GLUT4 TRANSLOCATION AUGMENTATION EFFECTS OF MEDICINAL PLANTS TRADITIONALLY USED FOR THE MANAGEMENT OF TYPE II *DIABETES MELLITUS*

by

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DECLARATION

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

Signature: _____

Date : _____

DEDICATION

I would like to dedicate this work to the almighty God and my loving family for all the support they have given me.

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder characterised by perpetual hyperglycaemia. Various oral pharmacological theraputic management strategies currently exist but are too expensive and having a host of undesirable side effects. Therefore people resort to the use of traditional medicinal plants as they offer a cost effective and readily available health care avenue. Despite the wide-spread use of traditional medicinal plants, several worrisome concerns about their effectiveness, clinical modes of action and safety have been raised.

Leaves of five selected plants (*Toona celliata, Seriphium plumosum, Schkuhria pinnata, Olea africana, Opuntia ficus-indica*) were collected from Mankweng area, Capricon Local Municipality, Limpopo province, South Africa. Ground plant materials were exhaustively extracted by maceration in methanol, acetone or hexane. The presence of different plant secondary metabolites in the crude extracts was determined using various standard chemical tests and thin layer chromatography (TLC). A myriad of compounds which represented various secondary plant metabolites groups were observed on the TLC plates and were best resolved in the non-polar (BEA) and intermediate (CEF) mobile phases. The total phenolic content and total flavonoids of the different extracts were determined spectrophotometrically using the Folin-Ciocalteu`s phenol reagent method and Aluminium chloride colorimetric assay respectively. The plants contained comparatively higher amounts of total phenolic compounds as compared to the flavonoids. The antiglycation activity of the plant extracts were determined using the bovine serum albumin assay. The acetone extract of *Seriphium plumosum* (SPlA) exhibited the most glycation inhibitory activity among all the examined extracts, as it resulted in 2,22% glycation. The antioxidant potential of each of the different extracts was quantitatively determined spectrophotometrically using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and the ferric ion reducing power assay. The methanol extract of *Seriphium plumosum* showed the best antioxidant activity among all the extracts in this study. It exhibited the lowest EC_{50} values of 0.72 mg/ml and 2.31 mg/ml for the DPPH scavenging activity and the ferric reducing power assay respectively. The cytotoxicity profiles of the different plant extracts on C2C12 cell line were determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

bromide (MTT) assay. It was concluded that since the all the extracts investigated had CC_{50} values greater than 50 µg/ml they were generally non-toxic. The amount of glucose taken up by differentiated C2C12 cells was quantified using the glucose uptake assay. Treatment of the C2C12 cells with the hexane extract of *Seriphium plumosum* resulted in the best glucose utilisation effect of 35,77% which was higher than that of insulin which was 26,06% after 6 hours. The translocation assay was used to determine the effect of the plant extract on GLUT4 translocation while the expression of various mitogen activated protein kinases in the cells was determined using the human MAPK profiler assay. It was established that treatment with *Seriphium plumosum* hexane extract resulted in increased GLUT4 translocation from the intracellular vesicular stores to the cell surface membrane. The increase in GLUT4 translocation may have resulted from the upregulation of expression of phosphorylated Akt-1, Akt-2, GSK3β, ERK1, ERK2 p70S kinase and MKK3 under the influence of *Seriphium plumosum* hexane extract.

The study documents a probable insulin-mimetic activity of the hexane extract of *Seriphium plumosum*. This activity may be responsible for its hypoglycaemic capability and may occur via the augmentation of proximal mitogen activated protein kinases involved in the GLUT4 translocation pathway. Further investigations need to be conducted to ascertain this novel finding which may help provide a cost-effective and readily available antidiabetic therapeutic agent.

LIST OF ABBREVIATIONS

μg/ml: Microgram per millilitre

µl: Microliter

4E-BP1: eIF4E-binding protein 1

A: Acetone

ADA : American Diabetes Association

ADP: Adenosine diphosphate

AGEs: Advanced glycation end-products

Akt1: Alpha serine/threonine protein kinase 1

Akt2: Alpha serine/threonine protein kinase 2

ATP: Adenosine triphosphate

BCA: Bicinchoninic acid

BMI: Body mass index

BSA: Bovine serum albumin

CC_{50}: Concentration exhibiting 50% cytotoxicity

CDCP: Center for Disease Control and Prevention

CREB: cAMP response element-binding protein

DMSO: Dimethylsulphoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EC_{50}: Concentration exhibiting 50% activity

ERK1: Extra-cellular signal regulated kinase 1

ERK2: Extra-cellular signal regulated kinase 2

FITC: Fluorescein isothiocyanate

GI: Gastro-intestinal

GLUT: Glucose transporter

GSK3β: Glycogen synthase kinase 3 beta

GWAS: Genome-wide association studies

H: Hexane

HIV: Human immune-deficiency virus

HRP: Horse radish peroxidase

IDF: International Diabetes Federation

IR: Insulin Receptor

IRS: Insulin receptor substrate

KCNJ11: Potassium inwardly rectifying channel, subfamily J, member 11

M: Methanol

MAPKs: Mitogen activated protein kinases

mg/dl: Milligrams per deciliter

mg/ml: Milligram per millilitre

mIU/L: milli-international units per litre

MKK3: Dual specificity activated protein kinase kinase

mM: millimolar

mmol/l: millimole/litre

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NEFAs: Non esterified fatty acids

nm: nanometres

OAfA: *Olea africana* (Acetone extract),

OAfH: *Olea africana* (Hexane extract),

OAfM: *Olea africana* (Methanol extract),

OFiA: *Opuntia ficus indica* (Acetone extract),

OFiM: *Opuntia ficus indica* (Methanol extract),

OH: Hydroxyl group

p38α: P38 mitogen activated protein kinases

p70S kinase: Ribosomal protein S6 kinase beta 1

PBS: Phosphate-buffered saline

PDK : 3'-phosphoinositide-dependent protein kinases

PGC1α: Proliferator-activated receptor gamma co-activator 1α

PIP_2 : Phosphatidylinositol 4,5-bisphosphate

PIP_3: Phosphatidylinositol 3,4,5-trisphosphate

PM: Plasma membrane

PPARs: Peroxisome proliferator–activated receptors

QE: Quercetin equivalents

RAGEs Receptors for advanced glycation end-products

ROS: Reactive oxygen species

S6K: Ribosomal protein S6 kinase

SGLT: Sodium glucose transporters

SNARE: Soluble NSF attachment protein receptor

SPiA: *Schkuhria pinnata* (Acetone extract),

SPiH: *Schkuhria pinnata* (Hexane extract),

SPiM: *Schkuhria pinnata* (Methanol extract),

SPlA: *Seriphium plumosum* (Acetone extract),

SPlH: *Seriphium plumosum* (Hexane extract)

SPlM: *Seriphium plumosum*, (Methanol extract)

TAE: Tannic acid equivalents

TB: Tuberculosis

TCA: Trichloroacetic acid

TCAM: Traditional, complementary and alternative medicine

TCF7L2: Transcription factor 7–like 2

TLC: Thin layer chromatography

TSC1/2: Tuberous sclerosis complex 1/2

ULK1: Unc-51-like kinase 1

USA: United States of America

USD: United States of America Dollar

USDHHS: United States Department of Health & Human Services

UV: Ultraviolet

WHO: World Health Organisation

CHAPTER 1

Introduction

1.1 Pathogenesis of diabetes mellitus

According to the American Diabetes Association (ADA, 2010), diabetes mellitus is a metabolic disorder characterised by the body's inability to maintain proper homeostatic levels of various biomolecules, particularly glucose. This disorder results in perpetually elevated blood glucose levels, a condition referred to as hyperglycaemia. Any individual with a fasting plasma glucose level ≥7.0mmol/l (126mg/dl) or a post-prandial plasma glucose level of ≥11.1mmol/l (200mg/dl) after 2 hours after consumption of a 75g load of glucose is considered to be diabetic (World Health Organisation) (WHO, 2006). Improper blood glucose levels in diabetics are known to emanate from poor utilisation of insulin, its relative or complete deficiency (ADA, 2014). Insulin enables cells to take up glucose from the bloodstream. In diabetic patients glucose remains in circulation within the blood. This translates to perpetually elevated blood glucose levels, a condition known as hyperglycaemia. Perpetual hyperglycaemia leads to the development and progression of a vast number of detrimental effects (Shrayyef and Gerich, 2010). Hyperglycaemia negatively changes the normal blood parameteres including the immune cell composition, density and nutrient milieu etc. This results in various acute and chronic signs and symptoms that are hallmarks of diabetes. Three major etiological categories of the condition are recognised namely type I, type II and gestational diabetes.

1.1.1 Type I diabetes mellitus

Type I diabetes mellitus results from the selective loss of β cells in the islets of Langerhans found within the pancreas. In type I diabetes, the β cells are selectively destructed by immune responsive cells due to an auto immune defect in genetically predisposed individuals (Bluestone, *et al.,* 2010). The underlying factor that triggers the process of this destruction still remains poorly understood. Several markers of autoimmune destruction are usually present at diagnosis, which includes autoimmune antibodies to insulin and the islets cells (American Diabetes Association) (ADA, 2010). Varying degrees of insulin deficiency result depending on

the extent of the destruction of β cell. Type I diabetes is usually of juvenile on-set, but may present itself at any stage later in life. The symptoms of this condition are usually acute with ketoacidosis being a hallmark sign. While other patients may have residual β cell function which may prevent ketoacidosis, those patients that have total β cell destruction cannot survive without insulin.

1.1.2 Type II diabetes mellitus

Type II diabetes mellitus on the other hand results from relative insulin deficiency and/or insulin resistance (American Diabetes Association) (ADA, 2010), which result in reduced insulin mediated uptake of blood glucose by insulin sensitive cells. The early stages in the progression of this chronic condition are characterised by mild symptoms. Most type II diabetes patients only seek medical assistance when the symptoms are more pronounced, at which time, irreversible damage at cellular level would have occurred. Contributing factors leading to the development of this condition include genetic predisposition, diet, obesity, age, physical inactivity, environmental factors and race (International Diabetes Federation) (IDF, 2013). Type II diabetes mellitus is known to co-occur with conditions such as hypertension and dyslipidaemia.

1.1.3 Gestational diabetes

Gestational diabetes is commonly seen in pregnant women with no previous history of diabetes. The elevated glucose levels during pregnancy are thought to be caused by impaired insulin function caused by hormones secreted by the placenta, usually during the second trimester of pregnancy (American Diabetes Association) (ADA, 2010). The condition has a reduced effect on the baby, since at the time of its development; all important features of the baby would have been fully developed. Uncontrolled hyperglycaemia during pregnancy may lead to foetal macrosomia which is associated with high risks particularly during child birth.

1.2 Predisposing factors of diabetes mellitus

The development of this condition is attributed to many factors which include genetic predisposition, environmental ills, insulin resistance and obesity (Ginter and Simko, 2013). The factors leading to the development of diabetes may broadly be categorised into controllable and uncontrollable risk factors (Alberti *et al.,* 2007).

Controllable risk factors are those factors that an individual may be able to take action and change, these including physical activity engagement, lifestyle and diet. Uncontrollable risk factors, such as genetic predisposition, age or ethnicity, on the other hand are beyond an individual's control.

1.2.1 Sedentary lifestyle and weight gain

People, particularly those in urban areas lead lives that are associated with reduced physical activity which results in gradual insulin resistance and weight gain. Regular non intensive and/or intensive physical activity is known to aid in proper blood glucose regulation (Jensen *et al*., 2011). An increase in body mass index (BMI) is highly associated with the development of various conditions including type II diabetes mellitus (Fowler, 2007). Non esterified fatty acids (NEFAs) have been implicated as an underlying cause of insulin resistance and impaired β-cell function (Kahn *et al*., 2006). Strong evidence exists suggesting that lipid oxidation also leads to insulin resistance as it results in increased reactive oxygen species (ROS) production (Yoon *et al*., 2006). Reactive oxygen species are implicated in the damage of insulin receptors on the surface membrane on various target cells which subsequently lead to insulin resistance.

1.2.2 Improper diet

Unhealthy and unbalanced diets that are high in sugar, fats, cholesterol, that are either refined or processed are particularly a risk factor when it comes to diabetes. These kinds of diets are particularly prominent in urban areas and are linked to insulin resistance with associated overall weight gain.

1.2.3 Genetic predisposition

Genome-wide association studies (GWAS) are currently being employed to correlate the link between diabetes and genetic predisposition (Sanghera and Blackett, 2012). Thus far, 75 susceptibility loci associated with diabetes have been identified and replicated (Sanghera and Blackett, 2012; Wu *et al.,* 2014). The identified susceptibility gene loci include the *KCNJ11* (potassium inwardly rectifying channel, subfamily J, member 11), *TCF7L2* (transcription factor 7–like 2), and *IRS1* (insulin receptor substrate 1) (Wu *et al.,* 2014). An estimated 40% of people whose family have had cases of diabetes are likely to develop the condition compared to the 6%

of individuals without a previous family history of the condition (Kobberling and Tillil, 1982).

1.2.4 Ethnicity and age

Different ethnic groups differ in their susceptibility to diabetes. A study in the United States of America (USA) assessed the incidence of the condition within different racial groups. Compared to non-Hispanic white adults, the risk of diagnosed cases of diabetes was 18% higher among Asian Americans, 66% higher among Hispanics, and 77% higher among non-Hispanic blacks. Among Hispanics compared to non-Hispanic white adults, the risk of diagnosed cases of diabetes was about the same for Cubans and for Central and South Americans, 87% higher for Mexican Americans, and 94% higher for Puerto Ricans as reported by: (Centers for Disease Control and Prevention) (CDCP, 2011). A separate study documents that people belonging to the Asian group are at the highest risk of developing diabetes globally (Yoon *et al*., 2006) Diabetes is a degenerative condition that is likely to develop as an individual grows old (Suastika *et al*., 2012). This is due to the inherent loss of function of different organs within the body which results in a host of different conditions including diabetes mellitus.

1.3 Acute signs and symptoms

Signs and symptoms of diabetes differ from individual to individual. In the early stages for this condition, the signs and symptoms may be mild or not evident at all (ADA, 2009). Some of the signs commonly experienced include the following: excessive thirst (Polydipsia) which results from the high blood glucose levels which change the blood osmolality prompting the individual to take up excessive fluids to normalise this situation (ADA, 2010). Frequent urination (Polyuria) results following excessive fluid intake: the urine contains some of the glucose as the kidney filter out the excessive glucose that would otherwise be retained in the body. Weight loss also occurs because the glucose is not taken up and stored as fat therefore the body uses its already stored energy reserves. The poor glucose uptake results in increased hunger (Polyphagia). Individuals with diabetes usually feel tired, have poor concentration and blurred vision due to lack of energy (ADA, 2010). Frequent infections, slow-healing wounds and other infections occur due to compromised immunity and increased blood glucose providing a good source of nutrition to pathogens (ADA, 2010). The development of type 1 diabetes is usually sudden and

dramatic while the symptoms can often be mild or absent in people with type 2 diabetes, making this type of diabetes hard to detect (ADA, 2009).

1.3.1 Chronic signs and symptoms

Prolonged hyperglycaemia results in more severe signs and symptoms. These are the causes of morbidity and motility in diabetic individuals. These occur due to the development of advanced glycation end-products (AGEs) where the high blood glucose concentrations spontaneously react with structural or functional protein moieties in different parts of the body (Peppa, *et al*.,2003). Glycosylated proteins are known to have disrupted molecular conformation, altered enzymatic activity, reduced degradative capacity and interfere with various receptor recognition processes (Aronson and Rayfield, 2002). Glycosylation reaction initially results in Schiff base formation which then rearranges itself into Amadori products product as time goes by (Aronson and Rayfield, 2002). In diabetes, the most apparent product is the glycated haemoglobin A_{1c} (A1C) (Peppa, *et al*., 2003).

1.3.1.1 Cardiovascular disease

Cardiovascular diseases are complications associated with altered morphology of the heart and its various associated blood vessels (WHO and UNAIDS, 2007). Cardiovascular disease is associated with coronary heart disease, hypertension, hyperglycaemia peripheral artery disease, and rheumatic heart disease. There is high co-occurrence of diabetes and different types of cardiovascular disease (Howard *et al*., 2002). Cardiovascular diseases are known to be the leading causes of morbidity and mortality in diabetic patients.

1.3.1.2 Diabetic nephropathy

Diabetic nephropathy is a condition in which the normal function of the kidneys is impaired (Tervaert *et al*., 2010).The condition results from chronic diminishing glomerular filtration rate. Severe cases are marked by heightened proteinuria and linked to fatal cases of uraemia if no steps are taken. Chronic hyperglycaemia effects several cellular changes on cells found within the kidney such as endothelial cells, smooth muscle cells, meningeal cells, podocytes, cells of the tubular and collecting duct system, inflammatory cells and myo-fibroblasts. The impairment of all these cellular components contributes to the complicated steps that lead to the development and progression of diabetic nephropathy (Tervaert *et al.,* 2010).

Diabetic nephropathy is also implicated in the development and progression of other macro-vascular complications such as atherosclerosis and cardiovascular disease.

1.3.1.3 Diabetic neuropathy

Diabetic neuropathy is a condition in which there is a progressively diminishing sensory perception caused by impaired function of somatic and autonomic divisions of the peripheral nervous system (Callaghan *et al*., 2012; Vinik *et al*., 2013). It is caused by the perpetually high blood glucose and blood pressure in diabetic individuals. It results in problems with digestion, slow wound healing, erectile dysfunction, and improper functions of extremities, in particular the feet and fingers (Callaghan *et al*., 2012). The patient usually feels pain, tingling sensations, or total loss of feeling (Van Dam *et al*., 2013). An incapacitated sense of feeling is dangerous as it allows injuries and infections to go unnoticed. Due to diabetic neuropathy diabetic patients are at high risk of than people without diabetes. However, with comprehensive management, a large proportion of amputations related to diabetes can be prevented (Vinik *et al*., 2013). People with diabetes are therefore urged to regularly examine their feet as a precautionary measure (Vinik *et al*., 2013).

1.3.1.4 Diabetic retinopathy

Diabetic retinopathy is characterised by lesion that occur on the retina of the eye. These lesions usually progress to render the affected individual to have reduced vision or blind in severe cases (Yau *et al*., 2012). These include changes in vascular permeability, capillary micro aneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization). Diabetic retinopathy is categorised into two stages which are non-proliferative and proliferative stages (Tarr *et al*., 2013). Non-proliferative diabetic retinopathy is characteristic of the early stages of retinal damage and is usually difficult to detect. Proliferative stages of diabetic retinopathy occur in more severe prognosis. Proliferative diabetic retinopathy results in the development of new blood vessels, visual impairment and blindness (Tarr *et al*., 2013). Consistently high levels of blood glucose, together with high blood pressure and high cholesterol, are the main causes of retinopathy. It can be managed through regular eye checks and keeping glucose and lipid levels at or close to normal (Chistiakov, 2011).

1.3.1.5 Pregnancy complications

Hyperglycaemia during pregnancy has been shown to result in various foetal developmental irregularities such excess weight gain, a condition known as diabetic embryopathy (Singh *et al*., 2013) . This proves to be a problem during delivery and may result in trauma to both the child and the mother. Women who are diabetic are argued to constantly check their blood glucose levels and keep them as close as possible to the normal ranges (Galtier-Dereure *et al*., 2000). Children born to diabetic mothers have an increases risk of developing the condition themselves (Ryckman *et al*., 2013).

1.3 Current mangement strategies of diabetes

Current management strategies of the condition include non-phamaological such as exercise, physical activity therapy and medical nutrition therapy. Pharmacological methods of diabetes managment involve the oral theraputics categorised broadly as biguanides,sulfonouearea, α-glucosidase inhibitors and thiazolidinediones(Molitch, 2013). .

1.3.1 Non-phamacological methods of diabetes management

1.3.1.1 Exercise and physical activity therapy

Physical activity and exercise are considered to be pivotal in both the prevention and management of type II diabetes mellitus (Jensen *et al*., 2011). Physical activity refers to any movements made by the body. These movements are a result of skeletal muscles contractions and result in an expenditure of energy (Knuttgen, 2013). Physical activity includes leisure and non-leisure daily activities. Exercise is defined as physical activity that is planned and intentionally carried out to enhance or maintain ones physical fitness, muscular strength or endurance and aerobic capacity (Colberg, 2010). Physical activity has been shown to increase insulin sensitivity and reduce the amount of glycated HbA1c levels (Zanuso *et al*., 2010).

1.3.1.2 Medical nutrition therapy

Medical nutrition therapy is defined as “nutritional diagnostic, therapy, and counselling services for the purpose of disease management, which are furnished by

a registered dietician or nutrition professional" (United States Department of Health & Human Services [USDHHS, 2001]). The amount of nutritional dietary intake directly influences the post-prandial blood glucose levels. As such, following a planned meal schedule prescribed by a registered dietician can better help manage the fluctuations in blood glucose level (Morris and Wylie-Rosett, 2010). With this type of therapy, patients are taught how to reduce their intake of foods that are rich in unsaturated fatty lipids, cholesterol and carbohydrates as part of the dietary intervention to manage diabetes. At diagnosis, the aim is to maintain a monitored blood glucose level rather than weight loss. As the patient continues to follow a strict guided diet schedule, a gradual decrease in body weight will occur and the normal recommended body weight will eventually be attained.

1.3.2 Pharmacological management

Pharmacological management is employed to manage diabetes mellitus when non-pharmacological methods have shown to be inefficient. The treatments fall under 4 main categories of drugs which are biguanides, sulfonylureas, glucosidase inhibitors and thiazolidinediones. Insulin may also be administered subcutaneously as a single therapeutic agent or in combination with other drugs (Molitch, 2013).

1.3.2.1 Biguanides

Biguanides and their derivatives are colourless organic compounds with the general formula $HN(C(NH)NH_2)_2$. This class of compounds are used in the treatment of cases of diabetes mellitus that show insulin resistance (Fowler, 2007; Molitch, 2013). They manifest their anti-diabetic property by inhibiting the hepatic glucose output, delaying glucose uptake from the small intestines and also sensitising the cells to insulin (Verspohl, 2012). Metformin remains the most popular biguanide on the market. It is highly recommended as a first line treatment in diabetes when non-pharmacological means have failed.

1.3.2.2 Sulfonylureas

Sulfonylureas are a group of compounds which exhibit their anti-diabetic activity by acting on β-cells to increase insulin release (Fowler, 2007). Sulfonylureas bind to the sulfonylurea receptor on the surface of β-cells and inhibit potassium efflux which depolarizes the β-cells and concurrently facilitate insulin release (Molitch, 2013).

Common sulfonylureas available on the market include glyburide, glipizde, and glimepiride.

1.3.2.3 α-Glucosidase inhibitors

This group of anti-diabetic agents exhibit their effect by slowing down the absorption of carbohydrates, particularly after meals. They act by inhibiting the action of enzymes that cleave polysaccharides into monosaccharaides (Verspohl, 2012). Polysaccharides are not easily absorbed into the blood as compared to monosaccharaides. The overall effect of these drugs is to reduce post-prandial blood glucose levels. Acarbose and miglitol are the most commonly available α-glucosidase inhibitors on the market (Verspohl, 2012).

1.3.2.4 Thiazolidinediones

Thiazolidinediones are insulin sensitising drugs. They exhibit their action by binding to peroxisome proliferator–activated receptors (PPARs) in cells. Once they are bound, the drug-PPAR complex then acts on response elements in promoter regions to affect the transcription of as many as 100 genes (Fowler, 2007). They may up-regulate the transcription of proteins that increase insulin sensitivity, such as adiponectin. They may also down-regulate the production of proteins responsible for insulin resistance or inflammation. Common thiazolidinediones available on the market include rosiglitazone and pioglitazone.

1.3.3 Drawbacks of current diabetes managent strategies

Non-phamacological methods of diabetes managent usually take self discipline and determination which many people lack and as such highly prone to failure. Pharmacological agents of diabetes management though being effective have a host of undesirable side effects (Verspohl, 2012). The major side effects include severe weight loss/gain, nausea, hepatotoxicity, diarrhoea, gastrointestinal upsets and some induce hypoglycaemic shock (Verspohl, 2012). They are also quite expensive and inaccessible to the majority of the people particularly those in low income countries.

1.4 Prevalence of diabetes mellitus

The global burden of diabetes is on an all-time high and is expected to rise in the future (Olokoba *et al*., 2012). The high incidence and prevalence of the condition is

a great cause for concern with other authors referring to the condition as a global epidemic (Narayan *et al*., 2006; Sierra, 2009; Zimmet *et al*., 2003). An estimated 382 million people mostly between the ages of 40-59 are currently suffering from the condition (IDF, 2013). An estimated 14 million more men suffer from diabetes than women. The number is projected to rise by 55% by the year 2035 bringing the total number to 592 million people affected globally (IDF, 2013). Furthermore, an estimated 5.1 million people succumb to this condition annually (IDF, 2013). About 80% of the diabetic community is found within the low to middle income group (IDF, 2013). The condition has had a pronounced detrimental impact particularly within Africa were 4 of the 5 people with diabetes globally reside.

A large number of diabetes cases go undiagnosed primarily due to the lack of adequate primary health care systems, traditional barriers such as religious and cultural beliefs or simply due to ignorance (Whiting *et al*., 2011; Shaw *et al*., 2010). In Africa the number of people suffering from diabetes is estimated to be about 19.8 million (IDF, 2013), while in South Africa, an estimated 2, 6 million people suffer from this condition and more than 50 % of the cases are believed to be undiagnosed (IDF, 2013).

1.5 Financial burden of diabetes and its associated complications

The global economic impact of the condition is enormous with an estimated 548 million USD in 2013 being channelled to fund the management of diabetes and its related complications (IDF, 2013). The International Diabetes Federation (2013) estimates this amount to rise to about 627 million by the year 2035. Noteworthy is that only 20% of this amount was used by the low to middle income countries (IDF, 2013). An estimated USD 4billion was spent on diabetes healthcare in 2013 within Africa. The total expenditure within Africa is expected to rise by 58% by 2035. Developing countries particularly those in Africa are most adversely affected by the detrimental effects of such a pandemic. These countries are economically disadvantaged and are unable to provide the necessary resources required to manage the conditions. Diabetes is the leading cause of lower limb amputations, blindness/eye cataracts and renal failure in developing and developed countries (WHO, 2013). Furthermore, there is a scarcity of effective primary health care facilities to cater for the bulk of the patients suffering from the condition.

CHAPTER 2

Literature Review

2.1 Traditional medicinal plants

The use of plants in traditional medicine is a wide-spread practise that is employed by people of distinct cultural origins globally (Newman and Cragg, 2012). The World Health Organisation (WHO) defines traditional medicine as the consolidated use of ancestral knowledge and practical experience in the prevention, diagnosis and treatment of physical, mental or social imbalance (Richter, 2003). A medicinal plant may be defined as any plant or plant part that possesses therapeutic properties which may be employed in traditional medicine. Traditional healers have the expertise in the administration of medicinal plants. However, diagnosis of ailments remains a challenge, compounded with dosage administered over prolong period of time. Medicinal plants are widely considered to be safe although toxicity studies over time remains controversial. Although concerns have been raised on the safety of traditional medicinal plants they remain quite popular (WHO, 1993). Plants may either be administered singularly or in combination with others; usually through decoctions that are taken orally by the patient (Otieno *et al*., 2008; Hogle and Prins, 2012). This knowledge has been passed down from generation to generation primarily by observation and orally during the training of the traditional healers by their predecessors (Hogle and Prins, 2012). The dawn of the 21st century has seen a reignited interest in the use of medicinal plants and their derivatives. This rejuvenated interest emanates from the fact that 74% of the 119 pure compounds that are used in western medicine are directly or indirectly derived from plants (Kumar *et al*., 2009, Marles and Farnsworth, 1995; Farnsworth *et al.,* 1985). Plant derived compounds are also known to have less undesirable side effects as they work harmoniously with the natural body processes (Atal,1983). Therefore it would be desirable to use these plants directly as a source of medicinal care system (Clark, 1996; Farnsworth, 1990).

2.2 The use of medicinal plants in developed countries

Western medicinal facilities in developed countries are well equipped and form the primary health care system in these countries. Bringing into perceptive the need for a harmonious relationship between western and traditional medicine. As such, in those

countries, traditional medicinal plants form part of alternative medicine. Alternative medicine is any other form of health care system that is used parallel to the dominant health care system within a country (WHO, 2002). In industrialized countries, almost half the population now regularly use some form of traditional, complementary and alternative medicine (TCAM). A survey by Barnes and colleagues in 2002 showed that about 19% of adults in the United States of America employ traditional, complementary and alternative medicine. About 52% of the population in South Australia above the age of 15 are reported to have used traditional medicine in 2000 (MacLennan, 2002). More so, over 50% of the currently prescribed medications in the USA are derived from medicinal plants (Grifo *et al*., 1997).

2.3 The use of medicinal plants in African countries

Traditional medicinal plants are currently being employed to meet the primary health care needs of about 80% of the population in Africa (WHO, 2002). The wide spread popularity in the use of traditional plants emanates from their cost effectiveness and availability (Hosseinzadeh *et al.,* 2015). This is particularly true for people who find conventional western health care as being either far too expensive or simply beyond their reach (Payyappallimana, 2010). The erratic availability of professional medical doctors and facilities in third world countries is also a major contributing factor. It is reported that the ratio of traditional healers to population in Africa is 1:500 versus the 1:40 000 medical doctors to population ratio (WHO, 2013).

2.4 Plant derived secondary metabolites

Plant secondary metabolites are compounds that are produced by higher plants that do not contribute to primary processes, such as photosynthesis, respiration, reproduction and growth (Bourgaud *et al.,* 2001). These secondary metabolites within the plants act as attractants to organisms such as pollinators, deterrents to predators and infectious organisms such as bacteria, fungi, viruses and other pests that might cause disease. Plants also produce secondary metabolites aimed at excluding competitors and protecting the plant from UV irradiation (Lattanzio *et al.,* 2009). Secondary metabolites are specie specific and vary greatly in their structural complexity. This variation in plant secondary metabolite complexity provides a bountiful number of compounds that can effect therapeutic properties in humans. Different types of plant secondary metabolites have long been recognised to help

manage various kinds of ailments such as chronic inflammation, cancer, tuberculosis (TB), human immune-deficiency virus HIV and diabetes.

2.5 Classification of plant secondary metabolites

Plant secondary metabolites are classified based on their structure, composition and synthetic pathway (Richardson, 1990). The biosynthetic pathway of classification is the most commonly employed system and it identifies three main categories of secondary plant metabolites i.e. phenolic, terpenes and steroids, and alkaloids (Salim *et al*., 2008).

2.5.1 Phenolic compounds

Plant phenolic compounds are defined as compounds that contain one (mono-phenolic) or more (polyphenolics) aromatic rings covalently bonded to one or more hydroxyl groups (Dai and Mumper, 2010). In plants, the range of the function of phenolic compounds includes protecting the leaves from ultraviolet-radiation and from browsers due to their bitter taste. Phenolic compounds are responsible for contributing to the colour of the different plant tissues (Dai and Mumper, 2010). The most remarkable property of phenolic compounds is their ability to act as antioxidant compounds. Antioxidant capacities of various secondary metabolites are associated with reduced rate of development and progression of several human diseases (Anderson *et al.,* 2001). They also exhibit their antioxidant function by electron/hydrogen donation, metal chelation and synergism with other antioxidant compounds such as ascorbic acid (Valentao *et al*., 2003). The anti-oxidant nature of many polyphenol is beneficial in scavenging free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Djeridane,2006). Phenolic compounds also contain large hydrophobic groups that are capable of strong interaction with protein moieties (Croft, 1998, Pereira *et al.,*2009).Phenolic compounds are sub-classified as phenolic acids, lignans, flavonoids, stilbenes and tannins according to their structure.

2.5.2 Terpenes and steroids

Terpenes are hydrophobic compounds characterised by long hydrocarbon chains of alternating double and single bonded carbon atoms. The essential oils of many

plants are characterised by a high amounts of terpenes and their derivatives, which play a major role in the defence of the plant from browsers (Heldt and Piechulla, 2010). Terpenes have a wide range of beneficial uses which includes their use as fragrances, food spices, food colourants, food additives and medicine (Newman *et al*., 2000). The most profound examples of drugs derived from terpenes include the anti-malaria drug artemisinin (Wang *et al*., 2005).

Steroids are lipid hydrocarbons that are derived from terpenes. They are characterised by a tetracyclic carbon skeleton consisting of three fused six-membered and one five-membered ring (Heldt and Piechulla, 2010). They are sub-categorised by the different conjugated groups on the tetracyclic carbon skeleton.

2.5.3 Alkaloids

Alkaloids are nitrogenous organic compounds found in many plant families. They have complex chemical structures that are derived from amino acids (Khan *et al.,* 2013). Their structures contain one or more nitrogen based cyclic rings (Bruneton, 2003). They are primarily involved in the defence system of plants against herbivores and other types of insults (Harborne, 1991). Alkaloids are known to have a range of therapeutic bio-activities in humans (Roberts, 2013). Several alkaloid based drugs are currently on the market; these include the anti-malaria drug quinine, muscle relaxants such as tubocurirane, anti-cancer agents such as taxol and vinblastine (Roberts, 20.13).

2.5.4 Antidiabetic modes of action of plant secondary metabolites

Over 800 plants have been reported to possess antidiabetic properties (Patel *et al*., 2012). These plants are known to synthesise different types of secondary metabolites that exhibit these properties. Various modes of action in different target tissues exist through which the plant secondary metabolites work towards regulating the blood glucose levels (Prabhakar and Doble 2011). Antidiabetic modes of action include: insulin release through pancreatic β-cell stimulation (insulin-secretagogues), increase in the number and sensitivity of insulin receptors, inhibition of hormones which rise blood glucose such as α-glucosidase and β-glucosidase, increase glucose usage and storage by tissues such as muscle, liver and adipose and free radicals scavenging (Li *et al*., 2004). The modes of action of compounds belonging to various major secondary metabolite classes are listed in table 1 below.

Table 1.1: Modes of action of various plant secondary metabolites categorised into alkaloids, flavonoids, terpenes and phenolics (Table adopted from Bahmani *et al*., 2014).

Plant secondary compound class	Name of compound	Mechanism of action and uses in diabetes
Alkaloids	Casuarine-6-O-α-glucoside	α-Glucosidase inhibitor
	Isoquinoline alkaloids: schulzeines A, B and C	α-Glucosidase inhibitor
	Tecomine 5β-hydroxyskitanthine Boschniakine	Stimulate basal glucose uptake rate in rat adipocytes
	Pyrrolidine alkaloids : radicamines A and B	α-Glucosidase inhibitor
	Quinolizidine alkaloids: javaberine A, javaberine A hexaacetate and javaberine B hexaacetate	Inhibitors of TNF-α production by macrophages and fat cells. Dietary supplement for prevention of diabetes
	Lupanine, 13-α-hydroxylupanine, 17-oxo-lupanine	Glucose-induced insulin release enhancement from isolated rat islet cells which was dependent on the glucose concentration
	Berberine Chloride berberine Sulfate berberine Palmatine sulfate Palmatine chloride	Aldose reductase inhibitor

Flavonoids	6-Hydroxyapigenin 6-hydroxyapigenin-7-O-β-D-glucopyranoside 6-hydroxyluteolin-7-O-β-Dglucopyranoside 6-hydroxyapigenin-7- O-(6-O-feruloyl)-β -D-glucopyranoside 6-hydroxyluteolin-7-O-(6-O-feruloyl)-β-D-glucopyranoside	α-Glucosidase inhibitor
	Myrciacitrin I, II, III, IV and V	Aldose reductase inhibitory activity.
	Quercetin 3-O-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside Kaempferol 3-O-β-Dglucopyranoside (astragalin) Quercetin-3-O-β-D-glucopyranoside (Isoquercitrin)	Glycation inhibitors
Terpenes	Lactucain A, B and C	Moderate lowering of plasma glucose
	3,22-Dihydroxyolean-12-en-29-oic acid, Tingenone Tingenine B Regeol A Triptocalline A Mangiferin	Aldose reductase inhibitory activity
	Centellasaponin A, Abietane-type diterpenoids: danshenols A and B Dihydrotanshinone I Tanshinone I, Cryptotanshinone, tanshinone IIA (-)-danshexinkun A	
	Triterpenedehydrotrametenolic acid	Insulin sensitizer
	Corosolic acid (GlucosolTM)	Glucose transport-stimulating activity

Phenolics	7'-(3',4'-Dihydroxyphenyl)-N-[(4 methoxyphenyl)ethyl] propenamide 7'-(4'-hydroxy-3'methoxyphenyl)-N-[(4butylphenyl)ethyl] propenamide (7S,8S)-syringoylglycerol 9-O-β-D-glucopyranoside(7S,8S)-Syringoylglycerol-9-O-(6'- O-cinnamoyl)-β-D- glucopyranoside	α-Glucosidase inhibitor
	Tetra- and penta-O-galloyl-β-D-glucose	Potent aldose reductase inhibitory activities.

2.6 Anti-diabetic and other beneficial properties of plants under study

The plants in this study have been reported on several accounts to possess anti-diabetic potential. Semenya *et al.,* (2012) reported that in Limpopo province of South Africa the root of *Opuntia ficus indica* is boiled in water for 20 minutes and the resulting infusion is taken orally to manage diabetes. In Mexico, capsules of the dried leaves and stems were taken orally to manage diabetes as well (Soumyanath, 2005). The anti-diabetic characteristics of *Opuntia ficus indica* are thought to result from the complex carbohydrates that are known to reduce the rate of glucose uptake in the intestines (Nuñez-López *et al*., 2013; Kaur *et al*., 2012). *In-vivo* tests on diabetic rat models by El-Razek and Hassan (2011) revealed the ability of *Opuntia-ficus indica* fruit juice extract to normalise various parameters.

In Kenya, the whole plant of *Schkuhria pinnata* is burnt and water is added to the ashes and the resulting infusion is taken orally for diabetes management (Kareru *et al*., 2007). Deutschländer *et al* (2009) documented the hypoglyceimic potential of this plant *in-vitro* using Chang liver, murine muscle (C2C12) and pre-adipocyte (3T3-L1) cells.

The leaves of *Olea africana* are used as hypoglycaemic agents by various communities throughout the world (Long *et al.,* 2010). Leaves, fruits/infusions and

macerations have been reported to have to help regulate blood glucose level by people in Morocco (Tahraoui *et al*., 2007). Leaf decoctions of *Olea* are reported to be used by people in Souk Ahras District in Algeria as antidiabetic and antihypertension agents (Amel, B., 2013). Oleanolic acid was reported to lower serum glucose and insulin levels in mice fed a high-fat diet, and it enhanced glucose tolerance (Sato *et al*., 2007).

Despite *toona ciliata* possessing various therapeutic effects its diabetic nature remains poorly studied. *Toona ciliata* was recently shown to have anti-hyperglycaemic activity in streptozotocin induced diabetes in rats (Rana *et al*., 2016). The above mentioned plants are known to possess various other therapeutic potential uses (Table 2 overleaf).

Seriphium plumosum is considered as an unwanted bush encroacher weed in various parts of South Africa. It is mainly used by the indigenous people for various non medicinal purposes such as a broom to sweep outside (Moffett, 2010). The Basotho people use this bush to ward off bugs by placing it under their bedding (Moffett, 2010). Other medicinal functions of this plant are listed in table 2 below. Currently no antidiabetic studies have been undertaken on this particular plant.

Table 2.1: The plants used in the current study and some of their beneficial traditional uses.

Scientific name, Family and Image	Common names	Traditional uses	Plant part used	Previously Isolated compounds	References
Olea europea sub.sp Africana (Olea Africana) (Oleaceae) 	Wild olive (English) Olienhout (Afrikaans) Mohlware (SSotho) umNquma (Zulu, Xhosa) Mutlhwari (Venda),	Kidney problems Backache Eye infections Sore throat Colic or urinary tract infections Hypertension Skin cleanser	Leaves Bark Seed extract Fruits	β-amyrin, Oleanolic acid Oleuropein Oleacein	(Hansen, K. *et al*.1996;Le Tutour and Guedon, 1992; Hashmi *et al*.,2015; Burdi *et al*., 2014)
***Opuntia ficus indica* (L.) Mill. (Cactaceae)** 	Prickly pear(English) Idolofiya (Ndebele) Mudorofia , Munanazi (Shona) Mudoro (Venda)	Alcohol hangover Anti-inflammatory Neuroprotective Anti-ulcer activity Anti-hyperlipidaemia Anti-hypercholesteraemic	Leaves Roots Fruits Flowers Stems	Penduletin, Luteolin Kaempferol, Quercetin, Rutin, Betalain,Orientin, Myricetin,Taxifolin	(Wolfram *et al*., 2002; Ginestra *et al*.,2009; Feugang *et al*.,2006)
***Schkuhria pinnata* (Lam.) Thell (Asteraceae)**	Dwarf marigold (English)	Eye infections Pneumonia, Stomach problems	Leaves Stems Flowers	Schkuhrianol, Schkuhrins,	(McGaw *et al*., 2008; Van der Merwe *et al*., 2000;Luseba *et al*., 2006; Muthaura *et al*., 2009;

	Ruhwahwa (Shona) Kleinkakiebos (Afrikaans) Gakuinini (Kikuyu) Canchalagua (Spain)	Diarrhoea Diarrhoea in cattle Wounds and retained placenta in livestock Chest and liver pains Malaria Antibacterial	Whole plant	Schkuhripinnatolides, Schkurianol, Thiarubrine A Thiophene, Tridecapentayne, Zaluzanin C	Ganzer, and Jakupovic, 1990; Pacciaroni *et al*.,1995)
Seriphium plumosum **(Asteraceae)** 	Slangbos (English) Vaalbos (Afrikaans) Khoikooigoed (Xhosa)	Gynaecological problems Stomach aches Intestinal worms Cardiac problems	Leaves Roots		(Synman, 2012; Snyman, 2009 http://www.plantzafrica.com)
Toona celliata **(Meliaceae)**	English(Moulmein cedar, Australian red cedar,	An astringent Tonic Anti-dysentery	Bark Leaves	Cedrelone 1,2-dihydro-cedrelone	(Rastogi, and Mehrotra, 1990; Kumar et al.,2012; http://www.mpbd.info

	Indian cedar,Indian mahogany)	Wound healing Rheumatism Antioxidant Activity	Flowers	Bergapten β-sitosterol Tetranortriterpenoid-6-deoxycedrelone Siderin Toonafolin	(Veiga *et al*.,2007;Karus *et al*., 1981)

2.7 Homeostatic maintenance of a normal glycaemic levels

The maintenance of normal blood glycaemic level is a delicate dynamic process that brings together various homeostatic control mechanisms (Pocock *et al*., 2013). These mechanisms include different organ systems such as the endocrine glands, hormonal and neural control as represented in figure 1.1 below (Pocock *et al*., 2013). These work hand in hand to strike a balance between glucose acquisition processes and glucose disposal mechanisms within the body (Pocock *et al*., 2013; Giugliano *et al*., 2008). Blood glucose levels are known to spike above normal homeostatic levels, a condition known as hyperglycaemia. Hyperglycaemia may occur due to small intestinal absorption following exogenous dietary carbohydrate intake, endogenous glyconenolysis and gluconeogenesis effected by the hormone glucagon. Blood glucose levels may also fall well below the normal homeostatic levels (hypoglycaemia). Hypoglycaemia occurs after extended periods of continued fasting, after intense exercise and as a result of insulin action. These states of hyperglycaemia and hypoglycaemia are normally short-lived (Pocock *et al*., 2013). The regulatory systems ensure a prompt restoration of normal homeostatic blood glucose levels. Prolonged states of either hypoglycaemia or hyperglycaemia which result from the improper functioning of these blood glucose level control mechanisms have adverse negative impacts on the body (Shrayyef and Gerich, 2010).

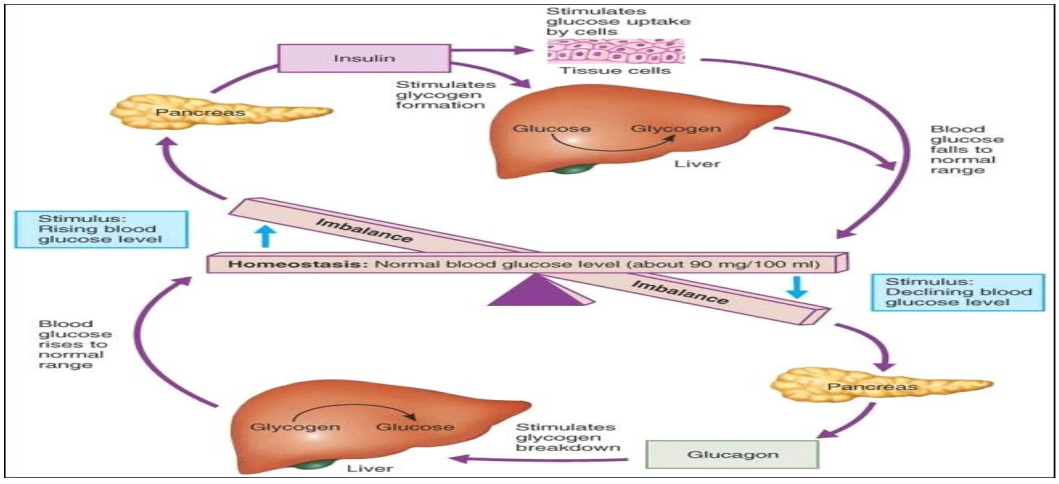

Figure 1.1: The processes that are involved in blood plasma glucose level regulation. (zhiyaobme.files.wordpress.com)

2.7.2 Blood sugar (glucose) and its importance

Glucose is a hexose aldose monosaccharide of un-paralleled importance within the body. This water soluble sweet reducing sugar naturally occurs in its D form. Glucose plays a critical role in the generation of ATP through its aerobic or anaerobic catabolism (Bergman, 1999). Certain cells within the body such as those of the brain are known to exclusively utilize glucose for ATP production (Cheeseman and Long, 2015). Glucose is also a prerequisite for the build-up of other carbohydrate conjugated moieties such as glycoproteins, proteoglycans, glycolipids and nucleic acids. There is therefore a constant demand for the supply of glucose to various cells of the body.

Glucose is acquired through various mechanisms. These help regulate blood glucose levels by increasing the amount of glucose within circulation (Rorsman and Braun, 2013). Sources of glucose include exogenous sources in which the glucose is obtained from outside the body especially from the diet. After a meal containing carbohydrates the complex polysaccharides are broken down into simpler oligosaccharides and further down into disaccharides and finally into monosaccharaides which is the end product of this digestion. The monosaccharaides are the ones which are then absorbed into the general blood circulation where they are transported to the different cells for metabolism or storage. (Shrayyef and Gerich, 2010). Glucose may also be acquired from within the body and this is termed endogenous glucose acquisition such as from glucose stores and other non-carbohydrate stores. During long fasting periods or after a bout of intense exercise the glycogen stores may be completely depleted. The body therefore has to find alternative glucose generating mechanisms. This occurs through a process known as gluconeogenesis. Gluconeogenesis is the synthesis of glucose from non-carbohydrate sources such as pyruvate, lactate, oxaloacetate, amino acids and glycerol. This process occurs primarily in the liver and in the kidneys.

2.7.3.4 Glucagon regulation of blood glucose levels

Glucagon is a hormone that is released by the alpha cells of the islets of Langerhans that are found in the pancreas. Glucagon has 29 amino acids and is derived from the proteolytic modification of pro-glucagon in a tissue specific manner (Jiang and

Zhang, 2003). It is secreted when the circulatory blood glucose levels drop below the normal range. It encourages gluconeogenesis from the liver by aiding the breakdown of the stored glycogen from the liver and making it available in the blood.

2.7.4 Glucose disposal mechanisms

Glucose disposal mechanisms are those system in the body that help regulate blood glucose levels by reducing the amount of glucose within circulation.

2.7.4.1 Glucose storage

When glucose is in abundance, after a meal with high amounts of carbohydrates, it is converted to glycogen and stored in skeletal muscles (~500g) and the liver (~100g) (Jensen *et al*., 2011). Any remaining glucose will then be stored in the adipose tissue as fat. During long fasting periods when there is shortage of glucose, these stores are utilised to provide the much needed glucose to meet the body's needs. The brain is particularly sensitive to the levels of glucose as it cannot utilise any other sugar to meet its energy requirements

2.7.4.2 Uptake of glucose on the cell surface membrane

The cell membrane is made up of a bi-lipid membrane layer which in its nature is highly hydrophobic. On the other hand, glucose has many OH groups conjugated to it making it a highly hydrophilic molecule. This poses a great challenge to the cell as its hydrophobic nature hinders the free flow of glucose into its interior. As such, glucose uptake by the cell is facilitated by a specialised group of integral proteins which span the cell membrane. These proteins create hydrophilic channels that allow the passage of glucose from the exterior of the cell to the interior of the cell (Zhao and Keating, 2007). These glucose transporters are categorised into two broad families namely the SGLT and GLUT family based on their modes of action and energy requirements (Wood and Trayhurn, 2003;Scheepers, 2004).

2.7.5 SGLT family

The sodium dependant glucose transporter family of proteins is one of the two main types of proteins that mediate the transport of glucose as well as other sugars

(Scheepers, 2004). The transport by this family of proteins is energy-coupled and is driven by the potential gradient of sodium within the cell. Glucose is co-transported into the cell using this gradient. This transporter system is mainly required for absorption and reabsorption of glucose by the body from food within the gastro-intestinal (GI) tract and from the urine in the kidneys (Wood and Trayhurn, 2003).

2.7.6 GLUT family

This is the second type of protein family that transports glucose and other related sugars. The GLUTs are a facilitative transport family that utilises the diffusion gradient of material being transported (Scheepers, 2004). They characteristically possess 12 transmembrane domains with both the N-terminus and the C-terminus on the cytosolic side (Uldry and Thorens, 2004). The different members of the GLUTs are known to exhibit different substrate specificities, kinetic properties and are expressed in different specific tissues. These proteins play a crucial role in the homeostasis of the body's blood glucose levels. Three main sub-classes (Class I, II and III) are recognised based on primary sequence similarities and characteristic elements (Wood and Trayhurn, 2003). The 14 known GLUT isoforms are categorised into these 3 classes as follows:

2.7.6.1 Class I

This facilitative class of transporters comprises the classical transporters GLUT1–4 and GLUT14. GLUT1 is the first member of the GLUTs to be purified (Baldwin,and Lienhard, 1989). It is highly expressed in the endothelial cells of the blood brain barrier and erythrocytes (Uldry and Thorens, 2004). It has also been reported to be expressed in the liver, adipose tissue and muscles in relatively smaller amounts (Mueckler and Thorens, 2013). On the other hand, GLUT2 is majorly expressed in the pancreatic beta cells where it plays a critical role in the glucose sensing mechanism which mediates the eventual secretion of insulin (Efrat and Lodish, 1994; Burcelin *et al*, 2000). It is also expressed in the kidney and liver where it mediates the transport of glucose into and out of these cells under hormonal control. GLUT3 is found in tissues which require glucose as a sole energy source such as the brain (Mantych, 1992). GLUT3 is particularly adapted for its function in these cells because of its high affinity for glucose, whereby it can still mediate glucose uptake

even in situations of low blood glucose levels (Mantych *et al*, 1992). GLUT4 is the major insulin responsive glucose transporter primarily expressed in the adipose tissue, skeletal muscle and cardiac muscle (Jewell, 2010; Fukumoto *et al*., 1989). It is found sequestrated in intracellular vesicles within the cells in which it is expressed (Richter and Hargreaves, 2013). Upon increase in blood glucose levels, insulin acts on the cell surface membrane thereby producing a cascade of events that lead to the translocation of GLUT4 intracellular vesicle to the cell surface membrane (Jewell, 2010). The vesicles immediately fuse with the membrane and produce a 10-20 fold increase in glucose uptake by the cell (Richter and Hargreaves, 2013).

