A comparative study of the *in vitro* antidiabetic properties, cytotoxicity and mechanism of action of *Albuca bracteata* and *Albuca setosa* bulb extracts

University of Fort Hare

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A comparative study of the *in vitro* antidiabetic properties, cytotoxicity and mechanism of action of *Albuca bracteata* and *Albuca setosa* bulb extracts

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DEDICATION

MY LOVING WIFE AND WONDERFUL CHILDREN

DECLARATION

I, Samuel Wale Odeyemi, declare that this thesis and the work therein submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences was carried out by me and it has never been submitted for any degree at this University or any other University.

I am completely aware of the University of Fort Hare policy on plagiarism and I have taken every necessary precaution to comply with the regulations of the University and that all sources of materials used for this thesis have been duly acknowledged.

I also declare that, I am aware of the University of Fort Hare Policy on research ethics and have taken every necessary precaution to comply with the regulations of the University. There was no need for ethical clearance for this work after ethical clearance application was filed.

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Signature: _____

Date: _____

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LIST OF ABBREVIATIONS

ABTS	-2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid
ADA	-American Diabetes Association
AGEs	-Advanced Glycation End Products
AMPK	- 5' Adenosine Monophosphate-activated Protein Kinase
ATCC	-American Type Culture Collection
ATP	-Adenosine Triphosphate
BHA	- Butylated Hydroxy Anisole
BHT	- Butylated Hydroxy Toluene
BSA	-Bovine Serum Albumin
CAP	-Cbl Associated Protein
Cbl	-Casitas B-lineage Lymphoma
CDA	-Canadian Diabetes Assciation
COX-2	-Cyclooxygenase-2
DMEM	-Dulbecco's Modification of Eagle's Medium
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
DNP	- 2,4-Dinitrophenol
DPPH	- 2, 2-diphenyl-1-picrylhydrazyl
EDTA	-Ethylenediaminetetraacetic acid
EMEM	-Eagle's Minimal Essential Medium
eNOS	-endothelial Nitric Oxide Synthase
Factor $-_{k}$B	- Nuclear factor kappa B
FBS	-Fetal Bovine Serum
FCS	-Fetal Calf Serum
FFA	-Free Fatty Acid

GIP	-Gastric Inhibitory Polypeptide or Glucose-Dependent Insulinotropic Polypeptide
GLP-1	-Glucagon-Like Peptide-1
GLUT	-Glucose Transporter
GPx	- Glutathione Peroxidase
GRE	-Griseofulvin
GSH	-Glutathione
HCl	-Hydrochloric acid
HEPES	-N-[2-Hydroxyethyl]piperazine-N- [2- ethanesulfonic acid]
HPLC	-High-performance liquid chromatography
IC50	- Inhibitory concentration at 50%
ICLAC	-International Cell Line Authentication Committee
IDDM	-Insulin-Dependent Diabetes Mellitus
INT	- 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium
IRS1	-Insulin Receptor Substrate 1
IRS2	-Insulin Receptor Substrate 2
$K_3Fe(CN)_6$	- Potassium Ferricyanide
LDH	-Lactate Dehydrogenase
m	-Milli
M	-Molar
MAPK	- Mitogen-Activated Protein Kinases
MTT	-3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
n	-Nano
NaCl	-Sodium chloride
$NaCO_3$	- Sodium carbonate
NADH	- Nicotinamide Adenine Dinucleotide
NDGA	-Nordihydroguaiaretic Acid

NIDDM	- Non insulin-dependent diabetes mellitus
NSAIDs	- Nonsteroidal anti-inflammatory drugs
P	- Probability
PBS	-Phosphate buffered saline
PBSA	-Phosphate buffered saline (without Ca^{2+} and Mg^{2+})
PI3K	- Phosphoinositide 3-kinase
PLA_2	- Phospholipase A2
PMS	- Phenazinemethosulfate
PPARγ	-Peroxisome Proliferator-Activated Receptor γ
R^2	- Correlation coefficient
ROS	-Reactive Oxygen Species
rpm	-Revolutions Per Minute
RPMI 1640	-Roswell Park Memorial Institute Culture Medium
SD	-Standard deviation
SOD	-Superoxide dismutase
T2DM	-Type 2 diabetes mellitus
TBARS	-Thiobarbituric acid Reacting Substance
TCA	-Trichloroacetic acid
TLC	-Thin Layer Chromatography
TNF – α	-Tumor Necrosis Factor
TNFα	-Tumor necrosis factor α
TPTZ	- 2, 4, 6-Tripyridyl-s-Triazine
Tris	-Tris (hydroxymethyl) aminomethane
TZDs	-Thiazolidinediones
µ	-Micro
UV	-Ultral violet
WHO	-World Health Organisation

ABSTRACT

The search for cheap, non toxic and readily available antidiabetic drugs has been a challenge for researchers and the pharmaceutical industries. Diabetes mellitus is a metabolic disease characterized by defects in the synthesis of insulin and/or insensitivity to the action of insulin at the target cells. The disease has been on the increase mostly in developing countries where large proportions of the population have little access to good medical care due to either accessibility or non availability of synthetic drugs. This has led to the use of medicinal plants to treat diabetes because it is safe, cheap and with few side effects. There is little scientific evidence on the dosages, active compounds, mechanisms of action and toxicity of these traditionally used plants.

Two of the most frequently used plants; *Albuca setosa* and *Albuca bracteata* were investigated in this study. The qualitative analysis of different extractions of these plants revealed the presence of phenolics, alkaloids, tannins and saponins. The antioxidant properties of aqueous, acetone and methanollic extracts of *Albuca setosa* and *Albuca bracteata* were investigated using models such as Diphenyl-1-Picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Ferric ion reducing antioxidant potential (FRAP), Nitric Oxide and Hydrogen Peroxide (H_2O_2). Both plants revealed inhibitions against DPPH in a concentration - dependent manner with *Albuca setosa* (0.330 mg/ml) showing higher activity than *Albuca bracteata* (0.647 mg/ml) determined from the IC_{50}. The aqueous extract of *Albuca setosa* showed a higher inhibition against DPPH radical compared to the *Albuca bracteata* aqueous extract at all concentrations investigated. The isolated saponins from *Albuca bracteata* had a higher DPPH scavenging activity than the crude methanolic extract of the plant in a concentration - dependent manner but are significantly different from each other at 0.4, 0.6 and 1.0 mg/ml only. The IC_{50} of the saponins was also observed to be higher than the crude extracts and standards.The *Albuca*

setosa aqueous extract showed a higher percentage inhibition of ABTS radicals than *Albuca bracteata* at all the concentrations investigated. Overall, the *Albuca setosa* aqueous extract (0.0809 mg/ml) showed maximum activity against ABTS radicals. The iron reducing power was significantly higher ($P < 0.05$) in the methanolic extract of both plants compared to the aqueous counterpart. Overall, the *Albuca bracteata* aqueous extract (0.344 mg/ml) showed maximum activity as indicated by the IC_{50}. The aqueous extracts of both plants also revealed percentage inhibitions in a concentration - dependent manner against NO_2. The aqueous extract of *Albuca bracteata* bulb was more active against nitric oxide and hydrogen peroxide inhibition. In this study, the cytotoxicity of the extracts was evaluated at a high dose of 100 µg/ml on Chang liver cells and determined using MTT, crystal violet, glucose consumption, lactate production and lactate dehydrogenase release and FRAP. The aqueous extracts of both *Albuca setosa* and *Albuca bracteata* were non-toxic on Chang liver cells at the concentrations investigated. The MTT revealed that the aqueous extract of *Albuca setosa* bulb had the optimum cell viability of 108.09% while the acetonic extract of *Albuca bracteata* showed the least cell viability (37.72%) compared with the control. The crystal violet test also revealed the acetone extract of *Albuca bracteata* to have the least percentage of cell viability at 31.47%, while the aqueous extract of *Albuca setosa* showed the maximum cell viability at 112.5%.The aqueous extracts of both plants showed higher percentage cell density on the second day of incubation from the proliferation assay. All the tested samples were observed to consume more glucose than the blank except for the methanollic and acetone extracts of *Albuca bracteata* bulb. The aqueous and methanolic extracts of *Albuca setosa* bulbs produced the highest lactate with 120.2 µg/ml and 113.7 µg/ml respectively. The acetone extracts of both *Albuca setosa* and *Albuca bracteata* revealed toxicity with a higher lactate dehydrogenase release compared to the control. The aqueous extracts of both *Albuca setosa* and *Albuca bracteata* bulbs showed reducing power of 18.7 and 22.1 µmol/l of $FeSO_4$

respectively. The *in vitro* antidiabetic potentials investigated revealed that the aqueous extracts of both plants inhibited the activity of α- glucosidase but have weak inhibition against α- amylase. The acetone extract of *Albuca bracteata* showed the highest inhibition against the alpha amylase followed by the acetone extract of *Albuca setosa.* Both plant extracts also showed weak inhibition against the dipeptidylpeptidase-IV (DPP-IV) at the concentration investigated. The glucose uptake in HepG2 for both aqueous and methanol extracts of *Albuca setosa* were not significantly different from the control but the MTT result of the treatments in HepG2 for both *Albuca setosa* and *Albuca bracteata* aqueous extracts revealed low cell density compared to the control. The aqueous extract of *Albuca setosa* demonstrated glucose utilization in L6 cells with a response of 188.93% of the control at 25 μg/ml while the aqueous extract of *Albuca bracteata* demonstrated glucose utilisation in L6 with a response of 89.17% of the control. The MTT of the treatments in L6 revealed no sign of cytotoxicity, as the cell density of all the treatments were not significantly different from the control. The aqueous extracts of both *Albuca setosa* and *Albuca bracteata* also showed high glucose utilization in 3T3-L1 cells with 123.21% and 131.56% respectively.

In conclusion, *Albuca setosa* possesses better *in vitro* strong antidiabetic activity compared to *Albuca bracteata.* The mechanism of glucose utilization in L6 and 3T3-L1 of *Albuca setosa* and *Albuca bracteata* may be due to the activation of some molecules in the insulin signaling pathway, while the *in vitro* antidiabetic activity of *Albuca bracteata* may also involve its inhibitory effect on α-glucosidase. The antioxidant properties of both plants may also play an important role in ameliorating the complications of diabetes. These findings therefore support the folkloric usage of these plants for the management of diabetes mellitus.

INTELLECTUAL PROPERTY AGREEMENT STATEMENT

COMPLIANCE STATEMENT

This thesis is meant to be used for information dissemination, therefore no part of this study in any form has been commercialized. The study elucidates on the medicinal potentials of *Albuca setosa* and *Albuca bracteata* to the immediate community and the entire Eastern Cape Province of South Africa.

_____ _____

Supervisor signature Student signature

"Life is short, art is long, the right moment soon speeds past, experience deceives, and judgment is difficult!"

- Hippocrates

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 Medicinal Plants and Disease Management

The aim of recent research in medicinal plants for the treatment of diseases has been to find alternative control strategies to reduce our dependence on synthetic drugs because plants have been used for medicinal purposes due to their diversity and the synthesis of various secondary metabolites such as phenols, flavonols, phenolic acids, quinones and tannins (Cowan, 1999). In 2000, the World Health Organization estimated that 80% of the world's population relied on traditional medicine mostly derived from medicinal plants for their health needs (WHO, 2000). In most developed countries traditional remedies are now used as alternatives to modern medicine (Schippmann, et al., 2002). India is one of the biodiversity hot region in the world; one fifth of the plants found in India are used for medicinal purposes (Schippmann et al., 2002). Plant based formulations are now on the increase in rural areas in India and plant export products have increased between 1995 and 2000 (Singh, et al., 2011). Singh et al., (2011) reported that there are about US $62 billion worth of sales of herbal remedies in the world and it is expected to rise to US $3 trillion by 2020.

Medicinal plants are important in pharmacological research and drug development, not only when the constituents are used directly as therapeutic agents but also as starting materials for drug synthesis or as models for pharmacologically active compounds (Mukherjee & Verpoorte, 2003). Sales of plant - derived chemicals such as those used as pharmaceutical, fragrance, flavour, and colouring ingredients in the world market exceed billions of dollars every year (Mukherjee & Verpoorte, 2003).

1.2 BACKGROUND TO DIABETES MELLITUS

It was the Egyptian physician Hesy-Ra of the 3rd Dynasty that makes the first known mention of diabetes (found on the Ebers Papyrus) and listed remedies to combat the ‘passing of too much urine (CDA, 2012).

Diabetes received its name from a Greek physician, Aretaeus of Cappadocia, after the word dia-bainein which means “to siphon” that is to pass through. This is because in diabetes excess sugar is found in the blood and urine and it was once referred to as “pissing evil” (Sattley, 2008).

Thomas Willis, an English physician in the 17th century, diagnosed his patients with diabetes by examining their urine for a sweet taste he diagnosed as “honeyed diabetes” He added the term “mellitus” to diabetes (Sattley, 2008). Bouchardat and Lancereaux (1880), differentiated between diabetes gras (Fat) and diabetes maigre (Thin). In 1921, Frederick Banting and Charles Best (Banting et al., 1922) treated a diabetic dog with a murky concoction of canine pancreas extract and kept it alive for 70 days and used a more refined extract to treat a young boy dying of diabetes, although the injected extract did not have resounding beneficial effects. This Banting and Best concoction was later found to contain insulin which led to the use of insulin for the treatment of diabetes. Ever since insulin was discovered, medical breakthroughs in research have continued to prolong and ease the lives of people with diabetes (Sattley, 2008).

In 1935, Roger Hinsworth differentiated between type I and typeII diabetes. He described Type I as "insulin sensitive" and Type II "insulin insensitive". By differentiating between the two types of diabetes, the Hinsworth discovery helped open up new treatment approaches to diabetes mellitus (Sattley, 2008).

It is more than three thousand years since Aretaeus spoke of diabetes as "the mysterious sickness." It has been a long and strenuous process of discovery, through a collective knowledge attempts to find a cure. At present, a lot is still to be done and another major breakthrough is awaited.

1.3 RATIONALE AND JUSTIFICATION OF THE STUDY

There is as yet no effective cure for diabetes and the available drugs and insulin currently used in managing the disease are associated with several undesirable side effects (Kumar, et al., 2006; Piédrola, et al., 2001; Yaryura-Tobias, et al., 2001). These undesirable side effects, coupled with the high cost of anti-diabetic drugs has led to the search for plants with hypoglycemic properties and consequently their use in the management of diabetes (Calixto, 2000; WHO, 2002).

Diabetes mellitus is an iceberg disease that is the disease show initial harmless symptoms which later have fatal consequences. This results in few early – stage patients seeking treatment while the majority of patients are either unaware of their condition or find alternative treatment methods. There is an increase in the prevalence of diabetes mellitus in the world, there were an estimated 285 million cases in 2010 and these are predicted to rise to 439 million adults cases by 2030 (Shaw, et al., 2010). In industrialized and developing countries such as South Africa the disease is generally acquired in the most productive period of life. There is inadequate awareness and increased susceptibility to diabetes mellitus among South Africans due to dietary and sedentary life style, such as in take of too much sugar,.

Diabetes treatment options are increasingly expensive, not readily available and frequently have side effects such as diarrhoea, weight gain, hypoglycaemia and abnormal liver function (ADA, 2006a).There is thus the need to search for cheaper sources of anti-diabetic agents in natural plants. *Albuca bracteata* and *Albuca setosa* has reportedly used by

traditional healers in the Eastern Cape Province to treat diabetes mellitus (Oyedemi at al., 2009; van Huyssteen et al., 2011).

1.4 THE CHOICE OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* JACQ. FOR THE STUDY

Albuca bracteata and *Albuca setosa* Jacq. have been reported to be used for the treatment of diabetes by traditional healers in the Eastern Cape Province, South Africa (van Huyssteen et al., 2011). *Albuca bracteata* and *Albuca setosa (*Hyacinthaceae) are both known as Ingwe beba(in Xhosa) and both are bulbs (monocot). Because they share the same local name there is confusion in differentiating both plants by the local people. *A. setosa* is moderately stout with about 6- 9 leaves. It occurs commonly from Cape Province to Kwazulu-Natal South Africa where it is widely used. *Albuca setosa* (Figure 1.1) is sometimes referred to as *Albuca pachychlamys* (Baker), *Ornithogalum setosum* (Jacq) inhabits terrestrial, rocky slopes and flats up to an altitude of 2400 m (Williams et al., 2008). It is used for traditional medicinal purposes and is available in some local markets across South Africa (Dold & Cocks, 2002). *A. setosa* can be propagated through the seeds and with the adult plant having bright coloured flowers (Figure 1.1).*A. setosa* can be pollinated by bees and shares the same mode of pollination with all other *albucas* (Johnson, et al., 2012).

Albuca bracteata (Figure 1.2), also referred to as *Ornithogalum brateatum* (Thunb.), *Ornithogalum caudatum* (Aiton) or *Ornithogalum longibracteatum* (Jacq) is found in terrestrial forest, fynbos, closed woodland and sheltered slopes (Raimondo et al. 2009; von Staden 2012). Despite the reported folkloric usage of *A. bracteata* and *A. setosa* for the management of diabetes, there is dearth of scientific information on *A. setosa*. Both plants are soaked in water and the concoction taken orally. Although the glucose uptake activity of *A. bracteata* has been reported there is little information about the phytochemical, toxicity and mechanism of action of the plant (van Huyssteen et al., 2011).

Therefore, this study is aimed at providing information on the antidiabetic, cytotoxicity, probable mechanisms of action of these plants with a view to validating its acclaimed use by the traditional healers of the Eastern Cape.

Figure 1.1: *Albuca setosa* plant

Figure 1.2: *Albuca bracteata* plant

1.4.1 Hypothesis

We hypothesize that

- *Albuca bracteata* and *Albuca setosa* will enhance glucose uptake in the cell lines
- *Albuca bracteata* and *Albuca setosa* have different medicinal potentials for the management of diabetes mellitus.

1.4.2 Aim of Study

The primary aim of this study was to validate the folkloric uses of *Albuca setosa* and to compare its antidiabetic property with that of *Albuca bracteata*.

1.4.3 Specific Objectives

1. To determine the anti-diabetic potentials of different solvent fractions of the plant extracts
2. To identify compounds for hypoglycaemic activities
3. To determine the mechanism of action of *Albuca bracteata* and *Albuca setosa*
    a. To determine the effect of the plant extracts on some enzymes involved in glucose metabolism
    b. To determine the *in vitro* antioxidant activity of the plant extracts
4. To compare the glucose uptake activity of *Albuca bracteata* with *Albuca setosa*
5. To carry out an *in vitro* cytotoxicity evaluation of the plant extracts using cell lines
6. To evaluate and compare this anti-diabetic potential of the plants with a reference drug

REFERENCES

ADA (American Diabetes Association). (2006a). Diagnosis and classification of diabetes mellitus. *Diabetes Care*, *29 Suppl 1*, S43–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16373932. Access date: 2014-08-17

ADA (American Diabetes Association). (2006b). Third-Party reimbursement for diabetes care, self-management education, and supplies. *Diabetes Care*, *29*(1): S68-S69.

Banting, F.G, Best, C.H, Collip, J.B, Campbell, W.R, and Fletcher A.A (1922). Pancreatic Extracts in the Treatment of Diabetes Mellitus. *Canadian Medical Association Journal*. 12 (3): 141-146

Bouchardat, A and Lancereaux, E (1880). Le diabetes maigre; ses symptoms, son evolution, son prognostic et son traitement; ses rapports avec les alterations du pancreas. *Union Medical.* 29: 161-168.

Calixto, J. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazillian Journal of Medical and Biological Research*, *33*, 179–189.

CDA. (2012). History of Diabetes. Retrieved September 8, 2013, from http://www.diabetes.ca/about-diabetes/history-of-diabetes#page-content Access date: 2013-09-08

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*(4), 564–82. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=88925&tool=pmcentrez&rendertype=abstract Access date: 2014-08-16

Dold, A., & Cocks, M. (2002). The trade in medicinal plants in the Eastern Cape Province, South Africa. *South African Journal of Science*, *98*(December), 589–597. Retrieved from http://reference.sabinet.co.za/sa_epublication_article/sajsci_v98_n11_12_a19 Access date: 2014-06-20

Johnson, S. D., Jürgens, A., & Kuhlmann, M. (2012). Pollination function transferred: modified tepals of Albuca (Hyacinthaceae) serve as secondary stigmas. *Annals of Botany*, *110*(3), 565–72. http://doi.org/10.1093/aob/mcs114

Kumar, G., Arulselvan, P., Kumar, D., & Subramannian, S. (2006). Anti-diabetic activity of fruits of *Terminalia chebula* on streptozotocin induced diabetic rats. *Journal of Health Science*, *52*(3), 283–291.

Mukherjee, P., & Verpoorte, R. (2003). *GMP for Indian systems of medicine. In: MUKHERJEE, P.K. and VERPOORTE, R. eds. GMP for Botanicals: Regulatory and Quality Issues on Phytomedicines*. (P. K. MUKHERJEE & R. eds. VERPOORTE, Eds.). New Delhi: Business Horizons.

Oyedemi, S. ., Bradley, G., & Afolayan, A. J. (2009). Ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe municipality of

South Africa. *Joournal of Medicinal Plants Research*, *3*(12), 1040–1044. Retrieved from http://www.academicjournals.org/JMPR

Piédrola, G., Novo, E., Escobar, F., & García-Robles, R. (2001). White blood cell count and insulin resistance in patients with coronary artery disease. *Annales D'endocrinologie*, *62*(1 Pt 1), 7–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11240402

Raimondo, D., von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. and Manyama, P.A. (2009). Red List of South African Plants.Strelitzia South African National Biodiversity Institute, Pretoria

Sattley, M. (2008). History of Diabetes. Retrieved from http://www.diabetes.ca/diabetes-and-you/what/history

Schippmann, U., Leaman, D., & Cunningham, A. (2002). Impact of cultivation and gathering of medicinal plants on biodiversity: Global trends and issues. *FAO*, 1–21. Retrieved from ftp://ftp.fao.org/docrep/fao/005/aa010e/aa010e00.pdf

Shaw, J. E., Sicree, R. A., & Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, *87*(1), 4–14. http://doi.org/10.1016/j.diabres.2009.10.007

Singh, R., Gangwar, S.P Singh, D., Singh, R., Pandey, R., and Kalra, A. (2011). Medicinal plant Coleus forskohlii Briq.: Disease and management. *Medicinal Plants, 3 (1) pp: 1-7.*

Van Huyssteen, M., Milne, P., Campbell, E., & van de Venter, M. (2011). Antidiabetic and cytotoxicity screening of five medicinal plants used by traditional african health practitioners in the Nelson Mandela Metropole, South Africa. *African Journal of Traditional,Complementary and Alternative Medicine*, *8*(2), 150–158. http://doi.org/PMC3252689

von Staden, L. (2012).*Albuca bracteata* (Thunb.) J.C.Manning & Goldblatt.National Assessment: Red List of South African Plants version 2014.1http://redlist.sanbi.org/species.php?species=3795-156

WHO. (2002). WHO Traditional Medicine strategy. *Bulletine World Health Organization*, *80*(7), 610–610. Retrieved from http://www.who.int/bulletin/archives/80(7)610.pdf

WHO, W. health O. (2000). WHO Traditional Medicine strategy. *World Health Organisation*, *80*(7), 610–610. Retrieved from http://www.who.int/bulletin/archives/80(7)610.pdf

Williams V Raimondo D Crouch N Cunningham A Scott-shaw C Lotter M Ngwenya A. (2008). *Albuca setosa* Jacq. National assessment: Red list of South African plants *Red list of South African plants version 2014.1*. Retrieved from http://redlist.sanbi.org/species.php?species=306-4

Yaryura-Tobias, J. A., Pinto, A., & Neziroglu, F. (2001). Anorexia nervosa, diabetes mellitus, brain atrophy, and fatty liver. *The International Journal of Eating Disorders*, *30*(3), 350–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11746296

CHAPTER 2

LITERATURE REVIEW

Part of this Chapter will be submitted for publication in South African Journal of Botany

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LITERATURE REVIEW

Diabetes Mellitus

Diabetes mellitus is a disease associated with sugar, a situation where the body is unable to control effectively the metabolism of glucose, the primary source of energy. It is described as a clinical syndrome characterized by inappropriate hyperglycemia caused by a relative or absolute deficiency of insulin or resistance to the action of the hormone at cellular level (Wadkar, et al., 2008). The cells either become insensitive to the action of insulin that regulates the body fuel or there is insufficient availability of the hormone. The deficient or diminished effectiveness in the endogenously synthesized insulin thereby increase glucose concentration in the blood and urine.

Diabetes mellitus at the advanced stages affect other metabolic pathways of lipids and manifests as hypercholesterolemia and hyperlipidemia which are risk factors in atherosclerosis (Krishnakumar et al., 1999; Ross, 1999; Schwartz, 2006). As the disease progresses vital organs are affected. For example, the liver may be damaged due to increased gluconeogenesis and ketogenesis, diabetic ketoacidosis, and non-ketotic syndrome (Feliget al., 1970). The kidneys may also be affected due to excessive thirst, polyuria and nephropathy, and the effect of advanced stage diabetes on eyes may be visual blurriness or retinopathy. Excessive weight loss, hypertension and neuropathy can also be seen in the later stages of diabetes (Knentz & Nattras, 1991; Kumar & Clark, 2002). There is also an increase in the concentration of advanced glycation end products (AGEs) due to the availability of glucose. Glucose reacts non-enzymatically with proteins, lipids and nucleic acids to form AGEs leading to the secondary complications of diabetes (Kumar & Clark, 2002) such as impaired wound healing and foot ulcers among others (Ge et al., 2009).

2.1 CAUSES OF DIABETES MELLITUS

To date the cause for diabetes mellitus is still not clear, but generally the disease is associated with the ingestion of high calorie foods, family history of the disease, obesity, race, genetic disorders, smoking, inactivity, viral infections and drugs or chemicals. Each type of diabetes mellitus has its own peculiarity, for instance type 2 is often associated more with genetic predisposition than the autoimmune form of type 1 diabetes mellitus (ADA, 2006a).

2.2 SYMPTOMS OF DIABETES MELLITUS

Common symptoms of diabetes mellitus includes: frequent urination, disproportionate thirst, intense hunger and fatigue irritability, blurred vision, wounds that do not heal properly or quickly, sexual dysfunction in men, and frequent gum infection (Perkins et al., 2006).

2.3 PREVALENCE

In the recent times, there has been an increase in the prevalence of diabetes mellitus worldwide. For instance, a study in 2000 estimated that about 171 million people (2.8%) were living with the disease and it was projected that about 366 million people (4.8%) will be affected with the disease by the year 2030 if necessary and adequate actions are not taken (Wild, et al., 2004). A recent study in 2015 however, suggests that 415 million adults are living with the disease and that this will rise to about 642 million by the year 2040 (IDF, 2015).

The African region has the highest proportion of undiagnosed diabetes, as investments, research, and health systems are slow to respond to this burden. Available information suggests that diabetes is emerging as a major health problem in Africa, including South Africa (Mbanya, et al., 1996).

In 2011, it was estimated that the most populous African countries have the highest number of people with diabetes with Nigeria having 3.0 million followed by South Africa with 1.9 million which make up just over half of the total number in the region (IDF, 2011).

It is imperative to constantly reassess glycemic control in people with diabetes due to the progressive nature of the disease which requires constant therapeutic regimen readjustment. Several drugs such as biguanides and sulfonylurea which are presently employed in the management of diabetes have side effects such as worsening of heart disease, increased body weight and hypoglyceamia (Dey, et al., 2002; Piédrola et al., 2001). These side effects coupled with the high cost of anti-diabetic drugs has led to the search for plants with anti-diabetic properties and consequently their use in the management of diabetes (Calixto, 2000; WHO, 2002a).

2.4 TYPES OF DIABETES

There are different types of diabetes, so assigning a type of diabetes to an individual usually depends on the circumstances present at the time of diagnosis (ADA, 2006a). There are three types of diabetes among others. These are type 1 (IDDM or insulin dependent diabetes mellitus), type 2 (NIDDM or non-insulin dependent diabetes mellitus), gestational diabetes and other specific types (ADA, 2006).

2.4.1 DIABETES MELLITUS TYPE 1

Type 1 diabetes or insulin dependent diabetes mellitus (IDDM) is caused by cellular-related autoimmune destruction of the beta cells where the body produces relatively low or no insulin and external insulin injection is required for survival. IDDM accounts for 5-10% of people living with diabetes (Ranjan & Ramanujam, 2002). This immune mediated diabetes mellitus is most common during childhood or adolescence but can occur at any age. It is

therefore referred to as juvenile diabetes mellitus. Common symptoms of type 1 diabetes include increased urination, thirst and life-threatening ketoacidosis may occur if diagnosis and treatment does not occur early enough (Loghmani, 2005). Individuals with type 1 diabetes are rarely obese. Ketoacidosis is common due to insulin injection and sufferers are prone to other autoimmune disorders such as Grave's disease, autoimmune hepatitis and Addison's disease (ADA, 2006b).

2.4.2 DIABETES MELLITUS TYPE 2

Type 2 diabetes mellitus or non-insulin dependent diabetes mellitus (NIDDM) is caused by a combination of factors primarily insulin resistance, a situation in which the body tissues such as muscles, liver and fats are insensitive to the action of insulin, and the fact that the body is unable to produce enough insulin to compensate for its impaired ability to utilize insulin (NIH, 2011). It is the most common form and accounts for 85 – 95% in all diabetic patients above the age of 40 years (Lanza et al., 1999). As the body does produce some insulin these individuals may not require insulin treatment to survive unlike in type 1 (ADA, 2006). Ketoacidosis is therefore not common in type 2 diabetes although it may occur as a result of stress of other illness such as infection. Obesity itself is a high risk factor in insulin resistance. Insulin resistance may improve with weight reduction and drug treatment of hyperglycemia but it is seldom restored to normal (ADA, 2006). The risk of type 2 diabetes increases with age, obesity and physical inactivity.

2.4.3 GESTATIONAL DIABETES MELLITUS

Gestational diabetes is associated with the metabolic demands and hormonal changes during pregnancy together with genetic and/or environmental factors. Gestational diabetes mellitus was formerly implicated in any degree of glucose intolerance with first recognition

during pregnancy which usually resolves after delivery (ADA, 2006). Simply put, gestational diabetes is when pregnant women without previously diagnosed diabetes develop a high blood glucose level. Several pregnancy- related factors, including the hormones produced by the placenta contribute to insulin resistance which occurs in all women during late pregnancy. This condition of insulin resistance requires more insulin synthesis needed to control blood glucose, and when the beta cells are unable to produce sufficient insulin due to defect or dysfunction, gestational diabetes occurs (NIH, 2011).

2.4.4 OTHER SPECIFIC TYPES

Other specific types of diabetes mellitus include genetic defects in B cell function, genetic defects of insulin action, diseases of the exocrine pancreas, drugs or chemical induced hyperglyceamia, infections and other genetic syndromes which are associated with diabetes (ADA, 2006).

2.5 ANTIOXIDANTS AND OXIDATIVE STRESS

2.5.1 Oxidative stress as a complication of diabetes

Oxidative stress can be defined as the imbalance between the systemic production of reactive oxygen species (ROS) and the body's capacity to defend or detoxify these biologically active intermediates (free radicals) against tissue damage, or the inability to repair the damage caused by them (Dare, et al., 2014). This condition therefore shifts the body towards stress as a result of the continuous damage caused by these reactive oxygen species. ROS are either produced during normal mitochondrial respiration, phagocytosis, nitric oxide synthase activity or through exposure to UV light, ionizing radiation and pollutants (Kunwar & Priyadarsini, 2011). These disturbances in the normal redox reaction of the cell result in the production of toxic

metabolites such as peroximes and free radicals that damage proteins, carbohydrates, lipids and DNA, which are all major cell components.

Oxidative stress therefore plays a major role in aging, diabetes, cancer, Alzheimer, angina, cataract, cardiovascular diseases and other pathological conditions (Rahimi, et al., 2005).

