Potential effect of *Senna italica* on glucose transport receptors- translocation of GLUT4 in NIH-3T3- L1 preadipocytes and C2C12 muscle cells

By

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DECLARATION

I Jane Choene Segolela hereby declare that the dissertation submitted to the University of Limpopo for the degree Master of Science in Biochemistry; have not been previously submitted by me at this University or any other University and that the information in this dissertation booklet is true and original. Where use was made of the work of others, it has been duly acknowledged in the text.

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DEDICATION

I am dedicating this project to my parents and my love (Herman) for being there for me when I needed their love, comfort and support throughout the challenges that I've met in my studies and lastly for believing in me, giving me hope and strength that I can do it no matter what comes my way. I would like to thank God for giving me strength and hope even when I felt like things were not going my way.

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LIST OF ABBREVIATIONS

A

A	Acetone
ADM	American Diabetes Association
AE	Acetone: ethyl acetate (5:5, v/v)
ATCC	American Type Culture Collection
A1c	Elevated haemoglobin
α	Alpha

B

BAW	Butanol: acetic acid: water (10:2:8, v/v/v)
BCA	Bicinchoninic acid protein assay
BEA	Benzene: ethanol: ammonium hydroxide (18:2:0.2, v/v/v)
BMI	Body mass index
BSA	Bovine serum albumin
β	Beta

C

C	Chloroform
CC_{50}	50% Cytotoxic concentration
CDC	Chronic Disease Prevention and Health Promotion Centre
CE	Chloroform: ethyl acetate (5:7, v/v)

CEF	Chloroform: ethyl acetate: formic acid (5:4:1, v/v/v)
CEM	Chloroform: ethyl acetate: methanol (8:5:9, v/v/v)
$^{\circ}$C	Degrees Celsius

D

DAPI	4', 6-Diamidino-2-Phenylindole
DASH	Dietary approaches to hypertension
DEX	Dexamethasone
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol

E

E	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
EMW	Ethyl acetate: methanol: water (8:2:10, v/v/v)
EPWM	Ethyl acetate: pyridine: water: methanol (8:3:5:1, v/v/v/v)

F

FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate

G

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDM	Gestational diabetes mellitus
GLUT's	Glucose transporters
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GOD	Glucose oxidase
GWASs	Genome wide association studies

H

H	Hexane
HbA1c	Glycosylated haemoglobin
HDE	Hexane: dichloromethane: ethyl acetate (8:1:6, v/v/v)
HE	Hexane: ethyl acetate (5:2, v/v)
HRP	Horse radish peroxidase
H_2SO_4	Sulphuric acid

I

I	Insulin
IBMX	Isobutylmethylxanthine
IDF	International Diabetes Federation
IUCN	International Union for Conservation of Nature

L

L	Leaf

M

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

P

PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PSN	Penicillin, Streptomycin, Neomycin

R

R	Root
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium

S

SA	South Africa
SDS	Sodium dodecyl sulphate
SI	*Senna italica*
SILA	*Senna italica* leaf acetone extract
SILE	*Senna italica* leaf ethyl acetate extract
SILH	*Senna italica* leaf hexane extract

SILM	*Senna italica* leaf methanol extract
SIRA	*Senna italica* root acetone extract
SIRE	*Senna italica* root ethyl acetate extract
SIRH	*Senna italica* root hexane extract
SIRM	*Senna italica* root methanol extract
SNPs	Single nucleotide polymorphisms

T

TAE	Tannic acid equivalents
TAG	Triacylglycerol
TBST	Tris buffered saline Tween-20
TLC	Thin layer chromatography
TNFα	Tumour necrosis factor alpha

U

UV	Ultraviolet
USA	United States of America

V

Vit C	Vitamin C
v/v	Volume per volume

W

WHO	World Health Organisation

Abstract

Diabetes mellitus is one of the major diseases worldwide that is life threatening and is reaching an epidemic proportion. The most important approach in reducing the burden of the disease worldwide is to search for effective, low cost hypoglycaemic drugs with fewer side effects. Past experimental evidence confirmed the hypoglycemic activity of many indigenous African medicinal plants. *S. italica* (Fabaceae family) is widely used by traditional healers to treat a number of diseases such as sexually transmitted diseases and other forms of intestinal complications traditionally. The current study was aimed at evaluating the *in vitro* effects of root and leaf extracts of *S. italica* on GLUT4 translocation in NIH-3T3-L1 preadipocytes and C2C12 muscle cells. In order to address the aim of the study various methods were undertaken. The roots and leaves of *S. italica* collected from Zebediela sub-region of the Limpopo province, South Africa, were ground to fine powder and extracted using acetone, methanol, ethyl acetate and *n*-hexane. The various extracts of the root and leaf material were subjected to fingerprint profiling using TLC plates and different mobile phases (BEA, CEF, EMW and BAW). The chromatograms were visualized with vanillin-H_2SO_4 reagent, *p*-anisaldehyde and iodine vapour. The extracts were assayed for the type of secondary metabolites contained in the studied plant parts using chemical text and by TLC analysis. The total phenolic content of the root and leaf material were also evaluated. Evaluation for antioxidant activity was performed using 0.2% DPPH qualitatively and quantitatively with vitamin C as a positive control. Toxicity study was performed on C2C12 muscle cells using the MTT assay, with Curcumin as a positive control and untreated cells as a negative control. The CC_{50} values of the acetone root and leaf extracts were determined by linear regression. The effect of acetone root and leaf extracts on glucose uptake by C2C12 muscle cells was evaluated, also on western blot and immunofluorescence for NIH-3T3-L1 preadipocytes. The solvents employed for extraction in this study are commonly used to extract various biological active compounds from plants in research settings. Methanol extracted more compounds followed by acetone, then ethyl acetate and *n*-hexane the least. The constituents extracted by methanol may be mostly sugars, amino acids and glycosides due to the polarity of this solvent. Hydro-alcoholic solvents extract a variety of compounds that are mostly polar. Acetone extracts

mostly alkaloids, aglycones and glycosides while *n*-hexane in general extracts mostly waxes, fats and fixed oils. High yield was obtained with leaf extracts with all the solvent used for extraction as compared to the root. The TLC finger-print showed that good separation was achieved with the methanol and acetone extracts in CEF mobile phase, ethyl acetate extracts in CEF and EMW and *n*-hexane extracts in BEA respectively, especially with the leaf extract. Most compounds present in *S. italica* extracts were UV active. Some compounds that were not reactive with vanillin-H_2SO_4 reagent were shown to be reactive with *p*-anisaldehyde reagent and iodine vapour which revealed the presence of sugars or aromatic compounds. Chemical analysis for secondary metabolites of the acetone root and leaf extracts revealed the presence of flavonoids, terpenes, tannins, steroids, reducing sugars and alkaloids while glycosides were detected only in the leaf extract. The results obtained using TLC analyses were consistent with the results obtained in the chemical analysis. Thin layer chromatography revealed the presence of glycoflavones in the acetone root extract, alkaloids in the root and leaf extracts; and phytosterols and flavonoid aglycones in root and leaf extracts. The acetone root and leaf extracts revealed the presence of phenols. The leaf extract was shown to contain high total phenolic content as compared to the root. The methanol and acetone root and leaf extracts were shown to possess antioxidant activity. However, the concentration of the activity was higher in the acetone root than in the leaf extract. The least activity was observed with the ethyl acetate root and leaf extracts as compared to other extracts. The *n*-hexane extracts however, was not shown to contain any antioxidant compounds. Although activity observed with the methanol extracts was comparable to that of the acetone extracts in the quantitative assay, the acetone extracts were shown to possess more antioxidant activity in the qualitative assay. The concentration of extracts increased with increase in scavenging activity. The root extract exhibited a more potent antioxidant activity compared to leaf extract. These extracts were evaluated for their cytotoxicity on normal cells. The highest cytotoxic concentration (CC_{50}) was obtained with the root extract with a CC_{50} value of 297 635 µg/ml at 48 hrs, followed by CC_{50} value of 21 544 µg/ml at 24 hrs. The CC_{50} value of the leaf extract at 24 hrs was 2 904 µg/ml with the least value at 48 hrs. The root extract at 24 and 48 hrs together with the leaf extract at 24 hrs were not toxic to C2C12 muscle cells at the concentration tested in this study. The acetone extracts were shown to possibly enhance proliferation of C2C12 muscle cells at a

concentration of 0.001–1000 µg/ml. The non-cytotoxic concentration of 25 µg/ml of the leaf extract in combination with insulin showed more glucose uptake as compared to other extracts as well as the control. Prolonged incubation time was shown to increase glucose uptake with leaf extract while increase in concentration of root extract decreased glucose uptake at 24 hrs. At incubation time of 3 and 24 hrs, glucose uptake results at concentration of 2.5 µg/ml were comparable with that of the root extract, with a similar trend observed at 25 µg/ml, although with decrease in uptake. The qualitative and quantitative fluorescence results showed GLUT4 to be translocated to the cell membrane. The leaf extract at a concentration of 25 µg/ml had more fold as compared to other extracts, indicative that more GLUT4 was translocated at this concentration of the leaf extract. The acetone root and leaf extracts were shown to increase protein expression of GLUT4 at 3 hrs incubation time as compared to other incubation times in insulin-stimulated C2C12 muscle cells. The plant constituents of *S. italica* was shown to contain a variety of secondary metabolites that maybe be acting alone or in concert with each other to exert the various activities observed in this study. Different solvents used for extraction may be responsible for the extraction of different constituents with antioxidant activity observed in the study. The acetone extracts enhanced proliferation of C2C12 muscle cells at concentrations used in the study. However, there was no significant reduction on viability of normal cells. In addition, the extracts were shown to enhance the differentiation of NIH-3T3-L1 preadipocytes into adipocytes and C2C12 muscle cells into myocytes. These in turn induced the translocation of GLUT4 to the cell membrane and as a consequence facilitate glucose transport. Hence, the differentiation of adipose cells as well as glucose uptake of muscle cells and GLUT4 expression might have been enhanced by constituents contained in the acetone extracts. In conclusion, the acetone leaf extract may have a beneficial role in glucose metabolism of differentiated C2C12 muscle cells. Therefore, further studies are however required to elucidate the molecular mechanism by which the acetone leaf extract of *S. italica* influences the translocation of GLUT4.

CHAPTER 1

1. Introduction

1.1. Metabolic diseases and their incidence leading to diabetes mellitus

Metabolic diseases occur in the body as a result of abnormal chemical reactions that disrupt metabolism. The clinical definitions of metabolic syndrome have been developed with a primary purpose of assisting in the identification of those at high risk of diabetes and cardiovascular diseases as a means of developing preventive measures that can reduce this risk (Balkau and Charles, 1999; Alberti *et al*., 2007). Metabolic diseases maybe a common factor leading to various chronic diseases. In light of this, individuals with metabolic syndrome are at a high risk of developing type II diabetes mellitus (DM) and cardiovascular diseases. This is due to the metabolic dysfunction commonly seen in individuals with insulin resistance (Cameron, 2010).

Metabolic syndrome is characterized by a cluster of factors including obesity, hypertension, hypertriglyceridemia, decreased plasma high-density lipoprotein cholesterol and elevated glucose (Bruce and Hanson, 2010). Individuals with metabolic syndrome are an important group that warrant education and early interventions prior to the development of DM (Dunkley *et al*., 2009). As such, in the prevention and control of metabolic syndrome, it is important to identify the causal risk factors that can help minimize the risk of getting type II DM. Dietary habits have a great impact on health. A dietary approaches to hypertension (DASH) may reduce the risk of metabolic syndrome (Azadbakht *et al*., 2005), while diets containing a high glycemic load may increase the risk (Lutsey *et al*., 2008).

Epidemiological studies have revealed that genetic factors play an important role in the pathogenesis of metabolic syndrome and in its development. Environmental factors such as high-fat food intake and lack of physical activity have been identified as major contributing factors (Benzinou *et al*., 2008; Zhao *et al*., 2014). Many single nucleotide polymorphisms (SNPs) associated with high body mass index (BMI) or increased risk of obesity have recently been identified by several genome wide association studies (GWAS) in populations of European ancestry (Meyer *et al*., 2010; Speliotes *et al*., 2010). This population ancestry may share a similar genetic

background, since obesity is highly correlated with metabolic syndrome (Zhao *et al*., 2014).

1.2. Predisposing factors to diabetes

An elevation of fasting or post-prandial plasma glucose levels (pre-diabetes) is one of the risk factors associated with type II DM and cardiovascular diseases. Integration of plasma glucose over time by elevated haemoglobin (A1c) or glycosylated haemoglobin (HbA1c), is promoted by some researchers as another indicator of pre-diabetes (Scott and Grundy, 2012). Pre-diabetes occurs commonly in combination with other cardiovascular risk factors that makes up the metabolic syndrome. In the development of metabolic syndrome, both genetic and environmental factors are considered to play a significant role. The chromosomal regions thought to be involved, have previously been linked to an increased risk of cardiovascular disease and Type II DM (Dunkley *et al*., 2009).

Obesity is a recognized risk factor that has been implicated in the development of type II diabetes and cardiovascular diseases (Liu *et al*., 2005). The extra fat stored in adipocytes, liver and muscle cells disrupts the response of these cells to insulin. Recent studies have shown that adipocytes are not only storage receptacles, but are also responsible for communication with other organs to maintain metabolic balance by secreting hormones and cytokines such as leptin, tumour necrosis factor α (TNFα) and adiponectin (Mora and Pessin 2002). The BMI is considered to be a good proxy measure for environmental factors. Its association with the adoption of poor lifestyle accompanied with less physical activity, higher consumption of sugar and fat makes obesity an epidemic. As a consequence pose a risk for the development of DM (Hossain *et al*., 2007).

A positive family history is widely accepted as a risk factor for many chronic diseases (Yoon *et al*., 2003). Oxidative stress is another factor that contributes to type II DM. In the development and progression of DM, oxidative stress is known to play a significant role (Henriksen *et al.*, 2010). The free radicals are formed disproportionately in diabetes by metabolic processes such as glucose oxidation,

non-enzymatic glycation of proteins and subsequent oxidative degradation of glycated proteins (Henriksen *et al*., 2010).

Decline in antioxidant defense mechanism due to abnormal high levels of free radicals can lead to damage of cellular organelles and enzymes as well as an increase in lipid peroxidation and *β*-cell apoptotic pathway activation (Valko *et al*., 2007). Indeed, ameliorating oxidative stress through treatment with antioxidants from plants might be an effective strategy for reducing diabetic complications (Johansen *et al*., 2005). Many medicinal plants including *Senna italica* (SI) possess antioxidant compounds (Gololo dissertation, 2008, Rahimi *et al*., 2007) and plants with antioxidant activity are considered to contribute to the management of various diseases such as diabetes (Elujoba *et al*., 2005).

1.3. Types of diabetes mellitus

1.3.1 Type I diabetes mellitus (Type I DM)

Type I DM is often referred to as immune-mediated diabetes, since it results from a cellular-mediated autoimmune destruction of the pancreas's *β*-cells. It accounts for about 5–10% of diabetic cases and is also termed insulin dependent diabetes (American Diabetes Association (ADM), 2012). The rate of *β*-cell destruction is often quite variable in this type of diabetes and develops rapidly in children that suffer from it and slow in adults. At the latter stage of the disease, there is low or undetectable level of plasma C-peptide as a result of little or lack of insulin secretion (ADM, 2012). The sufferers from this condition are often dependent on insulin administration for survival. The autoimmune destruction of *β*-cells is related to multiple genetic predispositions and environmental factors which are still poorly defined (Tollman *et al*., 2008).

1.3.2 Type II diabetes mellitus (Type II DM)

Type II DM is often referred to as non-insulin dependent diabetes and accounts for about 90–95% of people suffering from diabetes. Such individuals are insulin resistance and do not require insulin treatment to survive (ADM, 2012). Many causes have been associated with this type of diabetes, although the specific etiologies are

not known. The most common risk factor is its association with obesity. In addition, patients who are not obese may develop diabetes due to increase distribution of abdominal fat (Daar *et al*., 2007).

Type II DM usually goes undiagnosed for many years due to the gradual development of hyperglycemia, making the condition unnoticeable at an early stage (Groenewald *et al*., 2008). Late diagnosis puts patients at an increased risk of developing micro-vascular and macro-vascular complications. The frequency of type II DM varies among different ethnic groups and the risk of its development increases with age, obesity, lack of physical activity among others. The incidence is high in woman with prior gestational diabetes mellitus and hypertension or dyslipidemia (Tollman *et al*., 2008).

1.3.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is often defined as any degree of glucose intolerance with onset or first recognised during pregnancy. Although its limitations have been recognised for many years, the definition of GDM facilitates uniform strategy for its detection and classification (Groenewald *et al*., 2008). The number of pregnant women with undiagnosed type II DM has increased due to the high incidence of obesity and GDM. This results in an increased number of incidences of type II DM in women of childbearing age. However, most cases of GDM resolve following delivery. Annually, more than 200 000 cases are reported worldwide during pregnancies (about 7%) (Daar *et al*., 2007).

1.4. Available therapy for diabetes mellitus and their limitation

There are drugs that are in the market such as miglitol, voglibose, metformin and acarbose which are often used to manage diabetes. However, these drugs apart from their cost and unavailability to the rural poor, are often associated with undesirable side effects such as gastrointestinal disturbances, oedema and weight gain (Evans, 2007). There is no cure for DM, hence maintaining a good health requires a lifelong commitment to blood sugar level control. There is a report that also suggests that one of the therapeutic approaches to manage diabetes is to

decrease post-prandial hyperglycemia (Rhabaso-Lhoret and Chiasson, 2004). This is achieved by preventing the absorption of glucose through partial inhibition of carbohydrate-hydrolysing enzymes such as α-glucosidase and α-amylase in the digestive tract (Kwon *et al*., 2007).

1.5. Economic impact of diabetes mellitus (globally and in South Africa)

Diabetes Mellitus is wide spread and is considered a major problem worldwide (CDC/National Centre for Chronic Disease Prevention and Health Promotion, 2005) with about 346 million people suffering from the disease. It is estimated that diabetes will be the seventh leading cause of death and the number of people suffering from the disease will double by the year 2030 (WHO, 2011). Out of the two types, type II DM is the most prevalent and accounts for about 90–95% of all diagnosed cases of diabetes (Adefegha and Oboh, 2012).

A poorer health status and a higher prevalence of obesity which leads to type II DM is observed in rural dwellers of some developed countries that have been associated with unhealthy behaviour, less nutritious food and less exercise (Smith *et al*., 2008). On the contrary, in urban populations of these developed countries, there is a higher prevalence of obesity and metabolic syndrome which may be caused by higher fat diet and less physical activity (Assah *et al*., 2011; Chen *et al*., 2014). The complications and prevention of type II DM demands more attention due to the increase in its prevalence worldwide. The major complication of diabetes is cardiovascular diseases which includes both micro-vascular and macro-vascular diseases (Scott and Grundy, 2012), that have been associated with metabolic syndrome. It is estimated that the age adjusted prevalence of metabolic syndrome is 35% or 39% in the USA according to IDF definitions (Ford, 2005).

This figure is likely to increase due to rising levels of obesity and sedentary lifestyles (Ford, 2005).The prevalence of metabolic syndrome increases with age (Ford, 2005). It is higher in certain ethnic groups such as South Asians and Afro-Caribbean's and certain socio-economically disadvantaged groups (Wamala *et a*l., 1999; Dunkley *et al*., 2009; Siu *et al*., 2012). With the spread of western lifestyle, the prevalence of DM is predicted to increase in the coming decades (Siu *et al*., 2012).

Fortunately, reducing cardiovascular diseases risk factors and lifestyle modification has been shown to be effective in preventing DM (Siu *et al*., 2012). However, lifestyle modification is also cost effective. Type II DM has become a major public health challenge and is one of the fast growing diseases in Hong Kong (He *et al*., 2012). It has also been estimated that diabetic individuals are 25 times more likely than their non-diabetic counterparts to suffer severe, permanent vision loss (He *et al*., 2012).

Out of the two types of DM, there is a shortage of data on type I DM in Africa. From the South African perspective, estimates of type II DM vary between 3% and 28.7% amongst the various populations groups. The International Diabetes Federation (IDF) in 2003 report a prevalence of 3.4% for the 24 million South Africans between the ages of 20 and 79, with an expected increase to 3.9% by the year 2025. In addition, the current prevalence of 5.1% for the people living with diabetes may increase to 5.3% by the year 2035 in Africa (IDF, 2014). The increase in diabetes is linked to the worldwide increase in obesity. Consequent to this can lead to other forms of diseases such as heart diseases, stroke, kidney failure, *etc*., if not well managed resulting in a huge socio-economic impact.

This study therefore seek to investigate the potential effect of root and leaf extracts of *S. italica* on the translocation of glucose transport receptors (GLUT4) using NIH-3T3-L1 preadipocytes and C2C12 muscle cells as models *in vitro*. As a result, hoping to find bioactive extracts or compounds that can assist in enhancing *β*-cells to release insulin or that can aid in glucose transport.

CHAPTER 2

2. Literature review

2.1. Medicinal plants as a source of components with medicinal value

Medicinal plants as antimicrobial agents have been studied worldwide as to ascertain the specific information about the plant's antimicrobial activity (Thorshell *et al*., 2006). This specific information is then used as a complement to phytochemical analysis. Some medicinal plants may be useful in the management of diabetes mellitus, and have been used empirically as antidiabetic and anti-hyperlipidemic remedies (Malviya *et al.*, 2010). They have also been considered as natural products reservoirs with antidiabetic potential (Duarte, 2005). The anti-hyperglycemic effects of medicinal plants are attributed to their potential in restoring pancreatic tissues by inhibiting intestinal absorption or increasing insulin output or facilitating metabolites in insulin dependent processes, with the aim of aiding glucose transport (Malviya *et al.*, 2010).

Medicinal plants produce a diverse array of secondary metabolites which among others serve as defence against herbivores and plant diseases caused by virus, bacteria, moulds and parasites (Pieters and Vlietinck, 2005). Most of the plants that have been implicated as having antidiabetic properties contain secondary metabolites such as glycosides, alkaloids, terpenoids, flavonoids, cartenoids, *etc*. (Malviya *et al*., 2010). Screening of medicinal plants may help in understanding their toxic effects and ensuring their safety to humans and animals. The Secondary metabolites produced by plant may contain complex chemical structures which are not available in synthetic compound libraries (McGaw and Eloff, 2008).

These plants are extracted using organic solvents with the aim of isolating compounds which are then tested using different screening methods (McGaw and Eloff, 2008). The search for medicinal plants either as whole extract or single entities, which may serve as templates for the development of new drugs with fewer side effects, is desirable. Recent studies have shown that plants that contain antioxidative compounds are important in the prevention, management and treatment of various diseases (Heo *et al*., 2013).

2.1.1 *Senna italica* (*S. italica*)

S. italica (Fabaceae) is a small perennial shrub that grows from a woody base (figure 1) and is well known for its therapeutic properties in folk medicine (Al-Araidh *et al*., 2004). This plant is commonly known as *Morotwa ditšhotši* (Sepedi) and *Sebete* (Setswana). The leaves of this plant species from Venda in South Africa have shown antibacterial activity in many studies and are used as a treatment for sexually transmitted diseases due to the presence of anthraquinones (Tshikalange *et al*., 2005). A literature survey on chemical constituents of the genus *Senna* reveals the presence of alkaloids, quinones and anthraquinones, isolated from the heartwood, seeds, root bark, roots and leaves (Franz, 1993).

The plant is also a stimulant laxative which is mainly used for the treatment of constipation and bowel evacuation. It is also useful as a purgative, where habitual constipation, maybe one of the cases (Franz, 1993). The effect of the root extracts of *S. italica* has been examined against pathogenic bacteria (Masoko *et al*., 2010). Recently, the study on one of the therapeutic approach of managing DM by partial inhibition of carbohydrate digesting enzymes using plant extracts has been undertaken (Shai *et al*., 2010).

Figure 1: A representative of a flowering *S. italica* (International Union for Conservation of Nature (IUCN) Red List, 2011).