2.7.6.2 Class II

This class contains the ‘‘odd’’ isoforms GLUT5, 7, 9, and 11. The fructose transporter GLUT5 is primarily expressed in the human small intestine, adipose tissues, testes and the kidneys (Corpe *et al*., 2002; Cheeseman, and Long, 2015). GLUT7 is predominantly expressed at the apical membrane of the enterocytes in the small intestine (Cheeseman, and Long, 2015), while GLUT9 is evidently expressed in the liver and kidney (Mueckler and Thorens, 2013). Two isoforms of GLUT11 are recognised which are known to transport fructose in the heart, skeletal muscle, liver, lung, trachea and the brain.

2.7.6.3 Class III

The isoforms GLUT6, 8, 10, 12 and the proton driven myoinositol transporter HMIT (GLUT13) belong to this class. They characteristically have a targeting and a glycosylation site on loop 9. GLUT6 is expressed in the spleen, leucocytes and the brain (Cheeseman, and Long, 2015), while GLUT8 is present in the testis, brain and adipose tissue. GLUT10 on the other hand is reportedly expressed in the insulin-sensitive tissues of skeletal muscle and heart (Scheepers, 2004), and GLUT12 in heart, small intestine, prostate and insulin-sensitive tissues (Mueckler and Thorens, 2013).

2.8 Insulin regulated glucose uptake

Insulin is an important hormone within the body as it is responsible for normal growth, appropriate tissue differentiation and development, apoptosis and glucose

homeostasis. While in circulation, insulin is also responsible for supressing hepatic glucose output. It is secreted by the beta cells of the islets of Langerhans that are located within the pancreas. Insulin is secreted in an inactive form known as pro-insulin. Pro-insulin is a single chain amino acid composed of 148 amino acids divided into A,B and C sub-chains as illustrated in figure 2 below. The C chain is cleaved off during post-translational modification to yield equimolar amounts of an active insulin molecule. The molecule is composed of an A and a B chain that are bridged together by two disulphide bonds (Figure 2).

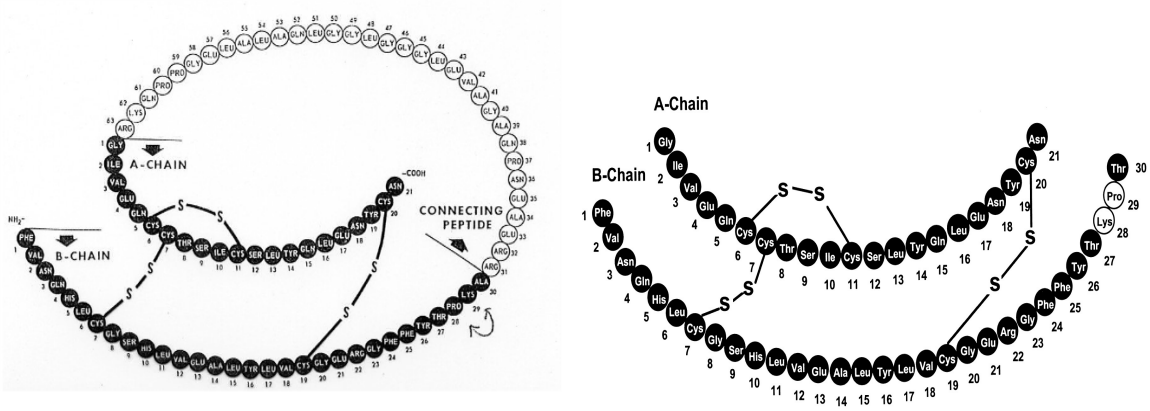

Figure 2: Pro-insulin with the A-chain and B-chain connected by the C-peptide (Left). Mature insulin after cleavage of the C-chain, showing the A-chain and B-chain held together by 2 di-sulphide bonds (Right).

2.9 Stimulation of insulin release

Insulin is secreted during high blood glucose levels and exhibits its function by performing two main activities (Rorsman and Braun, 2013). Insulin secretion is increased by elevated post prandial blood glucose concentrations, gastrointestinal hormones and β adrenergic stimulation. High glucose concentrations are sensed by a constitutively expressed glucose transporter GLUT 2 present on the cell surface membrane of the pancreas. GLUT 2 has a low affinity for glucose (KM ~11 mM) (Rorsman and Braun, 2013).It therefore transports glucose into the pancreas only during periods when there is high circulatory glucose concentrations, for example after a meal rich in carbohydrates (Rorsman and Braun, 2013). The increased intracellular glucose increases the ATP: ADP ratio via the mitochondrial oxidative phosphorylation pathway. Increased intracellular ATP concentration results in a closure of ATP-dependent potassium channels which in-turn depolarises the cell.

Cell depolarisation results in increases Calcium influx via voltage-dependent calcium channels. The overall effect is an increased intracellular calcium concentration which signals SNARE protein complex formation which facilitates insulin granule release as illustrated by figure 3 below (Jewell, 2010).

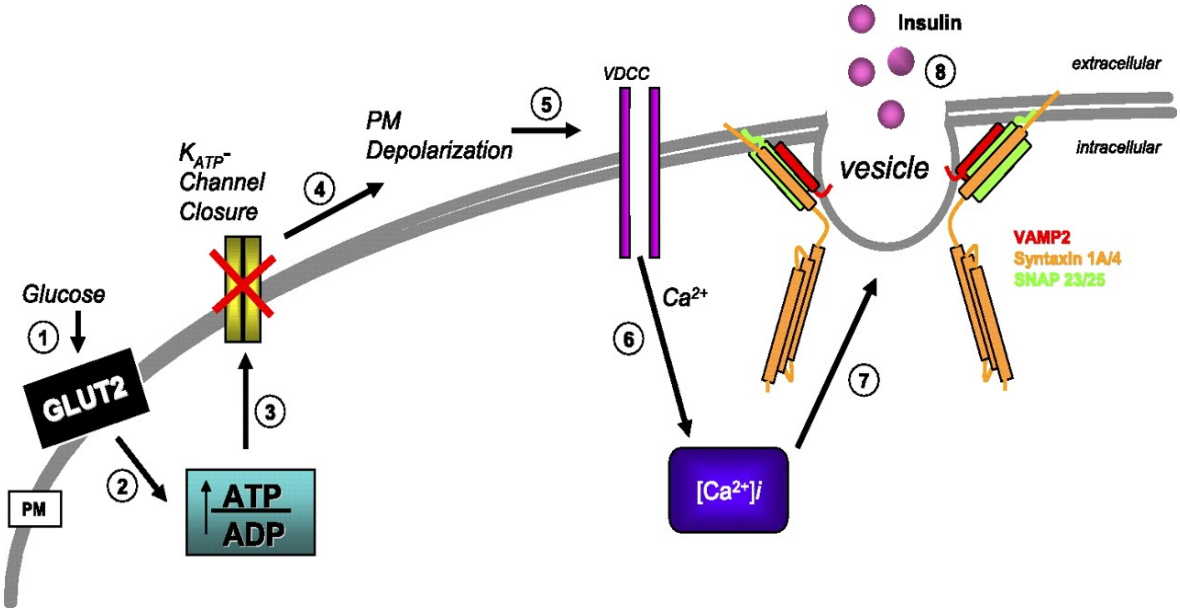

Figure 3: The calcium mediated insulin secretion pathway. The picture adopted from Jewell, 2010.

2.10 Insulin action/pathway

Insulin increases the uptake of blood glucose from circulation into mainly the muscle and adipose cells through an intrinsic pathway illustrated in figure 4 below (Jewell, 2010). Upon insulin binding to the extracellular alpha-subunits of the Insulin Receptor (IR) located on the surface membrane of its target cells, it causes auto-phosphorylation of its beta sub-unit thereby activating its intrinsic tyrosine kinase. Phosphorylated Insulin Receptor tyrosine kinase act on a number of proteins including the insulin receptor substrate (IRS1/2). Tyrosine-phosphorylated IRS proteins serve as docking proteins for the regulatory subunit (p85) of the phosphatidylinositol (PI) 3-kinase (Jewell, 2010). Once phosphatidylinositol (PI) 3-kinase is bound to IRS-1 or IRS-2, it activates the catalytic (p110) subunit of PI 3-kinase. Phosphatidylinositol PI 3-kinase is involved in the generation of a lipid product known as phosphatidylinositol 3,4,5-trisphosphate (PIP_3) which in-turn activates 3'-phosphoinositide-dependent protein kinases (PDK1/2). Activated 3'-phosphoinositide-dependent protein kinases (PDK1/2) are responsible for the

phosphorylation of phosphorylate Akt (Thr308 and Ser473) and PKC-zeta/lamda (Thr410). AKT phosphorylates AS160, and AS160 targets multiple Rabs present on GLUT4-containing vesicles, although the precise mechanisms beyond this remain unclear (Jewell, 2010). Vesicle fusion occurs via the SNARE proteins, resulting in GLUT4 integration into the plasma membrane (PM) to facilitate glucose uptake. Increased glucose uptake is directly linked to translocation of GLUT 4 to the cell surface membrane (Figure 4).

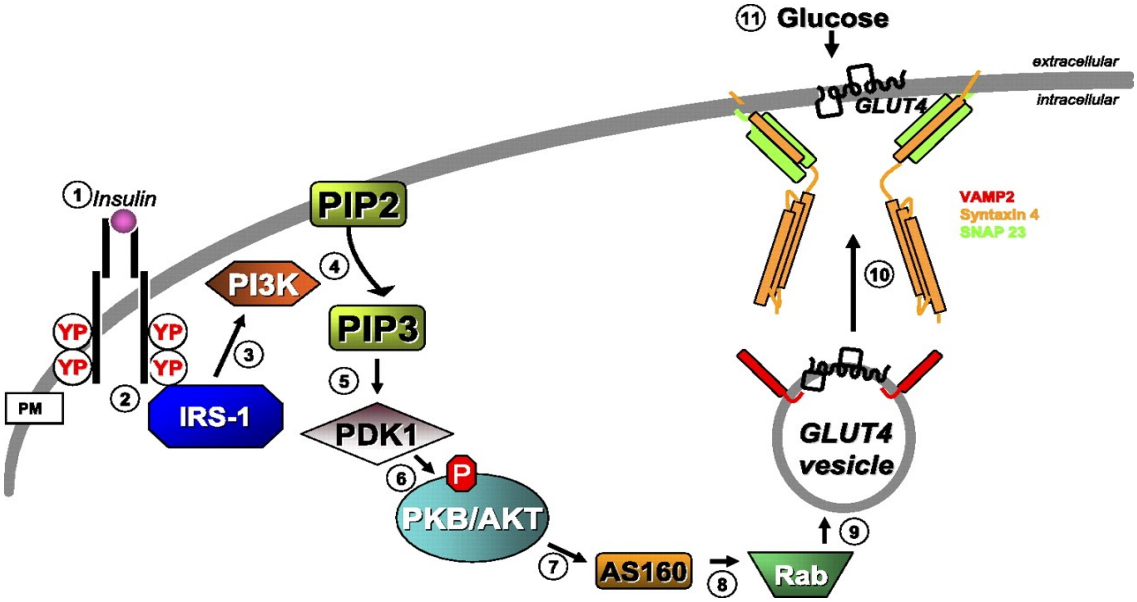

Figure 4: The insulin dependent GLUT4 translocation pathway. The picture adopted from Jewell, 2010.

Insulin acts primarily by mobilising the glucose transporter GLUT4 from its vesicular stores within the cell to the cell surface membrane were they facilitate the uptake of glucose from the blood into the cell where they are metabolised or stored. It suppresses the action of its counter hormone glucagon.

2.11 Problem statement

According to the American Diabetes Association (2010), diabetes mellitus is a metabolic disorder characterised by the body's inability to maintain proper homeostatic levels of various biomolecules, particularly glucose. The development of this condition is attributed to many factors which include genetic predisposition, environmental ills, insulin resistance and obesity (Ginter and Simko, 2013). The last two afore mentioned factors are largely inter-twined with the lifestyle of individuals

particularly those that reside in urban areas who are known to induldge more in unhealthy habbits such as heavy alcohol drinking, lack of physical activity, unhealthy diets and so forth (Peer *et al.,* 2013). The International Diabetes Federation (2011) reported that due to the rise in global urbanisation, the number of people affected by diabetes is projected to rise to about 552 milion globally by the year 2030 (Shaw *et al.,* 2010). The situation is further aggravated by the fact that there is currently no known cure for diabetes (Olokoba *et al.,* 2012). Current treatment of the condition either have several undesirable side effects, or are far too expensive for many people particularly in the underdeveloped and developing countries (Whiting *et al*., 2011). People therefore resort to more affordable and readily available management strategies such as the use of traditional medicinal plants. Regretably, worrisome concerns are raised as the modes of action and toxicology profiles of most medicinal plants remain largely unknown.

2.12 Rationale or motivation

Diabetes is one of the fastest growing epidemics. The management strategies currently being employed have proven to be ineffective, expensive and have many undesirable side effects (Marles and Farnsworth, 1995). Plants on the other hand have been employed for generations to help manage different conditions including diabetes. Plants have been used for effective management with relatively less side effects as they work in harmony with the natural metabolism of the body. It is hence prudent for research efforts to be directed towards the identification of phyto-compounds with possible potential as candidates for further development into commercial drugs which can be used in mono-therapy or in combination with other drugs to help effectively manage or treat diabetes.

2.13 Aim of the study

This study is aimed at determining the anti-diabetic effects of identified medicinal plants with particular emphasis directed on their ability to augment the translocation of GLUT4 molecules in C2C12 murine muscle cells.

2.13.1 The objectives of this study are to:

i. Identify and quantify the phyto-chemical constituents within the selected plants.

ii. Quantify the antioxidant and anti-glycation potential of the selected plants.

iii. Analyse the cytotoxic profiles of the plant extracts on mouse muscle (C2C12) cells.

iv. Investigate the effects of the plant extracts on glucose uptake by mouse muscle (C2C12) cells *in vitro*.

v. Determine the effect of the plant extracts on GLUT4 translocation from its cytosolic vesicular store to the cell surface membrane of mouse muscle cells C2C12 *in vitro*.

vi. Investigate the effects of the plant extracts on the phosphorylation of proteins involved in the glucose uptake pathway.

CHAPTER 3

Methods and Materials

3.1 Plant material

3.1.1 Plant collection and verification

Leaves of five selected plants (*Toona ciliata, Seriphium plumosum, Schkuhria pinnata, Olea africana, Opuntia ficus-indica*) were collected from Mankweng area, Capricon Local Municipality, Limpopo province, South Africa. The plants were selected based on literature surveys of reports of their anti-diabetic properties by traditional healers and village elders in the Limpopo province. One species of each of the selected plants was sampled; each was from the same or different soil strata. The identities of selected plants were authenticated by Dr B Egan a curator from the Larry Leach Herbarium, University of Limpopo.

3.1.2 Plant extracts preparation

Air dried plant materials were ground into fine powder using a domestic warring blender. Powdered plant material (1 g) was then exhaustively extracted using 10 ml each of methanol, acetone and hexane (Elloff, 1998). The supernatants were filtered using a Whatman No.1 filter paper into pre-weighed glass vials. The extracts were then dried under a stream of air. The quantity of plant materials extracted was determined and stored in air-tight glass vials in the dark until use. The dry plant extracts were reconstituted in dimethylsulphoxide (DMSO) (Sigma Aldrich ™, SA) for all cell based assays or in acetone for any other assay.

3.2 Qualitative phytochemical analysis

Qualitative phytochemical analyses of the different extracts were performed using thin layer chromatography according to Kotze and Eloff (2000). The presence of different phytochemicals in the methanol, acetone and hexane extracts of the different extracts were analysed by thin layer chromatography (TLC). Different mobile phases were prepared i.e. EMW (ethyl acetate, methanol and water-10:5.4:4, v/v/v), CEF (chloroform, ethyl acetate and formic acid-10:8:2, v/v/v) and TEA (toluene, ethanol and ammonium hydroxide-18:2:0.2, v/v/v).

Silica-gel coated aluminium plates (Sigma Aldrich ™, SA) were used as the stationary phase. Stock solutions of 10 mg/ml each of the extracts was prepared by re-dissolving the dry extract samples in acetone. TLC plates were spotted with 10 µl of the samples and eluted in the different mobile phases.

Following elution, plates were observed under an ultraviolet light at wavelengths of 254 and 365 nm in the dark and the fluorescent bands were marked with a pencil. The plates were then sprayed with freshly prepared acidic vanillin solution (0.1 g vanillin, 28 ml methanol and 1ml sulphuric acid) and heated in an oven at 110°C for maximum colour development (Stahl, 1969).

3.3 Qualitative antioxidant analysis using 2,2-diphenyl-1-picrylhydrazyl DPPH

Another set of plates were prepared as previously described in section 3.3. After drying, the plates were sprayed with 0.2% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol solution to detect the presence of compounds with antioxidant activity. A positive result was represented by the presence of yellow bands on a purple background. The images were captured using a scanner (CanoScan Lide 700F) (Deby and Magotteaux, 1970).

3.4 Plant secondary metabolites analysis

The presence of different plant secondary metabolites in the crude extracts was determined using various standard assays detailed in table 3.1 below (Harborne, 1973).

Table 3.1: Standard secondary metabolite identification assays.

Phyto-constituent	**Test**	**Observation**
Tannins *(Braymer's Test)*	2ml extract + 2ml H_2O + 2-3 drops $FeCl_3$ (5%)	Green precipitate
Flavonoids	1 ml extract + 1 ml $Pb(OAc)_4$ (10%)	Yellow coloration
Phenols	2ml extract 2ml of 2% $FeCl_3$.	Blue/black colouration
Saponins	(a) 5 ml extract + 5 ml H_2O + heat	Froth appears

	(b) 5 ml extract + Olive oil (few drops)	Emulsion forms
Steroids *(Salkowski Test)*	2 ml extract + 2 ml $CHCl_3$ + 2 ml H_2SO_4 (conc.)	Reddish brown ring at the junction
Phlobatannins (*Precipitate Test)*	2 ml extract + 2 ml HCl (1%) + heat	Red precipitate
Glycosides *(Liebermann's Test)*	2 ml extract + 2 ml $CHCl_3$ + 2 ml CH_3COOH	Violet to Blue to Green coloration
Coumarins	2 ml extract + 3 ml NaOH (10%)	Yellow coloration
Proteins *(Xanthoproteic Test)*	1 ml extract + 1 ml H_2SO_4(conc.)	White precipitate
Anthraquinones *(Borntrager's Test)*	3 ml extract + 3 ml Benzene + 5ml NH_3(10%)	Pink, Violet or Red coloration in ammonical layer
Anthocyanins	2 ml extract + 2 ml HCl (2N) + NH3	Pinkish red to bluish violet coloration
Leucoanthocyanins turns	5 ml extract + 5 ml Isoamyl alcohol	Organic layer into Red
Carbohydrates	2 ml extract + 2 ml iodine	A dark blue or deep purple coloration

3.5 Total phenolic content

The total phenolic content of the different extracts was determined spectrophotometrically using the Folin-Ciocalteu`s phenol reagent method according to (Humadi and Istudor, 2009*).* Stock solutions (100 mg/ml) of each of the different extracts were prepared. Folin-Ciocalteu reagent (50 µl) and distilled water (450 µl) were added to each of the extracts (100 µl) and left for 5 minutes in the dark at room

temperature. There after 7% sodium carbonate (500 µl) solution was added. Distilled water was added to make a final volume of 5000µl and the mixture allowed to stand for 90 minutes in the dark at room temperature. Absorbance of the mixture in triplicates was measured at 750 nm using a spectrophotometer (Beckman Coulter-DU730). The total phenolic content was determined by linear regression from a tannic acid calibration curve standard.

3.6 Total flavonoid content

Aluminium chloride colorimetric method was used for determination of total flavonoids (Chang *et al.,* 2002). A stock solution (10 mg/ml) of each of the different extracts was prepared. Each of the extracts (100 µl) was mixed with 10% aluminium chloride (100 µl), 1M potassium acetate (100 µl) and distilled water (2800 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.

3.7 Determination of anti-glycation activity

Antiglycation activity of the plant extracts were determined using the bovine serum albumin assay with slight modification (Matsuura *et al.,* 2002). Bovine Serum Albumin (Sigma–Aldrich) (500 µl) was incubated with glucose (400 µl), and plant extracts (100 µl), Phosphate buffer saline (100 µl) was used as the sample control and Arbutin (100 µl) (Sigma–Aldrich) as the reference standard. A negative control constituting of BSA (500 µl), phosphate buffer saline (400 µl) and plant extracts (100 µl) was included. The reaction was allowed to proceed at 60°C for 72 hours and was terminated by addition of 10 µl of a 100% (w/v) trichloroacetic acid (TCA) (Sigma–Aldrich). The TCA added mixture was kept at 4°C for 10 minutes and thereafter centrifuged for 4 minutes at 13000 rpm. The precipitate was re-dissolved with alkaline phosphate buffer saline (pH 10) and quantified for the relative amount of glycated BSA, based on fluoresce intensity, in black 96 well plates using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). The excitation

and emission wavelength used were at 370 nm and 440 nm respectively. Five concentrations of each sample was analysed in triplicate. Percentage of inhibition was calculated using the formula provided below and the sample concentration required for 50% inhibition of BSA glycation was calculated using linear regression.

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

3.8 Quantitative DPPH Radical-Scavenging Activity Assay

The antioxidant activity of each of the different extracts was quantitatively determined spectrophotometrically using the DPPH free radical scavenging assay (Deby and Magotteaux, 1970). Equal volumes of 0.2% DPPH in methanol and different concentrations (0 µg/ml to 1000 µg/ml) of the extracts were incubated in the dark at room temperature for 30 minutes. The DPPH in methanol solution was used as the experimental control, L-ascorbic acid (Vitamin C) as 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 Multi detection system). The degree of discoloration 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:

$$\text{Percentage inhibition} = \frac{((A_{490nm} \text{ of blank} - A_{490nm} \text{ of sample}) \times 100)}{(A_{490nm} \text{ of blank})}$$

3.9 Ferric ion reducing power

The ferric ion reducing power of the different extracts were determined. Various concentrations (0 µg/ml to 1000 µg/ml) of the extracts in deionised water (100 µl) were prepared. A blank was prepared without adding extract, while ascorbic acid was used as the reference standard. These were then mixed with phosphate buffer (250 µl) (pH 7.4 and concentration 0.2 M) together with potassium ferri-cyanide (250 µl) and incubated at 50°C for 20 minutes. After incubation, aliquots of trichloroacetic acid (250µl) were added to the mixture and centrifuged at 1000 x g for 10 minutes. The supernatant (250 µl) was mixed with distilled water (250 µl) and freshly prepared

ferric chloride solution (50 µl). The absorbance of the samples was measured at 700 nm. Substances, which have reduction potential, react with potassium ferri-cyanide (Fe^{3+}) to form potassium ferro-cyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric-ferrous complex that has an absorption maximum at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power which 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 formula:

$$\text{Percentage reducing power} = \frac{((A_{700nm} \text{ of sample} - 1) \times 100)}{(A_{700nm} \text{ of blank})}$$

The effective concentration (EC_{50}) values, which represent concentrations eliciting a 50% response, was determined by regression, from linear plots of concentration of the extract against the mean percentage of the antioxidant activity from three independent experiments. A low EC_{50} value represent a more effective reducing power. Expiriments were done in triplicates in three independent trials.

3.10 Cell culture

An immortalised mouse myoblast cell line (C2C12) was used in this study (ATCC, Rockville, USA).The cells were cultured and maintained in RPMI media (Lonza, BioWhittaker®), supplemented with 10% foetal bovine serum (Hyclone, Thermo Scientific) at 37 ˚C, in an atmosphere of 5% CO_2 in a humidified incubator (Heracell 150i CO_2 incubator, Thermo Scientific). The cells were differentiated by culturing them in RPMI media containing 2% horse serum for 4 days.

3.11 Cytotoxicity test – MTT cell viability assay

The cytotoxicity of the different plant extracts on C2C12 cell line were determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich ™, SA) as modified by (Ferrari *et al.,* 1990). Expiriments were done in triplicates in three independent trials. Cells were seeded at an initial cell density of $2x10^5$ cells/ml into 96-well cell culture plates (Nunc™, Roskilde, Denmark). The adherent cell lines were incubated overnight to allow the cells to attach. The cells were treated or not with different concentrations (0 µg/ml to 1000 µg/ml) of the different extracts. The untreated cells served as the experimental control.

Actinomycin (Sigma Aldrich ™) and DMSO served as positive and negative controls respectively. The plates were incubated at 37 °C for 24 hours after which MTT (10µl) was added to each well. The cells were further incubated at 37 °C for 2 hours. The medium was aspirated and the cells were washed once with pre-warmed PBS, pH 7.4. The insoluble purple coloured formazan formed intra-cellularly by the action of the mitochondrial dehydrogenase of viable cells following reaction with MTT was solubilised using DMSO (100 µl). The absorbance was measured at 490 nm using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). The percentage of viable cells was calculated according to the following formula:

$$\text{Percentage viability} = \frac{(A_{490nm} \text{ of sample}) \times 100)}{(A_{490nm} \text{ of control})}$$

3.12 Glucose uptake assay

The amount of glucose taken up by differentiated C2C12 cells was quantified using the glucose uptake kit according to the manufacturer's instructions (KAT Laboratories and Medicals (PTY) LTD). Cells at an initial seeding density of $5x10^4$ were treated for 1, 3 and 24 hours in the presence or absence of the different plant extracts. Untreated cells were used as the experimental control, while insulin and DMSO were used as positive and negative controls respectively. After treatment the media (supernatant) (1 µL) from each of the treatments, including the control was transferred into a new 96 well flat bottomed plate and then working reagent (100 µL) was added, protected from light. The mixture was incubated in the dark at 37°C for 5 minutes. Absorbance at 500 nm was immediately read using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). Experiments were done in triplicates in three independent trials.

3.13 GLUT4 translocation assay

The cells were differentiated by culturing them in RPMI media containing 2% horse serum for 4 days. The differentiated C2C12 cells were seeded at a density of $1x10^5$ in 6 well plates. The cells were treated with selected concentrations of the plant extracts for 3 hours. Insulin was used as a positive control and DMSO was used as a negative control. After treatment, the cells were washed three times with 1X phosphate-buffered saline (PBS) and fixed with 80% methanol for 15 minutes and washed three times with 1X PBS. Cells were then incubated with 4',6-diamidino-2-

phenylindole (DAPI) for 30 minutes and thereafter washed three times with 1X PBS. Cells were blocked for non-specific binding using Bovine Serum Albumin (1mg/ml) (Sigma-Aldrich, S.A) for 30 minutes and washed three times with 1X PBS. The cells were then incubated with rabbit-anti-GLUT4 primary antibody diluted 1:200 and thereafter washed three times with 1X PBS. The cells were then incubated with goat anti-rabbit-secondary antibody diluted 1:10000 conjugated to FITC for 1 hour and viewed using a fluorescence microscope and overlay images were captured.

3.14 MAPK profiler assay

The expression of various mitogen activated protein kinases in the cells was determined using the human MAPK profiler assay Kit according to the manufacturer's instructions (RnDSystems). Differentiated C2C12 cells at a density of $6x10^7$ cell/ml were seeded in 25 cm^3 cell culture flasks and treated for 3 hours in the presence or absence of plant extract at the given concentrations (0 µg/ml and 200 µg/ml). Insulin (50 ml/U) was used as a positive control. The cells were immediately rinsed with PBS after which lysis buffer 6 was added. The re-suspend cell lysates were then rocked gently at 2-8°C for 30 minutes. The lysates were thereafter, centrifuged at 14,000 x g for 5 minutes, and the supernatant transferred into a clean eppendorf tube. The total protein quantity was immediately determined using the BCA protein assay.

Following quantification array, buffer 5 (2 ml) was pipetted into each well of the 4 well plate where it served as a block buffer. Using flat-tip tweezers, the membranes were placed in separate wells of the 4 well plate with the number on the membrane facing upward and incubated for 1 hour on a rocking platform shaker. The samples were prepared by adding up to 400 µL of each sample to separate eppendorf tubes and adjusting the volume to 1.5 ml using array buffer 1. To each eppendorf tube reconstituted detection antibody cocktail (20 µL) was added and incubated for 1 hour. Array buffer 5 was carefully aspirated from the wells of the 4 well plate and the prepared sample/antibody mixtures were gently added and incubated overnight at 4°C on a rocking platform shaker.

The membranes were carefully removed and placed into individual plastic containers containing 1X wash buffer (20 ml) and washed 3X for 10 minutes on a rocking platform shaker. The membranes were carefully placed into each of the 4 well plate

containing the diluted Streptavidin-HRP (2 ml) and incubated for 30 minutes at room temperature on a rocking platform shaker. After incubation the membranes were washed 3 times with 1X wash buffer. Chemi Reagent Mix (1 ml) was added evenly onto each membrane. The membranes were then washed using TBST and the transferred proteins were detected using the Super Signal West Dura chemiluminescent substrate (Thermo scientific, U.S.A) and antigen antibody complex was visualised by photo-detection using the Syne-Gene Image analyser (Bio-Rad S.A).

3.15 Statistical analysis

The results obtained from three independent experiments were expressed as means ± standard deviation. The statistical significance of the results was tested using One-way Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p < 0,05$, two asterisks(**) for $p < 0,01$ and three asterisks(***) for $p < 0,001$.

CHAPTER 4

Plant extracts preparation and qualitative/quantitative phytochemical analysis

4.1 Introduction

The use of plants for therapeutic purposes has been traditionally practised since time immemorial. People that are well versed in the practise of traditional medicine are known as traditional healers. Traditional healers have the know-how of preparing various plant based remedies for their patients. This knowledge is acquired through word of mouth, practical experience and observation from their predecessors. For minor afflictions such as simple colds and headaches the traditional healers may prepare a remedy from a single plant extract. More complex conditions such as sexually transmitted infections and chronic diseases often warrant the preparation of a decoction from a mixture of various plants.

Herbal formulae containing multiple plants may have synergistic effects. These are administered in various ways; some may be taken in orally, applied externally, inhaled or infused directly into the blood stream. The medication is administered until there is remission of the apparent signs and symptoms. Although the efficacies of these medicines are tied to spirituality in many cultures, they are highly dependent on the experience of the traditional healer and the quality of the plants or parts used. The quality of the plant material is determined by various factors including geographical location, season and ecology (Liu *et al.,* 2015).

It is scientifically known that each plant consists of various compounds, only a few of which may be therapeutically effective (Atal, 1983; Farnsworth, 1990). The therapeutic nature of plants emanates from specialised plant products known as secondary metabolites. Most of the therapeutic secondary metabolites belong to the following groups of compounds; tannins, flavonoids, phenolic compounds, steroids and terpernoids. Different plant parts have different compound ingredient and concentration profiles (Prabhakar and Doble, 2011). Prior to being administered to a patient or being characterised and assayed for bioactivity in a laboratory set-up, these compounds have to be extracted from the plants (Sasidharan *et al.,* 2011). Different extraction methods exist and these include serial exhaustive extraction, maceration, steam distillation, sonification and soxhlet extraction (Sasidharan *et al.,* 2011). These extraction methods should take into account the thermal stability of the

various target compounds. The material to be extracted is firstly pre-washed, weighed and dried. It is then ground into fine powder for homogeneity and to increase the surface area to volume ratio for effective extraction. Different extraction solvents are employed which vary in their polarity. Polar solvents such as water, ethanol and methanol will extract hydrophilic compounds while none-polar solvents such as chloroform and hexane would extract lipophilic compounds (Azmir *et al.,* 2013). Water is by far the most commonly employed extraction solvent in traditional medicine. Different extraction solvents and methods yield different active ingredients. This chapter aims to outline how the extraction of selected plants (Table 2.1) was carried out. Following the extraction, both qualitative and quantitative phyto-chemical analysis was carried out. The antioxidant potential was then asseyed.

4.2 Objectives:

- **i.** Extraction of plant material using solvents of varying polarity (Methanol, Acetone and Hexane)
- **ii.** Qualitatively ascertain the presence or absence of various secondary metabolite
- **iii.** Determine the qualitative finger-print phytochemical profiles on thin layer chromatography plates
- **iv.** Quantify the total phenolic and total flavonoid content in the plant extracts
- **v.** Quantification of the anti-oxidant potential of the plant extracts using the DPPH and FRAP assay.

4.3 Methods

4.3.1 Plant extraction and yield quantification

After collection, the plants were pre-washed and extracted as explained in detail in section 3.2.2. Dry powdered plant material (1 g) was macerated using 10 ml each of methanol, acetone and hexane (Eloff, 1998). The supernatants were filtered using a Whatman No.1 filter paper into pre-weighed glass vials. The extracts were then dried under a stream of air. The quantity and percentage yield of plant materials extracted was determined.

4.3.2 Qualitative phytochemical analysis

4.3.2.1 Secondary metabolite analysis

The presence of different plant secondary metabolites in the crude extracts was determined using various standard chemical tests (Harborne, 1973) as explained in detail in section 3.5.

4.3.2.2 Thin layer chromatography

Qualitative phytochemical analyses of the different extracts were performed using thin layer chromatography according to Kotze and Eloff (2000) as explained in detail in section 3.3. The presence of different phytochemicals in the methanol, acetone and hexane extracts of the different extracts were analysed by thin layer chromatography (TLC). Stock solutions of 10 mg/ml of each of the extracts were prepared by re-dissolving the dry extract samples in acetone. TLC plates were spotted with 10 µl of the samples and developed in the different mobile phases.

4.3.3 Quantitative phytochemical analysis

4.3.3.1 Total phenolic content

The total phenolic content of the different extracts was determined spectrophotometrically using the Folin-Ciocalteu`s phenol reagent method according to (Humadi and Istudor, 2009) as described in section 3.6. The total

phenolic content was determined by linear regression from a tannic acid calibration curve standard.

4.3.3.2 Total flavonoid content

Aluminium chloride colorimetric method was used for determination of total flavonoids (Chang *et al*., 2002) as described in section 3.7. The total flavonoid content was interpolated from a linear quercetin calibration curve standard.

4.3.4 Qualitative antioxidant analysis

Qualitative antioxidant analyses of the different extracts were performed using thin layer chromatography according to Deby and Magotteaux, (1970) as explained in detail in section 3.4.

4.3.5 Quantitative antioxidant analysis

The effective concentration (EC_{50}) values, which represent concentrations eliciting a 50% response, were determined by regression, from linear plots of concentration of the extract against the mean percentage of the antioxidant activities described below from three independent experiments.

4.3.5.1 Quantitative DPPH radical-scavenging activity assay

The antioxidant activity of each of the different extracts was quantitatively determined spectrophotometrically using the DPPH free radical scavenging assay (Deby and Magotteaux, 1970) as described in section 3.9.

4.3.5.2 Quantitative Ferric reducing power assay

The ferric ion reducing power of the different extracts were determined as described in section 3.10.

4.4 Results

4.4.1 Plant extraction percentage yield

The percentage yields of extracts of different plant material using solvents of varying polarity namely; methanol, acetone and hexane are presented in the graph below (Figure 4.1). These were calculated by taking the dry mass extracted by each of the solvents as a percentage of the total initial dry plant material weight. The extraction was done by maceration of the plant in the solvent at a ratio of 1g of plant material to 10ml solvent. Methanol had the highest extraction percentage yield while hexane extracted the least amounts for all the plants. The methanol extract of *Toona ciliata* (TCiM) had the highest percentage yield of 20.83% while the hexane extract of *Schkuhria pinnata* (SPiH) had the least percentage yield of 0.64%.

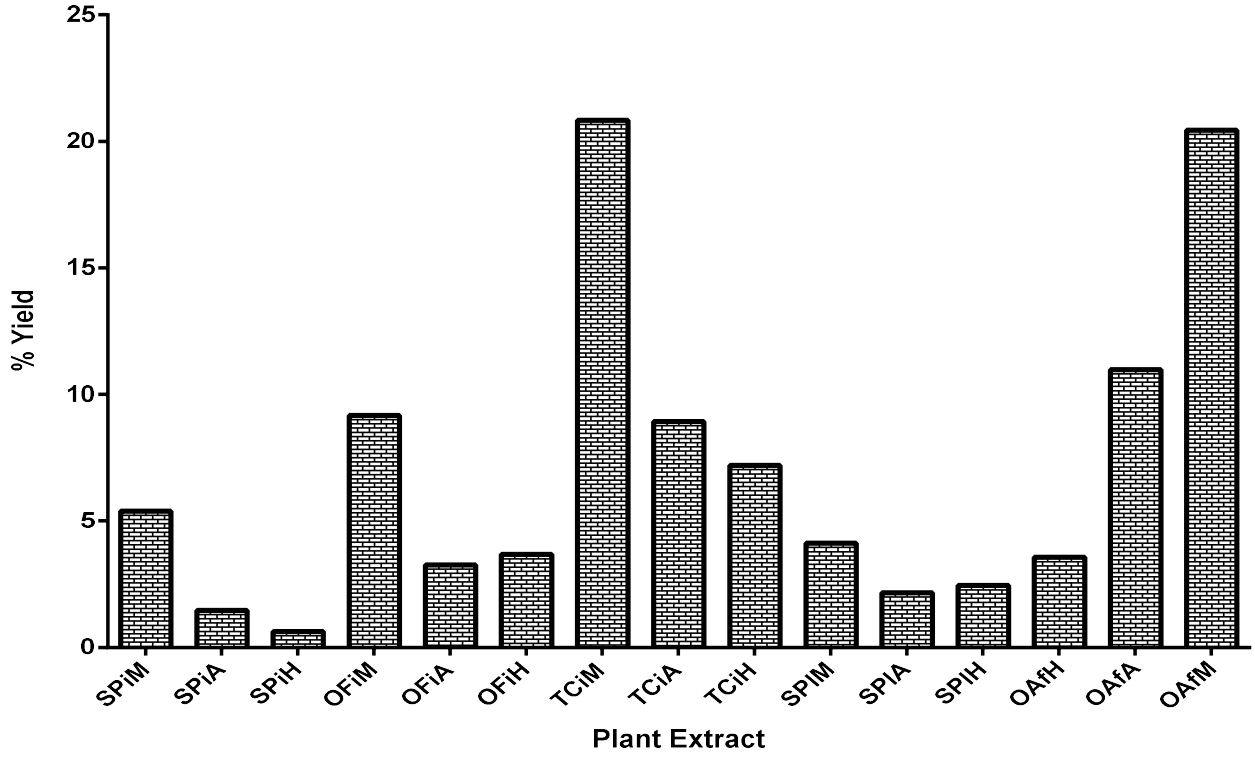

Figure 4.1: Percentage yields of the plant extracts obtained using solvents of varying polarity. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract) OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract),SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH *Schkuhria pinnata* (Hexane extract),OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract),TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract)

4.4.2 The qualitative finger-print phytochemical and UV active compound profiles on thin layer chromatography plates

Thin layer chromatography (TLC) plates below (Figure 4.2 to Figure 4.6) show the stable phyto-compounds that are present in each of the plant extracts. The compounds migrate according to their polarity in each of the mobile phases as well as their affinity to the stationary phase (silica gel). Plates labelled pA show the compounds that were developed by spraying the plates with vanillin sulphuric acid. Plates labelled pB show the compounds that are fluorescent in the presence of Ultra-Violet (UV) light at a wavelength of 360nm. Plates labelled pC show the compounds that are fluorescent in the presence of Ultra-Violet (UV) light at a wavelength of 254. The band intensities reflect the relative concentration of that compound within the extract. BEA and CEF better resolved the individual compounds within most of the extract as compared to EMW.

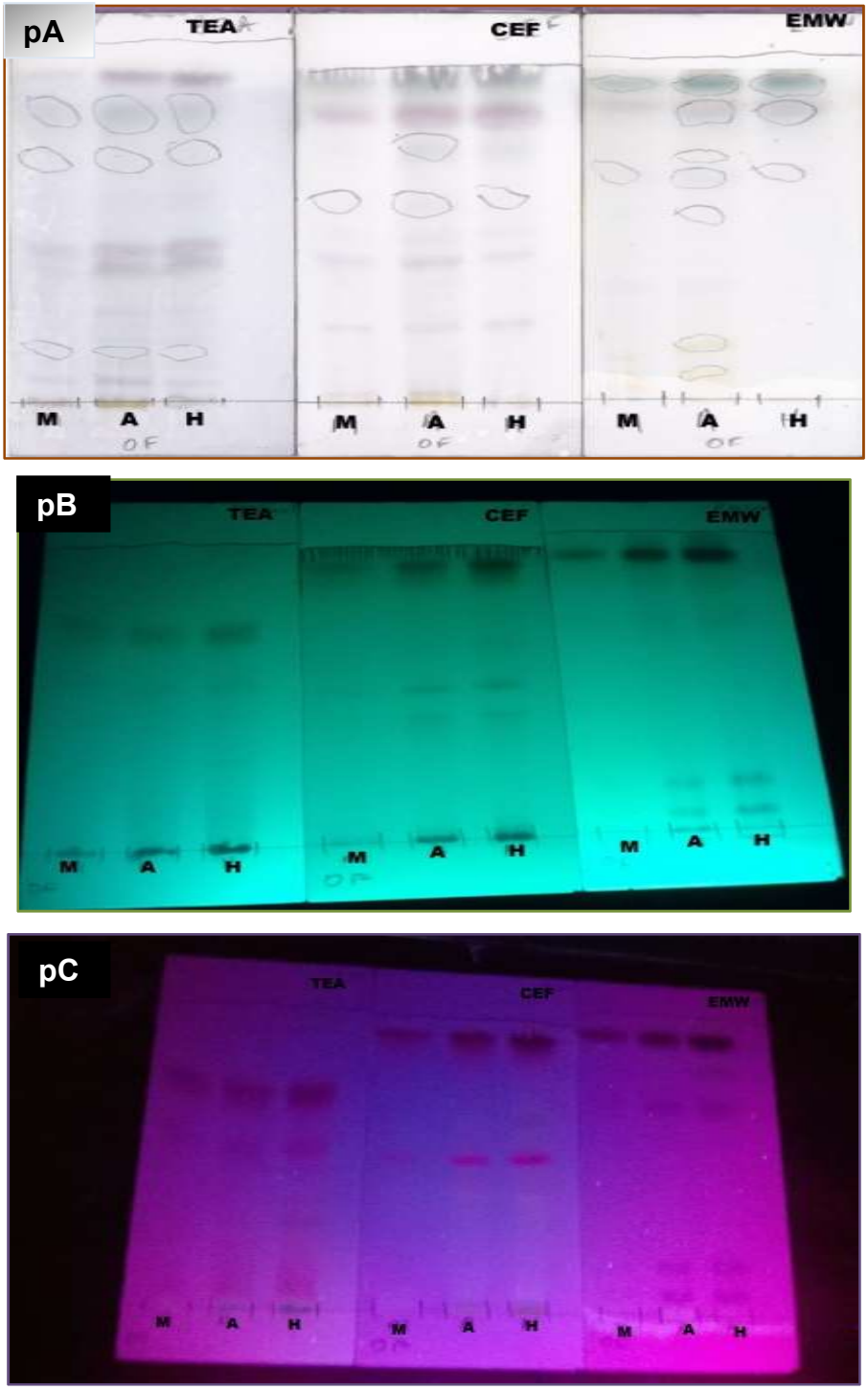

Figure 4.2:TLC finger print profile and UV active compounds of different extracts of *Opuntia ficus indica*. M=Methanol extrctact,A=Acetone extract,H=Hexane extract. pA= plates sprayed with acidic vanilin , pB= plates viewed using UV light at 254nm, pC= plates viewed using UV light at 365nm eluted in TEA, CEF and EMW solvent systems

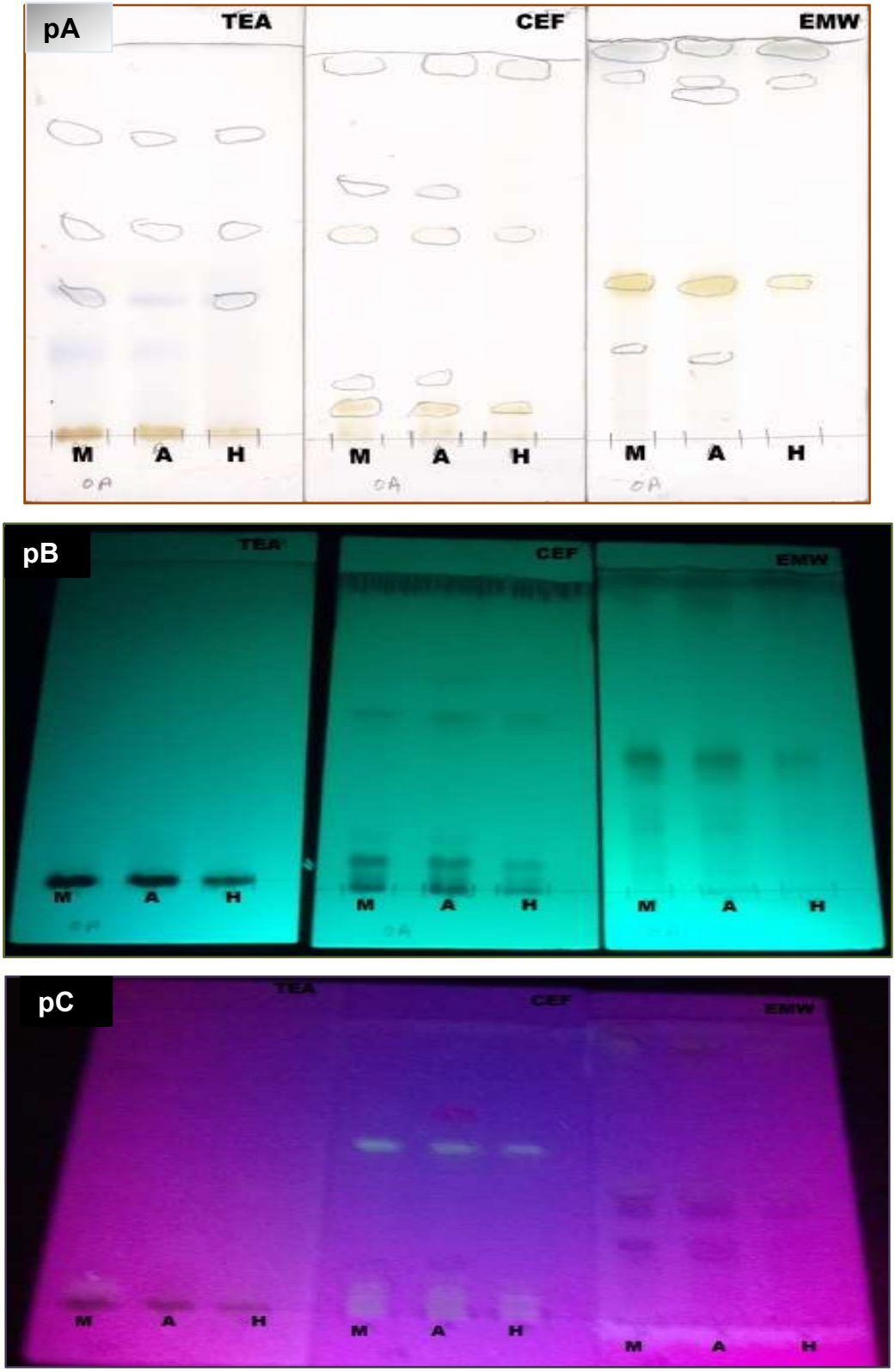

Figure 4.3 :TLC finger print profile and UV active compounds of different extracts of *Olea africana*.M=Methanol extrctact,A=Acetone extract,H=Hexane extract. pA= plates sprayed with acidic vanilin , pB= plates viewed using UV light at 254nm, pC= plates viewed using UV light at 365nm eluted in TEA, CEF and EMW solvent systems

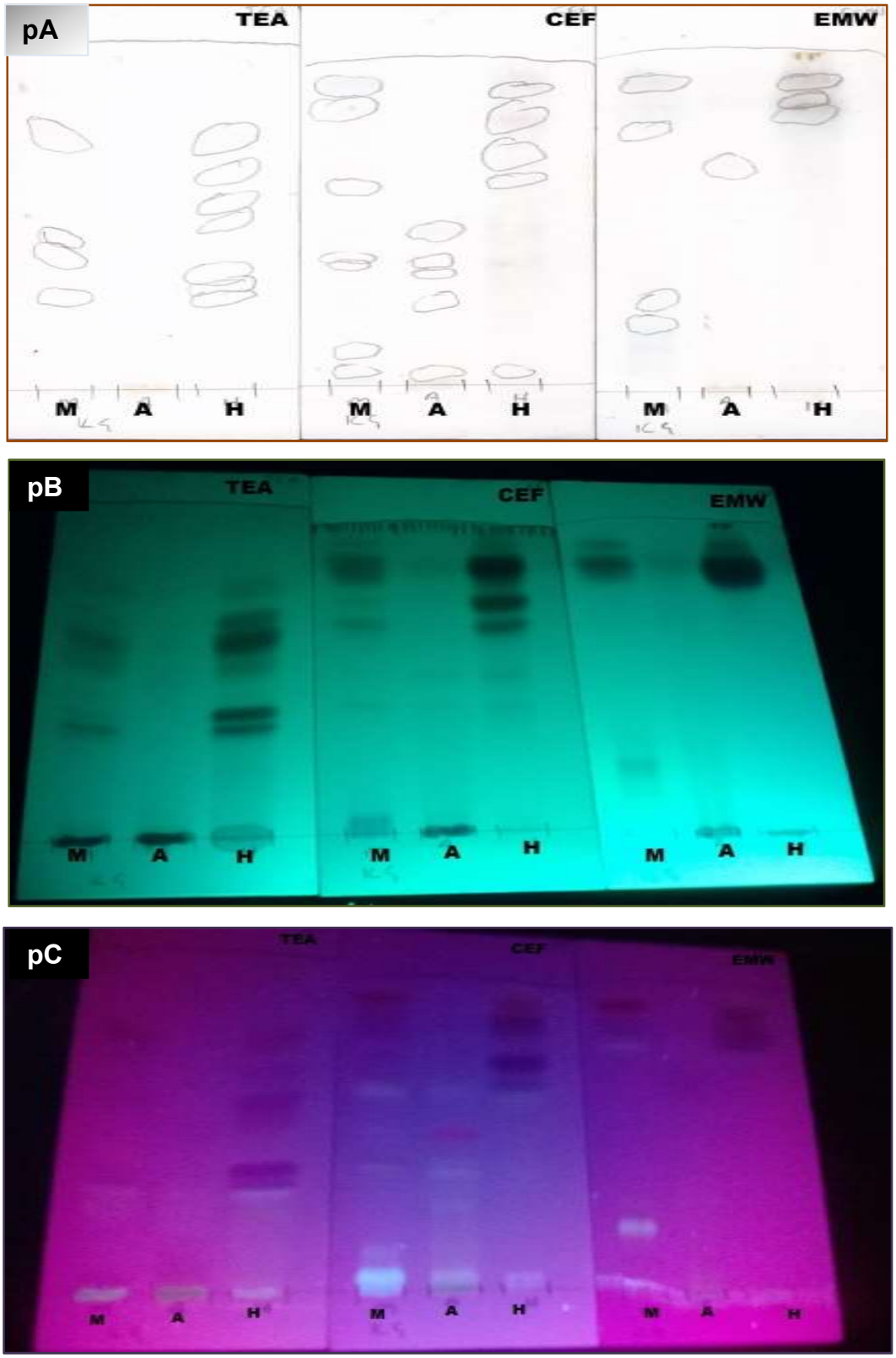

Figure 4.4 :TLC finger print profile and UV active compounds of different extracts of *Seriphium plumosum*.M=Methanol extrctact,A=Acetone extract,H=Hexane extract. pA= plates sprayed with acidic vanilin , pB= plates viewed using UV light at 254nm, pC= plates viewed using UV light at 365nm eluted in TEA, CEF and EMW solvent systems