Oxidative stress is involved in diabetes mellitus and associated complications. Oxidative stress can be induced during diabetes through the following ways:

1. Persistent hyperglycemia can result in glycation of proteins most especially antioxidant enzymes such as superoxide dismutase which will lead to oxidative stress (Wiernsperger, 2003).
2. High levels of insulin during type 2 diabetes mellitus generate H_2O_2 which contributes to further oxidative stress.
3. During diabetes the circulating catalase in the blood hampers the removal of peroxides and other ROS (Wiernsperger, 2003).
4. Insulin uptake and delivery to the cells are also affected by oxidative stress which may also be as a result of the glycation of the receptors involved in insulin signaling.
5. There is also impairment of GLUT-4 mediated glucose transport by ROS in the body (Bertelsen et al., 2001).
6. Hyperglyceamia induces mitochondrial dysfunction in both in vivo and in vitro studies and contributes to the production of high ROS levels in the cell (Evans & Rushakoff, 2007).

Recent studies suggest that common stress-activated signal pathways such as nuclear factor $-_k$B, MAPK, stress activated protein kinases underline the development of late diabetes

complications. In addition, the activation of these stress signal pathways by glucose and/or free fatty acids in diabetes type 1 and 2 leads to insulin resistance and impaired insulin secretion (Evans et al., 2002).

Vitamin C and E have been reported to defend against the high damaging effect of oxidative stress in diabetes through enzymatic, autoxidative glycosylation and reduction of metabolic stress (Baynes & Thorpe, 1999). Increasing evidence has shown that hyperglycemia is the initiating cause of tissue damage in diabetes mellitus and this can be either through long time accumulation of advanced glycosylation end products (AGEs) and glycated biomolecules or through repeated acute changes in glucose metabolism (Karasu, 2010). Furthermore, in addition to the elevated AGEs during hyperglycemia, other pathways that elevate intracellular ROS include:

a) Enhanced glucose auto-oxidation

b) Increased mitochondrial superoxide production

c) Uncoupled endothelial nitric oxide synthase activity (eNOS)

d) Activation of NADPH oxidase

e) Stimulation of eicosanoids metabolism

2.5.2 Role of antioxidants in insulin resistance

One of the primary forms of defence in the body against ROS is the use of antioxidants. These antioxidants occur in two groups, enzymatic and non-enzymatic. Examples of enzymatic antioxidants are superoxide dismutase, catalase, glutathione peroxidase and glutathione

reductase. Non enzymatic antioxidants are Vitamin C, E, urate and β- carotene (at low PO_2) and selenium.

Plants generally protect themselves from internal stresses (free radicals) and external stresses from insects and pests with the aid of antioxidants; this property of plants explains their potential to cure diseases and their benefits in traditional medicine.

Breaking the chain reaction of ROS and preventing the glycation of insulin receptors antioxidants alleviate insulin resistance in tissues making these receptors available for insulin uptake and signaling. One major consequence of oxidative stress is the activation of harmful genes products that cause cellular damage and are ultimately responsible for the late complications of diabetes (Lai, 2008). In an in vitro study, Vitamin E has been shown to improve insulin action and secretion by protecting peripheral tissues from free radical mediated damage (Liu et al., 2006). This is similar to a study which shows that chromium, Vitamin C and E reduced TBARS significantly (Lai, 2008).

2.6 INSULIN

The primary functions of insulin are the maintenance of body glucose homeostasis, growth and tissue development. Insulin, which is an anabolic hormone, is known to play a part in the fight or flight response during stressful situations, therefore its important activity in the maintenance of body fuels cannot be overlooked. Insulin concentration is increased primarily by the increase in glucose concentration in the blood. Insulin may also be secreted by certain amino acids, fatty acids, keto acids (which are products of fatty acids oxidation), and some hormones secreted by the intestinal tract. Examples of such amino acids include arginine and leucine (Cawston & Miller, 2010). Insulin regulates glucose homeostasis in the liver by reducing hepatic

glucose output through the inhibition of glycogenolysis and gluconeogenesis and also enhancing glucose uptake in satiated muscles and adipose cells. Insulin also enhances lipid synthesis in the liver and adipocytes by reducing the rate of fatty acid release from triacylglyceride in the liver and muscle cells (Pessin & Saltiel, 2000). Generally, insulin-dependent translocation of GLUT-4 to the plasma membrane enhances or controls the rate of clearance of circulating glucose in myocytes and adipocytes. The rate of clearance of circulating glucose is largely caused by the insulin-dependent translocation of GLUT-4 to the plasma membrane (Pessin & Saltiel, 2000).

2.6.1 Secretion

In response to high glucose concentration, pancreatic β – cells release insulin by exocytosis in granules at higher metabolic rates; insulin is released at a basal metabolic rate during low glucose concentration. These granules move to the plasma membrane in a series of cascade reactions. After a rich carbohydrate meal, the level of circulating plasma glucose concentration rises in the pancreas and the increased circulating glucose is taken up by plasma-bound glucose transporters called GLUT-2 on the β cells. Inside the cytosol of the β cells, glucose is phosphorylated by hexokinase IV (glucokinase) and undergoes glycolysis and is further metabolized to yield ATP via TCA and oxidative phosphorylation. The increased ATP inhibits the K^+-ATP sensitive channels thereby depolarizing the plasma membrane. This depolarization leads to influx of Ca^{2+} through the opening of voltage sensitive Ca^{2+} channels; it is the increased Ca^{2+} in the cytoplasm that agitates or activates the movement of insulin-stored vesicles to the plasma membrane and insulin is then secreted through exocytosis. Although some amino acids, fatty acids, ketone bodies, glucagon, and sulphulnurea drugs also initiate insulin secretion but it is primarily initiated by glucose concentration (De Marchi, et al., 2014).

2.6.2 Insulin Receptors

Insulin receptors are present in virtually almost all the tissues in the body, although with varied concentrations, from as little as 40 receptors in the circulating erythrocytes of the blood to as many as 200,000 receptors on hepatocytes and adipocytes (White, 1997). The insulin receptor is a heterotetrameric transmembrane protein, in that it is composed of two α sub-unit linked to β sub-units and also to each other by disulphide bonds, as shown in Figure 2.1.

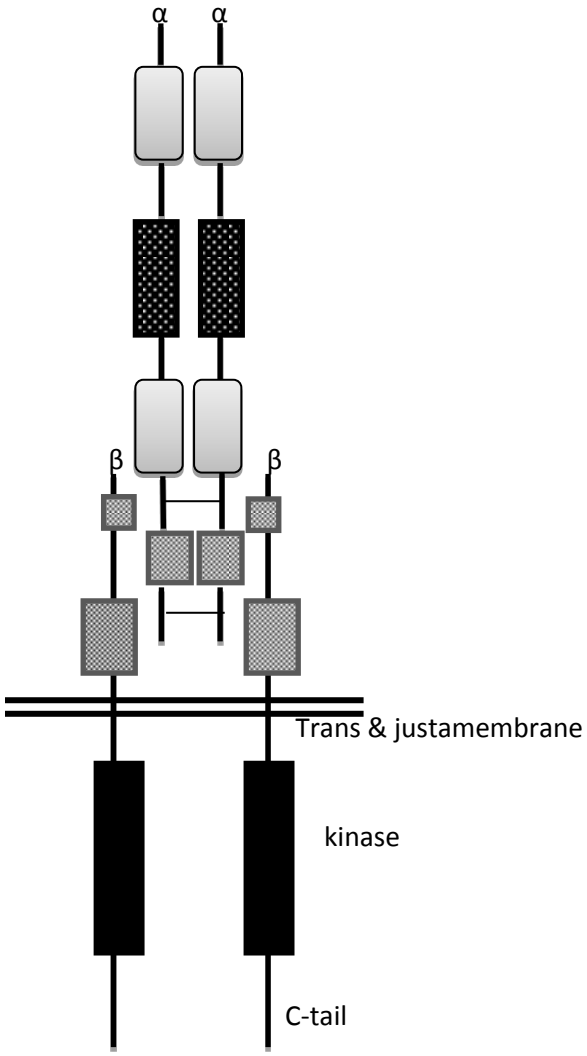

Figure 2.1: Structure of insulin receptor

(Li & Zhang, 2007)

The two α sub-units are located outside the cell and they contain the binding site for insulin. Two molecules of insulin must bind to this site to elicit a metabolic response, although the two α sub-units are joined together by a disulphide bond which stabilizes them. The two β sub-units, however, span through the cell with the intracellular portion containing insulin-regulated tyrosine protein kinase. Insulin binding to the two α sub-units activates the tyrosine kinase, causing autophosphorylation of the tyrosine residues in the intracellular β sub-units.

2.6.3 Insulin signaling and AMPK- dependent pathway

After insulin is released via exocytosis from the β-cells of the pancreas, insulin binds to its receptors on the plasma membranes of cells of the target tissues. The binding of insulin therefore activates the tyrosin kinase, leading to autophosphorylation of the tyrosin residues in the different regions of the intracellular β sub-units. The insulin receptors' autophosphorylation lead to the phosphorylation of different intracellular enzymes as shown in Figure 2.2.

The tyrosine kinase phosphorylates itself as well as insulin receptor proteins (IRS -1 and IRS -2), Cbl and $p52^{sho}$(Galic et al., 2005). IRS-1 role is more evident in muscles and adipose tissues while IRS-2 functions mainly in the liver. Studies show that IRS-2 enhances insulin synthesis by the pancreas (Alper, 2000).

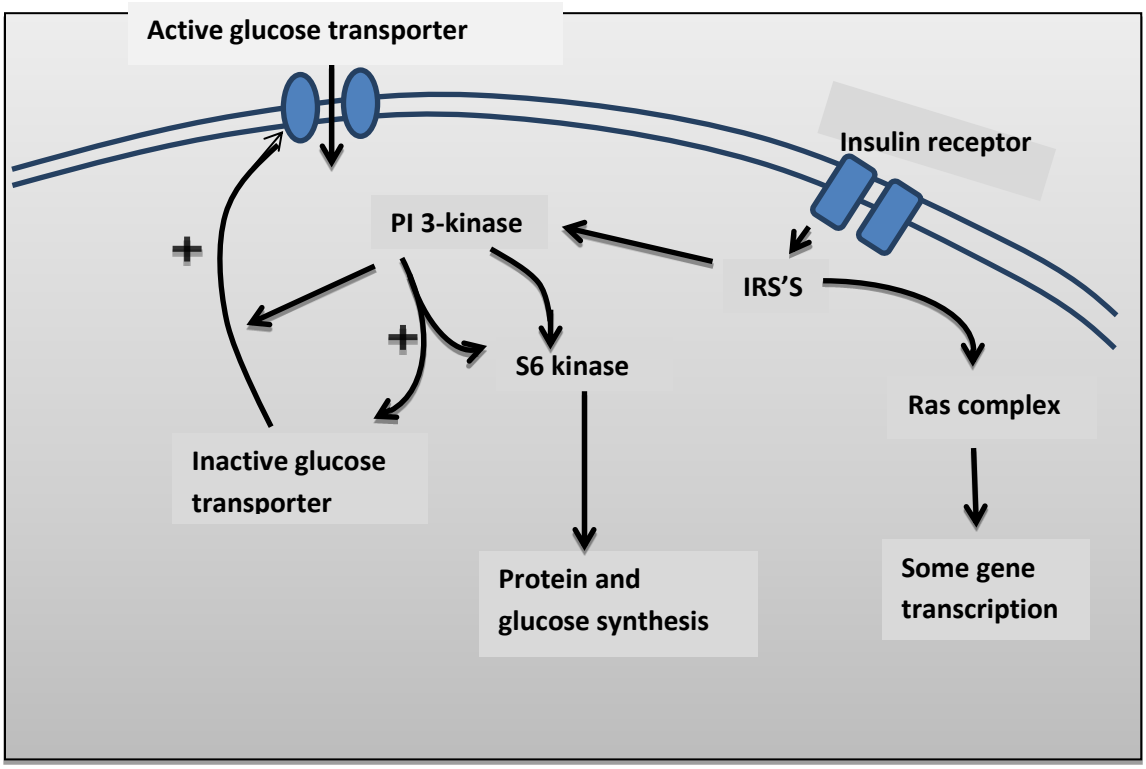

Figure 2.2: Pathways of insulin signaling and action

(Alper, 2000)

The signalling of insulin and activation of AMPK dependent pathway regulate glucose metabolism, fatty acid metabolism, cellular growth, differentiation and cell death (Gallagher & LeRoith, 2010). AMPK activates a catabolic process while insulin signalling activates an anabolic process

The two major enzymes phosphorylated are mitogen-activated protein kinase (MAPK) and phosphatydlinositol-3-kinase (PI-3-K), and are responsible for the expression of mitogenic and metabolic activities of insulin respectively, as shown in Figure 2.3

Activation of MAPK is involved in cell growth and gene proliferation. The activation of PI3K however, is involved in lipid, glycogen and protein synthesis. It also leads to cell

proliferation and survival It is the PI3K pathway that activates the mobilization of GLUT-4 to the plasma membrane.

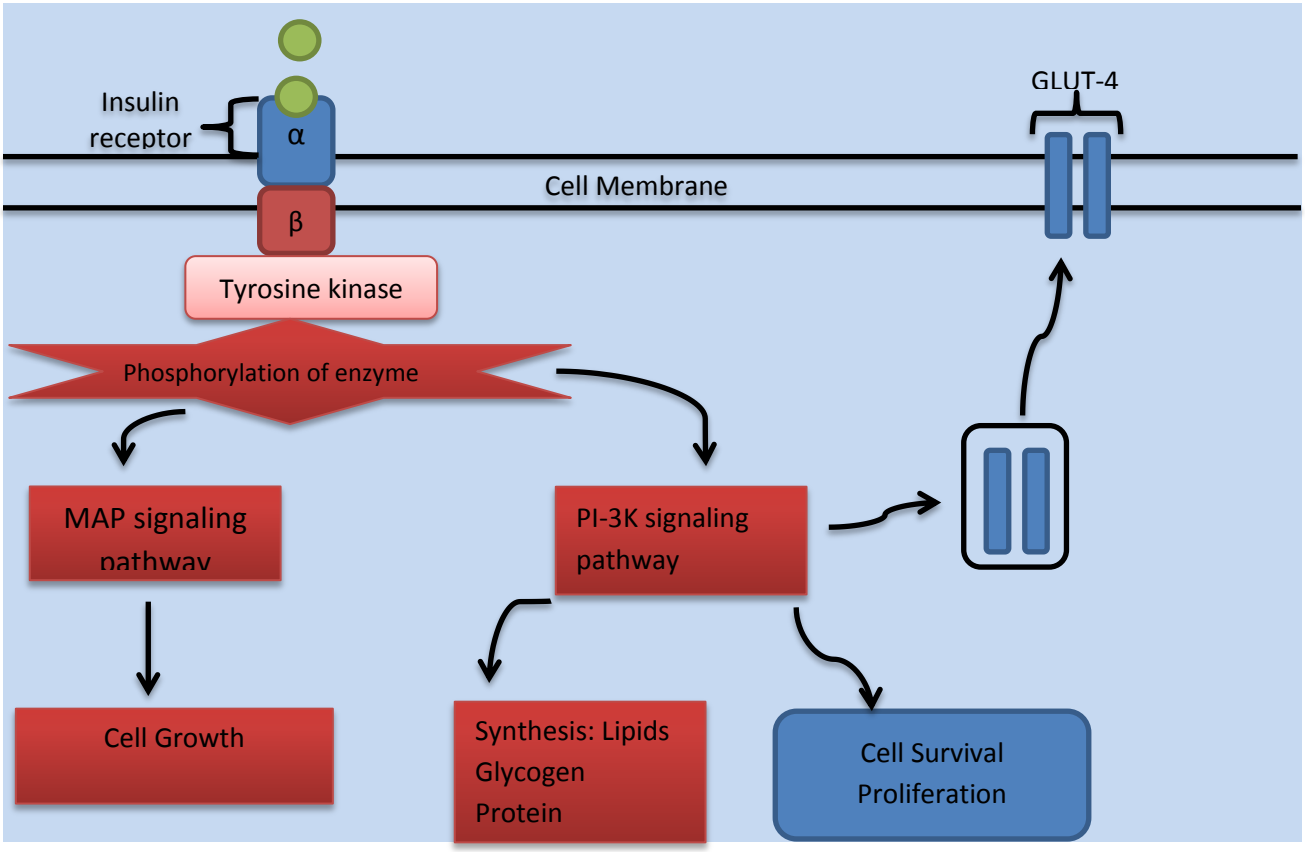

Figure 2.3: Illustrating the mechanism of action and signaling of insulin in target cells (Kumar et al., 2005)

2.7 Glucose metabolism

As glucose is a hydrophilic compound it cannot pass through the lipid bilayer of the plasma membrane by simple diffusion, and therefore requires a carrier protein to mediate its passage into the cell. Glucose entry into cells depends on different parameters such as the expression of glucose transporters on the plasma membrane of the target tissues, hormonal regulation and function (Gorovits & Charron, 2003). Cells therefore takes glucose by facilitated diffusion through glucose transporters (GLUTs) associated with the cells (Medina & Owen, 2002).

The process by which glucose enters the cell is very intricate and it is viewed as a four step process. The glucose transporter takes on two shapes, the initial shape that binds to glucose at the extracellular and the other at the intracellular compartment. Glucose first binds to the out-ward facing (extracellular) binding site, this then leads to conformational change of the transporter, making the occupied glucose binding site facing the intracellular site. The transporter therefore releases the glucose into the cytoplasm. In the last process the unoccupied transporter changes to its initial conformation where the binding site now faces the extracellular making it ready for another glucose and transport into the cell (Lienhard et al., 1992). There are different types of glucose transporters, the expression of each depends on hormonal and environmental control. These transporters are specific; according to their substrates and function in the tissues they are localized as shown the Table 2.1. Once the glucose is in the cell it is utilized according to the need of the cell. For instance, the liver and muscles convert excess glucose to glycogen, while adipocytesconvert excess glucose to fats.

Table 2.1: Glucose transporters expression and function

Protein	Alias	Expression	Function
GLUT-1		All tissues abundant in brain and erythrocytes	Basal glucose uptake
GLUT-2		Liver, pancreatic cells, retina	Glucose sensing
GLUT-3		Brain	Supplements GLUT-1 in tissues with high energy demand
GLUT-4		Muscle, fats, heart	Insulin responsive
GLUT-5		Intestine, testes, erythrocytes, kidney	Fructose transport

2.7.1 Major organs involved in glucose homeostasis

The major organs involved in glucose metabolism are the pancreas, liver, adipose and muscles. The effect of insulin on various tissues is summarized in Table 2.2.

The primary role of the adipose tissue in glucose homeostasis is the storage of energy in the form of triacylglycerides. Adipose tissue has a number of glucose transporters such as GLUT- 4, GLUT-8, and GLUT-12 that are responsible for the shuttling of glucose molecules into the cell. The GLUT-4 which is a major hexose transporter is highly expressed in adipocytes (Huang et al., 2007). The skeletal muscle stores glucose as glycogen which it utilizes when the body needs energy and accounts for about 75% of whole body insulin stimulated body intake (Perriott et al., 2001). The muscle glycogen cannot be used as a source of blood glucose because the tissue lacks glucose-6- phosphatase, an enzyme that is required to hydrolyse glucose-6-phosphate to phosphate and free glucose. The muscle therefore supplies the liver with lactate which can be converted to glucose. Glucose is transported into the myocytes through the transmemebrane transporter GLUT- 4.

One of the major effects of insulin in the muscles is the increased entry of glucose into the myocytes.

Table 2.2: Summary of the effects of insulin in different tissues

Adipose	Muscle	Pancreas	Liver
Increases glucose entry	Increases protein synthesis	Decreases beta cell growth proliferation	Decreases ketogenesis
Increases fatty acid synthesis	Increases glycogen synthesis	Increases glucose entry	Increases protein synthesis
Increases triglyceride deposition		Decreases insulin synthesis	Increases lipid synthesis
Activates lipoprotein lipase		Decreases insulin secretion	Decreases glucose output
Inhibits hormone sensitive lipase			

2.8 LIPID METABOLISM

The metabolism of lipids is also affected during diabetes especially at the advanced stage of the disease, which is evident by the presence of ketone bodies in the urine and accumulation in the blood. The primary source of body fuel is glucose, but the body can also source fuel in lipids when glucose is not readily available. Lipids are transported in the blood mostly as free fatty acids (FFA) in mammalian organisms and are the only form of fat released from the adipose tissue (Raz et al., 2005). Plasma FFA has a rapid turnover which indicates that even in non fasting conditions the body tissues can depend on FFA for the equivalent or greater energy than glucose (Raz et al., 2005). Insulin enhances glucose uptake in insulin-sensitive tissues and an inhibition of lipolysis thereby decreases FFA in the blood. However, in an insulin resistant state (hyperinsulinemia) usually seen in obesity and diabetes, there is an increased breakdown of fat leading to high levels of FFA and glycerol in the blood. This worsens the hyperglyceamic condition, as glucose and FFA compete for entry into oxidative pathways (Bohannon, 1992).

2.9 TREATMENT OPTIONS FOR DIABETES MELLITUS

There are several treatment options available for the management of diabetes and the primary aim is to attain a glycemic condition. Early treatment with combination therapy is usually needed to achieve this aim (McGill & Felton, 2006). Examples of these drugs are Biguanides, Sulphonylurea, Meglintinides, Thiazolidinediones and carbohydrate metabolizing inhibitors. These different classes of drugs exert glucose homeostasis effects via different mechanisms and sometimes a combination of these drugs may be used to achieve the normal glucose level in the blood. These treatment options are with side effects. Sulphonylureas for example, has been reported to worsen heart disease, induce hypoglycemia and increase body weight (Dey et al., 2002). Nausea, tiredness, palpitations and frequent hunger have been reported with insulin treatment. Liver toxicity has been observed in thiazolindodianes-treated patients, abdominal pain and discomfort has been associated with diabetic patients treated with carbohydrate hydrolyzing enzymes (De Fronzo, 1999).

2.10 HERBAL ALTERNATIVES

Modern antidiabetic drugs are not readily available, are expensive and adverse side effects require healthcare givers and scientists to search for herbal alternatives which are cheap, readily accessible and have fewer adverse effects.

Recent research showed that plants could be of great importance in the management of diseases.

REFERENCES

ADA (American Diabetes Association). (2006a). Diagnosis and classification of diabetes mellitus. *Diabetes Care*, *29 Suppl 1*, S43–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16373932

ADA (American Diabetes Association). (2006b). Third-Party reimbursement for diabetes care, self-management education, and supplies. *Diabetes Care*, *29*(1).

Alper, J. (2000). BIOMEDICINE: New Insights Into Type 2 Diabetes. *Science*, *289*(5476), 37–39. http://doi.org/10.1126/science.289.5476.37

Baynes, J. W., & Thorpe, S. R. (1999). Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes*, *48*(1), 1–9. http://doi.org/10.2337/diabetes.48.1.1

Bertelsen M, Anggard EE & Carrier MJ.(2001). Oxidative stress impairs insulin internalization in endothelial cells in vitro. *Diabetologia* 44, 605-613

Bohannon, N. (1992). Lipid metabolism in type 2 diabetes. *Postgraduate Medicine*, *92*(2), 1–4.

Calixto, J. . (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazillian Journal of Medical and Biological Research*, *33*, 179–189.

Cawston, E. E., & Miller, L. J. (2010). Therapeutic potential for novel drugs targeting the type 1 cholecystokinin receptor. *British Journal of Pharmacology*, *159*(5), 1009–1021. http://doi.org/10.1111/j.1476-5381.2009.00489.x

Dare, B., Oyeniyi, F., & Olaniyan, O. (2014). Role of Antioxidant in Testicular Integrity. *Annual Research & Review in BIology*, *4*(7), 998–1023. Retrieved from file:///C:/Users/USER/Downloads/Dare472013ARRB4453_1.pdf

De Fronzo, R. (1999). Pharmacology therapy for type 2 diabetes mellitus. *Annals of Internal Medicine*, *131*, 281–303.

De Marchi, U., Thevenet, J., Hermant, A., Dioum, E., & Wiederkehr, A. (2014). Calcium co-regulates oxidative metabolism and ATP synthase-dependent respiration in pancreatic beta cells. *The Journal of Biological Chemistry*, *289*(13), 9182–9194. http://doi.org/10.1074/jbc.M113.513184

Dey, L., Anoja, S., & Chun-Su, Y. (2002). Alternative therapies for Type 2 Diabetes. *Alternative Medicine Review*, *7*(1), 45–58. Retrieved from http://www.altmedrev.com/publications/7/1/45.pdf

Evans, J. L., Goldfine, I. D., Maddux, B. A., & Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocrine Reviews*, *23*(5), 599–622. http://doi.org/10.1210/er.2001-0039

Evans, J., & Rushakoff, R. (2007). Oral pharmacological agents for type 2 diabetes: Sulfonylureas, meglitinides, metformin, thiazolidinediones, α-glucosidase inhibitors and emerging approaches. In I. Goldfine & R. Rushakoff (Eds.), *Carbohydrate metabolism and diabetes mellitus*. EncloText.org. Retrieved from http://www.mdtext.com/diabetes/diabetes16/diabetesframe16.htm

Felig, P, Marliss, E, Ohman, J.L, Cahill, C.F (1970). Plasma amino acid levels in diabetic ketoacidosis.*Diabetes* 19 (10) 727-728

Galic, S., Hauser, C., Kahn, B. B., Haj, F. G., Neel, B. G., Tonks, N. K., & Tiganis, T. (2005). Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP. *Molecular and Cellular Biology*, *25*(2), 819–829. http://doi.org/10.1128/MCB.25.2.819-829.2005

Gallagher, E. J., & LeRoith, D. (2010). The proliferating role of insulin and insulin-like growth factors in cancer. *Trends in Endocrinology and Metabolism: TEM*, *21*(10), 610–618. http://doi.org/10.1016/j.tem.2010.06.007

Ge, K., Niu, Y., Xie, T., Lin, W., Tian, M., Xu, B., … Lu, S. (2009). [Influence of advanced glycosylation end products on wound healing of burn rats with diabetes]. *Zhonghua Shao Shang Za Zhi = Zhonghua Shaoshang Zazhi = Chinese Journal of Burns*, *25*(6), 433–436. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20193165

Gorovits, N., & Charron, M. J. (2003). What we know about facilitative glucose transporters: Lessons from cultured cells, animal models, and human studies. *Biochemistry and Molecular Biology Education*, *31*(3), 163–172. http://doi.org/10.1002/bmb.2003.494031030227

Huang S, Czech M, N., & Charron, M. J. (2007). The GLUT4 glucose transporter. *Cell metabolism, 5 (4): 237-52*. http://www.ncbi.nlm.nih.gov/pubmed/17403369

IDF. (2011). International Diabetes Federation. *IDF Diabetes Atlas, 5th Edn. Brussels, Belgium*.

IDF. (2015). International Diabetes Federation. *IDF Diabetes Atlas, 7th Edn. Brussels, Belgium*.

Karasu, C. (2010). Glycoxidative stress and cardiovascular complications in experimentally-induced diabetes: effects of antioxidant treatment. *The Open Cardiovascular Medicine Journal*, *4*, 240–256. http://doi.org/10.2174/1874192401004010240

Knentz, A., & Nattras, M. (1991). *Diabetic ketoacidosis, non ketotic hyperosmolar coma and lactic acidosis. In: Handbook of diabetes*. (J. Pickup & W. G, Eds.) (2nd ed.). Blackwell Science.

Krishnakumar, K., Augustii, K., & Vijayammal, P. (1999). Hypoglycaemic and anti-oxidant activity of salacia oblonga wall extract in streptozotocin-induced diabetic rats. *Indian Journal Physiology and Pharmacology*, *43*, 510–514.

Kumar, P., & Clark, M. (2002). *Textbook of clinical medicine*. (Parveen K and Michael C Eds) London: Saunders.

Kumar, V., Abbas, A. K., & Fausto, N. (2005). The Endocrine system. In Philadelphia (Ed.), *Robbins and Cotran Pathologic Basis of Disease* (7th ed., pp. 1191–1193). Elsevier Saundres.

Kunwar, A., & Priyadarsini, K. (2011). Free radicals, oxidative stress and importance of antioxidants in human health. *Journal of Medical Allied Sciences*, *1*(2), 53–60.

Lai, M.-H. (2008). Antioxidant effects and insulin resistance improvement of chromium combined with vitamin C and e supplementation for type 2 diabetes mellitus. *Journal of Clinical Biochemistry and Nutrition*, *43*(3), 191–198. http://doi.org/10.3164/jcbn.2008064

Lanza, R. P., Ecker, D. M., Kühtreiber, W. M., Marsh, J. P., Ringeling, J., & Chick, W. L. (1999). Transplantation of islets using microencapsulation: studies in diabetic rodents and dogs. *Journal of Molecular Medicine*, *77*(1), 206–210. http://doi.org/10.1007/s001090050337

Li, C., & Zhang, B. (2007). *Insulin signaling and action: glucose, lipids, protein*. (I. Goldfine & R. Rushakoff, Eds.). Endotext.com. Retrieved from http://diabetesmanager.pbworks.com/w/page/17680220/Insulin signaling and action%3A glucose%2C lipids%2C protein

Lienhard, G. E., James, D. E., Slot, J. W., & Mueckler, M. M. (1992). How cells Absorb Glucose. *Scientific American*, *266*(1), 86–91.

Liu, S., Lee, I.-M., Song, Y., Van Denburgh, M., Cook, N. R., Manson, J. E., & Buring, J. E. (2006). Vitamin E and risk of type 2 diabetes in the women's health study randomized controlled trial. *Diabetes*, *55*(10), 2856–2862. http://doi.org/10.2337/db06-0456

Loghmani, E. (2005). Diabetes Mellitis: Type 1 and Type 2. In J. Stang & M. Story (Eds.), *Guidelines for Adolescent Nutrition Services* (pp. 147–182). Retrieved from http://www.epi.umn.edu/let/pubs/adol_book.shtm

Mbanya, J., Bonnici, F., & Nagan, K. (1996). Guideline for the management of NIDDM in Africa. A consensus document. *Novo Nordisk A/s*, 1–35.

McGill, M., & Felton, A. (2006). On behalf of the global partnership for effective diabetes management. New global recommendations: a multidisciplanry approach to improving outcomes in diabetes. *Primary Care Diabetes*, *1*, 49–55.

Medina, Rodolfo A, Owen, Gareth I. (2002). Glucose transporters: expression, regulation and cancer.*Biological Research.*35(1), 9-26

NIH. (2011). Causes of diabetes. Retrieved August 18, 2014, from http://www.completemedicalhomecare.com/Health/Diabetes/download/Causes_of_Diabetes.pdf Date accessed 2014-08-18

Perkins, R. M., Yuan, C. M., & Welch, P. G. (2006). Dipsogenic diabetes insipidus: report of a novel treatment strategy and literature review. *Clinical and Experimental Nephrology*, *10*(1), 63–67. http://doi.org/10.1007/s10157-005-0397-0

Perriott, L. M., Kono, T., Whitesell, R. R., Knobel, S. M., Piston, D. W., Granner, D. K., … May, J. M. (2001). Glucose uptake and metabolism by cultured human skeletal muscle cells: rate-limiting steps. *American Journal of Physiology. Endocrinology and Metabolism*, *281*(1), E72–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11404224

Pessin, J. E., & Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. *The Journal of Clinical Investigation*, *106*(2), 165–169. http://doi.org/10.1172/JCI10582

Piédrola, G., Novo, E., Escobar, F., & García-Robles, R. (2001). White blood cell count and insulin resistance in patients with coronary artery disease. *Annales D'endocrinologie*, *62*(1 Pt 1), 7–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11240402

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomedicine & Pharmacotherapy = Biomédecine & Pharmacothérapie*, *59*(7), 365–373. http://doi.org/10.1016/j.biopha.2005.07.002

Ranjan, C., & Ramanujam, R. (2002). Diabetes and insulin resistance associated disorders: Disease and the therapy. *Current Science*, *83*(12), 1533–1538.

Raz, I., Eldor, R., Cernea, S., & Shafrir, E. (2005). Diabetes: insulin resistance and derangements in lipid metabolism. Cure through intervention in fat transport and storage. *Diabetes/metabolism Research and Reviews*, *21*(1), 3–14. http://doi.org/10.1002/dmrr.493

Ross, R. (1999). Atherosclerosis--an inflammatory disease. *The New England Journal of Medicine*, *340*(2), 115–126. http://doi.org/10.1056/NEJM199901143400207

Schwartz, S. L. (2006). Diabetes and dyslipidaemia. *Diabetes, Obesity & Metabolism*, *8*(4), 355–364. http://doi.org/10.1111/j.1463-1326.2005.00516.x

Wadkar, K., Magdum, C., Patil, S., & Naikwade, N. (2008). Anti-diabetic potential and Indian medicinal plants. *Journal of Herbal Medicine and Toxicology*, *2*(1), 45–50.