2.1.2 Plant Secondary metabolites

Most interesting biological activities come from plants as a rich source of secondary metabolites. These secondary metabolites are an important source of compounds with a variety of structural arrangements and properties (De-Fatima *et al*., 2006). Many of the secondary metabolites are distributed among a very limited number of plant species and can be diagnostic in chemotaxonomic studies (Crozier *et al*., 2006). Secondary metabolites can be divided into three major groups based on their plant biosynthetic origins: (i) flavonoids (allied phenolic and polyphenolic compounds), (ii) terpenoids and (iii) nitrogen-containing alkaloids together with sulphur-containing compounds (Crozier *et al*., 2006).

Their function in plants draw attention of researchers since they play a key role in protecting plants from herbivores, microbial infection, as attractants for pollinators, seed-dispersing animals, as allelopathic agents, UV protectants and signal molecules in the formation of nitrogen-fixing root nodules in legumes (Crozier *et al*., 2006). Other studies have demonstrated the importance of secondary metabolites in their use as dyes, fibres, glues, oils, waxes, flavouring agents, drugs, perfumes, *etc*. (Croteau *et al*., 2000; Dewick, 2002). The role of some secondary metabolites as protective dietary constituents has also become an important area of human nutrition research (Crozier *et al*., 2006), as well as their relevance in protecting against cancer and many chronic diseases (Crozier *et al*., 2006).

The benefit of knowing the chemical constituents of these medicinal plants is desirable for both the discovery of therapeutic agents and other uses. For instance, such information may be of great value in the discovery of new sources of economic phytocompounds for the synthesis of complex chemical substances and for the validation of folkloric remedies (Milne *et al*., 1993). Due to the complementary and overlapping mechanism of action of several phytochemicals, a thorough validation of herbal products has emerged (Sermakkani and Thangapandian, 2012). This new approach is used to emphasize and prioritize the standardization of natural drugs and products.

2.2. Role of antioxidants in reduction of oxidative stress or ameliorating the effect of diabetes

The accumulation of uncontrolled generation of reactive oxygen species (ROS) can lead to the development of oxidative stress. Cells have developed defense mechanisms for protection against ROS mediated oxidative damage (Kunwar and Priyadarsini, 2011). An antioxidant is a substance that is present at low concentrations and significantly delays or prevents oxidation of the oxidizable substrate (Vessby *et al*., 2002) and keeps in check the generation of ROS in the body. However, oxidative stress results from an imbalance between the generation of oxygen derived radicals and the organism's antioxidant potential (Abdollahi *et al*., 2004).

The effectiveness of antioxidants results from their ability to donate an electron to ROS, thereby neutralizing the adverse effects of the latter. This mechanism occurs in three different ways such as:

i. Keeping the formation of reactive species to a minimum level (prevention) e.g. desferrioxamine
ii. Scavenging reactive species either by using catalytic molecules (interception) e.g. ascorbic acid, α-tocopherol
iii. Repairing damaged target molecules (repair) e.g. glutathione (Kunwar and Priyadarsini, 2011).

Antioxidants systems are classified into two major groups, namely, enzymatic antioxidants that act as the body's first line of defense against ROS and non-enzymatic antioxidants. The report by Chanwitheesuk *et al*. (2005), have shown that plants contain a large variety of substances that possess antioxidant activity. In addition, the plants with high and strong antioxidant compounds play an important role in improvement of disorders involving oxidative stress (Rahimi *et al*., 2007).

2.3. Toxicity studies and its relevance in drug discovery

Various biological activities, including antioxidant activity of medicinal plants are due mainly to their polyphenol constituents (Surveswaran *et al*., 2007). Despite their desirable biological effects their toxicity profiles are not yet fully elucidated (Skotti *et*

al., 2014). Thus, toxicological studies include observational data gathering and utilization to predict outcome of exposure of these phytochemicals in human and animals are essential. Recently, attention has been focused on the use of herbs in therapy due to fewer side effects associated with their use as compared to allopathic drugs (Lobo *et al*., 2010). However, some plants have been categorised as harmful and some as safe by ancient humans (Pingale, 2008).

Furthermore, quality, safety and efficacy data on indigenous medicines is still lacking (Springfield *et al*., 2005). A proper check on quality and safety of medicinal plants is required (Mahmood *et al.,* 2013), coupled with the identification of plants with no or less toxic effects that can be used for the benefit of humans (Lobo *et al*., 2010). It is therefore important to investigate the toxic effect of plants *in vitro* and *in vivo*, alongside other relevant biological assays to harness their full medicinal potential.

2.4. Effect of insulin receptor on NIH-3T3-L1 preadipocytes and mechanism involved in translocation of GLUT4

The regulations of hormone and receptor number or affinity in response to ligand are well-documented mechanisms for modulating a cell's sensitivity to homologous hormone (Berger *et al*., 1999). In a study by Brent and Daniel (1980), the authors investigated the aspects of insulin receptor regulation by modifying the approach developed in Fambrough's laboratory. The method was applied to study insulin receptor regulation in NIH-3T3-L1 preadipocytes and NIH-3T3-C2 cell lines (Dell'Agli and Bosisio, 2002).

NIH-3T3-L1 preadipocytes cell line is a useful model for the examination of cell differentiation into adipocytes (adipocyte conversion) as well as for examination of the cellular physiology of triacylglycerol vesicle formation. During the process of adipocyte conversion, NIH-3T3-L1 preadipocytes acquire many of the enzymatic and biochemical characteristics of adipocytes (Berger *et al*., 1999) and initiate the storage of energy in the form of triacylglycerol (TAG)-rich lipid droplets. Hence, understanding the factors and mechanisms involved in adipocyte conversion and adipocyte lipid homeostasis is vital in understanding conditions such as obesity, hypertension, coronary artery disease, lipoatrophies and non-insulin dependent

diabetes (Berger *et al*., 1999). Furthermore the identification of the proteins involved in the process is an important step that can help in the understanding of the normal regulation of adipocytes or other lipogenic cells.

2.5. Glucose regulation

Glucose homeostasis requires the monitoring of blood glucose concentrations which can help modulate food intake and glucose use or production. It has been recognized that pancreatic *β*-cells possess a well-characterized glucose sensor, which is activated by high blood glucose. The rise in blood glucose induces the secretion of insulin, the hormone most important for the determination of glucose use. Similarly, secretion of glucagon to accelerate hepatic glucose production is triggered by a sensor, which responds to hypoglycemia, present in pancreatic α-cells. This secretory activity or regulation is also under the control of the autonomic nervous system (Moore and Cherrington, 1996). Discrete glucose sensors located in the hypothalamus and the brain stem modulates the activity of the autonomic nervous system which then regulates feeding, together with glucose homeostasis (Moore and Cherrington, 1996).

Plasma glucose levels increase after the absorption of glucose and stimulate insulin secretion by the pancreas. To normalise glucose levels, hyperglycemia suppresses hepatic glucose production and stimulate glucose uptake in the liver and peripheral tissues (adipose and muscle). The administration of oral glucose in humans leads to 30–40% uptake by the liver while the intravenous route results in 10–15% uptake (Abdul-Ghani *et al.*, 2006). In the fasting state, GLUT2 promotes the efflux of glucose following gluconeogenesis for maintaining whole body glucose homeostasis (Karim *et al*., 2012). After fasting, insulin levels are low and most glucose uptake occurs in insulin-insensitive tissues where the uptake is matched mainly by endogenous glucose uptake by the liver and a small amount by the kidney (Stumvoll *et al*., 1997).

Some factors that are rate limiting for glucose uptake are glucose transporters (GLUT's) which serves as important regulators for metabolism (Cheong *et al.*, 2014). The metabolism of adipose tissue and skeletal muscle have a common feature of an

increase in glucose influx, due to insulin response which results in translocation of GLUT4 from the storage vesicles into the plasma membrane (Wood and Trayhurn, 2003).

2.6. Mechanism of glucose uptake in the liver

Net hepatic glucose uptake is mainly determined by; (i) insulin and glucagon concentrations in the blood (net uptake increases with rise in insulin concentration and decrease in glucagon concentration), (ii) the glucose load reaching the liver (positively correlated with net uptake) and (iii) the route of glucose delivery (DiCostanzo *et al*., 2006). The liver removes excess glucose effectively from the blood using glucokinase (Agius, 2008).

This enzyme is also present in pancreatic *β*-cells, some endocrine enterocytes and hypothalamic neurons (Matschinsky, 2002). It helps regulate insulin secretion from the pancreas since it can act as a glucose sensor. Glucose homeostasis seems to require the association of the complex glucokinase-based signalling network containing all the four cell types (De Graaf-Roelfsema, 2014). Glucokinase gene mutations are associated with different type of DM (Osbak *et al*., 2009).

2.6.1 Mechanism of glucose uptake in peripheral tissues

The major consumer of glucose in the body after a meal or during an exercise is the skeletal muscle (C2C12 muscle cells). This glucose is taken up by GLUT1 and GLUT4 into the tissue through facilitated diffusion. The molar ratio of GLUT1:GLUT4 polypeptides in skeletal muscle plasma membranes is 0.6 to 1.0 (Klip and Marette, 1992). In skeletal muscle (C2C12 muscle cells) and adipose tissues (NIH-3T3-L1 preadipocytes) there are a number of glucose transporters that are involved in glucose disposal. In skeletal muscles, GLUT1 is responsible for low levels of glucose uptake since it is expressed at low levels in the plasma membrane (Annandale *et al*., 2004).

Translocation of intracellular GLUT4 to the cell surface is facilitated by glucose disposal in skeletal muscle and adipose tissue when stimulated by increase in insulin

concentration (Duehlmeier *et al*., 2010). In mouse models, an increase in the rate of glucose transport results from an increase in the density of GLUT4 on the cell plasma membrane (Huang and Czech, 2007). At low insulin levels, the vesicles carrying GLUT4 separate from the cell membrane within 3–5 min and return to the cell interior which will then be used again when needed (Guyton and Hall, 2006). The glycogen from skeletal muscle is formed at a rate which is determined by several factors such as the initial amount of muscle glycogen, carbohydrate availability, glucose transport into muscle and glycogen synthase activity (Waller and Lindinger, 2010). The enzyme is activated by insulin binding to its cell surface receptor and by muscle contraction (De Graaf-Roelfsema, 2014).

2.7. Relevance of GLUT4 in diabetes

GLUT4 is one of the glucose transporters that regulate the transport of glucose in the body. It is expressed solely in adipose and muscle tissues that has 12 membrane spanning regions with amino- and carboxyl- termini located intracellularly (Wood and Trayhurn, 2003). In the basal state, GLUT4 is enclosed in the vesicles of the cytoplasm of the cell (Rea and James, 1997; McCarthy and Elmendorf, 2007). The activation of insulin receptor triggers the increase in the rate of GLUT4 vesicle exocytosis and localization to the plasma membrane (Czech and Buxton, 1993; McCarthy and Elmendorf, 2007). This results in more than 10 fold increase in GLUT4 protein at the cell surface, for the overall insulin-dependent shift in the cellular dynamics (Malide *et al*., 2000).

Glucose transport activity in skeletal muscle is decreased with insulin resistance which is instrumental in the pathogenesis of type II DM (Garvey *et al*., 1998). Thus, insulin resistance occurs due to impaired translocation of intracellular GLUT4 in people with type II DM (Garvey *et al*., 1998). This will lead to accumulation of GLUT4 in a dense membrane compartment from which insulin is unable to recruit GLUT 4 to the cell surface (Olson and Pessin, 1996). The defect in translocation of GLUT4 could be due to either impaired insulin signal transduction or it could lie intrinsic to the glucose transporter system (Zhidan *et al*., 1998). Insulin stimulation triggers the release of GLUT4 from the intracellular vesicle to the plasma membrane, which then transports the glucose from the blood into the cells. When the level of insulin rises,

GLUT4 molecules are translocated to the cell membrane to increase the rate of glucose uptake by 15 fold or more. Nelson and Cox (2000) reported that phosphorylation of protein kinase B triggers the movement of GLUT4 from the vesicles to the plasma membrane, thereby stimulating glucose uptake from the blood. As such, glucose uptake mechanism in myocytes and adipocytes is regulated by the release of insulin.

2.8. Purpose of the study

2.8.1 Aim

The study was aimed at evaluating the *in vitro* effects of root and leaf extracts of *S. italica* on GLUT4 translocation in NIH-3T3-L1 preadipocytes and C2C12 muscle cells.

2.8.2 Objectives

The objectives of the study were to:

i. Extract the root and leaf material of *S. italica* using solvents of varying polarities
ii. Determine the fingerprint profile of the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane using TLC
iii. Determine the presence of secondary metabolites and total phenolic content in acetone root and leaf extracts using chemical and TLC analysis
iv. Determine presence of antioxidant constituents in the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane
v. Determine the cytotoxic effect of acetone root and leaf extracts on C2C12 muscle cells
vi. Evaluate the effects of acetone root and leaf extract on glucose uptake by non-differentiated and differentiated C2C12 muscle cells
vii. Evaluate the effects of acetone root and leaf extracts on translocation of GLUT4 receptor molecule from differentiated NIH-3T3-L1 preadipocytes and C2C12 muscle cells
viii. Determine the expression levels of GLUT4 protein using western blotting

CHAPTER 3

3. Materials and methods

3.1. Collection of plants

Roots and leaves of *S. italica* plant used in the study were collected from Zebediela sub-region in the Limpopo province, South Africa. The plant voucher specimen was deposited at the Larry Leach herbarium of the University of Limpopo. Following collection of the plant, the leaves were separated from tree branches. The roots and leaves of the plant were then dried at room temperature and ground to fine powder using ground miller then stored in bottles in a dark room until use.

3.1.1 Extraction of plant materials

The ground fine powders of the roots and leaves of *S. Italica* were weighed out (20 g) and extracted with 200 ml of the different solvents (acetone, methanol, ethyl acetate and *n*-hexane) (Sigma-Aldrich, S.A) in a ratio of 1:10 (w/v). The plant solvent mixture was tightly closed to avoid evaporation of solvents. The mixture was shaken overnight at room temperature in a shaker set at 200 rpm. The serial exhaustive extraction procedure was repeated three times to extract as much compounds as possible. The extracts was filtered with Whatman no.3 filter paper and poured into a pre-weighed erlenmeyer flask. The materials were dried under a stream of air. The dried materials were weighed to obtain mass of the extracts.

3.2. Thin layer chromatography

Finger print profile of the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane were analysed by thin layer chromatography (TLC) using silica-gel coated plates (Sigma-Aldrich, S.A) according to Kotze and Eloff (2002). The stock solutions of 10 mg/ml were prepared by dissolving 10 mg of the dried samples of the extracts in 1 ml of acetone, methanol, ethyl acetate and *n*-hexane then spotted on TLC plates. The TLC plates were eluted in four mobile phases namely BEA [benzene (18): ethanol (2): ammonium hydroxide (0.2)] which separates non-polar, CEF [chloroform (10): ethyl acetate (8): formic acid (2)] which separates intermediate

non-polar, EMW [ethyl acetate (8): methanol (2): water (10)] which has intermediate polarity and BAW [butanol (10): acetic acid (2): water (8)] which separates polar molecules. TLC plates were developed using the four mobile phases under saturated conditions. After eluting TLC plates, the solvent front was drawn and plates were allowed to dry in a fume hood before visualisation. The plates were visualized for fluorescing bands under UV light at wavelengths 254 nm and 366 nm followed by spraying of the plates with freshly prepared vanillin/H_2SO_4 (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagent, *p*-anisaldehyde and iodine crystals vapour (Sigma-Aldrich, S.A). The plates were then heated in an oven at 110°C for maximum colour development. The chromatograms were scanned.

3.3. Tests for secondary metabolites

3.3.1 Tannins

Briefly, 0.5 g of the root and leaf powders were dissolved in 5 ml of distilled water then boiled gently and cooled. A volume of 1 ml of this solution was aliquoted into a test tube and 3 drops of 1% ferric chloride solution was added. The greenish-black colour of the sample was observed to draw an inference, indicating the presence of tannins (Borokini and Omotayo, 2012).

3.3.2 Terpenes/ terpenoids

The Salkowski's test was used. Five ml of the root and leaf powders were mixed in 2 ml of chloroform and 3 ml concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. A reddish brown colour was observed to draw an inference, indicating the presence of terpenes (Borokini and Omotayo, 2012).

3.3.3 Steroids

About 2 ml of acetic anhydride was added to 0.5 g of each root and leaf, followed by addition of 2 ml of H_2SO_4. Blue colour was observed to draw an inference, indicating the presence of steroids (Borokini and Omotayo, 2012).

3.3.4 Cardiac glycosides

The Keller-Killani's test was used. About 5 ml of the root and leaf powders of the plant parts studied were treated with 2 ml of glacial acetic acid, containing one drop of ferric chloride solution. This was followed by addition of 1 ml concentrated H_2SO_4. The colour changes (brown interface, violet ring below and greenish ring at the lowest) were observed to draw inference, indicating the presence of cardiac glycosides (Borokini and Omotayo, 2012).

3.3.5 Test for reducing sugars (Fehling's test)

The root and leaf samples were prepared by dissolving 0.5 g of each plant powdered material into 5 ml of water. The solutions were added to a boiling 5 ml Fehling's solution (Fehling A was prepared by dissolving 7 g of hydrated copper (II) sulphate (Sigma-Aldrich, S.A) into 100 ml of distilled water). Fehling B was made by dissolving 35 g of potassium sodium tartrate and 10 g of sodium hydroxide (Sigma-Aldrich, S.A) in 100 ml of distilled water. Equal volumes of Fehling A and Fehling B were mixed to form a blue solution known as Fehling's solution in a test tube. The solution was observed for a blue-black colour reaction (Ayoola *et al*., 2008) to draw an inference, indicating the presence of reducing sugars.

3.3.6 Flavonoids

About 5 ml of diluted ammonia solution was added to a portion of the filtrate of each plant extract, followed by the addition of concentrated H_2SO_4. Yellow colour change was observed to draw an inference, indicating the presence of flavonoids (Borokini and Omotayo, 2012).

3.3.7 Saponins

The persistent frothing test for saponins was used. To 1 g of the powdered root and leaf samples, 30 ml of tap water was added. The mixture was vigorously shaken and heated. The sample was observed for the formation of froth to draw an inference, indicating the presence of saponins (Borokini and Omotayo, 2012).

3.3.8 Alkaloids

Drangendoff's reagent was used and the method of Harborne (1973). Briefly, 0.5 g powdered root and leaf samples were dissolved in 5 ml of distilled water, then boiled gently and cooled. The volume of 1 ml of this solution was aliquoted into a test tube and few drops of Drangendoff's reagent (Sigma-Aldrich, S.A) were added. The orange colour of the sample was observed to draw an inference, indicating the presence of alkaloids.

3.3.9 Tests for secondary metabolites using TLC

Other tests for secondary metabolites of the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane (Sigma-Aldrich, S.A) were analysed by thin layer chromatography (TLC) using silica-gel coated plates (Sigma-Aldrich, S.A). Stock solutions of 10 mg/ml were prepared by dissolving 10 mg of the dried samples of the extracts in acetone (1 ml) (Sigma-Aldrich, S.A). The TLC plates (10x10 cm) (Sigma-Aldrich, S.A) were used as the stationary phase and eluted in seven mobile phases namely EPWM [Ethyl acetate (8): Pyridine (3): Water (5): Methanol (1)] for Glycoflavones better, C [Chloroform (10)] for Petasines in Petasitidis species, CEM [Chloroform (8): Ethyl acetate (5): Methanol (9)] and AE [Acetone (5): Ethyl acetate (5)] for alkaloids, HE [Hexane (5): Ethyl acetate (2)] for phytosterols, HDE [Hexane (8): Dichloromethane (1): Ethyl acetate (6)] for coumarins and CE [Chloroform (5): Ethyl acetate (7)] for flavonoid aglycones. After developing the chromatograms, the solvent front was drawn and plates were allowed to dry in a fume hood before visualization with UV light and vanillin-H_2SO_4 reagent (Chakraborty *et al*., 2010). The R_f values were calculated from the plates.

3.4. Total phenolic content

The total phenolic content of the acetone root and leaf extracts was determined by Folin-Ciocalteau assay. Aliquots of extracts (0.1 mg/ml) or a standard solution of tannic acid (0.02, 0.04, 0.06, 0.08 and 0.10 mg/ml) of about 1 ml was added to a 25 ml volumetric flask, containing 9 ml of distilled water. Folin-Ciocalteau reagent (1 ml) was added to the mixture and shaken, then incubated for 5 min. Seven percent of

sodium carbonate solution (10 ml) was added to the mixture and diluted with distilled water to 25 ml followed by mixing. Reagent blank containing everything except extracts was also prepared. After 90 min incubation at room temperature, the absorbance of the standards and samples against the prepared reagent blank was determined at 750 nm using a UV-VIS Spectrophotometer DU 730 model (Coulter-Beckman, U.S.A). The results were expressed as milligrams of tannic acid equivalents per milligrams (TAE/mg). All samples were analysed in duplicates (Marinova *et al*., 2005).

3.5. Qualitative antioxidant activity assay

The TLC plates of the root and leaf extracts of acetone, methanol, ethyl acetate and n-hexane were prepared as described in section 3.2 above. To detect antioxidant compounds, the chromatograms were sprayed with 0.2% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, S.A) in methanol. The presence of yellow spots against a purple background was indicative of the presence of antioxidant constituents (Deby and Margotteaux, 1970).

3.5.1. Quantitative antioxidant activity assay

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane extract were determined by the method described by Katsube *et al*. (2004). The stock samples (10 mg/ml) was prepared and 50 µl of the extracts was pipetted into the first wells of 96-well plate. Two fold serial dilutions were performed to obtain concentrations ranging from 0.04–5 mg/ml. An equivalent volume of ascorbic acid (vit C) at a concentration of 2 mg/ml was used as a positive control. Fifty µl of 0.2% DPPH was added to each well and the plate gently shaken for 20 min at room temperature. The plates were read at wavelength of 550 nm by using a Glomax microtitre plate reader (Promega, U.S.A). The decrease in absorbance indicating an increase in radical scavenging activity was recorded. The extract that contained more antioxidant constituents was then used for further assays.

3.6. Cytotoxicity assay

The effect of acetone root and leaf extracts on viability of C2C12 muscle cells was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mossman, 1983). The cells were plated at a density of 5 x 10^4 cells/well in Dulbecco's minimum-eagles medium (DMEM) (Thermo-fisher Scientific, U.S.A) supplemented with 10% FBS. The plated cells were treated with various concentrations (0.01–1000 µg/ml) of both acetone root and leaf extracts in 96-well microtitre plates (Whitehead Scientific, S.A). Curcumin was used as a positive control at concentration of 200 mM. The cells without extracts were used as a negative control. The cells were incubated at 37°C in a humidified incubator (5% CO_2) at 24 hrs and 48 hrs. The treatment media was removed. Then 80 µl of fresh media plus 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4 hrs. The media with MTT was removed from the plate and 100 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, S.A) was added to each well and shaken well to dissolve the formazan product. The absorbance was recorded using a Glomax microtitre plate reader (Promega, U.S.A) at 560 nm. The concentration that reduced the cell viability by 50% (CC_{50} µg/ml) when compared to untreated controls was determined using the linear regression plot.

