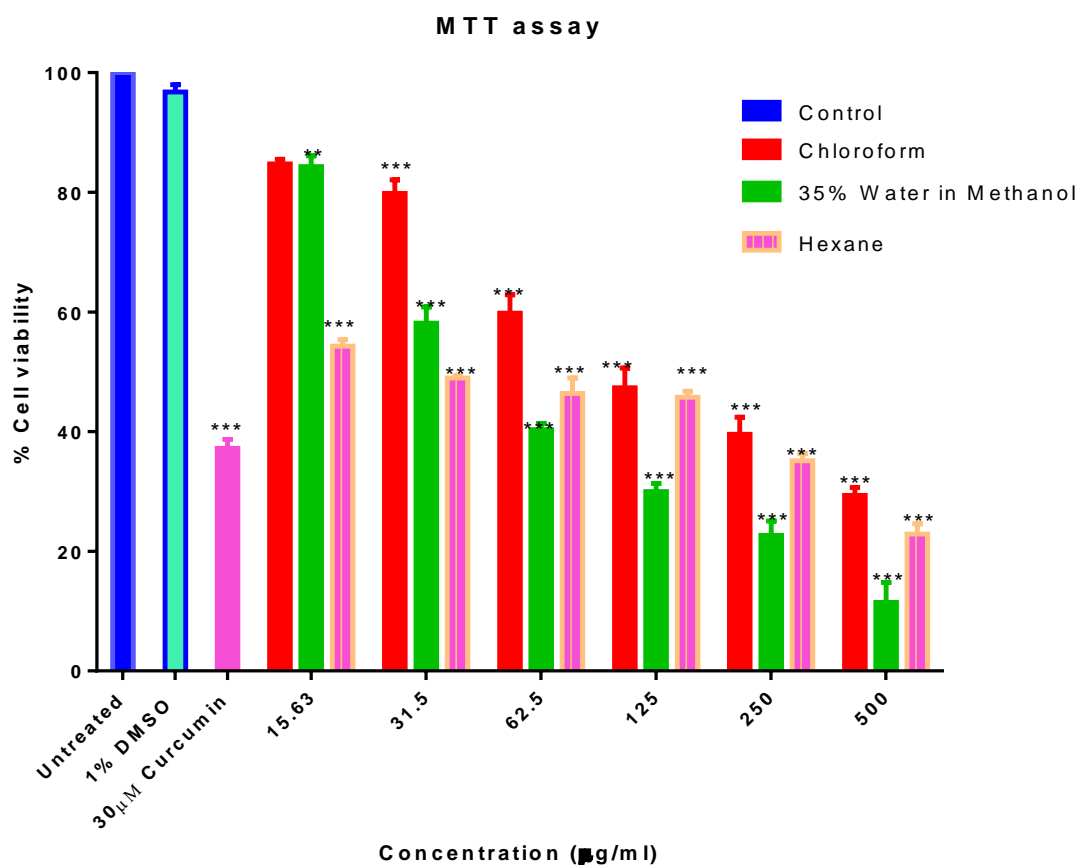

Figure 4.21: The effects of chloroform sub-fraction at a concentration of 15 µg/ml in stimulating glucose uptake in differentiated C2C12 cells at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Figure 4.22: The effects of *n*-butanol sub-fraction at a concentration of 15 µg/ml in stimulating glucose uptake in differentiated C2C12 cells at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Figure 4.23: The effects of water sub-fraction at a concentration of 15 µg/ml in stimulating glucose uptake in differentiated C2C12 cells at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Figure 4.24: The effects of crude methanol at a concentration of 15 µg/ml in stimulating glucose uptake in 3T3-L1 adipocytes at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.