White, M. F. (1997). The insulin signalling system and the IRS proteins. *Diabetologia*, *40 Suppl 2*, S2–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9248696

WHO. (2002). WHO Traditional Medicine strategy. *Bulletine World Health Organization*, *80*(7), 610–610. Retrieved from http://www.who.int/bulletin/archives/80(7)610.pdf

Wiernsperger, N. F. (2003). Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. *Diabetes & Metabolism*, *29*(6), 579–585. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14707886

Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, *27*(5), 1047–1053. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15111519

IN VITRO DETERMINATION OF ANTI-OXIDANT, FREE RADICAL AND ANTI-INFLAMMATORY ACTIVITIES OF *ALBUCA SETOSA* AND *ALBUCABRACTEATA* BULB EXTRACTS

CHAPTER 3

IN VITRO DETERMINATION OF ANTI-OXIDANT, FREE RADICAL AND ANTI-INFLAMMATORY ACTIVITIES OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS

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IN VITRO DETERMINATION OF ANTI-OXIDANT, FREE RADICAL AND ANTI-INFLAMMATORY ACTIVITIES OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA BULB EXTRACTS*

3.1 INTRODUCTION

Medicinal plants are reported to have antioxidant activities, which have been attributed to the presence of flavonols, flavones, anthocyanins, catechins and isocatechins (Aqil, et al., 2006). These antioxidants, prevent oxidative damage caused by free radicals due to their ability to chelate catalytic metals, scavenge reactive oxygen species and stop spontaneous reactions by acting as final electron acceptors (Shahidi & Wanasundara, 1992). Reactive oxygen species (ROS) are continuously generated in the body through enhanced glucose auto-oxidation, increased mitochondrial superoxide production and uncoupled endothelia nitric oxide synthase activity (Kunwar & Priyadarsini, 2011). Excess production of ROS and/or inadequate production of antioxidant defence lead to oxidative damage of various biomolecules including lipids, proteins and DNA (Farber, 1994). Oxidative damage is therefore a critical etiological factor involved in degenerative diseases such as diabetes, cancer, atherosclerosis, arthritis, neurodegenerative diseases and aging (Patel, et al., 2010).

Diabetes mellitus is a metabolic disease associated with persistent hyperglycaemia due to either the inability of the body to synthesize insulin or insensitivity to the action of insulin or both. Increasing evidence has shown that in poorly controlled or persistent hyperglycaemia, increased formation of advanced glycation end products (AGEs), ROS and lipid peroxidation products exacerbate intracellular oxidative stress.

The complications of diabetes mellitus such as chronic inflammation, glaucoma, cataract, neuropathy and nephropathy can therefore be ameliorated by antioxidants. For instance, a lot of inflammatory mechanisms that might induce insulin resistance, decrease in insulin

secretion and dysfunction of β-cells from islet cells have been described (Akash et al. 2013). The imbalance of cellular redox homeostasis through excessive production of reactive oxygen species (ROS) contributes to the pathogenesis of several diseases (Gupta et al., 2012). Although endogenous anti-oxidants such as SOD, GPx, and GSH are more powerful radical scavengers than those from the diets, under pathological conditions they are overwhelmed by free radicals. Available synthetic antioxidants such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) are not without side effects and have been reported to be unsafe (Patel et al., 2010), therefore, there is the need to substitute these synthetic antioxidants with naturally occurring ones obtained from plants.

Albuca bracteata and *Albuca setosa* are plants reported to have been used by traditional healers in the Eastern Cape Province of South Africa to treat diabetes mellitus (Oyedemi et al., 2009; van Huyssteen et al., 2011). These plants are widely distributed throughout the Eastern Cape. *Albuca setosa* has been reportedly used for ritual washing, protection against bad luck and to heal wounds in a similar manner to aloe vera (Cocks, 2006; Hutchings, 1996) while *Albuca bracteata* has been reportedly used as an aphrodisiac and to manage cancer (Kipkore, et al., 2014). The glucose uptake of fresh bulb aqueous extract has also been reported (van Huyssteen *et al*., 2011).

Before the commencement of this study, there has not been any report or information on the phytochemicals, antioxidants and comparative study of both plants in any scientific literature. The aim of this study is to investigate and compare the phytochemicals and antioxidant activities of these plants.

3.2 MATERIALS AND METHODS

Chemicals

2, 2, Diphenyl-2-picrylhydrazyl (DPPH), Ascorbic acid and BHT were obtained from Sisco Research Laboratories (Pvt. Ltd., Mumbai, India). Folin-Ciocalteu reagent and hydrogen peroxide (H_2O_2) were obtained from Merck Limited, (Mumbai, India), while methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade.

3.2.1 Collection of plant materials

Albuca bracteata and *Albuca setosa* were collected from a forest in Alice (Eastern Cape Province, South Africa) and identified and authenticated by Prof. DS Grierson of the Department of Botany, University of Fort Hare.

Plant materials obtained were separated into two groups; one was extracted fresh and the other was thinly sliced and oven dried at 40^{o}C for 72 h, then macerated in a Hamilton Beach Commercial Blender type GB27 model HBF 400-CE.

Eighty grams of the powdered sample was weighed for extraction in 1 L of distilled water, methanol, ethanol and acetone respectively and placed in an electric shaker for 48 h. The solutions were then filtered using Whatman No.1 filter paper in a vacuum pump. The aqueous filtrate was freeze dried while the methanolic, ethanolic and acetonic extracts were dried in a rotatory evaporator. The powdered plant material was stored at -4^{0}C and later reconstituted in the respective solvents just before the various analyses.

3.2.2 Determination of total phenol content

The total phenols in the extracts were determined with Folin-Ciocalteau reagent using the modified method of Wolfe et al. (2003). An aliquot of the extracts was mixed with 5 ml Folin-Ciocalteau reagent previously diluted with water (1:9 v/v). To this mixture, 4 ml of

sodium carbonate (75 g/L) was added, then the test tubes were vortexed for 10 s and allowed to stand for 30 min. at 40^{o}C for colour development. The absorbance was measured at 765 nm using the Hewlett Packard UV-Vis spectrophotometer. Samples of extract were evaluated at a final concentration of 1.0 mg/ml. Total phenolic content was expressed as mg/g of the extracts as tannic acid equivalent using the equation derived from the calibration curve:

$y = 0.1216x$, $R^2 = 0.9365$, where x is the concentration of the tannic acid equivalent and y is the absorbance. The experiment was conducted in triplicate and the results expressed as mean ± SD values. The total phenolic content was calculated as tannic acid equivalent (TAE) by the following equation:

$$T = \frac{C \times V}{M}$$

T is the total phenolic content in mg/g of the extracts as TAE, C is the concentration of tannic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract in g.

3.2.3 Determination of total flavonoids content

Total flavonoids content was measured using $AlCl_3$ as described by Ordonez et al. (2006). A 0.5 ml aliquot of the extracts was mixed with 0.5 ml of 2% aluminium chloride ($AlCl_3$) prepared in ethanol. The mixture was allowed to stand for 1 h at room temperature. A yellow colour indicates the presence of flavonoids. The absorbance was then measured at 420 nm. Total flavonoid was calculated as mg/g of the extracts as quercetin equivalent using the equation:

$y = 0.0255x$, $R^2 = 0.9812$, where y is the absorbance and x is the concentration of the quercetin equivalent.

3.2.4 Determination of total tannin contents

Tannin content was estimated using the method described by Wintola & Afolayan (2011). Into a 500 ml beaker, 0.2 g of the extract was measured, 20 ml of 50% methanol was added, covered and shaken on a mechanical shaker to ensure thorough mixing, then placed in a water bath at 78^{o}C for 1 hr. The mixture then filtered into a 100 ml volumentary flask, to which 20 ml of distilled water, 2.5 ml Folin - Denis reagent and 10 ml of 17 % $NaCO_3$ were then added and mixed thoroughly. The mixture was made up to mark with distilled water, and allowed to stand for 20 min to develop a bluish green colour. The absorbance was measured at 760 nm. Tannin standards were also prepared and the total tannin content was expressed as mg/g of the extracts as tannin standard equivalent using the equation derived from the calibration curve y = 0.087x- 0.06, R^2 = 0.9277, where y is the absorbance and x is the concentration of the tannin equivalent.

3.2.5 Determination of proanthocyanidin content

The total proanthocyanidin was determined using the procedure reported by Sun et al. (1998). 0.5 ml of 0.1 mg/ml of the extract solution was mixed with 3.0 ml of 4% vanillin-methanol solution, 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature, and the absorbance was measured at 500 nm. Total proanthocyanidin content was expressed as catechin equivalent (mg/gof the extracts) using the equation of the curve: y= 0.5825x, R^2 = 0.9277, where y is the absorbance and x is the quercetin equivalent.

3.2.6 Determination of total flavonols content

Total flavonols was estimated using the method of Kumaran & Karunakaran (2007). The reacting mixture consisted of 2.0 ml of the extract or standard, 2.0 ml of 2% $AlCl_3$ prepared in ethanol and 3.0 ml (50 g/L) sodium acetate solution. This was allowed to stand for 2 h at

20^{o}C. The absorbance was measured at 440 nm. Total flavonols content was calculated as quercetin (mg/g) equivalent using the equation derived from the calibration curve:

y = 0.0255x, R^2 = 0.9812, where y is the absorbance and x is the concentration of the quercetin equivalent.

3.2.7 Determination of saponins content

Saponins content was determined according to the method described by Otang et al. (2012). Briefly, 50 ml of 20% ethanol (in distilled water) was added to 5 g of the sample. The solution was heated for 4 h in a water bath with continuous stirring at 55^{o}C, this was then filtered and re-extracted in 50 ml of 20% ethanol (in distilled water). The extracts were combined and reduced to 10 ml at 90^{o}C. The concentrate was then transferred into a 250 ml separating funnel, 5 ml diethylether was added and vigorously shaken. The ether layer was discarded while the aqueous layer was repeatedly purified using 15 ml n – butanol and later washed with 10 ml of 5% aqueous NaCl. The solution was heated in a hot water bath to evaporation and was later oven dried to a constant weight at 40^{o}C.

The saponins content of the sample was calculated using the equation:

Saponins content (mg/g) = weight of residue/weight of sample

3.2.8 Determination of alkaloid content

The alkaloid contents of the plant extract was determined according to the method of Harborne (2013). Briefly, 200 ml of 20% acetic acid in ethanol was added to 5 g of the powdered sample and was allowed to stand for 4 hrs. This was then filtered and concentrated in a water bath until the volume was one quarter of the original volume. Concentrated ammonium hydroxide was then added drop wise until precipitation was completed. The solution was then filtered and the precipitate collected and weighed.

The alkaloid content was calculated using the equation:

Alkaloid content (mg/g) = $\frac{weight\ of\ precipitate}{weight\ of\ sample}$

3.2.9 Determination of diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

To determine the scavenging activity of DPPH free radical of the extract, the method of Liyana-Pathiranan & Shahidi (2005) was used. DPPH in methanol (0.135 mM) was prepared and 1.0 ml of this solution was mixed with 1.0 ml of the extract prepared in methanol containing 0.02 to 0.1 mg of the plant extracts and standard reference Vitamin C and BHT. The reaction mixture was then vortexed thoroughly and left in the dark at room temperature for 30 min. Absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant extract to scavenge DPPH radical was calculated from the equation:

DPPH radical scavenging activity = $\left(\frac{\mathbf{Abs}\text{control}-\mathbf{Abs}\text{sample}}{\mathbf{Abs}\text{control}}\right)$ X 100

Where $Abs_{control}$ is the absorbance of DPPH radical + methanol

Abs_{sample} is absorbance of DPPH radical + sample/standards.

3.2.10 Determination of ABTS scavenging activity

The -2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity of the plant extracts was determined according to the methods of Re et al. (1999). The working solution was prepared by mixing 7 mM of ABTS and 2.4 mM of potassium persulfate in the ratio 1:1 in distilled water. The mixture was allowed to react in the dark for 12 h at room temperature. After 12h, 3 ml of the working solution was further diluted with 150 ml methanol to obtain an absorbance of 0.706 ± 0.002 units at 734 nm using a spectrophotometer. This was adjusted by mixing of ABTS previously prepared drop wise. 1 ml of the working solution was then added to the extracts of varying concentrations (0.2 – 1.0

mg/ml) and allowed to react in the dark. The absorbance was measured at 734 nm after 7 min. The $ABTS^+$ scavenging capacity was compared with BHT and ascorbic acid. The percentage inhibition was calculated as follows:

$ABTS^+$ scavenging activity = $\left(1 - \frac{Abssample}{Abscontrol}\right) X\ 100$

Where Abs_{sample} is absorbance of $ABTS^+$ + sample (extract or standard)

$Abs_{control}$ is absorbance of $ABTS^+$ + methanol.

3.2.11 Ferric ion reducing antioxidant potential

The reducing power of the extract was estimated as described by Benzie & Strain (1999). The extract was prepared in different concentrations ranging from 0.02 – 0.1 mg/ml. To 1 ml of each concentration, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$) (1% w/v) were added. The resulting mixture was incubated for 20 min at 50^{o}C, 2.5 ml of trichloroacetic acid (10% w/v) was added and centrifuged at 3000 rpm for 10 min. (Labline Centrifuge model no. CF – 622). 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was measured at 700 nm against appropriate blank solution using a Vis/3000 spectrophotometer. Increase in absorbance of the reaction mixture is an indication of higher reducing power of the extract. BHT, ascorbic acid and rutin were used as positive controls.

3.2.12 Determination of nitric oxide scavenging activity

Nitric oxide scavenging activity of the extract was determined by the modified method of Ebrahimzadeh et al. (2008). 2 ml of 10 mM sodium nitoprusside prepared in 0.5 mM phosphate buffer saline (pH 7.4) was added to 0.5 ml of the extract and standards of varied concentrations (0.2 – 1.0 mg/ml) and then incubated for 2.5 h at 25^{o}C of the incubated mixture, 1 ml was then taken, mixed with 1 ml of Griess reagent (equal volume of 0.33% sulphanilic acid prepared in 20% glacial acetic acid and 0.1% (w/v)

naphthylenediaminedichloride) and incubated at room temperature for 30 min. The absorbance was measured at 540 nm and percentage nitric oxide inhibition by the extract was calculated using the equation:

NO scavenging activity (%) = $\left(\frac{Abscontrol - Abssample}{Abscontrol}\right) X\ 100$

Where Abs $_{control}$ is the absorbance of NO radicals; Abs $_{sample}$ is the absorbance of NO radical + sample or standard.

3.2.13 Determination of hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the extract was investigated using the modified method of Ruch et al. (1989). 4 mM hydrogen peroxide stock solution was prepared in 0.1 M phosphate buffer (pH 7.4). 2 ml of each sample was added to 0.6 ml of the hydrogen peroxide solution, incubated for 15 min at room temperature and then absorbance was measured at 230 nm. Percentage inhibition of hydrogen peroxide was calculated as:

Hydrogen peroxide scavenging activity % = $\left(\frac{Abscontrol - Abssample}{Abscontrol}\right) X\ 100$

Where the Abs $_{control}$ is the absorbance of H_2O_2 radicals; Abs $_{sample}$ is the absorbance of H_2O_2 radical + sample or standard.

3.2.14 Isolation of crude saponins extract

To obtain the crude saponin, the methanolic extract was fractionalized using the modified method of Fenwick et al., (1992); Igile, (1995). Briefly, the methanolic extract was spotted on a TLC plate using hexane : ethylacetate (1:1; 2:1; 1:2) and visualized under UV at 365 nm. Thereafter, it was re-spotted using hexane : ethylacetate : toluene (2:1:2) and visualised under UV at 365 nm. The crude methanolic extract was later loaded into a glass column containing silica gel 60 (0.063 – 0.200 nm) that had been previously washed with hexane :ethylacetate : toluene (2:1:2). The mobile phase was then run through the column and 176 fractions were

collected into test tubes in the order hexane : ethylacetate : toluene (2:1:2); hexane : ethylacetate (1:1); ethylacetate (300 ml); acetone (300 ml) and methanol. The fractions were subjected to thin layer chromatography (TLC) on silica gel plates (0.20 mm silica gel) using the solvent system hexane : ethylacetate : toluene (2:1:2). The developed plates were dried at room temperature. Visualization of saponins on developed plates were done by spraying with 0.5 ml anisaldehyde mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid mixed in that order. The TLC plates were then viewed under UV-365 nm. From TLC results fractions A1 – B5; B6 – C11; D1 – E6; E7 – H5; H6 – I5; I6 – P10; P11 were pooled together to get seven fractions and then concentrated.

3.2.15 Frothing test

Frothing was carried out as a confirmatory test of the presence of saponins, based on the fact that aqueous solutions of saponins form very stable foams. A 1 ml aliquot of each concentrated fraction was shaken with 5 ml of distilled water in a test tube. Stable foams would indicate the presence of saponins.

3.2.16 Evaluation of *in vitro* anti-inflammatory activity

The anti-inflammatory activity was carried out by modifying the method of Chandra, et al., (2012). 2.8 ml of phosphate buffer saline (pH 6.4) was added to 0.2 ml of egg albumin. 2 ml of the test solution which contained different concentrations of the test sample or diclofenac was then added. A control was prepared by adding distilled water instead of the test solution. This was then incubated at 37^{o}C for 5 min (160 L Economy incubator, model 227) before it was heated at 70^{o}C for 10 min in a water bath. The absorbance was read at 660 nm and the percentage protein inhibition was calculated by:

$$\% \text{ inhibition} = \left(1 - \frac{A}{A1}\right)$$

Where A = absorbance of test sample and A_1 = absorbance of control.

Statistical analysis

Statistical analysis was carried out with Minitab 12.1 1. The data were expressed as the mean ± standard deviation and a probability of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3.3 RESULTS

3.3.1 Phytochemical contents

Total phenol, flavonoids, flavonols and proanthocyanidin contents

The phytochemical contents of dried bulb extracts of *Albuca bracteata* and *Albuca setosa* are shown in Table 3.1.

Albuca setosa aqueous extract have the highest phenolic content (281.449± 1.687 mg/g) of all the solvents used for the plant, while the methanolic extract has the least (72.457± 0.235 mg/g). *Albuca bracteata* aqueous extract has the highest phenolic content (167.323±0.105mg/g) while the ethanol extract (84.491±0.138mg/g) with the least. Both plants extracts however, exhibited considerable phenolic contents with *Albuca setosa* aqueous extract (281.449±1.687 mg/g) having the overall highest phenol content and *A. bracteata* ethanol extract having the least content overall. The flavonols content of *Albuca setosa* is higher than its flavonoids content whereas the flavonoids content of *Albuca bracteata* is higher than its flavonols content. The aqueous extracts of both plants show the highest pronthocyanidins content and follows the order aqueous extract > acetone extract > methanol extract. *Albuca setosa* aqueous extract has the highest overall proanthocyanidins content while *Albuca bracteata* methanol extract has the least overall content. *Albuca bracteata* aqueous extract has the highest saponins content while *Albuca setosa* aqueous extract has the highest alkaloid content of the two plants. Comparing the aqueous extracts of the two plants (Figure 3.1) shows the major phytochemicals present being proanthocyanidins, phenols, saponins and alkaloids.

Table 3.1: Phytochemical constituents of *Albuca setosa* and *Albuca bracteata* bulb extracts

	AQUEOUS		ACETONE		METHANOL		ETHANOL
	A. setosa	*A. bracteata*	*A. setosa*	*A bracteata*	*A. setosa*	*A. bracteata*	*A. bracteata*
Total Phenols[1]	281.449±1.687	167.323±0.105	165.511±2.054	117.364±0.026	72.457±0.235	85.262±0.069 [a]	84.491±0.138 [a]
Flavonols[2]	124.31±0.126 [a]	164.09±0.029 [a]	2.066±0.287	26.282±0.029	1.59±0.064 [b]	1.386±0.015 [b]	1.097±0.026 [b]
Flavonoids[2]	128.39±0.029	43.4±0.026	2.542±0.153	5.355±0.026	1.853±0.039	0.68±0.015	1.063±0.015
Proantho cyanidins[3]	124.66±0.00	118.83±0.58	97.08±0.34	84.85±0.34	43.88±0.67	18.06±0.58	32.04±0.00
Tannin[1]	10.7±0.00245	7.4±0.000	0.103±0.0004	0.104±0.0001	0.095±0.0006	0.102±0.0002	0.095±0.02
Saponin	147.43±0.06	415.5±0.01	24.3±0.04	73.5±0.02	63.0±0.16	115.48±0.06	ND
Alkaloids	211.365±0.80	136.37±0.01	45.361±0.77	32.7±0.05	44.439±0.03	34.44±0.03	ND

- Data expressed as mean ± SD; n= 3, values with the same superscripts do not differ significantly ($P < 0.05$), the subscript indicates [1]Expressed as mg/g of the extracts as tannic acid equivalent, [2]Expressed as mg/g of the extracts as quercetin equivalent, [3]Expressed as mg/g of the extracts as catechin equivalent, ND - not determined

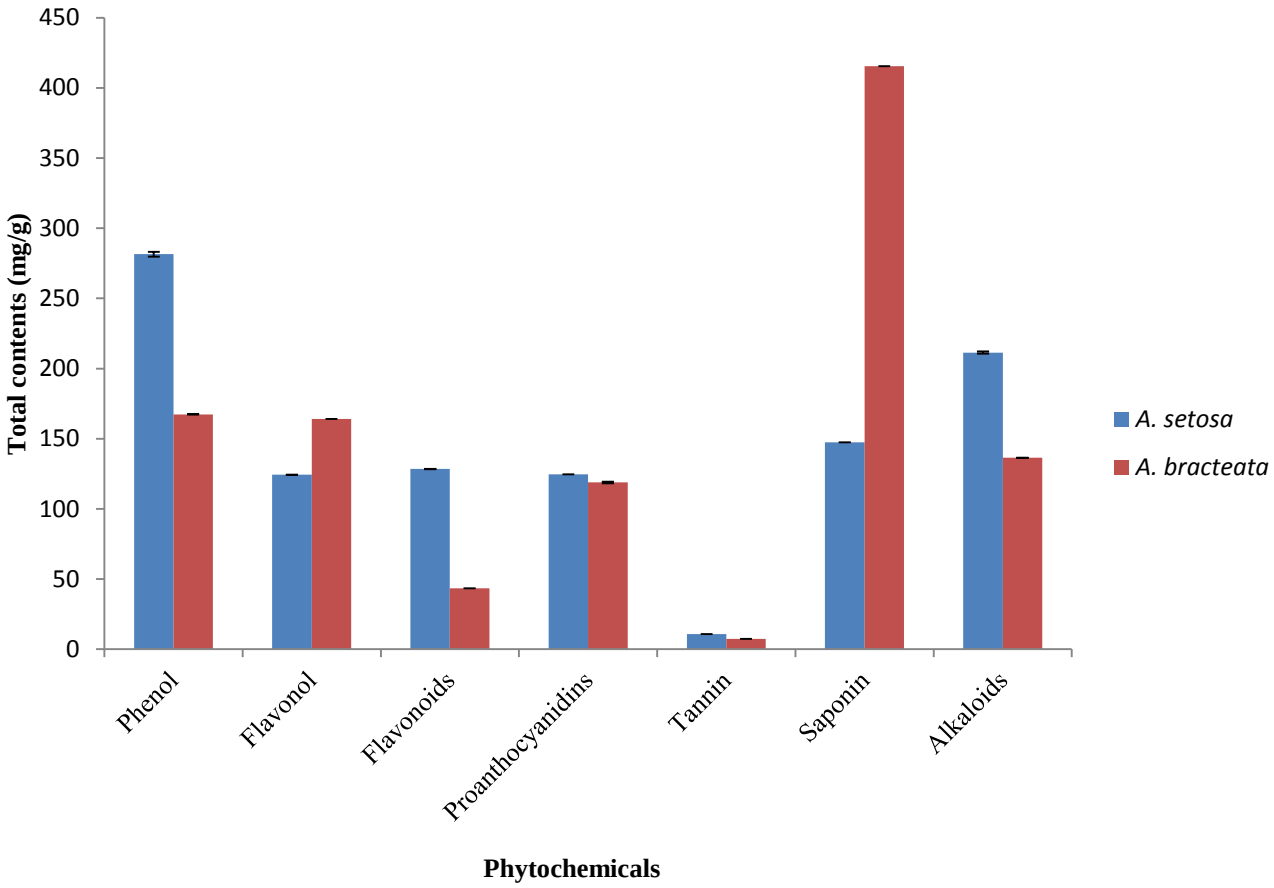

Figure 3.1 : Phytochemical constituents of aqueous bulb extracts of *Albuca setosa* and *Albuca bracteata*
Data expressed as mean ± SD, n=3

3.3.2 Antioxidant activity

The antioxidant activities of *Albuca setosa* and *Albuca bracteata* were determined by percentage inhibition of free radicals.

3.3.2.1 DPPH radical scavenging activity

The percentage inhibitory activity observed for DPPH of the aqueous and methanolic extract of *Albuca bracteata* and *Albuca setosa* compared with rutin and vitamin C is shown in Figures 3.2 and 3.3 respectively. *Albuca setosa* aqueous extract shows higher inhibition of DPPH radical compared to *Albuca bracteata* aqueous extract at all the concentrations investigated but lower than rutin (Figure 3.2). In contrast, the methanolic extracts of *Albuca bracteata* show higher inhibition of DPPH radical than *Albuca setosa* at all the concentrations

investigated but also lower than both vitamin C and rutin (Figure 3.3). From the percentage inhibitions of the extracts and the standards, a regression equation was generated and IC50 was calculated for each (Table 3.2). The value of IC_{50} is inversely related to activity and is the concentration required to attain 50% inhibitory effect. Both plants were found to show different activities in different solvent systems. Comparing the activity of both plants, *Albuca setosa* (0.330mg/ml) showed higher activity than *Albuca bracteata* (0.647mg/ml).

The isolated saponins from *Albuca bracteata* has a higher DPPH scavenging activity than the crude methanolic extract of the plant in a concentration dependent manner but they are significantly different from each other at 0.4, 0.6 and 1.0 mg/ml only (Figure 3.4). The scavenging activity of the saponins was significantly lower than that of the standards. The IC_{50} of the saponins was also observed to be higher than the crude extracts and standards (Table 3.3).

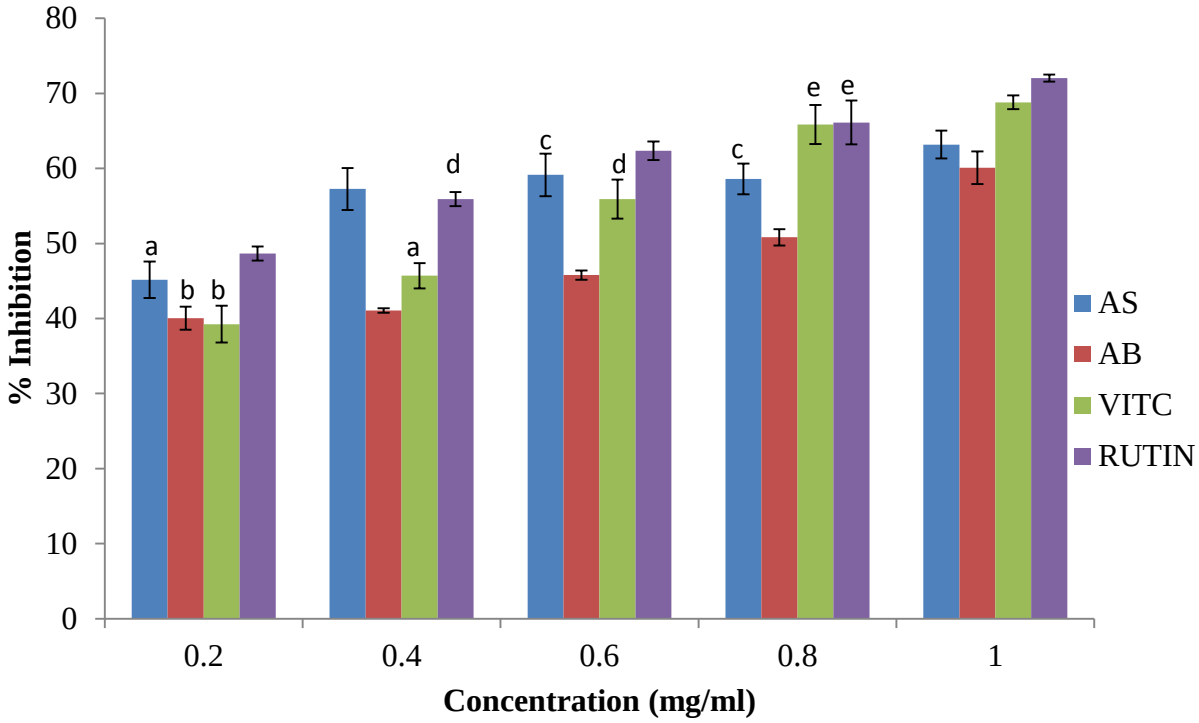

Figure 3.2: DPPH scavenging activity of aqueous bulb extracts of *Albuca setosa* and *Albuca bracteata.* Data expressed as mean ± SD, n= 3. All the bars with the same letters are not significantly different at (P < 0.01). AS: *Albuca setosa*; AB: *Albuca bracteata*. VIT C: Vitamin C.

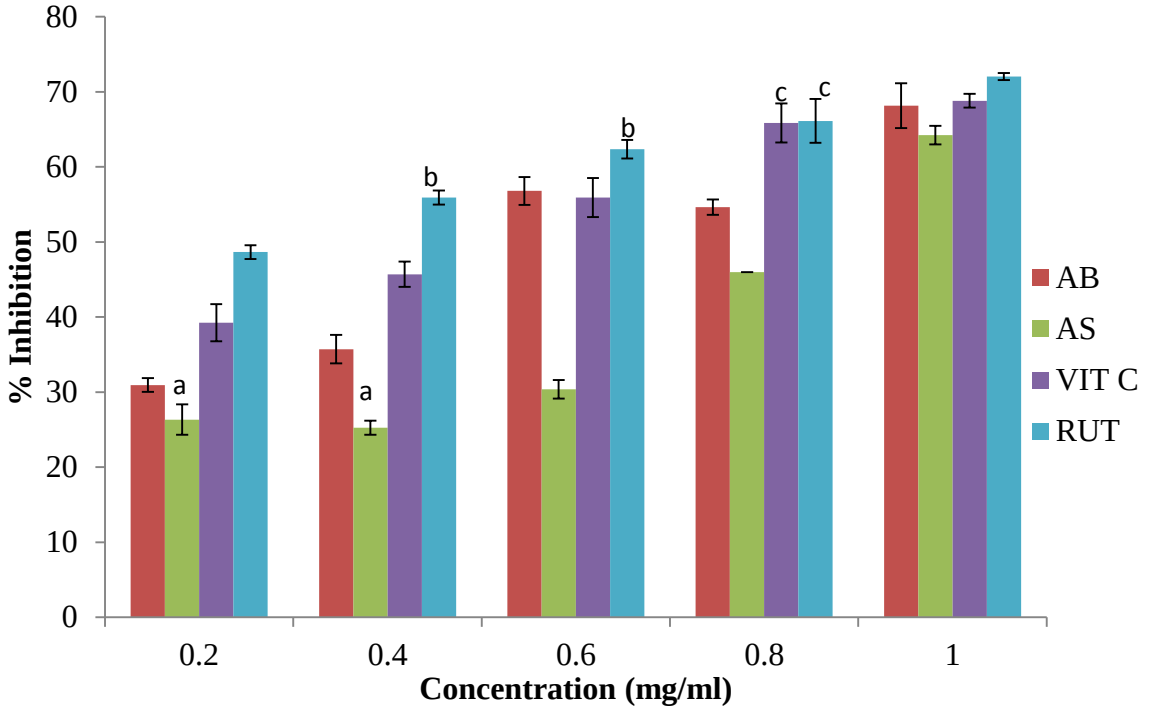

Figure 3.3: DPPH scavenging activity of methanollic bulb extract of *Albuca setosa* and *Albuca bracteata*.Data expressed as mean ± SD, n= 3. All the bars with the same letters are not significantly different at (P < 0.01). AS: *Albuca setosa;*AB: *Albuca bracteata*. RUT: Rutin.