3.7. Glucose uptake assay

Glucose uptake was measured using the method of Foley *et al*. (1983). Briefly the Roswell Park Memorial Institute medium (RPMI-1640) (Thermo-fisher Scientific, U.S.A), without phenol red, supplemented with 4.026 g/l sodium pyruvate, 2.05 mM glutamate and 11 mM glucose was used. About 100 µl of C2C12 muscle cells, at a density of 2.7 x10^4 cells/well were seeded in a 96 well plate (Whitehead Scientific, S.A) with media supplemented with 10% foetal bovine serum (FBS) and 1x penicillin, streptomycin, neomycin (PSN) (Thermo-fisher Scientific, U.S.A) for 48 hrs. The cells were serum starved for differentiation with 2% FBS for 96 hrs with the media changed every 48 hrs. The cells were exposed to acetone root and leaf extracts on day 7 at concentrations ranging from 2.5 µg/ml and 25 µg/ml for 1, 3 and 24 hrs respectively with insulin (1000 nM) (Roche, S.A) as a positive control. The medium without cells was used as a negative control. The glucose oxidase (Trinder-

GOD/PAP) kit (Kat Medical, S.A) which consists of glucose standard (5.56 mM) and glucose reagent was thereafter added and incubated for 5 min at 37°C in 5% CO_2. The absorbance was recorded using Glomax microtitre plate reader (Promega, U.S.A) at 560 nm including the blank (glucose reagent only). The standard curve was then plotted and glucose concentration utilized was extrapolated.

3.8. Cell differentiation and immunofluorescence of NIH-3T3-L1 preadipocytes cells and C2C12 muscle cells

3.8.1 Differentiation of NIH-3T3-L1 preadipocytes cells and C2C12 muscle cells

NIH-3T3-L1 preadipocytes and C2C12 muscle cells were seeded on coverslips to grow. The cells were maintained in DMEM (Thermo-fisher Scientific, U.S.A) supplemented with 10% FBS and 1x PSN (Thermo-fisher Scientific, U.S.A) at 37°C, 5% CO_2 incubator. The adipocyte conversion was induced by treating 48 hrs post-confluent cultures with DMEM containing 1.0 µM dexamethasone (DEX), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, S.A) and 0.5 mg/ml insulin (Roche, S.A) for 48 h. This was followed by treatment with 2.5 µg/ml and 25 µg/ml of the acetone root and leaf extracts for 24 hrs. Thereafter DMEM containing 0.5 mg/ml insulin and 1x PSN (post-differentiation media) was added for a further 48 hrs (Hou *et al*., 2013). The cells were stained with Oil-red O (Sigma-Aldrich, S.A) and viewed under fluorescence microscope for presence of fat droplets. The cells treated with only insulin (Roche, S.A) were used as the positive control and DMSO (0,2%) as the negative control. The C2C12 muscle cells were cultured in DMEM (Thermo-fisher Scientific, U.S.A) containing 2% FBS (Thermo-fisher Scientific, U.S.A) which was changed after every 2, 4 and 6 days of culturing. The experiment was then initiated on day 7 when myotubule differentiation was complete.

3.8.2 Qualitative and Quantitative Immunofluorescence assay of NIH-3T3-L1 preadipocytes and C2C12 muscle cells

The differentiated cells were treated with acetone root and leaf extracts as in section 3.8.1 above. For cell immunofluorescence, the differentiated cells were washed once with 1x phosphate buffered saline (PBS) (Thermo-fisher Scientific, U.S.A), followed

by fixation with 80% methanol (Sigma-Aldrich, S.A) at room temperature for 15 min. The cells were washed 3 times with 1x PBS and blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, S.A) for 10 min. The cells were washed 3 times with 1x PBS and then incubated with primary goat polyclonal antibody (anti-GLUT4) (Whitehead Scientific, S.A) for 1 hr. The primary antibodies were detected with FITC-conjugated rabbit anti-goat antibody (Whitehead Scientific, S.A) for 1 hr in the dark room. The washing steps were repeated and both cells were viewed under fluorescence microscope (using 20 x lenses) for qualitative immunofluorescence (Bruker, U.S.A) (Shigematsu *et al*., 2001). For quantitative immunofluorescence, the Glomax microtitre plate reader (Promega, U.S.A) was used to quantify the fluorescence of only C2C12 muscle cells using the blue filter (Excitation at 490 nm, Emission at 510–570 nm). The same controls as in section 3.8.1 above were used.

3.9. Western blot analysis

The cells were cultured at a density of 6 x 10^5 cells /ml overnight and then treated as in section 3.8.1 above. The Differentiated cells were washed once with 1x PBS and then suspended in a lysis buffer containing 50 mM Tris-triton (pH 8.0), 150 mM NaCl, 0.5% nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT and 5 mM EDTA (Sigma-Aldrich, S.A) for 30 min at 4°C. After centrifugation at 12 000 rpm for 20 min, the concentration of proteins in supernatant was measured using the Pierce bicinchoninic acid protein assay (BCA) (Thermo scientific, U.S.A). This was followed by separating 60 µg of the total protein from the supernatant by 12% SDS-PAGE before being transferred to a nitrocellulose membrane (Bio-Rad, S.A) using a semi-dry system (Bio-Rad, S.A). The GAPDH (Whitehead Scientific, S.A) was used as an internal positive control. The membranes were incubated with Tris-buffered saline Tween-20 (TBST) [150 mM/l sodium chloride, 50 mM/l Tris, pH 7.4] (Sigma-Aldrich, S.A) containing 1% BSA (Sigma-Aldrich, S.A) for 1 hr to block the non-specific binding sites. The membranes were incubated with 1:1000 dilutions of primary goat polyclonal antibody (anti-GLUT4) (Whitehead Scientific, S.A) at 4°C overnight. After incubation, the membranes were washed with TBST. The proteins were then detected by incubating with the corresponding HRP-conjugated rabbit anti-goat secondary antibodies (Whitehead Scientific, S.A) at 1:10 000 dilutions for 1h at room temperature. The membranes were washed using TBST and the transferred proteins

were detected using the Super Signal West Dura chemiluminescent substrate (Thermo Scientific, S.A). The antigen-antibody complex was then visualised by photodetection using the Syne-Gene Image analyser (Bio-Rad, S.A) (Saiki *et al.,* 2008).

CHAPTER 4

4. Screening for secondary metabolites, antioxidant activity and total phenolic content of *S. italica* plant

4.1. Introduction

Researchers have made considerable effort to find efficient extraction methods in order to get high efficiency (yield) and efficacy (magnitude of bioactivity). However, one has to optimize the methods for better efficiency of extraction of herbs. The extraction of plant materials is one of the most sustainable approaches for isolating biological components (Jadhav *et al*., 2009). Sampling, sample preparation, quantification and statistical evaluations are the most critical steps in analytical procedures (Gupta *et al*., 2012). The need for selection of most appropriate extraction methodology is evident from the fact that when different methods are applied on the same plant material using the same solvent or various solvents, extraction efficiency can vary significantly. Moreover, the choice of an appropriate solvent is of essential importance along with application of a compatible extraction method (Gupta *et al*., 2012).

The plant-derived products contain a diversity of phytochemicals such as phenolic acids, flavonoids, tannins, lignin and other small compounds (Cowan, 1999; Wendakoon *et al*., 2012). These compounds possess numerous health-related benefits such as anti-bacterial, anti-mutagenic, anti-carcinogenic activities. (Wendakoon *et al*., 2012). The type of solvent employed in the extraction procedure determines the biological active compounds present within the plant material. Organic solvents such as ethanol, acetone and methanol are often used to extract bioactive compounds (Eloff, 1998b; Wendakoon *et al*., 2012). Ethanol, however, is the most commonly used organic solvent by herbal medicine manufacturers because the finished products can be safely used internally by consumers of herbal extracts (Low Dog, 2009).

Secondary metabolites are phytochemicals produced by a plant which are used to defend the plant against herbivores and insects. As such, plants are a rich source of secondary metabolites with interesting biological activities. These secondary

metabolites are an important source of biological compounds with a variety of structural arrangements and properties (De-Fatima *et al*., 2006). Recently, plant-derived substances have become of great interest owing to their versatile applications. Hence, medicinal plants are considered the richest bio-resource of drugs in traditional systems of medicine in general. This may include modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al*., 2008). Their medicinal activity arises from bioactive compounds known as phytochemicals. However, bioactive phytochemicals may differ in various plant parts. In addition, they may also differ in composition and concentration depending on the ecological changes (Moure *et al*., 2001).

4.2. Objectives

i. To extract root and leaf materials from *S. italica* using four different solvents
ii. Determine the finger-print profile of the root and leaf extracts of the acetone, methanol, ethyl acetate and *n*-hexane using TLC
iii. Determine the presence of secondary metabolites and total phenolic content in the acetone root and leaf extracts
iv. Determine the antioxidant activity of the root and leaf extracts of the acetone, methanol, ethyl acetate and *n*-hexane

4.3. Materials and methods

4.3.1 Extraction of plant materials

The root and leaf of *S. italica* were extracted using four different solvents of varying polarities namely: acetone, methanol, ethyl acetate and *n*-hexane described in section 3.1.1.

4.3.2 TLC fingerprint profile

The phytochemical constituents present in the root and leaf extracts of *S. italica* were analysed on TLC plates according to the method of Kotze and Eloff (2002) described in section 3.2.

4.3.3 Tests for secondary metabolites

The root and leaf extracts of *S. italica* were qualitatively tested for presence of secondary metabolites using the method of Borokini and Omotayo (2012) described in section 3.3, as well as the method of Chakraborty *et al.* (2010) described in section 3.3.9.

4.3.4 Total phenolic content

The total phenolic content of the root and leaf extracts of *S. italica* was determined using the method of Marinova *et al*. (2005) described in section 3.4.

4.3.5 Qualitative and quantitative antioxidants activity

The qualitative and quantitative antioxidants activity was analysed using the method of Deby and Margotteaux (1970) and Katsube *et al*. (2004) described in section 3.5. and 3.5.1, respectively.

4.4. Results

4.4.1 Extraction

The amount of root and leaf extracts obtained after extracting all the plant materials exhaustively with acetone, methanol, ethyl acetate and *n*-hexane is represented in figure 2. The plant material (20 g) from the root and leaf of *S. italica* was exhaustively extracted with 200 ml of each solvent. The experiment was repeated three times. Methanol extracted the highest amount of extracts in both the root and leaf material (2.15 g and 4.81 g, SIRM; SILM) followed by acetone (0.61 g and 2.34 g, SIRA; SILA) respectively. The least amount of material extracted was observed with *n*-hexane in both the root and leaf extracts (0.4 g and 1.6 g, SIRH; SILH) respectively. More material was extracted from the leaf extract with all the solvents used.

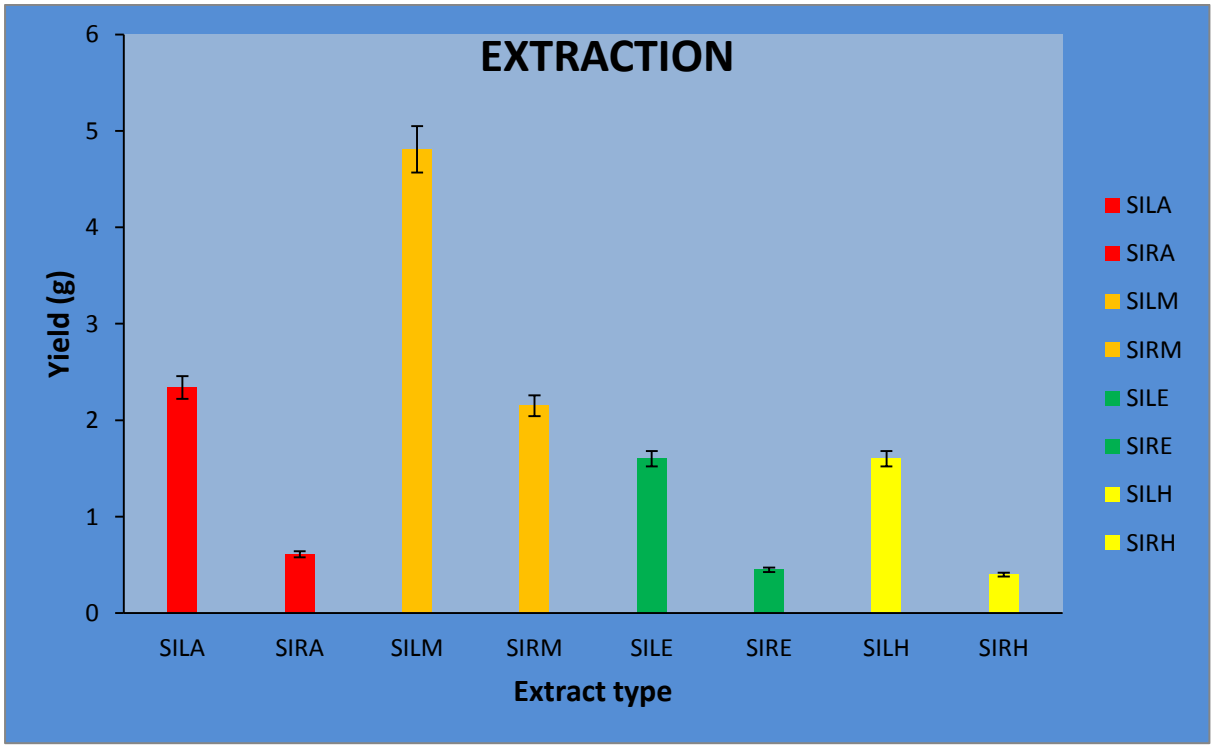


Figure 2: Yield obtained from the root (R) and leaf (L) extracts of *S. italica* (SI) using acetone, methanol, ethyl acetate and *n*-hexane as extractants. Results are expressed as the mean of three independent experiments relative to the control, which was set at 100% ± SD ($p < 0.001$).

Key: (SIRA, SILA= acetone extracts), (SIRM, SILM= methanol extracts), (SIRE, SILE= ethyl acetate extracts) and (SIRH, SILH= *n*-hexane extracts)

4.4.2 TLC profiles

The finger print profiles of the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane were performed using TLC plates developed in BEA, CEF, EMW and BAW mobile phases (figure 3–5). Good separation with most compounds was achieved with the methanol and acetone extracts in CEF mobile phase, while ethyl acetate extracts separated better in CEF and EMW. On the other hand, BEA mobile phase separated compounds better with *n*-hexane extracts although few were able to migrate to the solvent front. Most compounds present in *S. italica* extracts were UV active. The compounds that are marked by pencil represent those compounds that fluoresce under UV light at wavelengths 366 nm and 254 nm. The chromatograms were visualized using vanillin/H_2SO_4 reagent (figure 3), *p*-anisaldehyde (figure 4) and heated in an oven at 110°C for the development of colour. Most compounds were not reactive with vanillin/H_2SO_4 reagent. However, reactivity was enhanced on chromatograms that were sprayed with *p*-anisaldehyde reagent. Other compounds were also visualized following immersion in iodine vapors (figure 5) to show the presence of sugars.

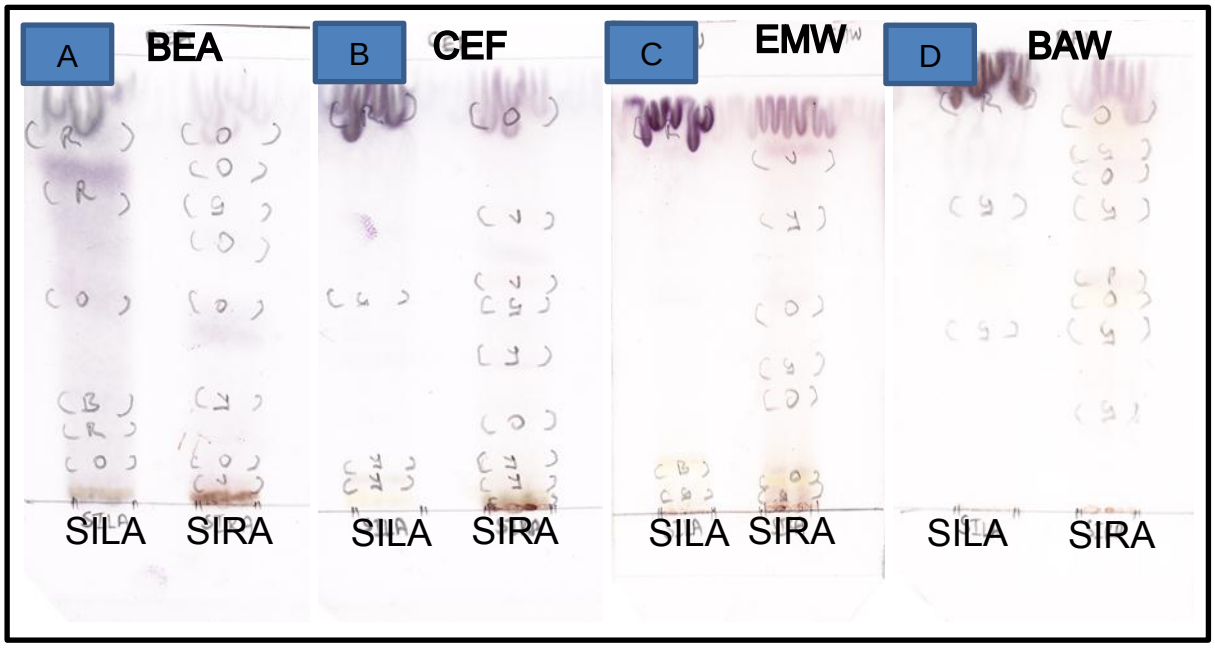


Figure 3: Thin layer chromatograms of acetone (A) root (R) and leaf (L) extracts obtained from *S. italica* (SI) developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with vanillin/H_2SO_4 and heated in an oven at 110°C for colour development.

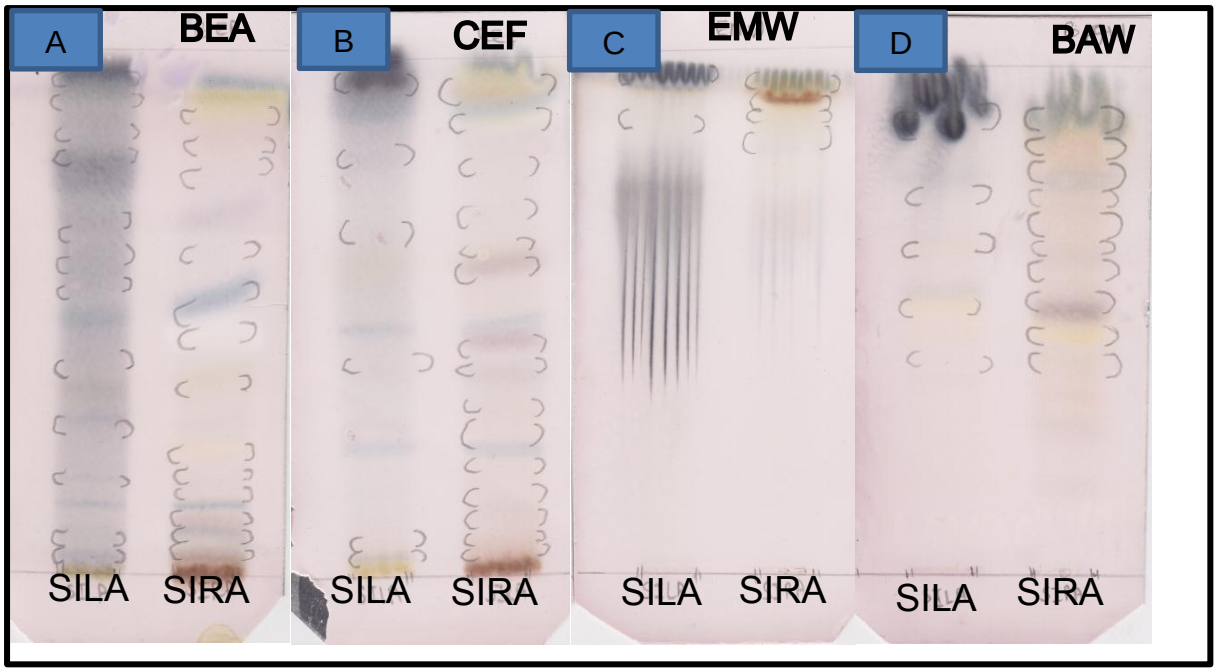


Figure 4: Thin layer chromatograms of acetone (A) root (R) and leaf (L) extracts obtained from *S. italica* (SI) developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with *p*-anisaldehyde and heated in an oven at 110°C for colour development.

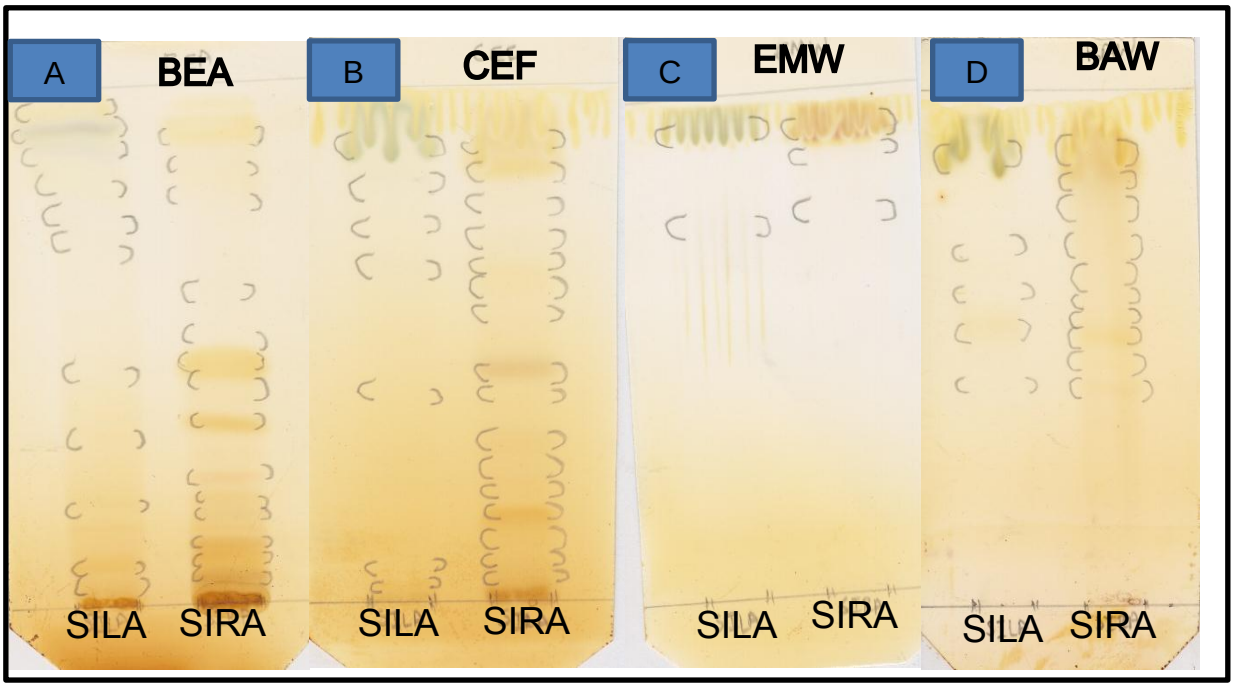


Figure 5: Thin layer chromatograms of acetone (A) root (R) and leaf (L) extracts obtained from *S. italica* (SI) developed in BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were immersed in vapours iodine crystals for colour development.

4.4.3 Tests for secondary metabolites

The test for secondary metabolites was conducted only on the acetone root and leaf extracts using the chemical methods, followed by the TLC method. The results are presented in table 1 and 2. The acetone root and leaf extracts showed the presence of flavonoids, terpenes, tannins, steroids, reducing sugars and alkaloids (table 1). However, saponins were not present in both the acetone root and leaf extracts. In addition, glycosides were present only in the acetone leaf extract. The presence of these metabolites was observed by the formation of various colours as a positive indicator. Flavonoids were observed by yellow precipitates, terpenes by reddish brown colour, tannins by greenish-black colour, steroids by blue colour, reducing sugars by blue-black colour and alkaloids by orange precipitates. However, saponins were absent in the root and leaf extracts, the presence of which, is indicated by the formation of froth. The obtained chromatograms were developed using seven different mobile phases (table 2). Alkaloids, phytosterols and flavonoid aglycones were present in both acetone root and leaf extracts. These results are consistent with those in table 1. However, glycoflavones were observed only in the acetone root extract. Other compounds such as coumarins and Petasines in *Petasitidis* species were not observed in either of the extracts.

Table 1: Detection of secondary metabolites in the acetone root and leaf extracts of *S. italica* using the chemical methods.