Figure 4.25: The effects of chloroform sub-fraction at a concentration of 15 µg/ml in stimulating glucose uptake 3T3-L1 adipocytes at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
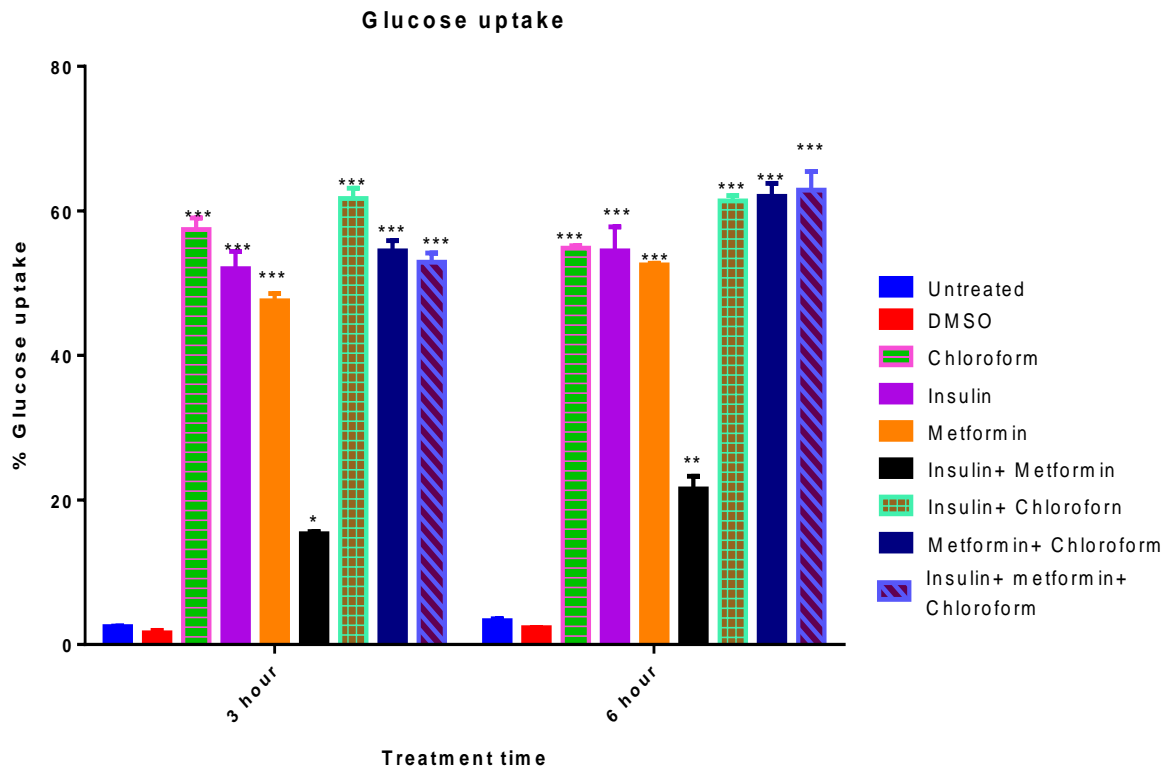
Figure 4.26: The effects of *n*-butanol sub-fraction at a concentration of 15 µg/ml in stimulating glucose uptake in differentiated 3T3-L1 adipocytes at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
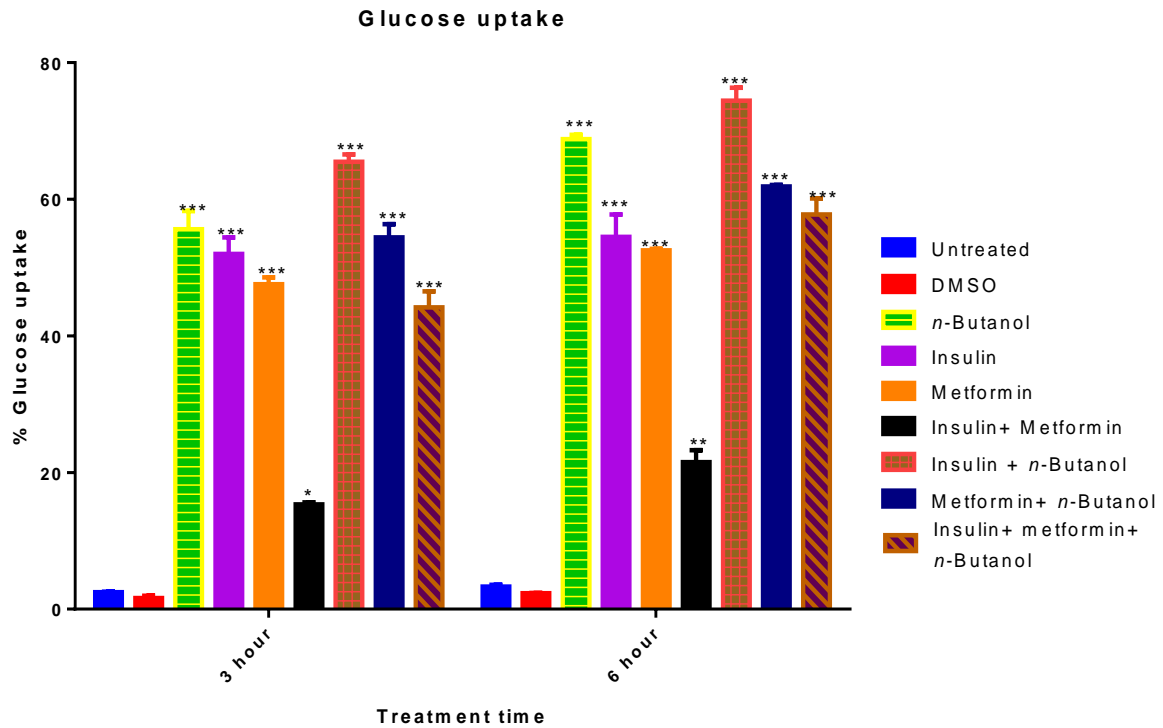
Figure 4.27: The effects of water sub-fraction at a concentration of 15 μg/ml in stimulating glucose uptake in 3T3-L1 adipocytes at 3 hours and 6 hours treatment. Untreated cells and cells treated with DMSO were used as experimental and negative controls respectively. Insulin and metformin were used as positive controls. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for $p < 0.05$, two asterisks(**) for $p < 0.01$ and three asterisks(***) for $p < 0.001$.
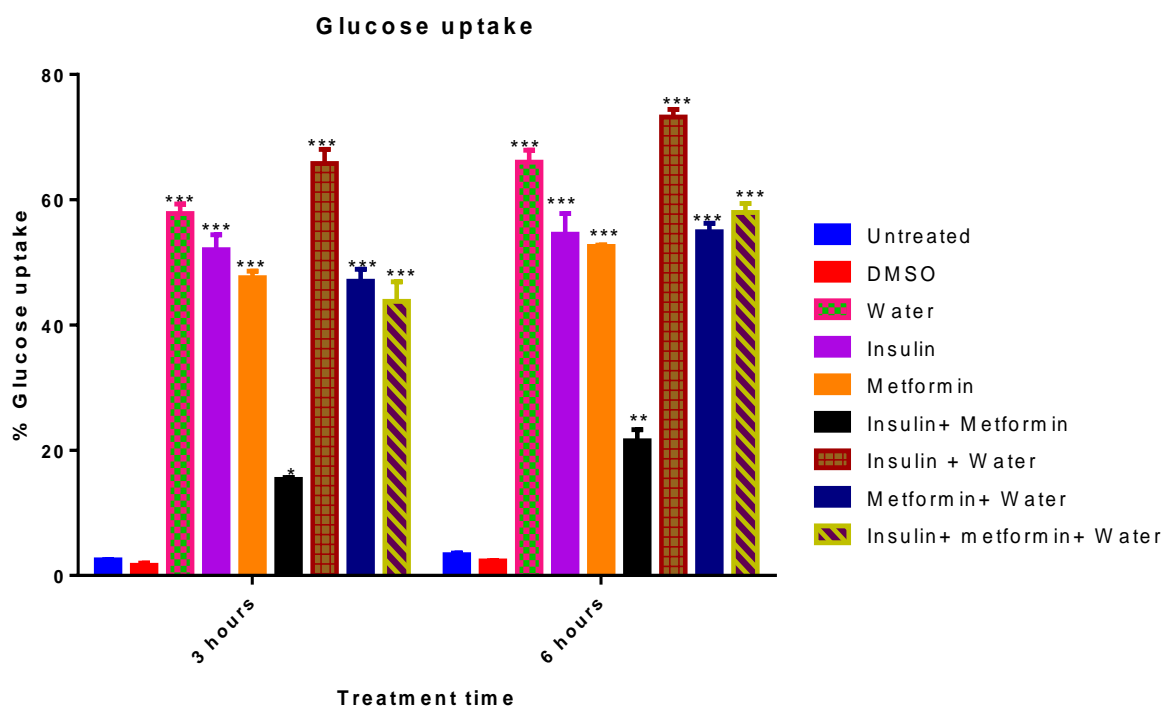
4.6 Adipogenesis assay.