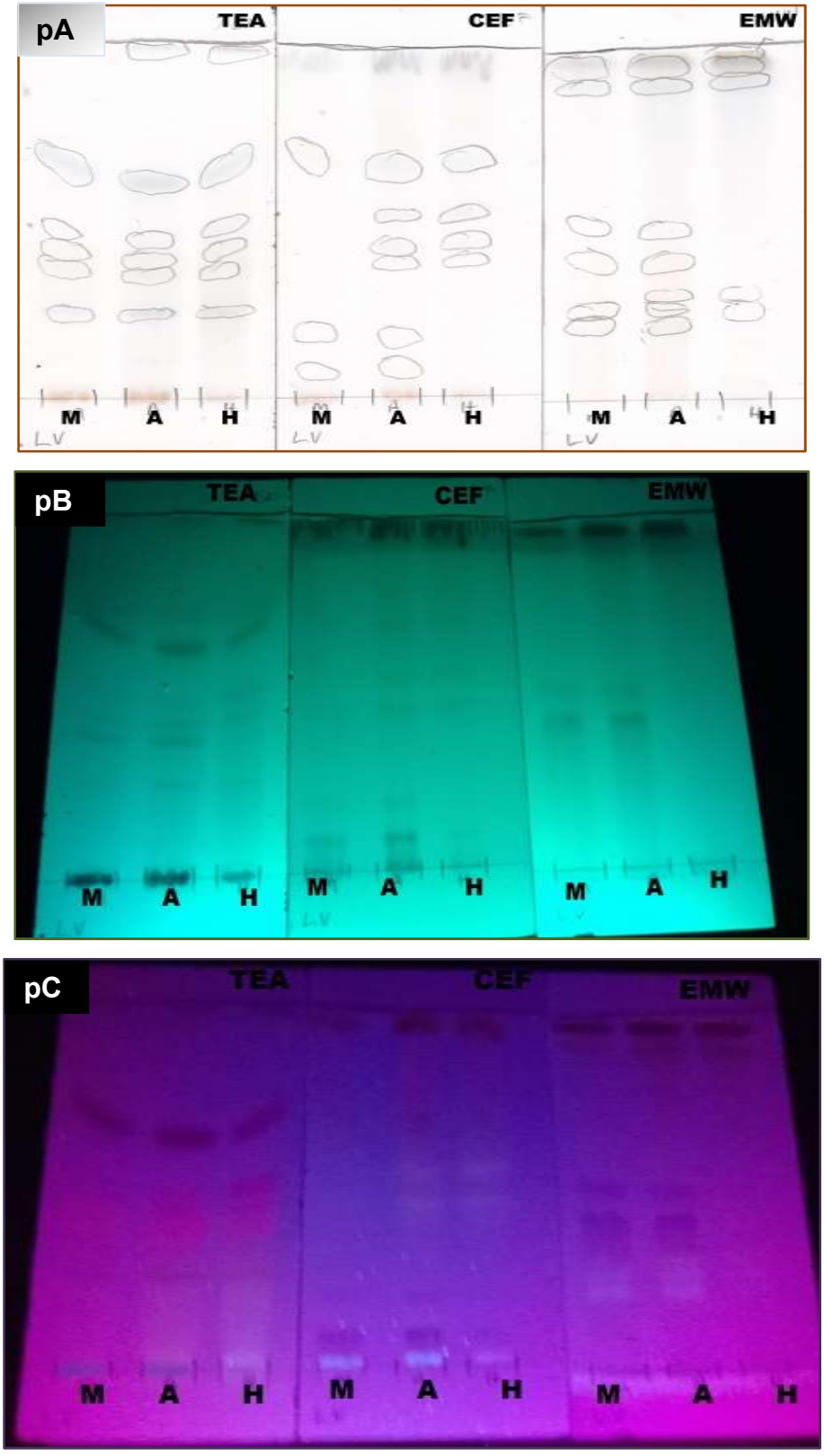

Figure 4.5 :TLC finger print profile and UV active compounds of different extracts of *Toona ciliata*.M=Methanol extrctact,A=Acetone extract,H=Hexane extract. pA= plates sprayed with acidic vanilin , pB= plates viewed using UV light at 254nm, pC= plates viewed using UV light at 365nm eluted in TEA, CEF and EMW solvent systems

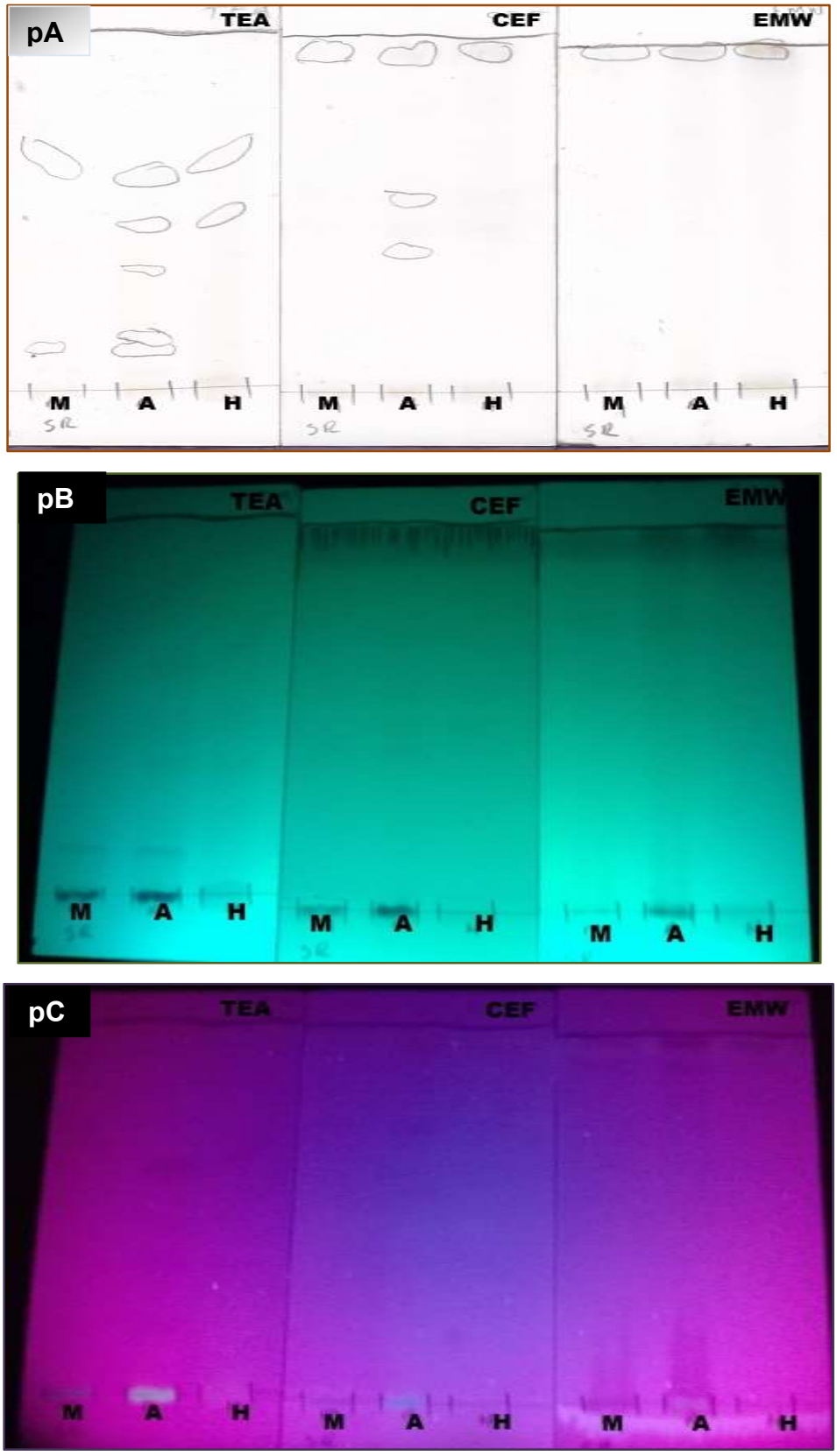

Figure 4.6 :TLC finger print profile and UV active compounds of different extracts of *Schkuhria pinnata* .M=Methanol extrctact,A=Acetone extract,H=Hexane extract. pA= plates sprayed with acidic vanilin , pB= plates viewed using UV light at 254nm, pC= plates viewed using UV light at 365nm eluted in TEA, CEF and EMW solvent system

4.4.3 Phytochemical analysis

4.4.3.1 Qualitative phytochemical analysis

Qualitative analysis of the phytochemicals was performed in order to determine the presence of tannins, flavonoids, phenols, saponins, steroids, phlobatannins, glycosides, coumarins, proteins, anthraquinones, anthocyanins, leucoanthocyanins turns and carbohydrates in all plant extracts. The test for these group of compounds was done using standardised calometric test as outlined in section 3.5. Tannins, flavonoids and phenols were present in all the plant extracts. Saponin, anthraquinones, anthocyanins, phlobatannins, leucoanthocyanins turns and carbohydrates were absent in all the extracts. Steroids, glycosides and coumarins were present in some of the plants, particularly the methanol and acetone extracts, while they were absent in others (Table 4.1).

Table 4.1: The present/ absence of various secondary metabolites in the different crude plant extracts of the different plants.

Metabolites	SPiM	SPiA	SPiH	OFiM	OFiA	OFiH	TCiM	TCiA	TCiH	SPIM	SPIA	SPIH	OAfM	OAfA	OAfH
Tannins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	+	+	-	-	-	-	+	+	-	+	+	+	-	-	-
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
Coumarins	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+
Proteins	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthocyanins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucoanthocyanins turns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-)= constituent absent, (+)= constituent present, SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract) OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract),SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH *Schkuhria pinnata* (Hexane extract),OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract),TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract)

4.4.3.2 Quantitative phytochemical analyis

The flavonoid and total phenolic content of each of the extract was determined as quercetin and tannic acid equivalence respectively (Figure 4.8). Tannic acid is a well-known poly-phenolic compound with several conjugated phenolic groups in its structure. Quercetin is a well-documented and characterised flavonol found in various plant parts particularly abundant in fruits. An amount of each of the extracts was taken and its flavonoid and total phenolic content was determined by linear regression from standard curves. The methanol and acetone extracts possessed the highest amounts of flavonoid and phenolic compounds while hexane extracts had the least. This indicates that the flavonoid and phenols contained in these plants are relatively of a polar nature. The plants contained comparatively higher amounts of total phenolic compounds as compared to the flavonoids.

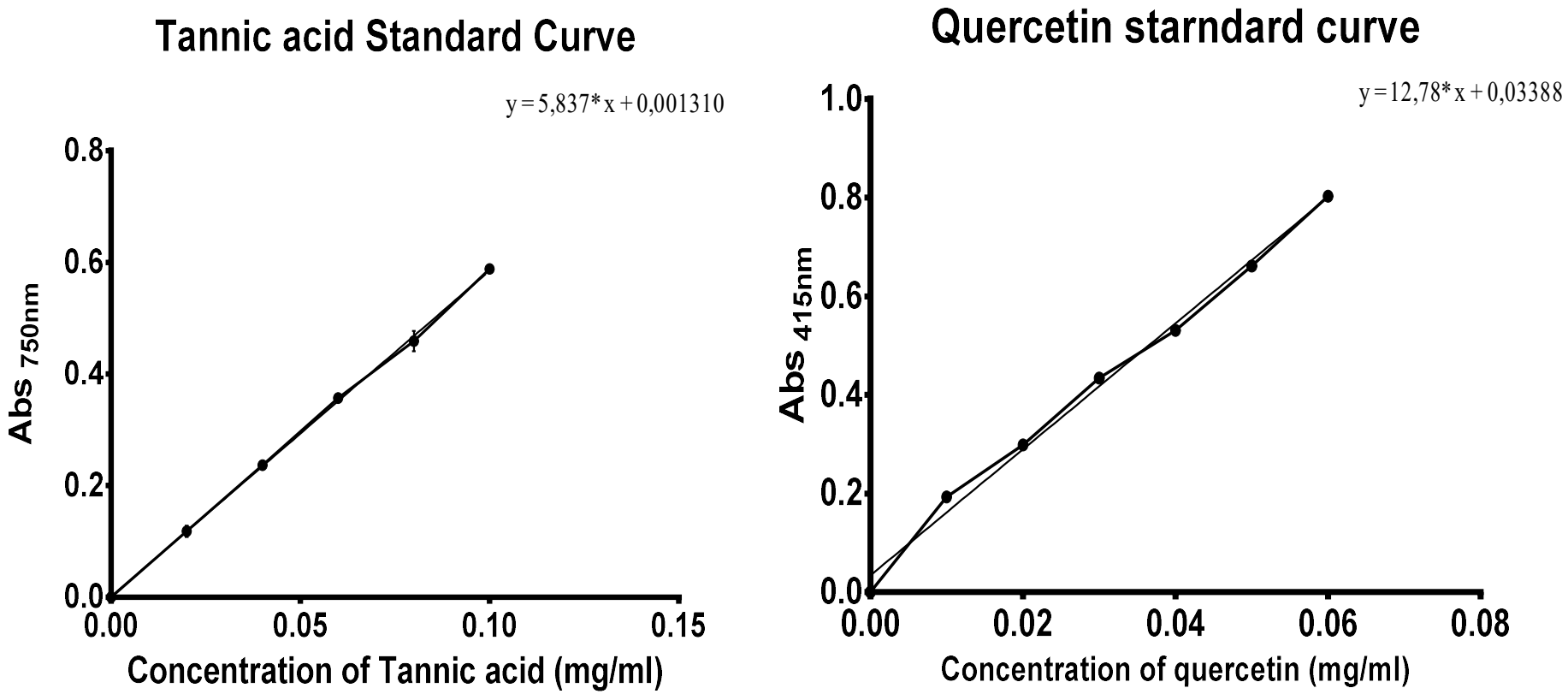

Figure 4.7: The tannic acid and the quercetin standard curves used for the quantification of the total phenolic compounds and the flavonoids respectively.

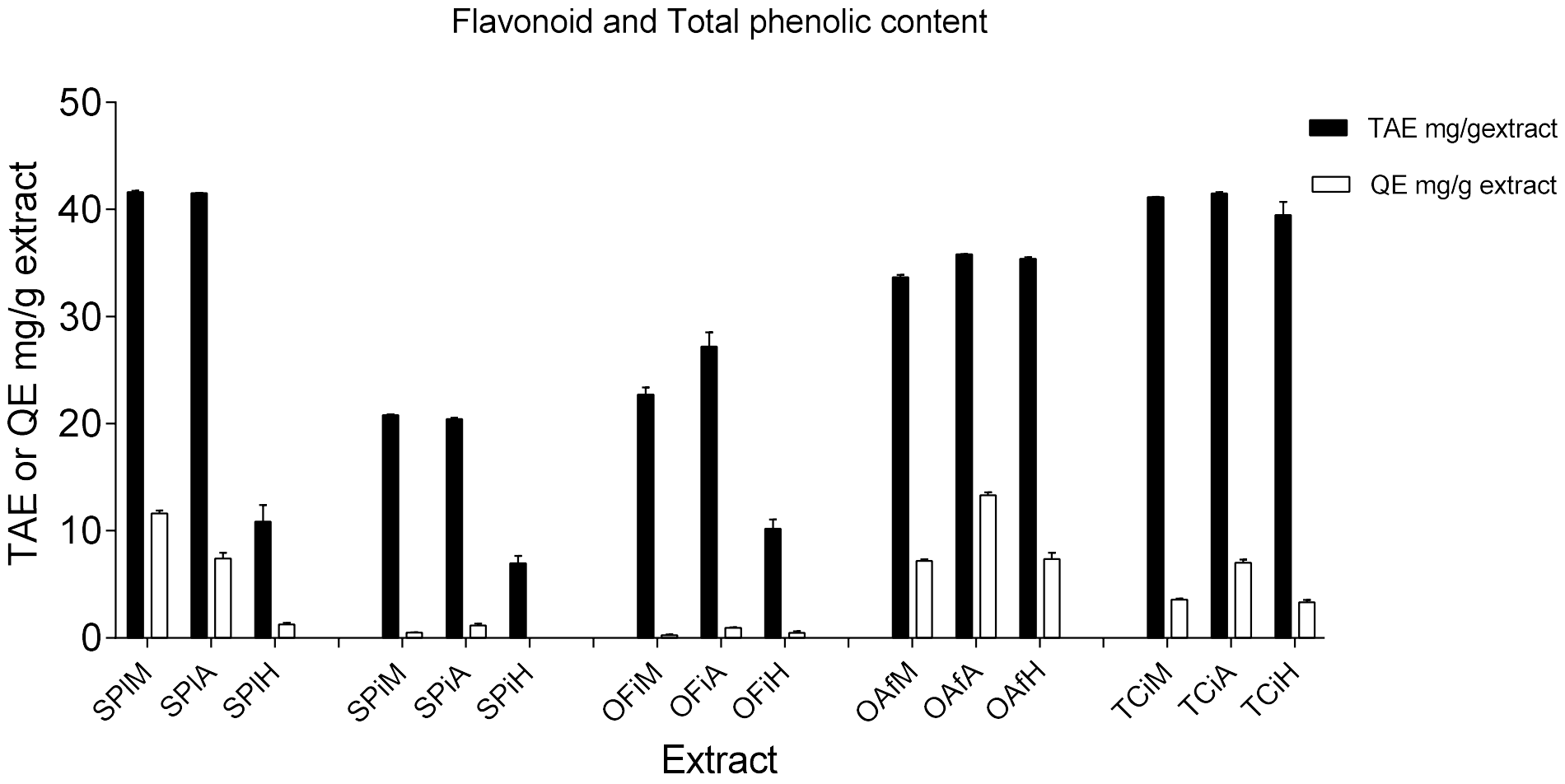

Figure 4.8: The total phenolic content of the different plant extracts represented as tannic acid equivalents (TAE mg/g) and the flavonoids in the different plant extracts represented as quercetin equivalents(QE mg/g). SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract) OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract),SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH *Schkuhria pinnata* (Hexane extract),OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract),TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract)

4.4.4 Antioxidant analysis

4.4.4.1 Qualitative antioxidant analysis

The plates (Figure 4.9 to Figure 4.11) were sprayed with 0.2% DPPH in methanol solution. The yellow bands on the purple backgrounds represent compounds that have antioxidant activity in terms of radical scavenging capability. The band intensity also reflects the relative concentration of that compound in the extract. Acetone (A) and methanol (M) extracts show the highest antioxidant activity. These bands migrated furthest in the polar (EMW), followed by CEF. The poor migration in the non-polar (BEA) indicates that the compounds with antioxidant activity are relatively polar in nature. The hexane (H) extracts contained relatively less compounds with antioxidant activity. *Opuntia ficus indica* showed no compounds with anti-oxidant activity while *Olea africana* showed the most bands of compounds with antioxidant activity.

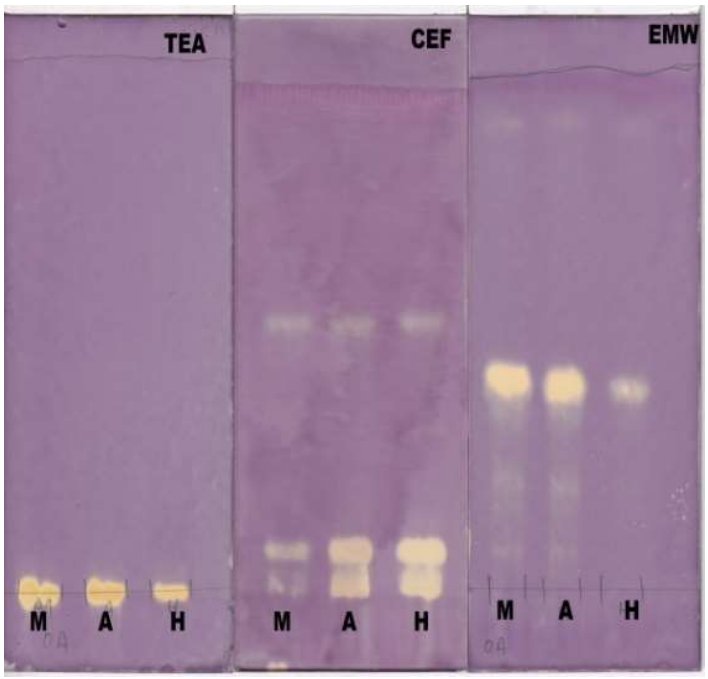

Figure 4.9:The anti-oxidant compounds of different extracts of *Olea africana*. M=Methanol extrctact,A=Acetone extract,H=Hexane extract the plates were sprayed with DPPH eluted in TEA, CEF and EMW solvent systems

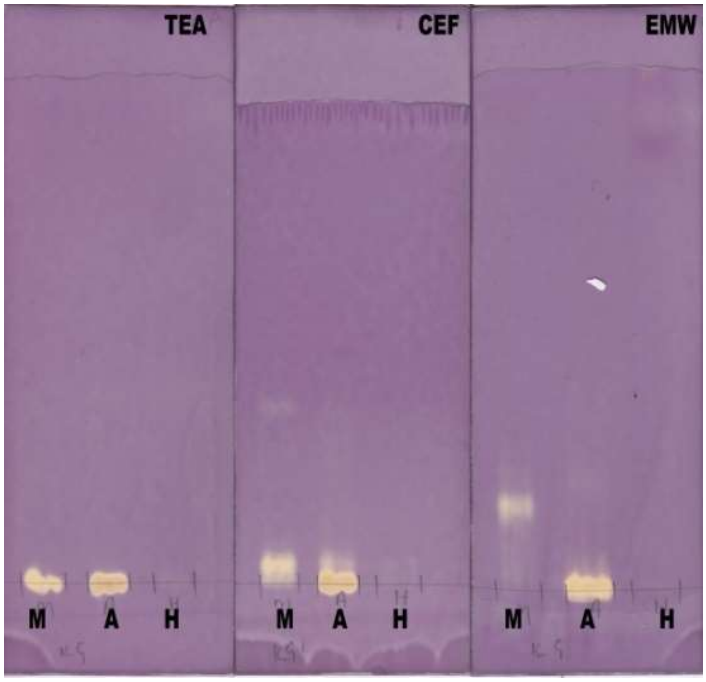

Figure 4.10:The anti-oxidant compounds of different extracts of *Seriphium plumosum*. M=Methanol extrctact,A=Acetone extract,H=Hexane extract the plates were sprayed with DPPH eluted in TEA, CEF and EMW solvent systems

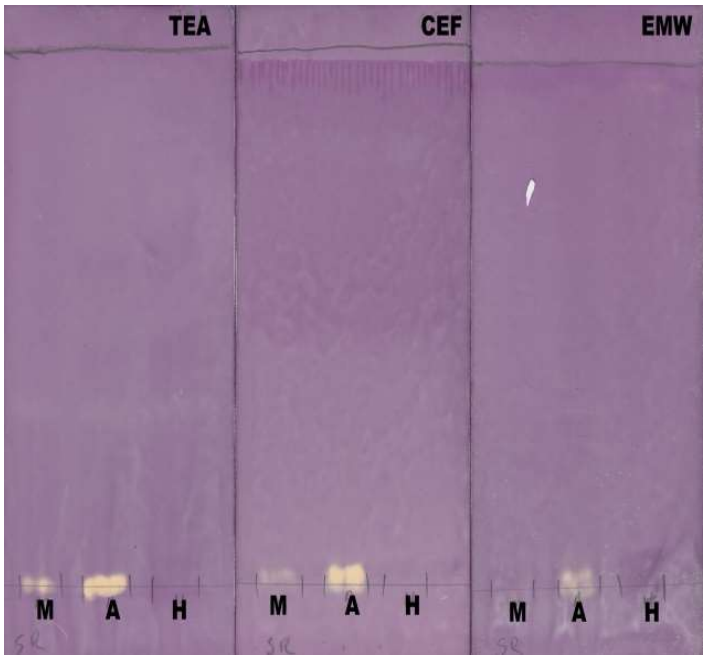

Figure 4.11:The anti-oxidant compounds of different extracts of *Schkuhria pinnata* . M=Methanol extrctact,A=Acetone extract,H=Hexane extract the plates were sprayed with DPPH eluted in TEA, CEF and EMW solvent systems

4.4.4.2 Quantitative antioxidant analysis

The DPPH scavenging assay takes advantages of the ability of compounds with antioxidant activity to turn DPPH from its hydrazyl (unstable) state which is purple to the hydrazine (stable) state which is yellow in colour. This is due to their capacity to donate a hydrogen atom which stabilises the DPPH free radical. The degree of discolouration which is indicative of the capacity of the extract to donate a hydrogen atom was measured for each plant extracts at different concentrations. Ascorbic acid was used as a positive control for comparative purposes. The percentage scavenging activity was calculated relative to the control. The methanol extract showed the highest DPPH scavenging activity and hexane showed the least scavenging activity.

The antioxidant activity of the extracts was also determined in terms of their ability to donate electrons by the ferric reducing power assay. The released electrons are accepted by Fe^{3+} in the complex potassium ferri-cynide $K_3[Fe(CN)_6$ to yield potassium ferro-cyanide (Fe^{2+}). This then reacts with ferric chloride ($FeCl_3$) to form ferric-ferrous complex which is prussian blue in colour with an absorption maximum at 700 nm. The intensity of the blue colour is indicative of the reducing power of the extract. The percentage reducing power is then determined relative to the control which is made up of the reaction mixture and water only. The methanol extract showed the highest activity and the hexane extract had the least activity. Ascorbic acid was used as the experimental control for comparative purposes.

The DPPH scavenging activity (Figure 4.12) and ferric reducing power (Figure 4.13) capabilities of the *Olea africana* crude extracts is represented. The methanol extract of *Olea africana* showed the best activity among all the extracts of this plant in both the DPPH scavenging activity and the ferric reducing power assay with EC_{50} values of 1.08 mg/ml and 2.54 mg/ml respectively. The acetone extract exhibited the second best activity in both assay while the hexane extract of *Olea africana* showed the least activity with EC_{50} values of 4.82mg/ml and 5.91mg/ml for the DPPH scavenging activity and ferric reducing power assay respectively. The antioxidant activity was shown to be dependent on the polarity of the extract. The more polar the extract, the better the antioxidant capability.

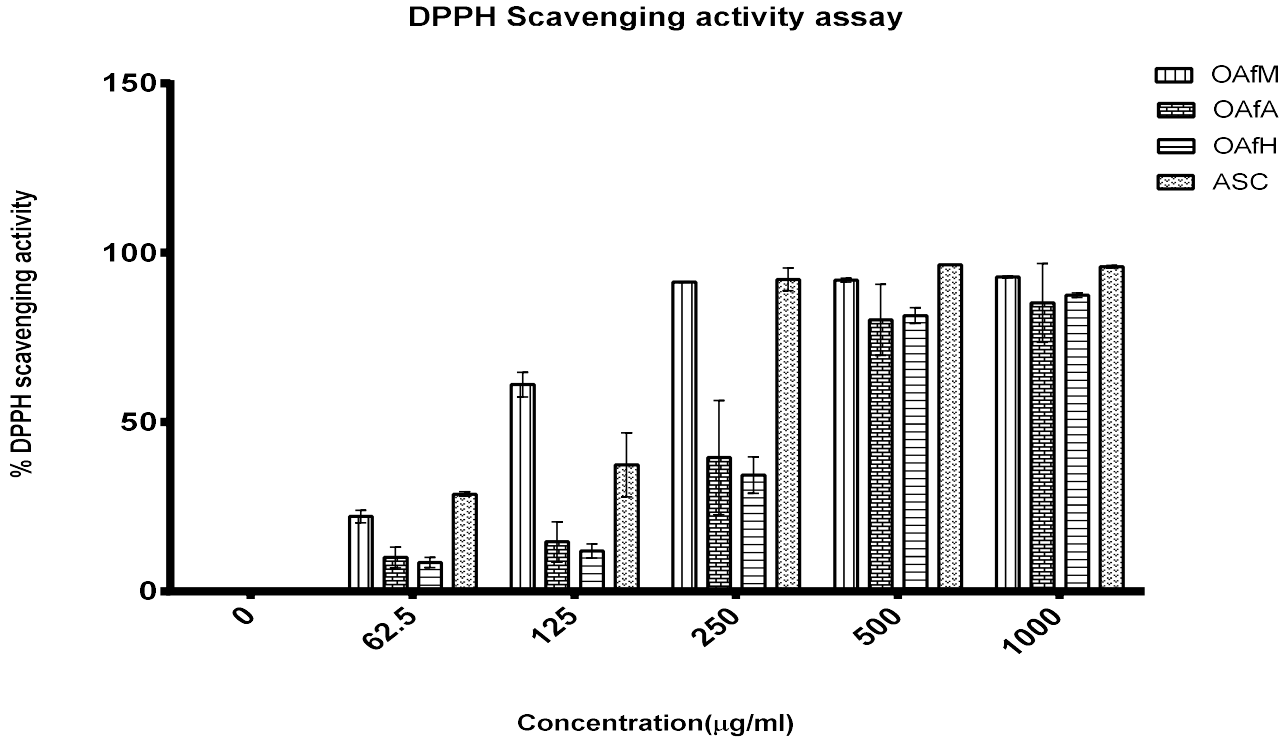

Figure 4.12: The percentage of the DPPH free radical scavenged by different concentrations of different *Olea africana* extracts. Ascorbic acid was used as the positive control. OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract), and ASC= Ascorbic acid.

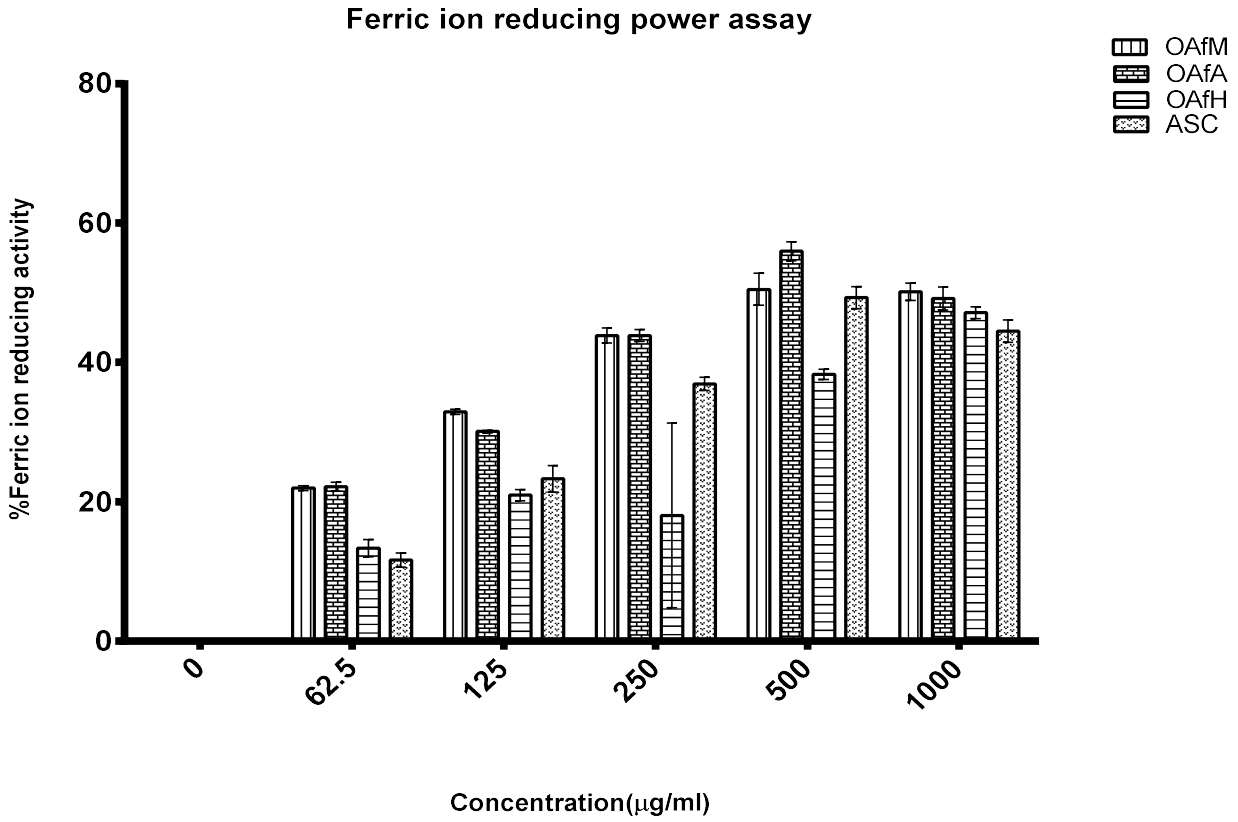

Figure 4.13: The percentage of the ferric ion reduced by different concentrations of different *Olea africana* extracts. Ascorbic acid was used as the positive control. OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract), and ASC= Ascorbic acid.

A similar trend as observed with the extracts of *Olea africana* for the extracts of *Seriphium plumosum* in the DPPH scavenging activity (Figure 4.14) and ferric reducing power assay (Figure 4.15). The methanol extract of *Seriphium plumosum* showed the best activity among all the extracts in this study for both the DPPH scavenging activity and the ferric reducing power assay. It exhibited the lowest EC_{50} values of 0.72 mg/ml and 2.31 mg/ml for the DPPH scavenging activity and the ferric reducing power assay respectively. These EC_{50} values were lower than those for ascorbic acid which were 1.62 mg/ml and 3.10 mg/ml for the DPPH scavenging activity and the ferric reducing power assay respectively.

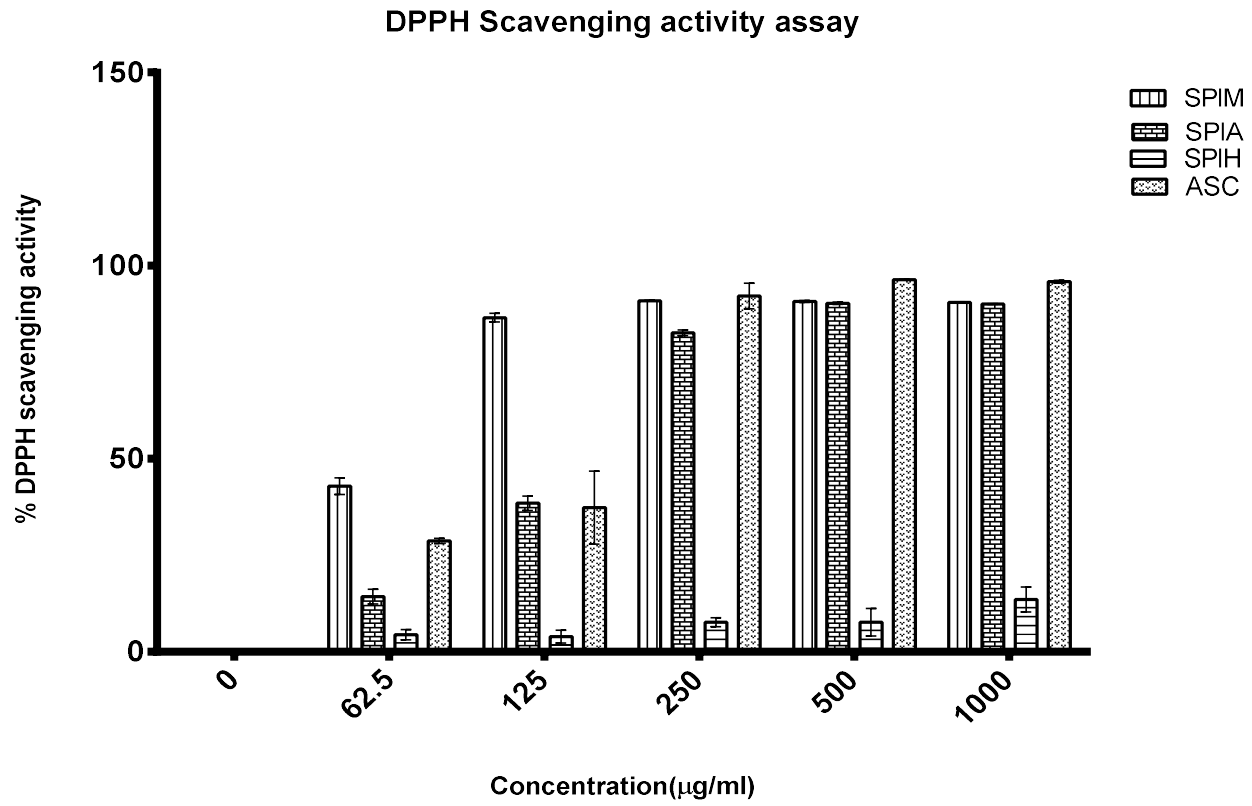

Figure 4.14: The percentage of the DPPH free radical scavenged by different concentrations of different *Seriphium plumosum* extracts. Ascorbic acid was used as the positive control. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract) and ASC= Ascorbic acid.

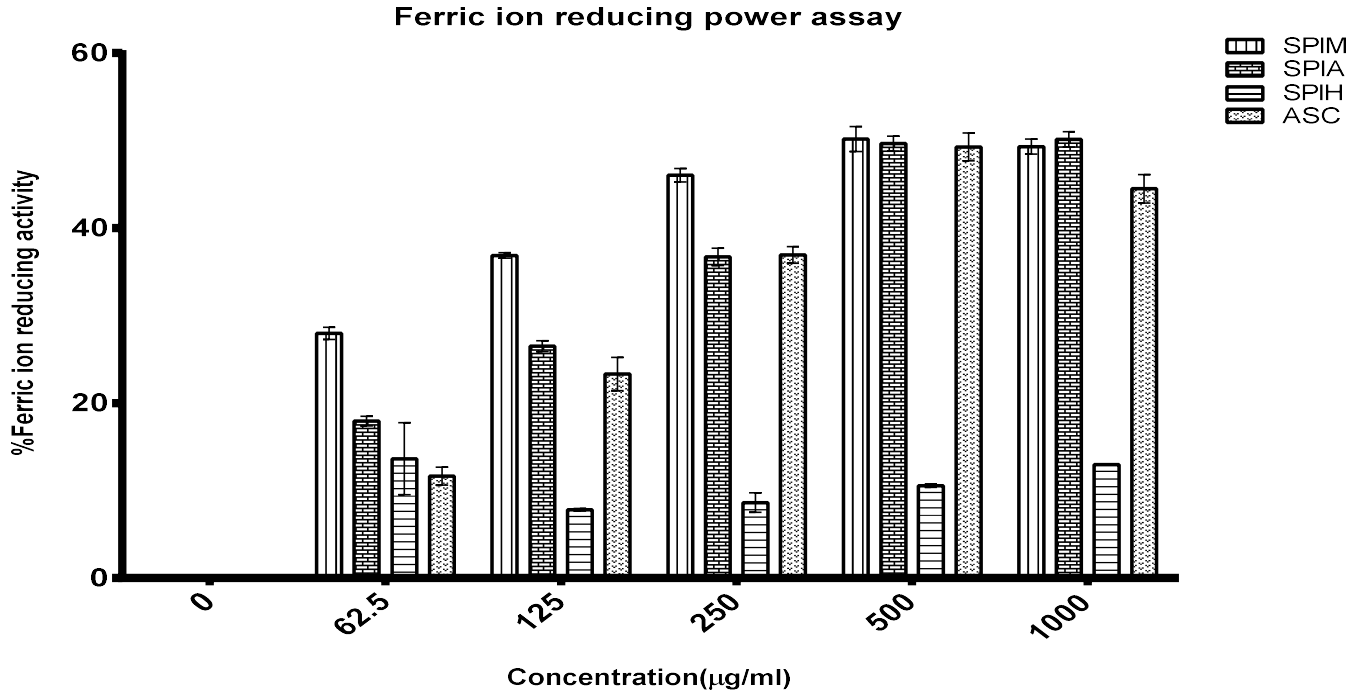

Figure 4.15: The percentage of the ferric ion reduced by different concentrations of different *Seriphium plumosum* extracts. Ascorbic acid was used as the positive control. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract) and ASC= Ascorbic acid.

The extract of *Schkuhria pinnata* on the other hand showed relatively low DPPH scavenging activity (Figure 4.16) and ferric reducing power (Figure 4.17) capabilities as compared to the afore mentioned *Olea africana* and *Seriphium plumosum* crude extracts. The plant extracts exhibited a lower DPPH scavenging activity as compared to their ability to reduce the ferric ion. An increase in concentration of the extracts particularly the acetone and hexane extract of *Schkuhria pinnata* did not result in marked activity as compared to the extracts of the previously described plant extracts. The hexane extract of *Schkuhria pinnata* however had the least activity among all the extracts in this study in the ferric reducing power assay with an EC_{50} value of 18.74 mg/ml.

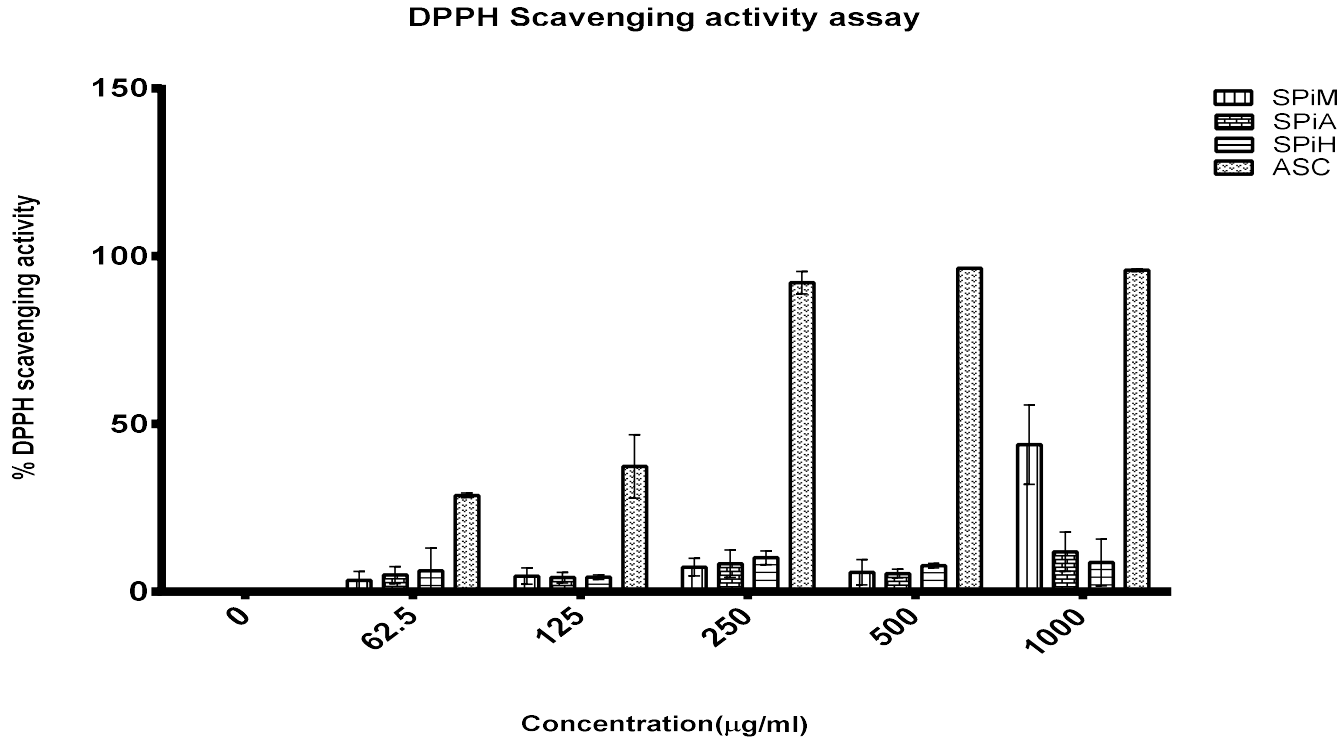

Figure 4.16: The percentage of the DPPH free radical scavenged by different concentrations of different *Schkuhria pinnata* extracts. Ascorbic acid was used as the positive control. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract), and ASC= Ascorbic acid.

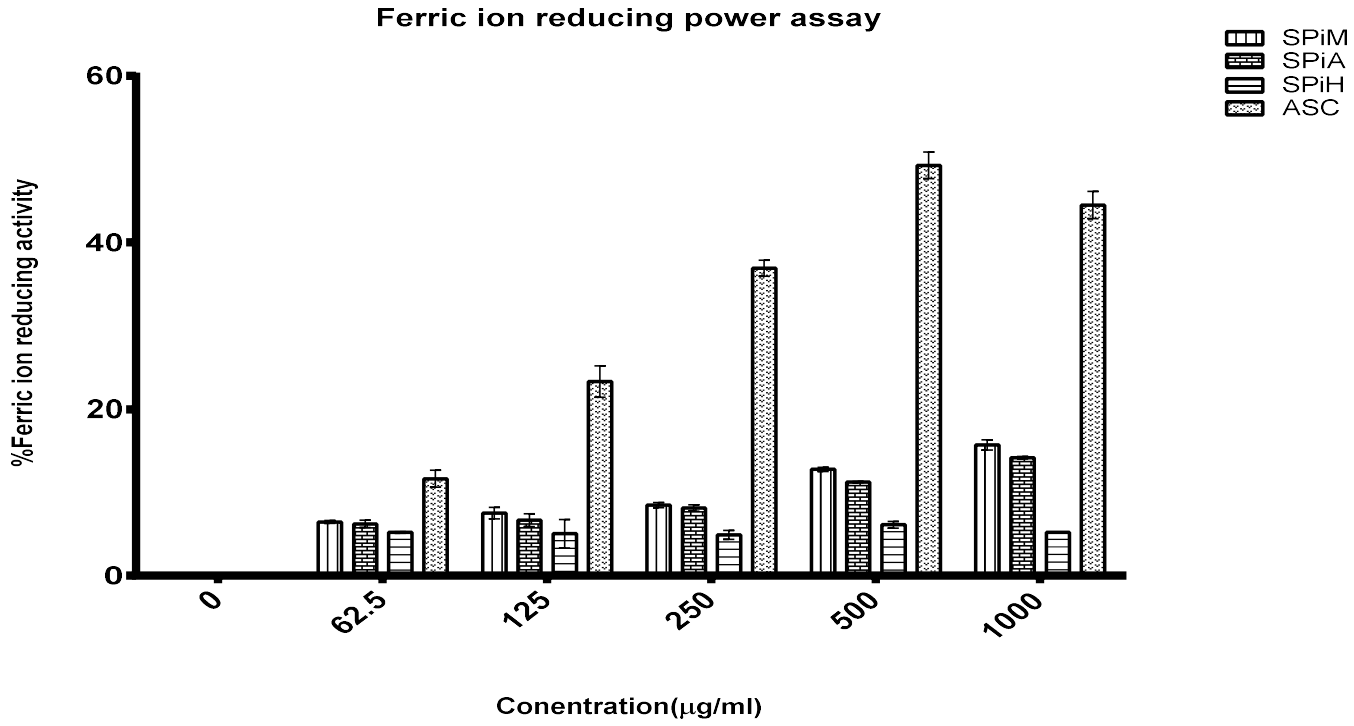

Figure 4.17: The percentage of the ferric ion reduced by different concentrations of different *Schkuhria pinnata* extracts. Ascorbic acid was used as the positive control. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract), and ASC= Ascorbic acid.

A similar trend observed for the extracts of *Schkuhria pinnata in* the DPPH scavenging activity and ferric reducing power capabilities was observed for the extracts of *Opuntia ficus indica.* The acetone extract of *Opuntia ficus indica* exhibited the least effectiveness in the DPPH assay (Figure 4.18) with an EC_{50} value of 12.35 mg/ml.