Type of secondary metabolites	Type of extract	
	SILA	SIRA
Flavonoids	+	+
Terpenes	+	+
Tannins	+	+
Saponins	-	-
Steroids	+	+
Glycosides	+	-
Reducing sugars	+	+
Alkaloids	+	+

Keynote: (+) presence; (-) absence; *S. italica* (SI) root (R) acetone (A) extract, *S. italica* (SI) leaf (L) acetone (A) extract.

Table 2: R_f values of secondary metabolites detected in the acetone root and leaf extracts of *S. italica* using TLC.

Type of extract	Mobile phases and ratio Used	Compounds detected	Rf values detected	UV Active (+/-)	Presence of active compounds (+/-)
SILA	Ethyl acetate: Pyridine: Water: Methanol (EPWM)(8:3:5:1)	**Glycoflavones**	**0.58**	**-**	**-**
SIRA			**0.54**	**+**	**+**
SILA	Chloroform (C)(10)	**Petasines in Petasitidis Species**	**0.88**	**-**	**-**
SIRA			**0.89**	**-**	**-**
SILA	Chloroform: Ethyl acetate: Methanol (CEM)(8:5:9)	**Alkaloids**	**No separation of compounds**		
SIRA			**0.80**	**-**	**+**
SILA	Acetone: Ethyl acetate (AE)(5:5)	**Alkaloids**	**0.70**	**+**	**+**
SIRA			**0.62**	**-**	**-**
SILA	Hexane: Ethyl acetate (HE)(5:2)	**Phytosterols**	**0.86**	**+**	**+**
SIRA			**0.86**	**+**	**+**
SILA	Hexane: Dichloromethane: Ethyl acetate (HDE)(8:1:6)	**Coumarins**	**0.75**	**-**	**-**
SIRA			**0.78**	**-**	**-**
SILA	Chloroform: Ethyl acetate (CE)(5:7)	**Flavonoid aglycones**	**0.81**	**-**	**+**
SIRA			**0.84**	**+**	**+**

Keynote: (+) presence, (-) absence, *S. italica* (SI) root (R) acetone (A) extract, *S. italica* (SI) leaf (L) acetone (A) extract.

4.4.4 Total phenolic content

The total phenolics of the acetone root and leaf extracts were determined. The values extrapolated from the tannic acid standard curve (figure 6) were represented as TAE/mg (table 3). Tannic acid was used as positive control at a concentration ranging from 0–0.1 mg/ml. The concentration of 1 mg/ml of the acetone root and leaf extracts was used. The acetone leaf extract had a high total phenol content of 0.052 TAE/mg as compared to the root extract which had the least (0.018 TAE/mg) (table 3).

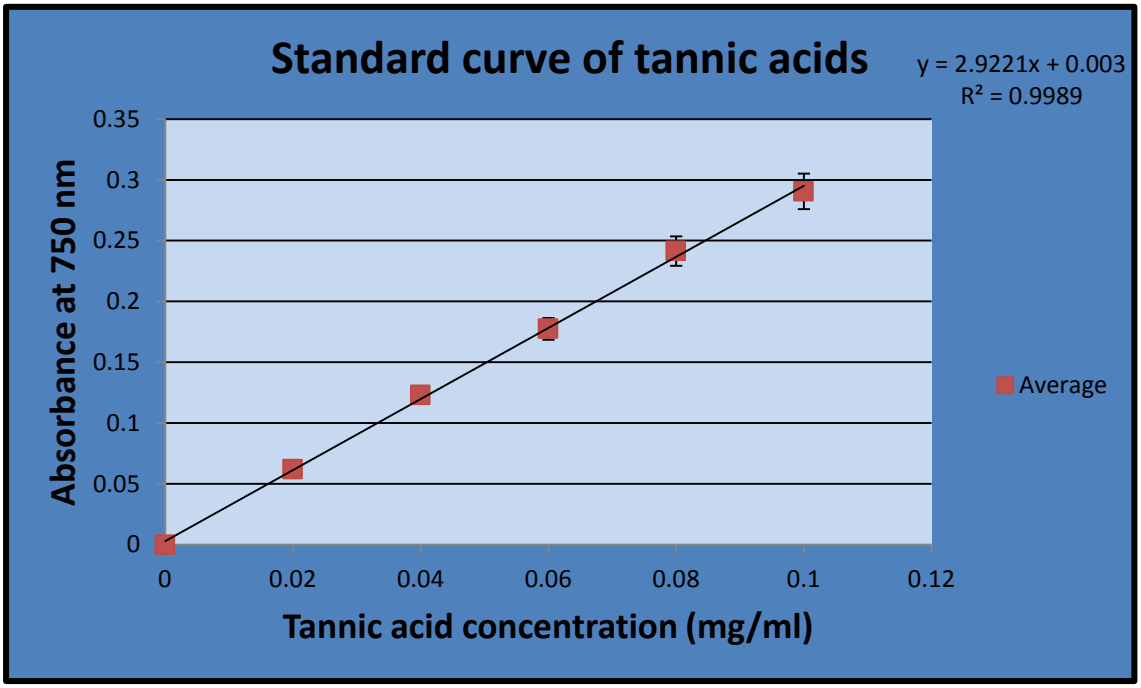


Figure 6: The standard curve of tannic acids used as positive control expressed as TA equivalents at a concentration ranging from 0–0.1 mg/ml.

Table 3: Total phenols detected from acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI).

Total phenols in extracts (TAE/mg)	
SILA concentration	**SIRA concentration**
0.052	**0.018**

4.4.5 Qualitative and Quantitative Antioxidant activity determination

The antioxidant activity of the root and leaf extracts of acetone, methanol, ethyl acetate and *n*-hexane was evaluated using qualitative and quantitative determination methods. The qualitative method determines the antioxidant compounds separated on TLC plates and a representative chromatogram of the acetone extracts eluted in the different solvent system is presented (figure 7). The yellow spots against the purple background represent compounds with antioxidant activity. The BEA mobile phase had antioxidant compounds of both the root extract of R_f 0.42, 0.87 and leaf extract of R_f 0.06. The antioxidant compound of the root extract which was not able to move in BEA and CEF was able to move in more polar mobile phases (EMW and BAW). The compound is not completely soluble in non-polar mobile systems. As the polarity of the mobile systems increases, compounds were able to move or separate. Other mobile phases contained antioxidant compounds of only the root extract (CEF, EMW and BAW). The CEF had R_f 0.54, EMW R_f 0.11; 0.91 and BAW with R_f 0.49; 0.84. However, *n*-hexane extracts did not show any activity.

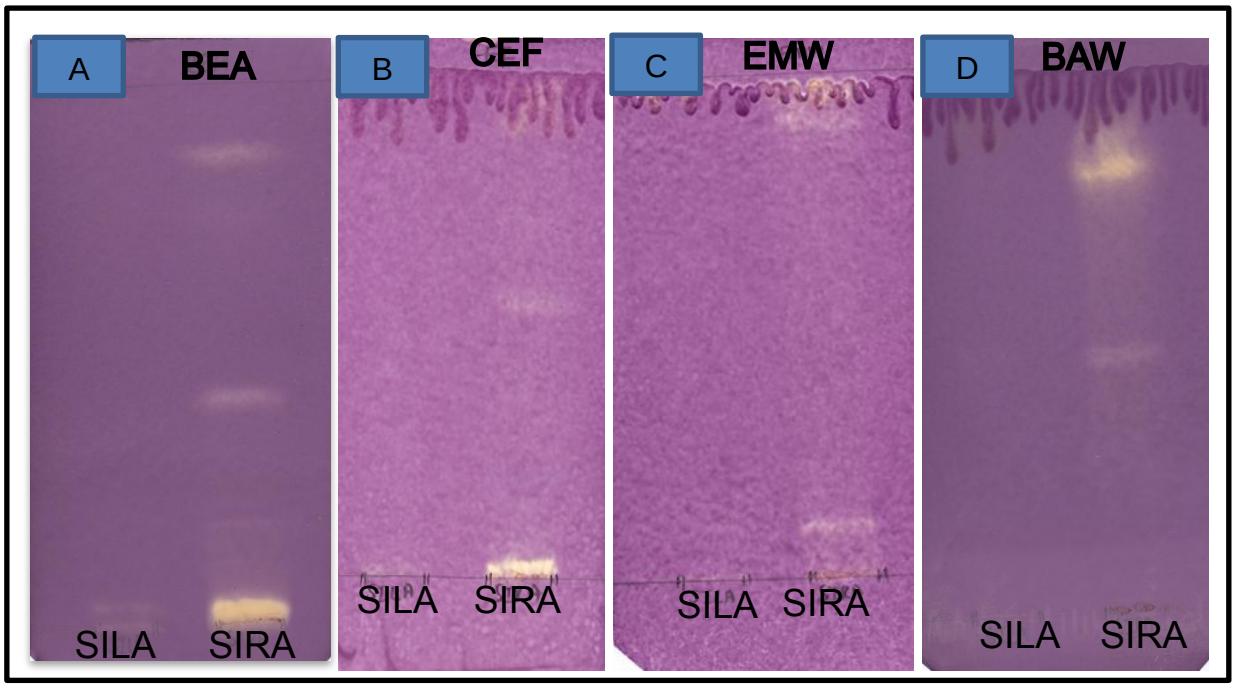


Figure 7: Thin layer chromatograms of acetone (A) root (R) and leaf (L) extracts obtained from *S. italica* (SI) developed with BEA (A), CEF (B), EMW (C) and BAW (D) as mobile phases. The chromatograms were sprayed with 0.2% DPPH.

The quantitative method determines the scavenging activity of the whole extract. The degree of discolouration indicates the scavenging activity of the sample. The vitamin C was used as a positive control and DMSO (0.2%) as a negative control in the quantitative assay. The acetone and methanol extracts showed more scavenging activity which was comparable in both the root and leaf extracts as compared to other extracts (figure 8). The ethyl acetate showed the least amount of scavenging activity in both the root and leaf extracts. The IC_{50} of the plant extracts is 3.5 mg/ml. Thus, as the concentration of the extracts increases the scavenging activity increases as well.

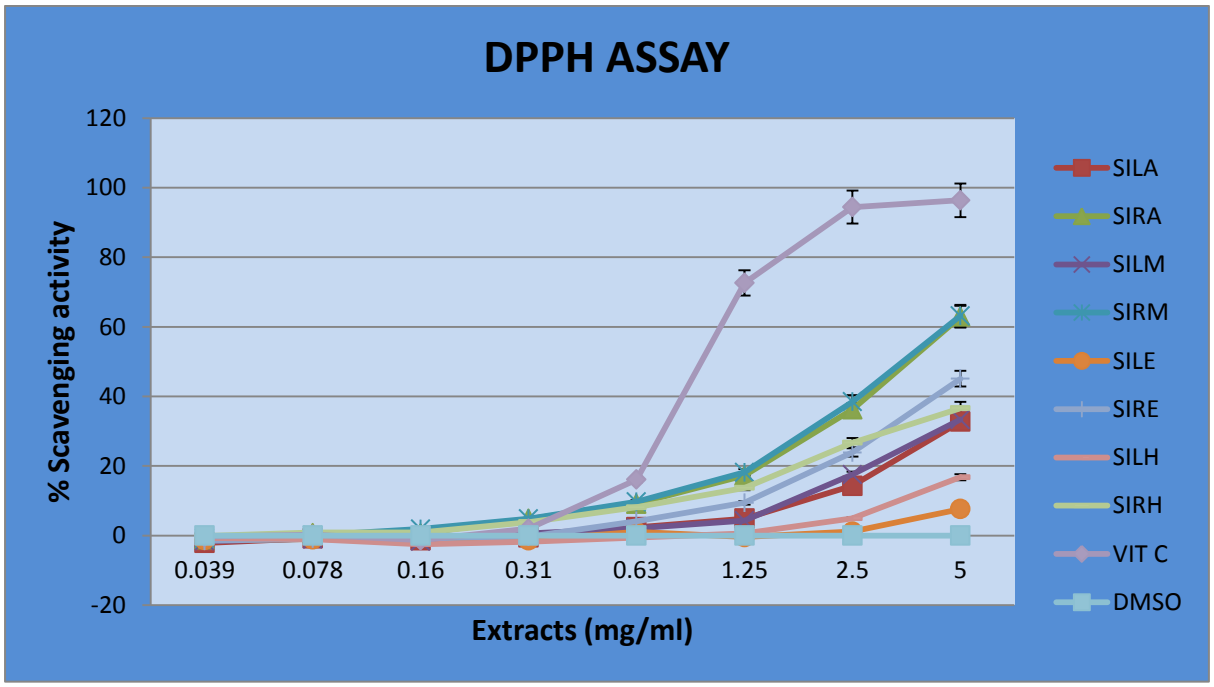


Figure 8: DPPH assay showing quantitative antioxidant activity of the root (R) and leaf (L) extracts of acetone (A), methanol (M), ethyl acetate (E) and *n*-hexane (H) at a concentration ranging from 0.04–5 mg/ml obtained from *S. italica* (SI). Vitamin C was used as a positive control at 2 mg/ml and DMSO (0.2%) as a negative control. Results are expressed as the mean of three independent experiments relative to the control, which was set at 100% ± SD ($p < 0.001$).

4.5. DISCUSSION

The choice of solvent used for extraction is very important for biological activity screening together with the ease of working with the particular solvent type. Boiled water is mainly used by traditional healers to extract bioactive compounds due to its nontoxic nature (Eloff, 1998b). However, most compounds volatilise when using boiled water as an extractant. In the present study, acetone, methanol, ethyl acetate and *n*-hexane were used to extract the plant materials. These solvents were used for extraction because of the wide range of compounds that can be extracted by them. The need for selection of an appropriate extraction method is evident from the fact that when different methods are applied on the same plant material with the same or different solvents, extraction efficiency can vary significantly. In addition, the method selected as the most appropriate one also needs to be standardized so as to achieve acceptable degree of reproducibility (Gupta *et al*., 2012).

Thus, the solvents employed are commonly used to extract various biological active compounds from plants. The yield of the extracts obtained from the root and leaf of *S. italica* was serial exhaustively extracted using acetone, methanol, ethyl acetate and *n*-hexane. Methanol extracted more compounds followed by acetone as compared to other solvents used in this study. Recent report (Mokgotho *et al*., 2013) from the same plant is consistent with results obtained in this study. Methanol extracts high amount of sugars, amino acids and glycosides while acetone extracts alkaloids, aglycones and glycosides. Hydro-alcoholic solvents extract a variety of compounds that are mostly polar. However, ethyl acetate and *n*-hexane extracted the least. The *n*-hexane in general extracts mostly waxes, fats and fixed oils.

The leaf extract gave more yields with all the solvents employed as compared to the root extract. Other authors in an attempt to evaluate the effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts demonstrated a high yield with *Moringa oleifera* leaf as compared to the root using ethanol and methanol extracts (Sultana *et al.,* 2009). The differences in the extract yields from the different plant part might be ascribed to the difference in the availability of extractable components present in the leaf and root material or

possibly due to the presence of high concentration of compounds present in the leaf, since most nutrients are synthesized in the leaf.

The finger print profile of constituents in *S. italica* extracts (acetone, methanol, ethyl acetate and *n*-hexane) were analysed by TLC using different mobile phases (BEA, CEF, EMW and BAW). Good separation was achieved with the methanol and acetone extracts in CEF mobile phase, ethyl acetate extracts in CEF and EMW and *n*-hexane extracts in BEA respectively, especially with the leaf extract. Most compounds present in *S. italica* extracts were UV active, possibly due to the presence of many fluorescent-quenching compounds which are usually contained within herbal extracts. The UV light identifies fluorescing compounds that mainly consists of aromatic rings which absorb at 254 nm thereby quenching the fluorescence of the pigment present in the silica gel (Masoko, 2006). More fluorescing bands were contained in the methanol, acetone and ethyl acetate extracts of the root extracts and *n*-hexane with the leaf extracts. However spraying with vanillin/H_2SO_4 reagent most compounds were shown to be non-reactive with the reagent. Hence *p*-anisaldehyde and iodine crystals vapor were used to visualise other compounds that may have not been detected by vanillin spray reagent.

Most compounds were reactive with *p*-anisaldehyde spray reagent, suggestive of the presence of terpenes. Reactive compounds with this reagent were more visible in acetone and ethyl acetate extracts of plates eluted in BEA and CEF mobile phases while those in methanol extract were more visible in CEF and BAW mobile phases. On the other hand compounds in *n*-hexane extract were more visible in BEA mobile phase only. The presence of sugars or organic compounds present in the extracts were detected by emersion in iodine vapor, with the appearance of brown bands on the developed chromatograms as a positive indicator, suggestive that some compounds contained in *S. italica* are organic in nature. Dave and Ledwani (2012) have reported the presence of organic compounds such as anthraquinones (aglycones and glycosides) in a related species (*Cassia alata*).

Various secondary metabolites were shown to be present in the acetone root and leaf extracts. Available report (Franz, 1993) indicates the presence of alkaloids, quinones and anthraquinones in *Senna* species. The acetone root and leaf extracts

revealed the presence of flavonoids, terpenes, tannins, steroids, reducing sugars and alkaloids while glycosides were detected only in the leaf extract. However, the presence of quinones was not evaluated for, in this study. In addition, Malviya *et al*. (2010) showed that most plants with antidiabetic activity contain secondary metabolites such as glycosides, alkaloids, terpenoids, flavonoids, cartenoids, *etc.* which is consistent with the present findings. Contrary to other findings, Dabai *et al*. (2012) after conducting phytochemical screening of *S. italica* root and leaf extracts, revealed the presence of secondary metabolites tested in this study, except glycosides in the leaf and tannins in both the root and leaf extract. The observed discrepancy may be related to the geographical location of the plant which is reported to influence its chemical constituents due to factors such as climate, soil, propagation method, *etc.* (Adoum *et al*., 1997). Odugbemi (2008) have also reported the influence of time of collection of plant and seasonal variations which are considered to affect the chemical composition of plants on its biological activity. Another reason could be the type of solvents used in the extraction process which can influence the type of phytochemicals extracted (Kawo, 2007).

The presence of other secondary metabolites were also analysed by TLC and developed in various mobile phases for each specific metabolite. The calculated R_f values suggest the presence of secondary metabolites that are contained within the acetone root and leaf extracts. Results obtained using TLC was consistent with the results obtained in the chemical analysis. Some of the compounds were shown to be UV active. Thin layer chromatography revealed the presence of glycoflavones in the acetone root extract, alkaloids in the root and leaf extracts; phytosterols and flavonoid aglycones in root and leaf extracts. The acetone root and leaf extracts revealed the presence of phenols. The total phenolic content was expressed as tannic acids equivalents in milligrams per millilitres. The acetone leaf extract was shown to contain high total phenolic content as compared to the root. This may be due to the presence of flavonoids, tannins and alkaloids which were detected in the extracts. Tomas-Barberan and Espin (2001) have reported the contribution of phenolic compounds towards the unique taste, flavour, aroma and health-promoting properties found in vegetables and fruits. The findings in this study are consistent with available report (Masoko *et al*., 2010) of the presence of phenolic compounds in acetone root extracts of the same plant.

Some of the developed chromatograms were evaluated for antioxidants activity using DPPH. The free radical scavenging activity of the root and leaf extracts (acetone, methanol, ethyl acetate and *n*-hexane) were evaluated qualitatively by spraying the developed chromatograms with DPPH. The appearance of yellow colour against a purple background on TLC plates was shown to be indicative of the presence of antioxidant constituents. The observed degree of decolouration is suggestive of hydrogen donating ability of antioxidant constituents in the extract (Es-Safi *et al*., 2007). Both the root and leaf extracts were shown to possess antioxidant activity with the methanol and acetone extracts. However, the concentration of the activity was higher in the acetone root than in the leaf extract. Silva *et al*. (2014) related the antioxidant activity of *Senna* species to be associated with the presence of anthraquinones and flavonoids. These compounds contain hydroxyl groups that are able to donate hydrogen, stabilizing the DPPH radical; thus forming substances with excellent antioxidant properties. The current study revealed the presence of flavonoids and anthraquinones (aglycones and glycosides) which may be responsible for observed activity. In addition, Mokgotho *et al*. (2013) reported the presence of resveratrol isolated from acetone root extract of *S. italica* with proven antioxidant activity. Antioxidant activity was shown to be low with the ethyl acetate root and leaf extracts as compared to other extracts. The *n*-hexane extracts however, was not shown to contain any antioxidant compounds. This was due to the absence of polar compounds in *n*-hexane extracts which tends to possess the antioxidant activity.

The extracts were then subjected to quantitative DPPH assay using 0.2% DPPH and vitamin C as a positive control. The higher the percentage of scavenging activity, the higher the antioxidant activity. Although activity observed with the methanol extracts was comparable to that of the acetone extracts, the acetone extract was shown to possess more antioxidant activity in qualitative assay. The concentration of extracts increased with increase in scavenging activity. The root extracts exhibited a more potent antioxidant activity compared to leaf extracts. However, the acetone and methanol extracts had more antioxidant activity in both the root and leaf as compared to other extracts. The recovery of antioxidant compounds from plant materials is said to be typically accomplished through different extraction techniques

and solvents employed (Sultana *et al*., 2009). This may be responsible for variation in antioxidant activity among the extracts.

4.5.1 Conclusion

The root and leaf extracts of *S. italica* showed the presence of various phytochemical constituents. TLC profile revealed the presence of more compounds in the root than in the leaf extracts. The root and leaf extracts were shown to contain high quantity of compounds when methanol was used as an extractant while ethyl acetate and *n*-hexane extracted the least. The root extract showed more antioxidant activity as compared to the leaf extract. This may be due to polar compounds that may be present in the root extract. However, total phenolic content was observed to be higher in the leaf extract than in the root extract, possibly due to the contribution of phenolic compounds towards the nutritional value of this plant in terms of flavor, taste, aroma and color. The acetone root and leaf extracts revealed the presence of various secondary metabolites. The compounds such as flavonoids, terpenes, tannins, steroids, reducing sugars, flavonoid aglycones, phytosterols and alkaloids were shown to be present in both the root and leaf extracts, with the absence of saponins. Moreover, glycosides were observed only in the leaf extract and glycoflavones only in the root extract. The presence of these constituents may contribute towards various activities associated with this plant. The acetone root and leaf extracts of *S. italica* revealed the presence of various secondary metabolites.

CHAPTER 5

5. Effects of acetone root and leaf extracts on glucose uptake, differentiation, cytotoxicity and western blot by adipose and muscle cells

5.1. Introduction

Bioassays are one of the methods that are used for the assessment of environmental pollution and chemical safety (Hitoshi, 2000). This chemical safety is estimated by monitoring biological responses to environmental pollutants and newly synthesized chemicals. An example being the evaluation of *in vitro* cytotoxicity programme for cell toxicology (Hitoshi, 2000). Such toxins may cause a serious health hazards to human life if not well attended. There is therefore a need for the proper checking and creating a balance between quality and safety in the use of medicinal plants (Mahmood *et al.,* 2013).

On the other hand, glucose uptake assays involves the glucose transporter (GLUT4) in the regulation of blood glucose homeostasis. Plasma glucose triggers pancreatic *β*-cells to release insulin into the blood after nutrient ingestion. This hormone then controls the cellular localization of GLUT4 in adipose and muscle cells which is vital in the management of blood glucose homeostasis. Under normal condition (basal state), GLUT4 resides in an intracellular membrane compartment called vesicles and upon insulin stimulation, it is translocated to the plasma membrane (McCarthy and Elmendorf, 2007). However, this metabolic process is impaired in individuals with insulin resistance due to the failure of peripheral adipose and muscle tissue respond to physiological levels of insulin. Initially, pancreatic *β*-cells will compensate for this resistance by releasing elevated levels of insulin into the blood (hyper-insulinaemia). Over time, *β*-cell function diminishes and blood glucose levels become elevated (McCarthy and Elmendorf, 2007). Thus, understanding the expression of GLUT4 is pivotal in regulation of glucose metabolism.