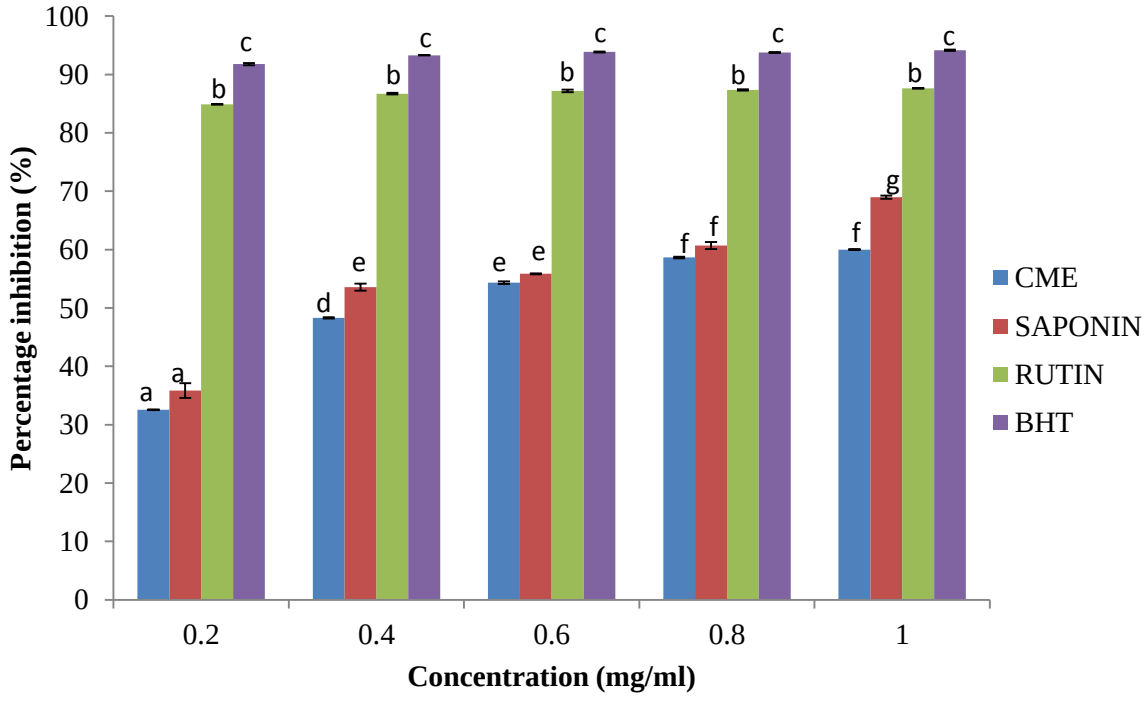

Figure 3.4: DPPH scavenging activity of saponins and methanolic bulb extract of *Albucabracteata*. Data expressed as mean ± SD, n=3. All the bars with the same letters are not significantly different at (P < 0.01). CME: Crude methanolic extracts; BHT: Butylated hydroxytoluene.

Table 3.2: IC_{50} of *Albuca setosa* and *Albuca bracteata* bulb extracts

	Sample	DPPH		ABTS		Reducing power		Nitric oxide		Hydrogen peroxide	
		IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2
Albuca setosa	Aqueous	0.330	0.9755	0.0809	0.9804	0.416	0.753	0.614	0.9942	1.3448	0.9279
	Methanol	0.5379	0.9017	0.3357	0.9859	0.4923	0.8921	0.570	0.9396	0.7708	0.9506
Albuca bracteata	Aqueous	0.6467	0.763	0.1326	0.9961	0.344	0.4463	0.564	0.9631	1.2306	0.8804
	Methanol	0.9027	0.6453	0.3817	0.9925	0.381	0.3991	0.655	0.8054	0.4092	0.9145
Rutin	-	0.2383	0.9761	0.222	0.9981	0.548	0.8287	-	-	-	-
BHT	-	-	-	0.170	0.9737	-	-	0.543	0.8876	0.3833	0.995
Vit. C	-	0.4004	0.9434	-	-	0.309	0.525	0.723	0.7939	0.2324	0.962

IC_{50} is defined as the concentration (mg/ml) sufficient to obtain 50% of a maximum scavenging capacity
R^2: coefficient of determination; values obtained from regression lines with 95% confidence level.
-: values not determined

Table 3.3: IC_{50} Scavenging activity of *Albuca bracteata* bulb extracts and standards

Sample	DPPH		ABTS	
	IC_{50}	R^2	IC_{50}	R^2
Saponin	0.530	90.17	0.526	94.67
CME	0.462	85.43	0.51	92.8
Rutin	0.439	78.19	0.620	99.49
BHT	0.442	76.15	0.617	98.07

IC_{50} is defined as the concentration (mg/ml) sufficient to obtain 50% of a maximum scavenging capacity
R^2: coefficient of determination; values obtained from regression lines with 95% confidence level.
CME: Crude methanolic extract

3.3.2.2 ABTS radical scavenging activity

The percentage inhibition of ABTS radicals of the aqueous and methanolic extracts of *Albuca setosa* and *Albuca bracteata* investigated is shown in Figures 3.5 and 3.6 respectively. *Albuca setosa* aqueous extract showed higher percentage inhibition of ABTS radical than *Albuca bracteata* at all the concentrations investigated but not significantly different from rutin as shown in Figure 3.5. *Albuca setosa* methanol extract also showed higher inhibition activity than *Albuca bracteata* as shown in Figure 3.6. From the percentage inhibitions of the extracts and the standards, a regression equation was generated and IC_{50} was calculated for each (Table 3.2). The value of IC50 is inversely related to activity and is the concentration required to attain 50% inhibitory effect. Overall, *Albuca setosa* aqueous extract (0.0809 mg/ml) showed maximum activity against ABTS radical as determined by the IC_{50} (Table

3.2). The activity follows the order *Albuca setosa* extract > *Albuca bracteata* aqueous extract > BHT > rutin >*Albuca setosa* methanol extract >*Albuca bracteata* methanol extract.

The ABTS radical scavenging activity of the isolated saponins compared favourably with the standards (Figure 3.7). The crude methanolic extract showed less scavenging activity when compared with the standards and saponins. All the tested samples and the standards showed an increase in percentage inhibition in a concentration-dependent manner. The IC_{50} of both saponin and crude methanolic extract were calculated and found to be lower than the values of the standards used (Table 3.3). The IC_{50} of saponins was higher than the crude methanolic extract but lower than the standards.

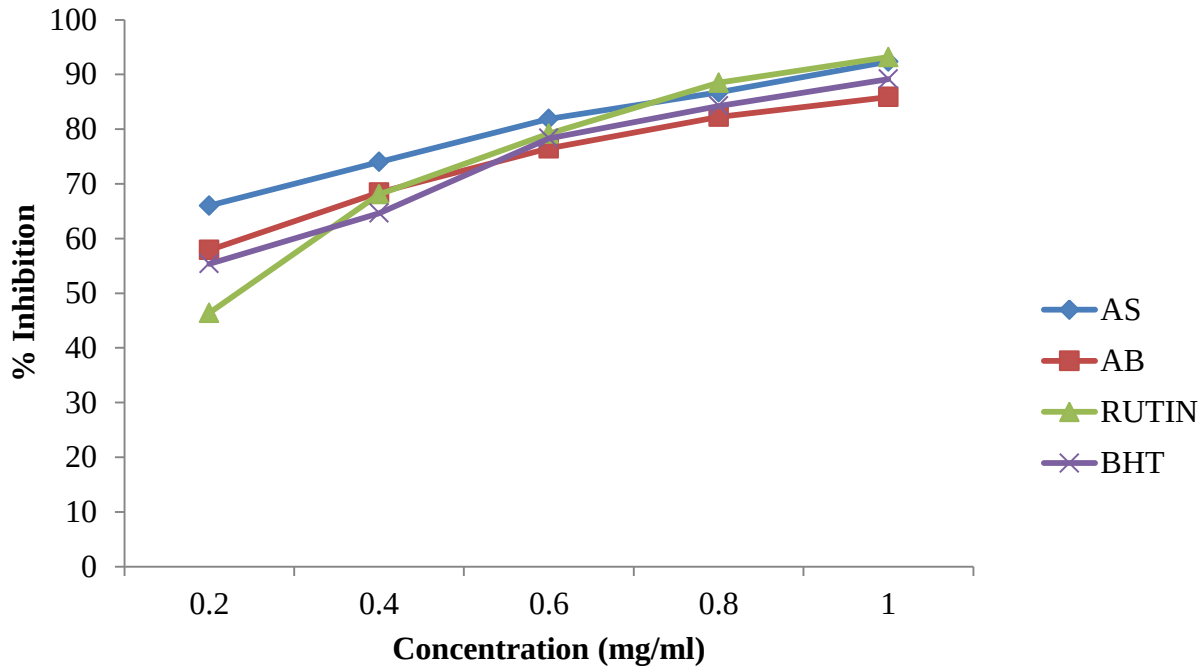

Figure 3.5: ABTS scavenging activity of aqueous bulb extracts of *Albuca setosa*
Data expressed as mean ± SD, n=3. *Albuca setosa*; AB: *Albuca bracteata* BHT: Butylated hydroxytoluene.

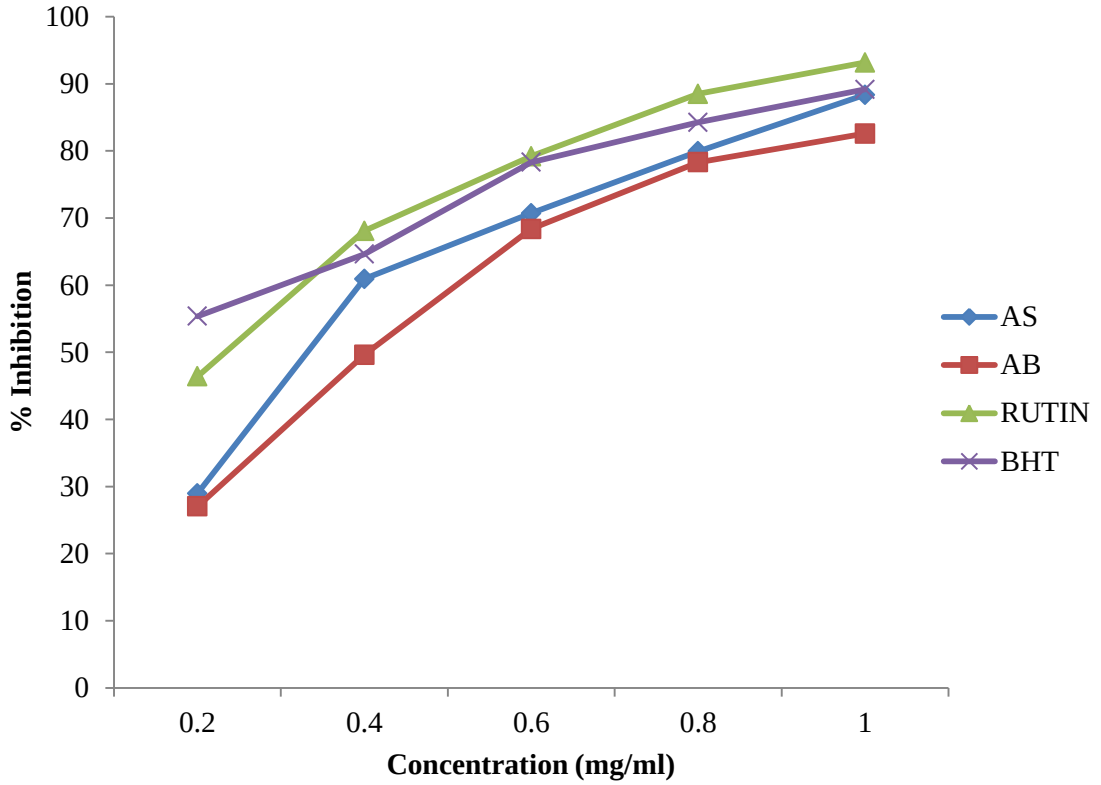

Figure 3.6: ABTS scavenging activity of methanolic bulb extracts of *Albuca setosa*
Data expressed as mean ± SD, n=3. AS: *Albuca setosa*; AB: *Albuca bracteata* BHT: Butylated hydroxytoluene.

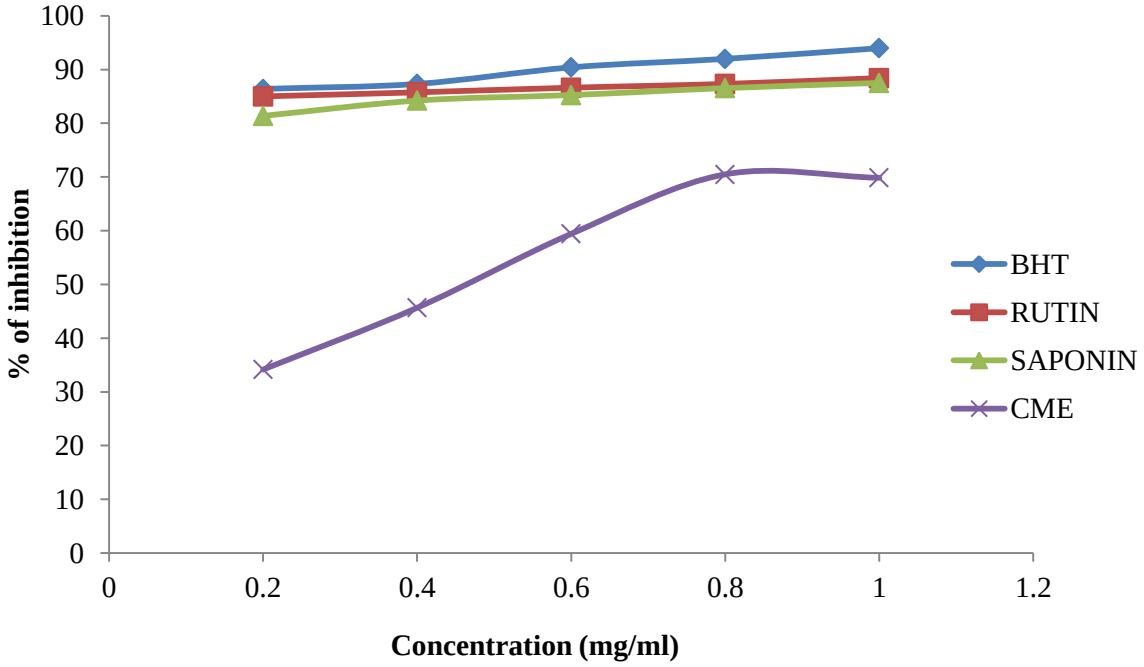

Figure 3.7: ABTS scavenging activity of saponin and crude methanolic bulb extractof *Albuca bracteata.*Data expressed as mean ± SD, n=3. CME: Crude methanolic extracts; BHT: Butylated hydroxytoluene.

3.3.2.3 Ferric ion reducing antioxidant potential

The reducing power of *Albuca setosa* and *Albuca bracteata* extracts shown in Figure 3.8, indicates that the iron reducing power was significantly higher ($P < 0.05$) in the methanolic extract of both plants compared to the aqueous counterpart. There is no significant difference in the percentage inhibition of Albuca *bracteata* methanolic extract (0.4 mg/ml) and *Albuca setosa* aqueous extracts at a concentration of 0.4 - 1.0mg/l. Both rutin and vitamin C have higher reducing power than both plants except for the *Albuca setosa* methanol extract (0.8 mg/ml) that does not differ significantly from rutin (0.2 mg/ml). From the percentage inhibitions of the extracts and the standards, a regression equation was generated and IC_{50} was calculated for each (Table 3.2). The value of IC_{50} is inversely related to activity and is the concentration required to attain 50% inhibitory effect. Overall *Albuca bracteata* aqueous extract (0.344 mg/ml) showed maximum activity (Table 3.2). The activity follows the order *Albuca bracteata* aqueous extract > *Albuca bracteata* methanol extract >*Albuca setosa* aqueous extract> *Albuca setosa* methanol extract.

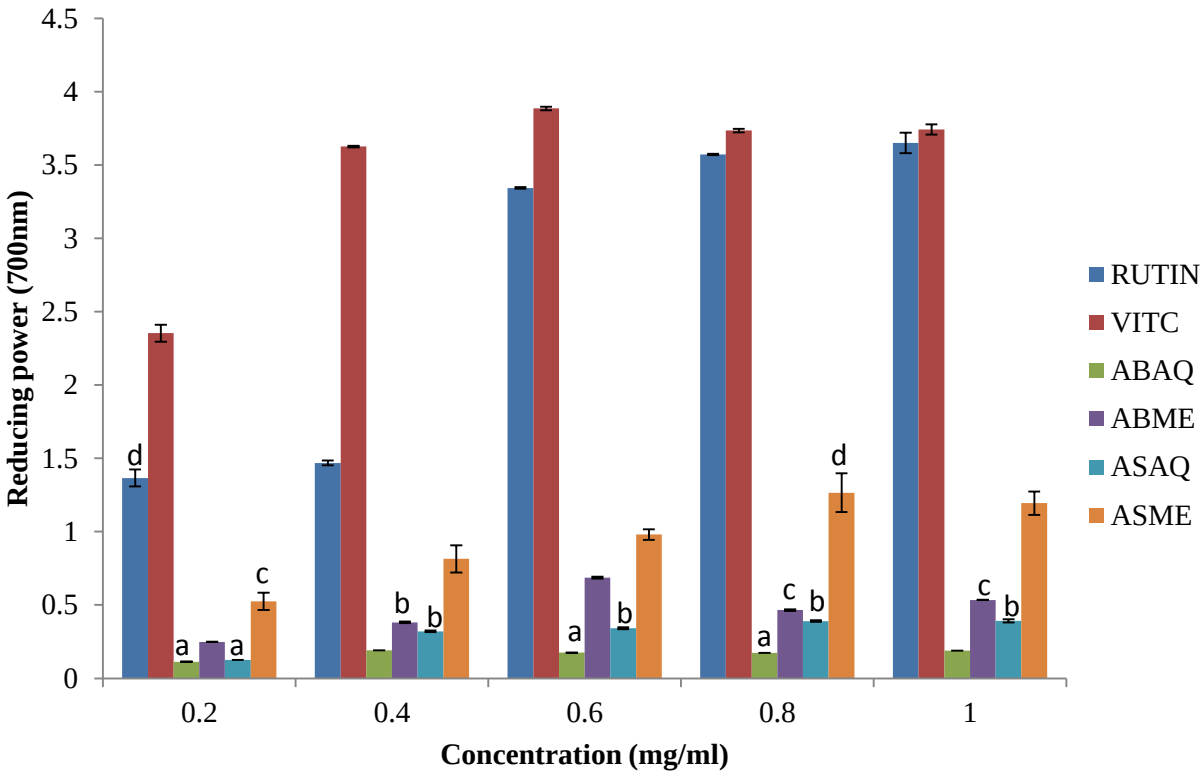

Figure 3.8: Reducing power of *Albuca setosa* and *Albuca bracteata* bulb extracts
Data expressed as mean ± SD, n=3. All the bars with the same letters are not significantly different at ($P < 0.01$). ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract, ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract. VIT C: Vitamin C.
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3.3.2.4 Nitric oxide inhibition activity

Nitric oxide inhibition activity of the extracts is shown in Figure 3.9. The percentage inhibitions of extracts were concentration-dependent and were not significantly different from each other at 0.4mg/ml of the methanolic extracts. The methanolic extracts of both plants were significantly higher than that of the aqueous extracts and compared favourably with the standards. A regression curve was generated from the percentage inhibitions of the extracts and the standards, and IC_{50} was calculated for each (Table 3.2). The value of IC_{50} is inversely related to activity and is the concentration required to attain 50% inhibitory effect. Overall *Albuca bracteata* aqueous extract (0.564 mg/ml) showed maximum activity as determined by the IC_{50} (Table 3.2). The activity follows the order *Albuca bracteata* aqueous extract

>*Albuca setosa* methanol extract > *Albuca setosa* aqueous extract> *Albuca bracteata* methanol extract.

The nitric oxide scavenging activity of both the saponins and crude extracts showed inhibitory activity in a concentration-dependent manner (Figure 3.10). However, the crude extract has more nitric oxide inhibition activity than the crude saponins extract but they are not significantly different from each other.

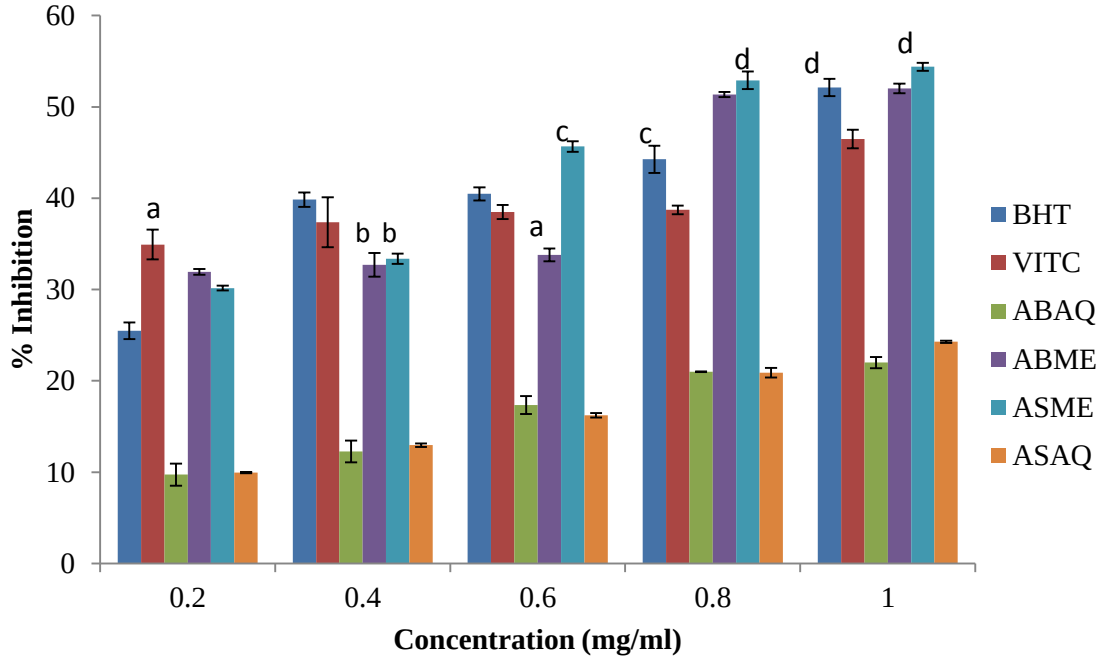

Figure 3.9: Nitric oxide scavenging activity of bulb extracts of *Albuca setosa* and *Albuca bracteata*.
Data expressed as mean ± SD, n=3. All the bars with the same letters are not significantly different at (P < 0.01).BHT: Butylated hydroxytoluene, VIT C: Vitamin C, ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract, ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract.

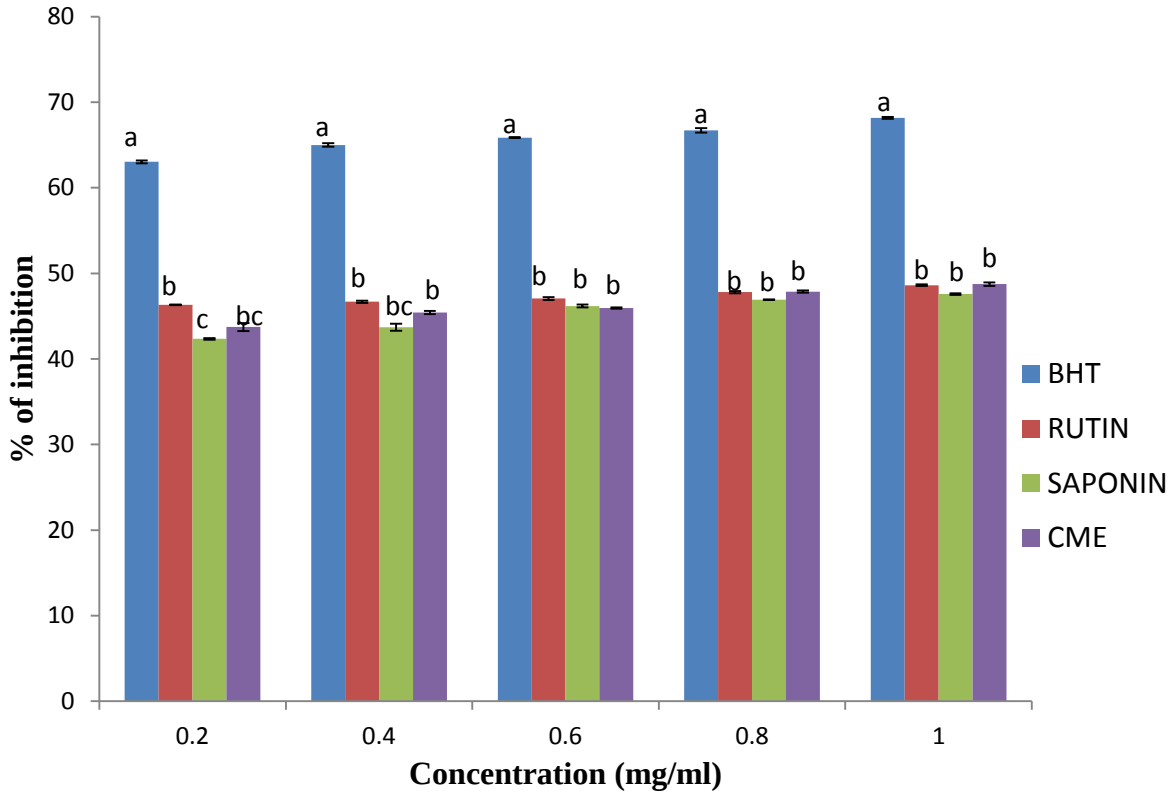

Figure 3.10: Nitric oxide scavenging activity of saponins and crude methanolic bulb extract of *Albuca bracteata*

Data expressed as mean ± SD, n=3. All the bars with the same letters are not significantly different at ($P < 0.01$).BHT: Butylated hydroxytoluene, CME: Crude methanolic extracts.

3.3.2.5 Hydrogen peroxide inhibition activity

The percentage inhibition of hydrogen peroxide of the extracts is presented in Figure 3.11. Among the extracts investigated, the methanolic extracts of both plants possess higher percentage inhibition compared to the aqueous extracts and compared favourably with the both BHT and Vit. C. There is no significant difference between the aqueous extracts of both plants at 0.2 and 0.4 mg/ml. The percentage of hydrogen peroxide inhibition follows the order Vit. C >*Albuca bracteata* ethanol extract > BHT >*Albuca setosa* methanol extract > *Albuca bracteata* aqueous extract >*Albuca setosa* aqueous extract. A regression equation was generated from the percentage inhibitions of the extracts and the standards and IC_{50} was calculated for each (Table 3.2). The value of IC_{50} is inversely related to activity and is the

concentration required to attain 50% inhibitory effect. Comparing both plants, *Albuca bracteata* methanolic extract (0.4092 mg/ml) showed maximum activity against hydrogen peroxide (Table 3.2). The activity follows the order Vit. C > BHT >*Albuca bracteata* methanolic extract > *Albuca setosa* methanol extract > *Albuca bracteata* aqueous extract> *Albuca setosa* aqueous extract.

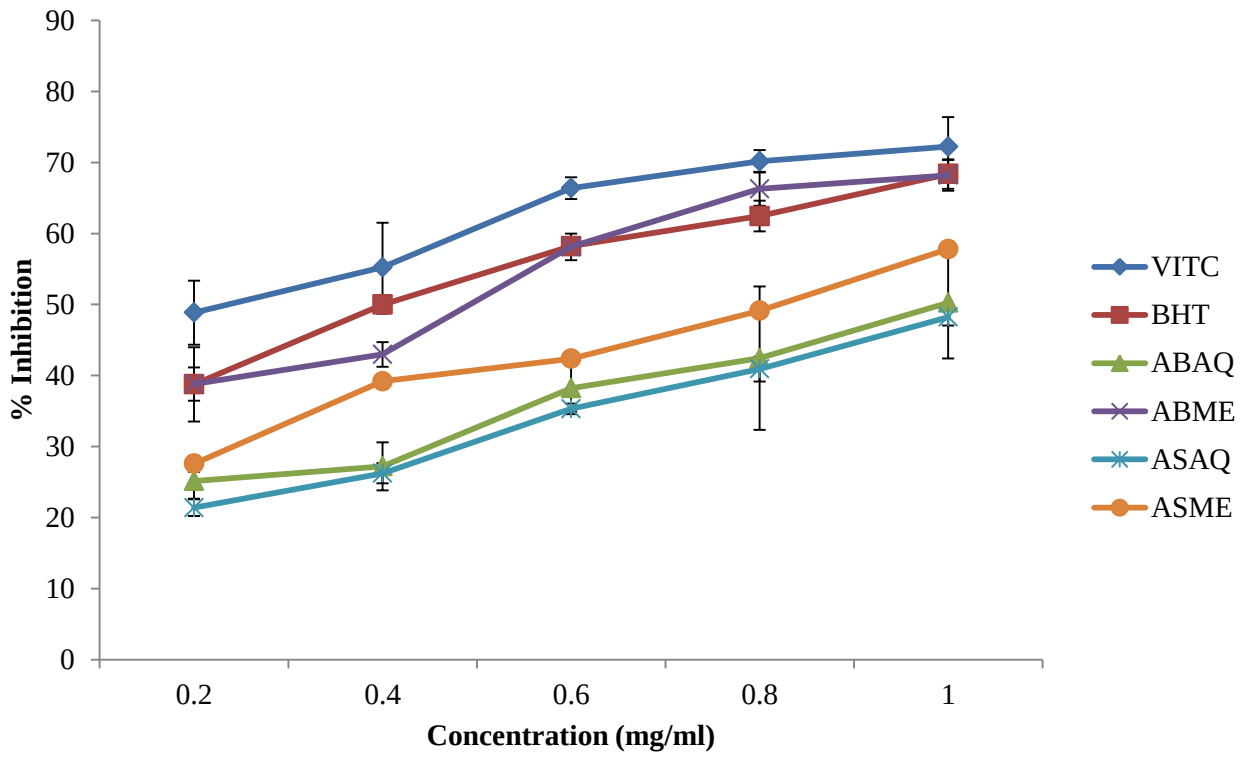

Figure 3.11: Hydrogen peroxide scavenging activity of bulb extracts of *Albuca setosa* and *Albuca bracteata*
Data expressed as mean ± SD, n=3. VIT C: Vitamin C; BHT: Butylated hydroxytoluene; ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract; ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract.

3.3.2.6 *In vitro* anti-Inflammatory activity

The *in vitro* anti-inflammatory activity of the saponins as shown in Figure 3.12 indicates that crude methanolic extract of *A. bracteata* has a higher percentage inhibition of protein denaturation than the saponins in a concentration-dependent manner. The inhibition of saponins compared favourably with that of diclofenac at the concentration investigated. The IC_{50} of crude methanolic extract was also lower compared with saponins and diclofenac sodium respectively (Table 3.4).