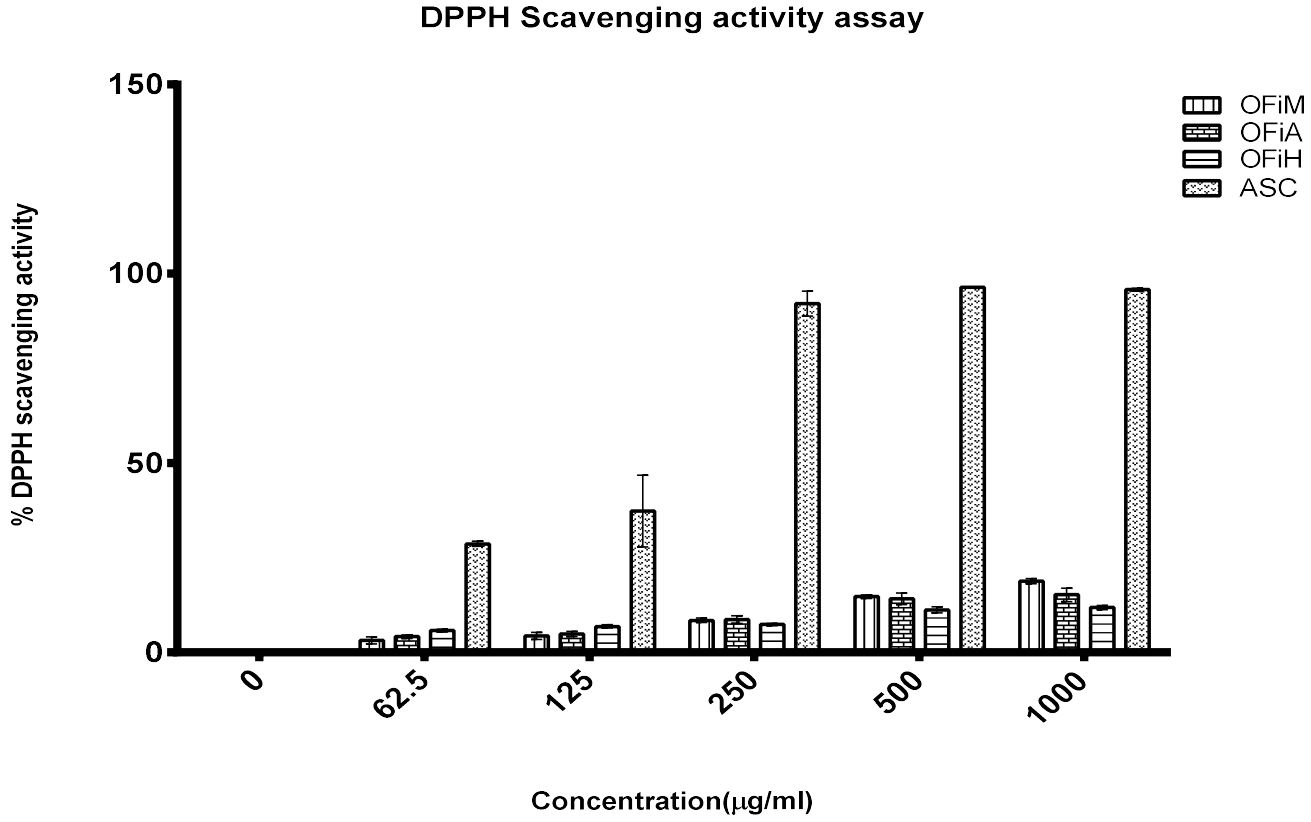

Figure 4.18: The percentage of the DPPH free radical scavenged by different concentrations of different *Opuntia ficus indica* extracts. Ascorbic acid was used as the positive control. OFiM=*Opuntia ficus indica* (Methanol extract), OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract) and ASC= Ascorbic acid.

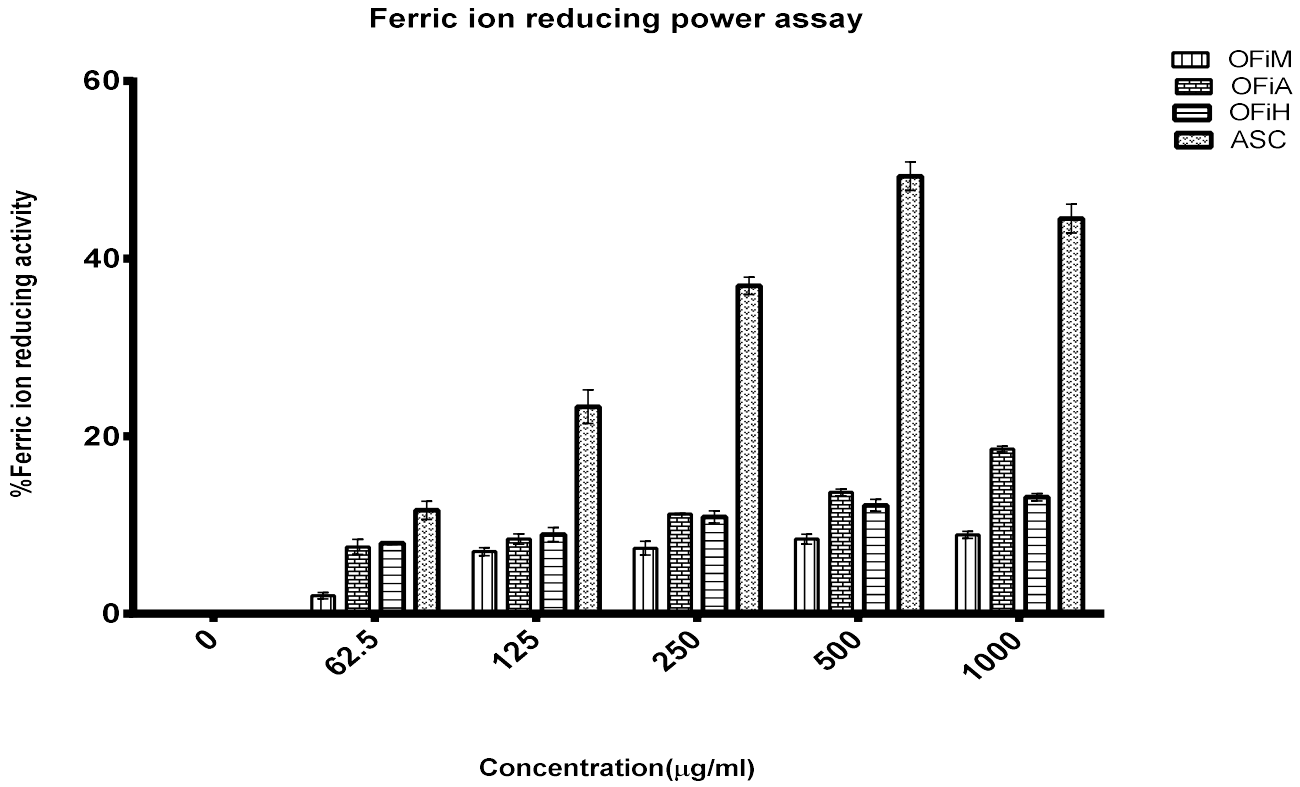

Figure 4.19: The percentage of the ferric ion reduced by different concentrations of different *Opuntia ficus indica* extracts. Ascorbic acid was used as the positive control. OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract) and ASC= Ascorbic acid

The DPPH scavenging activity (Figure 4.20) and ferric reducing power (Figure 4.21) capabilities of *Toona ciliata* crude extracts was shown to occur in a concentration dependant manner. However the antioxidant capability of this plant did not show correlation with the polarity of the extract as observed with the *Olea africana* and *Seriphium plumosum* extracts previously described. The acetone extract of *Toona ciliata* exhibited the best activity for both the DPPH scavenging activity assay as well as the ferric ion reducing power assay. It had EC_{50} values of 1.90 mg/ml and 5.26 mg/ml for the DPPH scavenging activity assay as well as the ferric ion reducing power assay respectively. These were lower than those of the methanol extract of *Toona ciliata* which were 2.36 mg/ml and 6.27 mg/ml for the DPPH scavenging activity assay as well as the ferric ion reducing power assay respectively.

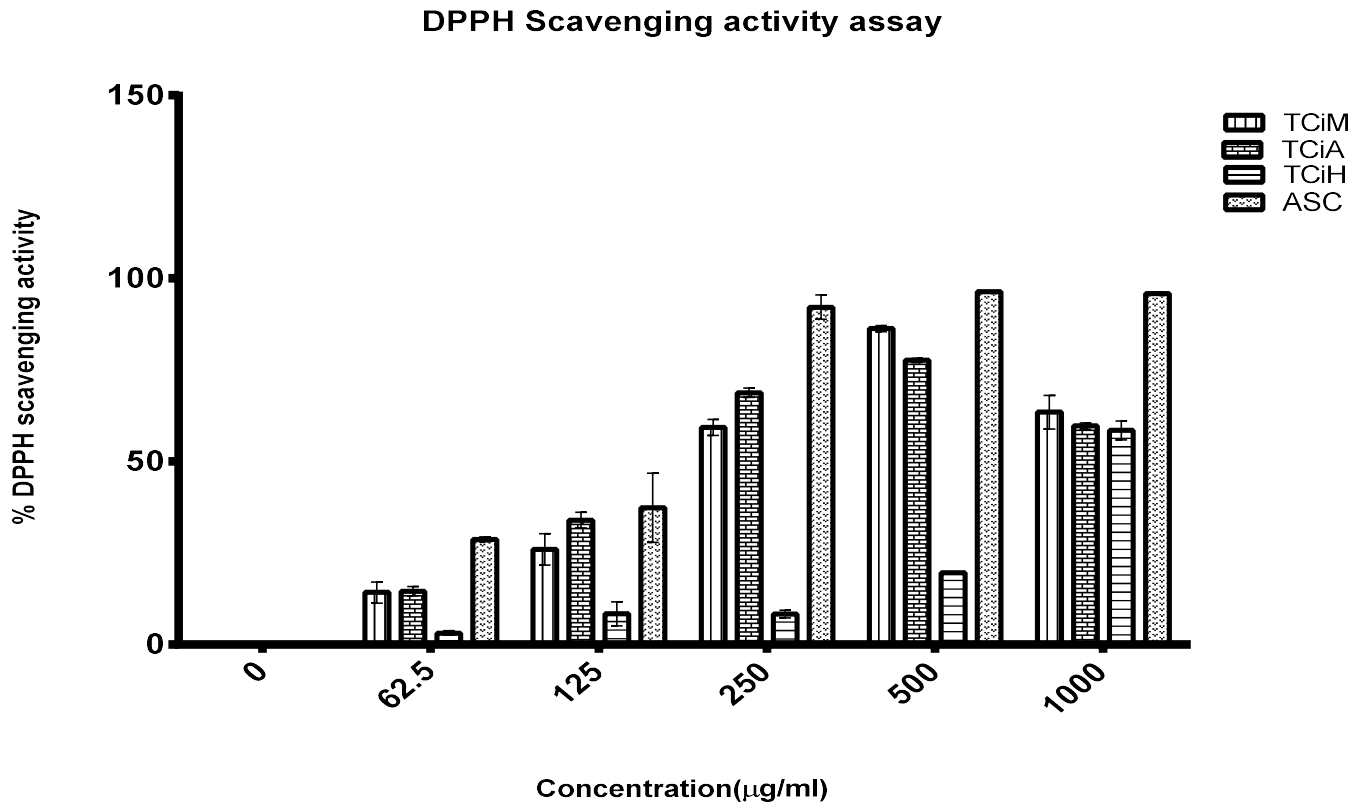

Figure 4.20: The percentage of the DPPH free radical scavenged by different concentrations of different *Toona ciliata* extracts. Ascorbic acid was used as the positive control. TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract) and ASC= Ascorbic acid.

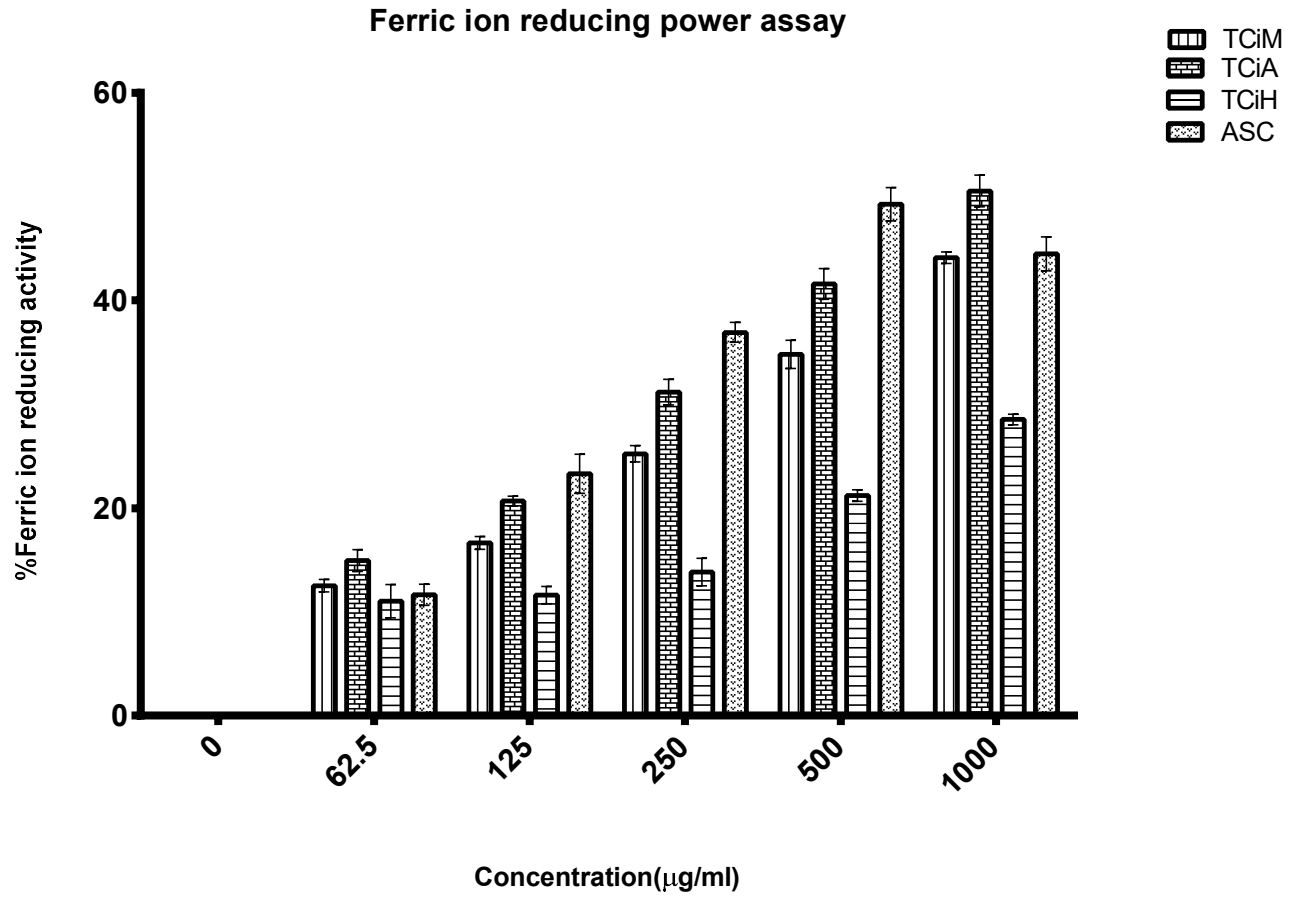

Figure 4.21: The percentage the ferric ion reduced by different concentrations of different *Toona ciliata* extracts. Ascorbic acid was used as the positive control. TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract) and ASC= Ascorbic acid.

The EC_{50} values for the DPPH scavenging assay and the ferric reducing power of the different plant extracts were calculated (Table 4.2). They were determined by plotting linear graphs of each of the extract concentrations against their percentage activity for each of the experiments. The concentrations that exhibit a 50 % activity were taken to be EC_{50} values. The lower the EC_{50} value the more effective that particular extract is. The methanolic extract of *Seriphium plumosum* showed the best activity in both the DPPH scavenging assay (0.72 mg/ml) and the ferric reducing power assay (2.31 mg/ml). The acetone extract of *Opuntia ficus indica* exhibited the least effectiveness in the DPPH assay (12.35 mg/ml) while the hexane extract of *Schkuhria pinnata* had the least activity in the ferric reducing power assay (18.74 mg/ml).

Table 4 .2: The EC_{50} values for the DPPH scavenging assay and the ferric reducing power of the extracts.

	DPPH scavenging assay EC_{50} (mg/ml)	**Ferric reducing power assay EC_{50} (mg/ml)**
SPlM	0.72	2.31
SPlA	1.71	3.02
SPlH	10.58	8.06
OAfM	1.08	2.54
OAfA	3.40	2.55
OAfH	4.82	5.91
SPiM	10.32	11.82
SPiA	11.30	12.68
SPiH	9.52	18.74
OFiM	8.89	12.31
OFiA	12.35	9.50
OFiH	10.13	12.47
TCiM	2.36	6.27
TCiA	1.90	5.26
TCiH	7.86	10.21
ASCORBIC ACID	1.62	3.10

SPlM=*Seriphium plumosum*, (Methanol extract) SPlA =*Seriphium plumosum* (Acetone extract), SPlH=*Seriphium plumosum* (Hexane extract) OAfM=*Olea africana* (Methanol extract),OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract),SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH *Schkuhria pinnata* (Hexane extract),OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract),TCiM=*Toona ciliata* (Methanol extract),TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract)

4.5 Discussion

Plants have been employed for therapeutic purposes since time immemorial. Traditional healers from across the globe practise the use of different plants and plant parts to heal various types of ailments. Traditional healers are endowed with the know-how of plant collection, preparation and administration. The success of any medicinal plant treatment regime is almost always based on how the medicinal plants are prepared after collection prior to administration. Plant extraction methods bridge the gap between the plants and the afflicted patient. Traditionally water is the most employed extraction solvent as it is readily available and non-toxic (Masoko *et al*., 2008; Parekh *et al*., 2006).

In this study methanol, acetone and hexane were employed as the extraction solvents for the different plants as they vary in polarity. The percentage yields of extracts of the different plants showed methanol extracts to have the highest percentage yield in all the plants. This finding is consistent with other previous studies (Njume *et al*., 2011; Ezekiel *et al*., 2009; Masoko *et al*., 2008; Ndip *et al*., 2007). This is because methanol has a small molecular weight which enables it to penetrate the plant material more effectively. Methanol although being highly polar in nature is amphipathic in nature and thus can extract most of the polar compounds and some of the intermediate and non-polar compounds. It has the added advantage of not being restricted as is the case with ethanol which requires a permit for purchase. The methanol extracts of *Toona ciliata* (TCiM) had the highest percentage yield of 20.83 % followed by the *Olea Africana* (OAfM) with 20.44% yield. Except for *Opuntia ficus indica* and *Seriphium plumosum* acetone had the second highest percentage yields after methanol. Acetone is of an intermediate polarity enabling it to extract compounds that are intermediate to polar. Eloff (1998) described acetone as being advantageous as an extraction solvent because of its ease of handling and increased range of polarity of extracted compounds. Hexane on the other hand yielded the least amount of mass for most of the plants in this study. The low percentage extraction yield of hexane results from its highly selective nature for non-polar compounds (Masoko *et al*., 2008). The non-polar compounds extracted by hexane form the least constituent in most plant material. The least amount of yield was obtained for the hexane extract of *Schkuhria pinnata* (SPiH)

which was 0.64%. The different percentage yields observed are due to different compounds dissolved in the extraction solvents. Polar solvents will extract hydrophilic compounds while non-polar solvents will extract hydrophobic compounds. Amphiphilic solvents on the other hand will extract a range of both hydrophilic and hydrophobic compounds. To this end, these compounds where separated on thin layer chromatography.

A myriad of compounds were separated on the thin layer chromatography plates in different mobile phases The separation of these compounds is based on both their ability to be carried along in the mobile phase which is mostly based on their affinity for the stationary phase. Compounds with a high adsorbent affinity to the stationary phase have reduced migration as compare to those with relatively less affinity to the stationary phase. The ability of the compounds to migrate also depends on their solubility in the mobile phase. Hydrophilic compounds are more soluble in polar mobile phases while hydrophobic compounds are more soluble in non-polar mobile phases. The compounds were visualised using vanillin sulphuric acid and ultraviolet light at wavelengths 254 and 365 nm. Vanillin sulphuric acid reacts with many organic compounds to give coloured bands characteristic of those compounds. The different coloured bands observed on plates represent different compounds within the plants in the study. These bands migrate differently on the plates to produce TLC profiles that are unique to each individual plant species commonly referred to as TLC fingerprint. The effectiveness of a mobile phase in TLC is based on how well the different compounds are resolve on the silica gel. UV light is used to visualise some of the compounds that are not reactive with the vanillin sulphuric acid. With plates visualised at 254 nm wavelength, the UV reactive compounds appear as black bands on a fluorescent green background. Compounds that appear under this wavelength are known as quenchers. The methanol and hexane extract of *Seriphium plumosum* showed the most compounds under this wavelength. The absence of compounds on the acetone extract of *Seriphium plumosum* may be attributed to the fact that acetone extracted compounds that are not active under this UV light probably due to thier polarity. *Schkuhria pinnata* extract showed the least active bands under 254 nm UV light, with just a single band for the methanol and the acetone extracts. For those plates that were visualised using UV light at 365 nm, active compounds are referred to as flourescers. These compounds contain

conjugated double bonds and or extended systems of delocalized π electrons. They fluoresce under this light and appear as brightly coloured blue, red, green violet or orange compounds. As can be observed in the different plates these compounds appear in some of the extracts but not in others. All these compounds that appear on the different plate discussed in the previous sections form part of the secondary metabolites that are usually a source of therapeutic remedies. The qualitative identities of the groups of secondary metabolites within the selected plants were then determined using different standard chemical tests.

The secondary metabolites in each of the extract were identified using different standardizes chemical tests. Tannins, flavonoids and phenols were present in all the plant extracts. Saponin, anthraquinones, anthocyanins, phlobatannins, leucoanthocyanins turns and carbohydrates were absent in all the extracts. Steroids, glycosides and coumarins were present in some of the plants, particularly the methanol and acetone extracts, while they were absent in others.

The quantification of the total phenolic compounds within the plant extracts was undertaken. Phenols form the largest group of secondary metabolites; they include phenolic acids, flavonoids, tannins, stilbenes and lignans. They are important as a significant percent of the plant derived drugs emanate from this group of secondary metabolites. Although all the plant extracts tested positive for total phenolic compounds, the amounts of these phenolic compounds in the different plants varied greatly. The total phenolic compounds were expressed as tannic acid equivalents for comparative purposes. The methanol and acetone extracts of *Seriphium plumosum* had the highest total phenolic content of 41.63 mg/g and 41.54 mg/g respectively. These were closely followed by the methanol and acetone extracts of *Toona ciliata* which had yields of 41.15 mg/g and 41.51 mg/g respectively. The lowest total phenolic compounds were observed for the hexane extract of *Schkuhria pinnata* which was 6.98 mg/g followed by the hexane extract of *Opuntia ficus indica* which was 10.21 mg/g.

The total flavonoids of the plant extract were then quantified as quercetin equivalents (Figure 4.8). As flavonoids fall under the category of phenolic compounds it was expected that the total yields of flavonoids will be less than that of the total phenolic compounds. The acetone extract of *Olea africana* yielded the highest amount of

flavonoids of 13.30 mg/g followed by the methanol extract of *Seriphium plumosum* of 11.60 mg/g. All the extracts of *Schkuhria pinnata* and *Opuntia ficus indica* yielded very low quantities of flavonoids with their hexane extracts yielding the lowest amounts of 0.02 mg/g and 0.47 mg/g respectively.

The antioxidant capabilities of the plants were determined qualitatively using thin layer chromatography plates spayed with DPPH free radical. TLC plates sprayed with DPPH free radical are used to qualitatively determine compounds that have free radical scavenging activity. These compounds appear as yellow bands on a purple background. This yellow colour is as a result of compounds' ability to reduce the purple hydrazyl to its yellow hydrazine state. This reducing capacity is achieved by the capability of such compounds to donate either electrons or hydrogen ions (H^+) that stabilise the DPPH. The yellow bands migrated furthest in the polar, followed by the intermediate and least in the non-polar mobile phase. Polar compounds have been shown to possess higher antioxidant potential as compared to non-polar compounds (Sharma and Bhat, 2009). The quantity of compounds showing antioxidant activity, indicated by the number of yellow bands, was polarity dependent. Methanol which is the most polar extractant had the highest antioxidant compounds (two bands) followed by acetone which had only one band while a hexane extracts had no band. The CEF mobile phase resolved more compounds with antioxidant activity.

As the DPPH free radical accepts electrons or hydrogen atoms it becomes stabilised. The stabilisation converts the purple hydrazyl to the yellow hydrazine which is accompanied by a decrease in absorbance that is quantified spectrophotometrically. The scavenging activity was observed to be in a polarity and concentration dependant manner. The methanol extract of *Seriphium plumosum* had the highest activity with an EC_{50} value of 0.72 mg/ml followed by the methanol extract of *Olea africana* which was 1.08 mg/ml (Table 4.2). The acetone extracts of *Opuntia ficus indica* exhibited the least effective radical scavenging activity with an EC_{50} value of 12.35 mg/ml, followed by that of the acetone extract of *Schkuhria pinnata* which was 11.30.

The electron donating potential of the extracts were determined by measuring their capability to reduce the ferric (Fe^{3+}) ion state to its ferrous (Fe^{2+}) ion state. The

ferrous ion then forms a blue ferric-ferrous complex in the presence of ferric chloride. The intensity of the blue colour increases as more of the ferric (Fe^{3+}) ion is reduced. The ferric reducing power was observed to be in a polarity and concentration dependant manner (Figure 4.14 to figure 4.18). Methanol extract of *Seriphium plumosum* showed the best ferric reducing ability with an EC_{50} value of 2.31 mg/ml followed by that of the methanol extract of *Olea africana* which was 2.54mg/ml and the acetone extract of *Olea africana* EC_{50} of 2.55 mg/ml. The hexane extract of *Schkuhria pinnata* showed the least activity with an EC_{50} value of 18.74 followed by the acetone extract of the same plant with an EC_{50} of 12.68 mg/ml. It is likely that the phenolic compounds might also be contributing to this ability of the extracts to reduce the ferric ion (Pereira *et al*., 2009).

The extracts showing the best DPPH scavenging activity namely the methanol extract of *Seriphium plumosum* had the highest activity with an EC_{50} value of 0.72 mg/ml followed by the methanol extract of *Olea Africana* which was 1.08 mg/ml having a better scavenging activity than that of ascorbic acid which was 1.62mg/ml. The same observation is evident in the ferric reducing power assay were methanol extract of *Seriphium plumosum* showed the best ferric reducing ability with an EC_{50} value of 2.31 mg/ml followed by that of the methanol extract of *Olea africana* which was 2.54 mg/ml and the acetone extract of *Olea africana* with an EC_{50} value of 2.55 mg/ml. Most of the extracts however showed reduced antioxidant capability when compared to ascorbic acid which was the positive control. This may be attributed to the fact that the ascorbic acid is a pure compound while the extracts contain an assortment of compounds. Those compounds that do not have antioxidant activity may mask the potential of those that have antioxidant activity. A comparison between the total number of bands on the TLC plates visualised using UV light and vanillin to those in the DPPH sprayed plates supports this suggestion. There is therefore a need to identify and isolate those compounds that have antioxidant activity, especially those in the extracts that had better activity than the ascorbic acid, which in their pure form maybe better antioxidant agents. In the following chapter the cytotoxicity and effects on glucose uptake on C2C12 cell of these plant extracts will be examined.

CHAPTER 5

The anti-glycation, cytotoxicity and glucose uptake effect of the plant extracts in differentiated C2C12 murine muscle cells

5.1 Introduction

Glucose and fatty acids are the most utilised metabolic fuels within the body. Glucose is particularly important for tissues such as the brain and muscles which preferentially use glucose in their function. Despite the overwhelming beneficial nature of glucose, above homeostatic concentrations, it results in pathological conditions within the body (Campos, 2012). Glucotoxicity is a term reserved for the structural and functional damage that occurs to a range of cellular components due to chronic hyperglycaemia (Campos, 2012). Hyperglycemia results in glycation, which is the spontaneous non-enzymatic reaction of reducing sugar with proteins, lipids or nucleic acid (Negre-Salvayre *et al*., 2009). The end result of chronic glycation is the accumulation of a heterogenous group of bio-molecules collectively termed advanced glycation end-products (AGEs) such as pentosidine, carboxymethyllysine, crossline and pyralline (Negre-Salvayre *et al*., 2009). Advanced glycation end-products , apart from directly compromising the function of glycated biomolecules, have been shown to interact with their specific plasma membrane localized receptors for AGEs (RAGE). Interaction of the AGEs with RAGE results in altered intracellular signaling, gene expression, release of pro-inflammatory molecules and free radicals (Negre-Salvayre *et al*., 2009; Singh *et al*., 2014). Glycation is therefore implicated as the major underlying cause of the host of complications observed in diabetic patients such as cardiovascular disease, nephropathy, neuropathy and retinopathy (Campos, 2012). Anti-glycation agents have been reported to result in delayed progression of the various complication associated with diabetes (Elosta *et al*., 2012). Anti-glycation agents are thought to function mainly by donating electrons to the different reactive products that emanate from glycation (Elosta *et al*., 2012). This is quite beneficial as it results in less damage to various biochemical surfaces and molecules. There is therefore a constant need to find highly potent anti-glycation agent as they help in delaying some of the detrimental effect observed in diabetes.

Glucose disposal mechanisms play a critical role in the lowering of blood glucose levels thereby keeping it in-check. Some diabetes medications are known to work by increasing glucose disposal within the body. These agents are known as hypoglycemic agents and provide an effective mechanism for the management of diabetes. The main glucose disposal mechanisms are grouped into those responsible for storage and metabolism. Glucose is primarily stored in the liver and muscle as glycogen and in the adipose tissue as fat. Process of glucose metabolism involves the contraction of muscles, function of the brain, heating of the body and so forth(Shrayyef and Gerich, 2010). Muscles play a pivotal role in the disposal of glucose. Agents that encourage increased glucose uptake by the muscle cells provide a proficient mechanism of the management of diabetes. Glucose uptake into the muscle cells is mediated by the hormone known as insulin (Shrayyef and Gerich, 2010).

Insulin is secreted by the pancreatic β cells of the islets of Langerhans. It is then transported via the blood stream to its different target organs. Upon reaching these sites, it causes a series of cascade reactions that eventually culminate in the translocation of a glucose transporter molecule (GLUT4). The fusion of the GLUT4 molecules with the cell membrane results in an increase in the influx of glucose from the extracellular environment into the cell. Muscles are responsible for an abundant insulin dependent glucose disposal. They possess an avenue that if harnessed correctly can be able to circumvent the issue of hyperglycemia and its accompanying ill effects. To this end various pharmacological therapies have been developed that mediate increased glucose uptake. There is still a need to develop more of these therapies as the current anti-diabetic therapies have various undesirable side effects (Abdel-Moneim and Fayez, 2015; Verspohl, 2012). Traditional medicinal plants are a lucrative avenue for the development of such therapies. They possess a magnitude of compounds that elicit anti-hyperglycaemic activity (Abdel-Moneim and Fayez, 2015). Some of the anti-hyperglycaemic modes of action include, but is not limited to, pancreatic β-cell stimulation, α-glucosidase and beta glucosidase inhibitors, insulin sensitisers and those that increase glucose uptake by various target organs (Li *et al*.,2004) .

Although plant derived compounds are currently being employed to manage various ailments, there are concerns that have been raised. These concerns were raised due

to the potential dangers that accompany the use of traditional medicinal. These dangers range from unknown toxicity profiles to unknown modes of action. People generally assume that medicinal plants do not have detrimental chronic and acute side effect on their well-being as they are natural agents. This misconception has resulted in a number of people being poisoned from administration of medicinal plants. Plants pose a high risk as they are administered as a crude extract with an array of compounds. While some of those compounds in the extracts may confer therapeutic properties the other compounds may have ill-effects (Patel *et al*., 2012). This is due to the fact that compounds interact with various receptors within the body. This is usually not the case with western medicine which mostly consist of a single purified chemical agent whose effects on different tissues is characterised and known. *In-vitro* cytotoxicity experiments provide a platform for the preliminary analysis of the toxicology profiles of various medicinal plants. The use of medicinal plants is also not standardised as observed in the differences in administration and dosage regimes from traditional healer to traditional healer. While certain concentrations of the plant extracts will have the desired efficacy, concentrations beyond that will result in toxic effects.

This chapter of this study therefore aims to investigate the cytotoxicity of medicinal plants employed in the current study. Once safe concentrations have been, determined it will further investigate the efficacy of the selected plants as hypoglycaemic agents which may play a focal role in the management of diabetes. The chapter will also assess the anti-glycation potential of the selected plants.

5.2 Objectives:

i. Quantitatively assess the extent to which the plant extracts inhibit glycation
ii. Differentiation of C2C12 from myoblasts to fibroblast
iii. Determine the cytotoxicity of the different plant extracts at various concentrations on C2C12 cells
iv. Quantify glucose uptake by C2C12 cells at selected concentrations of the plant extracts.

5.3 Materials and methods

5.3.1 Anti-glycation assay

Antiglycation activity of the plant extracts was determined using the bovine serum albumin assay with slight modification as detailed in section 3.8. Briefly Bovine Serum Albumin (BSA) was incubated with glucose, and plant extracts. Phosphate buffer saline was used as the sample control and Arbutin as the reference standard. The samples were incubated at 60°C for 72 hours. The samples were subjected to an excitation wavelength of 370 nm and the emission profiles at a wavelength of 440 nm were quantified.

5.3.2 Cell culturing and differentiation

An immortalised mouse myoblast cell line (C2C12) was used in this study. The cells were cultured and maintained in RPMI media, supplemented with 10% foetal bovine serum at 37°C, in an atmosphere of 5% CO_2 in a humidified incubator. The cells were differentiated by culturing them in RPMI media containing 2% horse serum for 4 - 6 days as detailed in section 3.11

5.3.3 Cytotoxicity test- MTT assay

The cytotoxic effect of the different plant extracts on C2C12 cell line were determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in section 3.12. Cells were seeded into 96-well cell culture plates and treated or not with different concentrations of the different extracts for 24 hours. The untreated cells served as the experimental control. Actinomycin and DMSO served as positive and negative controls respectively. The absorbance of the insoluble purple coloured formazan was measured at 490 nm using a microtiter-plate multimode detector.

5.3.4 Glucose uptake assay

The amount of glucose taken up by differentiated C2C12 cells was quantified using the glucose uptake kit as described in section 3.13. Cells were treated for 1, 3 and 6

hours in the presence or absence of the different plant extracts. Untreated cells were used as the experimental control, while insulin and DMSO were used as positive and negative controls respectively. After treatment, glucose concentration in the media in each of the wells was quantified spectrophotometrically using a microtiter-plate multimode detector.

5.4 Results

5.4.1 Anti-glycation Activity

Treatment with Arbutin, a known inhibitor of the glycation reaction, resulted in 7,40% glycation (Matsuura *et al.,* 2002). Arbutin was used as the comparative reference standard. Extracts that resulted in less percentage glycation than that of Arbutin were considered to be more potent in the inhibition of glycation.The acetone extract of *Seriphium plumosum* (SPIA) exhibited the most glycation inhibitory activity among all the examined extracts, as it resulted in 2,22% glycation (Figure 5.1). This extract was the most potent anti-glycation agent in this study. On the other hand treatment with the methanol and hexane extracts of *Seriphium plumosum* resulted in 7,30% and 4,90% glycation respectively. The acetone extract's activity surpassed that of the methanol and hexane extracts by a very wide margin.

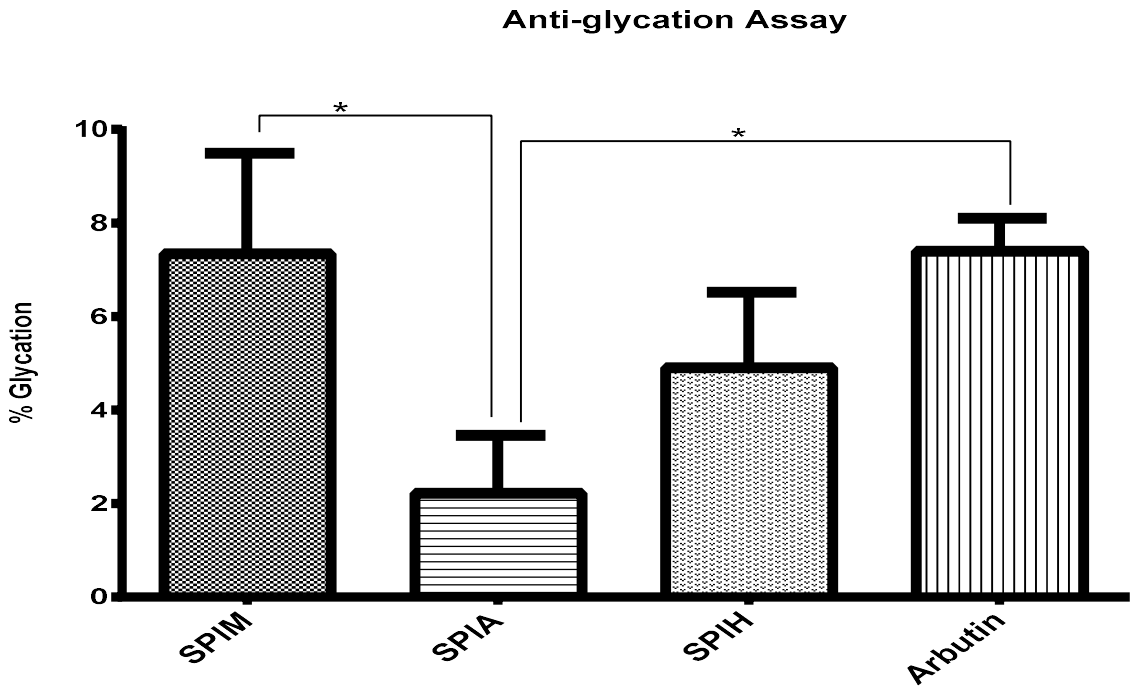

Figure 5.1: The effects of different extracts of *Seriphium plumosum* on the glycation of bovine serum albumin (BSA). Arbutin was used as the standard reference. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract).The 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 Tukey-Kramer Multiple Comparisons Test. The p value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

The extracts of *Toona ciliata* were all prominent anti-glycation agents (Figure 5.2). Treatments with the methanol, acetone and hexane extract of *Toona ciliata* resulted in 2,49%, 2,79% and 2,56% glycation respectively which were all within a comparable range as opposed to the previously mentioned trend observed for the *Seriphium plumosum* extracts(Figure 5.2). The anti-glycation activities of all the extract of *Toona ciliate* were significantly higher (p<0.01) than that of Arbutin.

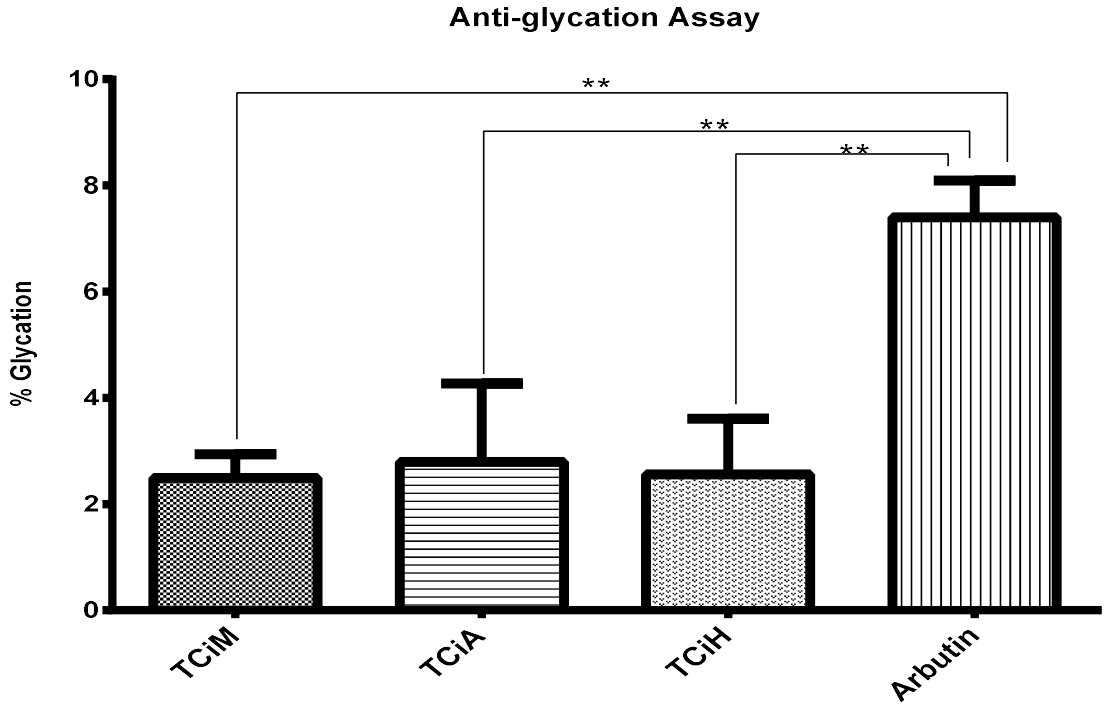

Figure 5.2: The effects of different extracts of *Toona ciliata* on the glycation of bovine serum albumin (BSA). Arbutin was used as the standard reference. TCiM=*Toona ciliata* (Methanol extract), TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The p value significance was represented an asterisk (*) for p<0,05, two asterisks(**) for p<0,01 and three asterisks(***) for p<0,001.

The methanol, acetone and hexane extract of *Opuntia ficus indica* resulted in 7,08%, 6,26% and 12,95% glycation respectively (Figure 5.3). While the methanol and acetone extracts of *Opuntia ficus indica* resulted in anti-glycation activity better than that of Arbutin they were however less effective than *Toona ciliata* extracts. The hexane extract of *Opuntia ficus indica* exhibited the least anti-glycation activity which was significantly lower than the rest of the other treatments.

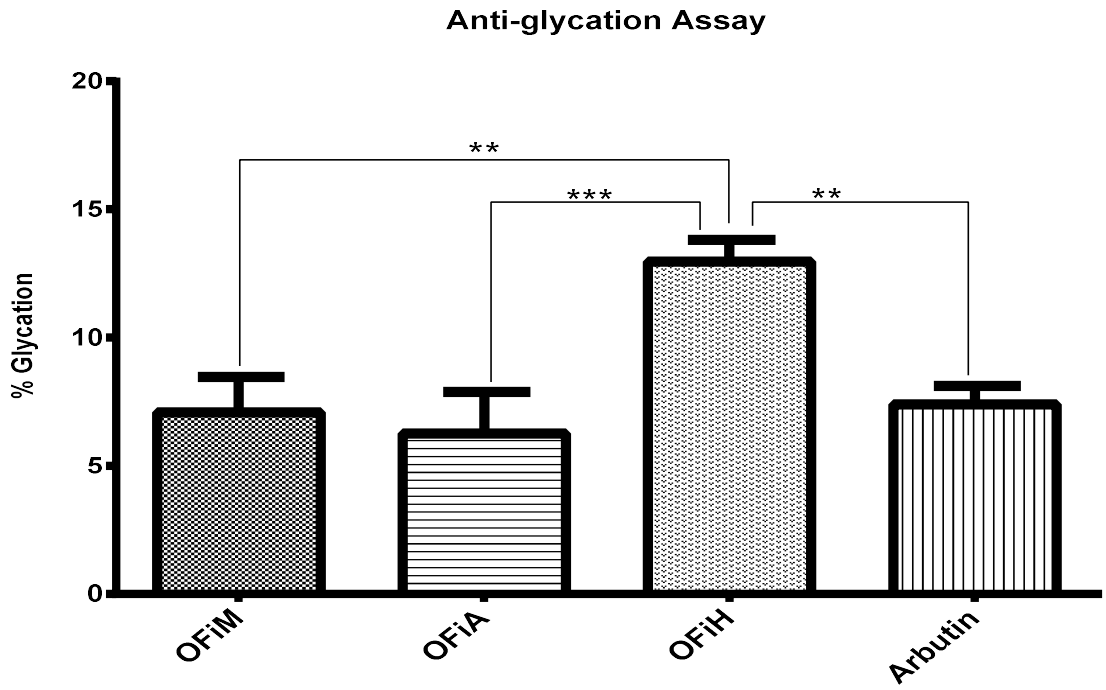

Figure 5.3: The effects of different extracts of *Opuntia ficus indica* on glycation of Bovine serum albumin (BSA). Arbutin was used as the standard reference. OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract). The 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 Tukey-Kramer Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

The rest of the remaining plants showed relatively poor anti-glycation activity having at most a single extract that showed better anti-glycation activity as compared to that of Arbutin. For instance the methanol, acetone and hexane extract of *Olea africana* resulted in 8,82%, 9,51% and 7,79% glycation respectively (Figure 5.4). Notably no statistical significance was obtained for all the *Olea africana* extracts.

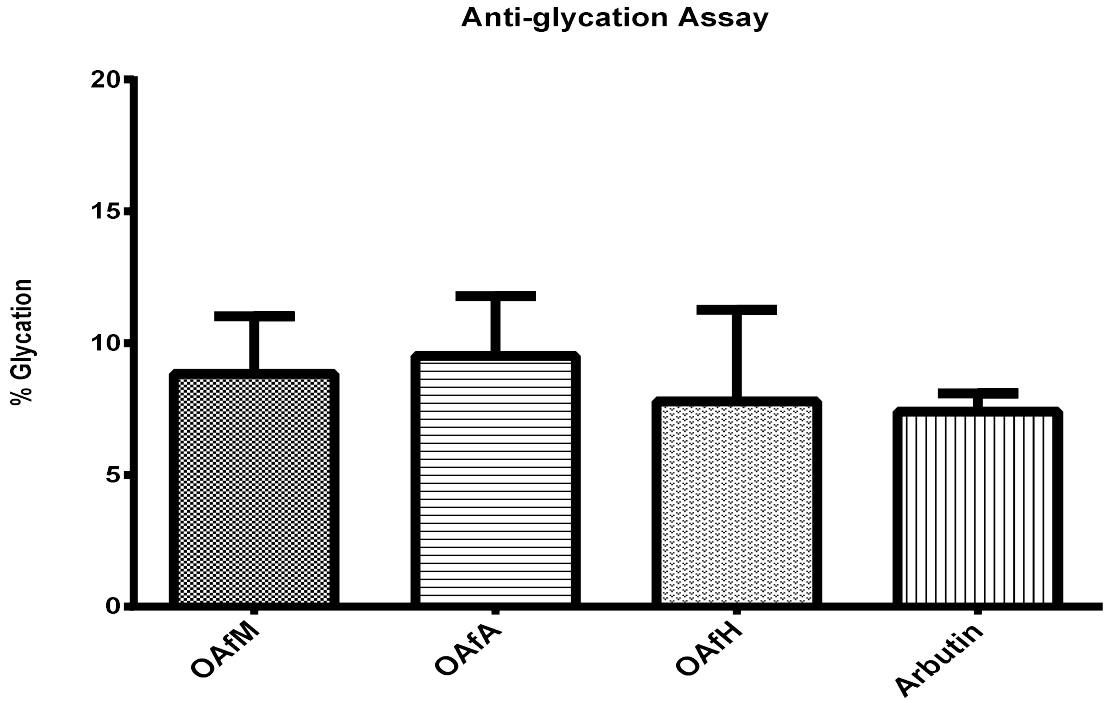

Figure 5.4: The effects of different extracts of *Olea africana* on the glycation of Bovine serum albumin (BSA). Arbutin was used as the standard reference. OAfM=*Olea africana* (Methanol extract), OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p < 0,05$, two asterisks(**) for $p < 0,01$ and three asterisks(***) for $p < 0,001$.

Both the acetone and hexane extracts of *Schkuhria pinnata* which resulted in 12,02% and 15,56% glycation respectively failed to reduce the extent to which the BSA was glycated as compared to Arbutin (Figure 5.5). Both the treatments resulted in statistically lower anti-glycation activities with respect to Arbutin. The least anti-glycation potentials among all the extracts were obtained for the hexane extract of *Schkuhria pinnata* (15,56%) followed by that of the hexane extract of *Opuntia ficus indica* (12,95%).

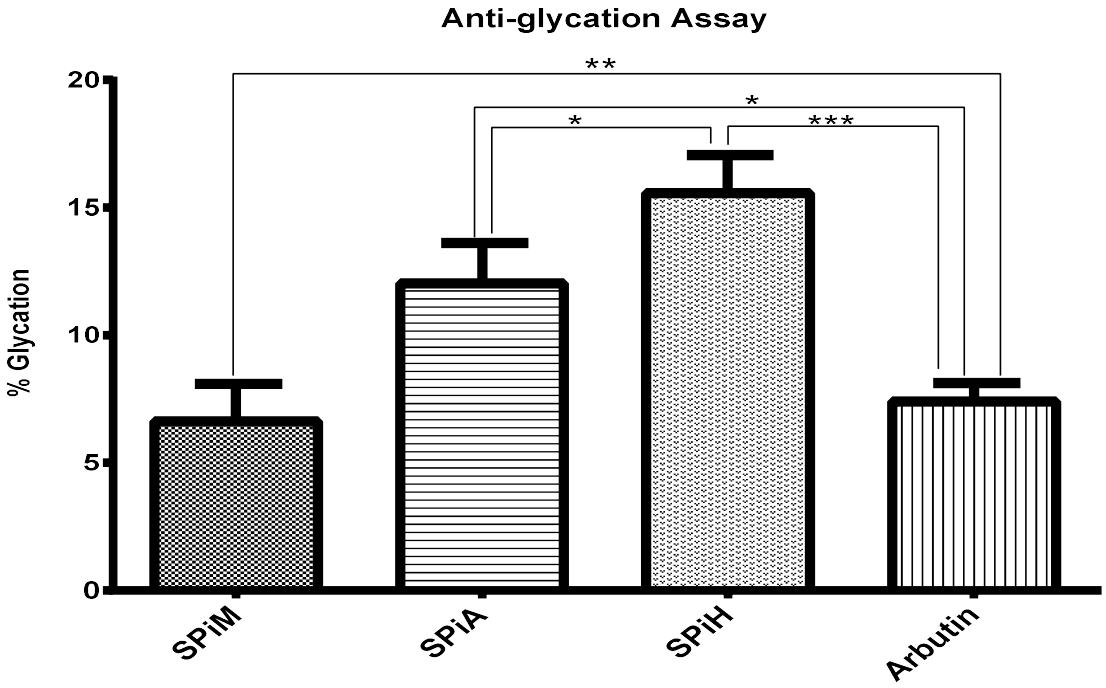

Figure 5.5: The effects of different extracts of *Schkuhria pinnata* on the glycation of Bovine serum albumin (BSA). Arbutin was used as the standard reference. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract). The results 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 Tukey-Kramer Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p < 0,05$, two asterisks(**) for $p < 0,01$ and three asterisks(***) for $p < 0,001$.

5.4.2 Differentiation of C2C12 cells from murine myoblast cells to fibroblast cells

The murine myoblast C2C12 cells were differentiated to fibroblast as indicated by the images below. Differentiation of the C2C12 cell line was induced by culturing the cells in reduced serum conditions (2% horse serum) for 6 days. Differentiation of these cells results in a wide range of functional and morphological changes. At day 0 (Figure 5.6) the cells are still un-differentiated and appear as multi-polar fibroblast-like cells, with a relatively low cell density. At day 2 the cell density has increased and a small population of elongated cells is observed. At day 4 the cells have increased in number and the majority of the cells have acquired the elongated fusi-form characteristic of the differentiated cells. At day 6 the cells are now confluent and have fully differentiated. The cells were observed to change from a mono-nucleated-fusiform nature to a more elongated poly-nucleated confluent mono-layer from day 0 to day 6. The differentiated C2C12 resemble the contractile muscle cell as compared to their un-differentiated counterparts.