Another assay is western blot analysis where researchers often use this technique to separate and identify proteins. The mixture of proteins is separated based on their molecular weight and type by the use of gel electrophoresis. The results are then transferred to a membrane producing a band for each protein which is then

incubated with labelled antibodies specific to the protein of interest (Shigematsu *et al.*, 2001). Protein expression plays crucial role in insulin-stimulated cells. Insulin triggers an increase in the rate of GLUT4 vesicle exocytosis resulting in an increase in cell surface GLUT4 protein and subsequent increase in glucose uptake (Shigematsu *et al.*, 2001). Recent report by Mazibuko *et al*. (2012) suggests AKT as a pivotal insulin-signalling protein responsible for GLUT4 expression and translocation to the cell membrane, thereby increasing glucose uptake in response to insulin-signalling. In the current study, the effect of the acetone root and leaf extracts was determined on the expression of GLUT4 in C2C12 muscle cells.

5.2. Objectives of the study

I. To evaluate the cytotoxic effects of the acetone root and leaf extracts of *S. italica* on C2C12 muscle cells
II. To evaluate the effects of acetone root and leaf extracts on glucose uptake by C2C12 muscle cells
III. To evaluate the effects of acetone root and leaf extracts on differentiation and immunofluorescence of NIH-3T3-L1 preadipocytes and C2C12 muscle cells
IV. To evaluate the effects of acetone root and leaf extracts on the expression of GLUT4 protein using western blotting

5.3. MATERIALS AND METHODS

5.3.1 Cytotoxicity assay

The cytotoxicity of acetone root and leaf extracts on C2C12 muscle cells was evaluated by MTT assay described in section 3.6.

5.3.2 Glucose uptake assay

Glucose uptake by C2C12 muscle cells exposed to the acetone root and leaf extracts was evaluated according to method of Foley *et al.* (1983) described in section 3.7.

5.3.3 Differentiation of NIH-3T3-L1 preadipocytes cells and C2C12 cells

The differentiation NIH-3T3-L1 preadipocytes and C2C12 muscle cells exposed to acetone root and leaf extracts of *S. italica* was evaluated described in section 3.8.1.

5.3.4 Qualitative and Quantitative Immunofluorescence

Immunofluorescence of NIH-3T3-L1 preadipocytes and C2C12 muscle cells exposed to acetone root and leaf extracts was evaluated, described in section 3.8.2.

5.3.5 Western blot analysis

The western blot analysis of GLUT4 protein expressed by C2C12 muscle cells following exposure to acetone root and leaf extracts of *S. italica* was evaluated according to method of Saiki *et al.* (2008) described in section 3.9.

5.4. RESULTS

5.4.1 MTT assay of C2C12 cells

The cytotoxic effects of acetone root and leaf extracts on C2C12 muscle cells was determined using MTT assay. The cells were treated with various concentrations (0.001–1000 µg/ml) of the acetone root and leaf extracts of *S. italica* for 24 and 48 hrs respectively. Curcumin was used as a positive control at concentration of 200 mM and cells without extracts as negative control. The linear regression plot was used to obtain the 50% cytotoxic concentration (CC_{50}) of the extracts and a representative plot is presented in figure 9. The CC_{50} values of the acetone root and leaf extracts are presented in table 4. The highest cytotoxic concentration (CC_{50}) was obtained with the root extract with the CC_{50} value of 297 635 µg/ml at 48 hrs, followed by CC_{50} value of 21 544 µg/ml at 24 hrs. The CC_{50} value of the leaf extract at 24 hrs was 2 904 µg/ml with the least value at 48 hrs of 884 µg/ml. The higher the CC_{50} value, the higher the concentration of the plant extracts required to kill 50% of cells. The root extract at 24 and 48 hrs together with the leaf extract at 24 hrs were not toxic to C2C12 muscle cells at the concentration used in the study. These plant extracts show that they may enhance proliferation of C2C12 muscle cells at a concentration of 0.001–1000 µg/ml. However, the leaf extract at 24 hrs seems to be rather toxic at a concentration of 1000 µg/ml. The acetone extracts employed had no significant reduction on viability of C2C12.

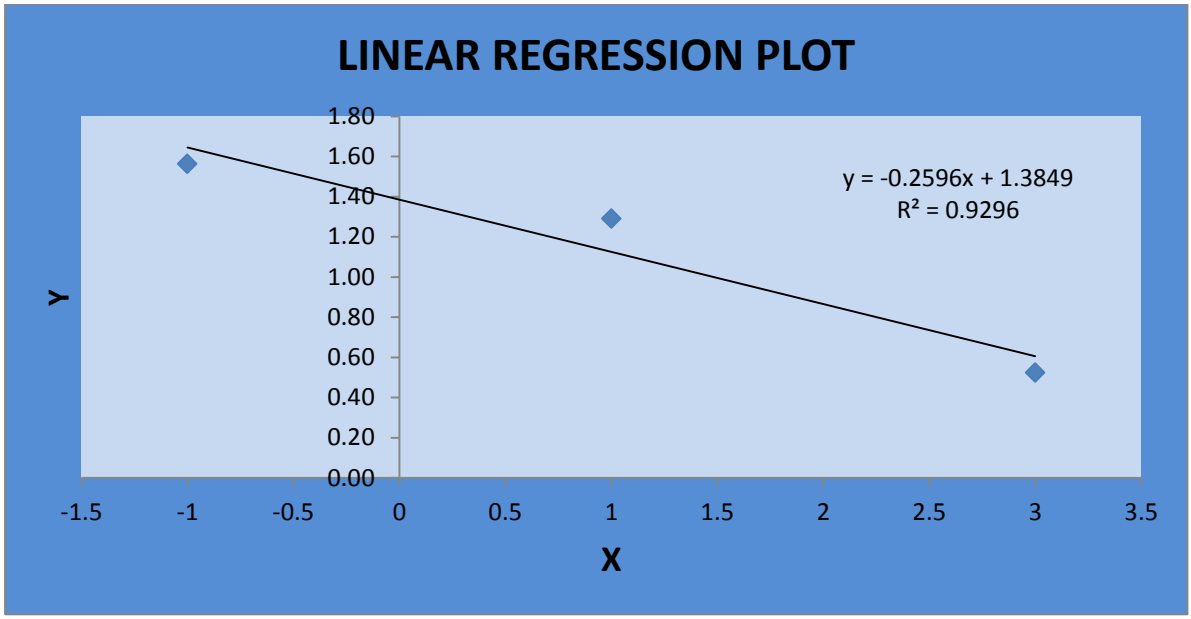


Figure 9: Linear regression plot obtained when C2C12 muscle cells were treated with various acetone (A) root (R) and leaf (L) extracts concentrations of *S. italica* (SI) (0.001–1000 µg/ml) for cytotoxicity. The experiment was carried out for 24 and 48 hrs respectively using MTT assay. Untreated cells were considered as a negative control and Curcumin as positive controls.

Table 4: Cytotoxic concentration (CC_{50}) of acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) on C2C12 muscle cells

Extract type	Time (hrs)	CC_{50} (µg/ml)
SILA	24	2904
SILA	48	884
SIRA	24	21544
SIRA	48	297635

5.4.2 Glucose uptake assays

The potential effect of acetone extracts on glucose uptake by non-differentiated and differentiated C2C12 muscle cells are presented in figure 10 and figure 11 respectively. The cells were treated with non-cytotoxic concentrations of 2.5 µg/ml and 25 µg/ml of the acetone root and leaf extracts of *S. italica* for 1, 3 and 24 hrs respectively. The ability of the extracts to facilitate glucose uptake by the cells was determined spectrophotometrically after adding glucose reagent. Insulin was used as

a positive control at a concentration of 1000 nM. Extracts alone did not have a significant effect on glucose uptake by the cells. However, extracts in combination with insulin was shown to facilitate glucose uptake into cells than insulin alone. This was observed in both cases with non-differentiated and differentiated cells. The concentration of 25 µg/ml of the leaf extract facilitated more glucose uptake as compared to other extracts. Glucose uptake was shown to increase with prolonged incubation time with leaf extract, while increase in concentration of root extract was shown to decrease glucose uptake at 24 hrs (figure 10). At 3 hrs of incubation glucose uptake by cells exposed to the extracts were comparable (figure 10). At 2.5 µg/ml concentration, the root extract was shown to increase glucose uptake while increase in concentration was shown to decrease glucose uptake (figure 11). At incubation time of 3 and 24 hrs glucose uptake results at concentration of 2.5 µg/ml were comparable with the root extract, with a similar trend at 25 µg/ml observed, although with decrease in uptake (figure 11).

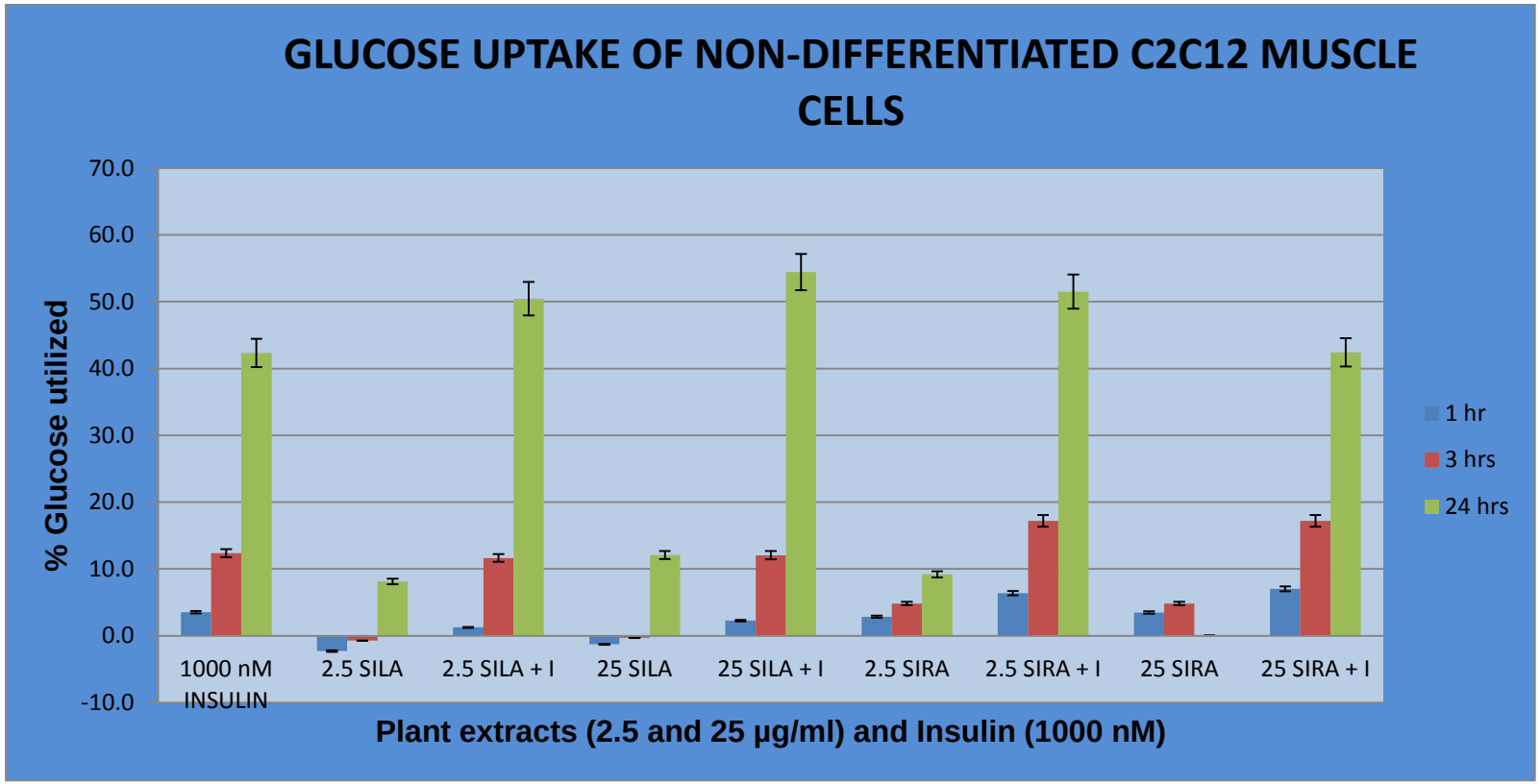


FIGURE 10: Effects of *S. italica* (SI) plant extracts on glucose uptake of non-differentiated C2C12 muscle cells. The acetone (A) root (R) and leaf (L) extracts of *S. italica* at concentrations of 2.5 µg/ml and 25 µg/ml plus 1000 nM Insulin were used. 1000 nM Insulin was used as a positive control. The experiment carried on for 1, 3 and 24 hrs respectively. Results are expressed as the mean of three independent experiments relative to the control, which was set at 100% ± SD ($p < 0.001$).

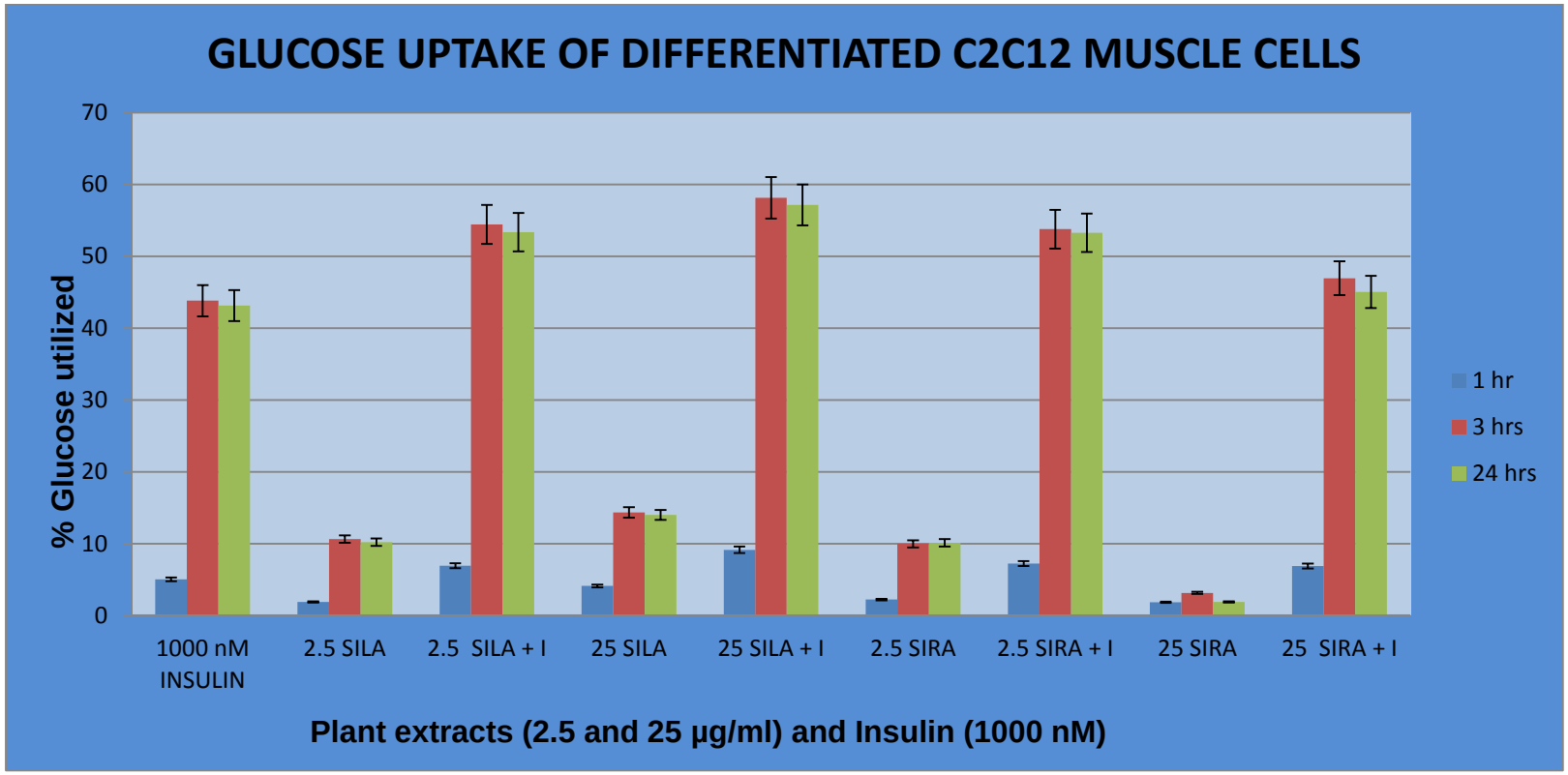


FIGURE 11: Effects of *S. italica* (SI) plant extracts on glucose uptake of differentiated C2C12 muscle cells. The acetone (A) root (R) and leaf (L) extracts of *S. italica* at concentrations of 2.5 µg/ml and 25 µg/ml plus 1000 nM Insulin were used. 1000 nM Insulin was used as a positive control. The experiment carried on for 1, 3 and 24 hrs respectively. Results are expressed as the mean of three independent experiments relative to the control, which was set at 100% ± SD ($p < 0.001$).

5.4.3 Differentiation of NIH-3T3-L1 preadipocytes and C2C12 muscle cells

The potential effects of acetone root and leaf extracts on the differentiation of NIH-3T3-L1 preadipocytes is represented in figure 12. The cells were treated with concentrations of 2.5 µg/ml and 25 µg/ml of the acetone root and leaf extracts of *S. italica* with combination of insulin at a concentration of 0.5 mg/ml used as a positive control. The extract alone was not used in this assay due to its poor ability to facilitate glucose uptake in the glucose uptake assay. Differentiated cells were stained with oil-red O to indicate the presence of fat droplets as a positive indicator of cell differentiation. The differentiation of NIH-3T3-L1 preadipocytes shows the ability of extract combination in facilitating the translocation of GLUT4 to the cell membranes. With the results of differentiation of C2C12 muscle cells, the morphology of cells was observed to ensure their transformation into myocytes.

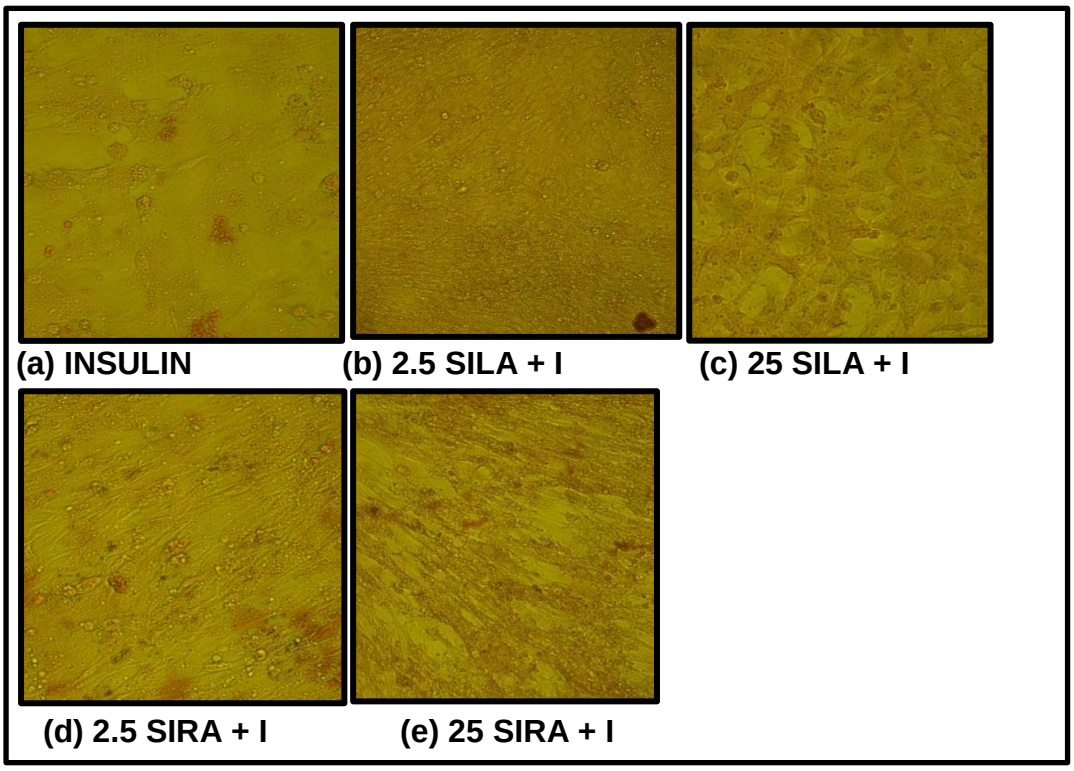


FIGURE 12: Effect of acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) on differentiation of NIH-3T3-L1 preadipocytes. The cells were treated with 2.5 µg/ml and 25 µg/ml (b–e) of extracts plus 0.5 mg/ml Insulin (I). Untreated cells with insulin only (a) were used as the positive control. The cells were then stained with oil-red O stain where the red droplets indicate a positive sign of differentiation.

5.4.4 Qualitative and quantitative immunofluorescence

The potential effects of acetone root and leaf extracts on translocation of GLUT4 to the plasma membrane of NIH-3T3-L1 preadipocytes and C2C12 muscle cells is presented in figure 13 and figure 14 respectively. The cells were treated with concentrations of 2.5 µg/ml and 25 µg/ml of both acetone root and leaf extracts of *S. italica* respectively with combination of insulin as a positive control. The cells were incubated with goat polyclonal anti-GLUT4 antibody for 1 hr at room temperature, followed by incubation with FITC-conjugated rabbit anti-goat antibody for a further 1 hr then visualized under a fluorescence microscope for qualitative immunofluorescence represented in figure 13 and 14. The increase in extracts concentration showed more fluorescence on the cell membrane indicative of GLUT4 translocation. The green fluorescence on the cell membrane (figure 13 and 14) indicates that GLUT4 has been successfully translocated. More fluorescence was observed with 25 µg/ml of the leaf extract than other extracts and insulin alone. Furthermore, the blue fluorescence that comes from the DAPI stain indicates the nucleus of the C2C12 muscle cells (figure 14). The quantified fluorescence (table 5) was obtained using a Glomax microtitre plate reader using blue light filter (Emits at 510 nm, Excites at 490–570 nm). DMSO (0.2%) was used as a negative control. The quantity of fluorescence was represented as fold with regard to the positive control. The leaf extract at a concentration of 25 µg/ml in combination with insulin had more fold (table 5) as compared to other extracts.

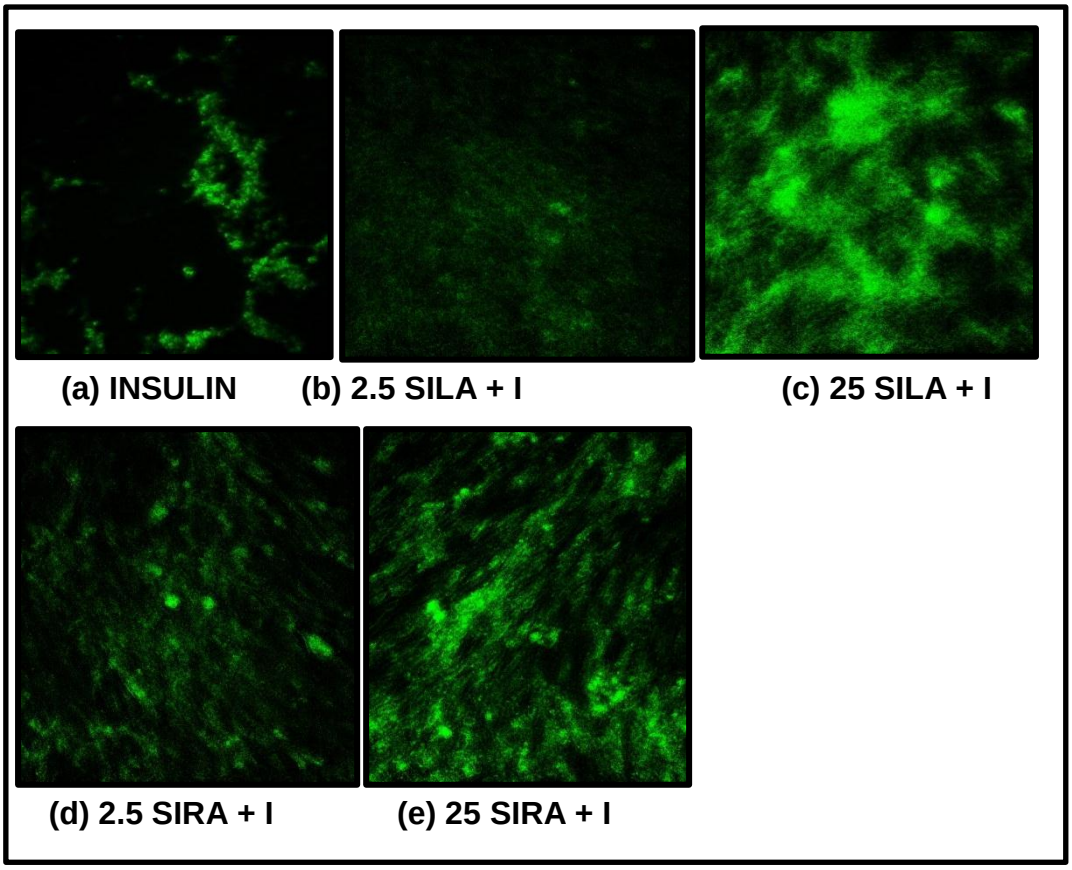


FIGURE 13: Effect of acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) on translocation of GLUT4 to the plasma membrane of NIH-3T3-L1 preadipocytes. The cells were treated with 2.5 µg/ml and 25 µg/ml (b–e) of acetone root and leaf extracts plus 0.5 mg/ml Insulin (I). Untreated cells with insulin only (a) were used as the positive control. The cells were then viewed with fluorescence microscope (20 x lenses).