The effect of *B. pilosa* on adipogenesis was determined using the adipogenesis kit on the differentiated 3T3-L1 adipocyte. Non cytotoxic concentrations were selected for treatment of cells for 48 hours. Untreated cells were used as an experimental control whereas adipocyte differentiation medium (ADM) served as a negative control. An increase in the concentration of the plant extracts resulted in an increase in the accumulation of triglycerides but not higher than ADM.
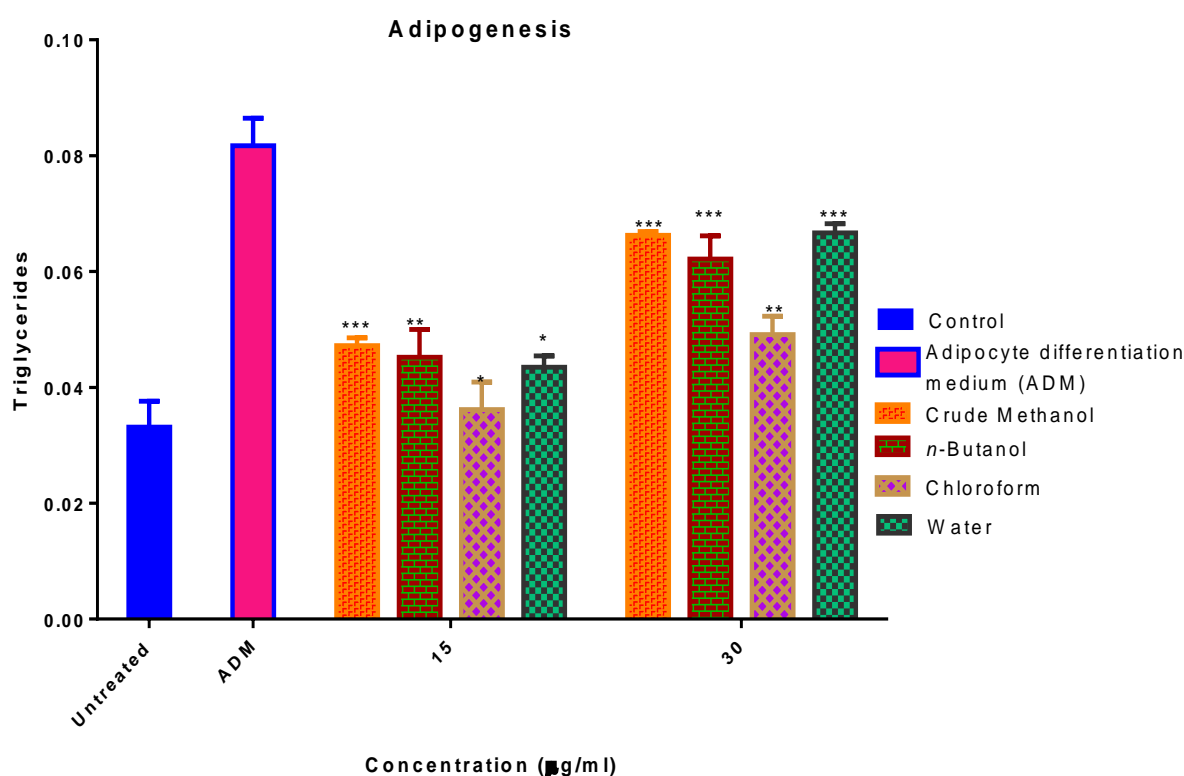
Figure 4.28: The effects of *B. pilosa* crude methanol extract and sub-fractions (chloroform, water and *n*-butanol) on adipogenesis