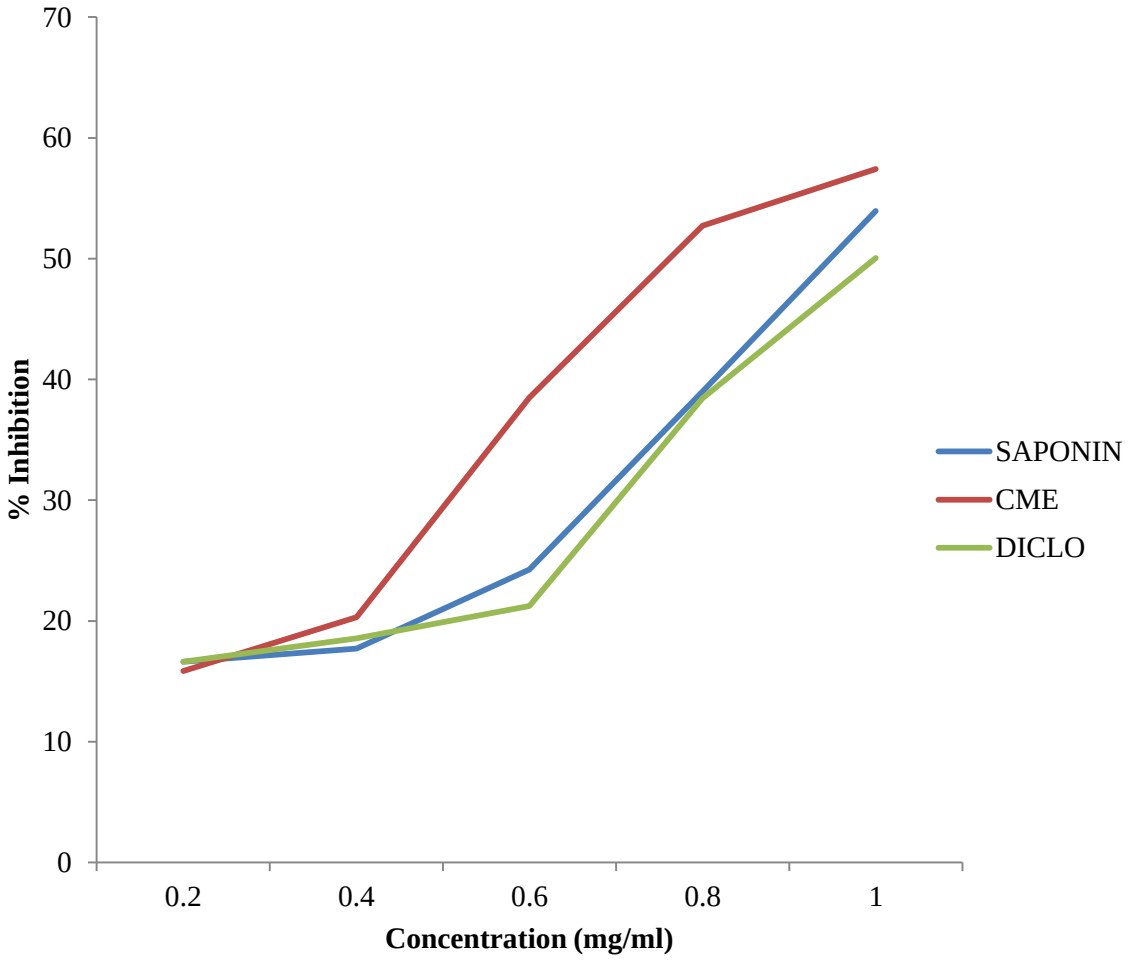

Figure 3.12: Anti-inflammatory activity of saponins and methanolic bulb extract of *Albuca bracteata.*
Data expressed as mean ± SD, n=3. CME: Crude methanolic extracts, DICLO: Diclofenac.

Table 3.4: IC_{50} activity of *Albuca bracteata* bulb extracts and standards

Sample	DPPH	
	IC_{50}	R^2
Saponin	0.704	90.49
CME	0.594	95.89
Diclofenac	70.04	88.01

IC_{50} is defined as the concentration [(µg/ml) for Diclofenac, (mg/ml) for CME and saponins] sufficient to obtain 50% of a maximum inhibition capacity
R^2: coefficient of determination; values obtained from regression lines with 95% confidence level.
CME: Crude methanolic extract

3.4 DISCUSSION

Phytochemicals are secondary metabolites that occur naturally in plants, and have been implicated in a variety of functions such as stimulation of protective enzymes, inhibition of nitrosation or formation of DNA adducts (Craig, 1999). The phytochemical analysis of solvent extractions of the bulb of *Albuca bracteata* and *Albuca setosa* revealed the presence of phenols, saponins, alkaloids, flavonols and flavonoids. The antioxidant capacity of these phytochemicals has been reported to be essential in ameliorating several diseases such as diabetes, cancer and arteriosclerosis (Olorunnisolaet al., 2011). The differences observed in the phytochemical contents may be attributed to microclimate change, nature of the soil (Choi et al., 2008) and processing methods and/or solvents of extraction. Polyphenols such as phenols, flavonols, flavonoids and proanthocyanidins have been reported to possess high antioxidant activity due to their redox properties. These are conjugated ring structures which enable them to absorb, scavenge, neutralise free radicals and inhibit lipid peroxidation (Duh, et al., 1999; Rice-Evans, et al., 1995). In this study it was found that *Albuca bracteata* possesses higher phenols and flavonols than *Albuca setosa,* which could account for its antioxidant potential; however, *Albuca setosa* possess higher tannin and proanthocyanidins content than *Albuca bracteata*. Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins (Ashok & Upadhyaya, 2012). This attribute of tannins could indicate *Albuca setosa* to be a good anti-inflammatory agent. Saponins are synthesized by plants and are used for protection against pathogens thus serving as natural antibiotics (Okwu and Emenike, 2006; Otunolaet al., 2010). This natural tendency for saponins in plants to ward off microbes may indicate that the presence of saponins in *Albuca bracteata* and *Albuca setosa* make it a good potential antifungal or antibacterial agent. Foam formation in aqueous solutions, lowering of cholesterol binding (thus interfering with cell growth and division), haemolytic activity, bitterness and the ability to inhibit or kill cancer cells (Okwu,

2005; Okwu, 2008; Okwu and Emenike, 2006) have also been associated with the presence of saponins. Recent reports have shown that saponins also have the ability to cure diabetes by reducing low grade and chronic subclinical inflammation associated with the increased risk of diabetes, as well as its hypolipidemic and increased insulin sensitization properties (Haffner, 2003; Bhavsaret al., 2009). Therefore, the presence of saponins in *Albuca bracteata* and *Albuca setosa* may justify their folkloric use in the treatment of diabetes.

Alkaloids in plants have been associated with analgesic effects and bactericidal activities (Okwu, 2004). Most plants reported to have medicinal values have been shown to contain traces of alkaloids (Okwu and Emenike, 2006), therefore, the presence of alkaloids in *Albuca bracteata* and *Albuca setosa* is an indication that the plants may have medicinal properties.

The DPPH scavenging activity of the extracts was used as a model for anti-oxidant capacity. Polyphenols, flavonoids, terpenes and tannins compounds present in plant and plant products have been reported to have anti-oxidative capacity (Dioufet al., 2009). These compounds donate electrons to DPPH radicals thereby making them stable molecules. These scavenging abilities have been attributed to the presence of phenolic compounds (Hasan et al., 2008). In this study, the high DPPH scavenging activity of *Albuca setosa* as compared to *Albuca bracteata* could be attributed to the high flavonoids, proanthocyanidins and alkaloids present in the plant. Similar reports have shown that flavonoids have antioxidant activities and exhibit this antioxidant property by scavenging free radicals (Gonçalves, et al., 2005; Pietta, 2000; Plumb, et al., 1998; Rice-Evans, et al., 1996). In addition, the antioxidant and antidiabetic activity of alkaloids have been reported (Tiong et al., 2013) and may contribute to the higher activity observed in *Albuca setosa* and thus its significance in diabetes treatment. The free radical scavenging activity of saponins observed to be higher than the methanolic extract could be due to the inhibitory effect of other compounds present in the

crude methanolic extract and may suggest that further purification of the saponins isolated could increase its free radical scavenging activity.

The ABTS scavenging method measures the antioxidant properties of the plant extracts using a blue chromophore. In this study, the plant extracts and standards scavenged the ABTS radical in a concentration-dependent manner. The high ABTS activity of the aqueous and methanolic extracts of *Albuca setosa* could be attributed to a high flavonoid and alkaloid content which is similar to previous reports (Gonçalves et al., 2005). The difference observed in the antioxidant activities of these plants may be due to solvents used for extraction, the mechanism of free radical scavenging activity and their solubility in different testing systems (Oyedemi, et al., 2010; Yu et al., 2002) and may be similar to a report which found that compounds with DPPH activity may not necessarily have ABTS activity (Wang, et al., 1998).

The reducing power measures the electron transfer ability of the plant extracts by reducing Fe^{3+} to Fe^{2+} and has been associated with the presence of a redundant molecule or complex that serves as the electron donor and/or free radical scavengers. Both plant extracts show antioxidant activity and this could be an indication that the plants have the ability to reduce oxidative damage in cells. The higher reducing activity of *Albuca bracteata* may be due to the presence of high phenolic compounds which may act as electron donors. This result supports the report of El-Hashasa, 2010; Mohamed, 2009 and Awika, 2003 that the reducing power of a plant correlates with its phenolic content.

Nitric oxides are generated by the endothelia cells, macrophages and neurons where they serve as important chemical messengers. Nitric oxide is also implicated in the regulation of various physiological processes but excess concentrations can lead to disease conditions (Ross, 1999). The nitric oxide scavenging activity of phenolics and flavonoids have been reported (Crozier et al., 2000; Kim, et al., 1999; Lindberg Madsen, et al. 2000), and could

explain the higher activity observed in *Albuca bracteata.* Inhibition of nitric oxide may suggest the mechanism for saponin activity against inflammation.

Saponins have been thought to exert their activity through the inhibition of TNF – α, a cytokine that is also involved in systemic inflammation (Chen, et al., 1994; Grivennikov & Karin, 2011).

The penetration of hydrogen peroxide into the cellular membrane accounts for its toxicity and could be more toxic if it is converted into hydroxyl radicals in biological cells (Oktay, et al., 2003). Hydroxyl radicals are thus more toxic than hydrogen peroxide and other reactive oxygen species and are capable of damaging biological molecules including DNA, proteins, and polyunsaturated fatty acids (Lobo et al., 2010). Alkaloids have been reported to decrease ROS formation and may therefore be involved in the cytoprotection against oxidative stress via the inhibition of hydrogen peroxide (Xie et al., 2013). The higher activity of *Albuca setosa* against hydrogen peroxide may be attributed to the high alkaloid and polyphenols content. In alternative medicine, hydrogen peroxide has been used when mixed with water to treat skin and mouth infections (Fang, 2002) due to its ability to penetrate cellular membranes.

Inhibit protein denaturation suggests that both the crude methanolic extract and the isolated saponins have significant anti-inflammatory activity that might be mediated through the inhibition of agents that cause inflammations. Although the percentage inhibition by the crude methanolic extract was higher than that of the saponins this may be attributed to the presence of other therapeutic agents in the crude methanolic extract. The ability of the extract and saponins to inhibit thermal and hypotonic protein denaturation may contribute to its anti-inflammatory properties similar to the mode of action of NSAIDs (Mizushima, 1964).

Ndebia et al. (2011) suggested that the precise mode of action of saponins could be through the inhibition of phospholipase A_2 (PLA_2) activity or the cyclooxygenase cascade, which blocks the release of vasoactive substances like histamine, serotonin and kinins.

Other modes of action have been shown to act through the inhibition of COX-2, or tumour necrosis factor-α (TNF-α) (Yaun, et al., 2006). Since nitric oxide is essential during cellular signalling, it is involved in many physiological and pathological processes including inflammation (Leiper & Nandi, 2011), protection of liver ischemic damage (Yang et al., 2011), carcinogenesis and rheumatoid arthritis (Melchers, et al., 2006).

Also, the numerous biological activities of saponins have also be suggested to be linked to their amphiphilic nature which could also explain their ability to intercalate into the plasma membrane resulting in changes in membrane fluidity that in turn may affect membrane function (Hassan et al., 2012).

In conclusion, the results of this present study show that both *Albuca setosa* and *Albuca bracteata* extracts possess high antioxidant activities which were comparable with the standards. These antioxidant properties could be as a result of the presence of polyphenols, saponins and alkaloids in both plants. The study also shows that saponin and methanolic extracts possess antioxidant and free radical scavenging activity and were effective at inhibiting thermal albumin denaturation induced inflammatory processes.

These plants could be useful in alleviating oxidative stress and degenerative diseases. This may indeed be the mode of action through which *Albuca bracteata* exerts its anti-diabetic properties as claimed by traditional healers.

REFERENCES

Akash, H. M. S., Rehman, K., & Chen, S. (2013). Role of inflammatory mechanisms in pathogenesis of type 2 diabetes mellitus. *Journal of Cellular Biochemistry*. http://doi.org/10.1002/jcb.24402

Aqil, F., Ahmad, I., & Mehmood, Z. (2006). Antioxidant and Free Radical Scavenging Properties of Twelve Traditionally Used Indian Medicinal Plants. *Turkish Journal of Biology*, *30*, 177–183.

Ashok, P., & Upadhyaya, K. (2012). Tannins are Astringent. *Journal of Pharmacognosy and Phytochemistry*, *1*(3), 45–50.

Awika, Joseph M, Rooney, Lloyd W, Wu, Xianli, Prior, Ronald L, Cisneros-Zevallos, Luis. (2003).Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products.*Journal of Agricultural and Food Chemistry*. 51 (23): 6657-6662

Benzie, I. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, *299*, 15–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9916193

Bhavsar, S. K, Föller, M., Gu, S., Vir, S., Shah, M. B, Bhutani, K.K, Santani, D. D, Lang, F. (2009). Involvement of the PI3K/AKT pathway in the hypoglycemic effects of saponins from *Helicteres isora*.*Journal of Ethnopharmacology.* 126(3): 386-396

Chandra, S., Chatterjee, P., Dey, P., & Bhattacharya, S. (2012). Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pacific Journal of Tropical Biomedicine*, *2*. http://doi.org/10.1016/S2221-1691(12)60154-3

Chen, Y. L., Le Vraux, V., Giroud, J. P., & Chauvelot-Moachon, L. (1994). Anti-tumor necrosis factor properties of non-peptide drugs in acute-phase responses. *European Journal of Pharmacology*, *271*(2-3), 319–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7705432

Choi, D.J., Lee, S.J., Kang, M.J., Cho, H.S., Sung, N.J., Shin, J.H. (2008). Physicochemical Characteristics of Black Garlic (*Allium sativum* L.). *Journal of the Korean Society of Food Science and Nutrition* 37(4): 465-471

Cocks, M. L., & Dold, A. P. (2006). Cultural Significance of Biodiversity: The Role of Medicinal Plants In Urban African Cultural Practices In The Eastern Cape, South Africa. *Journal of Ethnobiology*, 26(1), 60-81.

Craig, W J. (1999). Health-promoting properties of common herbs.*The American Journal of Clinical Nutrition.* 70(3 Suppl): 491S-499S

Crozier, A., Burns, J., Aziz, A. A., Stewart, A. J., Rabiasz, H. S., Jenkins, G. I.,Edwards, C A., Lean, M E. (2000). Antioxidant flavonols from fruits, vegetables and beverages:

measurements and bioavailability. *Biological Research*, *33*(2), 79–88. http://doi.org/10.4067/S0716-97602000000200007

Diouf, P. N, Stevanovic, T., Cloutier, A. (2009). Study on chemical composition, antioxidant and anti-inflammatory activities of hot water extract from *Picea mariana* bark and its proanthocyanidin-rich fractions. *Food Chemistry*. 113(4): 897-902

Duh, P. ., Tu, Y. ., & Yen, G. . (1999). Antioxidant activity of water extract of Harng Jyur (Chrssanthemum morifolium Ramat). *Lebnesmittel-Wissenschaft Und Technologie*, *32*, 269–277.

Ebrahimzadeh, M. A., Pourmorad, F., & Hafezi, S. (2008). Antioxidant Activities of Iranian Corn Silk. *Turkish Journal of Biology*, *32*(1), 43–49.

El-Hashash, M. M, Abdel-Gawad, M. M, El-Sayed, M. M, Sabry, W. A, Abdel-Hameed, E. S, Abdel-Lateef, E. E (2010). Antioxidant properties of methanolic extracts of the leaves of seven Egyptian Cassia species.*Acta pharmaceutica (Zagreb, Croatia)* 60(3):361-367

Fang, Y, Yang, S, Wu, G. (2002). Free radicals, antioxidants, and nutrition.*Nutrition (Burbank, Los Angeles County, Calif.)* 18(10): 872-879

Farber, J. L. (1994). Mechanisms of cell injury by activated oxygen. *Environmental Health Perspectives*, *102*(10), 17–24.

Fenwick GR, Price KR, TsuKamoto C, O. K. (1992). *Institute of Food Research, Norwich Laboratory*. Norwich: Institute of Food Research, Norwich Laboratory.

Gonçalves, C., Dinis, T., & Batista, M. T. (2005). Antioxidant properties of proanthocyanidins of Uncaria tomentosa bark decoction: a mechanism for anti-inflammatory activity. *Phytochemistry*, *66*(1), 89–98. http://doi.org/10.1016/j.phytochem.2004.10.025

Grivennikov, S. I., & Karin, M. (2011). Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. *Annals of the Rheumatic Diseases*, *70 Suppl 1*, i104–8. http://doi.org/10.1136/ard.2010.140145

Gupta, S. C., Hevia, D., Patchva, S., Park, B., Koh, W., & Aggarwal, B. B. (2012). Upsides and Downsides of Reactive Oxygen Species for Cancer: The Roles of Reactive Oxygen Species in Tumorigenesis, Prevention, and Therapy. *Antioxidants & Redox Signaling*. http://doi.org/10.1089/ars.2011.4414

Haffner, S. M. (2003). Pre-diabetes, insulin resistance, inflammation and CVD risk. *Diabetes Research and Clinical Practice*. 61 Suppl 1: S9-S18

Harborne, J. (2013). *Phytochemical methods. A guide to modern techniques of plant analysis* (4th ed.). Dordrecht, Netherlands: [S.I]: Springer.

Hasan, S.M, Hossain, M.M, Faruque, A, Mazumder, M.E.H, Rana, M.S, Actor, R, Alam, M.A (2008). Comparison of antioxidant potential of different fractions of Commelina benghalensis Linn. Bang. *Journal of Life Sciences.* 20(2): 9 - 16

Hassan, H., Sule, M., Musa, A., Musa, K., Abubakar, M., & Hassan, A. (2012). Anti-inflammatory activity of crude saponin extracts from five Nigerian medicinal plants. *Afr J Tradit Complement Altern Med*, *9*(2), 250–255.

Hutchings, A. (1996). Zulu medicinal plants: an inventory. University of Natal Press.

Igile GO. (1995). Phytochemical and biological studies on some constituents of Vernonia amygdalina (composite) leaves. Ph.D thesis . 1995.

Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y., & Kim, H. P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochemical Pharmacology*, *58*(5), 759–765.

Kipkore, W., Wanjohi, B., Rono, H., & Kigen, G. (2014). A study of the medicinal plants used by the Marakwet Community in Kenya. *Journal of Ethnobiology and Ethnomedicine*, *10*, 24. http://doi.org/10.1186/1746-4269-10-24

Kumaran, A., & Karunakaran, R. (2007). In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. *LWT - Food Science and Technology*, *40*(2), 344–352. http://doi.org/10.1016/j.lwt.2005.09.011

Kunwar, A., & Priyadarsini, K. (2011). Free radicals, oxidative stress and importance of antioxidants in human health. *Journal of Medical Allied Sciences*, *1*(2), 53–60.

Leiper, J., & Nandi, M. (2011). The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. *Nature Reviews. Drug Discovery*, *10*(4), 277–91. http://doi.org/10.1038/nrd3358

Liyana-Pathiranan, F., & Shahidi, C. (2005). Antioxidant activity of commercial soft and heat wheat (Triticumaestivum L) as affected by gastric pH conditions.*Journal of Agricultural and Food Chemistry, 53:* 2433 – 2440.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: *Impact on Human Health. Pharmacognosy reviews.* 4(8): 118.

Madsen H Andersen C Jørgensen L Skibsted L (2000). Radical scavenging by dietary flavonoids. A kinetic study of antioxidant efficiencies. *European Food Research and Technology* 211 (4): 240-246. http://doi.org/10.1007/s002170000189

Melchers, I., Blaschke, S., Hecker, M., & Cattaruzza, M. (2006). The -786C/T single-nucleotide polymorphism in the promoter of the gene for endothelial nitric oxide synthase: insensitivity to physiologic stimuli as a risk factor for rheumatoid arthritis. *Arthritis and Rheumatism*, *54*(10), 3144–51. http://doi.org/10.1002/art.22147

Mizushima, Y. (1964). Inhibition of protein denaturation by antirheumatic ao antiphlogistic agents. *Archives Internationales de Pharmacodynamie et de Thérapie 149*, 1–7.

Mohamed, H.M. Ons, E.T. Yosra, S. Rayda, G. Neji and N. Moncef, (2009). Chemical composition and antioxidant and radical-scavenging activities of *Periploca laevigata* root bark extracts .*Journal of the Science and Food and Agricultural.* 89(5): 897-905

Ndebia, E., Umapathy, E., Nkeh-Chungag BN, I., & Puto, J. (2011). Anti-inflammatory properties of Albuca setosa and its possible mechanism of action. *Journal of Medicinal Plant Research*, *5*(18 4658-4664), 4658–4664. Retrieved from http://www.academicjournals.org/article/article1380730621_Ndebia et al.pdf

Oktay, M., Gülçin, İ., & Küfrevioğlu, Ö. İ. (2003). Determination of in vitro antioxidant activity of fennel (Foeniculum vulgare) seed extracts. *LWT - Food Science and Technology*, *36*(2), 263–271. http://doi.org/10.1016/S0023-6438(02)00226-8

Okwu, D. E. (2004). Phytochemicals and vitamin content of indigenous spices of South Eastern Nigeria.*Journal of Sustainable Agriculture Environment.* 6(1): 30-37.

Okwu, D. E. (2005). Phytochemicals, vitamins and mineral contents of two Nigerian medicinal plants. *International Journal of Molecular Medicine and Advance Sciences*. 1(4): 375-381.

Okwu, D. E., & Nnamdi, F. U. (2008). Evaluation of the chemical composition of *Dacryodes edulis* and *Raphia hookeri* Mann and Wendl exudates used in herbal medicine in south eastern Nigeria. *African Journal of Traditional, Complementary and Alternative Medicines.* 5(2): 194-200.

Okwu, D. E., & Emenike, I. N. (2006). Evaluation of the phytonutrients and vitamins content of citrus fruits. *International Journal of Molecular Medicine and Advance Sciences.* 2(1): 1-6.

Olorunnisola, O. S., Bradley, G., & Afolayan, A. J. (2011). Antioxidant properties and cytotoxicity evaluation of methanolic extract of dried and fresh rhizomes of Tulbaghia violacea. *African Journal of Pharmacy and Pharmacology*. 5(22), 2490-2497.

Ordonez, A., Gomez, J., Vattuone, M., & LSLA, M. (2006). Antioxidant activities of Sechium edule (Jacq.) Swartz extracts. *Food Chemistry*, *97*(3), 452–458. http://doi.org/10.1016/j.foodchem.2005.05.024

Otang, W. M., Grierson, D. S., & Ndip, R. N. (2012). Phytochemical studies and antioxidant activity of two South African medicinal plants traditionally used for the management of opportunistic fungal infections in HIV/AIDS patients. *BMC Complementary and Alternative Medicine*, *12*(1), 43. http://doi.org/10.1186/1472-6882-12-43

Otunola, G. A., Oloyede, O. B., Oladiji, A. T., & Afolayan, A. J. (2010). Comparative analysis of the chemical composition of three spices–*Allium sativum* L. *Zingiber officinale* Rosc. and *Capsicum frutescens* L. commonly consumed in Nigeria. *African Journal of Biotechnology*. 9(41): 6927-6931.

Oyedemi, S. ., Bradley, G., & Afolayan, A. J. (2009). Ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe municipality of South Africa. *Joournal of Medicinal Plants Research*, *3*(12), 1040–1044. Retrieved from http://www.academicjournals.org/JMPR

Oyedemi, S., Bradley, G., & Afolayan, A. (2010). In vitro and in vivo antioxidant activities of aqueous stem bark extract of Strychnos henningsii Gilg. *African Journal of Pharmacy and Pharmacology*, *4*(2), 070–078.

Patel, V., Patel, P., & Kajal, S. (2010). Antioxidant Activity of Some Selected Medicinal Plants in Western Region of India. *Advances in Biological Research*, *4*(1), 23–26.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*(7), 1035–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10924197

Plumb, G., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., Williamson, G. (1998). Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Radical Research 29 (4): 351-8.*

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231–1237. http://doi.org/10.1016/S0891-5849(98)00315-3

Rice-Evans, C. ., Miller, N. ., Bolwell, P. ., Bramley, P. ., & Pridham, J. . (1995). The relative activities of plant-derived polyphenolic flavonoid. *Free Radical Reshearch 22*, 375–383.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*(7), 933–956. http://doi.org/10.1016/0891-5849(95)02227-9

Ross, R. (1999). Atherosclerosis--an inflammatory disease. *The New England Journal of Medicine*, *340*(2), 115–26. http://doi.org/10.1056/NEJM199901143400207

Ruch, R. J., Cheng, S. J., & Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, *10*(6), 1003–1008.

Shahidi, F., & Wanasundara, P. (1992). Phenolic antioxidants. *Critical Review of Food Science and Nutrition 32*, 67–103.

Sun, J.-S., Tsuang, Y.-H., Chen, I.-J., Huang, W.-C., Hang, Y.-S., & Lu, F.-J. (1998). An ultra-weak chemiluminescence study on oxidative stress in rabbits following acute thermal injury. *Burns*, *24*(3), 225–231. http://doi.org/10.1016/S0305-4179(97)00115-0

Tiong, S. H., Looi, C. Y., Hazni, H., Arya, A., Paydar, M., Wong, W. F., … Awang, K. (2013). Antidiabetic and antioxidant properties of alkaloids from Catharanthus roseus (L.) G. Don. *Molecules (Basel, Switzerland)*, *18*(8), 9770–84. http://doi.org/10.3390/molecules18089770

Van Huyssteen, M., Milne, P., Campbell, E., & van de Venter, M. (2011). Antidiabetic and cytotoxicity screening of five medicinal plants used by traditional african health practitioners in the Nelson Mandela Metropole, South Africa. *African Journal of Traditional, Complementary and Alternative Medicine 8* (2), 150–158. http://doi.org/PMC3252689

Wang, M., Li, J., Rangarajan, M., Shao, Y., & Lavoie, E. J. (1998). Antioxidative phenolic compounds from sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry*, *46*(732), 4869–4873. http://doi.org/10.1021/jf980614b

Wintola, O. A., & Afolayan, A. J. (2011). Phytochemical constituents and antioxidant activities of the whole leaf extract of *Aloe ferox* Mill. *Pharmacognosy Magazine*, *7*(28), 325–33. http://doi.org/10.4103/0973-1296.90414

Wolfe, K., Wu, X., & Liu, R. H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*, *51*(3), 609–14. http://doi.org/10.1021/jf020782a

Xie, Y., Zhang, Y., Zhang, L.-T., Zeng, S.-X., Guo, Z.-B., & Zheng, B.-D. (2013). Protective effects of alkaloid compounds from *Nelumbinis Plumula* on tert-butyl hydroperoxide-induced oxidative stress. *Molecules (Basel, Switzerland)*, *18*(9), 10285–300. http://doi.org/10.3390/molecules180910285

Yang, L.-Q., Tao, K.-M., Liu, Y.-T., Cheung, C.-W., Irwin, M. G., Wong, G. T. C., Lv, H., Song, J.G,Wu, F, Yu, W.-F. (2011). Remifentanil preconditioning reduces hepatic ischemia-reperfusion injury in rats via inducible nitric oxide synthase expression. *Anesthesiology*, *114*(5), 1036–47. http://doi.org/10.1097/ALN.0b013e3182104956

Yaun, G. ., Wahlqvist, M., He, G., Yang, M., & Li, D. (2006). Natural products and anti-inflammatory activity. *Asia Pacific Journal of Clinical Nutrition*, *15*(2), 143–152. Retrieved from http://apjcn.nhri.org.tw/server/apjcn/15/2/143.pdf

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J., & Qian, M. (2002). Free radical scavenging properties of wheat extracts. *Journal of Agricultural and Food Chemistry*, *50*(6), 1619–1624.

CHAPTER 4

CYTOTOXICITY OF *ALBUCA BRACTEATA* AND *ALBUCA SETOSA* BULB EXTRACTS

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CYTOTOXICITY OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS

4.1 Introduction

The extracts of medicinal plants are known to possess bioactive compounds for disease treatment and prevention. This potential of plants in disease management is significant (Mosaddegh et al., 2012). People who live in rural areas have traditionally depended on a wide range of plants to maintain their health. It is estimated that 80% of people living in developing countries depend on alternative medicine to maintain their health (WHO, 2002b). This reliance on herbal treatment among these populations may be attributed to their easy accessibility, low cost and fewer side effects (da Costa Lopes et al., 2000); however, there is little scientific evidence on the correct dosages and toxicity of these plants. These plants have been increasingly used for medication over many generations based on folkloric traditional usage without any supporting data. A challenge in the detection of pharmacological activity in plants is that extracts of a single plant may contain mixtures of compounds which can vary in concentration of the active principles depending on its location and ecology (Farnsworth, 1993). It is also worthy of note that traditional healers sometimes combine different plants or plant parts for treatments. This practice is well appreciated by the patients and is a sign of deep knowledge of traditional herbal treatment (Deutschländer, et al., 2009). Concoctions however, pose a problem of identification and expression during pharmacological investigation to determine which of the many constituents of a single plant in the mixture is active (Deutschländer et al., 2009). Generally plants synthesize toxic substances that serve as a defence against infections and insects. These toxic compounds are known to affect animals that feed on them (Teixeira et al., 2003), and highlight the need for an assessment of their cytotoxicity potential in humans to ensure the safety of these plants during disease management. Several reports in developed and developing countries in recent times have

indicated adverse effects arising from the use of medicinal plants (Elvin-Lewis, 2001). Adverse effects such as diarrhoea, vomiting, abdominal pains, abortions, loss of appetite, dizziness, ulcers, sexual dysfunction and death to the use of these plants can arise from incorrect and/or the presence of cytotoxic compounds in the plant (Azaizeh, et al., 2003; Gessler et al., 1995). The liver is the primary target organ for toxicants and due to the complexity of many bioactive compounds in medicinal plants; the toxicity of the plant will depend on which bioactive compound reaches the liver first. An in vitro investigation is therefore only a first-line experiment in determining the toxicity of these plants and such studies should be followed up by further *in vivo* studies. The toxicity of plants can be influenced by plant parts, route of administration and methods of preparation.

4.1.1 In vitro cytotoxic assay

In vitro cytotoxic investigation is an inexpensive approach for short term testing (Tshikalange & Hussein, 2010), usually carried out in cell lines to study individual compounds of known structures and concentration (Otang, et al., 2014). Cytotoxicity investigation of medicinal plants only considers a single cell type, metabolic pathway or enzyme, which reduces the possibility of identifying an antidiabetic compound. *In vitro* assays also only measure acute or immediate effects but does not consider the effect after chronic exposure to these bioactive compounds (van de Venter et al., 2008). In this study, the Chang liver cell line was used to evaluate the cytotoxicity of *Albuca setosa* and *Albuca bracteata* extracts. The Chang liver cell line which was originally established in 1954, was thought to be derived from normal liver tissue (Chang, 1954). It has been associated with controversies arising in the literature about the authenticity based on the isoenzyme analysis, presence of HeLa cells biomarkers (DNA sequence) and the absence of PCR amplifiable Y chromosome DNA. It was therefore assumed that Chang liver cell was contaminated with HeLa cells before deposition to the cell bank (Gao, et al., 2011; Nelson-Rees &Flandermeyer,

1976) and has been listed by International Cell Line Authentication Committee (ICLAC) among contaminated cell lines (Capes-Davis et al., 2010; ICLAC, 2013). Even if reportedly contaminated, Chang liver (since not described here as hepatocytes) can still be used as a model to explore general cytotoxicity due to the fact that many of the known drug- induced cytotoxicity mechanisms such as activation of cell death, oxidative stress, inhibition of mitochondrial function, disruption of intracellular homoeostasis and synthesis of metabolites that cause toxicity are common to most cells. This investigation in Chang liver is therefore based on this premise, to identify toxicity and to flag unsuitable extracts prior to specific anti-diabetic screening assays. It is important to emphasise that in this study, *in vitro* cytotoxicity assays using Chang liver cell line is of low concordance with human liver toxicity, thus predictions of the clinical hepatotoxicity potential will be unreliable. In order to maximise the predictive capacity of this pre-anti-diabetic cytotoxicity screening, different endpoints are assessed so as to more comprehensively characterise the nature of the plant extracts.