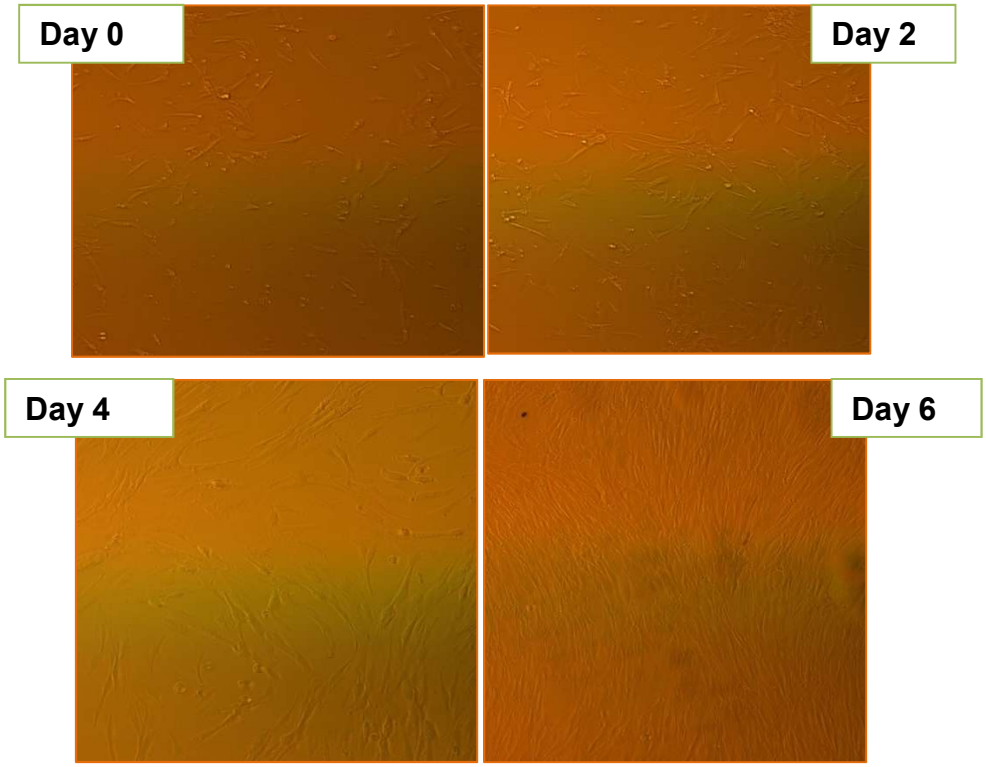

Figure 5.6: The progression of differentiation of murine myoblast cells to myo-tubules in differentiation media from day 0 to day 6. The myoblast C2C12 cells changed during differentiation from fusiform cells to elongated confluent multinucleated myo-tube cells.

5.4.3 Cytotoxic effects of the different plant extracts on C2C12 cells

The viability of C2C12 cell line was assessed at increasing concentrations of the different extracts using the MTT cell viability assay. The mitochondrial dehydrogenase of viable cells converts MTT from its tetrazolium salt (yellow) to the insoluble formazan salt (purple) within the cells. The insoluble formazan is then dissolved in DMSO and quantified spectrophotometrically. The amount of the formazan formed intracellularly is directly proportional to the number of cells that are metabolically active. The extracts decreased the viability of the C2C12 cells in a concentration dependant manner albeit concentrations below 125 µg/ml had no negative effect on cell viability. Actinomycin and DMSO were used as positive and negative controls respectively. Non-cytotoxic concentrations (125 µg/ml and below) determined from this assay were used in subsequent experiments.

The different concentrations of *Schkuhria pinnata* extracts (Figure 5.7a and Figure 5.7b) resulted in concentration dependant viability of cells after the 24 hour treatment. The highest concentrations of the methanol, acetone and hexane extracts of *Schkuhria pinnata* resulted in 50,77%, 39,91% and 33,52% cell viability respectively while the lowest concentrations of the same extracts resulted in 84,00%, 88,75% and 96,49% cell viability respectively(Figure 5.7a).

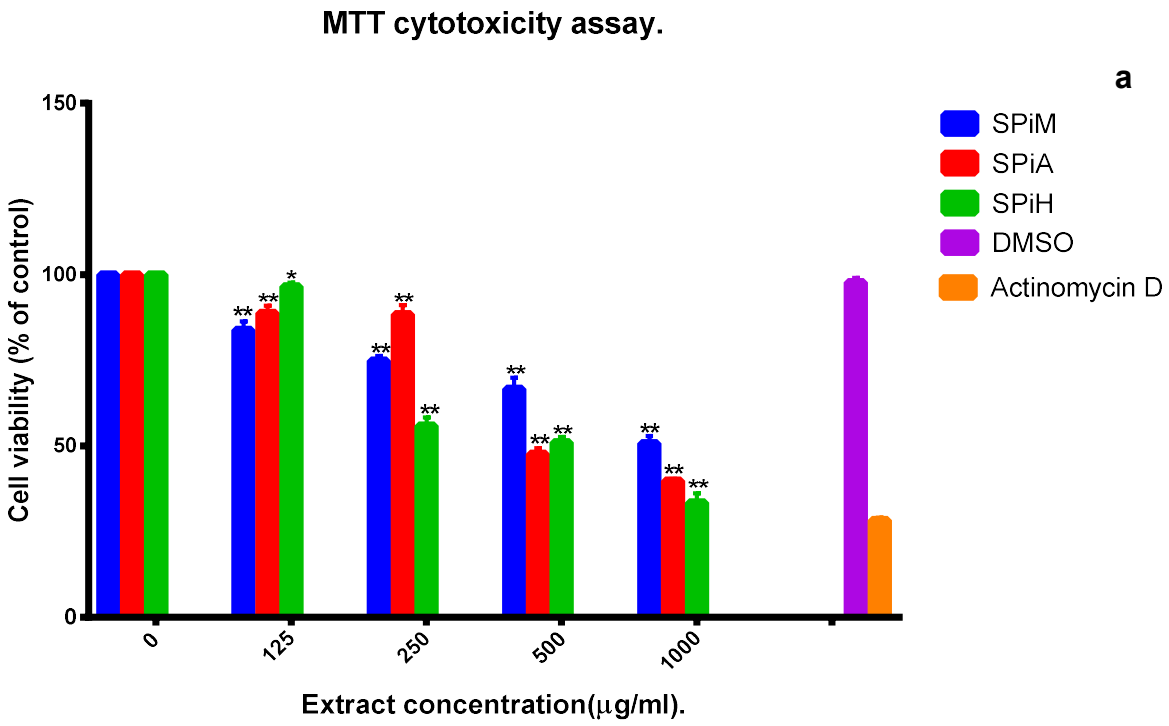

Figure 5.7a: The effects of different extract concentrations of *Schkuhria pinnata* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as-concentration vs cell viability. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for *p* <0,05, two asterisks(**) for *p*<0,01 and three asterisks(***) for *p* <0,001.

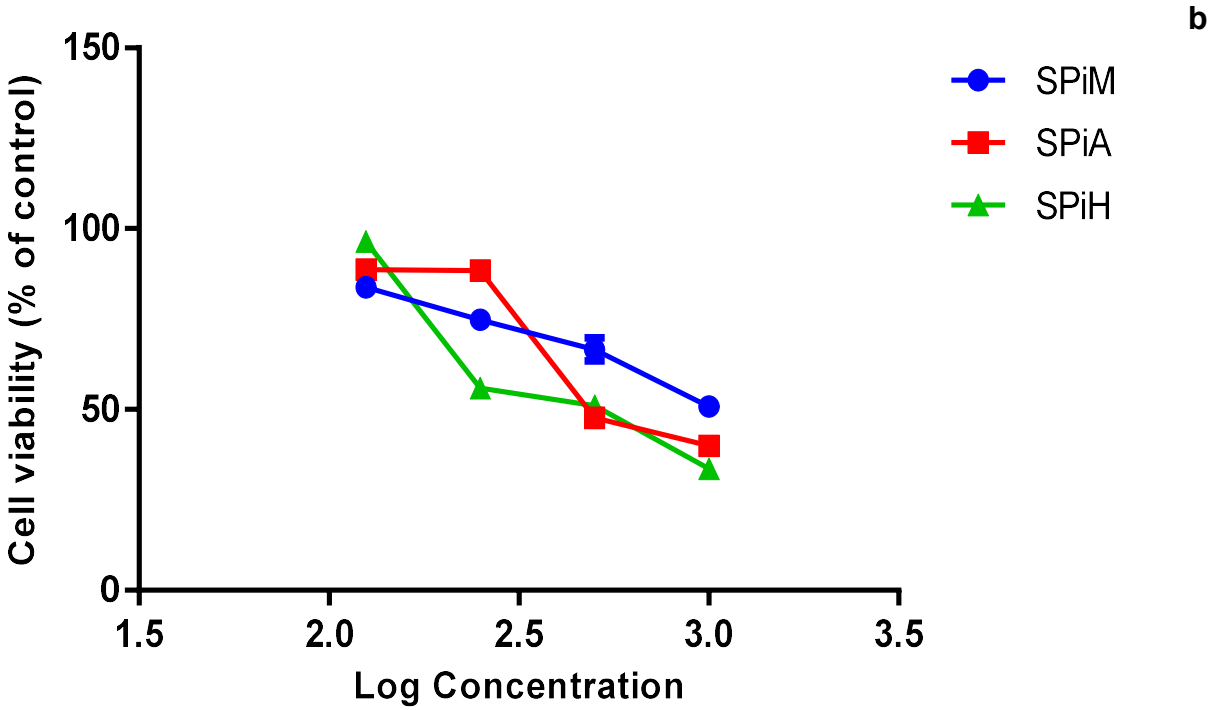

Figure 5.7b: The effects of different extract concentrations of *Schkuhria pinnata* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as Log concentration vs. cell viability. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation.

A similar trend was observed for the methanol, acetone and hexane extracts of *Seriphium plumosum, Toona ciliata and Olea Africana* (Figure 5.8a, Figure 5.9a, Figure 5.10a). However an interesting observation was made wherein the lowest concentrations of *Seriphium plumosum* methanol, acetone and hexane extracts resulted in 104,22%, 122,68% and 126,37% cell viability respectively which were all higher than that of the untreated control (Figure 5.8a).

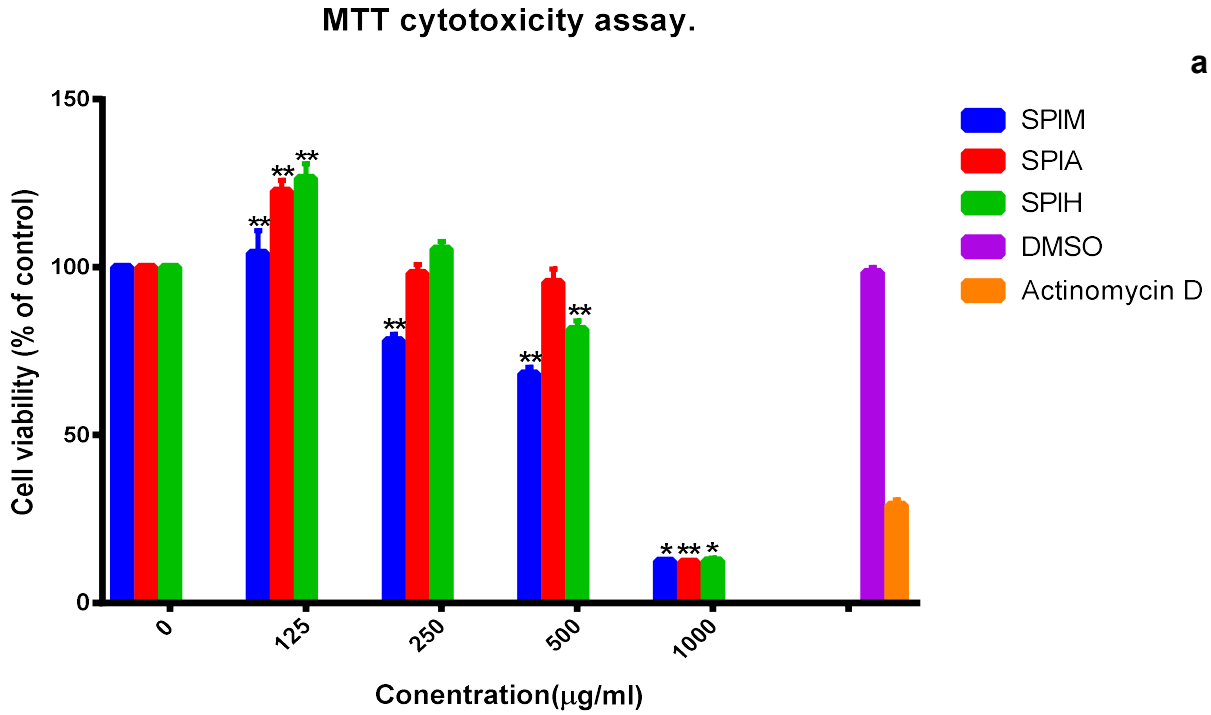

Figure 5.8a: The effects of different extract concentrations of *Seriphium plumosum* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as-concentration vs cell viability. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for *p*<0,05, two asterisks(**) for *p* <0,01 and three asterisks(***) for *p* <0,001.

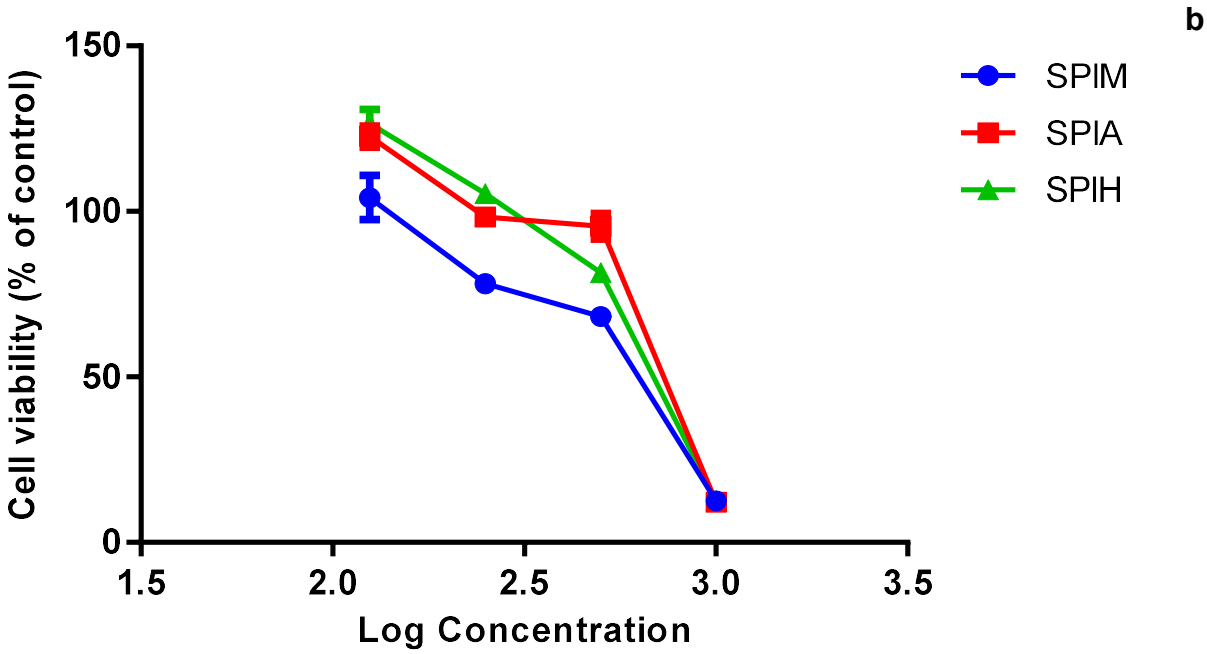

Figure 5.8b: The effects of different extract concentrations of *Seriphium plumosum* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as Log concentration vs. cell viability. SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation.

The hexane extract of *Toona ciliata* resulted in the least CC_{50} value of 402,16 µg/ml and was therefore concluded to be the most cytotoxic extract in this study. The lowest concentration of the hexane extract was however not cytotoxic as it resulted in 98,07% cell viability and was therefore employed in subsequent experiments.

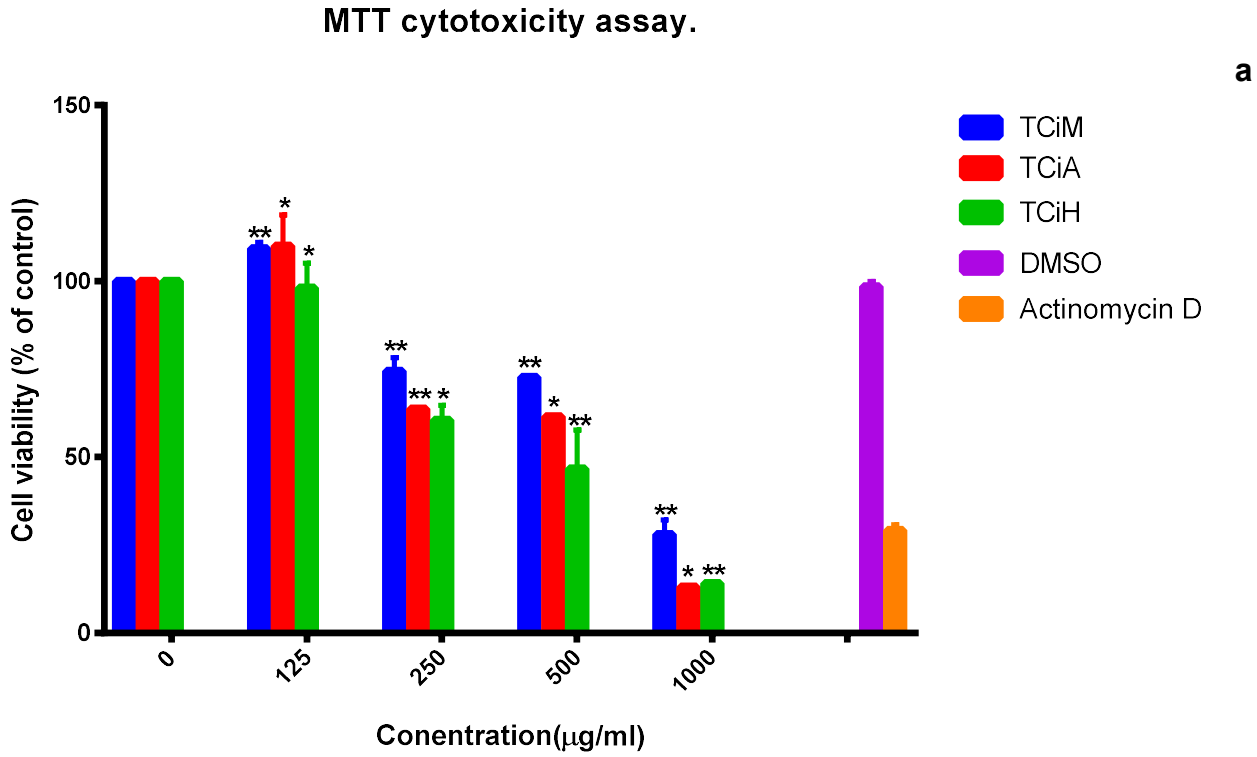

Figure 5.9a: The effects of different extract concentrations of *Toona ciliata* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as-concentration vs cell viability. TCiM=*Toona ciliata* (Methanol extract), TCiA=*Toona ciliata*(Acetone extract),TCiH=*Toona ciliata* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

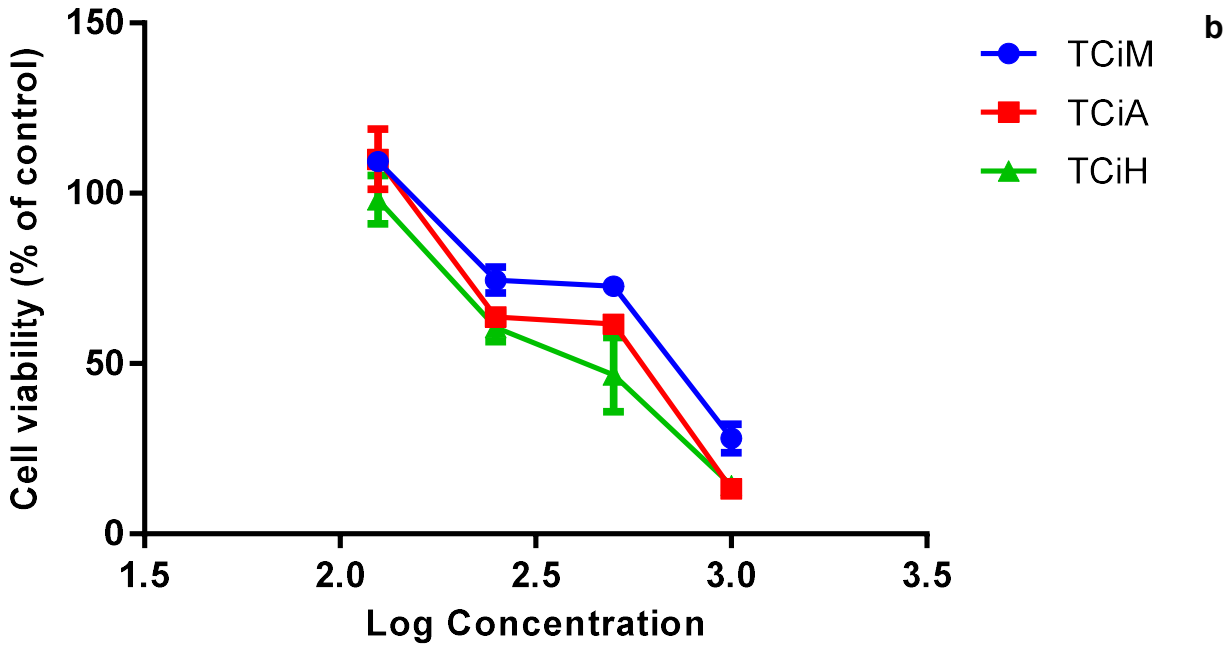

Figure 5.9b: The effects of different extract concentrations of *Toona ciliata* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as log concentration vs. cell viability. TCiM=*Toona ciliata* (Methanol extract), TCiA=*Toona ciliata*(Acetone extract),TCiH=*Toona ciliata* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation.

The hexane extract of *Olea Africana* was not used in the following experiments as it was cytotoxic even at low concentrations which resulted in over 40% cytotoxicity (Figure 5.10a).

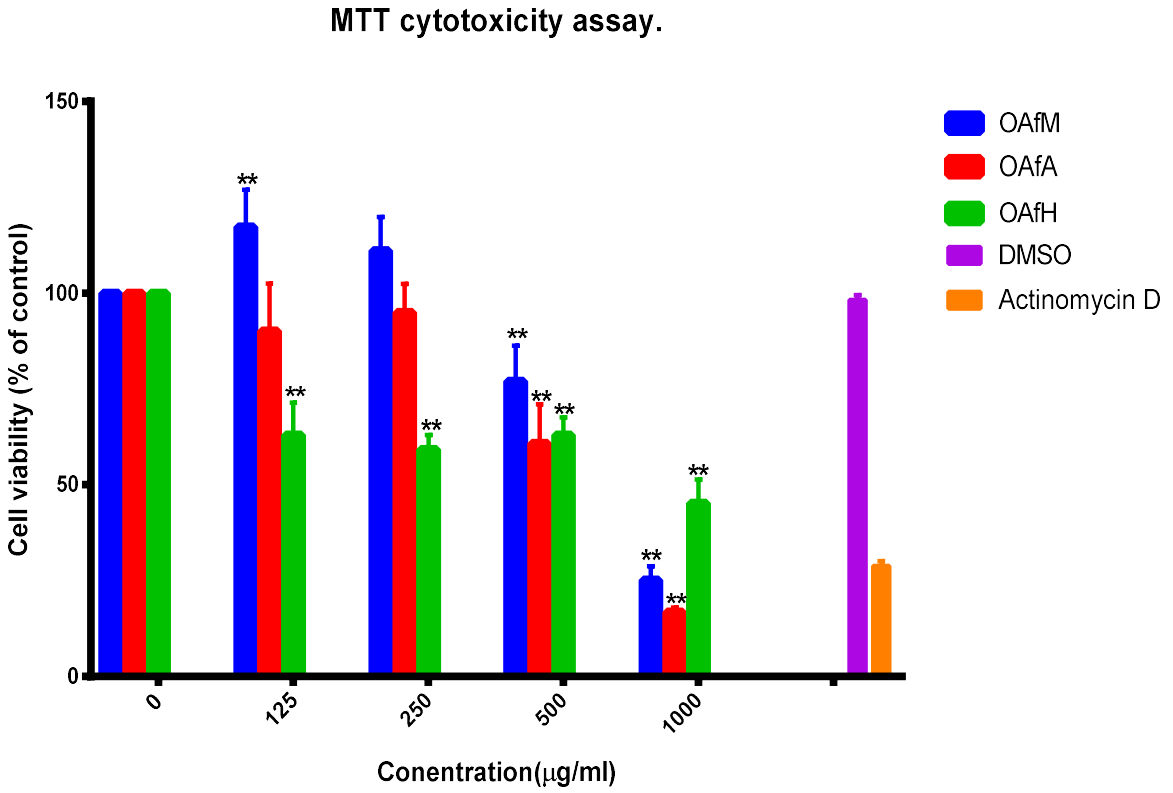

Figure 5.10a: The effects of different extract concentrations of *Olea africana* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as concentration vs cell viability. OAfM=*Olea africana* (Methanol extract), OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test.The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

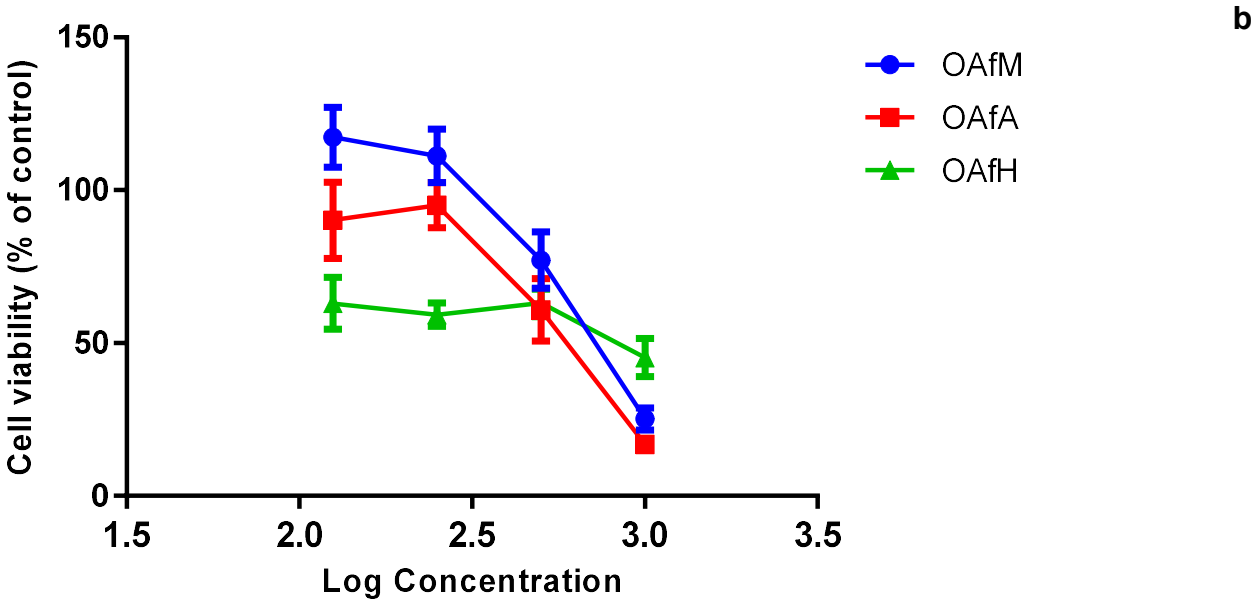

Figure 5.10b: The effects of different extract concentrations of *Olea africana* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as Log concentration vs. cell viability. OAfM=*Olea africana* (Methanol extract), OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation.

The *Opuntia ficus indica* extracts exhibited the least cytotoxicity as they had the highest CC_{50} values as compared to all the other extracts under investigation in this study (Table 5.1). The methanol, acetone and hexane extracts of *Opuntia ficus indica* resulted in CC_{50} values > 1000µg/ml respectively.

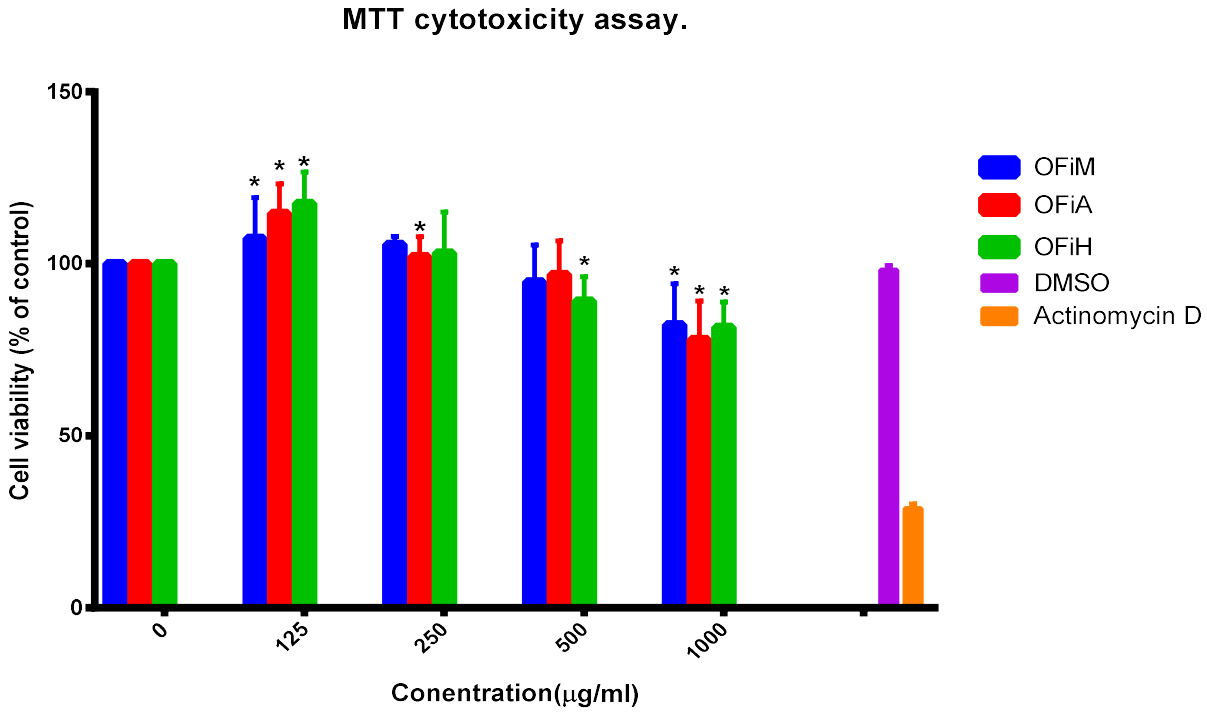

Figure 5.11a: The effects of different extract concentrations of *Opuntia ficus indica* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as- concentration vs cell viability. OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

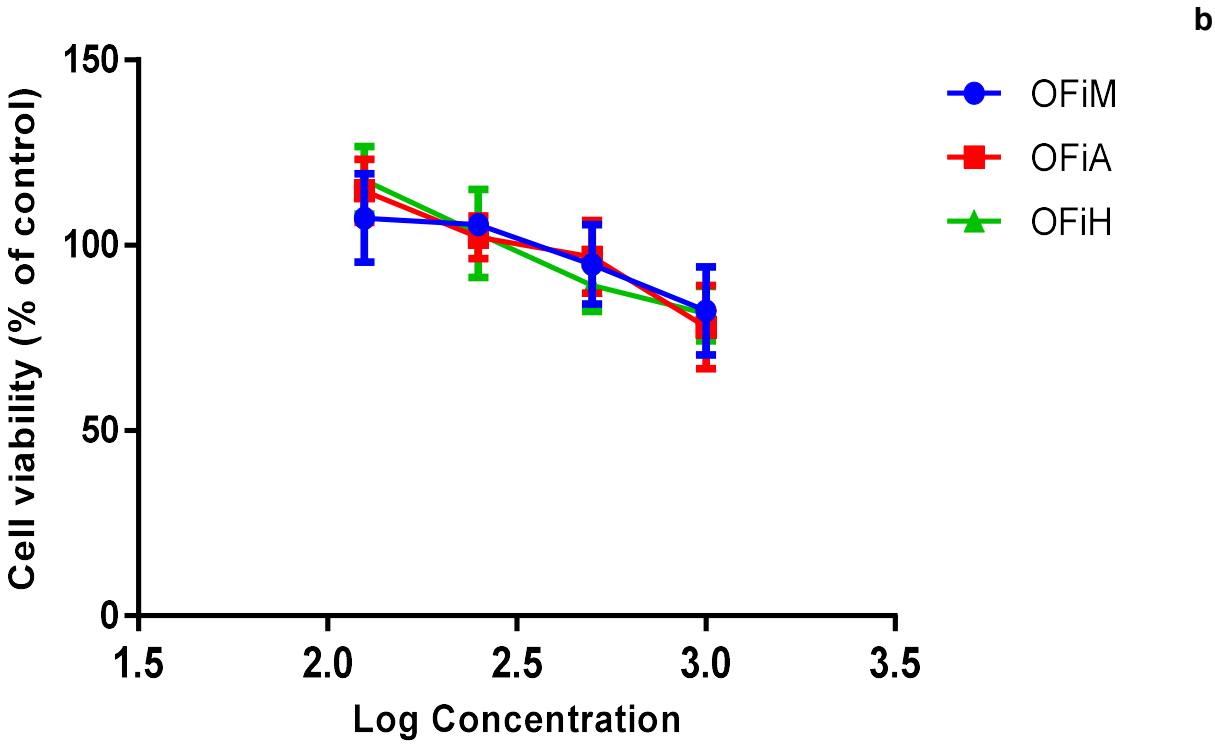

Figure 5.11b: The effects of different extract concentrations of *Opuntia ficus indica* on the proliferation of murine myoblast cells (C2C12) as a percentage of the untreated control represented as Log concentration vs. cell viability. OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation.

The CC_{50} values for the MTT cell viability assay of the different plant extracts were calculated (Table 5.1). They were determined by linear regression from the graphs of each of the extracts' log concentrations against their percentage activity for each of the experiments. The concentrations that exhibit a 50 % activity were taken to be CC_{50} values. The lower the CC_{50} value the more cytotoxic that particular extract is. The methanolic extract of *Opuntia ficus indica* showed the least cytotoxic effect with a CC_{50} value of 16409,68 µg/ml. while the hexane extract of *Toona ciliata* exhibited the most cytotoxic effect on the C2C12 cells with a CC_{50} value of 402,16 µg/ml.

Table 5.1: The values indicated in the table below represent concentrations of the various plant extracts that result in 50% cytotoxicity termed the CC_{50} value. The values were obtained by linear regression. Higher CC_{50} values mean that the extract is less toxic than those with lower CC_{50} values.

Extract	**CC_{50}(µg/ml)**
SPiM	>1000
SPiA	644,76
SPiH	491,36
SPlM	518,80
SPlA	691,03
SPlH	641,80
TCiM	641,80
TCiA	470,98
TCiH	402,16
AOfM	734,68
OAfA	541,87
OAfH	>1000
OFiM	>1000
OFiA	>1000
OFiH	>1000

KEY: SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract). SPlM=*Seriphium plumosum*, (Methanol extract) SPlA =*Seriphium plumosum* (Acetone extract), SPlH=*Seriphium plumosum* (Hexane extract) TCiM=*Toona ciliata* (Methanol extract) TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract) OAfM=*Olea africana* (Methanol extract), OAfA=*Olea africana* (Acetone extract),OAfH=*Olea africana* (Hexane extract)). OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract)

5.4.4 Effects of plant extracts on glucose uptake by the C2C12

The amount of glucose utilized by the differentiated C2C12 cells under different treatment conditions was quantified by the glucose uptake assay. The percentage glucose utilised was calculated with respect to the untreated control for 1, 3 and 6 hours. Dimethylsulphoxide (DMSO) and insulin were the negative and positive controls respectively. The glucose utilisation in the DMSO treated cells was comparable with the untreated control. The cells were treated with plant extracts in the presence or absence of insulin.

The effect of different extracts of *Olea Africana* in the presence or absence of insulin on the glucose uptake by the C2C12 cells at different time intervals is represented below (Figure 5.12). Only the hexane extract of O*lea africana* was not used in the glucose uptake experiment as it was cytotoxic to the cells even at low concentrations. Glucose uptake increased over time for all the different treatments. The highest glucose utilisation is observed after 6 hours. The combination of *Olea Africana* acetone extract with insulin resulted in the second highest glucose utilisation of 24,94% after that of insulin which was 26,06% after 6 hours (Figure 5.12). The least glucose utilisation was observed for *Olea Africana* methanol extract which resulted in only 7,90% glucose utilisation after 6 hours and was lower than that of the untreated control which was 13,52%(Figure 5.13)

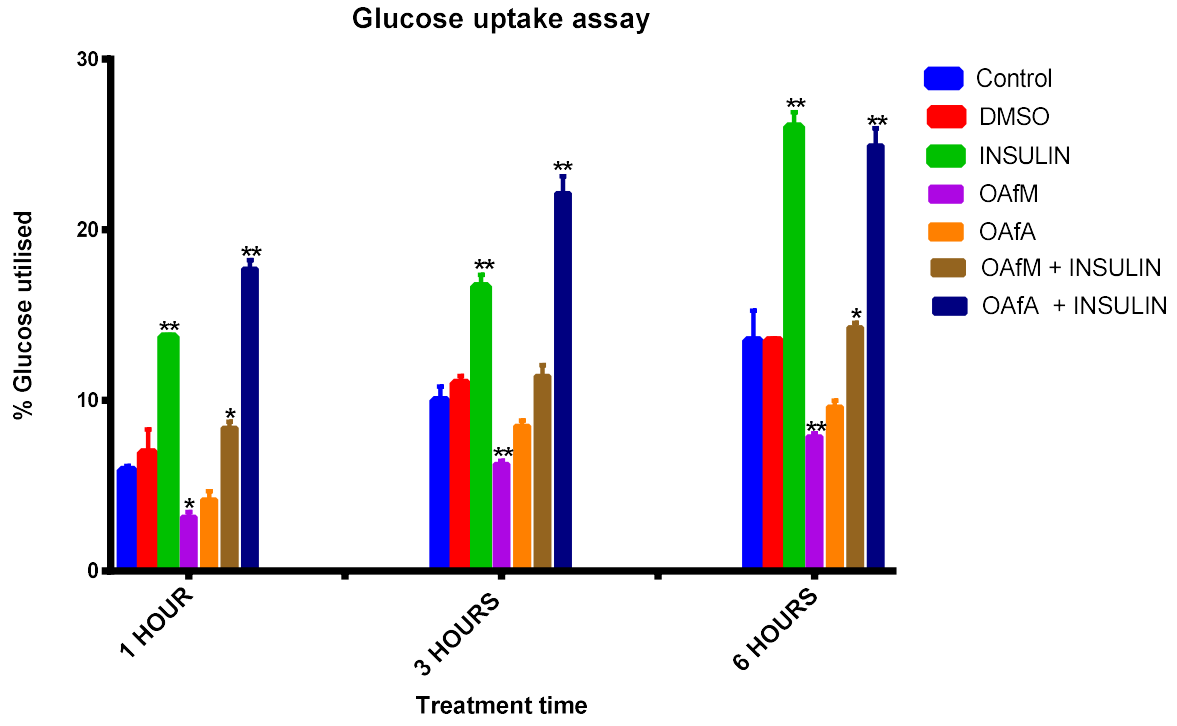

Figure 5.12: The effects of different extract of *Olea Africana* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. OAfM=*Olea africana* (Methanol extract), OAfA=*Olea africana* (Acetone extract), DMSO= Dimethylsulphoxide. The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using One-way Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

The acetone extract of *Opuntia ficus indica* resulted in the least glucose uptake of 4, 99% after a 6 hour period when compared to the rest of the other treatments in this study (Figure 5.13). In contrast the treatment which had the combination of the acetone extract of *Opuntia ficus indica* and insulin resulted in the highest glucose uptake of 31,51% . This was closely followed by the treatment of the cells with the hexane extract of *Opuntia ficus indica* which had a glucose utilisation of 30,04%. These two previously mentioned treatments resulted in glucose utilisation higher than that of insulin only (26,06%).

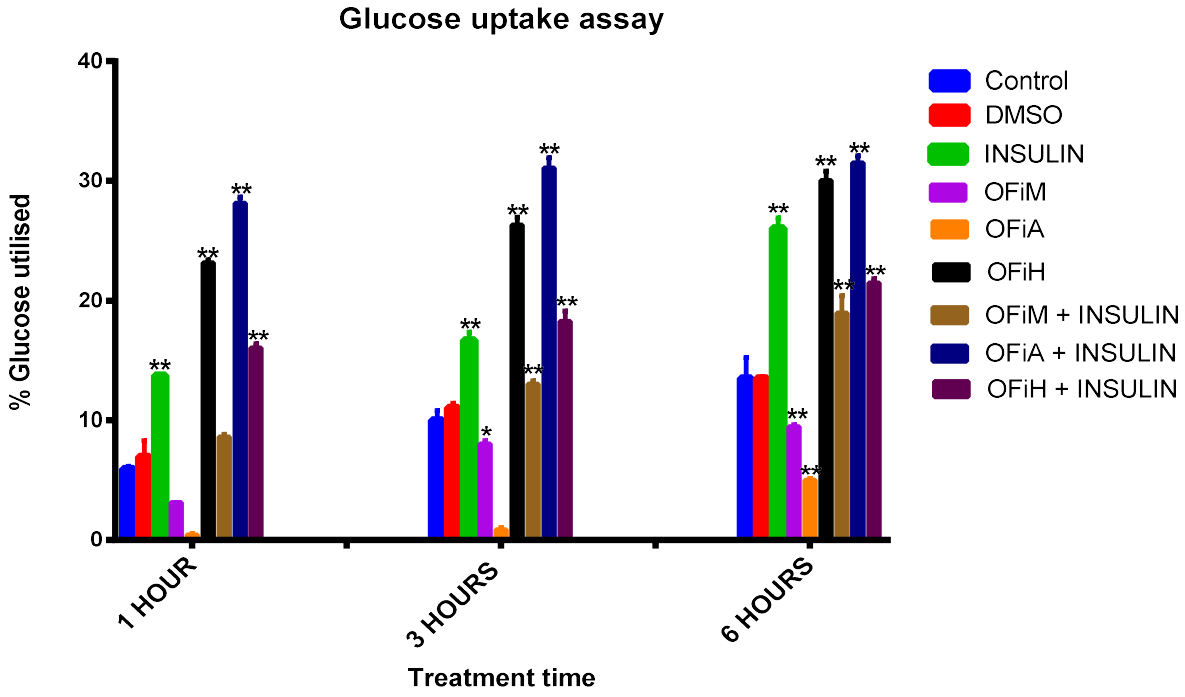

Figure 5.13: The effects of different extracts of *Opuntia ficus indica* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. OFiM=*Opuntia ficus indica* (Methanol extract),OFiA=*Opuntia ficus indica* (Acetone extract), OFiH=*Opuntia ficus indica* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

All the extracts of *Schkuhria pinnata* whether or not in combination with insulin resulted in glucose utilisation greater than that of the untreated control (Figure 5.14). This was a different trend as compared to the extracts of *Opuntia ficus indica* and those of *Olea Africana* previously described. While that was the trend, only the hexane extract of *Schkuhria pinnata* resulted in glucose utilisation of 28, 56% which was higher than that of insulin (26,06%) after 6 hours (Figure 5.14).

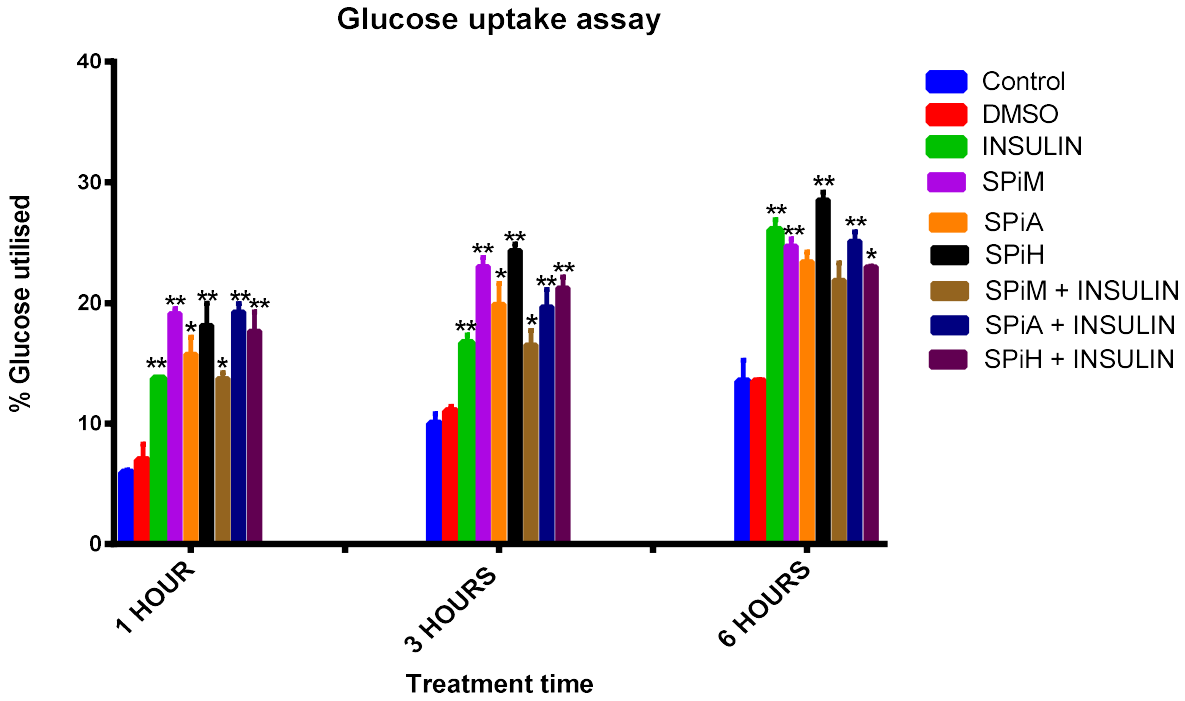

Figure 5.14: The effects of different extract of *Schkuhria pinnata* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. SPiM=*Schkuhria pinnata* (Methanol extract), SPiA=*Schkuhria pinnata* (Acetone extract),SPiH=*Schkuhria pinnata* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

On the other hand none of the treatments with different extract of *Toona ciliata* resulted in glucose utilisation that was lower than that of insulin only (Figure 5.15). The highest glucose utilisation resulted from the treatment which had the acetone extract of *Toona ciliata* in combination with insulin which resulted in 21,37% glucose utilisation after 6 hours (Figure 5.15). This was closely followed by the treatment with the hexane extract of *Toona ciliata* which had a glucose utilisation of 19,63% followed by that of the methanol extract with 16,98% (Figure 5.15). The rest of the treatments resulted in glucose utilisation less than that of the untreated control (Figure 5.15). Except for the hexane extract of *Toona ciliata* the combination of the plant extracts with insulin resulted in increased glucose uptake.

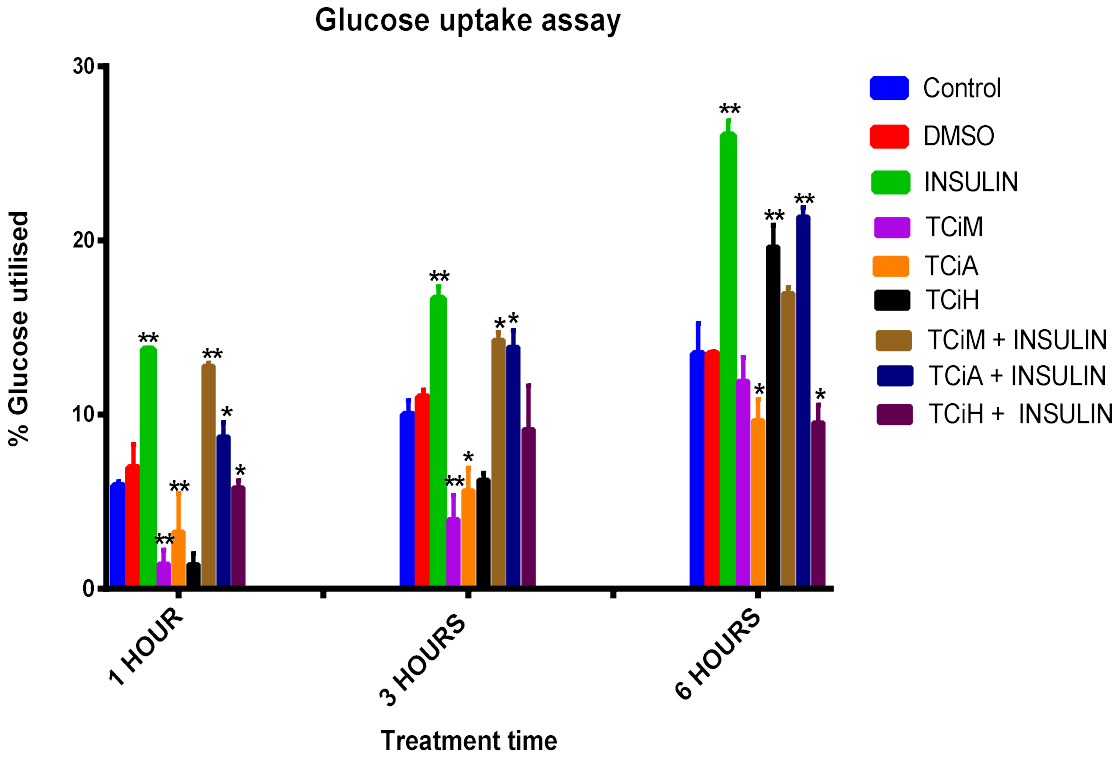

Figure 5.15: The effects of different extract of *Toona ciliata* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. TCiM=*Toona ciliata* (Methanol extract), TCiA=*Toona ciliata* (Acetone extract),TCiH=*Toona ciliata* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

When the effects of the different extract of *Seriphium plumosum* in the presence or absence of insulin on the glucose uptake by C2C12 were examined a unique trend was observed (Figure 5.16). The combination of the plant extracts with insulin resulted in less glucose uptake as compared to the plant extract alone. This was observed for all the extracts of this plant particularly the hexane extract of *Seriphium plumosum*, which not only resulted in the highest glucose uptake of 35, 77% but was also shown to have more potent glucose uptake ability than insulin when used alone than in combination with insulin after 6 hours of exposure (32, 23 %).The percentage glucose utilisation increased as the time increased.