using 3T3-L1 adipocytes. Cells were treated with non-cytotoxic concentrations of the extracts. Adipocyte differentiation medium was used as a positive control. Untreated cells were used as experimental control. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Turkey-Kramer Multiple Comparisons Tests. The results were considered significant at an sterisk(*) for p < 0.05, two asterisks(**) for p < 0.01 and three asterisks(***) for p < 0.001.
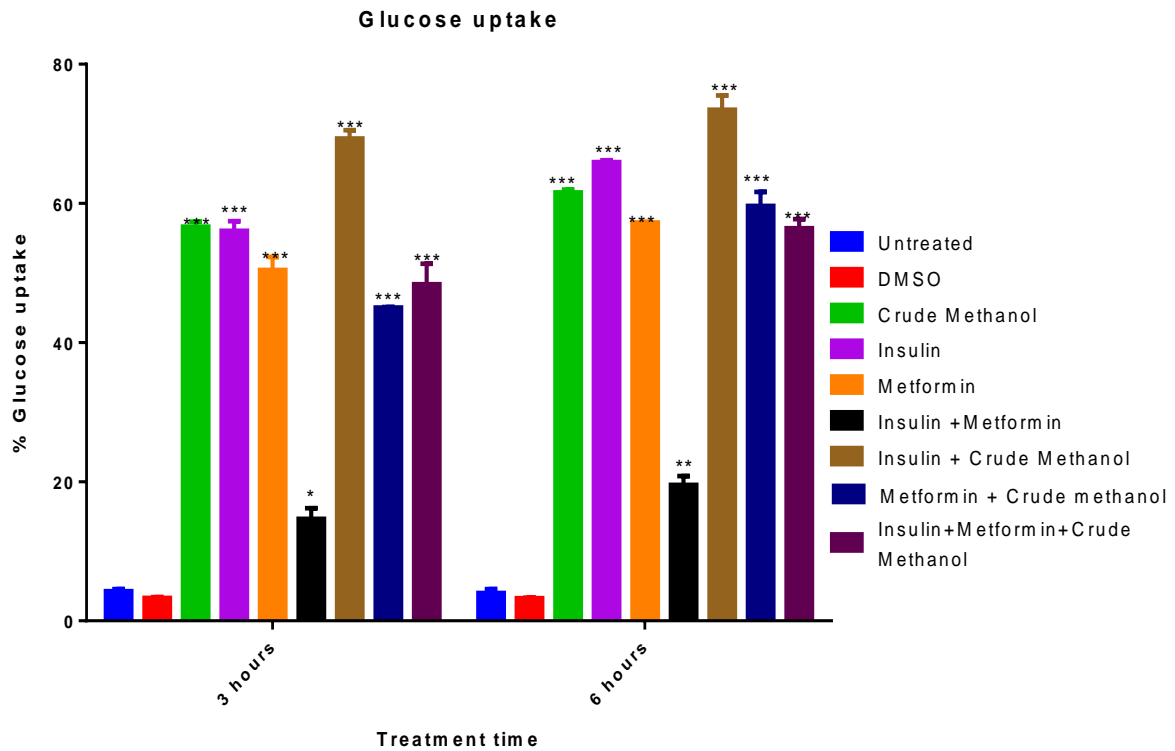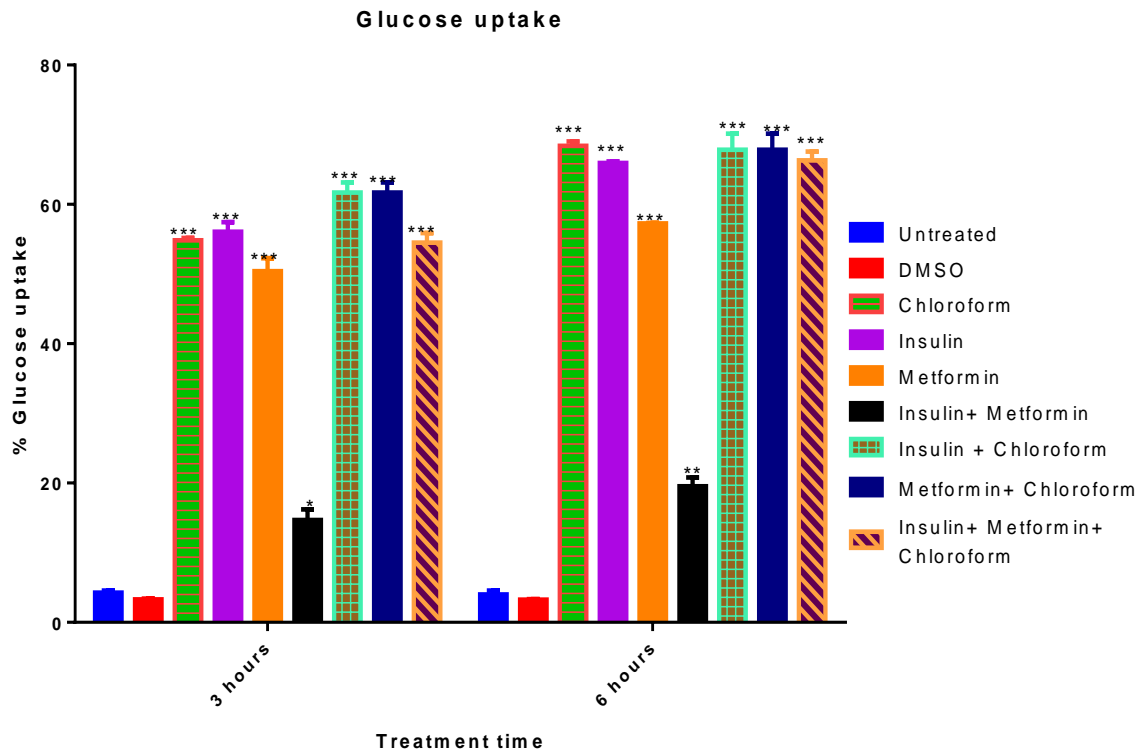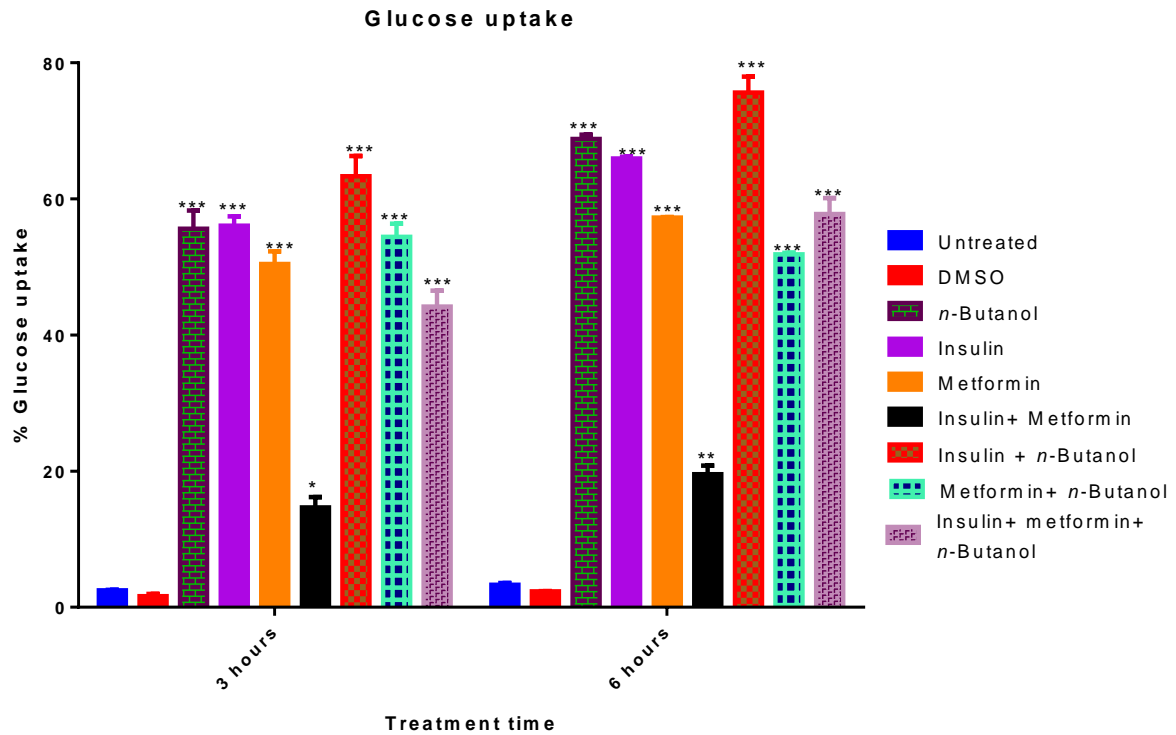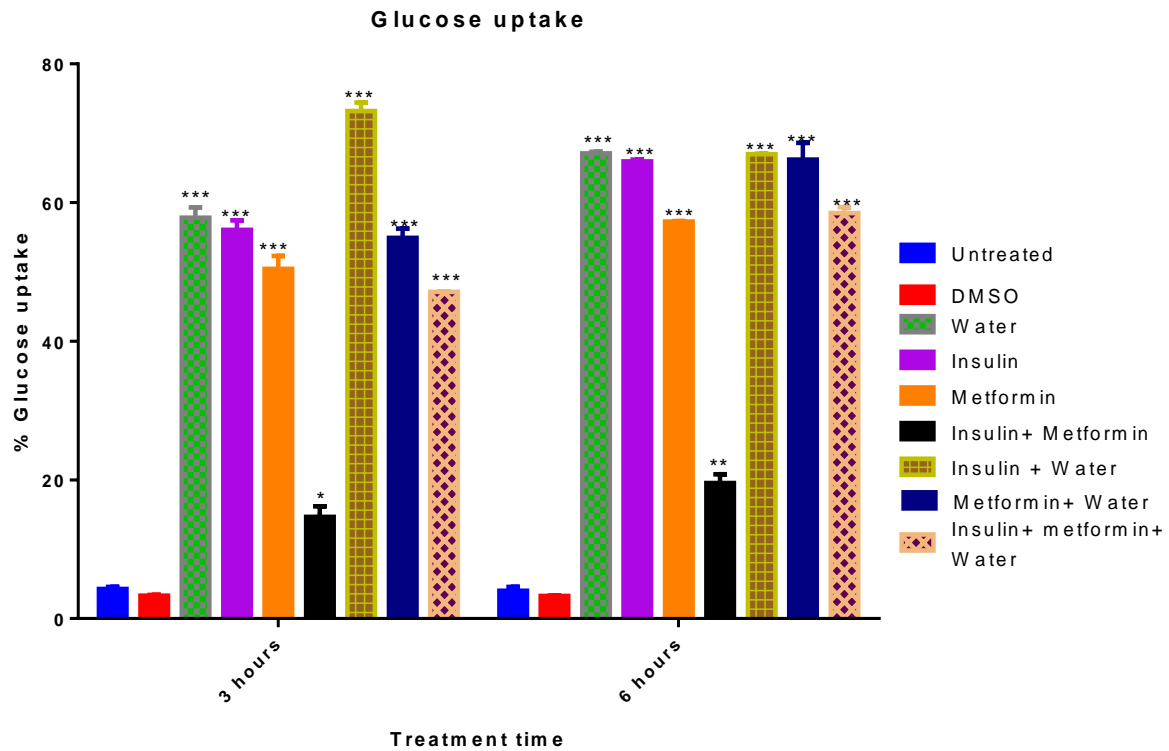
Chapter 5: Discussion and conclusion