4.2 Materials and methods

This study used MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, USA), Chang liver, MHRF, INS-1 (ATCC), EMEM (Eagle's minimum essential medium) (Sigma Aldrich), (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline), formaldehyde , glucose oxidase (SIGMA G0543), Peroxidase (SIGMA P8375) , FCS, PBS. All other chemicals used are of good quality and analytical grade.

4.2.1 The plant material

The bulbs of *Albuca setosa* and *Albuca bracteata* were thinly sliced and oven-dried at 40^{o}C in the laboratory. The dried materials were further ground into powder form using an electric blender (Hamilton Beach Commercial Blender type GB27 model HBF 400-CE). About 80 g of the powdered plant material was extracted in 1000 ml of distilled water, methanol, and

acetone respectively. The extracts were placed on a mechanical shaker (Stuart Scientific Orbital Shaker, SOS1, Essex, UK) for 48 h. The extracts were then filtered using Whatman No.1 filter paper in a vacuum pump. The aqueous filtrate was quickly frozen at -40^{0}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapour Trap, RV T41404, USA) to give a yield of 18.6 g and 15.2 g dry extract of *Albuca setosa* and *Albuca bracteata* respectively. The methanol and acetone extracts were dried using rotary evaporator. Both the methanolic and acetonic extracts were resolubilised in distilled water and freeze dried before been used for cytotoxicity experiments. All the dried extracts were then reconstituted in diluted DMSO to a concentration of 100 µg/ml, sonicated and left for at least 15 min before further dilution with the respective growth medium before use.

4.2.2 Basal cytotoxicity assay:

The Chang liver cells were cultured in EMEM containing 10% FCS seeded into two 96-well plates at a density of 6000 cells per well, with a volume of 100µl, and one 24-well plate at 12000 cells per well at a volume of 500µl. The first two rows of the 96-well plates were devoid of cells to serve as controls for later end-point assays. The cells were left for 12-16 h to attach overnight and then treated by adding 100µl of standards and plant extracts at a doubled concentration dose of 100µg/ml each. After being incubated for 48 h, 150µl each of the spent culture media from the two 96-well plates were transferred into a new 96-well plate C (Figure 4.1).

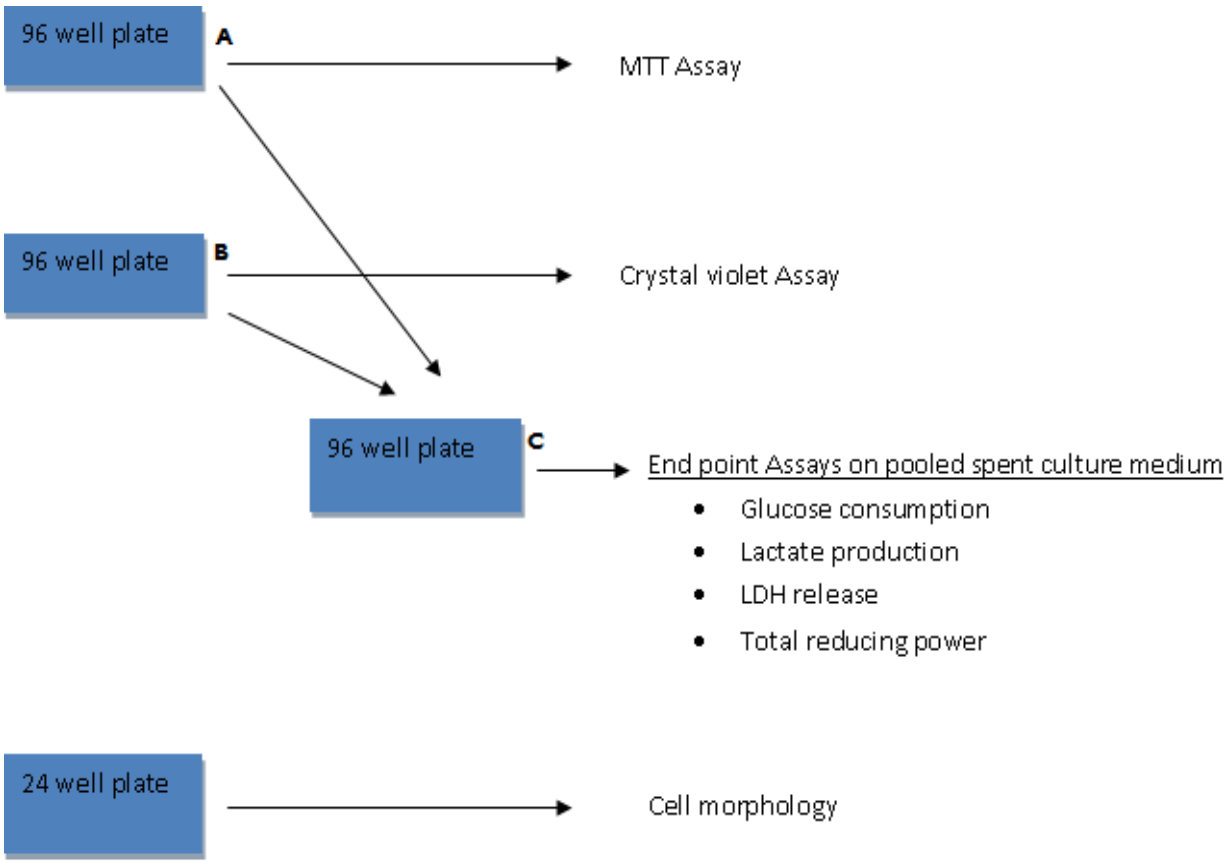

Figure 4.1: Schematic representation of the multicomplex cytotoxicity assays

4.2.3 MTT cytotoxicity assay

The MTT assay was determined by the modified method of (Mosmann, 1983). To one of the cell plated 96-well plates (A in Figure 4.1), 100µl EMEM medium supplemented with 10% FBS and 0.5mg/ml MTT was added and further incubated for 3 h at 37^{o}C.

The medium was then removed and the purple formazan products was disolved in DMSO (200µl/well). The absorbance was read at 540 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37^{o}C humidified incubator with 5% CO_2.

Data manipulation

Each sample had six replicates in the microtiter plate and individual experiments were repeated twice. Percentage growth inhibition was calculated for each replicate using the 100% viability values of the blank.

% Growth inhibition = $\left(\frac{Aver\ sample\ reading}{Aver\ Blank\ reading}\right)\ X\ 100$

4.2.4 Crystal Violet assay

Crystal violet cell viability assay was determined using a modifed method of Itagaki et al. (1991). To the second 96-well plate B (Figure 4.1), the cells were fixed for 1 h at room temperature with 100µl of 4% formaldehyde in PBS. The fix solution was removed and 100µl of crystal violet solution, which was previously prepared by dissolving 0.1g of crystal violet in 100ml distilled water and filtred through whatman No.1 filter paper, was added. It was incubated for 30 min. and the excess dye removed by washing with water before being incubated at 37^{o}C in an oven for 10-12 h. The dye was extracted with 100µl per well of 10% acetic acid and then absorbance was measured at 595 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems).

Data manipulation

Each sample had six replicates in the microtiter plate and individual experiments were repeated twice. Percentage growth inhibition was calculated for each replicate using the 100% viability values of the blank.

% Growth inhibition = $\left(\frac{Aver\ sample\ reading}{Aver\ Blank\ reading}\right)\ X\ 100$

4.2.5 Medium based cytotoxicity assays

To the spent culture medium of the two 96-well plates that were pooled together into a new 96-well plate C (Figure 4.1), the following assays were carried out. Chloroquine, griseofulvin, 2,4 – dinitophenol, nordihydroguaiaretic acid were used as controls.

4.2.6 Glucose consumption assay

5µl of the spent culture medium from 96-well plate C (Figure 4.1) was transferred into a new 96 – well plate and 200µl of glucose reagent was added before it was incubated for 10 min at 37^{o}C. The glucose reagent was prepared by adding 0.028 g of phenol, 0.008 g of 4-aminoantipyrine, 0.074 g EDTA, 65µl of glucose oxidase and 0.001 g of peroxidase to 100 ml of phosphate buffer. The absorbance was read at 510 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). The glucose consumption was calculated from the equation:

Glucose consumption= Abs(blank) – Abs(sample)

Where

Abs (blank) is the absorbance of wells without cells

Abs (sample) is the absorbance of wells with treated cells

Glucose consumption rate is the glucose consumption for each treatment compared to the glucose consumption of the normal control group, which was calculated using the equation

$y = 0.0608x$; $R^2 = 0.9973$ from the glucose consumption standards curve.

4.2.7 Lactate production assay

For the lactate production assay, 5µl of the spent culture medium from 96-well plate C (Figure 4.1) was transferred into a new 96-well plate and another 96-well plate was also

prepared containing lactate standard. To both 96-well plates 200µl of assay reagent that was prepared fresh was added. The assay reagent was prepared by dissolving 100mg BSA, 0.028 g phenol, 0.008 g 4-aminoantipyrine, 20 µl lactic oxidase (5U) and 0.01 g horseradish peroxidise in 100 ml of assay buffer. To prepare the buffer, 0.1% CaC_{l2} and 0.02% NaN_3 were added to 0.1 M citric acid (pH was adjusted to 6.0 using 1 M Na_2HPO_4. BSA was then added to a final concentration of 1 mg/ml. The 96-well plates were incubated at 37^{o}C for 60 min. and absorbance read at 510 nm.

The lactate produced was calculated form the equation:

Lactate production = Abs(blank) – Abs(sample)

Where

Abs (blank) is the absorbance of wells without cells

Abs (sample) is the absorbance of wells with treated cells

Lactate production rate was calculated using the equation $y = 0.0021x$; $R^2 = 0.9973$ from the lactate assay standard curve.

4.2.8 Lactate dehydrogenase release assay

To determine the lactate dehydrogenase released, a coupled enzymatic reaction whereby tetrazolium salt (INT) was reduced to water soluble formazan was used (Decker & Lohmann-Matthes, 1988; Legrand et al., 1992). Briefly, to 50 µl of the medium in 96-well plate C (Figure 4.1), 50 µl of Tris – HCl buffer and 50µl of lactate solution were added in a new 96 – well plate. 50µl of freshly prepared PMS/INT/NAD solution was then added and incubated for 20 min. in the dark. The absorbance was read at 492nm.

4.2.9 Total reducing capacity (Ferric reducing antioxidant potential) assay

The total reducing antioxidant activity of the spent culture medium potential was carried out with a modified method described by Benzie & Strain (1999). The FRAP reagent was

prepared by mixing 20 mL acetate buffer (300 mM, pH 3.6), 2 mL TPTZ solution, 2mL $FeCl_3$ solution and 2.4 mL of distilled water. The solution was kept in a water bath at 37°C for 10 min prior to use. 50 µl of the spent tissue culture medium was transferred into a 96-well plate after which 200 µL of freshly prepared FRAP reagent was added and was incubated for 30min. at 37°C. The absorbance was then measured 593 nm.

4.2.10 Cell proliferation assay

The cell proliferation assay was carried out using MRHF and INS-1 cells. The MRHF cells were cultured in DMEM containing 5% FCS while the INS-1 cells were cultured in RPMI containing 5% FCS. Both cells were seeded into three different 96-well plates at a density of 5000 cells per well, with a volume of 100µl, The cells were left for 12 – 16h to attach overnight and then treated by adding 100µl of plant extracts at a doubled concentration dose of 100µg/ml each. One plate each of both cells were stained with crystal violet as previously described after 8 , 24, 48 h of incubation at 37^oC and 5% CO_2.

Data manipulation

Each sample had six replicates in the microtiter plate and individual experiments were repeated twice. Percentage cell density was calculated for each replicate using the 100% viability values of the control.

% cell density = $\left(\frac{A-B}{A}\right) X\ 100$

Where

A = the absorbance of the untreated cells (control).

B = the absorbance of the treated cells

4.3 RESULTS

4.3.1 MTT cytotoxicity

The cytotoxic effect of *Albuca setosa* and *Albuca bracteata* extracts were examined using MTT assay as shown in Figure 4.2. DNP, GRE, NDGA and Chloroquine were used as positive controls. The aqueous extract of *Albuca setosa* shows the maximum cell viability with 108.09%, while the acetonic extract of *Albuca bracteata* shows the least cell viability with 37.72%. Comparing both plants, it was observed that the aqueous extracts showed higher cell viability than the methanol and acetone extracts of both plants. Griseofulvin shows the least cell viability of all the controls investigated but was significantly higher than the *Albuca bracteata* acetone extract.

4.3.2 Crystal Violet

The cytotoxic effect of *Albuca setosa* and *Albuca bracteata* extracts examined by crystal violet assay is shown in Figure 4.3. The acetone extract of *Albuca bracteata* shows the least percentage of cell viability with 31.47% while the aqueous extract of *Albuca setosa* showed the maximum cell viability of 112.5%. The percentage of cell viability follows the order DNP >*Albuca setosa* aqueous extract >*Albuca bracteata* aqueous extract > NDGA > Chloroquine >*Albuca setosa* methanolic extract > GRE >*Albuca setosa* acetone >*Albuca bracteata* methanol >*Albuca bracteata* acetone. It was observed that cell viability of the extracts follows the order aqueous > methanol > acetone for both plants.

4.3.3 Glucose consumption

The glucose consumption of the cells was monitored after treatment with plant extracts and standard drugs as shown in Figure 4.4. The result of the glucose consumption assay of *Albuca setosa* and *Albuca bracteata* extract treated cell medium was calculated from the glucose standard curve shown in Figure 4.5. All the tested samples were observed to consume more

glucose than the blank except for the methanol and acetone extracts of *Albuca bracteata* and Griseofulvin. The *Albuca bracteata* methanolic extract showed the least glucose consumption but was not significantly different from griseofulvin. The aqueous extract of *Albuca setosa* showed the highest glucose consumption but was not significantly different from the *Albuca setosa* methanol extract, DNP and NDGA.

4.3.4 Lactate production

The cytotoxic effect of *Albuca setosa* and *Albuca bracteata* extracts were examined using lactate production assay as shown in Figure 4.6. The result of the lactate production after the treatment with *Albuca setosa* and *Albuca bracteata* extracts was calculated from the lactate standard curve shown in Figure 4.7. The methanolic extracts of *Albuca bracteata* showed the least concentration of lactate while NDGA showed the highest concentration, with 145.4 µg/ml. Considering both plants, the aqueous and methanolic extracts of *Albuca setosa* produced the highest lactate with 120.2 µg/ml and 113.7 µg/ml of lactate respectively, but they are not significantly different from each other. It was observed that the aqueous extracts of both plants were higher than their methanol and acetone counterparts.

4.3.5 Lactate dehydrogenase release

The cytotoxic effect of *Albuca setosa* and *Albuca bracteata* extracts were examined using lactate dehydrogenase assay as shown in Figure 4.8. Lactate dehydrogenase release was observed in griseofulvin and acetonic extractions of both *Albuca bracteata* and *Albuca setosa.* The lactate dehydrogenase release that was observed in the *Albuca bracteata* acetone extract was higher than the *Albuca setosa* acetone extract but not significantly different. Both *Albuca bracteata* and *Albuca setosa* acetone extracts were not significantly different from the griseofulvin.

4.3.6 Ferric reducing antioxidant potential (FRAP)

The cytotoxic effect of *Albuca setosa* and *Albuca bracteata* extracts in the medium were examined using ferric ion reducing antioxidant potential assay as shown in Figure 4.9, while the standard curve is shown in Figure 4.11. NDGA has the highest ferric ion reducing potential with 68.9 µmol/l of $FeSO_4$. Among the extracts, the methanolic extract of *Albuca setosa* showed the highest reducing potential followed by the acetonic extract of *Albuca* setosa. The aqueous extracts of both *Albuca setosa* and *Albuca bracteata* showed a reducing power of 18.7 and 22.1 µmol/l of $FeSO_4$ respectively that was higher than the blank. The *Albuca setosa* methanolic extract showed the highest reducing potential with 122.9 µmol/l of $FeSO_4$ followed by the *Albuca setosa* acetonic extract with 106.4µmol/l of $FeSO_4$.

The reducing potential of the extracts shown in Figure 4.10 revealed that the methanolic extract of *Albuca setosa* has the highest reducing potential with 3887.8 µmol/l of $FeSO_4$, while the aqueous extract of *Albuca bracteata* has the least reducing potential with 511.2 µmol/l of $FeSO_4$. Comparing both plants, the methanolic extracts have a higher reducing potential than that of the aqueous extracts.

4.3.7 Cell proliferation

The effect of the aqueous extracts of both *Albuca setosa* and *Albuca bracteata* on MRHF and INS-1is shown in the Figure 4.11 and 4.12 respectively. The addition of the extracts caused reduction in cell density after 24 h which later increased significantly after 48 h in both MRHF and INS-1 cells

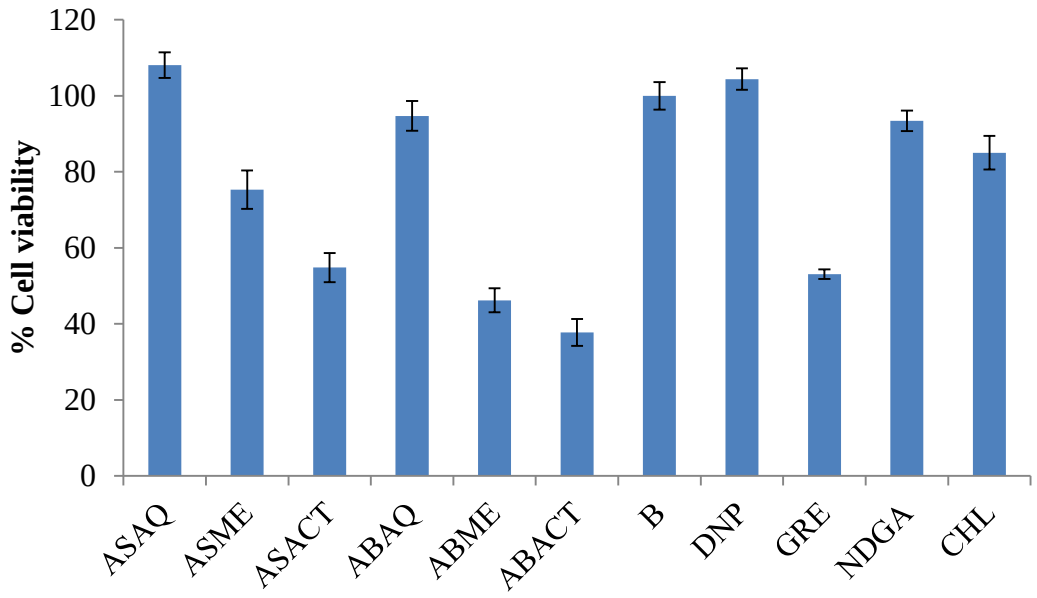

Figure 4.2: MTT cell viability activity of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa acetone extract;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata;* DNP: 2, 4-dinitrpphenol; B: Blank; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CHL: Chloroquine.

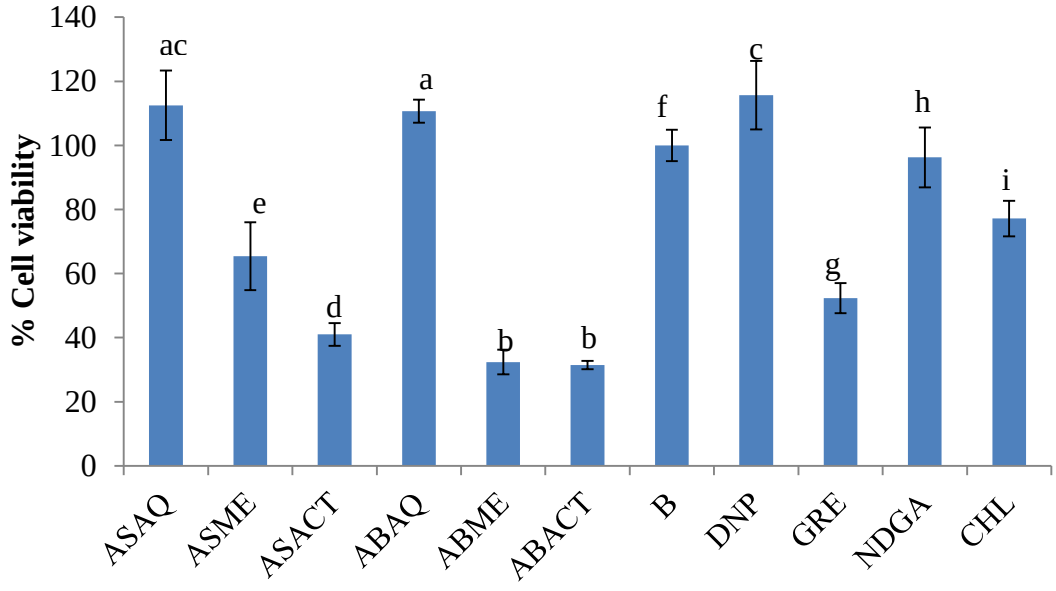

Figure 4.3: Crystal Violet cell viability activity of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa acetone extract;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata;* DNP: 2, 4-dinitrpphenol; B: Blank; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CHL: Chloroquine.

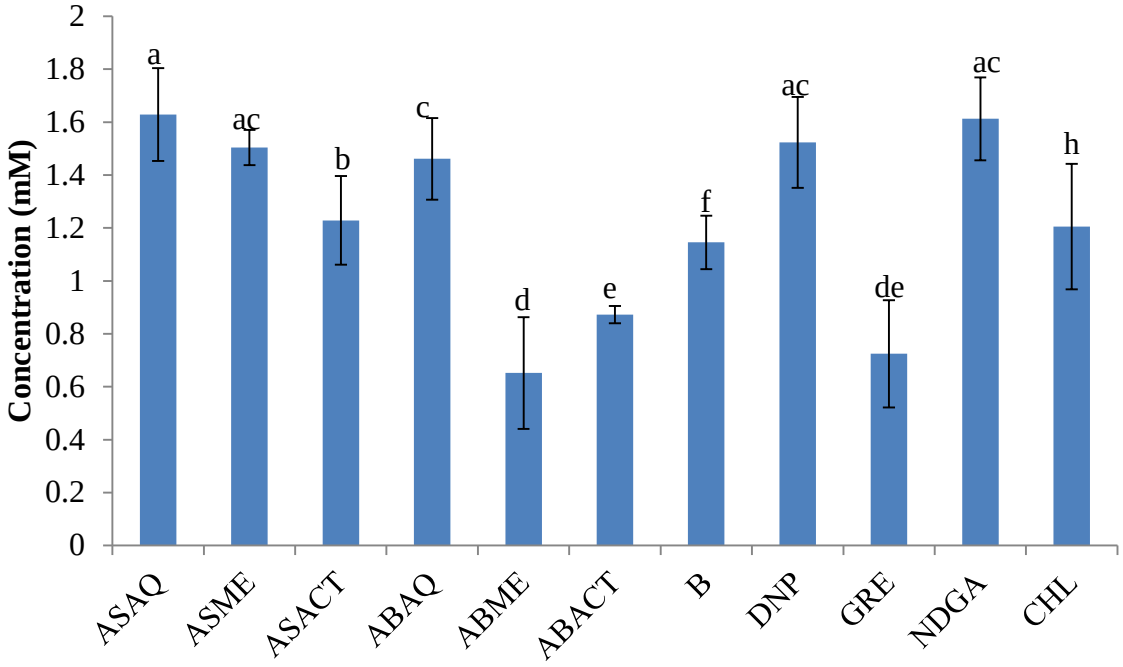

Figure 4.4: Glucose consumption activity of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa acetone extract;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata;* DNP: 2, 4-dinitrpphenol; B: Blank; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CHL: Chloroquine.

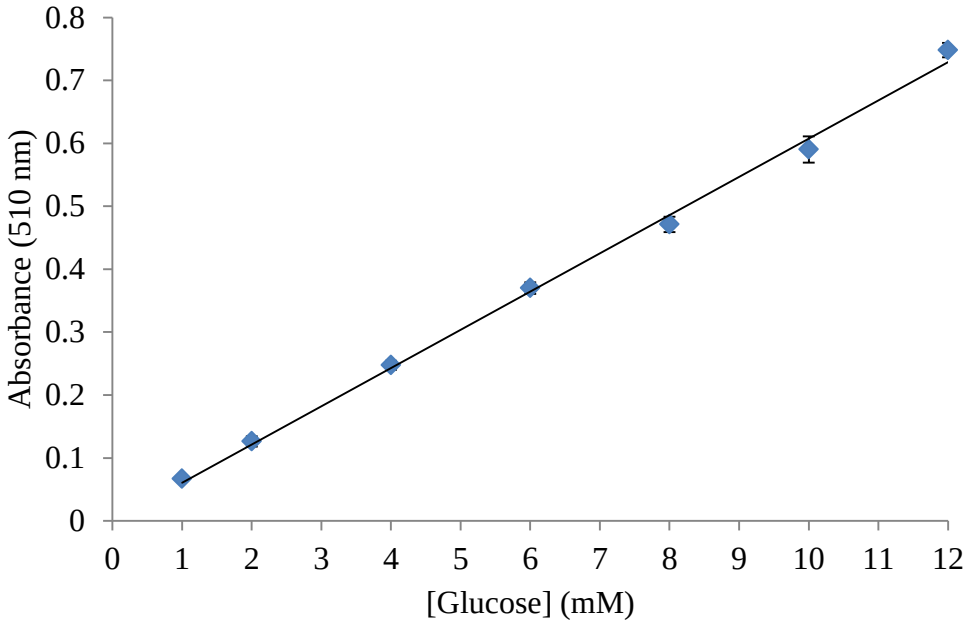

Figure 4.5: Glucose assay standard curve

Data expressed as mean ± SD, n= 6.y = 0.0608x; R^2 = 0.9973

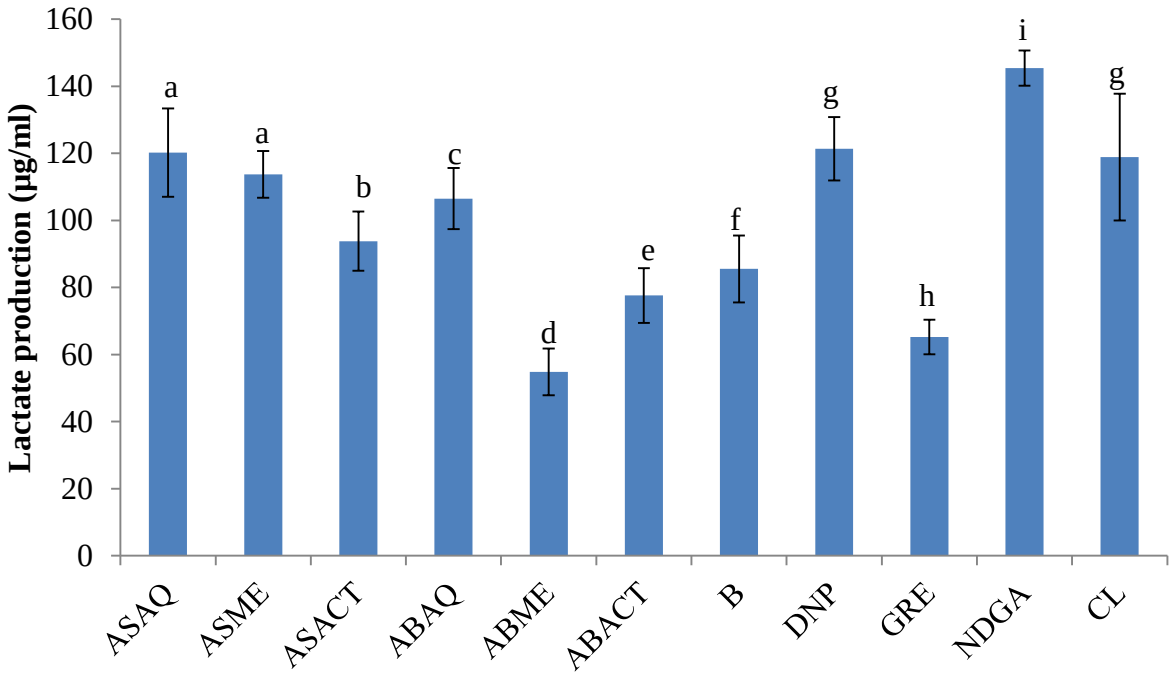

Figure 4.6: Lactate production activity of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01). ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract*;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata;* DNP: 2, 4-dinitrpphenol; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CL: Chloroquine.