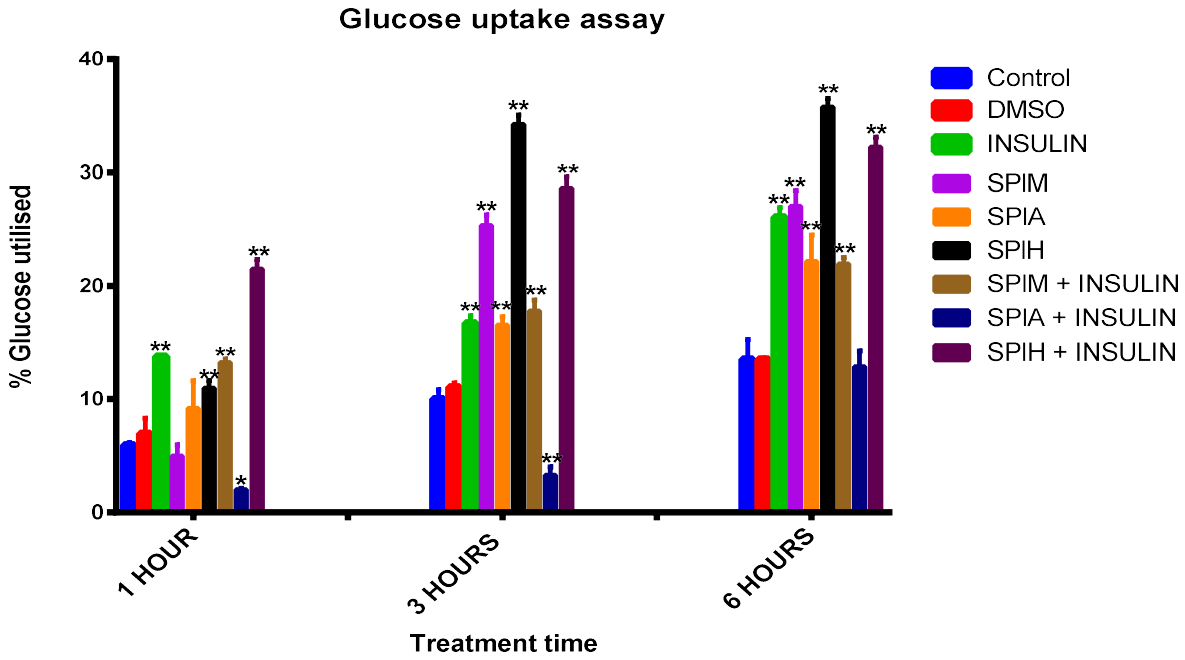

Figure 5.16: The effects of different extract of *Seriphium plumosum* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. . SPIM=*Seriphium plumosum*, (Methanol extract) SPIA =*Seriphium plumosum* (Acetone extract), SPIH=*Seriphium plumosum* (Hexane extract), DMSO= Dimethylsulphoxide. 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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$, two asterisks(**) for $p<0,01$ and three asterisks(***) for $p<0,001$.

5.5 Discussion

Increased glucose disposal by various tissues results in a direct lowering of the blood glucose in circulation. This is beneficial for people suffering from diabetes mellitus as they are known to have perpetual hyperglycaemia. Various medications are therefore aimed primarily at increasing glucose disposal by different target organs. In this study, the cytotoxic effects of the selected plants were established to determine which concentrations were non-toxic to the cell line used. Thereafter the extent to which the selected plant extracts affect glucose disposal was investigated. A murine myoblast cell line was used as an *in-vitro* muscle cell model due to its ability to mimic the action of muscles in the body. Since hyperglycaemia is a hallmark feature observed in diabetic patients, the ability of the plant extract to inhibit glycation was also investigated. Glycation which is implicated as the major cause of the debilitating signs and symptoms in diabetes patients is a disruptive spontaneous reaction that occurs mainly between proteins and reducing sugars. A previous study by Jedsadayanmata (2005) has shown that arbutin inhibits glycation of BSA in the presence of glucose. Arbutin is therefore a known inhibitor of the glycation reaction which was therefore used as a reference standard to assess the anti-glycation potency of the plant extracts in the current study. In the presence of Arbutin 9,73% of the BSA was shown to be glycated in this study. Some of the plant extracts assayed showed more potent anti-glycation activity as compared to Arbutin at the concentrations tested. The acetone extract of *Seriphium plumosum* showed the most BSA glycation inhibitory activity as it resulted in 2,22% glycation followed by the methanol, acetone and hexane extract of *Toona ciliata* which resulted in 2,49%, 2,79% and 2,56% glycation of BSA respectively. These plants in chapter 4 were shown to contain the highest amount of total phenolic compounds in this study (section 4.4.3.2). Available reports (Sadowska-Bartosz and Bartosz, 2015) suggests plants with high concentrations of total phenolic compounds to possess high anti-oxidant and anti-glycation activity This report is consistent with findings in this study because the hexane extracts of *Schkuhria pinnata* and *Opuntia ficus indica* that were shown to contain the least amount of total phenolic content in this study are also shown to exhibit poor glycation activity of BSA (section 4.4.3.2). Although the precise mode by which these plants exhibit their anti-glycation activity have not been established, previous study suggests that anti-glycation agents may

act by delaying the formation of AGEs by preventing further oxidation of Amadori product and metal-catalyzed glucose oxidation (Ramkissoon *et al*., 2013). Healthy individuals and diabetic patients that drink herbal teas of plants that have anti-glycation activity have been shown to have delayed onset of diabetes (Elosta, *et al*., 2012).

Establishment of cytotoxic and non-cytotoxic concentrations of different plant extracts is a crucial step in ascertaining the use and safety of the plant extract as a therapeutic agent (Ernst and Pittler, 2002). While some plants may have therapeutic uses at lower concentrations, intake of these plants above these concentrations may be as dangerous as an overdose of the approved drugs. The viability of the cells in this study was shown to decrease as the concentration of the plant extracts increased. A study by Deutschländer *et al* (2009) have shown the methanolic and acetone extracts of *Schkuhria pinnata* to be cytotoxic on 3T3-L1 preadipocytes. Another study by Muthaura *et al* (2007) showed low cytotoxicity of the methanolic and water extracts of *Schkuhria pinnata* to Vero E6 cells even though in vivo test in mice did not show any adverse effect. Rahman *et al* (2015) showed that *Olea africana* ethanolic extract exhibited significant cytotoxic effect in the brine shrimp lethality bioassay. Oleuropein which is one of the main active ingredients found within this species has also been shown to be a potent anti-cancer agent as it was shown to be cytotoxic to MCF7 breast cancer cells (Hamdi and Castellon, 2005). Oleuropein is also reported to limit the growth and induce apoptosis in HT-29 human colorectal adenocarcinoma cells via p53 pathway activation adapting the HIF-1α response to hypoxia (Cárdeno *et al*., 2013). Kim *et al* (2015) showed that SW480 human colorectal cancer cells were more sensitive than the MCF7 human breast cancer cells to the cytotoxic effect of various *Opuntia ficus-indica* extracts. Cell death by various *Opuntia ficus-indica* extract treatment caused significant inhibition of cyclooxygenase-2 and increased the Bax/Bcl2 ratio in both SW480 and MCF7 cell lines (Kim *et al*., 2015). Siderin isolated from *Toona celiata* was shown to have cytotoxic principles in the brine shrimp lethality bioassay (Chowdhury *et al*., 2003). Isolates from *Toona celiata* were evaluated for their cytotoxic properties on six cancer cell lines and were shown to have moderate cytotoxicity (Zhang *et al*., 2012). No documented report is available on cytotoxic effect of *Seriphium plumosum*. In this study the plant was shown to be non-cytotoxic at lower concentrations as opposed to

the highest concentrations of *Seriphium plumosum* methanol, acetone and hexane extracts which resulted in 12.53%, 12,25% and 12,64% cell viability respectively. The lowest concentrations of *Seriphium plumosum* methanol , acetone and hexane extracts resulted in 104,22%, 122,68% and 126,37% cell viability respectively. These results suggest that the plant may have compounds that encourage cell proliferation of C2C12 cells.

Apart from the *Seriphium plumosum* extracts, similar activity was observed for lower concentrations of the other plant extracts in this study as they too resulted in increased cell proliferation. This may be due to the fact that the crude plant extract contains an array of compounds which have different targets within the cell. Some of the compounds may therefore be pro-proliferation agents which may be responsible for the observed effect. Although concentrations below 125 µg/ml were observed to increase cell number, concentration of 100 µg/ml employed in the glucose uptake assay would not affect the cell density of C2C12 exposed to extracts for 6 hours since the doubling number of this cell is known to be between 19-24 hours. Differences in results obtained in this study and those reported in the literature could also be ascribed to differences in extraction procedures and the natural variability in plants. The toxicology information obtained in this section can be invaluable in the interpretation of drug safety signals, alteration in posology, or removal of the substance (Kamsu-Foguem and Foguem, 2014).

In this study all the plant extracts that resulted in increased glucose utilisation when compared to untreated control suggests their potential as a source of anti-diabetic agents. Previous studies show that substances that increase glucose disposal by various peripheral organs including the muscles has a hypoglycaemic effect (Shrayyef and Gerich, 2010). The untreated control resulted in 13,52% glucose utilisation after 6 hours.

Olea africana has been shown to improve glucose homeostasis in animal models by reduction of starch digestion and absorption (Wainstein *et al*., 2012).Polyphenols from *Olea europaea L.* a close relative of *Olea africana* has been shown to significantly improve insulin sensitivity and pancreatic β-cell secretory capacity in overweight middle-aged men at risk of developing *diabetes mellitus* (de Bock *et al*., 2013). In the current study, the combination of *Olea Africana* acetone (OAfA) extract

with insulin resulted in glucose utilisation of 24,94% which was less than that of insulin which was 26,06% after 6 hours . The use of this extract was therefore discontinued together with the methanol extract of *Olea Africana* acetone (OAfM) which resulted in even less activity. The increased antidiabetic effect of *Olea Africana in-vivo* maybe due to the fact that the plant enhances other anti-diabetic factors which were not examined by this study (Wainstein *et al*., 2012; de Bock *et al*., 2013).

Treatment of diabetic rats with a single or repeated dose of *Opuntia ficus indica* fruit juice could restore homeostatic glucose levels (El-Razek and Hassan, 2011). The acetone extract of *Opuntia ficus indica* resulted in the least glucose uptake of 4,99% after a 6 hour period (Figure 5.8). The glucose utilization fold of the acetone extract of *Opuntia ficus indica* was calculated to be 0,37 and was therefore concluded to be inhibiting glucose uptake by the cell. Insulin was used as a positive control and resulted in a glucose utilisation of 26,06% after 6 hours. The low activity of the *Opuntia ficus indica* extract are supported by a previous study by Frati-Munari *et al*., (1988) which suggest that dehydration of the leaves results in reduced activity. The study found that dehydrated extract of nopal (*Opuntia ficus-indica*) did not show acute hypoglycaemic effect, although it could attenuate postprandial hyperglycaemia Frati-Munari *et al*., (1988).

A study by Deutschländer *et al* (2009) showed that acetone and methanol extracts of *Schkuhria pinnata* resulted in increased glucose uptake in C2C12 and 3T3-L1 cells. The study however discontinued further assessments as there were cytotoxicity concerns. In the current study however, the methanolic, acetone and hexane extracts of *Schkuhria pinnata* is shown to increase glucose uptake in a time dependent manner. The hexane extract of *Schkuhria pinnata* resulted in the highest glucose utilisation of 28, 56% among all the *Schkuhria pinnata* treatments. The cytotoxicity concerns previously raised by Deutschländer *et al* (2009) did not affect the results in the current study as the cells were treated for a maximum of 6 hours only. Studies with this plant were however not carried forward as it did not result in the most potent glucose uptake activity.

Findings of a previous study by Rana and colleagues (2016) suggest that *Toona ciliata* has significant antihyperglycemic activity in streptozotocin induced diabetes in

rats. The highest glucose utilisation in this study resulted from treatment with acetone extract of *Toona ciliata* in combination with insulin which resulted in 21,37% glucose utilisation after 6 hours (Figure 5.10). This activity was however the highest activity obtained among all the plant extracts in this study and the use of the plant for further experiments was dis-continued. In addition, the study found that the rest of the treatments resulted in glucose utilisation less than that of the untreated control.

The hexane extract of *Seriphium plumosum* showed the best glucose utilisation effect of 35,77% after 6 hours. The highest glucose utilisation fold was however calculated to be greater at 3 hours with a value of 3, 42 fold as opposed to the 6 hour period which resulted in a 2,65 fold increase in glucose . The hexane extract of *Seriphium plumosum* in combination with insulin resulted in 32,23 % glucose uptake after 6 hours. To the best of my knowledge no studies on the antidiabetic activity of *Seriphium plumosum* have been reported. The impressive glucose uptake activity of this plant is novel and warrants further study.

The extracts which increased glucose utilisation may work by being insulin mimetics. Insulin mimetic help regulate glucose uptake by the muscle cells by providing compounds that mimic that of insulin thereby eliciting a similar cascade of reaction to that of insulin that result in increased glucose uptake (Manukumar *et al*., 2016). The compounds may not necessarily bind to insulin receptor on the cell surface membrane but to any other protein within the cascade. These compounds are helpful particularly to patients that produce relatively low amounts of insulin. Most of the plant extracts were observed to enhance glucose uptake activity more when in combination with insulin as opposed to the extract alone. This is possibly because these plants extracts fall into a separate class of hypoglycaemic compounds known as inulin sensitisers (Manukumar *et al*., 2016). In the current study, the hexane extract of *Seriphium plumosum* that showed the highest glucose uptake activity may contain compounds that have more of an insulin mimetic effect as opposed to insulin sensitizers. This is due to the fact that the compounds in the extract resulted in more glucose uptake than when used in combination with insulin. This observation is in concurrence with a previous study carried out by Kalekar and colleagues, (2013) which showed increased glucose uptake in 3T3 L1 cells through an insulin sensitizing effect of selected plant extracts. A probable mode of action for this plant extract is that it helps to increase GLUT4 translocation from their intracellular

vesicular store to the cell surface membrane. The GLUT4 molecule is a well characterised insulin dependent glucose transporter. The hexane extract of *Seriphium plumosum* which resulted in the highest glucose uptake after 3 hours was used in further experiments. The next chapter therefore aims to ascertain whether or not *Seriphium plumosum* hexane extract upregulates glucose utilisation through the upregulation of insulin sensitive GLUT4 molecule.

CHAPTER 6

The effect of *Seriphium plumosum* hexane extract on GLUT4 translocation and phosphorylation of various mitogen activated protein kinases

6.1 Introduction

Chronic hyperglycaemia that results from defective glucose metabolism is a hallmark characteristic in diabetic patients. Return of elevated blood glucose levels to normal is based on several cellular mechanisms that dispose of exogenous glucose. The major cellular mechanism involves insulin stimulated glucose uptake into various peripheral tissues. Once the glucose has been taken up by these tissues it may either be stored or immediately oxidised to provide the energy needs of the body. Insulin mediated glucose uptake is primarily mediated by insulin responsive glucose transporter molecule termed GLUT4. GLUT4 is a member of the sugar transporter proteins containing 12-transmembrane domains from the SLC2A4 gene (Huang and Czech, 2007). It is one of the many recognised sugar transporters that catalyse sugar transport across cell membranes through an ATP-independent, facilitative diffusion mechanism. The GLUT4 plays a pivotal role in the removal of glucose from the circulation and is therefore a key regulator of whole-body glucose homeostasis (Jewell, 2010; Huang and Czech, 2007; Fukumoto *et al*., 1989).

The GLUT4 molecules are usually found sequestrated within vesicles in the cytoplasm of cells under normoglycemic conditions. When glucose levels rise beyond the normal levels, the rise is detected and a series of cascade reactions occur which culminate in the secretion of insulin. Insulin is then carried to its various target tissues through the blood stream. At the target tissues insulin will bind to its specific receptor. The binding of insulin to its specific receptor triggers a series of reactions that culminate in the translocation of GLUT4 molecules from their vesicular cytoplasmic stores to the membrane. The GLUT4 molecules fuse with the membrane thereby creating channels that facilitate increased glucose uptake in to the cells (Richter and Hargreaves, 2013). The trafficking of GLUT4 molecules in response to insulin is mediated by a host of proteins collectively known as the mitogen activated protein kinases (MAPK).

Mitogen activated protein kinases (MAPK) are a family of proteins at the heart of various important signal transduction pathways (Robinson and Cobb, 1997; Arbabi and Maier, 2002; Šamajová, *et al*., 2013). This group of proteins control processes such as the activation of other protein kinases, production of second messengers and the appropriate subcellular redistribution of those transducers to bring them into contact with their various targets. This enables the MAP kinases to greatly influence processes such as embryogenesis, cell differentiation, cell proliferation, gene expression, metabolism, cell morphology and cell survival (Šamajová, *et al*., 2013). MAP kinases are activated by a wide range of stimuli which in-turn phosphorylate numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes. These stimuli results in the activation of a three tiered kinase cascade comprising of a MAP kinase kinase kinase (MAPKKK,MAP3K, MEKK or MKKK), a MAP kinase kinase (MAPKK, MAP2K, MEK or MKK) and the MAPK (Šamajová, *et al*.,2013). The effects that various compounds have on these proteins are of paramount importance in the understanding of any pharmacological mode of action of these compounds. The chapter therefore is aimed at determing the effects of *Seriphium plumosum* hexane extract in the presence or absence of insulin on the translocation of GLUT4 molecules from their intra-cellular vesicles to the cell surface membrane. This was achieved by examining the extent to which the extract influenced the expression of mitogen activated proteins involved in both GLUT4 translocation pathway and glucose metabolism in general.

6.2 Objectives:

i. To qualitatively analyse the expression of GLUT4 molecules using fluorescence microscopy.
ii. To quantify the GLUT4 molecules on cell surface membrane using fluorescence microscopy.
iii. To analyse the extent to which various mitogen activated protein kinases involved in the GLUT4 translocation pathway are phosphorylated.

6.3 Materials and methods

6.3.1 GLUT4 translocation assay

The cells were differentiated by culturing them in RPMI media containing 2% horse serum for 4 days. The differentiated C2C12 cells were seeded overnight on microscope glass cover slips in 6 well plates. The cells were treated with selected concentrations of the plant extracts for 3 hours. Insulin was used as a positive control and DMSO was used as a negative control. After treatment, the cells were fixed with 80% methanol. Cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) followed by incubation with the anti-GLUT4 primary antibody .The cells were then incubated with secondary antibody conjugated to FITC and viewed using a fluorescence microscope images were captured as detailed in section 3.14.

6.3.2 MAPK profiler assay

The expression of 26 mitogen activated protein kinases in the cells was determined using the human MAPK profiler assay Kit according to the manufacturer's instructions (RnDSystems) as detailed in section 3.14. Differentiated C2C12 cells were seeded and treated for 3 hours in the presence or absence of plant extract. Insulin was used as a positive control. After treatment the cells were immediately rinsed with PBS and lysed. The total quantity of protein in the lysates was immediately determined using the BCA protein assay.

Following quantification, the samples were diluted appropriately and the reconstituted detection antibody cocktail was added. The prepared samples were then added to the 4 well plate with the membranes and incubated overnight on a rocking platform shaker. The membranes were carefully washed and Streptavidin-HRP was added after which Chemi Reagent Mix was added evenly onto each membrane. The membranes were then washed using TBST and the transferred proteins were detected using the Super Signal West Dura chemiluminescent

substrate (Thermo scientific, U.S.A) and antigen antibody complex was visualised by photo-detection using the Syne-Gene Image analyser (Bio-Rad S.A).

6.4 Results

6.4.1 Glut 4 translocation assay

In this study the GLUT4 translocation assay was used to determine the localisation of GLUT 4 molecules under various treatment conditions. The cells were treated or not with the hexane extract of *Seriphium plumosum* (100 µg/ml) in the absence or presence of insulin. The untreated cells served as the negative control while DMSO and insulin served as solvent and positive controls respectively. The cellular localization of GLUT4 was determined by first using a primary antibody specific for GLUT4 molecule, thereafter staining with an FITC conjugated secondary antibody to detect the areas to which the primary antibody had bound. The FITC fluoresces green; therefore the green fluorescence indicates the area where GLUT4 molecules are located.

Higher fluorescence intensity means that more of GLUT4 molecules have translocated to the membrane where the primary antibodies would subsequently have greater access and bind more. On the other hand, lower green fluorescence intensities means that more of GLUT4 molecules remain sequestrated in their cytoplasmic peri-nuclear vesicular stores. The Houestch stain fluoresces blue when it is bound to DNA material. The Houestch stain was therefore used to stain the DNA of the cells so as to pinpoint the position of the nucleus in relation to both the cytoplasm and the cell membrane. This was done by overlaying the images of the green fluorescence and those of the blue fluorescence. Furthermore the intensity profiles of the green fluorescence which represented GLUT4 molecules were quantified. The results of an average of 4 focal points per treatment were graphed. Each of the florescence intensity profile from the various treatments was compared to that of the control.

The untreated cells represented below (Figure 6.1) resulted in a fluorescence intensity profile characterised by cells with relatively low intensity. These cells were taken to represent cells that had basally translocated levels of GLUT4 molecules bound to their membrane. In the overlay images (Figure 6.1C) of the untreated cells the blue colour overshadows the green colour. This is due to the fact that the green fluorescence in B is concentrated within the cytoplasm as compared to that on the membrane. The heavily stained nucleus in A is shown to be more dominant in the overlay images in C.

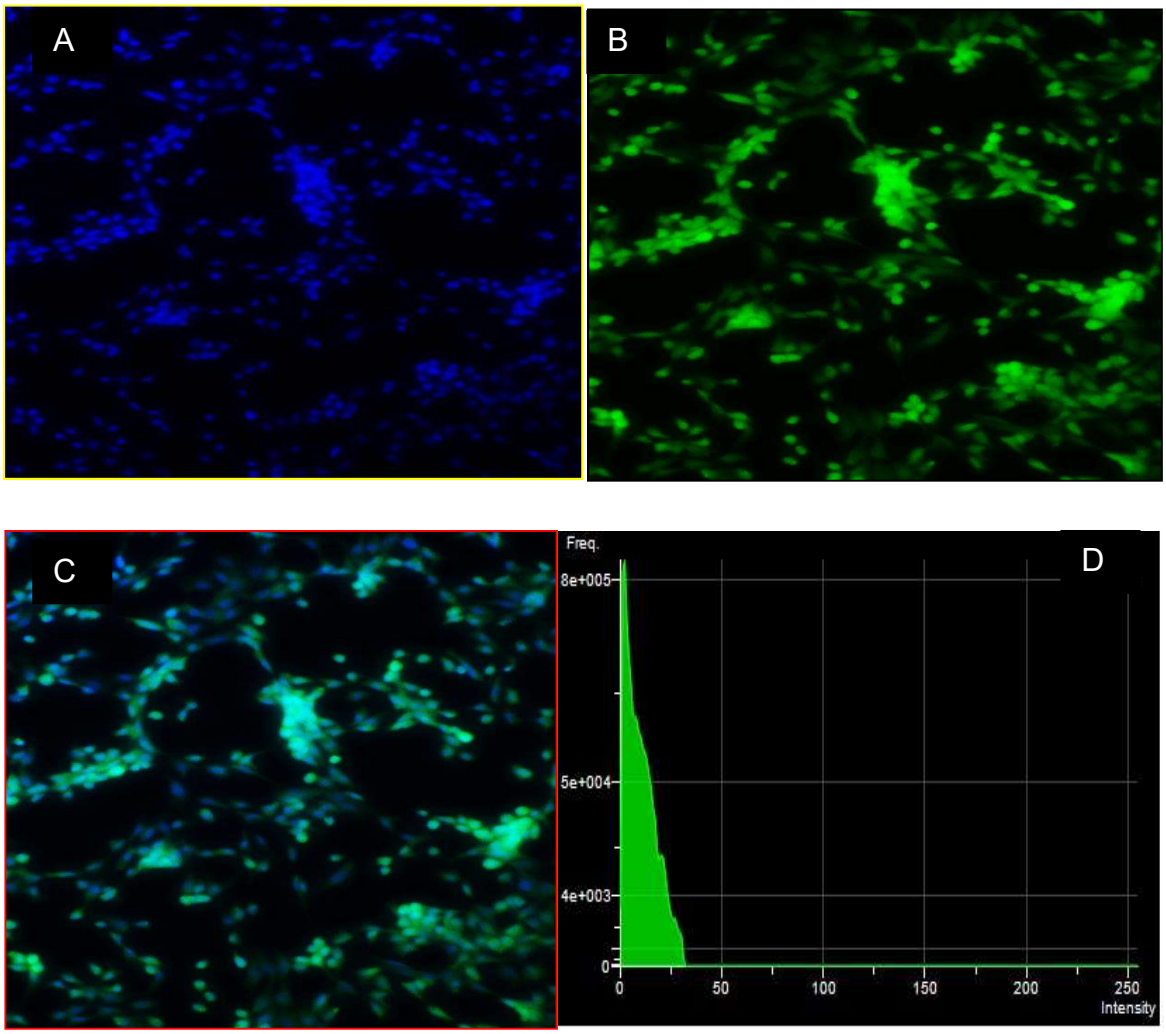

Figure 6.1:The flourescence profiles of the untreated differentiated C2C12 cells. The images above were taken by the X100 objective of an inverted flourescence microscope at a X60 magnification.A= images of the nucleas stained with DAPI which flouresce blue. B= the green flourescence represents the FITC bound GLUT4 molecules.C= An overlay of Images A and B to show the the position of the nuleas with respect to the distribution of the GLUT4 molecules. D=The flourescence intensity profile of the green flourescence from the FITC conjugated secondary anti-body which was used to stain the GLUT4 molecules of the untreated differentiated C2C12 cells.

A similar trend to that observed in the control was observed in the cells that were treated with DMSO. DMSO which was the solvent control did not increase or decrease the translocation of GLUT4 molecules to the membrane. This was further confirmed by the overlay of the intensity profile of the cells treated with DMSO.

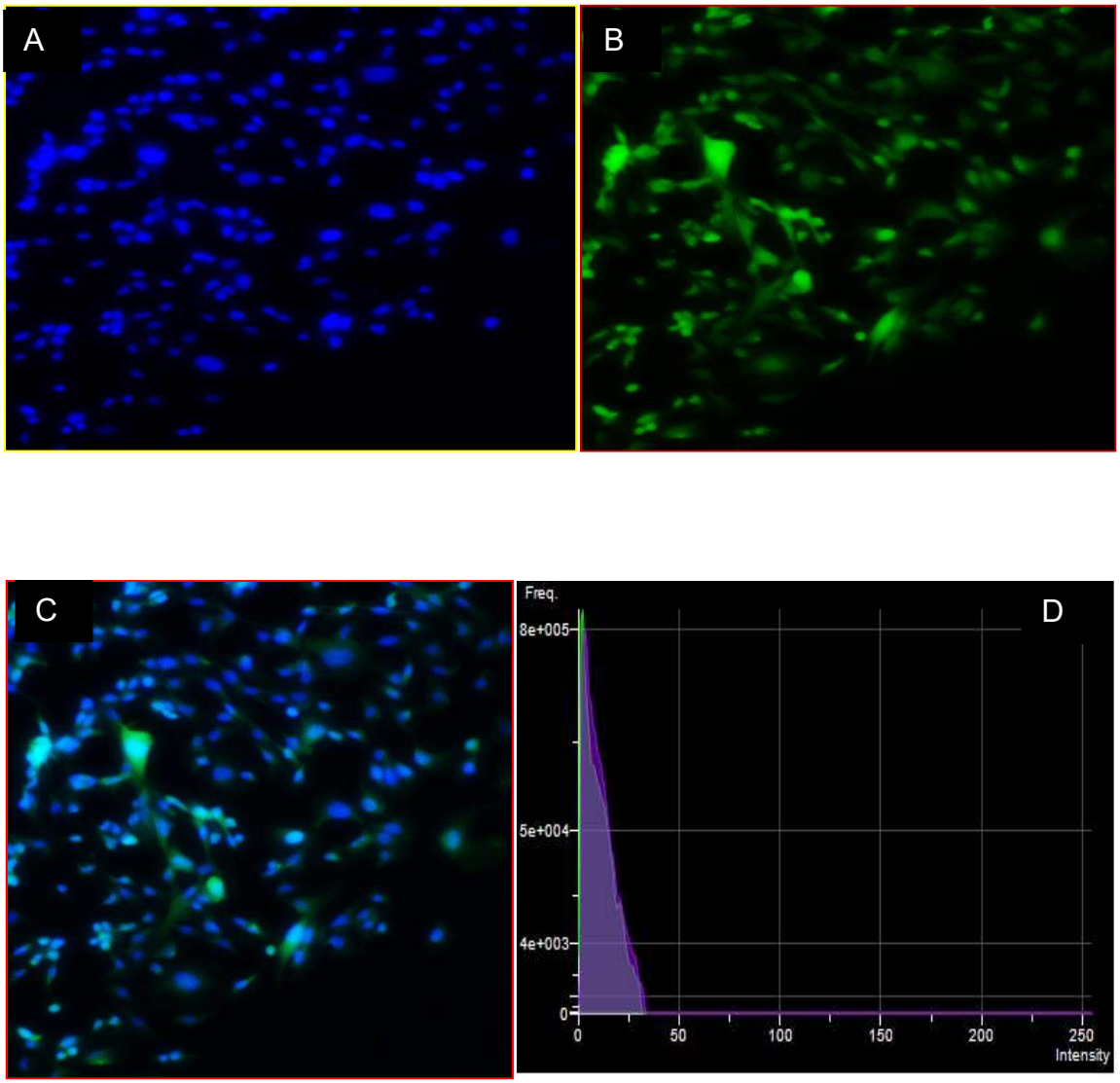

Figure 6.2:The flourescence profiles of the differentiated C2C12 cells treated with DMSO. The images above were taken by the X100 objective of an inverted flourescence microscope at a X60 magnification.A= images of the nucleas stained with DAPI which flouresce blue. B= the green flourescence represents the FITC bound GLUT4 molecules.C= An overlay of Images A and B to show the the position of the nuleas with respect to the distribution of the GLUT4 molecules. D=The flourescence intensity profile of the green flourescence from the FITC conjugated secondary anti-body which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with DMSO represented by the purple graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

It was observed in figure 6.3D below that there was a shift in the fluorescence intensities profiles of the cells treated with the hexane extract of *Seriphium plumosum.* This treatment resulted in more cell numbers with relatively higher intensities as compared to those found in the control. This means that more of the GLUT4 molecules have translocated to the cell membrane as compared to those in the untreated control. The nucleus is clearly observed in the middle of the cells (Figure 6.3C) while the green fluorescence is more aligned to the periphery of the cells. This is due to increased translocation of GLUT4 molecules to the membrane of the cells.

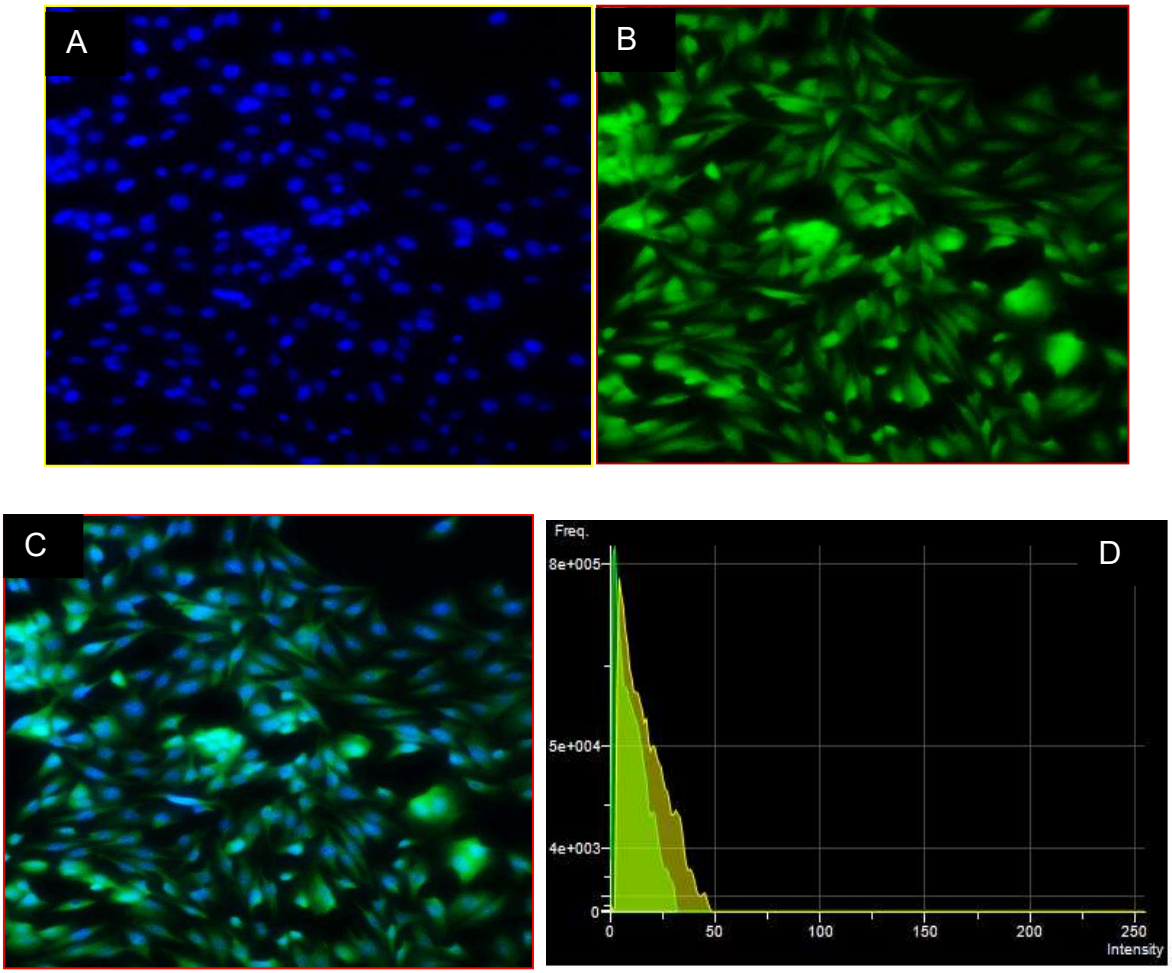

Figure 6.3:The flourescence profiles of the differentiated C2C12 cells treated with the hexane extract of *Seriphium plumosum*(SPlH). The images above were taken by the X100 objective of an inverted flourescence microscope at a X60 magnification.A= images of the nucleas stained with DAPI which flouresce blue. B= the green flourescence represents the FITC bound GLUT4 molecules.C= An overlay of Images A and B to show the the position of the nuleas with respect to the distribution of the GLUT4 molecules. D=The flourescence intensity profile of the green flourescence from the FITC conjugated secondary anti-body which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with the hexane extract of *Seriphium plumosum*(SPlH) respresented by the yellow graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

An unexpected observation was made for the cells that were treated with the combination of insulin and the hexane extract of *Seriphium plumosum* represented in figure 6.4 below. This treatment resulted in an intensity profile similar to that of the control. When insulin and the hexane extract of *Seriphium plumosum* were used as singular treatments, they both resulted in an increase in GLUT4 translocation but failed to do so when in combination.

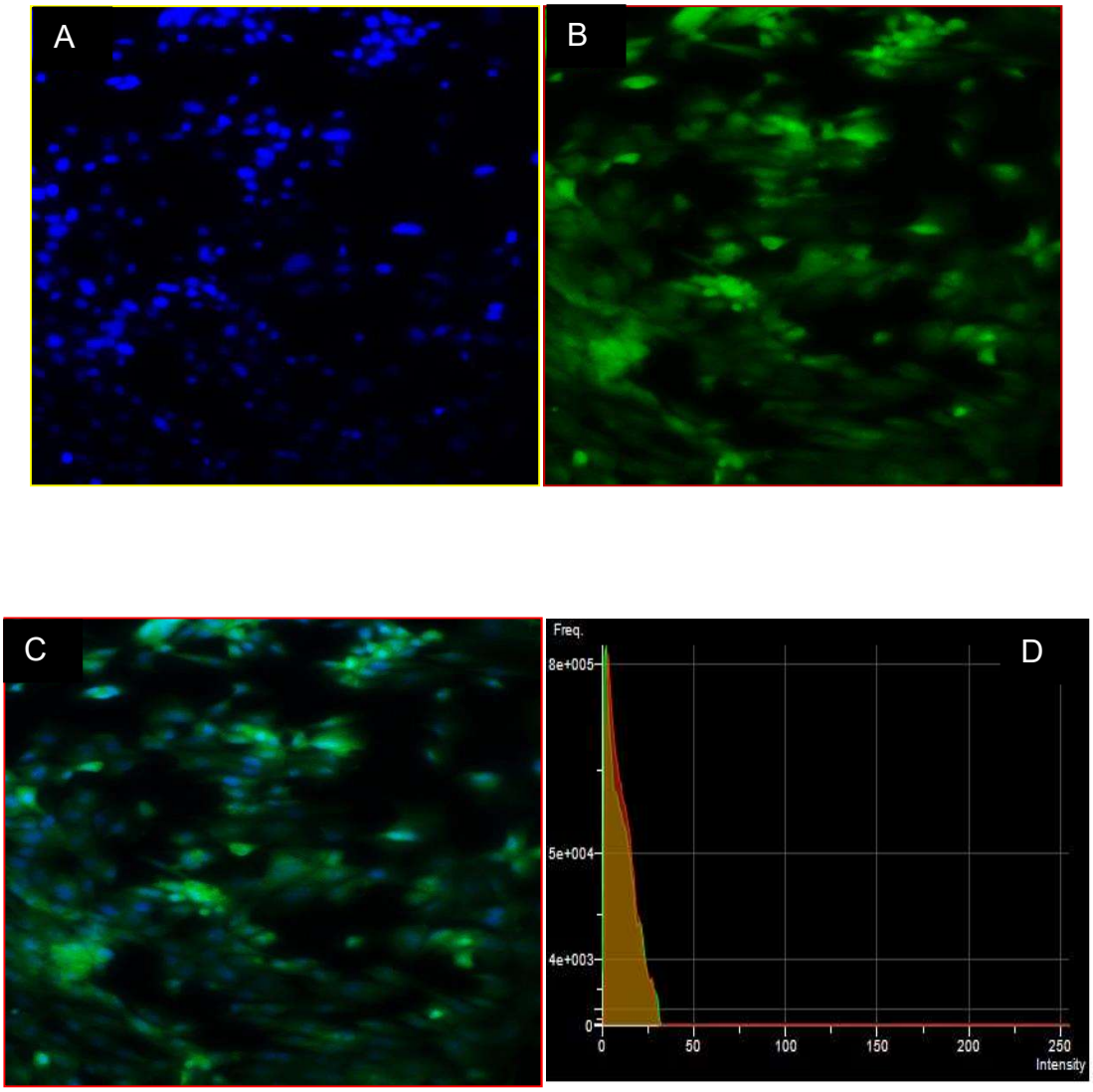

Figure 6.4:The flourescence profiles of the differentiated C2C12 cells treated with the combination of the hexane extract of *Seripium plumosum*(SPlH) and insulin. The images above were taken by the X100 objective of an inverted flouresence microscope at a X60 magnification.A= images of the nucleas stained with DAPI which flouresce blue. B= the green flourescence represents the FITC bound GLUT4 molecules.C= An overlay of Images A and B to show the the position of the nuleas with respect to the distribution of the GLUT4 molecules. D=The flourescence intensity profile of the green flouresence from the FITC conjugated secondary anti-body which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with the hexane extract of Seriphium plumosum(SPlH) in combination with insulin respresented by the red graph superimposed on the green grapgh representing the untreated differentiated C2C12 cells.

Although a shift in the fluorescence intensities profile of the cells treated with insulin was observed, the treatment did not however supersede that of hexane extract of *Seriphium plumosum.* Insulin was employed as the positive control for these experiments. As in the cells treated with the extract alone the nucleus is clearly observed in the middle of the cells in the overlay images (Figure 6.5 C). It can be observed that the green fluorescence representing GLUT4 molecules is more dominant than the blue fluorescence representing the nucleus. The green fluorescence is more aligned to the periphery of the cells (Figure 6.5 C), indicative of increased translocation of GLUT4 molecules to the membrane of the cells.

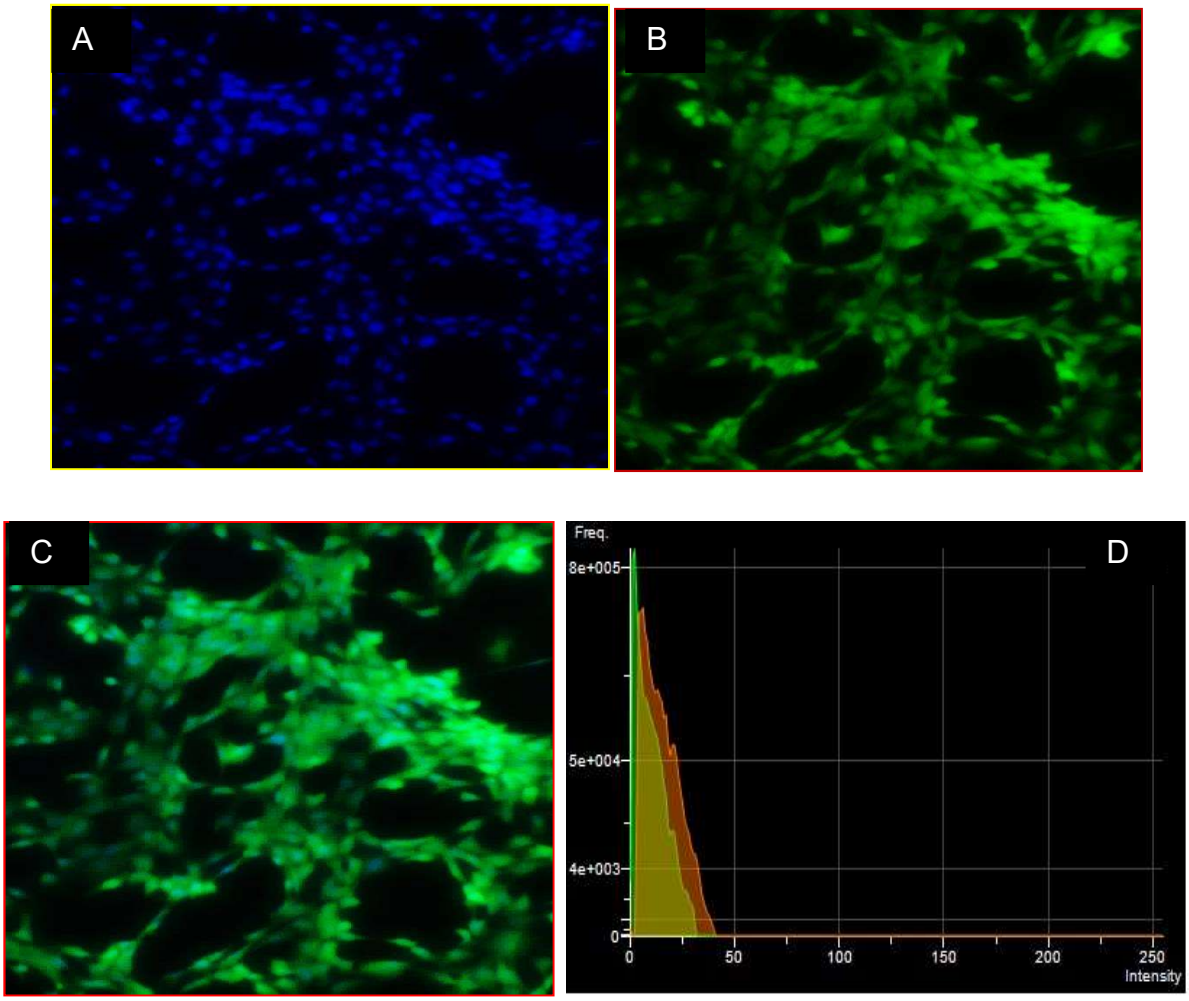

Figure 6.5:The flourescence profiles of the differentiated C2C12 cells treated with insulin. The images above were taken by the X100 objective of an inverted flourescence microscope at a X60 magnification.A= images of the nucleas stained with DAPI which flouresce blue. B= the green flourescence represents the FITC bound GLUT4 molecules.C= An overlay of Images A and B to show the the position of the nuleas with respect to the distribution of the GLUT4 molecules. D=The flourescence intensity profile of the green flourescence from the FITC conjugated secondary anti-body which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated insulin respresented by the orange graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

6.4.2 The Human Phospho-MAPK Array

The Human Phospho-MAPK Array was used to determine the effect of various treatments on the phosphorylation of various mitogen activated protein kinases involved in the glucose uptake signal transduction pathway. Total whole cell lysates were extract after the cells were subjected to a 3 hour treatment with the hexane extract of *Seriphium plumosum* (SPIH), SPIH in combination with insulin, insulin only and untreated cells served as the control. The extracted cell lysates were then incubated with membranes coated with various anti-bodies specific for different phosphorylated mitogen activated protein kinases (MAPKs). The anti-body-protein complexes which were observed as spots on the membranes were visualised using chemiluminescent reagents. The relative pixel densities of these spots on the membranes were quantified. Since the spots and their relative pixel densities represent particular phosphorylated mitogen activated protein kinases, the darker the spot, the higher the concentration of the protein for which the particular spot represents. The various spots represent intensities of different phosphorylated mitogen activated protein kinases (MAPKs) of interest after the various treatments were analysed and graphed. The spots which represented the proteins of interest which were quantified are clearly marked (Figure 6.6).

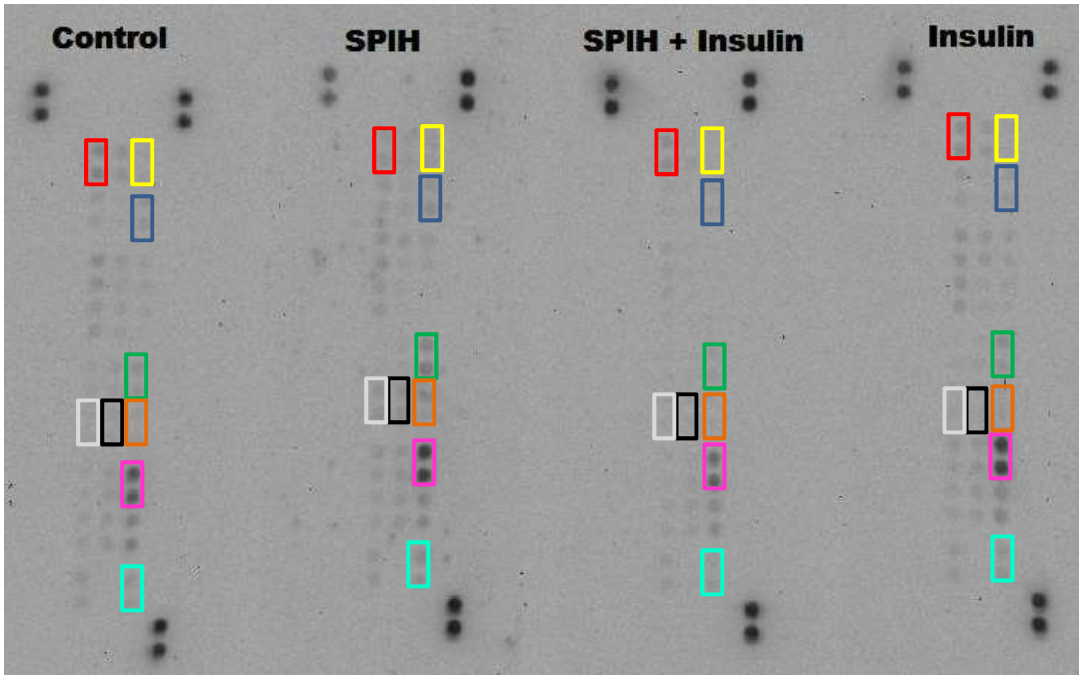

Figure 6.6: The spot on the membranes represent the expression of various mitogen activated protein kinases following various treatments for 3 hours as anotated . The un-treated cells served as the control while insulin was the positive control. The hexane extract of *Seriphium plumosum* =(SPIH). The spots on the membranes representing the genes of interest are marked and the identies of the marked proteins are represented by the following key. = Akt1 (B3,B4), = Akt2 (B5,B6) = CREB (B11,B12), = ERK1 (B13,B14), = ERK2 (B15,B16), = GSK3β (B19,B20), = MKK3 (C13,C14), = p38α (D3,D4), = p70S kinase (D13,D14).

Expression of Akt1(Figure 6.7) was significantly higher in the cells treated with the hexane extract of *Seriphium plumosum* (SPIH) as compared to the control, insulin and the combination of the hexane extract of *Seriphium plumosum* and insulin. Additionally the combination of the hexane extract of *Seriphium plumosum* and insulin resulted significantly lower amounts of Akt as compared to insulin and the hexane extract of *Seriphium plumosum* only.

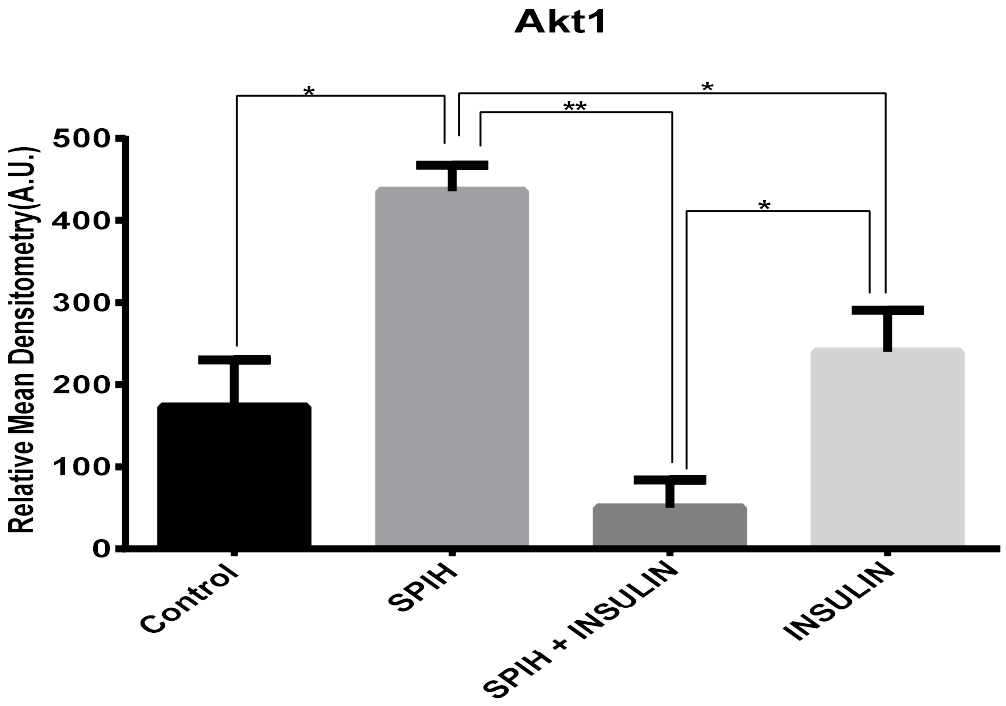

Figure 6.7: The expression of Akt1 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

A similar trend is observed in the expression of Akt1/2(Figure 6.7-6.8), cAMP response element binding protein (CREB) (Figure 6.9), extracellular signal–regulated kinase 2 (ERK2) (Figure 6.10) and glycogen synthase kinase 3 beta (GSK3β) (Figure 6.11) the hexane extract of *Seriphium plumosum* resulted in the highest expression of these proteins. The next best expression levels were observed for the positive control then the untreated cells had the third best expression levels. Interestingly however the treatment that had the combination of hexane extract of *Seriphium plumosum* and insulin resulted in the least expression of the afore mentioned proteins. Both the hexane extract of *Seriphium plumosum* and insulin resulted in significantly higher amounts of Akt2 exepression with respect to the combination of hexane extract of *Seriphium plumosum* and insulin.