Plants have been used all over the world for therapeutic reasons. All parts of the plants are used as an ingredient in folk medicine by traditional healers all over the world. Different plant parts of different species have different pharmacological activities such as antidiabetic, anti-cancerous, antioxidant, antimicrobial, etc. Plant based drugs play an important role in the primary health care system (Cos *et al.*, 2006). Plants used as traditional medicine constitute a wide variety of phytochemicals that are responsible for the treatment of various diseases. Medicinal plants also provide an opportunity in the discovery of new drugs due to their chemical diversity. Preparation of plant samples and choice of solvents is the first and most important step in analysing the plants. It is of major significance to extract the desired chemical compounds from the herbal materials for further separation and analysis for their mode of action (Poole and Poole, 1996). The characteristic nature of phytoconstituents has provided an opportunity for specific compounds of interest to be extracted. Different solvents of varying polarities are employed for extraction of plant derived phytochemicals. Analysis of medicinal plants play a major part in the overall effort of ensuring and providing high quality herbal products, and chemical compounds which are responsible for regulation of certain metabolic pathways involved in diseases (Majors, 1996).

For this current study, *B. pilosa* leaves were extracted using absolute methanol and then subjected to solvent-solvent fractionation to yield the chloroform, water, *n*-butanol, hexane, 35% water in methanol and ethyl acetate sub-fractions. The choice of the solvent-solvent fractionation procedure was influenced by the desire to allow compounds to be separated according to their polarities. Furthermore, this assists to attain therapeutically desired phyto-compounds and to compare and choose the best sub-fraction, which will result in the most active anti-diabetic properties and can equally induce adipogenesis. Water sub-fraction resulted in the highest percentage yield of 19.48%, whereas ethyl acetate sub-fraction resulted in a very low percentage yield of 1.3%. High percentage yield of water sub-fraction results from its highly selective nature for polar compounds, indicating that the constituents of the plant are

mostly polar compounds. Water is the most used extraction solvent traditionally because of its availability and non-cytotoxic effects (Parekh *et al.*, 2006).

Methanolic crude extract and its sub-fractions were separated using TLC plate of different mobile phases. Thin layer chromatography was used to verify the identity of plant extracts by determining the chemical fingerprint of the extracts. The migration of the phytoconstituents depends on their solubility in the mobile phase. Generally, the EMW separation system exhibited better separation of compounds compared to the TEA and CEF systems after visualization. This indicates that the plant extract mostly constitutes polar and intermediate compounds. In addition, a larger number of compounds were observed viz. selected order of increasing chloroform>35% water in methanol >*n*-butanol sub-fractions. The TLC separated phytochemicals were visualised under ultraviolet (UV) light of 365 nm wavelength. Under this spectrum, electrons in compounds undergo transition from a ground state to an excited state. When the electrons are de-excited to the ground state, they release energy in the form of an electromagnetic radiation wavelength of a lower energy than the UV light that ranges within the visible spectrum. Structurally diverse compounds fluoresce and are visualised in different colours. In this study, the TLC plates indicated different compounds fluorescing, hence a variety of compounds were successfully separated by solvent-solvent fractionation. The fluorescence indicated that the compounds consisted of conjugated double bonds and/or extended pi (π) electron configurations (Ahmed *et al.*, 2014). Polyphenols have been reported to have lengthy conjugated aromatic systems in their chemical structures (Dai and Mumper, 2010). Therefore, the various fluorescing compounds in Figure 4.2 may be due to the presence of polyphenolic compounds or their analogues. The vanillin-sulphuric acid spray was used to establish the phytochemical profiles of compounds that were unable to fluoresce under the UV light. The TLC plates were further sprayed with p-Anisaldehyde to visualise compounds present in the plant extract, such as phenols, sugars, steroids and terpenes. Vanillin sulphuric acid reagent reacted with many organic compounds to give the compounds coloured bands characteristics. Major chemical classes detected were terpenoids (purple or bluish-purple bands), flavonoids (pinkish, yellow or orange) and proanthocyanidins (pink) (Ahmed *et al.*, 2014; Taganna *et al.*, 2011; Dai and Mumper, 2010). Therefore,

qualitative phytochemical analysis was performed using different standard chemical tests in order to identify the type of secondary metabolites in the crude extract and sub-fractions. The crude extract tested positive for the presence of secondary metabolites (phenols, flavonoids, saponins, steroids, tannins and glycosides). Similarly, these compounds were found to be present in chloroform fraction, except for terpene. There was a variation in the secondary metabolites in the other sub-fractions. The findings on the presence of secondary metabolites present in *B. pilosa* correlates with other previous studies (Lima Silva *et al.*, 2011; Chen *et al.*, 1975). Phenolic and flavonoid compounds are a group of secondary metabolites associated with anti-diabetic and anti-obesity properties (Ghasemzadeh and Ghasemzadeh 2011). Therefore, identification of medicinal plants which are rich in the compounds could help in the regulation of pathways involved in diabetes and obesity. The results obtained indicate that *n*-butanol extract has the highest phenolic content (10.4 TAE mg/g), followed by water, chloroform and crude extracts, respectively. Hexane and 35% water in methanol extracts resulted in the lowest amount of phenolic content (3.13 and 3.29 TAE mg/g). There was a slight variation in flavonoid content, where chloroform extract had the highest flavonoid content of 1.73 QE mg/g, followed by *n*-butanol and 35% water in methanol and crude extracts, respectively. These findings on phenolic and flavonoids content of *B. pilosa* correlates with the previous studies (Bartolome *et al.*, 2013; Dimo *et al.*, 2001).