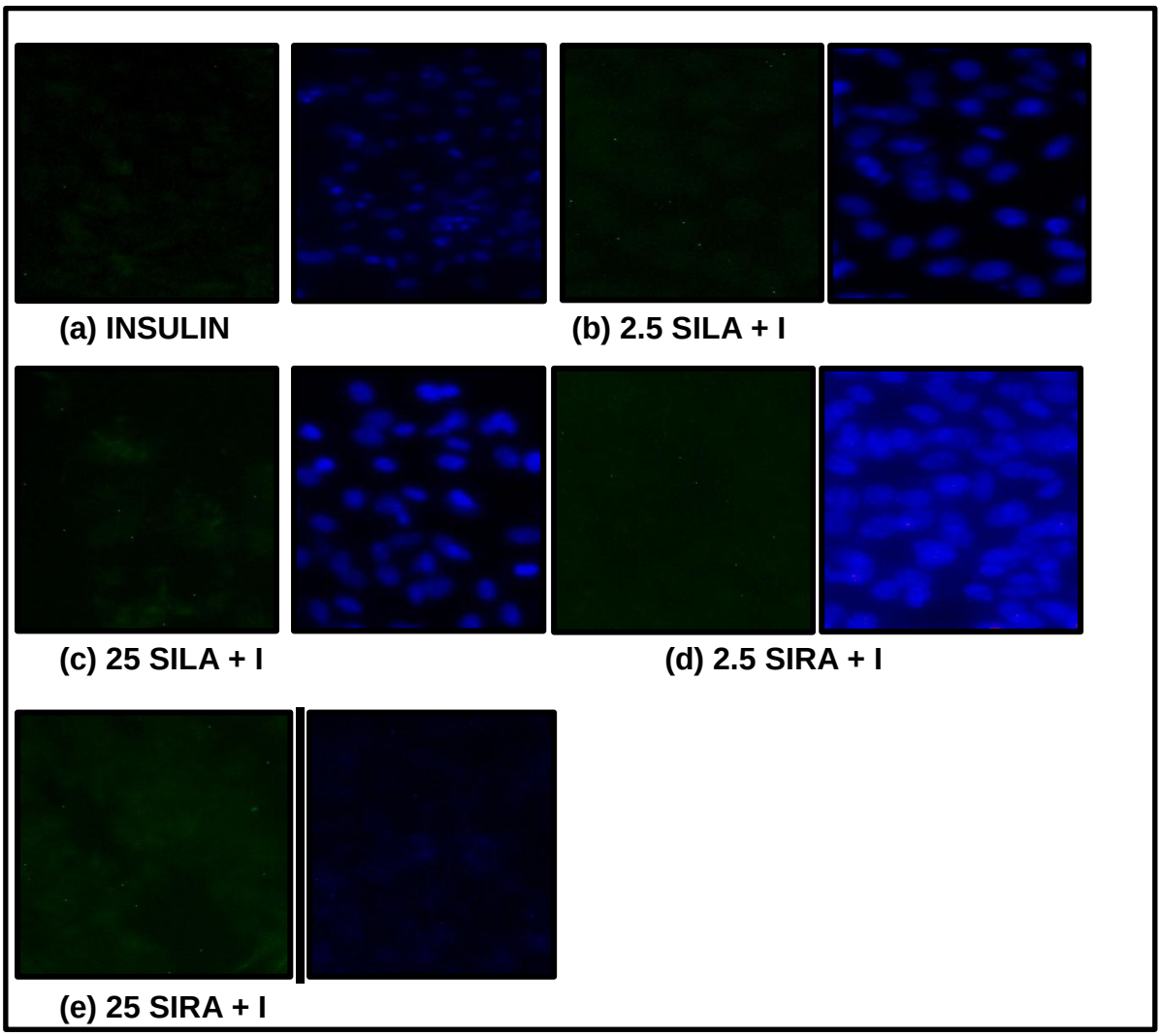


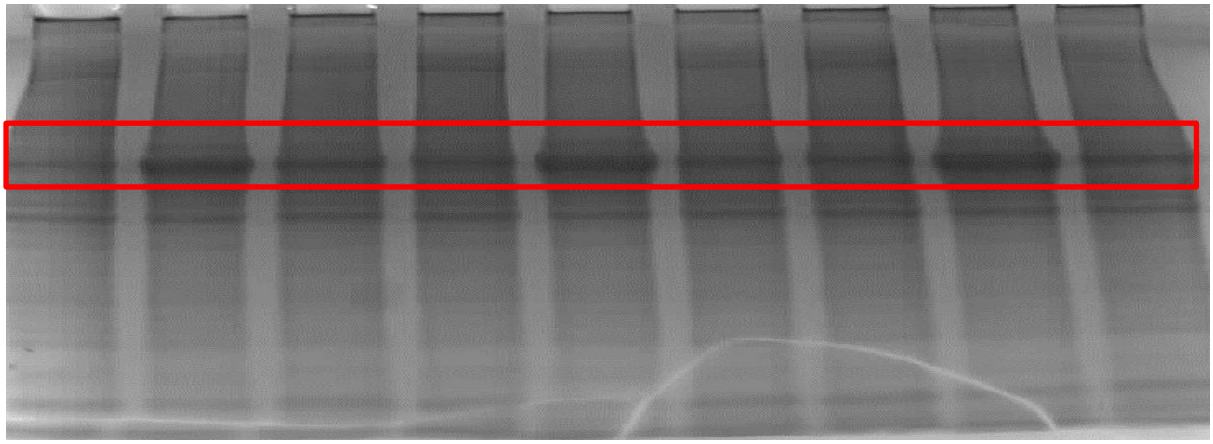
Figure 14: Effects of acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) on translocation of GLUT4 to the plasma membrane of C2C12 muscle cells. The cells were treated with 2.5 µg/ml and 25 µg/ml (b–e) of the root and leaf extracts plus 1000 nM Insulin (I). Untreated cells with insulin only (a) were used as the positive control. Cells were stained with DAPI stain to visualize the nucleus of the C2C12 muscle cells (Blue fluorescence).

Table 5: Quantitative immunofluorescence of GLUT4 from C2C12 muscle cells treated with 2.5 µg/ml and 25 µg/ml of the acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) with combination of insulin (1000 nM) as a positive control and DMSO (0.2%) as a negative control.

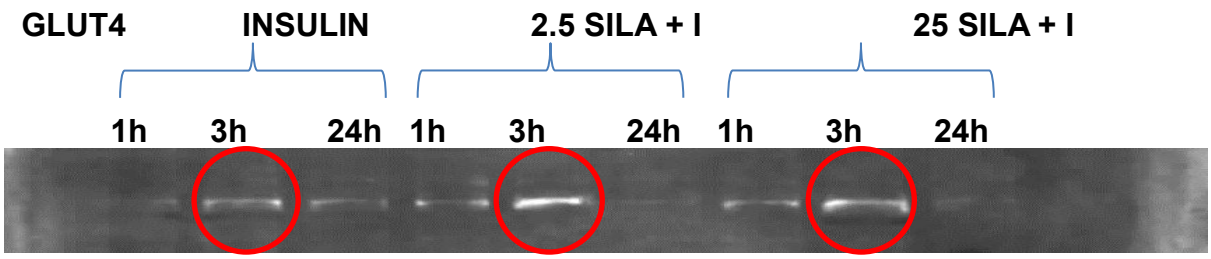
CONTROLS USED	CONCENTRATION OF CONTROLS (nM or %)	EXTRACTS USED	EXTRACTS CONCENTRATION (µg/ml)	FOLD
INSULIN	**1000**			**1.00**
DMSO	**0.2**			**1.03**
		SILA	**2.5**	**1.00**
		SILA	**25**	**1.07**
		SIRA	**2.5**	**1.02**
		SIRA	**25**	**0.98**

5.4.5 Western blot analysis

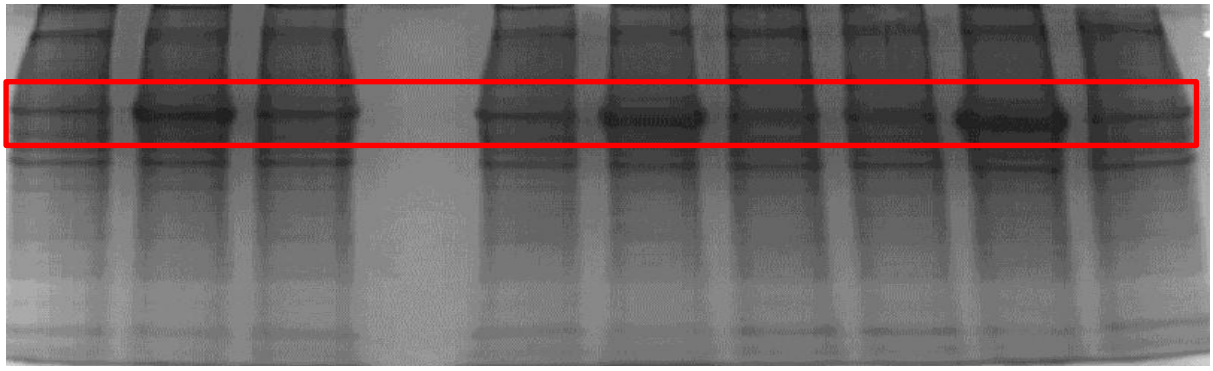
The cells were treated with concentrations of 2.5 µg/ml and 25 µg/ml of both acetone root and leaf extracts of *S. italica* respectively in combination with insulin (100 nM). The cells were incubated with a goat polyclonal anti-GLUT4 antibody overnight at 4°C in the dark, followed by incubation with HRP-conjugated rabbit anti-goat antibody for a further 2 hrs. The immunoblots were incubated with Dura west chemiluminiscence substrate to detect the protein of interest (GLUT4). The area highlighted in red indicates the expected region of the protein of interest (GLUT4) on SDS-PAGE gel (figure 15 A and C) for both insulin-stimulated acetone root and leaf extracts following exposure of C2C12 muscle cells. GAPDH was used as an internal control. GLUT4 protein was isolated successfully from C2C12 muscle cells treated with acetone root and leaf extracts (figure 15 B and D). In addition, acetone root and leaf extracts increased protein expression of GLUT4 at 3 hrs incubation time as compared to other incubation times in insulin-stimulated C2C12 muscle cells.



A Proteins isolated from insulin-stimulated C2C12 muscle cells treated with leaf extract on SDS-PAGE gel (12%). The estimated area of the protein of interest is highlighted in red.



B



C Proteins isolated from insulin-stimulated C2C12 muscle cells treated with root extract on SDS-PAGE gel (12%). The estimated area of the protein of interest is highlighted in red.

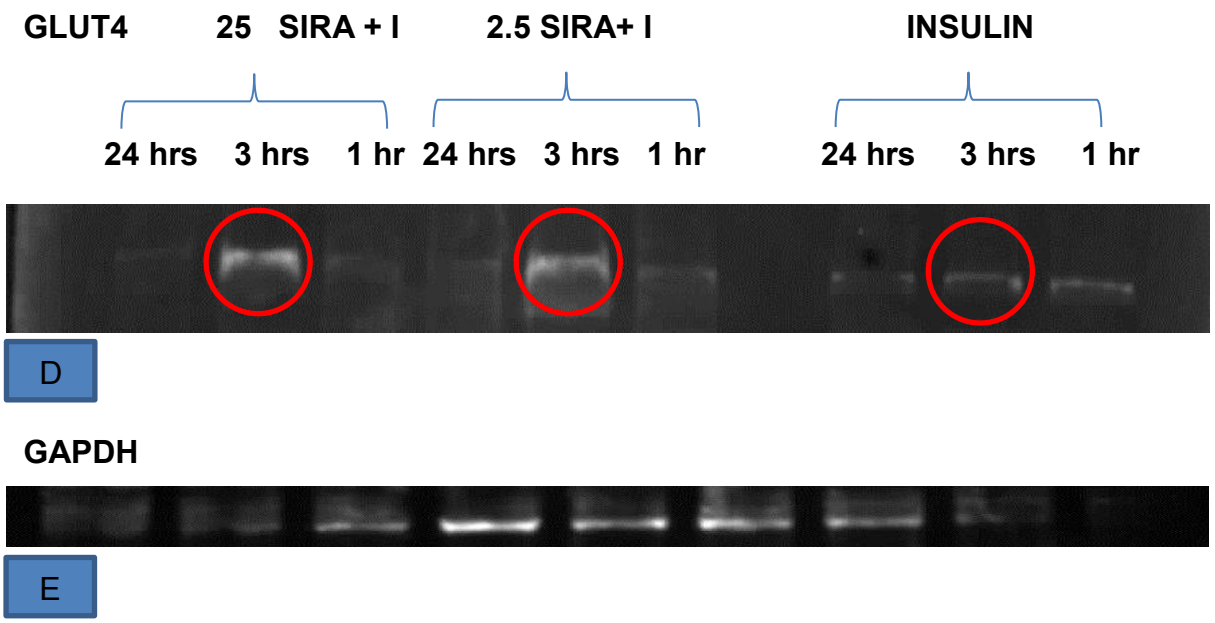


Figure 15: Expressed GLUT4 protein of differentiated C2C12 muscle cells treated with acetone (A) root (R) and leaf (L) extracts of *S. italica* (SI) at a concentration of 2.5 µg/ml and 25 µg/ml plus 1000 nM insulin (I). The cells were treated for 1, 3 and 24 hrs respectively. The proteins were first separated on SDS-PAGE gel (12%) (B and D) before being transferred to a nitrocellulose membrane (A and C). GAPDH was used as an internal control (E).

5.5. Discussion

Toxicity studies are essential in discovering and developing safe products such as new drugs. Thus, *in vitro* studies are required as a first step in toxicological studies to achieve this. In this study, MTT assay was undertaken to determine the cytotoxic effects of acetone root and leaf extracts on C2C12 muscle cells. The MTT method is based on the ability of the mitochondrial enzyme succinate-dehydrogenase to transform the MTT tetrazolium salt to a formazan product, which is proportional to the number of viable cells present (Saravanan *et al*., 2013). The CC_{50} values of the acetone root and leaf extracts were determined by linear regression plot. The higher the CC_{50} value, the higher the concentration of the plant extracts which require being toxic to 50% of the cells. The highest cytotoxic concentration (CC_{50}) was obtained with the root extract with the CC_{50} value of 297 635 µg/ml at 48 hrs, followed by CC_{50} value of 21 544 µg/ml at 24 hrs. The CC_{50} value of the leaf extract at 24 hrs was 2 904 µg/ml with the least value at 48 hrs. The root extract at 24 and 48 hrs together

with the leaf extract at 24 hrs were not toxic to C2C12 muscle cells at the concentration used in the study. The plant extracts were shown to possibly enhance proliferation of C2C12 muscle cells at a concentration of 0.001–1000 µg/ml. The findings by Masoko *et al.* (2010) showed the presence of compounds in the acetone root extract of *S. italica* with antiproliferative activities while Stivala *et al*. (2001) confirmed the antiproliferative effect of resveratrol which is contained in the acetone root extract. However, the leaf extract at 48 hrs seems to be rather toxic at a concentration of 1000 µg/ml or it might be due to the cells trying to adapt to the new conditions employed. The acetone extracts employed had no significant reduction on viability of C2C12 muscle cells. The effect of acetone extracts on glucose uptake was also evaluated. Glucose uptake studies can be conducted on either adipose, muscle or liver cells. However, in this study only C2C12 muscle cells were used for the glucose uptake. This has been employed on non-differentiated and differentiated C2C12 muscle cells. The percentage of glucose utilized by the cells was recorded. The extracts alone had no significant effect on glucose uptake by C2C12 muscle cells.

It has been observed that glucose uptake is enhanced when extracts in combination with insulin is used. This was observed in both cases with non-differentiated and differentiated cells. Mazibuko *et al*. (2013) have reported the combination of rooibos extracts with insulin to be more effective than insulin alone. The authors further suggested that high polyphenols level is important in improving glucose uptake and metabolism in palmitate-induced IR C2C12 skeletal muscle cells. It may be likely that the high phenolic content in the leaf extract may be responsible for high glucose uptake observed in this study. The concentration of 25 µg/ml of the leaf extract in combination with insulin showed more glucose uptake as compared to other extracts as well as the control. Natural compounds with insulin mimicking effects have been proposed as potential therapeutic agents in the treatment of diabetes mellitus (Lee *et al*., 2006; Pinent *et al.,* 2008). They would act by promoting or enhancing glucose transport and glucose metabolism (Alberti *et al*., 2006) which is consistent with the present findings where the extracts enhanced glucose uptake. Furthermore, Son *et al*. (2012) have shown that aspalathin increases glucose uptake in L6 muscle cells, promoted AMPK activation and increase GLUT4 translocation into the plasma membrane. Similarly, Abesundara *et al*. (2004) reported that *Cassia auriculata*

extracts exhibit potential antihyperglycemic effects in Sprague-Dawley rats. Prolonged incubation time increased glucose uptake with leaf extract while increase in concentration of root extract decreased glucose uptake at 24 hrs. This might be due to some compounds within the root extract that may possess antagonistic effects at high concentration in combination with insulin at prolonged incubation time. At 3 hrs of incubation glucose uptake of cells exposed to the plant extracts were comparable. At incubation time of 3 and 24 hrs glucose uptake results at concentration of 2.5 µg/ml were comparable with the root extract, with a similar trend observed at 25 µg/ml, although with decrease in uptake. The antagonistic effects of the root extracts with insulin might have interfered with glucose uptake, hence the slight decrease was observed. In addition, the effect of acetone extracts on translocation of GLUT4 to the cell membrane was evaluated using qualitative and quantitative immunofluorescence. Firstly, both the NIH-3T3-L1 preadipocytes and C2C12 cells allowed to differentiate in order for the cells to be able to translocate GLUT4 to the cell membranes. The qualitative and quantitative fluorescence results showed GLUT4 which have been translocated to the cell membrane. For the quantitative study, the quantity of fluorescence is represented as fold with regard to the positive control. The leaf extracts at a concentration of 25 µg/ml had more fold as compared to other extracts, indicative that more GLUT4 was translocated at this concentration of the leaf extracts. This might be due to AKT which is a pivotal insulin-signalling protein responsible for GLUT4 expression and translocation (Mazibuko *et al*., 2012).

Western blot analysis was used to determine the expression of GLUT4 protein. C2C12 muscle cells were used in this study since skeletal muscle is a major tissue involved in peripheral insulin-stimulated glucose disposal and accounts for 75–80% of glucose disposal from the circulation (Abdul-Ghani and DeFronzo, 2010). GLUT4 protein was isolated from C2C12 muscle cells treated with acetone root and leaf extracts. The acetone root and leaf extracts increased protein expression of GLUT4 at 3 hr incubation time as compared to other incubation times in insulin-stimulated C2C12 muscle cells. However, the mechanism by which the acetone leaf extract stimulates the expression of GLUT4 warrants further studies.

5.5.1 Conclusion

The acetone extracts employed induced no significant reduction on the viability of C2C12 muscle cells. The root extract at 24 and 48 hrs together with the leaf extract at 24 hrs were not toxic to C2C12 muscle cells at the concentration used in the study. The extracts were shown to enhance proliferation of the C2C12 muscle cells. Furthermore, differentiation of adipose cells as well as glucose uptake of muscle cells might have been enhanced by acetone plants extracts. At incubation time of 3 and 24 hrs, glucose uptake results at concentration of 2.5 µg/ml were comparable with that of root extract. A similar trend was observed at 25 µg/ml but with decrease in glucose uptake. The antagonistic effects of the root extract with insulin combination might have interfered with glucose uptake and as such a slight decrease was observed. The expression level of GLUT4 protein determined in insulin-stimulated C2C12 muscle cells treated with plant extracts in combination with insulin was shown to be enhanced at 3 hr incubation. The acetone leaf extract was shown to have good effect on differentiation, glucose uptake and GLUT4 expression of glucose storage cells. Therefore, further studies are however required to elucidate the molecular mechanism by which the acetone leaf extract of *S. italica* influences the translocation of GLUT4.

CHAPTER 6

6. General discussion

Diabetes mellitus is a major problem worldwide and is one of the leading causes of death. There are current drugs that are being used to manage this disease. However, apart from their costs and unavailability to the rural poor, there are a lot of side effects associated with them. For this reason and many more, the use of medicinal plants in health care are of primary importance in the socio cultural and ethno-pharmacology of different culture in many parts of the world (Awodele *et al*., 2013). The search for new treatment with fewer side effects from medicinal plants is pivotal due to the various biological activities attributed to biological compounds that are present within these medicinal plants. These compounds are extracted using solvents of varying polarities (Eloff, 1998b). In the current study, different solvents such as acetone, methanol, ethyl acetate and *n*-hexane, were used for extraction of plant material due to the wide range of compounds that can be extracted by these solvents. However, boiled water is mainly used by traditional healers to extract bioactive compounds because of its nontoxic nature towards human beings (Eloff, 1998b). In addition, most compounds tend to volatilise when using boiled water as an extractant. Thus, the need for selection of the most appropriate extraction method is evident from the fact that when different methods are applied on the same plant material with the same or different solvents, extraction efficiency can vary significantly. In addition, the method selected as the most appropriate, also needs to be standardized so as to achieve acceptable degree of reproducibility (Gupta *et al*., 2012). The current study was aimed at evaluating the *in vitro* effects of the root and leaf extracts of *S. italica* on GLUT4 translocation in NIH-3T3-L1 preadipocytes and C2C12 muscle cells.

The masses of the extracts obtained were extracted from different solvents in a serial exhaustive extraction as to extracts as much as possible from the plant materials. Hence, the solvents employed are commonly used to extract various biological active compounds from plants in research settings. Methanol extracted more compounds followed by acetone with *n*-hexane as the least. The constituents extracted by methanol may be mostly sugars, amino acids and glycosides due to the

polarity of this solvent. Hydro-alcoholic solvents extract a variety of compounds that are mostly polar. Acetone extracts mostly alkaloids, aglycones and glycosides. *n*-Hexane in general extracts mostly waxes, fats and fixed oils. More yields were obtained with leaf extract with all the solvent used for extraction as compared to the root extract. Similarly, others have obtained a high yield of antioxidants with *Moringa oleifera* leaf as compared to the root using ethanol and methanol extracts (Sultana *et al*., 2009). The differences in the extract yields from the different plant part might be ascribed to the difference in the availability of extractable components present in the root and leaf material. Alternatively, this might be due to the presence of high concentration of compounds present in the leaf since most nutrients are synthesized in the leaf.