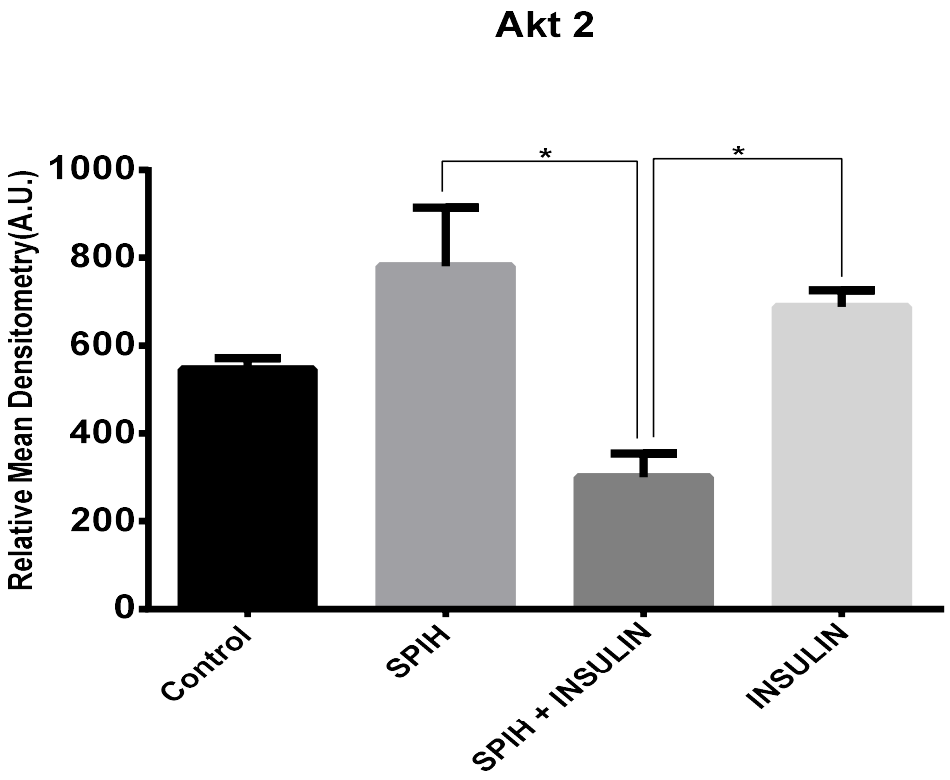

Figure 6.8: The expression of Akt2 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

The expression of the cAMP (Figure 6.9) response element binding protein (CREB) was highest in the treatment with the hexane extract of *Seriphium plumosum*. This was significantly higher than that of the control and the combination of the hexane extract of *Seriphium plumosum* and insulin. The next best expression levels were observed for the positive control then the untreated cells had the third best expression levels

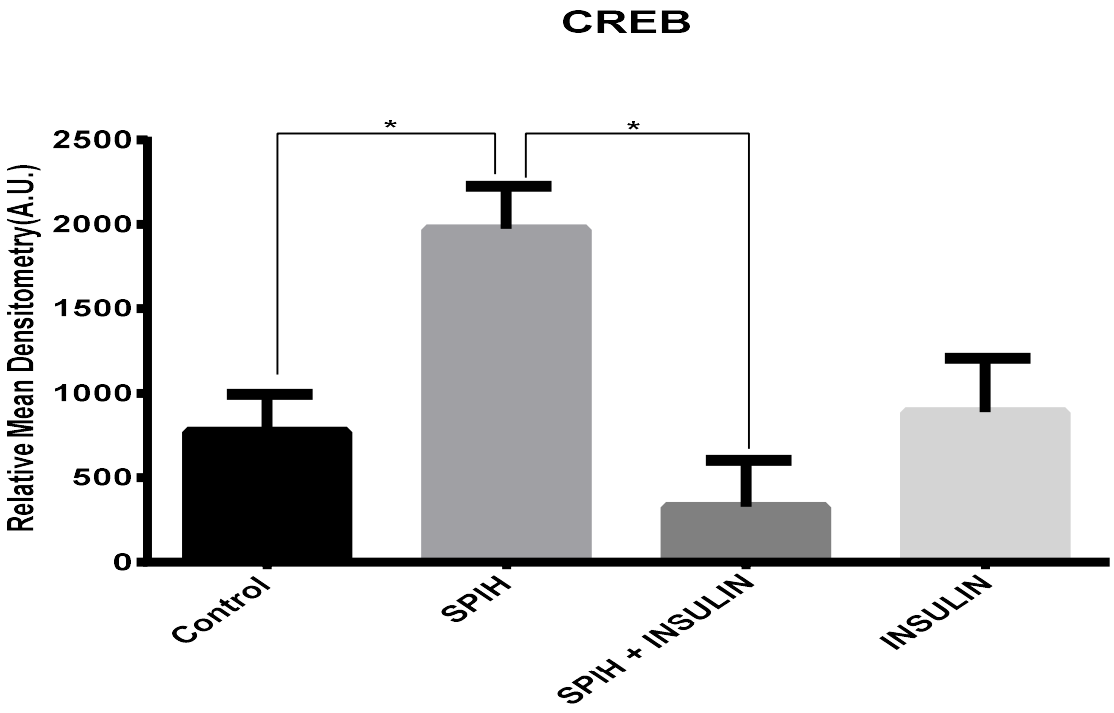

Figure 6.9: The expression of CREB following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

The expression of the extracellular signal–regulated kinase 2 (ERK2) (Figure 6.10) was relatively the highest of all the mitogen activated protein kinases examined. Treatment with the hexane extract of *Seriphium plumosum* resulted in the highest expression levels of the ERK2. These were significantly higher than the rest of the other treatments.

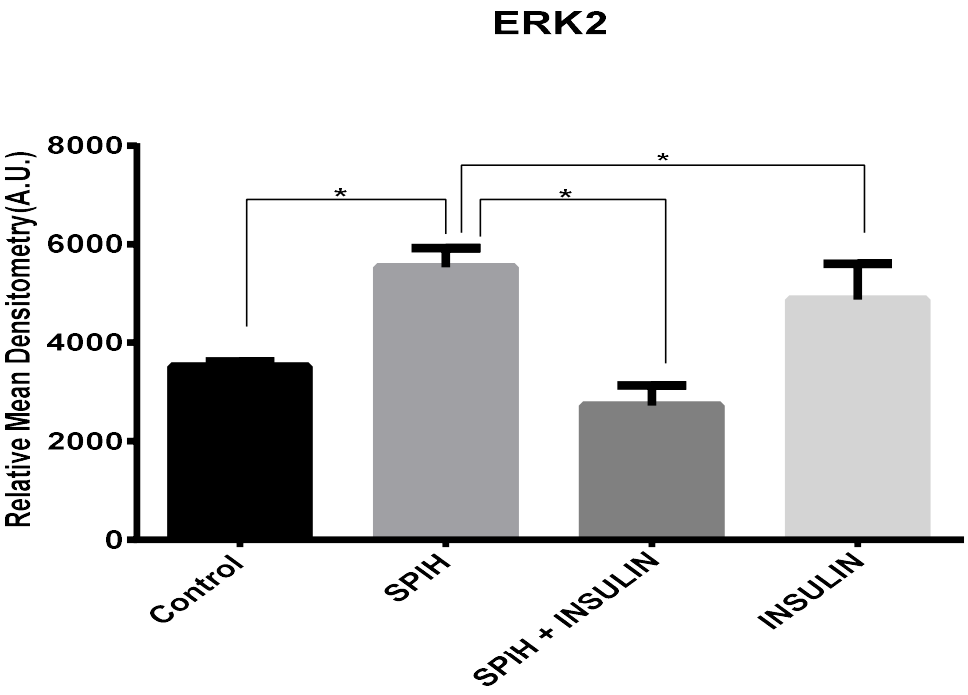

Figure 6.10: The expression of ERK2 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

A similar trend is observed in the expression of glycogen synthase kinase 3 beta (GSK3β) (Figure 6.11) as with the Akt1/2 (Figure 6.7-6.8), cAMP response element binding protein (CREB) (Figure 6.9), extracellular signal–regulated kinase 2 (ERK2) (Figure 6.10) and wherein the hexane extract of *Seriphium plumosum* resulted in the highest expression of these proteins. The next best expression levels were observed for the positive control then the untreated cells had the third best expression levels. Interestingly however the treatment that had the combination of the hexane extract of *Seriphium plumosum* and insulin resulted in the least expression of the afore mentioned proteins. The expression of glycogen synthase kinase 3 beta (GSK3β) was significantly higher than that of the combination of the hexane extract of *Seriphium plumosum* and insulin.

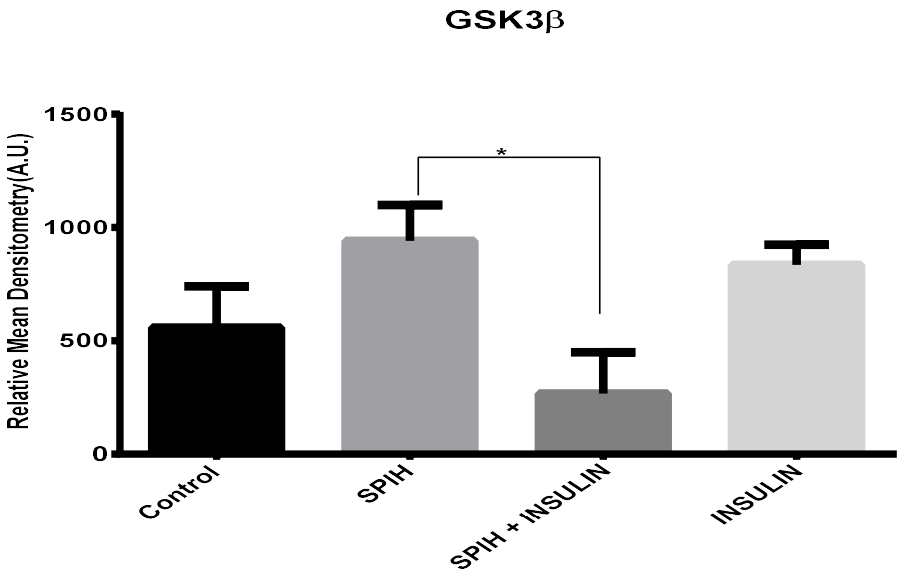

Figure 6.11: The expression of GSK3β following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

The ribosomal protein S6 kinase beta-1 (p70S kinase) (Figure 6.12) was highly expressed in the treatment with the hexane extract of *Seriphium plumosum.* The expression of p70S kinase was significantly higher in the cells treated with the hexane extract of *Seriphium plumosum* as compared to the untreated cells.

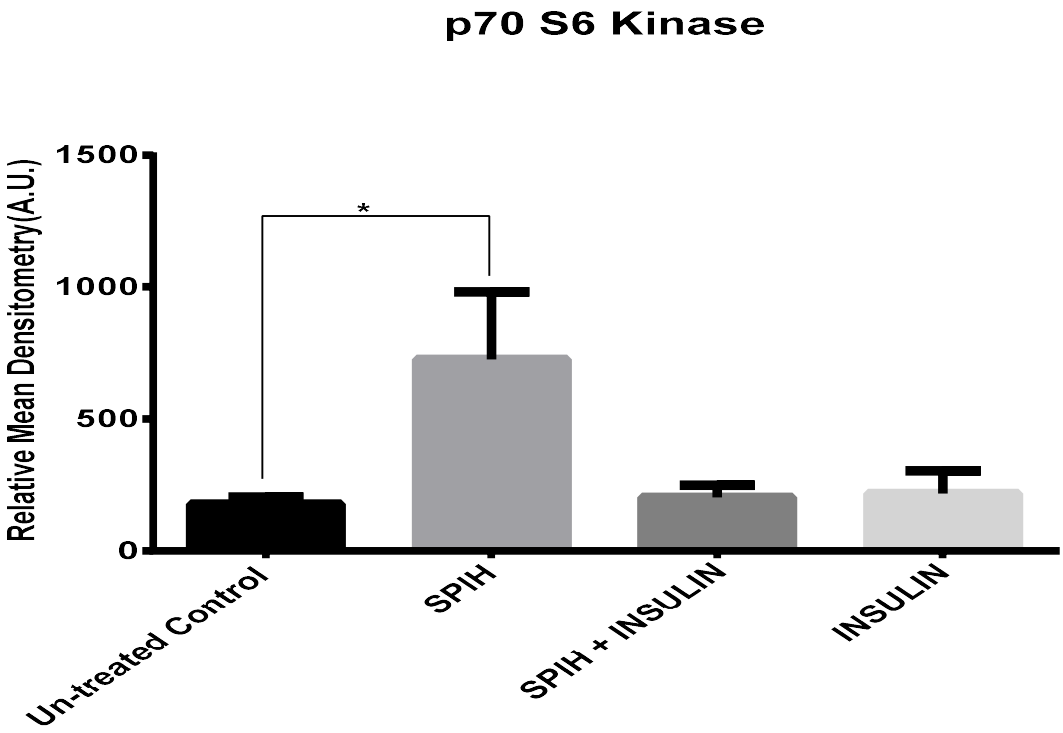

Figure 6.12: The expression of p70 S6 Kinase following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

The expression of the extracellular signal–regulated kinase 1 (ERK1) (Figure 6.13) was highly expressed in the treatment with the hexane extract of *Seriphium plumosum*. the hexane extract of *Seriphium plumosum* resulted in expression of ERK1 that was significantly higher than the untreated control,the combination of insulin and the hexane extract of *Seriphium plumosum.* The expression of the ERK1 after treatment with the hexane extract of *Seriphium plumosum* surpassed the other treatment by a large margin while the other treatment resulted in relatively comparable expression.

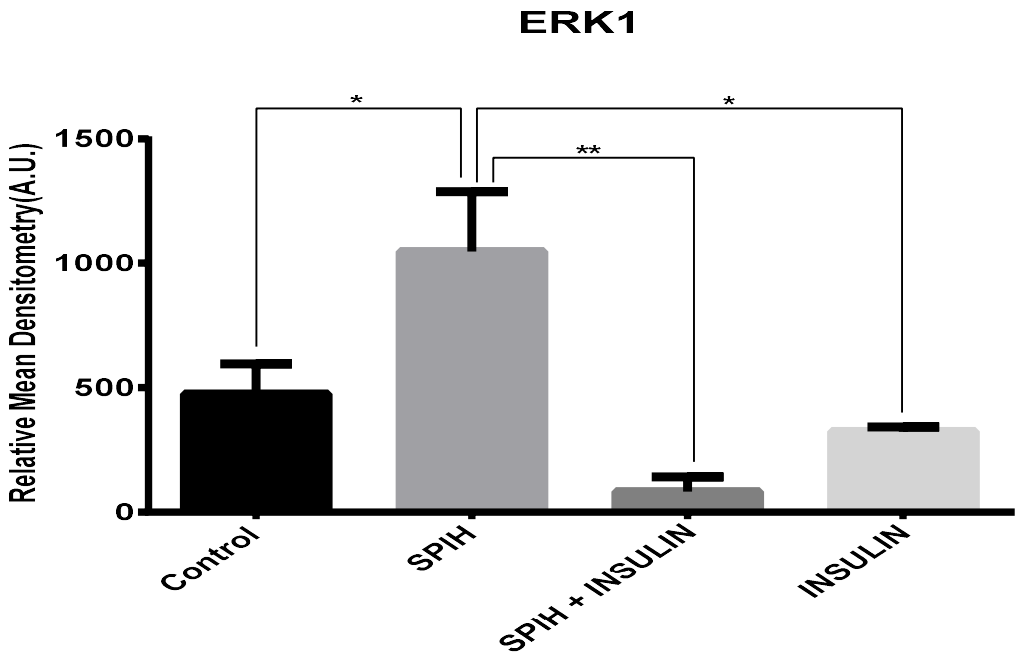

Figure 6.13: The expression of ERK1 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

A similar trend was observed for the expression of the mitogen-activated protein kinase kinase 3 (MKK3) (Figure 6.14) and extracellular signal–regulated kinase 1 (ERK1) (Figure 6.13). Treatment with the hexane extract of *Seriphium plumosum* resulted in significantly higher amounts of the MKK3 as compared to the rest of the treatments. In this case however the insulin resulted in the least expression of the MKK3.

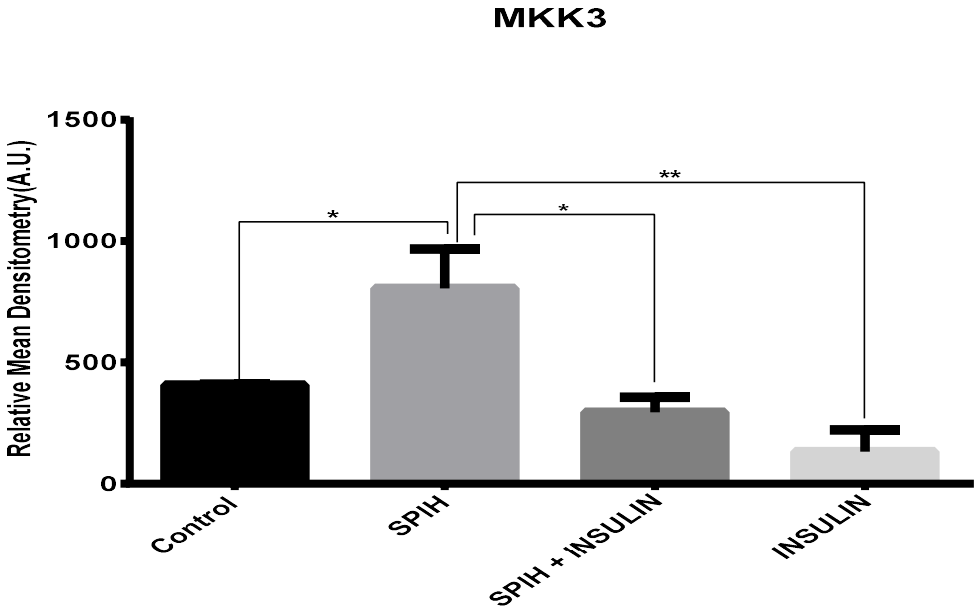

Figure 6.14: The expression of MKK3 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

A different trend in expression motif was observed for p38α (Figure 6.15) in which cells treated with the hexane extract of *Seriphium plumosum* (SPIH) resulted in the lowest expression levels of this protein. The amount of p38α produced by treatment with the hexane extract of *Seriphium plumosum* and that of the combination of insulin and the hexane extract of *Seriphium plumosum* were significantly lower with respect to the control. The untreated cell resulted in the highest expression of this protein.

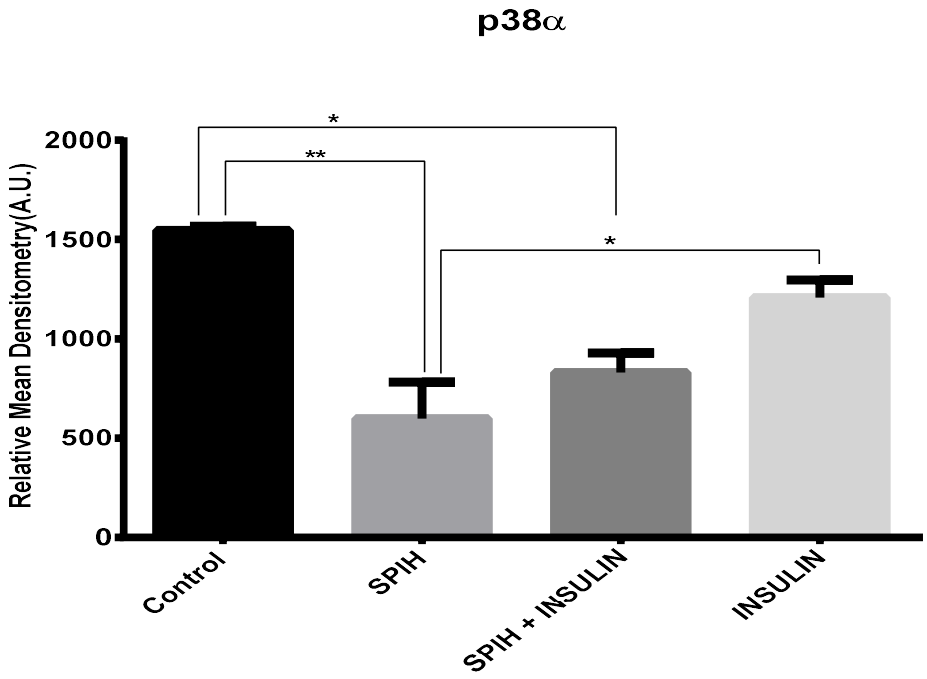

Figure 6.15: The expression of p38α following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *Seriphium plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The P value significance was represented an asterisk (*) for P <0,05, two asterisks(**) for P<0,01 and three asterisks(***) for P<0,001.

6.5 Discussion

Upon an increase in blood glucose levels beyond the normal range, insulin is released by β-pancreatic cells. The released insulin mediates the decrease in blood glucose to homeostatic levels. Insulin achieves this by increasing glucose uptake in various target tissues through initiating a cascade of reactions that culminate in the translocation of insulin responsive glucose transporter molecules such as GLUT4 (Huang and Czech, 2007; Leto and Saltiel , 2012). Translocation of GLUT4 molecule from its cytoplasmic vesicles to the membrane results in increased glucose transport into the cell. This is due to the fact that fusion of GLUT4 with the membrane results in increased transmembrane channels through which glucose can enter the cell (Leto and Saltiel , 2012). The GLUT4 translocation assay performed in this study showed that there is a shift in the distribution of GLUT4 molecules between the membrane and the cytosolic vesicle in the given cells following different treatments. Cells in which GLUT4 molecules are sequestrated within their vesicular stores have lower fluorescence intensity as compared to those in which the GLUT4 molecules have translocated to the membrane. It was observed that in the control and DMSO treated cell there are relatively more cells with relatively lower intensity and less cells with higher intensity. These were the basal expression levels of the GLUT4 molecules on the cell surface membrane and to some extent those that are within the intracellular vesicles. Treatment with insulin resulted in an increase in cells with higher intensity profiles which imply that those cells had relatively more GLUT4 molecules on the membranes as compared to the untreated cell. In the treatment with the *Seriphium plumosum* hexane extract a similar trend was observed in which more of the cells showed higher intensity. It can therefore be concluded that an increase in cells with higher intensity profiles show the cells to have relatively more GLUT4 molecules on the membranes as compared to their counterparts that have lower intensity profiles. An increase in the number of GLUT4 molecules that translocated to the membrane is partly responsible for the increased glucose uptake observed in the previous chapter.

The insulin blood glucose regulation transduction pathway is one of those pathways that is under the control of the MAPK proteins. An increase in the blood glucose level prompts the secretion of insulin by the β cells of the pancreas. The secreted insulin

is transported to various target organs where it specifically binds to the extracellular α-subunits of its hetero-tetrameric receptor on the cell surface membrane, termed the insulin receptor (IR). The binding of insulin to the insulin receptor activates it by inducing conformational changes which facilitate the auto-phosphorylation of tyrosine residues on the intracellular part of membrane-spanning β-subunits. The phosphorylated β-subunits in turn attract and phosphorylate a family of adapter molecules, the insulin receptor substrate (IRS). The phosphorylation attracts various other downstream signalling molecules. One such molecule is the phosphoinositide-3-kinase (PI_3K). Once activated, PI_3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) at the plasma membrane. Akt then binds to the PIP_3 which in turn facilitates activation of Akt by upstream kinases as the activation of Akt requires that it undergoes phosphorylation at two sites. The PI_3K/Akt pathway is activated downstream of the insulin receptorand is known to mediate several levels of glucose metabolism including insulin dependent systemic and cellular metabolic activities. This protein kinase is activated by insulin and various growth and survival factors to function in a wortmannin-sensitive pathway involving PI_3 kinase. The activated Akt molecule is released from the plasma membrane and translocates to cellular compartments, such as the cytoplasm, mitochondria and nucleus, where it is responsible for the phosphorylation of many of its substrates (Schultze *et al*., 2012).

It enhances glucose-uptake in various insulin-responsive tissues by inducing the expression of GLUT1 and GLUT3 and the translocation of GLUT4 to the plasma membrane. In this study *Seriphium plumosum* hexane extract resulted in the expression of an increased amounts of the phosphorylated Akt-1 and Akt-2 with respect to the untreated control and insulin. The Akt-1 isoform was phosphorylated at the Serine at position 473 while the Akt-2 isoform was phosphorylated at the Serine at position 474. Phosphorylation of these different isoforms precedes their translocation into the cytoplasm where they mediate a host of functions by further phosphorylating other downstream molecules. The Akt isoforms are also referred to as PKB or Rac are known to play pivotal roles in controlling survival and apoptosis. Therefore an increase in the amounts of phosphorylated Akt molecules translates to an increase in the amount of the molecules which function downstream of Akt. The Akt mediated cascade is known to mediate an increase in the translocation of

GLUT4 molecules via the activation of the Akt substrate 160 (AS160) (Brewer *et al*., 2011). The AS160 is to date the only known Akt substrate identified that shows a phosphorylation-dependent effect on GLUT4 trafficking (Brewer *et al*., 2011). A study by Karlsson *et al*., (2005) suggest that aberrant insulin signalling to AS160 via Akt contributes to defects in GLUT4 translocation and glucose uptake in skeletal muscle in insulin-resistant type 2 diabetic patients. Akt also activates mTORC1 by inhibiting tuberous sclerosis complex 1/2 (TSC1/2). Activated mTORC1 upregulates mitochondrial biogenesis, inhibits autophagy and induces protein synthesis by regulation of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), unc-51-like kinase 1 (ULK1), and ribosomal protein S6 kinase (S6K) and eIF4E-binding protein 1 (4E-BP1), respectively (Schultze *et al*., 2012). Akt has since emerged as a crucial transducer of the insulin-signalling cascade leading to GLUT4 translocation and glucose uptake.

Apart from directly increasing glucose metabolism through increased GLUT4 molecule translocation Akt induces glycolysis through the phosphorylation and activation of Phosphofructokinase 2 (PFK2), which in turn activates Phosphofructokinase 1 (PFK1) (Deprez *et al*., 1997). These enzymes mediate the conversion of fructose-6-phosphate into fructose-1, 6-bisphosphate, a key step in glucose metabolism. Akt is involved in the regulation of forkhead box protein O1 (FOXO1) which is involved in gluconeogenesis (Schultze *et al*., 2012). Akt also activates glycogen synthesis by phosphorylating and inactivating GSK3β, which leads to the activation of glycogen synthase (Patel *et al*., 2008; Schultze *et al*., 2012).

Akt therefore plays another essential function in the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β. Although the Akt isoforms induces glycogen synthesis through inhibition of GSK-3β , treatment in this study with *Seriphium plumosum* hexane extract resulted in an increased expression of the phosphorylated GSK3β at position S9. Phosphorylated GSK3β is directly responsible for the phosphorylation of glycogen synthase but has no direct bearing on the translocation of GLUT4 molecules. Phosphorylation of glycogen synthase by phosphorylated GSK3β inactivates its glycogen synthesis capability. In this study it was shown that there is an increase in glucose uptake coupled with an increase in the expression of phosphorylated GSK3β. This may indicate that the increased

uptake of glucose was not due to an increase in the storage of glucose within the cell. A probable explanation for this is that the cells have an increased metabolic pathway to compensate for this. The cells are therefore catabolising the glucose within the media as opposed to utilising their internal glucose stores that are in the form of glycogen which opposing pathways cooperate in promoting.

Akt may also be involved in activation of the nutrient-dependent Thr/Ser kinase, mTOR (Tzatsos and Kandror, 2006). Activation of mTOR further results in the phosphorylation of ribosomal protein S6 kinase (p70S6K). p70 S6 kinase is a mitogen activated Ser/Thr protein kinase required for cell growth and G1 cell cycle progression (Le *et al*.,2003). Additionally, it phosphorylates the S6 component of the 40S ribosomal subunit and is involved in translational control of 5' oligopyrimidine tract mRNAs on a mitogen activated signalling pathway downstream of phosphoinositide-3 kinase (PI_3K). Targets for the p70S6K pathway may include the ribosomal proteins S6 and S17. In the current study the *Seriphium plumosum* hexane extract resulted in the upregulation of the phosphorylated p70S kinase which means that more energy will be directed to protein synthesis (Le *et al*., 2003). Presumably the cell therefore compensates for this increased protein synthesis by increased glucose uptake.

Another target for Akt is the cAMP Response Element-Binding Protein (CREB). Phosphorylation at Ser133 of this transcription factor by Akt causes an increased affinity of CREB for its co-activator protein, CRB (Crumbs). The heterodimer, now an active transcription factor, promotes transcription of genes that contain CREs (cAMP responsive elements) in their promoter resulting in further increase expenditure of energy by the cell. Insulin activates a number of signalling pathways including the extracellular regulated kinase 1 and 2 (ERK1/ERK2) pathways. ERK1/ERK2, also called p44 and p42 MAP kinase, phosphorylate a number of transcription factors in vitro and may be in the pathway responsible for the phosphorylation of the initiation factor eIF4E. Thus ERK1/ERK2 in combination with p70S6K signalling pathways may mediate in the regulation of protein synthesis by insulin. Therefore the increase in the amount of phosphorylated ERK1/ERK2 and p70S6K that resulted from the treatment of cells with the *Seriphium plumosum* hexane extract may result in increased protein synthesis.

The *Seriphium plumosum* hexane extract resulted in an increase in the amount of phosphorylated MMK3 protein. Although there was an increase in the amount of phosphorylated MMK3 this however did not directly result in the upregulation of phosphorylation of its down-stream p38α molecule. p38 mitogen-activated protein kinase (MAPK), functions downstream of MAPK kinase (MKK) 6 and MKK3. p38α is activated by mitogenic or stress-inducing stimuli, as well as by insulin. Activation of the MKK6/3-p38 MAPK pathway markedly enhances glucose transport by up-regulating GLUT1 expression, irrespective of the stimulus. This effect may help cells meet the increased energy demands incurred when they are under stress, perhaps determining whether cells survive or succumb to apoptosis, a process in which the MKK6/3-p38 MAPK pathway also plays a role. Therefore the increased glucose uptake observed in this study was not due to p38α activation as it was down regulated by the *Seriphium plumosum* hexane extract.

The combination of insulin and *Seriphium plumosum* hexane extract resulted in an antagonistic relationship. It was observed that the combination resulted in lower expression of the different phosphorylated mitogen active protein kinases as compared to when the treatments were single. This may be due to the fact that the extract may contain some compounds that mask the action of insulin in the array of compounds that it contains. A way of circumventing this is to isolate the pure compound in this extract that elicits the desired function which may not negatively affect the normal action of insulin.

CHAPTER 7

7.1 General discussion and conclusion

Diabetes is a severe debilitating metabolic disorder characterised by chronic hyperglycaemia. The global prevalence of this condition has reached an all-time high with an estimated 592 million people are projected to suffer from it by the year 2035 (IDF, 2013). The worrisome up-surge in the occurrence of this condition is partly attributed to lack of cure for the condition. Current management strategies include biguanides, sulfonylureas, meglitinides and thiazolidinediones which are known to have a host of accompanying ill-side effects (Verspohl, 2012). Resource scarce, middle to low income countries are hardest hit by the financial burden that accompanies this plight. These countries are mostly within Africa, including South Africa. The majority of people in these areas generally do not have access to western medicine as a source of primary health care and if they do, many of them cannot bear the financial costs associated with it. People therefore resort to the use of more affordable primary health care system which comes in the form of traditional medicinal plants. An estimated 80% of people living in impoverished African countries rely on traditional medicinal plants to take care of their primary health care needs (WHO, 2002). Although the use of traditional medicinal plant has been practised since time immemorial, it use have gained renewed interest. The renewed interest emanates from the fact that plants provide a cheap source of therapeutic compounds. Therapeutic plant derived compounds are generally thought to have relatively less side effects (Farnsworth, 1990). This is due to the fact that they can work more harmoniously with the normal metabolic system as opposed to the synthetic compounds currently being employed (Atal,1983). Scientists around the globe have therefore taken it upon themselves to document the modes of action of different medicinal plant and determine their safety.

In the current study, various bioactivities of five plants that are traditionally used for the management of diabetes were investigated and documented. The investigated bioactivities included phytochemical analysis, anti-glycation and antioxidant activities, cytotoxicity analysis, glucose uptake and GLUT4 translocation

augmentation capabilities. Various crude extracts were prepared from the dried plant material of the selected plants by maceration in organic solvents of varying polarity.

Although water is the most commonly used traditional extractant, some traditional healers are known to use whisky in their preparation of extracts. The alcohol in the whisky may have similar extraction capabilities as those observed for the methanol extract in this study. The methanol extract resulted in the highest percentage yield of extraction from all the plants in the study. The methanol extracts of *Toona ciliata* (TCiM) had the highest percentage yield of 20.83 % followed by the *Olea Africana* (OAfM) with 20.44% yield. This observation may primarily be due to two parameters, the first being that the plants contain compounds of a highly polar nature as opposed to compounds of relatively less polar nature. This would therefore result in the polar solvent extracting the highest amount of compounds as opposed to its less polar counterparts. The second parameter is the molecular size of the methanol itself. Methanol was also the smallest molecular sized extractant in this study, making it capable of penetrating the plant material more effectively as compared to the rest of the other solvents to obtain, the high extraction yields observed in this study. On the other hand the nonpolar hexane extractant had the lowest extraction yield. Despite the low extraction yield the hexane extract are useful as they may contain non-polar active compounds which may not be available in the methanolic extracts. To this end some traditional healers are known to use oil in the preparation of their extract. The oil will extract mainly the nonpolar compounds.

The qualitative secondary metabolite analysis carried out on the plant reveal the presence and absence of many different compounds within the different plant extracts. Of importance, it determined the presence of phenolic, tannic acid and flavonoid compounds in almost all the extracts of the different plants extracts which are known to be templates for the development of various therapeutic agents. Hence the availability of these compounds in the extracts provides promising step in the acquisition of therapeutic agents from the plants under study. Another important observation is that most of the plant did not contain starch. Plant extracts containing carbohydrates are not recommended as antidiabetic agents because the carbohydrates would add on the amount of glucose in the body thereby causing further hyperglycaemic episodes in the patient. The different compounds that were qualitatively identified to be present in the different plant extracts were further

confirmed and separated on thin layer chromatography plates. These compounds had various R_f values and also were responsive to different wavelengths of UV-light and also resulted in different coloured bands in the presence of acidic vanillin. All these observations concurred with the fact that there were many different compound classes qualitatively identified in the different plant extracts.

Some of the compounds within the plant extract were shown to possess antioxidant activity through qualitative DPPH assay. The antioxidant capabilities of these compounds were further quantified spectrophotometrically. The methanol extract of *Seriphium plumosum* had the highest DPPH scavenging activity and ferric reducing power with EC_{50} values of 0.72 mg/ml and 2.31 mg/ml respectively. Antioxidant activity is an important bioactivity that is associated with the overall metabolic well-being of an individual. Various antioxidant enzymes within the body such as glutathione peroxidase, catalase, and superoxide dismutase are known to combat the harmful effects of free radicals that may be deleterious to various biomolecules within the body. Compounds with antioxidant capabilities aid these enzymes by also functioning to ameliorate these toxic free radical intermediates. Antioxidant activities are known to reduce the risk of many conditions such as cancer, cardiovascular diseases, Alzheimer's disease, Parkinson's disease and rheumatoid arthritis.

The extent to which a therapeutic agent affects the oxidative status of an individual has also been linked to anti-glycation ability of that particular agent. Glycation is an oxidative process and is in particular a huge challenge in diabetic patients due to chronic hyperglycaemia. Chronic hyperglycaemia is implicated as the major driving force behind the spontaneous glycation of proteins by sugars within the body. Continued glycation results in the accumulation of advanced glycation end-products (AGEs). Advanced glycation end-products (AGEs) form cross-links with long-living tissue proteins to form products such as crosslinked collagen which in turn result in pathological conditions such as arterial stiffness and decreased myocardial compliance, resulting from the loss of collagen elasticity (Singh *et al*., 2002; Aronson, 2003). Advanced glycation end-products accumulate in the body with age but have been shown to accumulate up to four times faster in diabetic patients with uncontrolled blood glucose levels (Alikhani *et al.,* 2005). This has the overall effect of increased occurrence of the debilitating complications observed in diabetic patients. Although the methanolic extract of *Seriphium plumosum* exhibited the highest

antioxidant potential it however did not result in the best anti-glycation ability as expected. The acetone extract of *Seriphium plumosum* exhibited the most glycation inhibitory activity among all the examined extracts, as it resulted in 2,22% glycation. This observation may have resulted from the high amounts of total phenolic compounds found within *Seriphium plumosum*. The methanol and acetone extracts of *Seriphium plumosum* had the highest total phenolic content of 41.63 mg/g and 41.54 mg/g respectively. Furthermore *in-vivo* studies to ascertain whether these plants extracts may help in blocking the interaction of AGEs with their specific receptors would be extremely helpful in determining the extent to which the plants under study exert their therapeutic properties.

Although plant derived compounds are currently being employed to manage various ailments there are worrisome causes of concerns that have been raised. These concerns are raised due to the potential dangers that accompany the use of traditional medicine. These dangers range from unknown toxicity profiles to unknown modes of action. The use of medicinal plants is not standardised as observed in the differences in administration and dosage regimes from traditional healer to traditional healer. Previous studies have shown that people are under the assumption that since plants are naturally occurring, they are therefore automatically safe (Kamsu-Foguem, and Foguem, 2014). A number of casualties result each year from the improper use of plants for medicinal purposes (Kamsu-Foguem, and Foguem, 2014). For this reason, the cytotoxicity of the extracts was evaluated. The methanol, acetone and hexane extracts of *Opuntia ficus indica* resulted in CC_{50} values >1000µg/ml respectively and were shown to be the least cytotoxic extracts under investigation. In this study, the hexane extract of *Toona ciliata* with an CC_{50} value of 402,16 µg/ml was the most cytotoxic extract. It was therefore concluded that since all the extracts investigated had CC_{50} values greater than 50 µg/ml they were generally non-toxic.

Glucose disposal mechanisms play a critical role in the lowering of blood glucose levels which helps to keep glucose levels in-check. Some of the diabetes medications are known to work by increasing glucose disposal within the body thereby reducing the extent of hyperglycaemia and its associated complications. These agents are known as hypoglycaemic agents and provide an effective mechanism for the management of diabetes. The main glucose disposal

mechanisms are grouped into storage and metabolism. Glucose is primarily stored in the liver and muscle as glycogen and in the adipose tissue as fat. Glucose metabolism involves the contraction of muscles, function of the brain, heating of the body and so forth. Muscles play a pivotal role in the disposal of glucose. Agents that encourage increased glucose uptake by the muscle cells provide a proficient mechanism of the management of diabetes. In this study the hexane extract of *Seriphium plumosum* resulted in the highest glucose uptake of 35, 77%. This is a promising result as the crude extract can further be purified and the compound exhibiting this activity may be isolated and employed for the management of diabetes. The glucose activity that resulted from treatment of the cells with this plant extract was even better than the activity observed when the cells were treated with insulin (26, 06%) which was the positive control.

Muscles are responsible for an abundant insulin dependent glucose disposal. The insulin dependent blood glucose level regulation relies on the ability of GLUT4 molecules to cycle between the plasma membrane and their vesicular store in the cytoplasm. In the current study the hexane extract of *Seriphium plumosum* which resulted in the highest glucose uptake was investigated for its effects on GLUT4 translocation. Treatment with the hexane extract of *Seriphium plumosum* resulted in increased GLUT4 molecule translocation to the plasma membrane. The translocation of GLUT4 molecules to the plasma membrane is regulated by a signal transduction cascade composed of a host of proteins termed mitogen activated protein kinases. Various pharmacological therapies that activate or deactivate one or more of these proteins may be developed to mediate increased glucose uptake in diabetic patients. Traditional medicinal plants, by virtue of them possessing magnitudes of compounds represent an untapped store of such therapeutic compounds (Abdel-Moneim and Fayez, 2015). These plant derived therapeutic compounds may act by augmenting the phosphorylation of different mitogen activated protein kinases involved in GLUT4 translocation pathway. It was observed that the plant extract resulted in the upregulation of the amount of phosphorylated Akt molecules. Akt is known to function downstream of PIP_3 in the insulin dependent pathway (Schultze *et al*., 2012). Activation of Akt results in the phosphorylation of its various downstream proteins in this pathway. The eventual result of increased Akt phosphorylation is an increase in the amount of GLUT4 translocation. The extract

may also increase glucose metabolism by increasing various energy requiring processes within the cells. This realisation was made due to the fact that treatment of the cells resulted in the up regulation of MKK3 and pS70 kinase. These protein kinases are directly linked to protein synthesis and increased transcription as well as cell growth which are all energy requiring processes.

Further studies especially on the hexane extract of *Seriphium plumosum* is required to ascertain its precise mode of action. The main active ingredient may be isolated and identified. This may help to produce a novel anti-diabetic compound with relatively less side effects. The same applies for the compounds that had other bio-activities such as antioxidant and anti-glycation capabilities. *In-vivo* studies may also be carried out in order to determine the effects of the plant within a living system. Other hypoglycaemic modes of action such as pancreatic β-cell stimulation, α-glucosidase and beta glucosidase inhibition and presence of insulin sensitisers may also be further investigated.

Chapter 8

8.1 References

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Appendix

Antioxidant, anti-glycation and hypoglycaemic effect of *Seriphium plumosum* crude plant extracts.

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Abstract

Diabetes is a severely debilitating metabolic disorder characterised by chronic hyperglycaemia. Traditional medicinal plants provide an important avenue for the development of novels antidiabetic agents. The anti-diabetic potential of the methanol, acetone and hexane extracts of *S. plumosum* was assessed using different parameters. These included secondary metabolite quantification, hypoglycaemic, cytotoxic effects and GLUT4 translocation augmentation on C2C12 cells. The methanol extract contained the highest amount of total phenolic and flavonoid compounds and showed enhanced antioxidant activity. The methanol extracts had the best DPPH scavenging (EC_{50} = 0.72 mg/ml) and ferric reducing powers (EC_{50} = 2.31 mg/ml). The hexane extract resulted in the highest glucose uptake activity of 35,77% with respect to all other treatments after a 6 hour exposure period. Immunocytochemistry technique further revealed that the increased glucose utilisation may be due to increased membrane fused GLUT4 molecules in C2C12 cells. The hexane extract was also shown to up-regulate the phosphorylation of p70 S6 kinase and Akt1/2. The study highlights a probable insulin-mimetic activity of the hexane extract via the augmentative Akt1/2 phosphorylation which is involved in GLUT4 translocation pathway. Furthermore, the study represents the first report on the cytotoxic effect, GLUT4 translocation and glucose uptake potential of *S. plumosum*.

Introduction

The incidence and prevalence of diabetes is on an all-time high and is expected to rise drastically in the near future unless strategic counter measures are implemented immediately (Olokoba *et al*., 2012; Narayan *et al*., 2006; Sierra, 2009; Zimmet *et al*., 2003). An estimated USD4 billion was spent on diabetes healthcare in 2013 within Africa (IDF, 2013). In South Africa alone it is estimated that the cost of managing diabetes per individual annually was approximately R5 000 in 2010 but has risen to R26 743.69 in 2015 (IDF, 2015). Diabetes mellitus is a multifactorial metabolic disorder characterised by chronic hyperglycaemia. Perpetual hyperglycaemia results in exasperated rates of glycation, which is defined as the spontaneous non-enzymatic reaction of reducing sugars with

proteins, lipids or nucleic acids (Negre-Salvayre *et al*., 2009). The end result of chronic glycation is the accumulation of a heterogeneous group of bio-molecules collectively termed advanced glycation end-products (AGEs) such as pentosidine, carboxymethyllysine, crossline and pyralline (Negre-Salvayre *et al*., 2009). Further evidence suggests that advanced glycation end-products interact with their specific plasma membrane localised receptors for AGEs (RAGE). Interaction of the AGEs with RAGE results in altered intracellular signaling, gene expression, release of pro-inflammatory molecules and free radicals (Negre-Salvayre *et al*., 2009; Singh *et al*., 2014). Glycation is therefore implicated as the major underlying cause of the host of complications observed in diabetic patients such as cardiovascular disease, nephropathy, neuropathy and retinopathy (Ahmed, 2005). Diabetes is known to cause oxidative stress which in turn results in increased progression of its associated complications (Giacco and Brownlee, 2010). Antioxidants therefore can help reduce these ill effects by chelating the free radicals, donation of electrons and donation of protons that stabilise these oxidative stress products that would otherwise have deleterious effects.

Some hypoglycaemic anti-diabetic therapeutic agents are known to work by mediating an increasing glucose disposal within the body. GLUT4 is the major insulin responsive glucose transporter primarily expressed in adipose tissues, skeletal muscle and cardiac muscle (Fukumoto *et al*., 1989; Jewell, 2010). It is found sequestrated in intracellular vesicles within the cells in which it is expressed (Richter and Hargreaves, 2013). Upon increase in blood glucose levels, insulin acts on the cell surface membrane thereby producing a cascade of events that lead to the translocation of GLUT4 from intracellular vesicle to the cell surface membrane (Jewell, 2010). The cascade of signal transduction events leading to the translocation and trafficking of GLUT4 molecules in response to insulin is mediated by a host of proteins collectively known as the mitogen activated protein kinases (MAPKs). Mitogen activated protein kinases (MAPKs) are a family of proteins at the heart of various important signal transduction pathways (Arbabi and Maier, 2002; Robinson and Cobb, 1997; Šamajová, *et al*., 2013).

Seriphium plumosum is commonly known as the bankrupt bush or *slangbos* (Synman, 2009). It is a perennial woody dwarf shrub which can grow up to 1 m high. It has characteristically intricate branched slender stems from the ground which bear the feathery greyish small leaves (Synman, 2009). It is considered as an unwanted bush encroacher weed in various parts of South Africa (Synman, 2009) and mainly used by the indigenous people for various non-medicinal purposes such as a broom (Moffett, 2010). The Basotho people use this bush to ward off bugs by placing it under their bedding and as an anti-diabetic agent (Moffett, 2010). This study was therefore conducted to determine the toxicology profile of *S. plumosum* and its effects on GLUT4 translocation in C2C12 muscle cells. The study further assessed the anti-glycation and antioxidant effects of the plant as well as the presence of various phytochemicals contained in the crude extract.

Materials and Methods

Plant collection and verification

Leaves of *S plumosum* were collected from Mankweng area, Capricorn Local Municipality, Limpopo Province, South Africa. The plant was selected based on literature surveys of reports of its anti-diabetic properties by traditional healers and village elders in the Limpopo Province. The plant was sampled from the same soil strata. The identity of the plant was authenticated by Dr B Egan, a curator at the Larry Leach Herbarium, University of Limpopo (Voucher specimen number UNIN 121065).

Plant extract preparation

Air-dried whole plant materials were ground into a fine powder using a domestic warring blender. Powdered plant material (1 g) was exhaustively extracted using 10 ml each of methanol, acetone and hexane (Elloff, 1998). The supernatants were filtered using a Whatman No.1 filter paper into pre-weighed glass vials and air-dried under a stream of cold air. The quantity of plant materials extracted was determined and stored in air-tight glass vials in the dark until use. The dry plant extracts were reconstituted in dimethylsulphoxide (DMSO) (Sigma Aldrich ™, SA) for all cell based assays or in acetone for any other assay.

Determination of secondary metabolites

The presence of different plant secondary metabolites in the crude extracts was determined using various standard chemical tests (Harborne, 1973).

Phyto-constituent	Test	Observation
Tannins *(Braymer's Test)*	2 ml extract + 2 ml H_2O + 2-3 drops $FeCl_3$ (5%)	Green precipitate
Flavonoids	1 ml extract + 1 ml $Pb(OAc)_4$ (10%)	Yellow colouration
Phenols	2ml extract 2ml of 2% $FeCl_3$.	Blue/black colouration
Saponins	(a) 5 ml extract + 5 ml H_2O + heat	Froth appears
	(b) 5 ml extract + Olive oil (few drops)	Emulsion forms
Steroids *(Salkowski Test)*	2 ml extract + 2 ml $CHCl_3$ + 2 ml H_2SO_4 (conc.)	Reddish brown ring at the junction
Phlobatannins (*Precipitate Test)*	2 ml extract + 2 ml HCl (1%) + heat	Red precipitate
Glycosides	2 ml extract + 2 ml $CHCl_3$ + 2 ml CH_3COOH	Violet to Blue to Green colouration

(Liebermann's Test)		
Coumarins	2 ml extract + 3 ml NaOH (10%)	Yellow colouration
Proteins *(Xanthoproteic Test)*	1 ml extract + 1 ml H_2SO_4 (conc.)	White precipitate
Anthraquinones *(Borntrager's Test)*	3 ml extract + 3 ml Benzene + 5 ml NH_3 (10%)	Pink, Violet or Red colouration in ammonical layer
Anthocyanins	2 ml extract + 2 ml HCl (2N) + NH3	Pinkish red to bluish violet colouration
Leucoanthocyanins turns	5 ml extract + 5 ml Isoamyl alcohol	Organic layer into Red
Carbohydrates	2 ml extract + 2 ml Iodine	A Dark blue or Deep purple colouration

Total phenolic content

The total phenolic content of the different extracts were determined spectrophotometrically using the Folin-Ciocalteu`s phenol reagent method as described (Humadi and Istudor, 2009). Stock solutions (100 mg/ml) of each of the different extracts were prepared. Folin-Ciocalteu reagent (50 µl) and distilled water (450 µl) were added to each of the extracts (100 µl) and left for 5 minutes in the dark at room temperature. Thereafter 7% sodium carbonate (500 µl) solution was added. Distilled water was added to make a final volume of 5000 µl and the mixture allowed to stand for 90 minutes in the dark at room temperature. Absorbance of the mixture in triplicates was measured at 750 nm using a spectrophotometer (Beckman Coulter-DU730). The total phenolic content was determined by linear regression from a tannic acid calibration curve standard.