Diabetes mellitus and obesity are highly related with other metabolic disorders. Oxidative stress is one of the major leading causes of these metabolic disorders. Biochemical studies have revealed an increase in the generation of ROS in the cells and tissues of diabetic patients (Inoguchi *et al.*, 2003). Hence, the consumption of potent antioxidants in the body of a patient is necessary in order to tackle the ROS (Sen *et al.*, 2010). In this study, the antioxidant activity of the plant was determined qualitatively by spraying the TLC plates with 0.2% DPPH in methanol. The antioxidant compounds appear as yellow bands on the purple background of TLC plates. The presence of the yellow bands is based on the principle that the compound in the plant will donate hydrogen ions or electrons to the DPPH radical, thus the reduction of the purple hydrazyl to its yellow hydrazine state. The migration of the bands is based on the polarity of the compounds. Polar compounds migrate to the top of the plate, followed by intermediate compounds which are mostly found in the middle of the plates, the least being polar compounds. The number of bands and intensity of the yellow bands indicate the quantity of the antioxidant present in plants (Sharma and Bhat, 2009). The CEF mobile phase best resolved compounds with antioxidant activity, followed by EMW, and fewer compounds with antioxidant activity were observed on TEA. *n*-butanol resulted in a lot of yellow intense bands followed by 35% water in methanol, chloroform and crude. No antioxidant compounds were observed in the hexane sub-fractions in all the mobile phases, which indicates that this sub-fraction does not have any compounds with antioxidant activity. The stability of the purple hydrazyl to the yellow hydrazine can be quantified spectrophotometrically by measuring a decrease in absorbance of the plant according to the different concentrations (Sreelatha and Padma, 2009). The free radical scavenging activity was observed to be in a concentration dependent manner. A concentration of 1 mg/ml resulted in the best antioxidant activity of the crude extract and sub-fractions. Chloroform had the highest antioxidant activity followed by crude, *n*-butanol, water and 35% water in methanol sub-fractions. Whereas, hexane sub-fraction resulted in the least antioxidant activity. An EC_{50} value of chloroform, water and *n*-butanol sub-fractions were found at 0.125 mg/ml, while an EC_{50} value of crude extract, hexane and 35% water in methanol sub-fractions was observed at 0.25 mg/ml. At the highest concentration of 1 mg/ml crude extract and chloroform sub-fraction resulted in a higher percentage inhibition of free radical scavenging activity as compared to ascorbic acid.

Furthermore, the electron donating potential, that reduces the ferric ion state to its ferrous ion state, is used to measure the quantity of the antioxidant present in plants. Ferric reducing power (FRP) measures the formation of Pearl's Prussian blue complex as a result of the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidant compounds. The ferric reducing power was observed to be in a concentration and polarity dependent manner. DMSO was used as a negative control however the results obtained indicate that there was a reduction of Fe^{3+} to Fe^{2+} on DMSO, study has shown that polar solvents are capable of hydrogen bonding (Foti, 2007). It has been reported that DMSO is known for its effectiveness in the ability to suppress radiation induced transformation in vitro. Furthermore, DMSO is known to be extremely effective OH (Kennedy *et al.*, 1987). Therefore, this could be the reason why DMSO shows ferric reducing power potency. A concentration of 1 mg/ml resulted in the highest ferric reducing power in the controls, crude extract and sub-fractions. Ascorbic acid is known to be a good antioxidant and has resulted in the highest absorbance. Water sub-fraction results showed the highest reducing ability power at 1mgl at the compared to other sub-fractions indicating its ability to reduce the ferric ion to its ferrous form, followed by *n*-butanol, chloroform and crude, whereas hexane sub-fraction consistently resulted in no antioxidant activity. Phenolic compounds are known for contributing to the ability of the plant extracts to reduce the ferric ion to its ferrous form (Periera et al., 2009). This correlates with results obtained on total phenolic compounds, where it was indicated the water, *n*-Butanol, chloroform has high phenolic compounds and Hexane and 35% water in methanol showed lower phenolic compound.

The most active extract and/ or sub- fractions, which resulted in the paramount antioxidant activity, were found to be chloroform, *n*-butanol, crude extracts and water sub-fractions. The DPPH scavenging activity data indicated that chloroform sub-fraction and crude extract results showed the most active antioxidant activity. Quantification of antioxidant activity in crude extract and sub-fractions resulted in high antioxidant activity, as compared to the yellow bands observed on TLC plates sprayed with DPPH. This is due to the different strains and mechanisms the assays use pattering antioxidant activity. On TLC plates, compounds separate according to

their polarities in varying mobile phases, whereas DPPH scavenging activity quantifies all the antioxidant compounds found in the crude extract and subfractions. This also results in synergistic effects of the compounds which is indicated by the high antioxidant compounds.

However, assessment of the antioxidant potential of the sub-fractions and crude extract, using the ferric reducing power method, showed that ascorbic acid had higher antioxidant activity than the crude extract and all the sub-fractions. The reason for this could be attributed to the fact that plant extracts contain a lot of different compounds, with some having antagonist effects toward the mode of antioxidant activity, whereas ascorbic acid is a pure compound (Pereira *et al.*, 2009). Nonetheless, the antioxidant activity observed of *B. pilosa* is consistent with previous studies (Chiang *et al.*, 2004; Muchuweti *et al.*, 2007) where the findings in this study indicated that *B. pilosa* possesses high antioxidant activity through DPPH free radical scavenging activity.

The antioxidant activity observed in the DPPH and FRAP assays indicates that plant extracts have different mechanisms through which they exert their antioxidant activity; they can donate both protons (DPPH assay) and electrons (FRAP assay). Furthermore, these results demonstrate that different plant extracts can have different strengths depending on the various mechanisms it uses to prevent oxidative damage. The combined effect of the different mechanisms may have the effect of amplifying the therapeutic effect of the crude extract and sub-fractions. Therefore, identification and isolation of compounds, which are responsible for different modes of action pertaining to antioxidant activity, should be considered to better understand the chemistry and nature of the compounds. This might help in identifying the mechanism by which the plant regulates certain metabolic pathways in relation to discovery of anti-diabetic and anti-obesity therapeutic drugs (Klein *et al.*, 2007).