The finger print profile of constituents in *S. italica* extracts (acetone, methanol, ethyl acetate and *n*-hexane) were analysed by TLC using different mobile phases (BEA, CEF, EMW and BAW). Good separation was achieved with the methanol and acetone extracts in CEF mobile phase, ethyl acetate extracts in CEF and EMW and *n*-hexane extracts in BEA respectively, especially with the leaf extract. Most compounds present in *S. italica* extracts were UV active, possibly due to the presence of many fluorescent-quenching compounds which are usually contained within herbal extracts. Ultra violet light identifies fluorescing compounds that mainly consists of aromatic rings which absorb at 254 nm thereby quenching the fluorescence of the pigment present in the silica gel (Masoko, 2006). More fluorescing bands were contained in the acetone, methanol and ethyl acetate extracts of the root material and *n*-hexane with the leaf material. However, following spraying with vanillin/H_2SO_4 reagent most compounds were shown to be non-reactive with the reagent. Hence, *p*-anisaldehyde and iodine crystals vapor were used to visualize other compounds that may have not been detected by vanillin/H_2SO_4 reagent.

Most compounds were reactive with *p*-anisaldehyde reagent, suggestive of the presence of terpenes. The reactive Compounds with this reagent were more visible in acetone and ethyl acetate extracts of the TLC plates eluted in BEA and CEF mobile phases. Those in methanol extract were more visible in CEF and BAW mobile phases. On the other hand compounds in *n*-hexane extract were more visible

in BEA mobile phase only. The presence of sugars or organic compounds present in the extracts were detected by emersion of TLC plates in iodine vapor, with the appearance of brown bands on the developed chromatograms as a positive indicator, suggestive that some compounds contained in *S. italica* are organic in nature. Dave and Ledwani (2012) have reported the presence of organic compounds such as anthraquinones (aglycones and glycosides) in a related species (*Cassia alata*). The presence of secondary metabolites was evaluated by chemical analysis. The acetone root and leaf extracts revealed the presence of flavonoids, terpenes, tannins, steroids, reducing sugars and alkaloids while glycosides were detected only in the leaf extract. However, the presence of quinones was not evaluated in this study. In addition, Malviya *et al.* (2010) showed that most plants with antidiabetic activity contain secondary metabolites such as glycosides, alkaloids, terpenoids, flavonoids, *etc*. which is consistent with the present findings. Contrary to other findings Dabai *et al.* (2012), in phytochemical screening of *S. italica* root and leaf extracts, revealed the presence of secondary metabolites tested for in this study. However, apart from the glycosides in the leaf and tannins in both the root and leaf extract which was only observed in the current study. The observed discrepancy may be related to the geographical location of the plant which is reported to influence its chemical constituents due to factors such as climate, soil, propagation method, *etc*. (Adoum *et al*., 1997). Odugbemi (2008) have also reported the influence of time of collection of the plant and seasonal variations which are considered to affect the chemical composition of plants on its biological activity. Another reason could be the type of solvents used in the extraction process which can influence the type of phytochemicals extracted (Kawo, 2007).

The presence of other secondary metabolites were also analysed by TLC and developed in various mobile phases for each specific metabolite. The calculated R_f values suggest the presence of secondary metabolites that are contained within the acetone root and leaf extracts. The results obtained using TLC was consistent with the results obtained in the chemical analysis. Some of the compounds were shown to be UV active. Thin layer chromatography revealed the presence of glycoflavones in the acetone root extract, alkaloids in the root and leaf extracts; phytosterols and flavonoid aglycones in root and leaf extracts. The acetone root and leaf extracts revealed the presence of phenols. The total phenolic content was expressed as

tannic acids equivalents in milligrams per millilitres. The leaf extract was shown to contain high total phenolic content as compared to the root. This may be due to the presence of flavonoids, tannins and alkaloids which were detected in the extracts. Indeed, Tomas-Barberan and Espin (2001) have reported the contribution of phenolic compounds towards the unique taste, flavour, aroma and health-promoting properties found in vegetables and fruits. A finding in this study is consistent with available report (Masoko *et al*., 2010) of the presence of phenolic compounds in acetone root extract of the same plant. These compounds mostly posses various biological activities from different plant parts such as antioxidant, anti-inflammatory, antiproliferative, antimicrobial, *etc*. There are studies that show that the antioxidant activity correlates to the phenolic content in plants. For instance, Wang and Jiao (2000) showed that blueberries contain high level of phenolic compounds with high *in vitro* antioxidant capacities compared with other fruits. Developed chromatograms were evaluated for antioxidants activity using DPPH. The free radical scavenging activity of the root and leaf extracts (acetone, methanol, ethyl acetate and *n*-hexane) were evaluated qualitatively by spraying the developed chromatograms with DPPH. The appearance of yellow colour against a purple background on TLC plates was shown to be indicative of the presence of antioxidant constituents. The observed degree of decolouration is suggestive of hydrogen donating ability of antioxidant constituents in the extract (Es-Safi *et al*., 2007).

Both the root and leaf extracts were shown to possess antioxidant activity with the acetone and methanol extracts. However, the concentration of the activity was higher in the acetone root than in the leaf extract. Silva *et al*. (2014) related the antioxidant activity of *Senna* species to be associated with the presence of anthraquinones and flavonoids. These compounds contain hydroxyl groups that are able to donate hydrogen, stabilizing the DPPH radical, thus forming substances with excellent antioxidant properties. The current study revealed the presence of flavonoids and anthraquinones (aglycones and glycosides) which may be responsible for the observed activity. In addition, Mokgotho *et al*. (2013) have reported the presence of resveratrol isolated from acetone root extract of *S. italica* with proven antioxidant activity. Antioxidant activity was shown to be low with the ethyl acetate root and leaf extracts as compared to other extracts. *n*-Hexane extracts however, were not shown to contain any antioxidant compounds.

The extracts were then subjected to quantitative DPPH assay using 0.2% DPPH and Vitamin C as a positive control. The higher the percentage of scavenging activity, the higher the antioxidant activity. Although activity observed with the methanol extract was comparable to that of the acetone extract, the acetone extract was shown to possess more antioxidant activity in the qualitative assay. The concentration of extracts increased with increase in scavenging activity. The root extract exhibited a more potent antioxidant activity compared to the leaf extract. However, the acetone and methanol extracts had more antioxidant activity in both the root and leaf as compared to other extracts. The recovery of antioxidant compounds from plant materials is said to be typically accomplished through different extraction techniques and solvents employed Sultana *et al*. (2009). This may be responsible for variation in antioxidant activity among the extracts. Antioxidants have been shown to prevent the destruction of *β*-cells and to inhibit the oxidation processes in the human body (Diaz *et al*., 1997; Fernandes de Oliveira *et al*., 2012). Raphael *et al*. (2002b) reported that most medicinal plants with antidiabetic activity have been found to possess antioxidant activity. The plant extracts were then tested for their cytotoxic effect on C2C12 muscle cell line.

Toxicity studies such as *in vitro* assays are required as the first step to discover and develop safe products such as new drugs. In this study, MTT assay was undertaken to determine the cytotoxic effects of acetone root and leaf extracts on C2C12 muscle cells. However, the acetone extracts employed had no significant reduction on the viability of C2C12 muscle cells. The effect of acetone extracts on glucose uptake was also evaluated. Glucose uptake studies can be conducted on either adipose, muscle or liver cells. C2C12 muscle cells were used in this study since skeletal muscle is a major tissue involved in peripheral insulin-stimulated glucose disposal and accounts for 75–80% of the glucose disposal from the circulation (Abdul-Ghani and DeFronzo, 2010). This has been employed on non-differentiated and differentiated C2C12 muscle cells which were observed as percentage glucose utilized by the cells. The extracts alone had no significant effect on glucose uptake by C2C12 muscle cells. As such, natural compounds with insulin mimicking effect have been proposed as potential therapeutic agents in the treatment of diabetes mellitus (Lee *et al*., 2006; Pinent *et al.,* 2008). They would act by promoting or

enhancing glucose transport and glucose metabolism (Alberti *et al*., 2006). The glucose transport in skeletal muscle is regulated by two distinct pathways including phosphatidylinositol-3 kinase (PI 3-kinase) and 5-AMP-activated protein kinase (AMPK). The PI 3-kinase pathway includes activation of Akt leading to activation of glycogen synthesis and the other proteins necessary for the acute metabolic effects of insulin, which promotes GLUT4 translocation from the intracellular pool to the plasma membrane (Smith and Muscat, 2005). AMPK is a phylogenetically conserved intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism (Carling, 2004). Both pathways also increase the phosphorylation and activity of mitogen-activated protein kinase (MAPK) family components, which participates in the full activation of insulin-stimulated glucose uptake via GLUT4 translocation (Konrad *et al.*, 2001). It has been observed that glucose uptake is more enhanced when extracts in combination with insulin is used. This was observed in both cases with non-differentiated and differentiated cells. Mazibuko *et al*. (2013) have reported the combination of rooibos extracts with insulin to be more effective than insulin alone.

The authors further suggested that high polyphenols level is important in improving glucose uptake and metabolism in palmitate-induced IR C2C12 skeletal muscle cells. It may be likely that the high phenolic content in the leaf extract may be responsible for high glucose uptake observed in this study. The concentration of 25 µg/ml of the leaf extract in combination with insulin showed more glucose uptake as compared to other extracts as well as the control. Insulin mimetics would act by promoting or enhancing glucose transport and glucose metabolism (Alberti *et al*., 2006), a mechanism that is consistent with the present findings where the extracts enhanced glucose uptake. Furthermore, Son *et al*. (2012) have shown that aspalathin increases glucose uptake in L6 muscle cells, promoted AMPK activation and increase GLUT4 translocation into the plasma membrane. Abesundara *et al*. (2004) reported that *Cassia auriculata* extracts exhibit potential antihyperglycemic effects in Sprague-Dawley rats.

Prolonged incubation time increased glucose uptake with leaf extract while increase in concentration of root extract decreased glucose uptake at 24 hrs. This might be due to some compounds within the root extract that may possess antagonistic

effects at high concentration in combination with insulin at prolonged incubation time. At 3 hrs of incubation glucose uptake of cells exposed to the plant extracts were comparable. At incubation time of 3 and 24 hrs glucose uptake results at concentration of 2.5 µg/ml were comparable with the root extract, with a similar trend observed at 25 µg/ml, although with decrease in uptake.

The antagonistic effects of the root extracts in combination with insulin might have interfered with glucose uptake, hence the slight decrease observed. In addition, the effect of the acetone extracts on the translocation of GLUT4 to the cell membrane was evaluated using qualitative and quantitative immunofluorescence. Firstly, both the NIH-3T3-L1 preadipocytes and C2C12 muscle cells were subjected to differentiate in order for the cells to be able to translocate GLUT4 to the cell membranes. The qualitative and quantitative fluorescence results showed GLUT4 to be translocated to the cell membrane. For the quantitative study, the quantity of fluorescence was represented as fold with regard to the positive control. The leaf extract at a concentration of 25 µg/ml had more fold as compared to other extracts, indicative that more GLUT4 was translocated at this concentration of the leaf extract. This might be due to AKT which is a pivotal insulin-signalling protein responsible for GLUT4 expression and translocation (Mazibuko *et al*., 2012). The expression of GLUT4 protein was determined using western blot analysis. GLUT4 protein was isolated from C2C12 muscle cells treated with acetone root and leaf extracts in combination with insulin. The acetone root and leaf extracts increased protein expression of GLUT4 at 3 hrs incubation time as compared to other incubation times in insulin-stimulated C2C12 muscle cells.

6.1. General conclusion

The root and leaf extracts of *S. italica* showed the presence of various phytochemical constituents. TLC profile revealed the presence of more compounds in the root than in the leaf extracts. The root and leaf extracts were shown to contain high quantity of compounds when methanol was used as an extractant while ethyl acetate and *n*-hexane extracted the least. The root extract showed more antioxidant activity as compared to the leaf extract. This may be due to polar compounds that may be present in the root extract. However, total phenolic content was observed to be

higher in the leaf extract than in the root extract, possibly due to the contribution of phenolic compounds towards the nutritional value of this plant in terms of flavor, taste, aroma and color. The acetone root and leaf extracts revealed the presence of various secondary metabolites. Compounds such as flavonoids, terpenes, tannins, steroids, reducing sugars, flavonoid aglycones, phytosterols and alkaloids were shown to be present in both the root and leaf extracts, with the absence of saponins. More so, glycosides were observed only in the leaf extract and glycoflavones only in the root extract. The presence of these constituents may contribute towards various activities associated with this plant. The acetone extracts employed had no significant reduction on the viability of C2C12 muscle cells. The root extract at 24 and 48 hrs together with the leaf extract at 24 hrs were not toxic to C2C12 muscle cells at the concentration used in the study. The extracts were shown to enhance proliferation of the C2C12 muscle cells. Furthermore, differentiation of adipose cells as well as glucose uptake of muscle cells might have been enhanced by acetone plants extracts. At incubation time of 3 and 24 hrs glucose uptake results at concentration of 2.5 µg/ml were comparable with that of root extract, with a similar trend at 25 µg/ml observed, although with decrease in glucose uptake. The antagonistic effects of the root extract with insulin combination might have interfered with glucose uptake and as such a slight decrease was observed. The expression level of GLUT4 protein determined in insulin-stimulated C2C12 muscle cells treated with plant extracts in combination with insulin was shown to be enhanced at 3 hr incubation. The acetone leaf extract was shown to have good effect on differentiation, glucose uptake and GLUT4 expression of glucose storage cells. Further studies are however required to elucidate the molecular mechanism by which the acetone leaf extract of *S. italica* influences the glucose uptake and translocation of GLUT4.

CHAPTER 7

7. References

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APPENDIX

TLC profiles

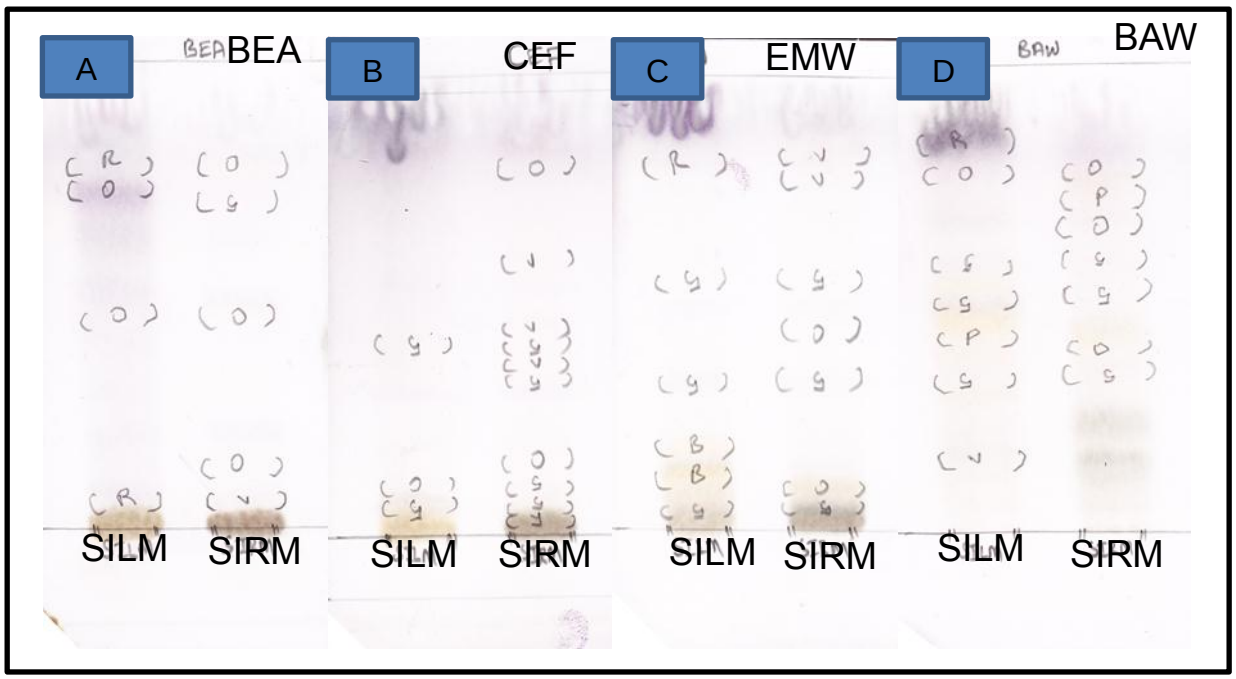

Figure 16: Thin layer chromatograms of methanol root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with vanillin/H_2SO_4 and heated in an oven at 110°C for colour development.

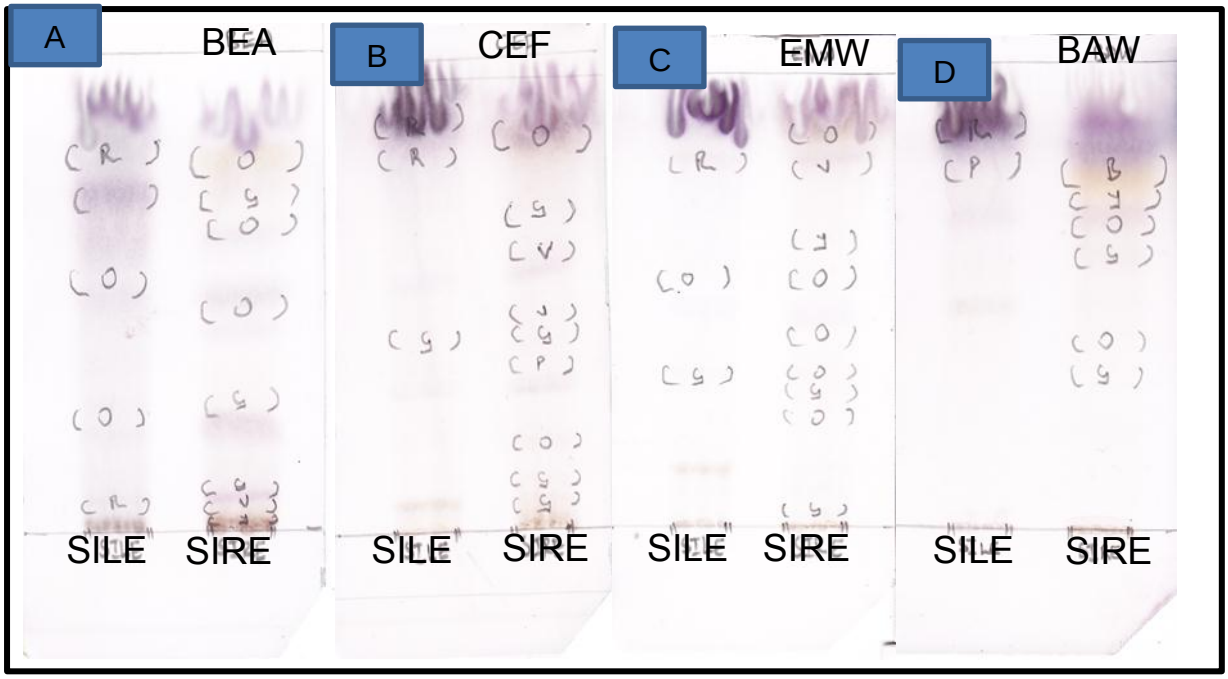

Figure 17: Thin layer chromatograms of ethyl acetate root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with vanillin/H_2SO_4 and heated in an oven at 110°C for colour development.

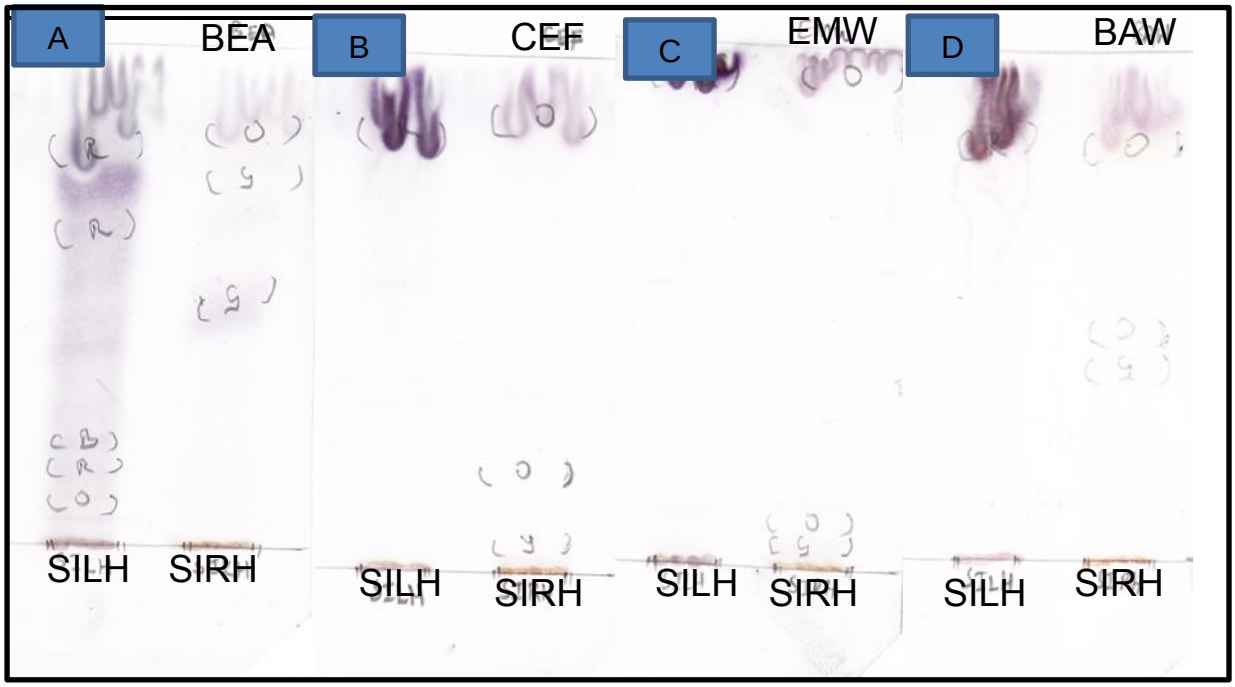

Figure 18: Thin layer chromatograms of *n*-hexane root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with vanillin/H_2SO_4 and heated in an oven at 110°C for colour development.

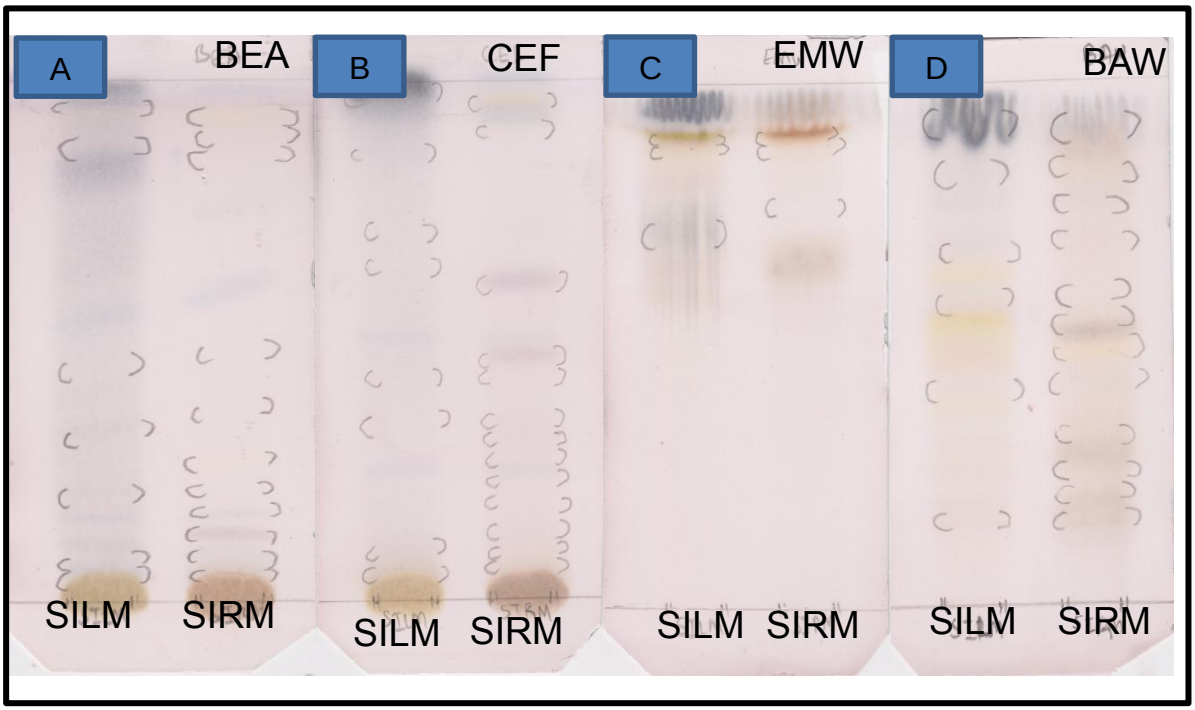

Figure 19: Thin layer chromatograms of methanol root and leaf extracts obtained from *S. italica* developed in BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with *p*-anisaldehyde and heated in an oven at 110°C for colour development.