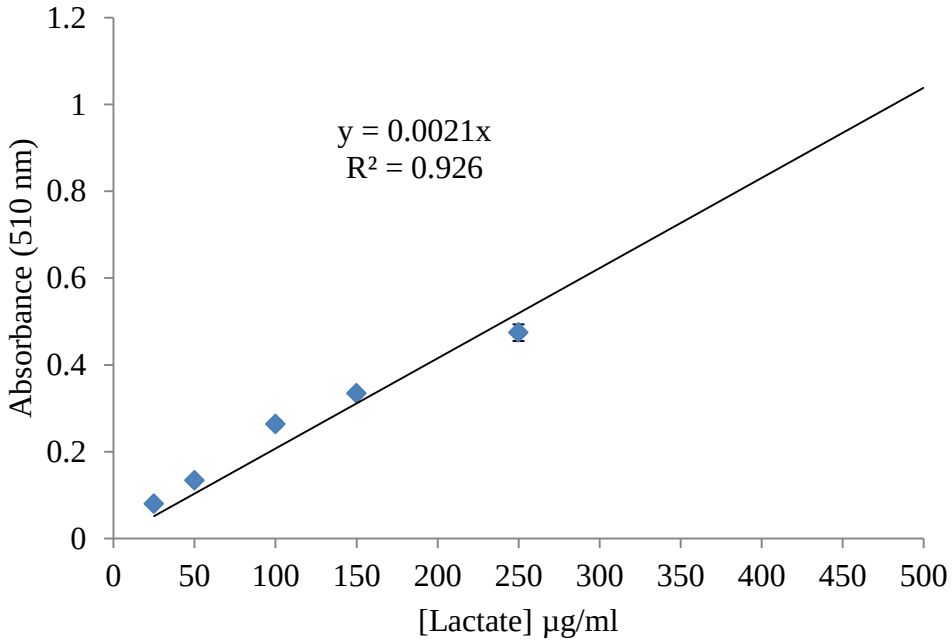

Figure 4.7: Lactate assay standard curve

Data expressed as mean ± SD, n= 6. y = 0.0021x; R^2 = 0.926

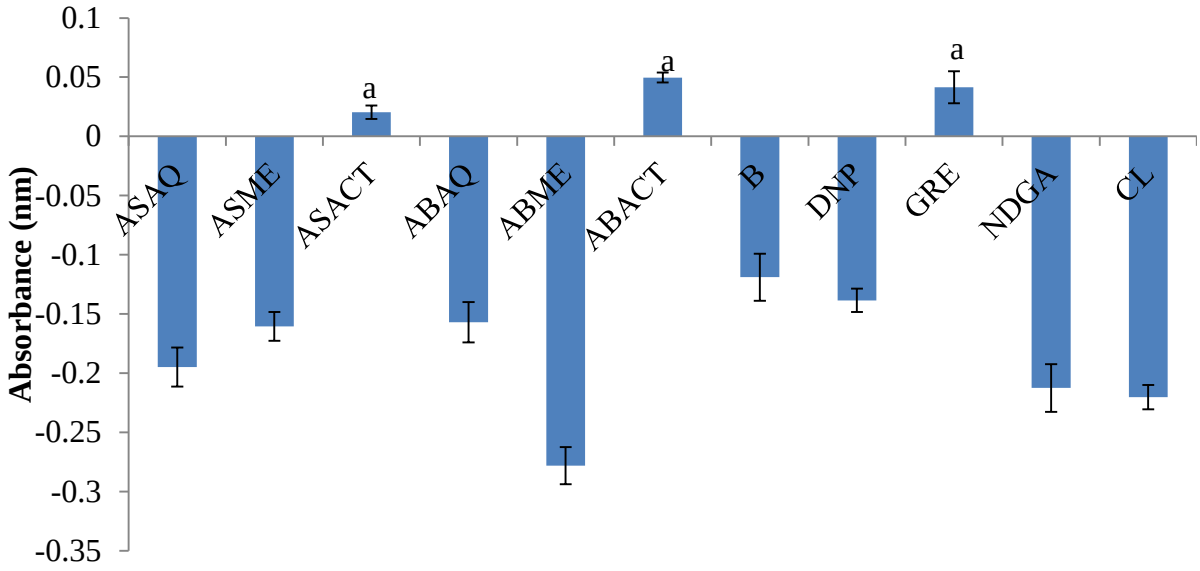

Figure 4.8: Lactate dehydrogenase release activity of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract*;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract*;* B: Blank; DNP: 2, 4-dinitrpphenol; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CL: Chloroquine

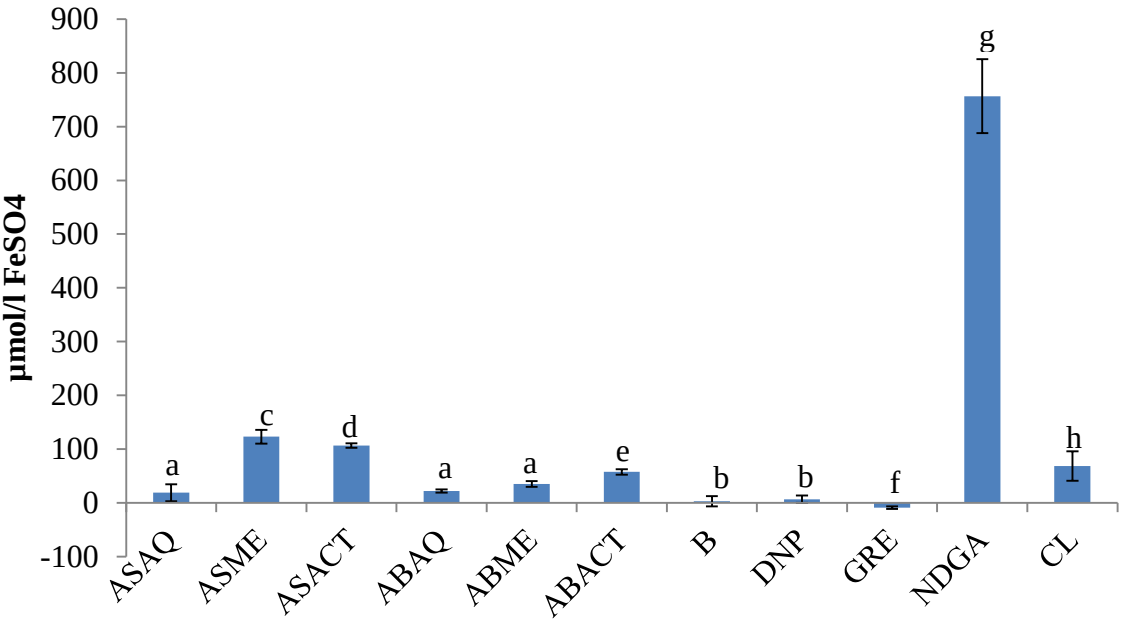

Figure 4.9FRAP of *Albuca setosa* and *Albuca bracteata* extracts on Chang cells

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract*;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata;* DNP: 2, 4-dinitrpphenol; GRE: Griseofulvin; NDGA: Nordihydroguaiaretic Acid; CL: Chloroquine

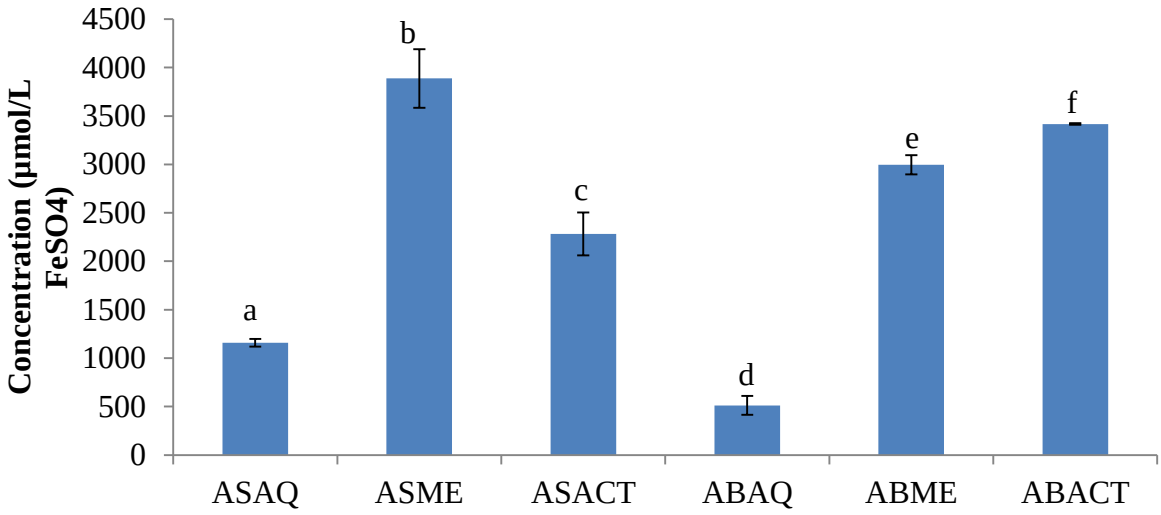

Figure 4.10: FRAP of *Albuca setosa* and *Albuca bracteata* extracts

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01). ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract*;* ABAQ: *Albuca bracteata* aqueous extract*;* ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract

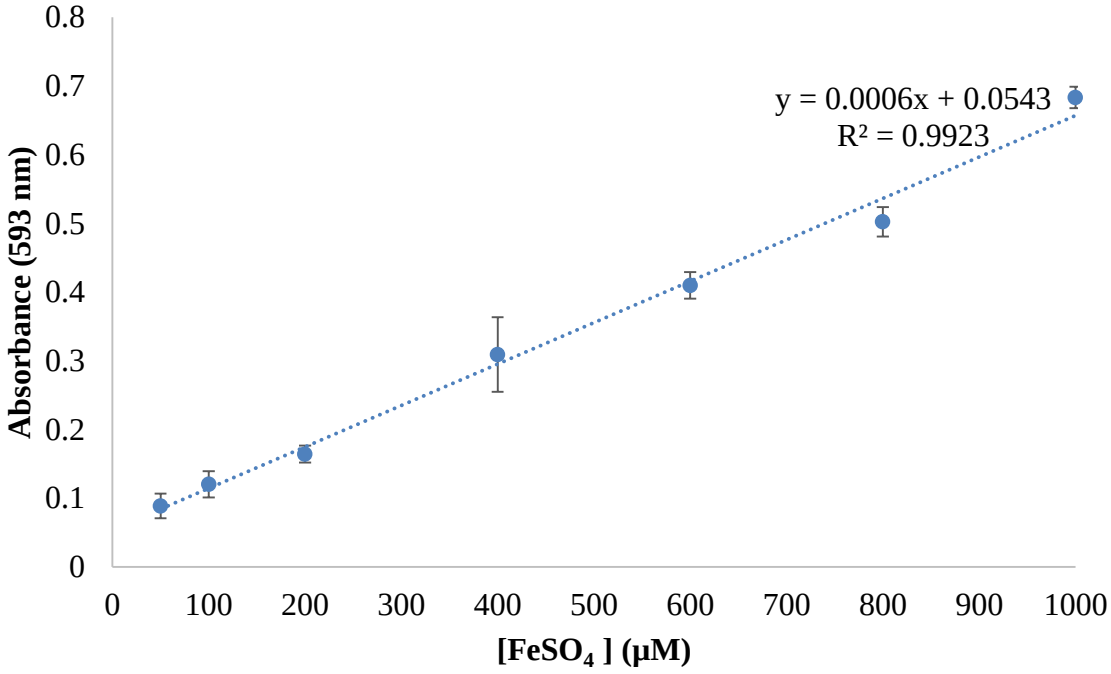

Figure 4.11: FRAP standard curve

Data expressed as mean ± SD, n= 6. y = 0.0006x + 0.0543; R^2 = 0.9923

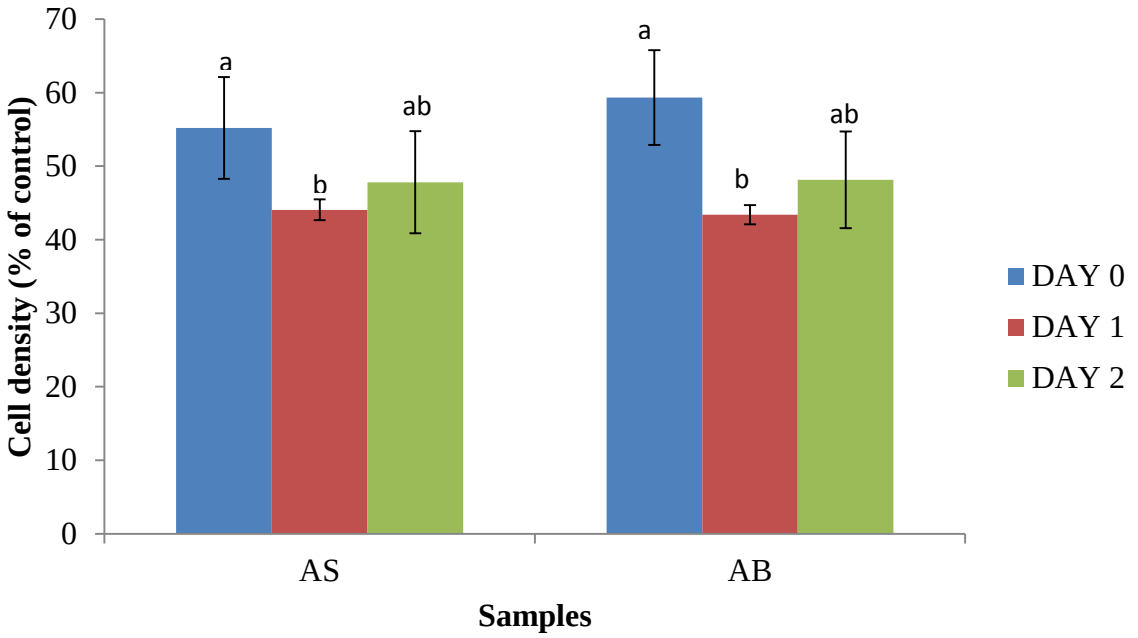

Figure 4:12Proliferative effect of *Albuca setosa* and *Albuca bracteata* on MRHF cells.
Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).AS: *Albuca setosa;* AB: *Albuca bracteata*.

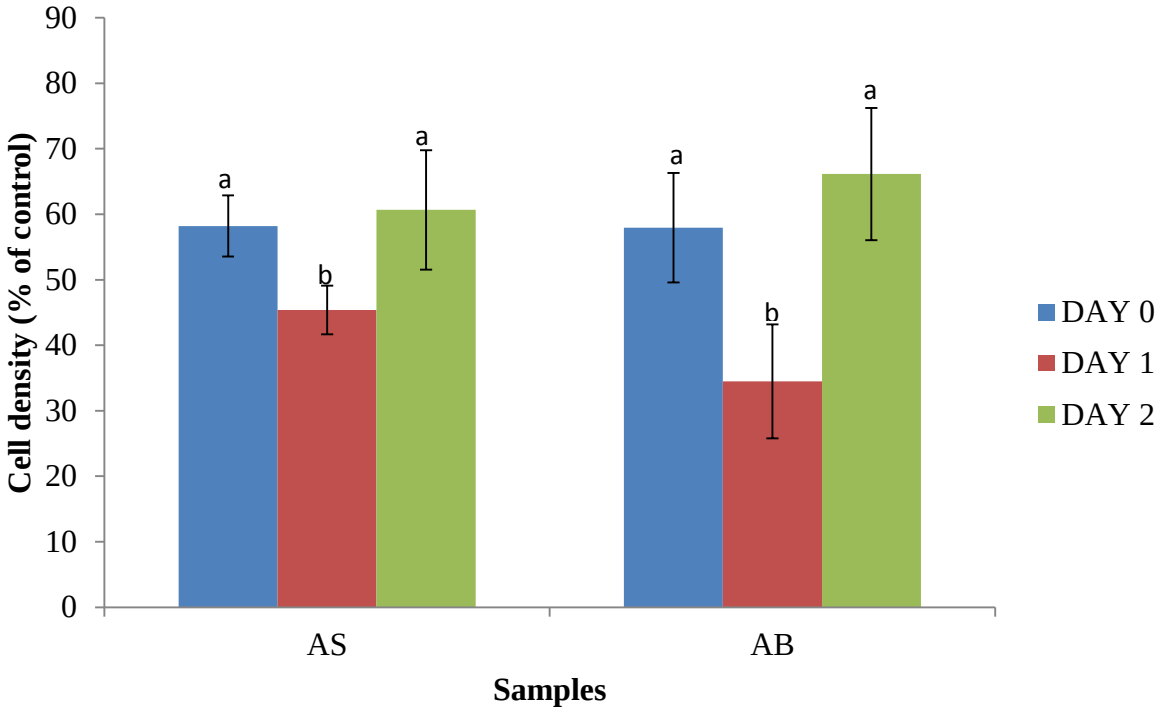

Figure 4:13Proliferative effect of *Albuca setosa* and *Albuca bracteata* on INS-1 cells.
Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).

4.4 DISCUSSION

Drugs and herbal formulations must be evaluated for their therapeutic activity in terms of their toxicity and tolerability (Gosenca, et al., 2010). The toxicity of medicinal plants is often neglected as usage of herbal products is widely believed to be safe (WHO, 2002), however, prolonged traditional usage of medicinal plants may not be a reliable guarantee of safety due to unmonitored delayed adverse effects of these plants.

The MTT assay to test for cytotoxicity is a quantitative and reliable colorimetric test that is based on the enzymatic reduction of yellow water-soluble MTT dye by mitochondrial succinate dehydrogenase to form a purple formazan product (insoluble in water) which measures viability, proliferation and the activation of cells. The MTT assay measures cell membrane integrity by determining mitochondrial activity through an enzymatic reaction on the reduction of MTT to formazan (Azurah et al., 2011). The amount of formazan produced is assumed to be directly proportional to the cell number in a range of cells lines (Gerlier & Thomasset, 1986; Mosmann, 1983). In addition, crystal violet has been reportedly used to quantify cell numbers in monolayer cultures due to the absorbance of the dye taken up by the cells (Gillies, et al., 1986; Naseer, et al., 2009).

Cellular viability and proliferation are considered to be important functional characteristics of healthy and actively growing cells. Therefore an increase in cell viability indicates cell proliferation in a non-toxic environment while a decrease in cell proliferation indicates cell death due to toxicity. In this study, it was observed that *Albuca setosa* aqueous extract showed the highest percentage of cell viability at the tested concentration as indicated by both MTT and crystal violet assays, which could indicate that the extract is both non-toxic and may possess active principle(s) with mitogenic effect or is able to induce the expression of growth stimulating factors. This is similar to the report of Bagla, (2012).

Temin et al. (1972) reported that growth stimulating substances of cell lines exhibit a high degree of specificity with varying cell types. However, the acetonic extract of *Albuca bracteata* indicated the least cell viability which indicates caution in the usage of this plant in alcohol. The higher percentage cell viability of both plants, aqueous extracts from the MTT and crystal violet assays indicate that these plants may be safe as traditionally used in the management of diabetes.

Glucose represents a major source of both carbon and energy for cell growth which indicates that the rate of glucose consumption correlates well to cell proliferation. Therefore changes in cellular glucose metabolism provide some information regarding the mechanism of toxicity (Rossetti, et al., 1990).Similarly, changes in glucose metabolism for non-toxic samples may also provide an indication of the presence of compounds with potential anti-diabetic activity that could be similar to metformin. The increase in glucose consumed by the aqueous extracts of *Albuca setosa* and *Albuca bracteata* as compared to the blank indicated no sign of toxicity and could indicate the presence of compounds with antidiabetic activity.

In the case of mitochondrial impairment, the decline in ATP production is compensated for by accelerated glycolysis as glucose metabolism is channelled towards the production of lactate via anaerobic respiration. Therefore, elevated lactate production observed in the extracts of *Albuca setosa and Albuca bracteata* indicates potential injury to the mitochondria. However, the elevated lactate production observed in the standard drugs suggests a similar effect of these extracts to 2, 4-dinitrpphenol, Nordihydroguaiaretic and Chloroquine. Lactate dehydrogenase is a cytosolic enzyme released into the medium as a consequence of cell membrane rupture or permeability of the plasma membrane, which is a key sign of cell death. The elevated concentration of lactate dehydrogenase observed in the acetone extracts of both plants confirms the toxicity observed in the MTT and crystal violet assays, which suggests the potential toxicity of these plants in acetonic extractions.

The results of the other extractions, which did not indicate lactate dehydrogenase release, corroborates earlier reports on berberine (Xu et al., 2014; Yin, et al., 2008; Yin et al., 2002). There are several reports of antihyperglycemic and antidiabetic activities of medicinal plants based on their free radical scavenging and antioxidant properties (Zhang & Tan, 2000). The methanolic extract of *Albuca setosa* showed the highest ferric ion reducing potential which was similar to our report in the previous chapter. It was also observed that the phytochemical analysis of these plants showed that the methanolic extract indicated higher total phenol contents than the aqueous extracts. This corroborates with recent reports which suggests a high positive relationship between total phenols and antioxidant activity (Oktay, et al., 2003). Therefore, the high ferric reducing potential of the methanolic extract could be a result of higher total phenolic contents.

In conclusion, the results of this study indicate that the aqueous extracts of both *Albuca setosa* and *Albuca bracteata* are not cytotoxic at the doses investigated. The methanolic extractions indicate little cytotoxicity, which is similar to the standard drugs routinely used but caution must be taken with the acetone extraction. However, as these extracts were resolubilised in DMSO prior to the cytotoxic experiments, the potential toxicity attributed to acetone has been ruled out.

REFERENCES

Azaizeh, H., Fulder, S., Khalil, K., & Said, O. (2003). Ethnobotanical knowledge of local Arab practitioners in the Middle Eastern region. *Fitoterapia*, *74*(1-2), 98–108. http://doi.org/10.1016/S0367-326X(02)00285-X

Azurah, N., Akhir, M., Chua, L. S., Adibah, F., Majid, A., & Sarmidi, M. R. (2011). Cytotoxicity of Aqueous and Ethanolic Extracts of Ficus deltoidea on Human Ovarian Carcinoma Cell Line. *British Journal of Medicine & Medical Research*, *1*(4), 397–409.

Bagla, V. P. (2012). *Isolation and characterization of compounds from Podocarpus henkelii (Podocarpaceae) with activity against bacterial, fungal and viral pathogens*. University of Pretoria. Retrieved from etd-05082012-101645

Benzie, I. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, *299*, 15–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9916193

Capes-Davis, A., Theodosopoulos, G., Atkin, I., Drexler, H. G., Kohara, A., MacLeod, R. A. F., … Freshney, R. I. (2010). Check your cultures! A list of cross-contaminated or misidentified cell lines. *International Journal of Cancer. Journal International Du Cancer*, *127*(1), 1–8. http://doi.org/10.1002/ijc.25242

Chang, R. S. M. (1954). Continuous subcultivation of epithelial-like cells from normal human tissues. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *87*(2), 440–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13237268

Da Costa Lopes, L., Albano, F., Augusto Travassos Laranja, G., Marques Alves, L., Fernando Martins e Silva, L., Poubel de Souza, G., … Kovary, K. (2000). Toxicological evaluation by in vitro and in vivo assays of an aqueous extract prepared from Echinodorus macrophyllus leaves. *Toxicology Letters*, *116*(3), 189–98. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10996480

Decker, T., & Lohmann-Matthes, M.-L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*, *115*(1), 61–69. http://doi.org/10.1016/0022-1759(88)90310-9

Deutschländer, M. S., van de Venter, M., Roux, S., Louw, J., & Lall, N. (2009). Hypoglycaemic activity of four plant extracts traditionally used in South Africa for diabetes. *Journal of Ethnopharmacology*, *124*(3), 619–24. http://doi.org/10.1016/j.jep.2009.04.052

Elvin-Lewis, M. (2001). Should we be concerned about herbal remedies. *Journal of Ethnopharmacology*, *75*(2-3), 141–164. http://doi.org/10.1016/S0378-8741(00)00394-9

Farnsworth, N. . (1993). Biological approaches to the screening and evaluation of natural products. In P. Rasoanaivo & S. Ratsimamanga-Urverg (Eds.), (pp. 35–43). Madagaskar: Monograph from the IFS-NAPRECA Workshop on Bioassays.

Gao, Q., Wang, X.-Y., Zhou, J., & Fan, J. (2011). Cell line misidentification: the case of the Chang liver cell line. *Hepatology (Baltimore, Md.)*, *54*(5), 1894–5. http://doi.org/10.1002/hep.24475

Gerlier, D., & Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods*, *94*(1-2), 57–63. http://doi.org/10.1016/0022-1759(86)90215-2

Gessler, M. C., Msuya, D. E., Nkunya, M. H., Mwasumbi, L. B., Schär, A., Heinrich, M., & Tanner, M. (1995). Traditional healers in Tanzania: the treatment of malaria with plant remedies. *Journal of Ethnopharmacology*, *48*(3), 131–44. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8719974

Gillies, R. J., Didier, N., & Denton, M. (1986). Determination of cell number in monolayer cultures. *Analytical Biochemistry*, *159*(1), 109–13. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3812988

Gosenca, M., Obreza, A., Pečar, S., & Gašperlin, M. (2010). A new approach for increasing ascorbyl palmitate stability by addition of non-irritant co-antioxidant. *American Association of Pharmaceutical Scientists*, *11*(3):1485–92. http://doi.org/10.1208/s12249-010-9507-8

ICLAC. (2013). Database of Cross-Contaminated or Misidentified Cell Lines. Retrieved August 31, 2014, from http://standards.atcc.org/kwspub/home/the_international_cell_line_authentication_committee-iclac_

Itagaki, H., Hagino, S., Kato, S., Kobayashi, T., & Umeda, M. (1991). An in vitro alternative to the Draize eye-irritation test: Evaluation of the crystal violet staining method. *Toxicology in Vitro*, *5*(2), 139–143. http://doi.org/10.1016/0887-2333(91)90034-B

Legrand, C., Bour, J. M., Jacob, C., Capiaumont, J., Martial, A., Marc, A., … Hache, J. (1992). Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. *Journal of Biotechnology*, *25*(3), 231–243. http://doi.org/10.1016/0168-1656(92)90158-6

Mosaddegh, M., Esmaeili, S., Naghibi, F., Hamzeloo Moghadam, M., Haeri, A., Pirani, A., & Moazzeni, H. (2012). Ethnomedical Survey and Cytotoxic Activity of Medicinal Plant Extracts Used in Kohgiluyeh and Boyerahmad Province in Iran. *Journal of Herbs, Spices & Medicinal Plants*, *18*(3), 211–221. http://doi.org/10.1080/10496475.2012.671801

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55–63. http://doi.org/10.1016/0022-1759(83)90303-4

Naseer, M. I., Zubair, H., Ikramullah, & Kim, M. ok. (2009). Effect of Fetal Calf Serum on Cellular Proliferation of Mouse Y1 Adrenocortical Cells in vitro. *Pakistan Journal of Medical Sciences*, *25*(3), 500–504.

Nelson-Rees, W. A., & Flandermeyer, R. R. (1976). HeLa cultures defined. *Science (New York, N.Y.)*, *191*(4222), 96–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1246601

Oktay, M., Gülçin, İ., & Küfrevioğlu, Ö. İ. (2003). Determination of in vitro antioxidant activity of fennel (Foeniculum vulgare) seed extracts. *LWT - Food Science and Technology*, *36*(2), 263–271. http://doi.org/10.1016/S0023-6438(02)00226-8

Otang, M. W., Grierson, D. S., & Ndip Ndip, R. (2014). Cytotoxicity Of Three South African Medicinal Plants Using The Chang Liver Cell Line. *African Journal of Traditional, Complementary & Alternative Medicine*, *11*(2), 324–329. http://doi.org/10.4314/ajtcam.v11i2.16

Rossetti, L., Giaccari, A., & DeFronzo, R. A. (1990). Glucose toxicity. *Diabetes Care*, *13*(6), 610–630. http://doi.org/10.2337/diacare.13.6.610

Teixeira, R. de O., Camparoto, M. L., Mantovani, M. S., & Vicentini, V. E. P. (2003). Assessment of two medicinal plants, *Psidium guajava* L. and *Achillea millefolium* L., in in vitro and in vivo assays. *Genetics and Molecular Biology*, *26*(4), 551–555. http://doi.org/10.1590/S1415-47572003000400021

Temin, H. ., Pierson (Jnr), R. ., & Dulak, C. (1972). *Growth, Nutrition and metabolism of cells in culture*. (G. . Rotfblatt & V. . Cristofalo, Eds.) (Vol. 1). New York: Academic press.

Tshikalange, T. E., & Hussein, A. (2010). Cytotoxicity activity of isolated compounds from Elaeodendron transvaalense ethanol extract. *Journal of Medicinal Plants Research*, *4*(16), 1695–1697.

Van de Venter, M. Roux, S. Bungu, L. Louw, J. Crouch, N. Grace, O. Maharaj, V. Pillay, P. Sewnarian, P. Bhagwandin, N.Folb P (2008). Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. *Journal of Ethnopharmacology*, *119*(1), 81–86.

WHO, W. health O. (2002). WHO Traditional Medicine strategy. *World Health Organisation*, *80*(7), 610–610. Retrieved from http://www.who.int/bulletin/archives/80(7)610.pdf

Xu, M., Xiao, Y., Yin, J., Hou, W., Yu, X., Shen, L., … Jia, W. (2014). Berberine Promotes Glucose Consumption Independently of AMP-Activated Protein Kinase Activation. *PloS One*, *9*(7), e103702. http://doi.org/10.1371/journal.pone.0103702

Yin, J., Gao, Z., Liu, D., Liu, Z., & Ye, J. (2008). Berberine improves glucose metabolism through induction of glycolysis. *American Journal of Physiology. Endocrinology and Metabolism*, *294*(1), E148–56. http://doi.org/10.1152/ajpendo.00211.2007

Yin, J., Hu, R., Chen, M., Tang, J., Li, F., Yang, Y., & Chen, J. (2002). Effects of berberine on glucose metabolism in vitro. *Metabolism: Clinical and Experimental*, *51*(11), 1439–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12404195

Zhang, X. F., & Tan, B. K. (2000). Antihyperglycaemic and anti-oxidant properties of Andrographis paniculata in normal and diabetic rats. *Clinical and Experimental Pharmacology & Physiology*, *27*(5-6), 358–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10831236

CHAPTER 5

SCREENING FOR ANTI – DIABETIC ACTIVITY OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS

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Part of this Chapter will be submitted for publication in Journal of Ethnopharmacology

CHAPTER 5

SCREENING FOR ANTI – DIABETIC ACTIVITY OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS

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SCREENING FOR ANTI – DIABETIC ACTIVITY OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS

5.1 Introduction

Screening using a variety of *in vitro* test systems or *in vivo* (animal models) has been the standard practice in studying the antidiabetic effects of herbal remedies. While the *in vivo* studies mimic the effect of the drug in humans and are necessary to study the mechanism of action under clinical or pathophysiological conditions (Thorat, et al., 2012), *in vitro* models are considered to be first line screening tools as the model is quick and provides a cell/ tissue specific determination of the cells' response to the drug. Models provide insights into the pathogenesis of some human diseases (Ekwall, et al., 1990) and are therefore commonly used in the study of diabetes, cancer and other diseases. Sensitivity, contamination and transformation of cell lines are the major challenge of *in vitro* investigations and researchers should work with care and high throughput techniques. Another important limitation of *in vitro* based testing is that it does not take into account the biotransformation of drugs that may occur during oral ingestion of drugs, chemical kinetics such as rates of absorption, distribution and the excretion which are all believed to influence the exposure of the biologically active compounds at the target cells *in vivo*(Ekwall et al., 1990). Cell-based assay however, can provide useful information on the mechanism of action of therapeutic agents and can predict these effects under clinical or pathophysiological conditions.

The recent surge of antidiabetic drugs on the market exploits a wide range of therapeutic targets due to the variety of pathogenic abnormalities associated with diabetes and its related complications. Examples of different mechanistic classes of oral drugs used in the first line treatment of diabetes include inhibitors of carbohydrate digestion which delay postprandial absorption of monosaccharides (α-glucosidase and α-amylase inhibitors), inhibition of

hepatic glucose production by the inhibition of gluconeogenesis (metformin), insulin secretagogues or insulin releasers which increase the release of insulin from pancreatic β-cells (sulfonylureas), insulin sensitizing agents or insulin sensitizers which increase the insulin responsiveness in peripheral tissues (thiazolidenediones), incretinpotentiators such as inhibitors of incretin degradation (DPP IV inhibitors), enhancement β-cell function and inhibition of protein glycation as illustrated in Figure 5.1.

The numerous phytochemical contents which medicinal plants could possess allow for different mechanisms of action in the management of specific diseases. This fact may point to the success traditional healers have experienced when using medicinal plants to treat diseases.

In this study *Albuca setosa* and *Albuca bracteata* extracts were screened for antidiabetic potentials using HepG2 and L6 as models for liver, muscle, fat and pancreatic cells respectively. These cell lines were chosen due to the importance of these tissues in diabetes because they more so possess different glucose transporters which respond differently to insulin stimulation.

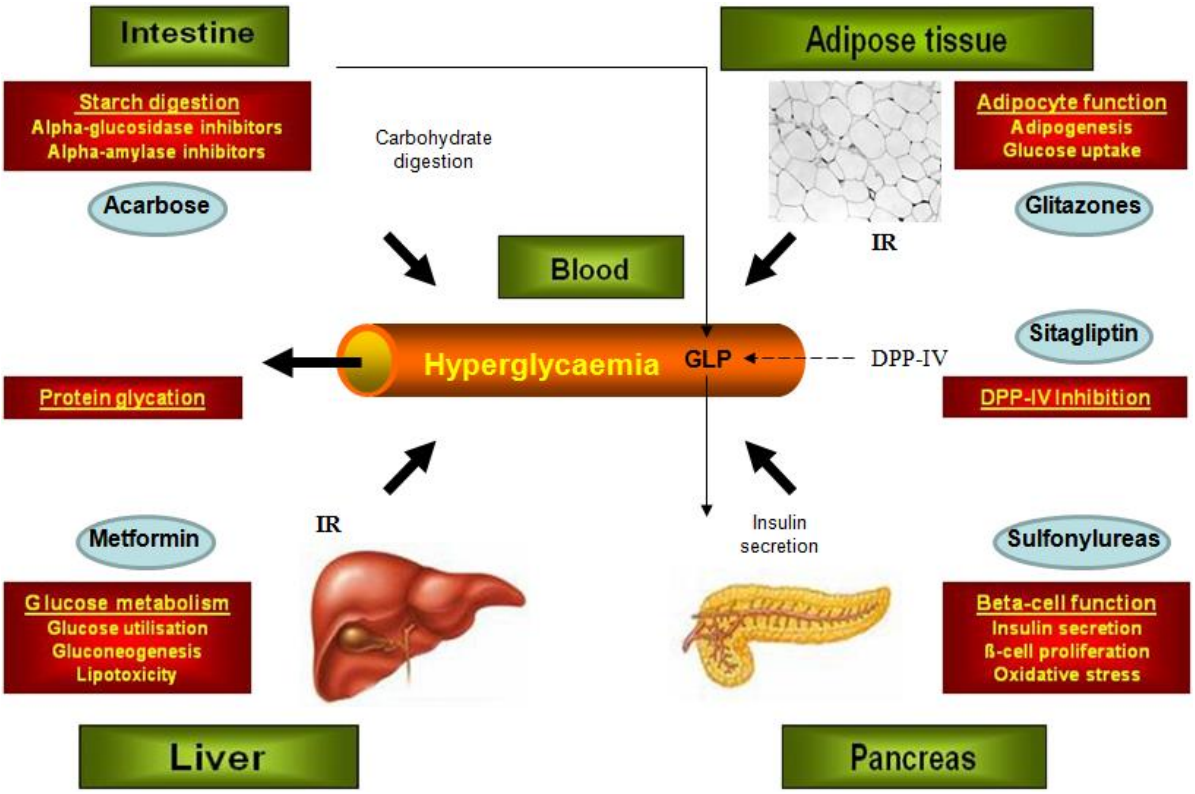

Figure 5.1: Schematic representation of the direct therapeutic targets used in conventional treatment of diabetes

IR = insulin resistance, DPP-IV= dipeptidyl peptidase IV and GLP = glucagon like peptide 1).