An increase in glucose in the bloodstream is a hallmark factor in diabetic patients. Glycation is regarded as a key molecular feature of diabetes complications since it results in a non-enzymatic reaction which occurs mainly between proteins and reducing sugar and might lead to microvascular and macrovascular diseases (Wijetunge and Perera, 2014). Hence, the anti-glycation activity of *B. pilosa* crude extract and sub-fractions was performed using the bovine serum albumin assay. Arbutin was used as a positive control since it is an inhibitor of glycation reaction. The results obtained indicated that an increase in the concentration of the plant extracts resulted in a significant increase in the anti-glycation activity, which was however not higher than that of arbutin. A concentration of 10mg/ml resulted in the best inhibitory potency in the crude extract and all the sub-fractions. At the highest concentration of 10mg/ml the water sub-fraction resulted in 91% anti-glycation activity, surpassing all the other sub-fractions, followed by chloroform sub-fraction with 90% crude extract with 88% and *n*-butanol with 76% anti-glycation activity. The hexane and 35% water in methanol sub-fractions resulted in the least anti-glycation activity of 65% and 78% respectively at 10mg/ml which. This in general indicate that *B. pilosa* crude extract and sub-fractions have anti-glycation inhibitory potential with an increase in the concentration. The presence of secondary metabolites and high phenolic and flavonoid content could be responsible for the high anti-glycation activity of the crude extract and sub fractions. Studies have revealed that the amount of secondary

metabolites present in plant extracts plays a big role in regulations of metabolic pathways associated with anti-diabetic properties. High phenolic and flavonoid content possess high antioxidant and anti-glycation activity (Ramkissoon *et al.*, 2013). Medicinal plants with anti-glycation activity could reasonably serve as adjuvants for promoting the health of diabetics. It has been reported that anti-glycation compounds might act by preventing the production of AGEs through inhibition of oxidation of Amadori products (Beseni *et al.*, 2019).

Carbohydrate digesting enzymes play a major role in the post-prandial blood glucose levels. Alpha amylase enzyme catalyses the hydrolysis of carbohydrates into disaccharides and oligosaccharides. Alpha glucosidase further breaks down the disaccharides and oligosaccharides into absorbable glucose molecules, leading to increased glucose in the bloodstream (Picot *et al.*, 2014), which serves as a problem to diabetic patients. Partial inhibition of carbohydrate digesting enzymes can help in managing the amount of glucose entering the bloodstream and can then help in lowering the post prandial blood glucose level. Hence, the ability of the plant extracts to partially inhibit the alpha amylase was determined calorimetrically (Olaokun *et al.*, 2003). Acarbose, a positive control, is a standard drug used in diabetic patients to reduce high blood glucose. It works by delaying digestion of ingested carbohydrates, thereby resulting in a lower rise in blood glucose concentration following meals. *Bidens pilosa* crude extract and sub-fractions showed significant inhibitory effect on α- amylase enzyme at different concentrations, with 35% water in methanol sub-fraction inhibiting the enzyme by 63%. The *n*-butanol sub-fraction was the least active at 54%. Plant extracts with high polyphenols and antioxidant properties are known to have an inhibitory potential on carbohydrate digesting enzymes (Kim *et al.*, 2000). Partial inhibition by water and chloroform sub-fractions was found at an IC_{50} of 0.25 mg/ml and 0.5 mg/ml, respectively. The results suggest that *B. pilosa* extracts can be used to partially inhibit the carbohydrate digesting enzymes. The observed effect can thus help in the regulation of the amount of glucose in the bloodstream, the phenomenon of hypoglycemic effects. Medicinal plants with hypoglycemic effects can serve as an adjuvant for management of diabetes mellitus.

Plant derived compounds are not yet known, identified or fully understood by their mechanisms of action or how they alter metabolic pathways in searching for drug

discoveries. Safety concerns of medicinal plants are very important in pharmacological studies. Some compounds might be toxic for human consumption and might lead to serious complications in primary health care. Hence, plant extracts must be evaluated for their toxic effects and characterisation of test substances (Arome and Chinedu, 2013). In this study, the toxicity effects of *Biden pilosa* crude extract and sub fractions were evaluated using MTT assay on C2C12 muscle cells and 3T3-L1 adipocytes. Curcumin was used as an internal control to measure the success of the assay. The cytotoxicity profiles of *Biden pilosa* crude extract and sub-fractions were observed to be in a dose dependent manner. The water and chloroform sub fraction had lower noncytotoxic effects for both cell lines at different concentrations. The findings on these nontoxicity profiles show that different cell lines respond differently to the toxic effects of the substance. The most active extract / sub-fractions, which possess anti-diabetic properties with less toxicity effects, were selected for further analysis which are chloroform, water, *n*-butanol and crude. Traditionally, the use of *B. pilosa* as an ingredient in teas and herbal medicine has been reported to be safe (Zhou and Yan, 1989). However, findings in this study indicate that *B. pilosa* is toxic when used at very high concentrations of above 500 μg/ml. These findings are consistent with the study of Liang *et al*. (2019) where a high dosage *of B. pilosa* extracts resulted in toxicity in animal models. The study of Frida *et al*. (2008) have highlighted that there is a need for further safety verification and clinical trials before *B. pilosa* can be considered for medicinal use. In this study, the less active sub-fractions exhibited enhanced activity in MTT assay.

The C2C12 muscle cells and 3T3-L1 pre-adipocytes were differentiated prior to glucose uptake assay. Differentiation of cells was performed in order to mimic the mature cells of a typical mammal. The ability of cells to take up glucose occurs best in differentiated cells as compared to undifferentiated cells. The success of differentiated C2C12 cells was confirmed by transition from fusiform cells to elongated confluent multinucleated myotube cells (Wang *et al*., 2012). The 3T3-L1 preadipocytes were fully differentiated on day 8. The accumulated fat droplet resembled adipocyte morphology (Zebisch *et al*., 2012). The uptake of glucose in these tissues is a target for anti-diabetic drug discoveries as this will result in hypoglycaemia and maintain a balanced glucose level in the bloodstream (Singh, 2011). The *B. pilosa* crude extract and sub-fractions was then evaluated for

stimulating the uptake of glucose in C2C12 muscle cells and 3T3-L1 adipocytes for 3- and 6-hours treatment. Metformin and insulin were used as positive controls because they are well-known anti-diabetic drugs. Metformin and insulin were further used in combination with the crude extract and sub-fractions to evaluate the effects of the plant on patients who take these drugs and to understand the mode of action the plant use partnering their blood glucose lowering properties. Results show a significant increase in glucose uptake in crude extract and sub-fractions as compared to both untreated cells and cells treated with DMSO. The combination of the crude extract and sub-fractions with insulin resulted in a significant increase in glucose uptake as compared to treatment with the crude extract and insulin alone. This signifies that *B. pilosa* extracts, when used in combination with insulin, have synergistic effects in facilitating glucose uptake in both C2C12 myotubes and 3T3-L1 adipocytes. This suggests that there might be some binding compounds found in the plant extracts that are responsible for stimulation of expression of a number of genes that encode for proteins involved in the metabolism of glucose, which may be responsible for the increase in glucose uptake (Adam *et al.*, 2009). However, treatment with the crude extract and sub-fractions, on both cell lines in combination with metformin, resulted in a decrease in the amount of glucose taken up the cells. This suggests that there are compounds found in the plant that may be contributing to the insulin-mediated glucose transport signalling pathway. It has been reported that the structural alterations of these factors could lead to the blocking of the insulin signalling pathway at one point (Michael *et al.*, 2010). Hence there is an observed decrease in glucose uptake in both cell lines when treated with the crude extract in combination with metformin. Nonetheless, treatment with the crude extract and sub-fractions on C2C12 myotubes and 3T3-L1 adipocytes resulted in increased glucose utilisation. Treatment with crude extract and sub-fractions resulted in a higher glucose uptake at 3 hours treatment as compared to 6 hours. The fast active insulin mimetic will help regulate glucose uptake by the muscle cells and adipocytes, by providing active compounds found in the plant that mimic that of insulin, by eliciting the same cascade of reaction to that of fast active insulin that result in increased glucose uptake within a period of 3 hours (Manukumar *et al.*, 2017). Therefore, *B. pilosa* crude extract and sub-fractions could serve as a potential anti-diabetic drug which will help in reducing the post-prandial blood glucose and maintain glucose homeostasis. Plant extracts with increased glucose utilisation when compared to