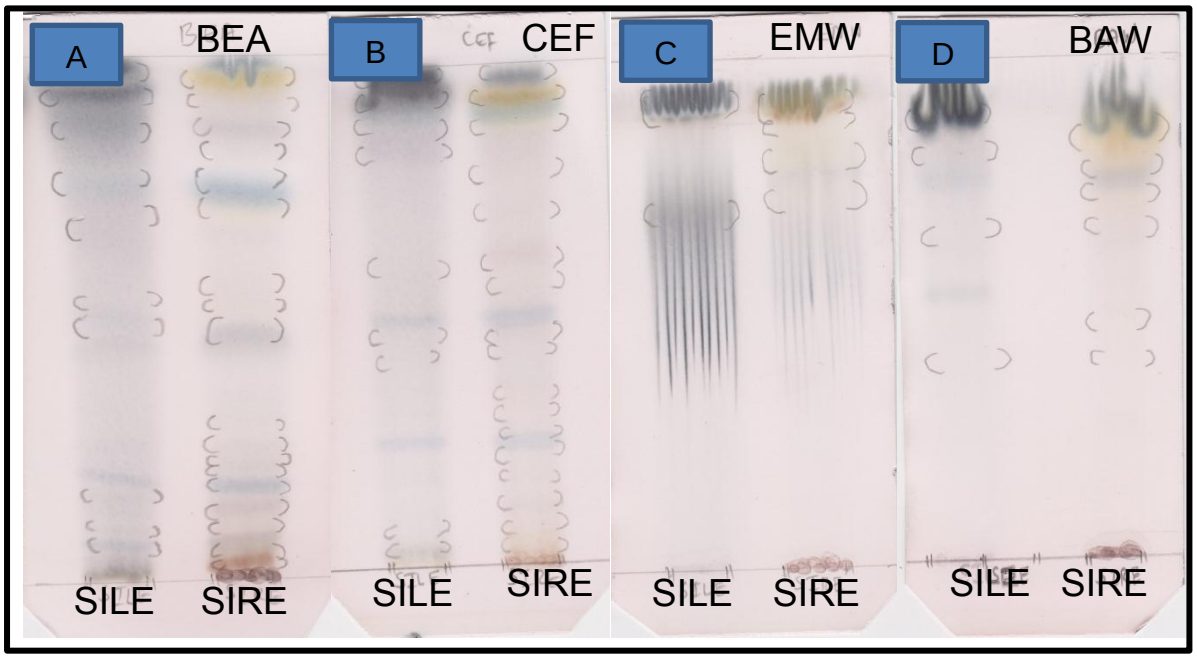

Figure 20: Thin layer chromatograms of ethyl acetate root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with *p*-anisaldehyde and heated in an oven at 110°C for colour development.

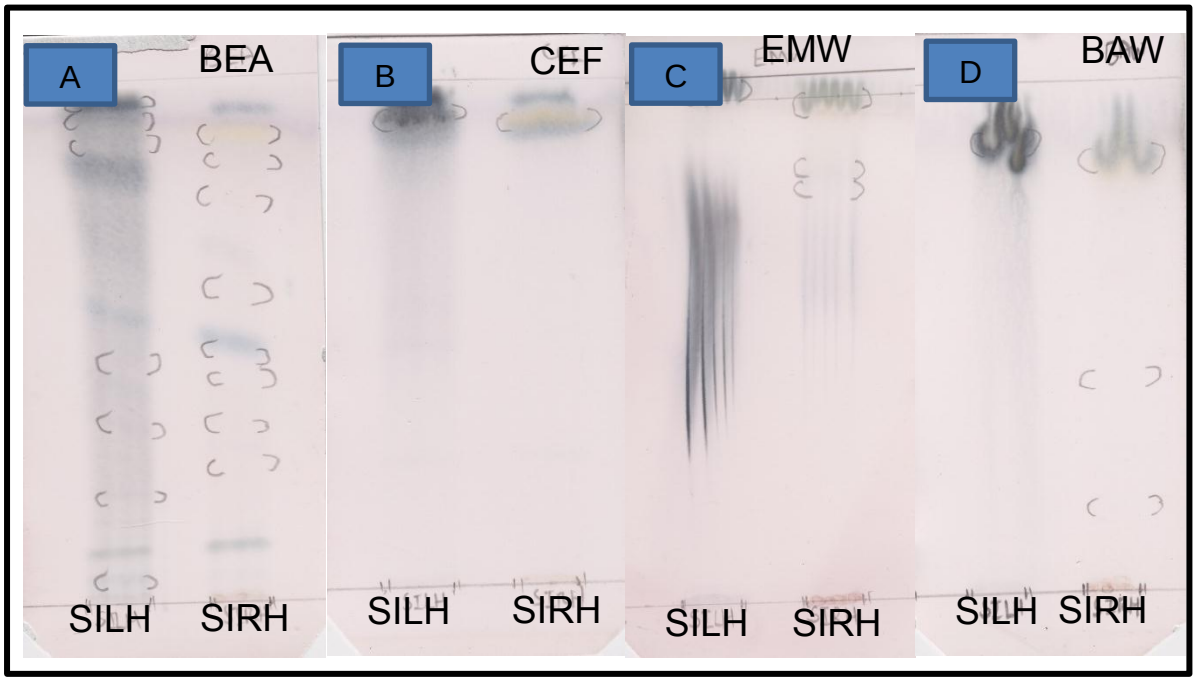

Figure 21: Thin layer chromatograms of *n*-hexane root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were sprayed with *p*-anisaldehyde and heated in an oven at 110°C for colour development.

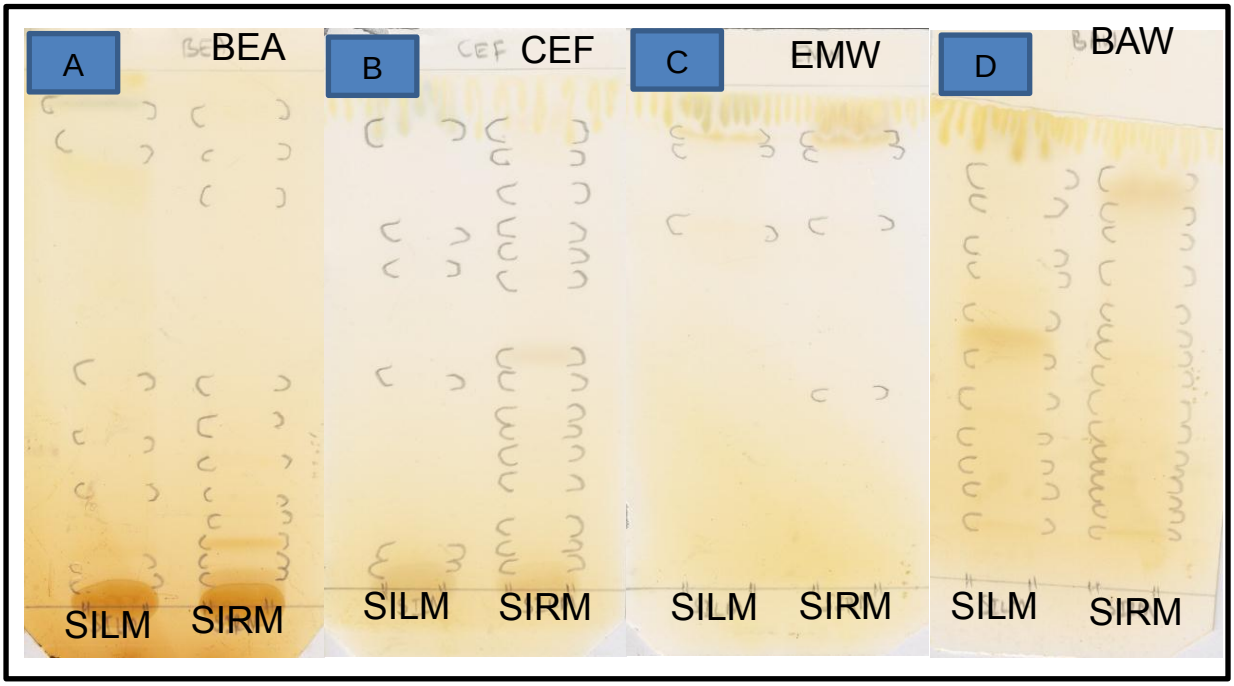

Figure 22: Thin layer chromatograms of methanol root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF(B), EMW (C) and BAW (D) mobile phases. The chromatograms were immersed in vapours of iodine crystals for colour development.

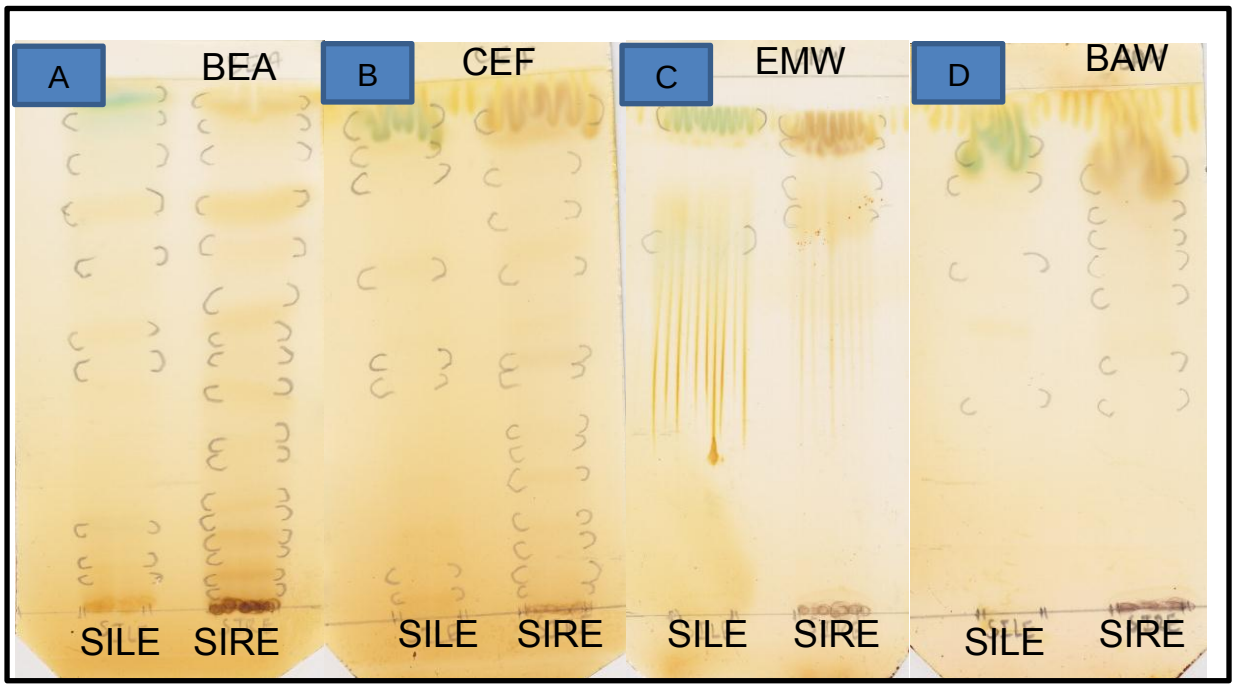

Figure 23: Thin layer chromatograms of ethyl acetate root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) mobile phases. The chromatograms were immersed in vapours of iodine crystals for colour development.

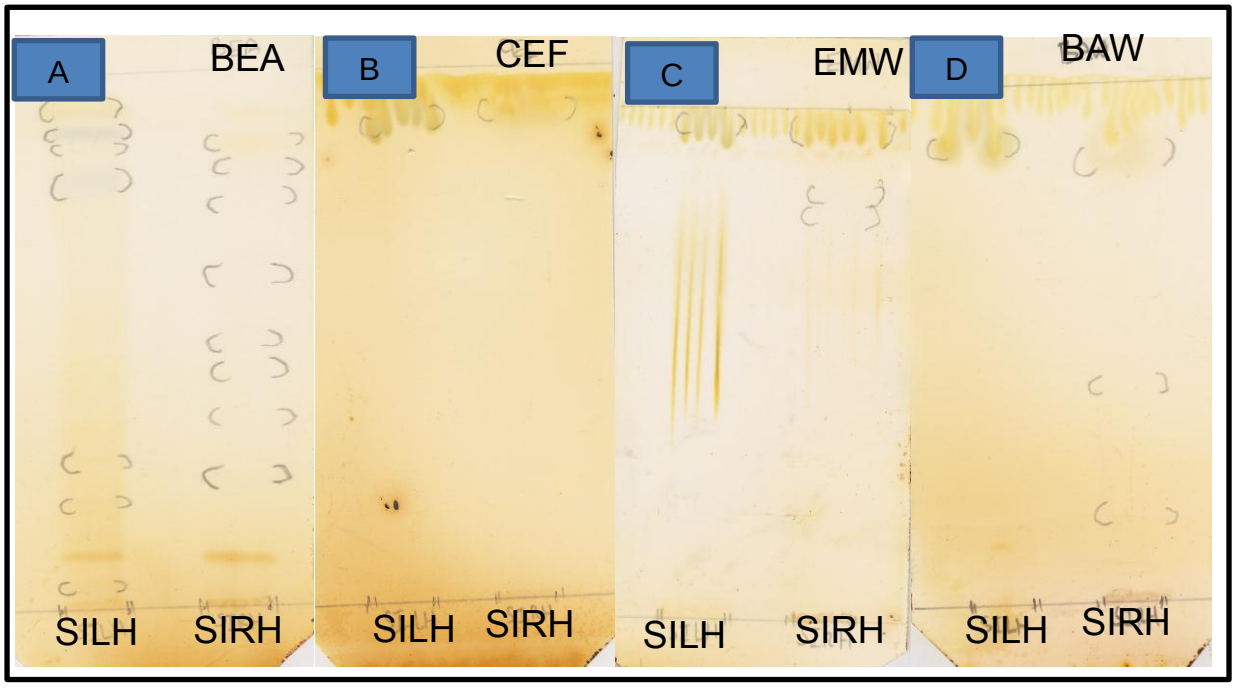

Figure 24: Thin layer chromatograms of *n*-hexane root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW(C) and BAW (D) mobile phases. The chromatograms were immersed in vapours of iodine crystals for colour development.

Qualitative antioxidant activity

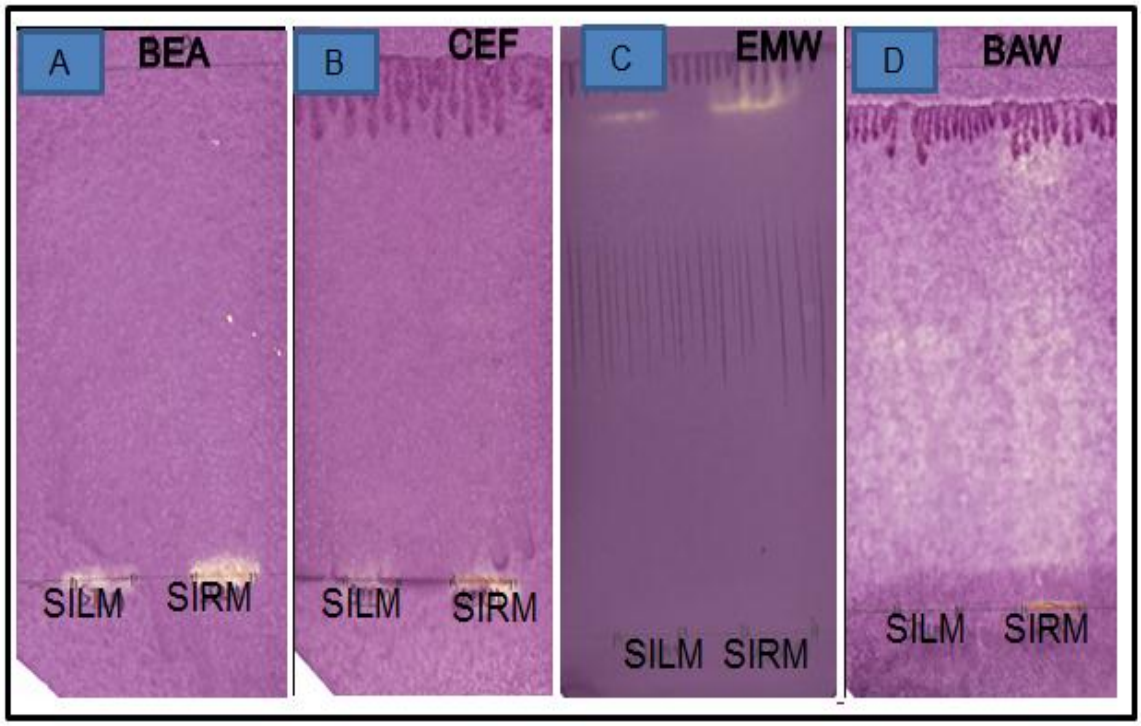

Figure 25: Thin layer chromatograms of methanol root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) as mobile phases. The chromatograms were sprayed with 0.2% DPPH.

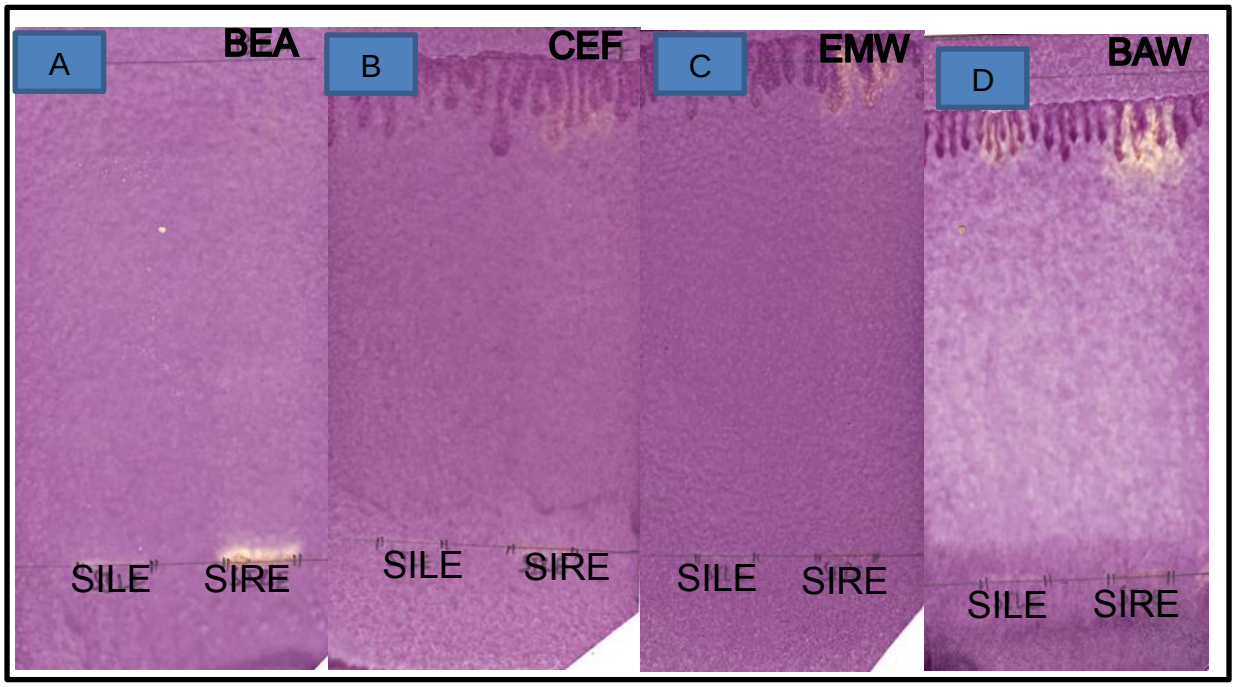

Figure 26: Thin layer chromatograms of ethyl acetate root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) as mobile phases. The chromatograms were sprayed with 0.2% DPPH.

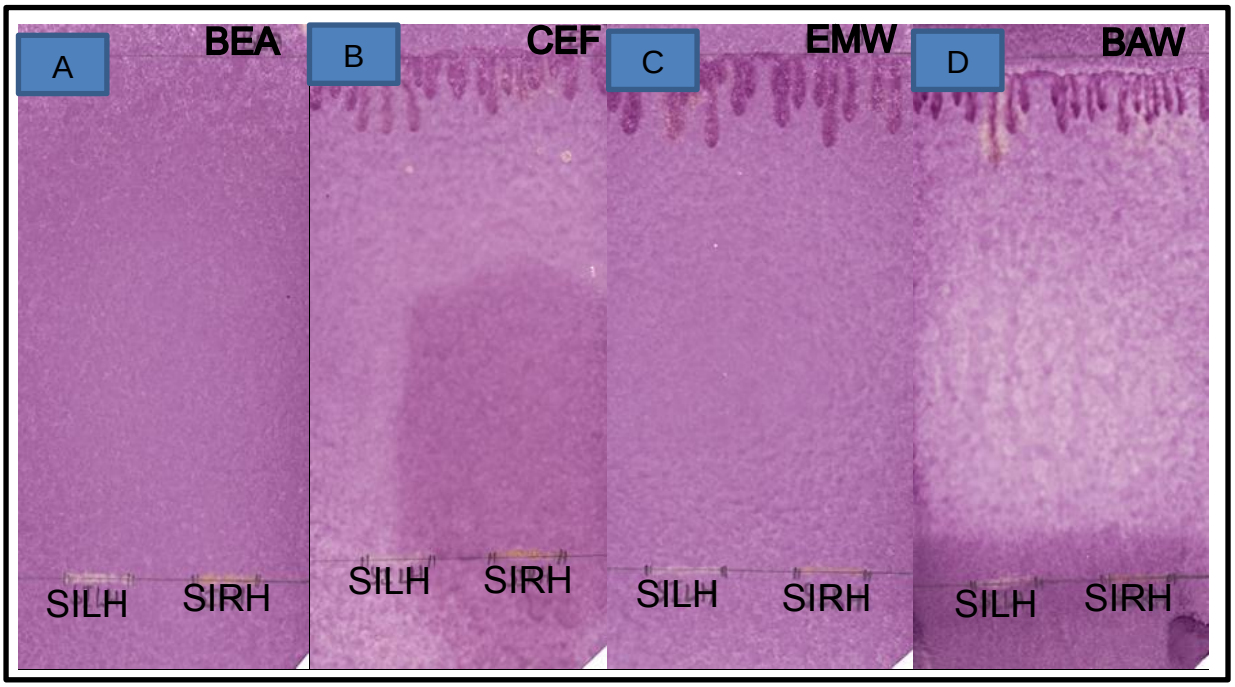

Figure 27: Thin layer chromatograms of *n*-hexane root and leaf extracts obtained from *S. italica* developed with BEA (A), CEF (B), EMW (C) and BAW (D) as mobile phases. The chromatograms were sprayed with 0.2% DPPH.