Total flavonoid content

Aluminium chloride colorimetric method was used for determination of total flavonoids (Chang *et al.,* 2002). A stock solution (10 mg/ml) of each of the different extracts was prepared. Each of the extracts (100 µl) was mixed with 10% aluminium chloride (100 µl), 1 M potassium acetate (100 µl) and distilled water (2800 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.

Determination of anti-glycation activity

Anti-glycation activity of the plant extracts were determined using the bovine serum albumin assay with slight modification (Matsuura *et al.,* 2002). Bovine Serum Albumin (Sigma–Aldrich) (500 µl) was incubated with glucose (400 µl) and plant extracts (100 µl). Phosphate buffer saline (100 µl) was used as the sample control and Arbutin (100 µl) (Sigma–Aldrich) as the reference standard. A negative control constituting of BSA (500 µl), phosphate buffer saline (400 µl) and plant extracts (100 µl) was included. The reaction mixture was allowed to proceed at 60°C for 72 hours and terminated by addition of 10 µl of 100% (w/v) trichloroacetic acid (TCA) (Sigma–Aldrich). The TCA added mixture was kept at 4°C for 10 minutes and thereafter centrifuged for 4 minutes at 13000 rpm. The precipitate was re-dissolved in alkaline phosphate buffer saline (pH 10) and quantified for relative amount of glycated BSA, based on fluoresce intensity in 96 well plates using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). The excitation and emission wavelength used were at 370 nm and 440 nm, respectively. Five concentrations of each sample 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:

$$\% \text{ inhibition} = \frac{\text{OD blank} - (\text{OD sample} - \text{OD sample negative})}{\text{OD blank}} \times 100$$

Quantitative DPPH radical-scavenging activity assay

The antioxidant activity of each of the different extracts was quantitatively determined spectrophotometrically using the DPPH free radical scavenging assay (Deby and Magotteaux, 1970). Equal volumes of 0.2% DPPH in methanol and different concentrations (0 µg/ml to 1000 µg/ml) of the extracts were incubated in the dark at room temperature for 30 minutes. The DPPH in methanol solution was used as the experimental control, L-ascorbic acid (vitamin C) as 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 Multi detection 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:

$$\% \text{ inhibition} = \frac{((A_{490nm} \text{ of blank} - A_{490nm} \text{ of sample}) \times 100)}{(A_{490nm} \text{ of blank})}$$

Ferric ion reducing power

The ferric ion reducing power of the different extracts was determined (Benzie and Strain, 1996). Various concentrations (0 µg/ml to 1000 µg/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 phosphate buffer (250 µl) (pH 7.4 and concentration 0.2 M) together with potassium ferri-cyanide (250 µl) and incubated at 50˚C for 20 minutes. After incubation,

aliquots of trichloroacetic acid (250 µl) were added to the mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant (250 µl) was mixed with distilled water (250 µl) and freshly prepared ferric chloride solution (50 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 formula:

$$\text{Percentage reducing power} = \frac{((A_{700nm} \text{ of sample} - 1) \times 100)}{(A_{700nm} \text{ of blank})}$$

The effective concentration (EC_{50}) values, which represent concentrations eliciting a 50% response, were determined by regression analysis, from linear plots of concentration of the extract against the mean percentage of the antioxidant activity from three independent experiments. A low EC_{50} value, represent a more effective reducing power. Experiments were done in triplicates in three independent trials.

Maintenance of cell culture

An immortalised mouse myoblast cell line (C2C12) was used in this study (ATCC, Rockville, USA). The cells were cultured and maintained in RPMI media (Lonza, BioWhittaker®), supplemented with 10% foetal bovine serum (Hyclone, Thermo Scientific) at 37°C, in an atmosphere of 5% CO_2 in a humidified incubator (Heracell 150i CO_2 incubator, Thermo Scientific). The cells were differentiated by culturing in RPMI media containing 2% horse serum for 4 days.

Cytotoxicity assay

The cytotoxicity of the different plant extracts on C2C12 cell line were determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich™, SA) as modified by (Ferrari *et al.,* 1990). Experiments were done in triplicates in three independent trials. Cells were seeded at an initial cell density of 2 x 10^5 cells/ml into 96-well cell culture plates (Nunc™, Roskilde, Denmark). The adherent cell lines were incubated overnight to allow the cells to attach. The cells were treated or not with different concentrations (0 µg/ml to 1000 µg/ml) of the different extracts. The untreated cells served as the experimental control. Actinomycin (Sigma Aldrich™, SA) and DMSO served as positive and negative controls, respectively. The plates were incubated at 37°C for 24 hours after which MTT (10 µl) was added to each well. The cells were further incubated at 37°C for 2 hours. The medium was aspirated and the cells were washed once with pre-warmed PBS, pH 7.4. The insoluble purple coloured formazan formed intra-cellularly by the action of the mitochondrial dehydrogenase of viable cells following reaction with MTT was solubilised using DMSO (100 µl). The absorbance was measured at 490 nm using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). The percentage of viable cells was calculated according to the following formula:

$$\text{Percentage viability} = \frac{(A_{490nm} \text{ of sample} \times 100)}{(A_{490nm} \text{ of control})}$$

Glucose uptake assay

The amount of glucose taken up by differentiated C2C12 cells was quantified using the glucose uptake kit according to the manufacturer's instructions [KAT Laboratories and Medicals (PTY) LTD]. Cells at an initial seeding density of $5x10^4$ were treated for 1, 3 and 24 hours in the presence or absence of the different plant extracts. Untreated cells were used as the experimental control, while insulin and DMSO were used as positive and negative controls, respectively. After treatment the media (supernatant) (1 µl) from each of the treatments, including the control was transferred into a new 96 well flat bottomed plate and then working reagent (100 µl) was added, protected from light. The mixture was incubated in the dark at 37°C for 5 minutes. Absorbance at 500 nm was immediately read using a microtiter-plate multimode detector (Promega-Glomax Multi detection system). Experiments were done in triplicates in three independent trials.

GLUT4 translocation assay

The cells were differentiated by culturing in RPMI media containing 2% horse serum for 4 days. The differentiated C2C12 cells were seeded at a density of 1 x 10^5 cells per well in 6 well plates. The cells were treated with selected concentrations (0 µg/ml and 100 µg/ml) of the plant extracts for 3 hours. Insulin was used as a positive control and DMSO was used as a negative control. After treatment, the cells were washed three times with 1 x phosphate-buffered saline (PBS) and fixed with 80% methanol for 15 minutes and washed three times with 1 x PBS. Cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes and thereafter washed three times with 1 x PBS. Cells were blocked for non-specific binding using Bovine Serum Albumin (1mg/ml) (Sigma-Aldrich, SA) for 30 minutes and washed three times with 1 x PBS. The cells were then incubated with anti-GLUT4 primary antibody diluted 500x and thereafter washed three times with 1 x PBS. The cells were then incubated with secondary antibody conjugated to FITC for 1 hour and viewed using a fluorescence microscope and overlay images were captured at 100x magnification.(Nikon Ti microscope).

MAPK profiling assay

The expression of 26 mitogen activated protein kinases in the cells was determined using the human MAPK profiler assay Kit according to the manufacturer's instructions (RnD Systems). Differentiated C2C12 cells at a density of 6 x 10^7cell/ml were seeded in 25 cm^3 cell culture flasks and treated for 3 hours in the presence or absence of plant extract at the given concentrations (0 µg/ml and 100 µg/ml). Insulin (50 mI/U) was used as a positive control. The cells were immediately rinsed with PBS after which lysis buffer 6 was added. The re-suspend cell lysates were then rocked gently at 2-8°C for 30 minutes. The lysates were thereafter, centrifuged at 14,000 x g for 5 minutes, and the supernatant transferred into a clean Eppendorf tube. The total protein quantity was immediately determined using the BCA protein assay.

Following quantification array, buffer 5 (2 ml) was pipetted into each well of the 4 well plate where it served as a block buffer. Using flat-tip tweezers, the membranes were placed in separate wells of the 4 well plates and incubated for 1 hour on a rocking platform shaker. The samples were prepared by

adding up to 400 µl of each sample to separate Eppendorf tubes and adjusting the volume to 1.5 ml using array buffer 1. To each Eppendorf tube reconstituted detection antibody cocktail (20 µl) was added and incubated for 1 hour. Array buffer 5 was carefully aspirated from the wells of the 4 well plate and the prepared sample/antibody mixtures were gently added and incubated overnight at 4°C on a rocking platform shaker.

The membranes were carefully removed and placed into individual plastic containers containing 1x wash buffer (20 ml) and washed 3x for 10 minutes on a rocking platform shaker. The membranes were carefully placed into each of the 4 well plates containing diluted Streptavidin-HRP (2 ml) and incubated for 30 minutes at room temperature on a rocking platform shaker. After incubation the membranes were washed 3 times with 1x wash buffer. Chemi Reagent Mix (1 ml) was added evenly onto each membrane. The membranes were then washed using TBST and the transferred proteins were detected using the Super Signal West Dura chemiluminescent substrate (Thermo Scientific, USA) and antigen antibody complex was visualised by photo-detection using the Syn-Gene Image analyser (Bio-Rad, SA).

Statistical analysis

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 Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same group. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The *p* value significance is represented as asterisk (*) for $p < 0,05$, two asterisks (**) for $p < 0,01$ and three asterisks (***) for $p < 0,001$.

Results

Plant material extraction

The percentage yields of the different crude extracts obtained using solvents of varying polarity namely; methanol, acetone and hexane are presented in Figure 1. Methanol had the highest extraction percentage yield of 4,12% and acetone the least (2,16%).

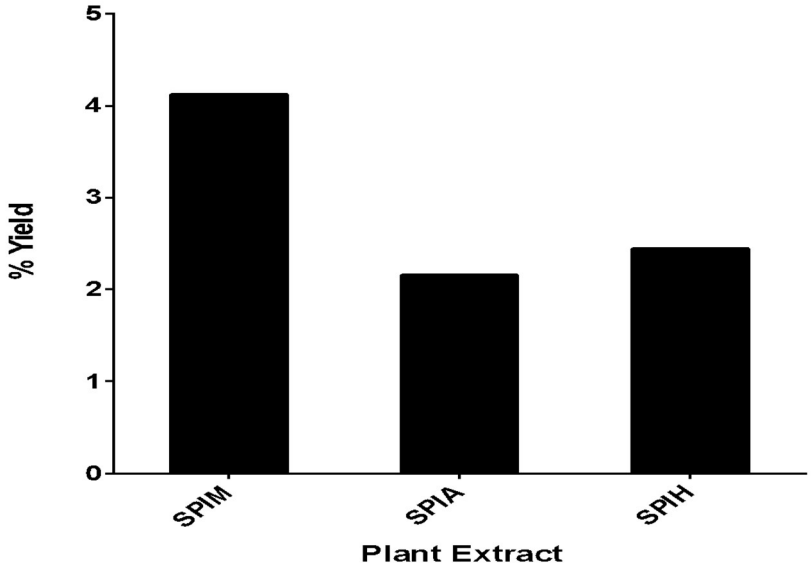

Figure 1: Percentage yields of the plant extracts obtained using solvents of varying polarity. SPIM = *S. plumosum*, (Methanol extract) SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract).

Secondary metabolite analysis

Qualitative analysis of the phytochemicals was performed in order to determine the presence of tannins, flavonoids, phenols, saponins, steroids, phlobatannins, glycosides, coumarins, proteins, anthraquinones, anthocyanins, leucoanthocyanins turns and carbohydrates in all the crude plant extracts. Tannins, flavonoids, phenols and steroids were present in all the extracts. Saponins, anthraquinones, anthocyanins, phlobatannins, glycosides, leucoanthocyanins turns and carbohydrates were absent in all the extracts. Coumarins on the other hand were present in the methanol and acetone extracts and absent in the hexane extract (Table 1).

Table 1: The presence / absence of various secondary metabolites in the different crude plant extracts of the different solvents.

		SPIM		SPIA		SPIH
Tannins		+		+		+
Flavonoids		+		+		+
Phenols		+		+		+
Saponins		-		-		-
Steroids		+		+		+
Phlobatannins		-		-		-

Glycosides		-		-		-
Coumarins		+		+		-
Proteins		-		-		-
Anthraquinones		-		-		-
Anthocyanins		-		-		-
Leucoanthocyanins Turns		-		-		-
Carbohydrates		-		-		-

(-) = constituent absent, (+) = constituent present, SPIM = *Seriphium plumosum* (Methanol extract), SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract).

Quantitative Phenolic and flavonoid analysis

The flavonoid and total phenolic content of each of the extract was determined as quercetin and tannic acid equivalents, respectively (Figure 2) using linear regression from standard curves. The methanol and acetone extracts showed the highest amount of flavonoid and phenolic contents, while hexane extracts showed the least.

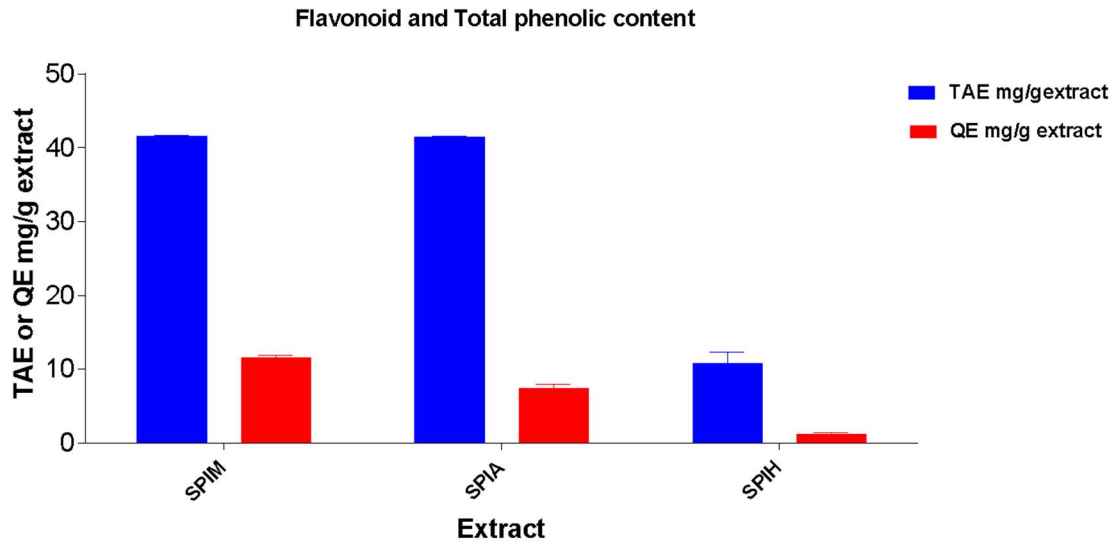

Figure 2: The total phenolic content of the different plant extracts represented as tannic acid equivalents (TAE mg/g) and flavonoids content in the different plant extracts represented as quercetin equivalents(QE mg/g). SPIM = *S. plumosum* (Methanol extract), SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract).

Quantitative FRAP and DPPH

The EC_{50} values for DPPH scavenging assay and ferric reducing power of the different plant extracts were calculated using linear regression (Table 2). The methanol extract showed the best activity

among all the extracts in both assays. It exhibited the lowest EC_{50} values of 0.72 mg/ml and 2.31 mg/ml for the DPPH scavenging activity and the ferric reducing power assay, respectively. These EC_{50} values were lower than those for ascorbic acid which were 1.62 mg/ml and 3.10 mg/ml for the DPPH scavenging activity and the ferric reducing power assay, respectively.

Table 2: The EC_{50} values for the DPPH scavenging assay and the ferric reducing power of the extracts.

	DPPH scavenging activity EC_{50} (mg/ml)	Ferric reducing power EC_{50} (mg/ml)
SPIM	0.72	2.31
SPIA	1.71	3.02
SPIH	10.58	8.06
Ascorbic acid	1.62	3.10

Anti-glycation activity

The ability of the extracts to inhibit the glycation of bovine albumin serum was conducted (Figure 3). The acetone extract exhibited the most glycation inhibitory activity among all the examined extracts, as it resulted in 2,22% glycation compared to Arbutin, a known anti-glycation (7,40%) agent which was used as the positive control. On the other hand, treatment with the methanol and hexane extracts resulted in 7,30% and 4,90% glycation, respectively.

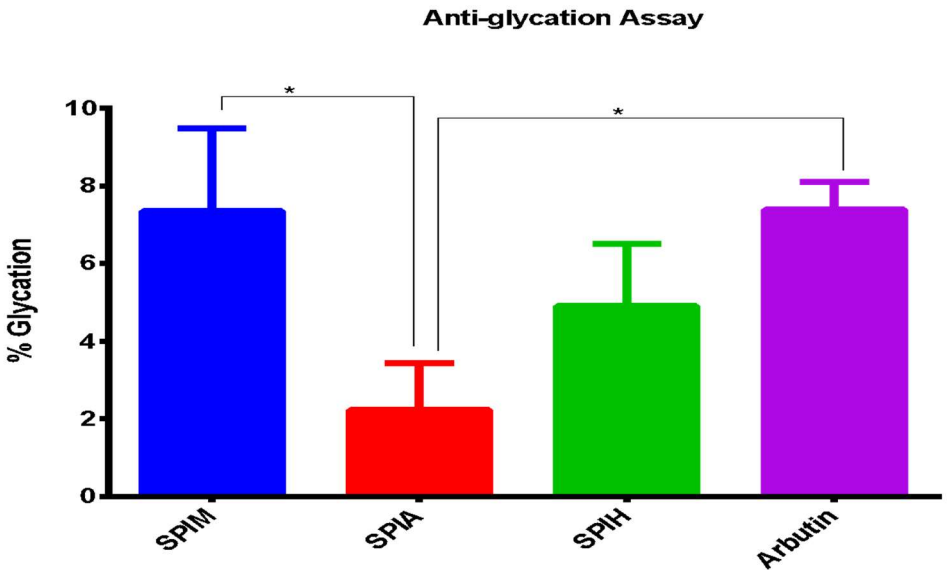

Figure 3: The effects of different extracts of *S. plumosum* on the glycation of bovine serum albumin (BSA). Arbutin was used as the standard reference. SPIM = *S. plumosum* (Methanol extract), SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract). The results were obtained from three independent experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The p-value significance was represented an asterisk (*) for $p<0,05$.

Cytotoxicity analysis

The viability of C2C12 cell line was assessed at increasing concentrations of the different extracts using the MTT cell viability assay. The percentage cell viability was calculated relative to the untreated control. The cell viability decreased as the concentration of the various extracts increased. Actinomycin and DMSO were used as positive and negative controls, respectively. Non-toxic concentrations (125 µg/mg to 500 µg/mg) obtained from the assay were chosen for use in subsequent experiments. The methanol extract was shown to relatively reduce the viability of the cells as compared to the other extracts.

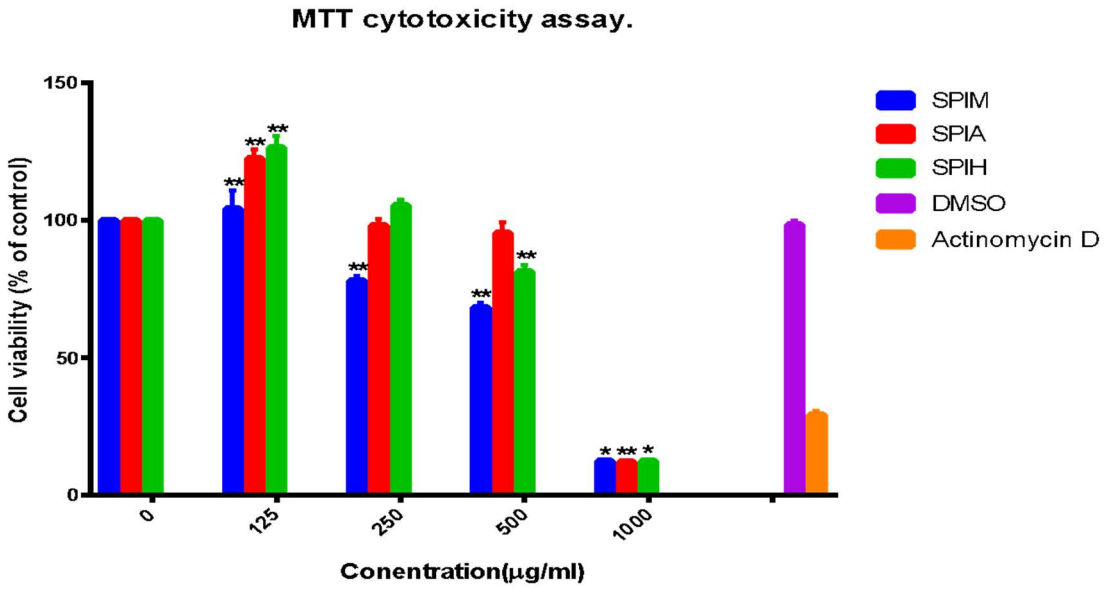

Figure 4: The effects of different extract concentrations of *S. plumosum* on the proliferation of murine myoblast cells (C2C12).. SPIM = *S. plumosum* (Methanol extract), SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract), DMSO = Dimethylsulphoxide. The results were obtained from three independent replicate experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using One-way Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test .The *p* value significance was represented an asterisk (*) for $p < 0,05$ and two asterisks (**) for $p < 0,01$

Glucose uptake assay

The amount of glucose utilised by the differentiated C2C12 cells exposed to different treatment conditions was quantified by the glucose uptake assay. The percentage glucose utilised was calculated with respect to the untreated control for 1, 3 and 6 hours. DMSO and insulin were used as negative and positive controls, respectively. The glucose utilisation in the DMSO treated cells was comparable with the untreated control. The combination of the plant extracts with insulin resulted in less glucose uptake as compared to the plant extract alone. This was observed for all the extracts particularly the hexane extract, which not only resulted in the highest glucose uptake of 35,77% but

was also shown to have more potent glucose uptake ability than insulin when used alone than in combination with insulin after 6 hours of exposure (32,23%).The percentage glucose utilisation is shown to increase incubation time.

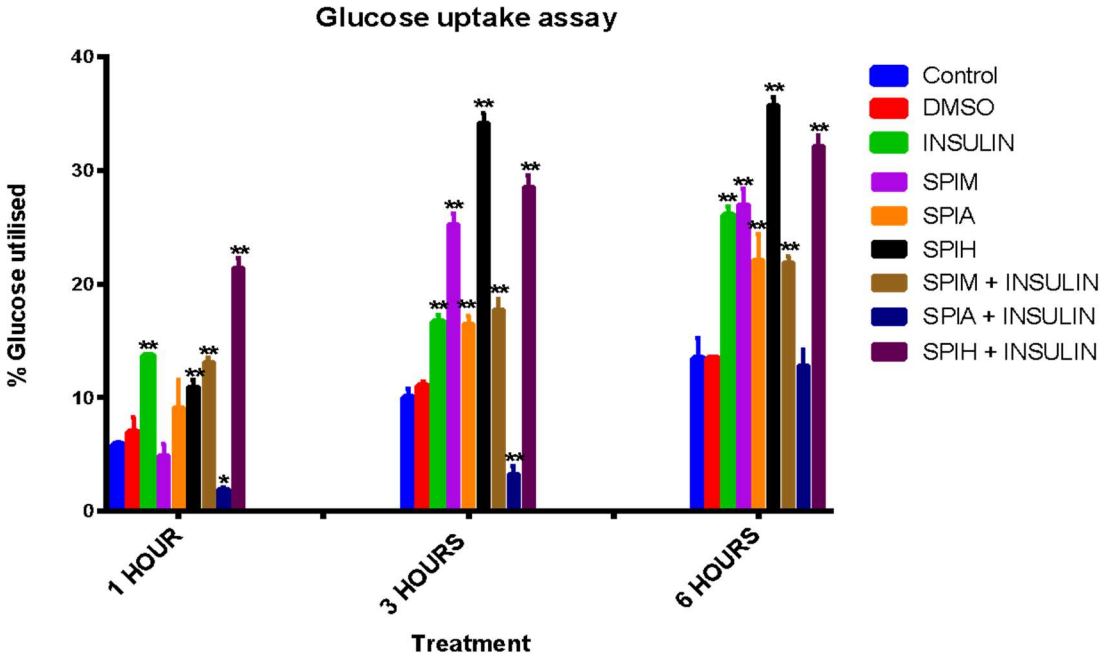

Figure 5: The effects of different extracts of *S. plumosum* in the presence or absence of insulin on the glucose uptake by murine myoblast cells (C2C12) at different time intervals. SPIM =*S. plumosum* (Methanol extract), SPIA = *S. plumosum* (Acetone extract), SPIH = *S. plumosum* (Hexane extract), DMSO = Dimethylsulphoxide. The results were obtained from three independent replicate experiments and expressed as means ± standard deviation. The statistical significance of the results was tested using One-way Analysis of Variance (ANOVA) employing the Dunnett Multiple Comparisons Test between the control and the different treatments within the same time group. The *p* value significance was represented an asterisk (*) for $p<0,05$ and two asterisks (**) for $p<0,01$.

Qualitative GLUT4 translocation assay

The GLUT4 translocation assay was used to determine the localisation of GLUT4 molecules under various treatment conditions. The cells were either treated with or not with the hexane extract (100 µg/ml) since it was this extract that showed the highest glucose utilisation potential at 3 and 6 hours of exposure in the absence or presence of insulin. The untreated cells served as the negative control while DMSO and insulin served as solvent and positive controls, respectively. The cellular localization of GLUT4 was determined by first using a primary antibody specific for GLUT4 molecule, thereafter staining with an FITC-conjugated secondary antibody to detect the areas to which the primary antibody had bound. The green fluorescence indicates the area where GLUT4 molecules are located. Higher fluorescence intensity shows the translocation of GLUT4 molecules to the membrane, which indicates where the primary antibodies had greater access and bind more. On the other hand, lower

green fluorescence intensities shows more GLUT4 molecules sequestration in their cytoplasmic peri-nuclear vesicles. The fluorescence intensity profiles from an average of 4 focal points per treatment were quantified and graphed (Figures 6-10). Each of the florescence intensity profile from the various treatments was compared to that of the control by overlay analysis (Figures 7-10).

The fluorescence intensity profile of untreated cells is represented in Figure 6. This fluorescence intensity profile was taken to represent cells that had basally-translocated levels of GLUT4 molecules bound to their membrane.

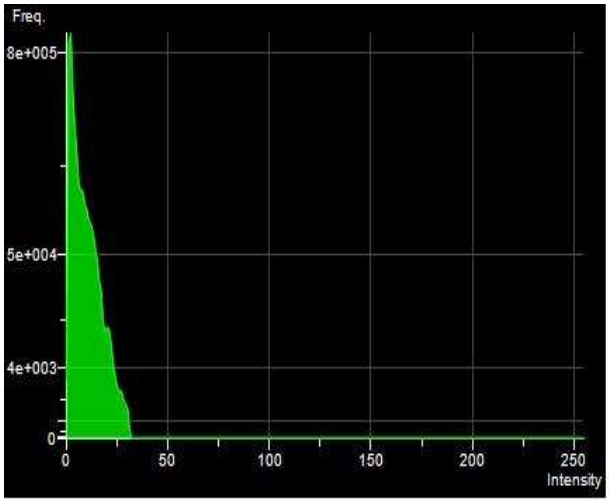

Figure 6: The flourescence intensity profile of the green flourescence emitted from the FITC-conjugated secondary antibody which was used to stain the GLUT4 molecules of the untreated differentiated C2C12 cells.

An overlay of the fluorescence intensity profiles of untreated cells and cells treated with DMSO is represented in Figure 7. DMSO, which was the solvent control, did not have an effect on the translocation of GLUT4 molecules to the membrane. The overlay of the intensity profiles following these two treatments are almost super-imposable to show their similar effect on the translocation of GLUT4 molecules to the cells membrane.

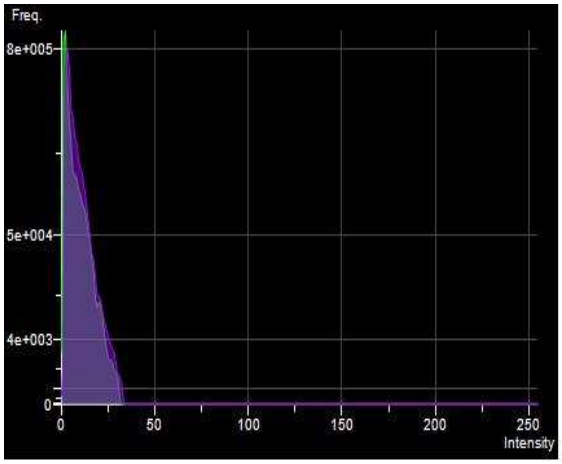

Figure 7: The flourescence intensity profile of the green flourescence from the FITC-conjugated secondary antibody which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with DMSO represented by the purple graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

It was observed in Figure 8 that there was a shift in the fluorescence intensity profiles of the cells treated with hexane extract. This treatment resulted in more cells with relatively higher intensities as compared to those found in the control. This means that more of the GLUT4 molecules have translocated to the cell membrane as compared to those in the untreated control.

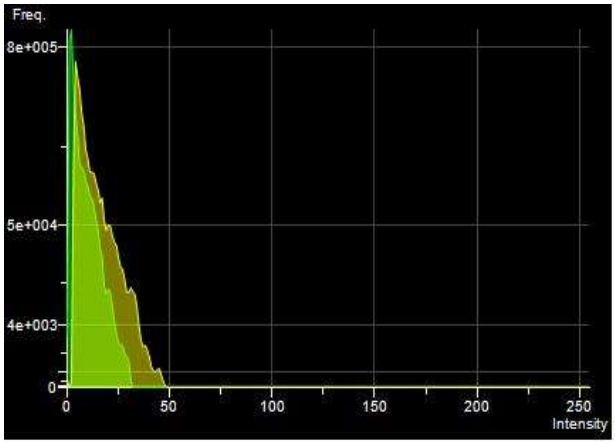

Figure 8: The flourescence intensity profile of the green flourescence from the FITC-conjugated secondary antibody which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with the hexane extract of *S. plumosum* (SPlH) represented by the yellow graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

The fluorescence intensity profile of the cells treated with the hexane extract in combination with insulin is represented in Figure 9. When insulin and the hexane extract of *S. plumosum* were used as individual treatments, they each resulted in an increase in GLUT4

translocation but failed to do so in combination. This treatment resulted in an intensity profile similar to that of the control.

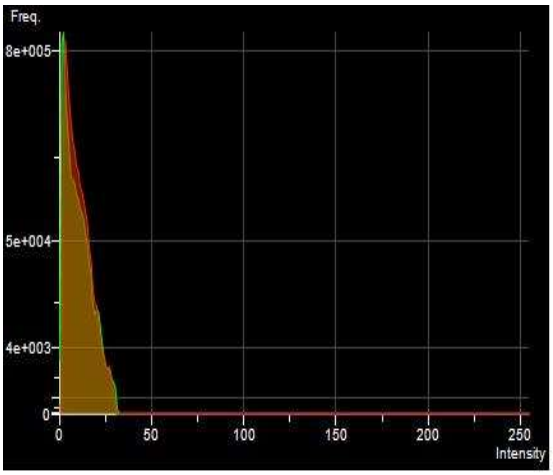

Figure 9: The flourescence intensity profile of the green flourescence from the FITC-conjugated secondary antibody which was used to stain the GLUT4 molecules of the differentiated C2C12 cells treated with the hexane extract of *S. plumosum* (SPlH) in combination with insulin represented by the red graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

Insulin was employed as the positive control for these experiments and the resulting fluorescence intensity profile was compared to that of the control in the figure below (Figure 10). Although a shift in the fluorescence intensities profile of the cells treated with insulin was observed, the treatment did not however supersede that of the hexane extract.

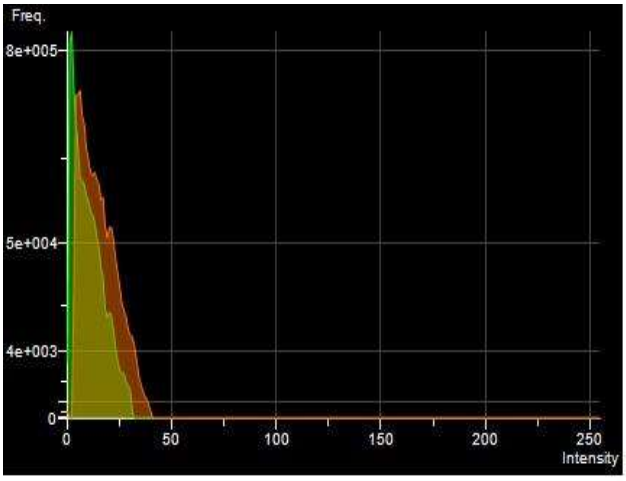

Figure 10: The flourescence intensity profile of the green flourescence from the FITC-conjugated secondary antibody which was used to stain the GLUT4 molecules of the differentiated C2C12 cells

treated insulin represented by the orange graph superimposed on the green graph representing the untreated differentiated C2C12 cells.

Mitogen activated protein kinase proteome profile analysis

The Human Phospho-MAPK Array was used to determine the effect of various treatments of the hexane extract on the phosphorylation of various mitogen activated protein kinases involved in the glucose uptake signal transduction pathway. Total whole cell lysates were extracted after the cells were subjected to a 3 hour treatment with the hexane extract in combination with insulin, insulin alone and untreated cells served as the control. The antibody-protein complexes which were observed as spots on the membranes were visualised using chemiluminescent reagents. The relative pixel densities of the spots on the membranes were quantified and the graphs were plotted for various treatments and their effects on the expression of different MAPKs that were analysed (Figures11-13).

Expression of Akt1 was significantly higher in the cells treated with the hexane extract as compared to the control, insulin and the combination of the hexane extract with insulin (Figure 11). Additionally, the combination of the hexane extract of *S. plumosum* and insulin resulted in significantly lower amounts of phosphorylated Akt1 as compared to insulin and the hexane extract only.

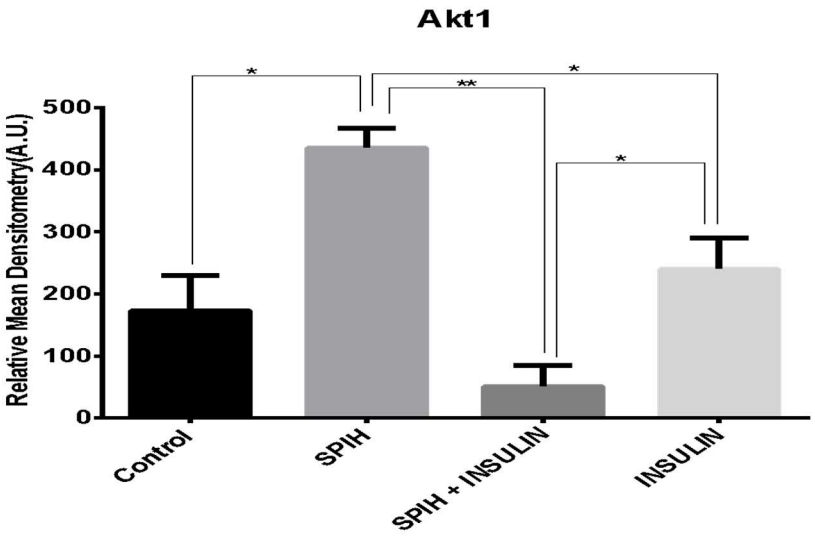

Figure 11: The expression of Akt1 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *S. plumosum* only (SPIH) and in combination with insulin. The results were obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The p value significance was represented an asterisk (*) for p<0,05, two asterisks(**) for p<0,01.

A similar trend as observed for Akt1 was also observed in the expression profile of Akt2 following different treatments (Figure 12). The hexane extract resulted in the highest expression of phosphorylated Akt2 followed by that for the positive control (insulin) and the untreated cells respectively, whereas the combination of hexane extract and insulin resulted in the least expression of the afore mentioned protein.

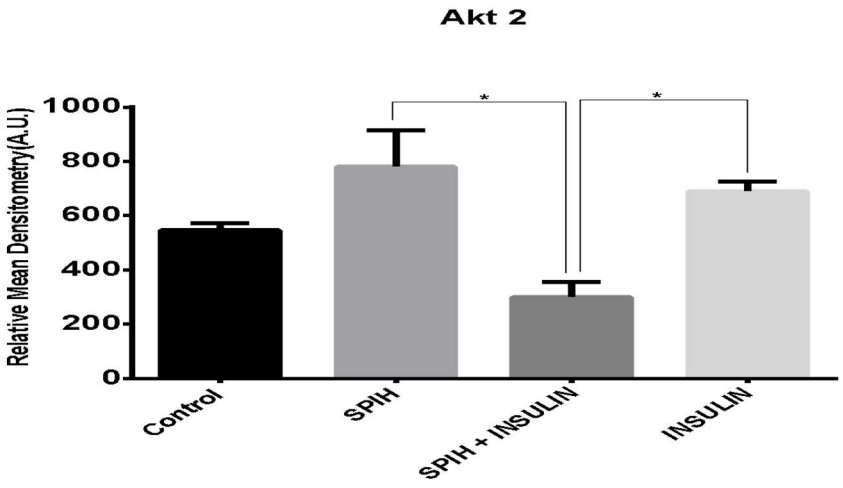

Figure 12: The expression of Akt2 following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *S. plumosum* only (SPIH) and in combination with insulin. The results obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The p value significance was represented an asterisk (*) for p<0,05.

The ribosomal protein S6 kinase beta-1 (p70S kinase) was highly expressed in the treatment with hexane extract (Figure 13). The expression of p70S kinase was significantly higher in the cells treated with hexane extract as compared to the untreated cells.

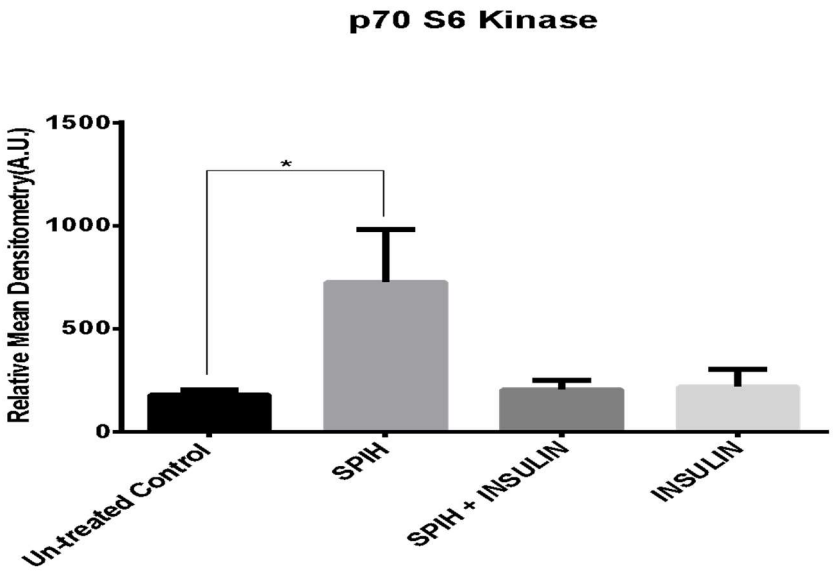

Figure 13: The expression of p70 S6 Kinase following various treatments indicated above for a period of 3 hours. The untreated cells served as the control while insulin was the positive control. The cells were also treated with the hexane extract of *S. plumosum* only (SPIH) and in combination with insulin. The results obtained were expressed as means ± standard deviation. The statistical significance of the results was tested using one way ANOVA employing the Tukey-Kramer Multiple Comparisons Test. The p value significance was represented an asterisk (*) for $p<0,05$.

Discussion

Plants have been employed for therapeutic purposes since ancient time. Traditional healers from across the globe practise the use of different plants and plant parts to aid in the alleviation of afflictions that result from various types of ailments. In this study methanol, acetone and hexane were used as extraction solvents due to differences in their polarities. Methanol resulted in the highest percentage yield of 4,12%. This finding is consistent with other previous studies where methanol resulted in the highest percentage extraction yields relative to other solvents (Njume *et al.*, 2011, 2009; Masoko *et al.*, 2008; Ndip *et al.*, 2007). This finding is mainly attributed to the small molecular weight of methanol which enables it to penetrate the plant material more effectively. Although acetone resulted in the least extraction yields of 2,16%, it is of an intermediate polarity enabling it to extract compounds that are intermediate to polar. Eloff (1998) described acetone as being advantageous as an extraction solvent because of its ease of handling and it is known to extract compounds that have a broader spectrum of polarity. The qualitative phytochemical analysis conducted revealed that tannins, flavonoids and phenols were present in all the plant extracts. These groups of phytochemicals and their derivatives were targeted in this study as they are known to be employed as

templates for the manufacture of various novel therapeutic agents. The methanol extract contained slightly higher amounts of total phenolic compounds (41.63 mg/g) as compared to the acetone extracts (41.54 mg/g) while the hexane extract had the least amount (10.87 mg/g) suggesting that the majority of phenolic compounds in this plant were more polar in nature. A similar quantification profile was observed for the total flavonoids of the extracts. As flavonoids fall under the category of phenolic compounds, it was expected that the total yields of flavonoids will be less than that of the total phenolic compounds.

Polar compounds have been shown to possess higher antioxidant potential as compared to non-polar compounds (Sharma and Bhat, 2009). This was also observed when the antioxidant potential of the plant extracts was quantified by both the DPPH free radical assay and the ferric ion reducing power assay. The scavenging activity was observed to be in a polarity and concentration dependant manner. The methanol extract had the highest activity with an EC_{50} value of 0.72 mg/ml which was better than that of ascorbic acid which was 1.62mg/ml. The electron donating potential of the extracts were determined by measuring their capability to reduce the ferric (Fe^{3+}) ion to its ferrous (Fe^{2+}) ion state. The ferric reducing power was observed to be in a polarity and concentration dependant manner. Methanol extract showed the best ferric reducing ability with an EC_{50} value of 2.31 mg/ml which was lower than that of ascorbic acid which was 3.10 mg/ml. It is likely that the phenolic compounds might also be contributing to this ability of the extracts to reduce the ferric ion to its ferrous state (Pereira *et al*., 2009). There has not been any report on the anti-oxidant potential of *S. plumosum* but several studies have shown a high degree of correlation between enhanced anti-oxidant activity in plants with high amounts of phenolic compounds (Cai *et al*., 2004; Pourmorad *et al*., 2006). Glycation which is implicated as the major cause of the debilitating signs and symptoms in diabetic patients is a disruptive spontaneous reaction that occurs mainly between proteins and reducing sugars. The acetone extract showed the most potent BSA glycation inhibitory activity as it resulted in 2, 22% glycation as compared to Arbutin which resulted in 9,73% glycation. Available reports (Sadowska-Bartosz and Bartosz, 2015) suggest plants with high concentrations of total phenolic compounds to possess high anti-oxidant and anti-glycation activity. This report is consistent with findings in this study because the hexane extract which contained the least amount of total phenolic content also exhibited the least BSA glycation inhibitory activity. Although the precise mode by which these plants exhibit their anti-glycation activity have not been established, previous study suggests that anti-glycation agents may act by delaying the formation of AGEs by preventing further oxidation of Amadori product and metal-catalysed glucose oxidation (Ramkissoon *et al*., 2013).

Establishment of cytotoxic and non-cytotoxic concentrations of different plant extracts is a crucial step in ascertaining the use and safety of plant extracts as a therapeutic agents (Ernst and Pittler, 2002). While some plants may have therapeutic uses at lower concentrations, intake of these plants above these concentrations may be as dangerous as an overdose of western medicines. No documented report is available on the cytotoxic effect of *S. plumosum*. The methanol, acetone and hexane extracts resulted in CC_{50} values of 518,80 µg/ml, 691,03 µg/ml and 641,80 µg/ml, respectively on C2C12 cells. It was therefore concluded that since all the extracts investigated had CC_{50} values greater than

50 µg/ml they were generally non-cytotoxic. A non-cytotoxic concentration of 100 µg/ml was determined from this analysis and used in all subsequent experiments.

Increased glucose disposal by various tissues results in a direct lowering of blood glucose in circulation. Since the hexane extract was shown to exhibit the highest glucose utilisation potential at 3 and 6 hours of exposure, it was further used in subsequent assays. Previous studies show that substances that increase glucose disposal by various peripheral organs including the muscles have hypoglycaemic effect (Shrayyef and Gerich, 2010). Despite studies available studies (Ghasemzadeh and Ghasemzadeh, 2011; Ali-Asgar, 2013) which suggests the predominant phenolic nature of most anti-diabetic compounds, the hexane extract which contained the least amount of phenolic and flavonoid content, showed the best glucose utilisation effect of 35,77% which was better than that of insulin which was 26,06% after 6 hours. On the other hand this observation is in concurrence with a study by Qi *et al* (2010) which documents the anti-diabetic activity of several non-phenolic phyto-compounds, a report that is consistent with the present findings in this study. Treatment with the hexane extract in combination with insulin resulted in 32,23% glucose uptake after 6 hours which was lower than when the extract was used alone. This observation strongly concurs with an observation documented by (Manukumar *et al*., 2016) suggesting that the hexane extract acts more as an insulin mimetics rather than as an insulin sensitizer. Insulin mimetics help regulate glucose uptake by the muscle cells by producing effects that mimic that of insulin, thereby eliciting a similar cascade of reaction that result in increased glucose uptake (Manukumar *et al*., 2016). The compounds may not necessarily bind to insulin receptor on the cell surface membrane but to any other protein within the cascade. These compounds can be helpful particularly to patients that produce relatively low amounts of insulin.

Upon an increase in blood glucose levels beyond the normal range, insulin is released by β-pancreatic cells. The released insulin mediates a cascade of reactions that culminate in the translocation of insulin responsive glucose transporter molecules known as GLUT4 (Huang and Czech, 2007; Leto and Saltiel, 2012). Translocation of GLUT4 molecule from its cytoplasmic vesicles to the membrane results in increased glucose transport into the cell. This is due to the fact that fusion of GLUT4 with the membrane results in increased transmembrane channels through which glucose can enter the cell (Leto and Saltiel, 2012). The GLUT4 translocation assay was performed to investigate the shift in the distribution of GLUT4 molecules between the membrane and the cytosolic vesicle in C2C12 muscle cells following different treatments. Fluorescence intensity profiles of the different treatments against the control were used to determine these distribution patterns. The untreated cells (control) resulted in a fluorescence intensity profile that was taken to represent the basal translocation levels of GLUT4 molecules to the cell surface membrane. The fluorescence intensity profiles of the untreated cells and those treated with DMSO (negative control) were quite comparable. They both resulted in more cells with relatively low intensity and less cells with higher intensity. On the other hand, treatment with insulin alone and the hexane extract resulted in cells with higher fluorescence intensity profiles. Cells treated with the hexane extract however showed higher fluorescence intensity profile. The increase in fluorescence intensity implies that those cells had

relatively more GLUT4 molecules translocating to the membranes as compared to the untreated cell. An increase in the number of GLUT4 molecules that translocated to the membrane is partly responsible for the increased glucose uptake observed in the glucose uptake assay. The effect of *S. plumosum* on both GLUT4 translocation and glucose uptake has thus far not been investigated.

The translocation of GLUT4 to the plasma membrane and the expression of GLUT1 and GLUT3 which are known to enhance glucose-uptake in various insulin-responsive tissues are under the control of protein kinase B (Akt) (Hernandez *et al*., 2001). Akt is known to function downstream of phosphoinositide-3 kinase (PI_3K) in the PI_3K-Akt signal transduction pathway activated by insulin. In this study the hexane extract resulted in the expression of increased amounts of phosphorylated Akt1 and Akt2 with respect to the untreated control and insulin. The Akt1 isoform was phosphorylated at the Serine at position 473 while the Akt2 isoform was phosphorylated at the Serine at position 474. Phosphorylation of these different isoforms precedes their translocation into the cytoplasm where they mediate a host of functions by further phosphorylating other downstream molecules. The Akt-mediated cascade is known to mediate an increase in the translocation of GLUT4 molecules via the activation of the Akt substrate 160 (AS160) (Brewer *et al*., 2011). The AS160 is to date the only known Akt substrate identified that shows a phosphorylation-dependent effect on GLUT4 trafficking (Brewer *et al*., 2011). A study by Karlsson *et al.* (2005) suggest that aberrant insulin signalling to AS160 via Akt contributes to defects in GLUT4 translocation and glucose uptake in skeletal muscle in insulin-resistant type 2 diabetic patients. Akt has since emerged as a crucial transducer of the insulin-signalling cascade leading to GLUT4 translocation and glucose uptake. Akt may also be involved in activation of the nutrient-dependent Thr/Ser kinase, mTOR (Tzatsos and Kandror, 2006). Activation of mTOR further results in the phosphorylation of ribosomal protein S6 kinase (p70S6K). p70 S6 kinase is a mitogen activated Ser/Thr protein kinase required for cell growth and G1 cell cycle progression (Le *et al*., 2003). In the current study the hexane extract resulted in the upregulation of phosphorylated p70S kinase which means that more energy will be directed to protein synthesis (Le *et al*., 2003). Among the proteins that are synthesised by activation of p70 S6 kinase is the GLUT4 protein. The increased amounts of p70 S6 kinase therefore may result indirectly in increased glucose uptake by both increasing the amount of GLUT4 molecules and increased energy expenditure.

Conclusion

The study reveals the anti-oxidant, anti-glycation and hypoglycaemic potential of crude plant extracts of *S. plumosum.* Furthermore, the study documents a probable anti-diabetic mode of action of the hexane extract of *S. plumosum* through enhanced glucose uptake. The increased amounts of glucose taken up by cells treated with the hexane extract were shown to result from the increase in the amount of GLUT4 molecules translocated to the cell membrane, presumably via the Akt-mediated pathway. The combination of insulin and the hexane extract of *S. plumosum* resulted in an antagonistic relationship as it resulted in lower expression of the different phosphorylated mitogen active protein kinases as compared to when the treatments were single. This may be due to the fact that the extract may contain some compounds that mask the action of insulin in the array of compounds that it contains. A plausible way of circumventing this is to isolate the pure compound in

this extract that elicits the desired function which may not negatively affect the normal action of insulin. Purification and identification of compounds, which is currently under consideration, that will exhibit the observed anti-oxidant, anti-glycation and hypoglycaemic activities may assist in the development of more potent antidiabetic pharmaceuticals.

Conflict of interest

The authors declare that they have no conflicts of interest.

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