untreated control suggests their potentials as a source of anti-diabetic agents. Studies have indicated that substances that result in increased glucose disposal by various peripheral organs, such as muscles and adipocytes, have an hypoglycaemic effect (Shrayyef and Gerich, 2010). These result of the ability of *B. pilosa* on the uptake of glucose in cells correlates with previous studies (Ubillas *et al.*, 2000; Khan *et al.*, 2001).

Obesity is an increasing problem worldwide and is known to be a major risk factor of type II diabetes mellitus due to its link with induced insulin resistance (Jung *et al.*, 2004). The search for anti-diabetic drugs that can equally mitigate obesity related complications is desired. Phytochemicals are potential agents to inhibit differentiation of preadipocytes, stimulate lipolysis and induce apoptosis of existing adipocytes, thereby reducing the amount of adipose tissue (Andersen *et al.*, 2010). Dysregulation of lipid metabolism is a major key factor in some pathological conditions such as the development of diabetes mellitus, insulin resistance and obesity.This led to the evaluation of the effect *B. pilosa* crude extract and sub-fractions on adipogenesis using 3T3-L1 adipocytes. Adipocyte differentiation medium (ADM) was used as a positive control because it is known to induce accumulation of adipocytes.The lipid accumulation was measured based on the triglycerides content of differentiated cells at various conditions. The results indicate that an increase in the concentration of the crude extract resulted in an increase in the accumulation of triglycerides. At the highest concentration of 30 µg/ml, the crude extract and sub-fractions resulted in a higher accumulation of triglycerides, better than untreated cells and ADM indicating that the crude extract and sub-fractions induce adipogenesis. This corresponds with the results obtained from glucose uptake where cells resulted in the stimulation of glucose uptake. The glucose taken up by the cells is stored in adipose tissues in the form of triglycerides. This then signifies that the plant has the ability to induce adipogenesis. Adipocytes have three primary functions; fat cells are insulin sensitive; they store lipid and they secrete hormones that act in distant tissues (Gastaldelli, 2011). On that note, this elaborates the importance of plant extracts which induce adipogenesis. Inhibition of any of the pathways leading to adipogenesis might result in an unhealthy metabolic disease state that increases the risk of type II diabetes. Inhibition of adipogenesis results in lipid storage in other tissues or cells which results in lipotoxicity which might lead to

cellular dysfunction (Greenfield and Campbell, 2004). Hormones that are produced exclusively in adipocytes, such as leptin and adiponectin, are responsible for regulation of carbohydrate intake and stimulation of sensitivity to insulin. Insulin is required for glucose homeostasis and loss of insulin responsiveness results in insulin resistance, which characterises the development of type II diabetes mellitus. Therefore, identification of medicinal plants with adipogenesis properties helps in the prevention of disruptive or metabolic pathways, which might lead to the formation of diabetes mellitus and its associated diseases. The results obtained in this study reveals the nature and characteristic of *B. pilosa*. *Bidens pilosa* possess insulin-like characteristics, in the sense that insulin stimulates the uptake of glucose, and in this case 3T3-L1 adipocytes. Plants with insulin-like characteristics may serve as adjuvant for anti-diabetic drug with fewer side effects (Kuppusamy *et al.*, 2014). However, it is known that high accumulation of triglycerides might lead to the development of obesity. It is observed that the plant extract when tested on the adipocytes at high concentrations result in high triglycerides accumulation. Therefore, standardization of concentrations within the ranges, that support both anti-diabetes and have minimal effects on adipogenesis, are of a major importance.

In conclusion, this study has revealed different mechanisms of anti-diabetic properties that *B. pilosa* crude extract and sub-fractions possess. *Bidens pilosa* crude extract sub-fraction contain phytoconstituents and antioxidant compounds which are capable of exhibiting different pharmacological activities, such as partial inhibition of α-amylase enzyme, which suggest its hypoglycaemic effects. In addition, *B. pilosa* crude extract and sub-fractions have glycation inhibitory potential which can help in reducing diabetes complications. Furthermore, this study has revealed the non-cytotoxic effects of *B. pilosa* crude extract and sub-fractions which were found at lower concentrations of 31.5µg/ml ± 62.5µg/ml on C2C12 muscle cells and 3T3-L1 adipocytes. The results on glucose uptake further elaborate the hypoglycemic effects of *B. pilosa*. The plant extract and sub-fractions stimulate the uptake of glucose in the C2C12 myotubules and 3T3-L1 adipocytes and exhibited high glucose utilization in combination with insulin. This is beneficiary to people living with type I diabetes, particularly people living in the rural areas who depend on traditional medicine even when taking western anti-diabetic medication. Therefore, *B. pilosa* might serve as an adjuvant for treatment or management of diabetes

mellitus. This study further documents the probable properties and characteristic the nature of *B. pilosa* as a plant with insulin-mimetic activities. This is indicated by the stimulation of glucose uptake in cells by *B. pilosa* and its further induction of the process of adipogenesis.

Bidens pilosa could be a source of a good anti-diabetic drug. However, further studies need to be carried out in order to verify the specific mechanism of action that *B. pilosa* uses for hypoglycaemic activity. The results obtained on inhibition of α-amylase digesting enzyme should be confirmed by determining enzyme kinetics. Inhibition of α- glucosidase enzyme should be investigated to fully understand the mode of action the plant use pertaining to inhibition of carbohydrates digesting enzymes. Translocation of glucose transporters, such as GLUT4, should be determined to understand the mode of action that *B. pilosa* uses to stimulate the uptake of glucose in C2C12 myotubules and 3T3-L1 adipocytes. Furthermore, assays, such as lipolysis, should be carried out to determine the anti-obesity properties of the plant. *In-vivo* studies should be considered to determine the effect of *B. pilosa* within a living system, and if they correspond with the in vitro results obtained in this study. Isolation and verification of biologically active compounds should be performed to ensure the active compounds responsible for regulation of anti-diabetic and anti-obesity pathways.

Chapter 6: References

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