Source: (Koekemoer, et al., 2009)

5.2 Materials and methods

5.2.1 Cell lines, media and chemicals

HepG2 (Hepatocyte) and L6 (Myoblast) cell lines were obtained from the American Type Culture Collection (Highveld Biological, Johannesburg, South Africa). DMEM and EMEM were obtained from Sigma Aldrich, South Africa. Glucose oxidase reagent (SERA-PAK Plus, Bayer), and RPMI 1640 medium was obtained from Sigma Aldrich, S. Africa, and foetal calf serum (FCS) was purchased from Highveld Biological, South Africa. The chemicals and other reagents used in this study were of analytical grade and were purchased from Merck Chemicals (Pty) Ltd, S. Africa.

5.2.2 Preparation of plant extracts

The bulbs of *Albuca setosa* and *Albuca bracteata* were thinly sliced and oven-dried at 40^{o}C in the laboratory. The dried materials were further ground into powdery form using an electric blender (Hamilton Beach Commercial Blender type GB27 model HBF 400-CE). About 80 g of the powdered plant material were extracted in 1000 ml of distilled water, methanol, and acetone respectively. The extracts were maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, SOS1, Essex, UK) for 48 h. The extracts were then filtered using Whatman No.1 filter paper in a vacuum pump. The aqueous filtrate was quickly frozen at -40^{o}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 18.6 g and 15.2 g dry extract of *Albuca setosa* and *Albuca bracteata* respectively. The methanol and acetone extracts were dried using a rotary evaporator. Both the methanolic and acetonic extracts were resolubilised in distilled water and freeze dried. All the dried extracts were then reconstituted in diluted DMSO, sonicated and left for at least 15 min before further dilution with the respective growth medium before use. The final DMSO concentration did not exceed 0.25%.

5.2.3 Alpha amylase inhibition assay (Starch digestion assay)

15 µl of the plant extracts that was previously diluted in a phosphate buffer was added to 5µl of enzyme porcine pancreatin solution in the wells of a 96-well plate and then incubated for 10 min. at 37°C. The reaction was initiated by adding 20µlof 2% starch solution and incubated for exactly 30 min. To stop the reaction, 10µl 1M HCl was added to each well followed by 75 µl of iodine reagent. A blank containing phosphate buffer (pH 6.9) instead of the extract, and a positive control containing acarbose (20mM) were prepared. No enzyme control and no starch control were included for each test sample. The absorbance was read at 580nm and the percentage inhibition calculated by using the equation:

$$\% \text{ inhibition} = \left(\frac{A - B}{A}\right) X\ 100$$

Where

A = the absorbance of the activity of the enzyme with the test solution and

B = the absorbance of the activity of the enzyme without the test solution.

A standard curve for starch was prepared. Briefly, 40 µl of different concentrations of starch as shown in Table 5.1 were prepared into a 96–well plate in triplicates per concentration. 10 µl of HCl was then added followed by 75 µl of iodine reagent. The absorbance was read at 580 nm and the dose response graph was plotted for acarbose. The concentration of starch was determined using the equation $y = 0.033x + 0.0841$; $R^2 = 0.9996$.

Table 5.1: Starch dilutions

Volume Starch solution (µl)	Volume phosphate buffer (µl)	Concentration Starch in assay (µg/well)
0	1000	0
125	875	10
250	750	20
375	625	30
500	500	40
625	375	50
750	250	60
1000	0	80

5.2.4 Alpha glucosidase inhibition assay

5 µ of the plant extract was added to 20µl of 50µg/ml α-Glucosidase enzyme inside a 96-well plate. 60 µl of 67 mM Potassium Phosphate buffer (pH 6.8) was then added before being incubated at 37°C. After 5 min, 10 µl of 10 mM ρ-Nitrophenyl-α-D-Glucoside solution (PNP-GLUC) was then added. This was incubated for 20 min at 37°C. After incubation, 100 µl of 100 mM sodium carbonate solution was added and the absorbance read at 405 nm. A blank and sample blank were also prepared by adding 5 µl of deionised water instead of plant extracts and 20 µl of deionised water instead of enzyme respectively. The percentage inhibition was calculated using the equation:

$$\% \text{ inhibition} = \left(\frac{A-B}{A}\right) X\ 100$$

Where

A = the absorbance of blank solution

B = the absorbance of the extract solutions.

5.2.5 DPP-IV Inhibition assay

The DPP – IV inhibition assay was carried out according to a modified method of Al-masri et al. (2009) using a chromogenic substrate H-Gly-Pro-para-Nitroaniline to release para-nitroaniline (pNA) measured at 410 nm. Briefly, the assay mixture contained 50 mMTris/HCl buffer (pH 8.0), 0.8 mU/µl DPP – IV and plant extracts at a final concentration of 50 µg/ml. Positive control was also prepared using Diprotin A instead of the plant extracts. The reaction was initiated by adding 0.2 mM pNA substrate and incubated for 30 min at 37^{o}C. 25 µl of 25% acetic acid was added to stop the reaction and the absorbance was read at 410 nm. Percentage inhibition was calculated with the equation:

$$\% \text{ inhibition} = \left(\frac{A-B}{A}\right) X\ 100.$$

Where A = activity of the control in the absence of inhibitor

B = activity of the sample

Maintenance of cell cultures

All the cell cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO2. The HepG2 was fed fresh with growth medium every 2– 3 days, consisting of RPMI 1640 (Highveld Biological, South Africa) medium supplemented with 10% foetal bovine serum. L6 cells were fed fresh in EMEM (1.5g/l NaHCO3) (Highveld Biological, South Africa) supplemented with 10% foetal bovine serum under careful rigorous aseptic techniques to avoid cell culture contamination. All the cell lines were sub-cultured after 90% confluence was reached.

5.2.6 Glucose utilisation assay in HepG2

HepG2 cells were seeded into a 96-well culture plate to a density of 6 000 cells and allowed to adhere and grow for 3 days in a humidified incubator with 5% CO2 at 37°C. Two rows

were left free to serve as blanks for glucose utilisation assay. Without changing the medium, a 10 µl aliquot of the plant extract was added to each well to a final extract concentration of 25 µg/ml. The cells were incubated in the presence of the extract for a further 48 h. The spent culture medium was removed and replaced with a 25 µl incubation buffer containing RPMI medium diluted with PBS, 0.1% BSA and 8 mM glucose and was incubated for 3 h at 37°C. 1 µM metformin was used as the positive control while the control contained only the incubation medium without treatment. After incubation, 5 µl of the incubation medium was then transferred from each well onto a new 96-well plate. The glucose concentration in the medium was determined using a glucose oxidase assay by adding 200 µl of glucose oxidase reagent (SERA-PAK Plus, Bayer) into each well and incubating for 15 min at 37^{o}C. The absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Lab systems). The amount of glucose utilized was calculated as the difference between the cell free and cell containing wells (Yin, et al., 2008).

5.2.7 Glucose utilisation in L6 myoblasts

The L6 cells were seeded into 96-well culture plates at a density of 3 000 cells/well and allowed to adhere until 90% confluence was reached. Two rows were left free to serve as blanks for glucose utilisation assay. After 90% confluence, the culture medium was removed and replaced with EMEM containing 2% FBS and cultured for an additional 5 days. On day three of incubation (48 h prior to the glucose utilisation assay), the culture medium was replaced and a 10 µl aliquot of the plant extract was added to each well to a final extract concentration of 50 µg/ml. A column was also treated with insulin (1 µM) and berberine (50µM) instead of the plant extracts to serve as a positive control. The cells were incubated in the presence of the extract for a further 48 h. On the fifth day, the spent medium was removed and replaced with a 25 µl incubation buffer containing RPMI medium diluted with PBS, 0.1% BSA and 8mM glucose and incubated for 3 h at 37°C. 5 µl of the incubation medium

was then transferred from each well onto a new 96-well plate. The glucose concentration in the medium was determined using a glucose oxidase assay by adding 200 µl glucose oxidase reagent (SERA-PAK Plus, Bayer) into each well and incubating for 15 min at 37^{o}C. The absorbance was measured at 520 nm using a Multiscan MS microtitre plate reader (Lab systems). The amount of glucose utilized was calculated as the difference between the cell free and cell containing wells. The cell density in each well was then determined using MTT assay as described earlier.

5.2.8 Glucose utilisation in 3T3-L1Adipocytes

The 3T3-L1 cells were seeded into 96-well culture plates at a density of 3 000 cells/well in DMEM containing 10% FBS and the medium replaced every two to three days. Two rows were left free to serve as blanks for glucose utilisation assay. 48 h prior to the glucose utilisation assay, the culture medium was replaced and a 10 µl aliquot of the plant extract was added to each well to a final extract concentration of 25µg/ml. The cells are incubated in the presence of the extract for a further 48 h. A column was also treated with insulin (1 µM)instead of the plant extracts to serve as a positive control and another column without treatment serves as the negative control. The cells were incubated in the presence of the extract for a further 48 h. after which the spent medium was removed and replaced with 25µl incubation buffer containing 8mM glucose (RPMI medium diluted with PBS containing 0.1% BSA). Incubate for 3 hours at 37°C.5µl of the incubation medium was then transferred from each well into a new 96-well plate and the glucose concentration in the medium was determined using a glucose oxidase assay into which 200 µl glucose oxidase reagent (SERA-PAK Plus, Bayer) was added. After incubation for 15 min at 37^{o}C, the absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Lab systems). The glucose utilisation was calculated as the difference between the cell free and cell containing wells and is expressed as the percentage of control.

5.3 Results

5.3.1 Alpha amylase inhibition

The inhibitory effect of *Albuca setosa* and *Albuca bracteata* on α-amylase shows acarbose with the highest inhibition. Among the extracts tested, the acetonic extract of *Albuca bracteata* shows the highest inhibition against alpha amylase enzyme followed by the acetonic extract of *Albuca setosa* and then the methanolic extract of *Albuca bracteata* as shown in Figure 5.2. The inhibition observed in the aqueous extracts of *Albuca setosa, Albuca bracteata* and the methanolic extract of *Albuca setosa* are lower and do not significantly differ from each other. The amount of starch remaining was calculated from the starch standard curve (Figure 5.3), the percentage inhibition of alpha amylase enzyme compared with acarbose as shown in Table 5.2 while Figure 5.4 shows the acarbose dose response used for the calculation of the IC_{50}.

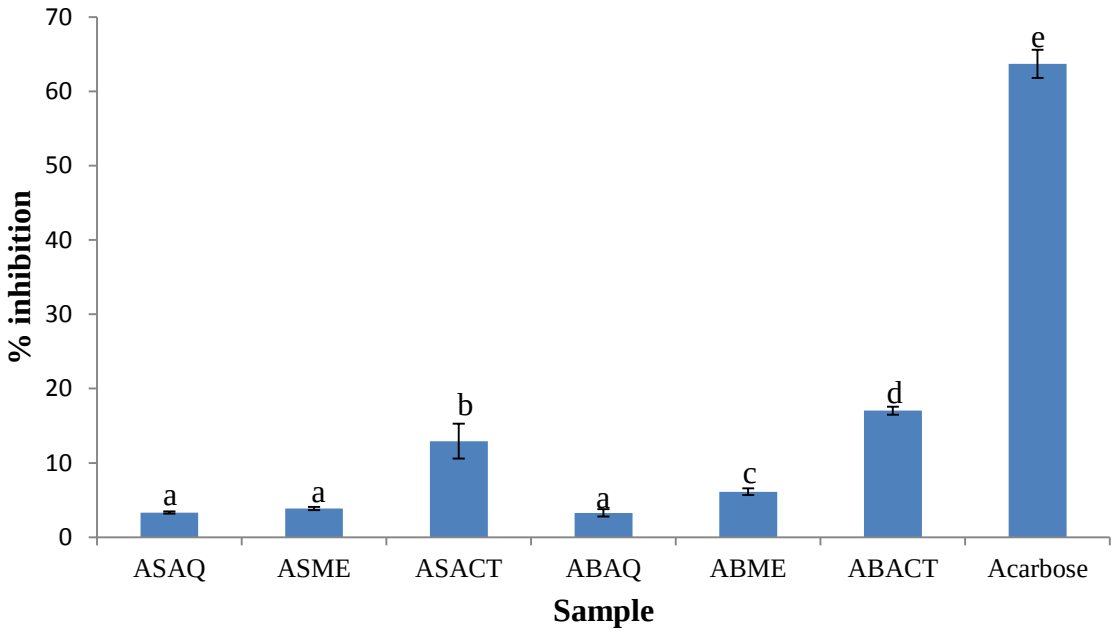

Figure 5.2: Percentage inhibition of alpha amylase
Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at ($P < 0.01$). ASAQ = *Albuca setosa* aqueous extract; ASME = *Albuca setosa* methanol extract; ASACT = *Albuca setosa* acetone extract; ABAQ= *Albuca bracteata* aqueous extract; ABME = *Albuca bracteata* methanol extract; ABACT = *Albuca bracteata* acetone extract.

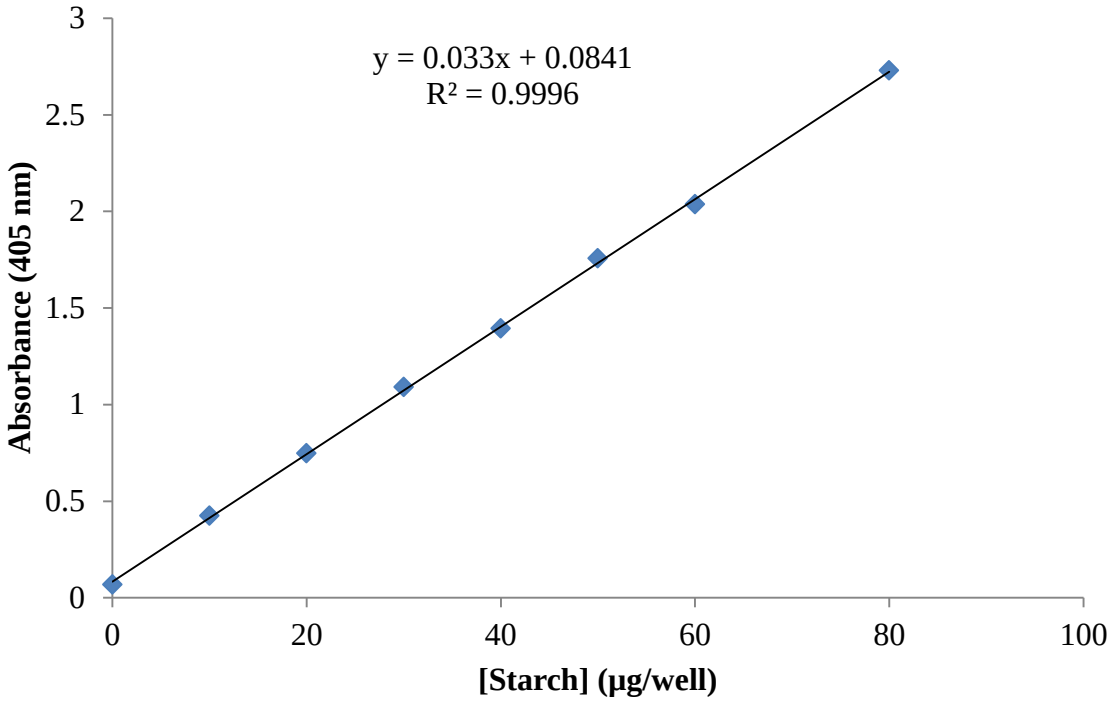

Figure 5.3: Starch standard curve

Data expressed as mean ± SD, n= 6. y = 0.033x + 0.0841; R^2 = 0.9996

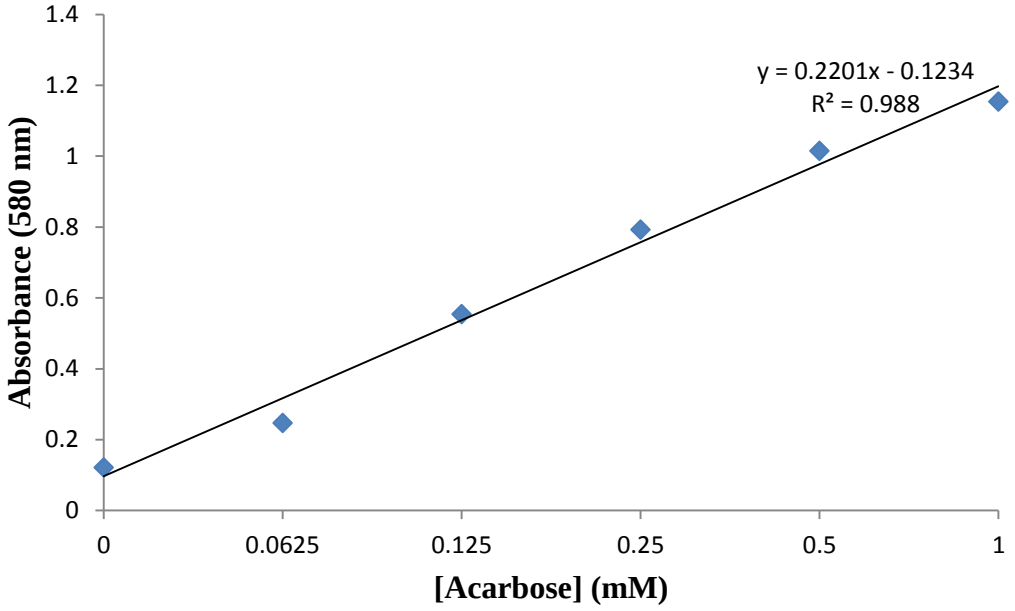

Figure 5.4: Acarbose dose - response curve

Data expressed as mean ± SD, n= 6. y = 0.2201x – 0.1234; R^2 = 0.988

5.3.2 Alpha glucosidase inhibition

The inhibitory effect of *Albuca setosa* and *Albuca bracteata* on α-glucosidase shows acarbose with the highest percentage inhibition at 80.73% (Table 5.2). Among the extracts the acetonic extract of *Albuca bracteata* showed the highest inhibition with 68.88%, followed by the methanolic extract of *Albuca setosa*, while the methanolic extract of *Albuca bracteata* showed the least inhibition against α-glucosidase (Figure 5.5). Comparing the aqueous extracts of both plants, that of the *Albuca setosa* showed a higher percentage inhibition with 28.35% as opposed to 8.02% for *Albuca bracteata*.

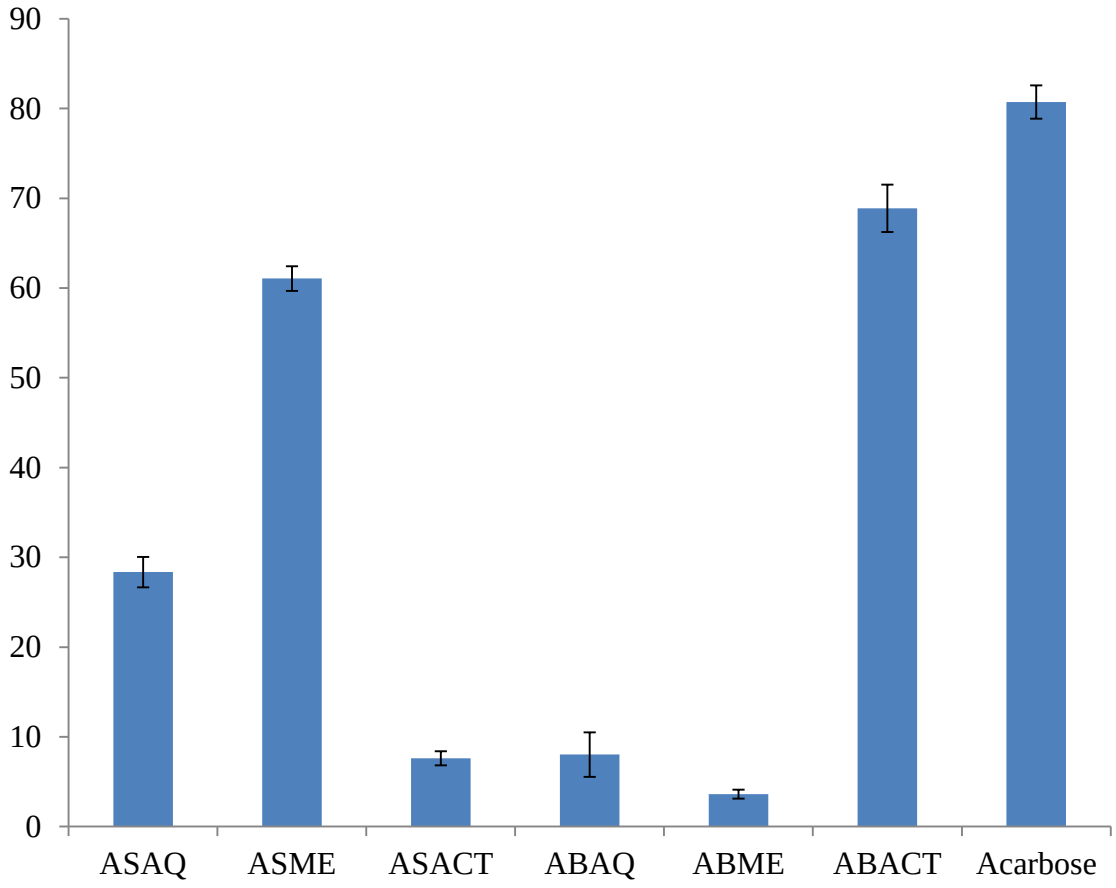

Figure 5.5: Percentage inhibition of alpha glucosidase

Data expressed as mean ± SD, n= 6. ASAQ = *Albuca setosa* aqueous extract; ASME = *Albuca setosa* methanol extract; ASACT = *Albuca setosa* acetone extract; ABAQ= *Albuca bracteata* aqueous extract; ABME = *Albuca bracteata* methanol extract; ABACT = *Albuca bracteata* acetone extract

5.3.3 Inhibition of Dipeptidylpetidase IV

The inhibitory effect of *Albuca setosa* and *Albuca bracteata* extracts on DPP – IV is shown in Figure 5.6.

Both plant extracts showed weak inhibition against DPP-IV enzyme. Diprotein A showed the maximal inhibition with 98.23% of the control. Among the extracts, the *Albuca setosa* aqueous extract showed the highest with 1.89% but this was not significantly different from that of *Albuca bracteata* aqueous and *Albuca bracteata* methanol extracts with 1.60% and 1.65% respectively. Other extracts such as the *Albuca setosa* methanol, *Albuca setosa* acetone and the *Albuca bracteata* acetone extracts were not significantly different from the blank.

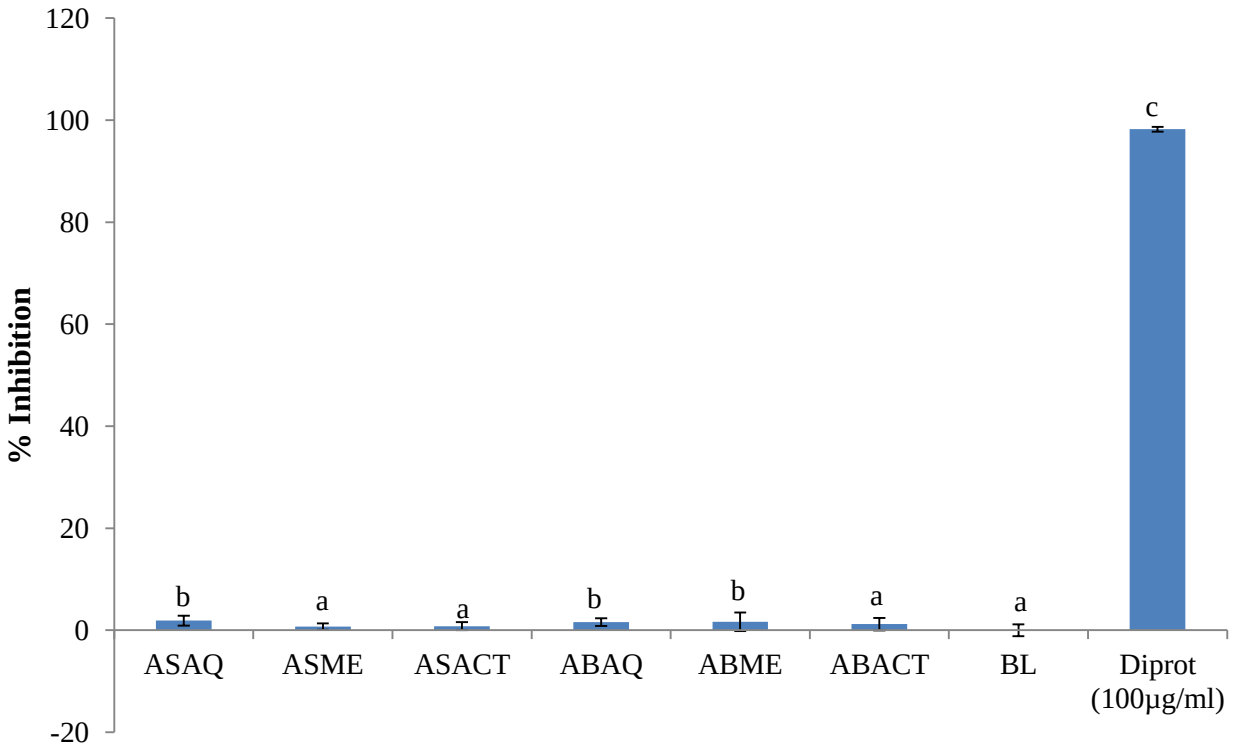

Figure 5.6: Inhibitory effect of *Albuca setosa* and *Albuca bracteata* bulb extracts on DPP-IV

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at ($P < 0.01$). ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract; ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata*; BL: Blank; Diprot: Diprotein A.

5.3.4 Glucose uptake in HepG2 Cells

The effect of the *Albuca setosa* and *Albuca bracteata* extracts on glucose uptake in HepG2 cells is revealed in Figure 5.7. Berberine showed the highest glucose uptake followed by metformin. Both aqueous and methanol extracts of *Albuca setosa* were not significantly different from the control but the MTT result of both *Albuca setosa* and *Albuca bracteata* have low cell density compared to the control. The glucose uptake of all the extracts of *Albuca bracteata* was significantly lower than the control, together with the acetone extract of *Albuca setosa.* The ratio of the glucose uptake compared with the cell density is shown in Figure 5.8.

From Figure 5.8 it was observed that the glucose uptake/MTT ratio of the *Albuca bracteata* acetone extract was the highest compared to the control, metformin and berberine. The aqueous extract of *Albuca setosa* was higher than the aqueous extract of *Albuca bracteata* but not significantly different from that of the control. In the same manner, the methanol extract of *Albuca setosa* was higher than both the methanol extract of *Albuca bracteata* and the control but not significantly different from metformin. The methanol extract of *Albuca bracteata* was higher but not significantly different from the control.

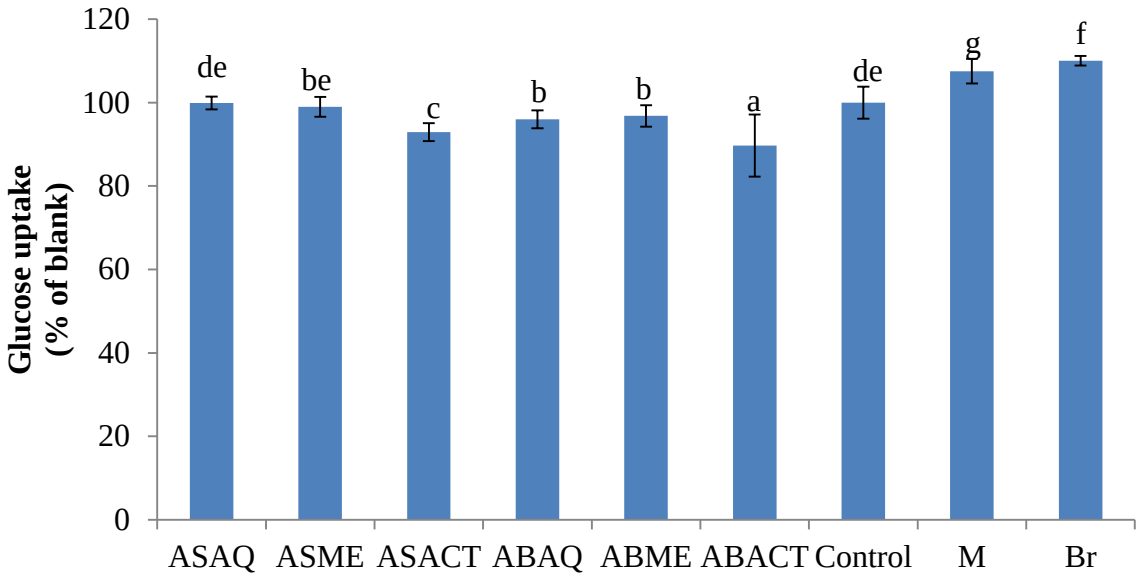

Figure 5.7: Effect of *Albuca setosa* and *Albuca bracteata* bulb extracts on glucose uptake in HepG2 cell

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01). ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract; ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract, Br: berberine; M: Metformin.

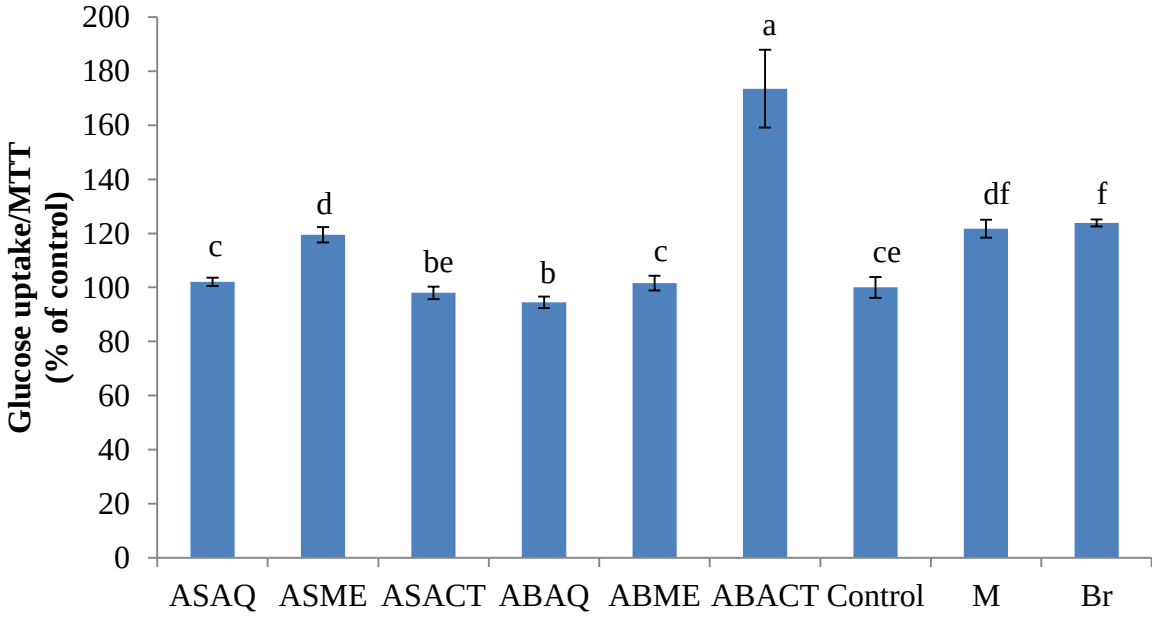

Figure 5.8: Effect of *Albuca setosa* and *Albuca bracteata* bulb extracts on glucose uptake in HepG2 cell per cell density

Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract; ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract; M: Metformin; Br: berberine.

Table 5.2: Percentage Inhibition of starch digestion enzymes

	ASAQ	ASME	ASACT	ABAQ	ABME	ABACT	Acarbose
α-amylase (%)	3.33 ± 0.15^{a}	3.86 ± 0.21^{a}	12.93 ± 2.33^{b}	3.27 ± 0.5^{a}	6.13 ± 0.47^{c}	17.03 ± 0.54^{d}	63.69 ± 1.90^{e}
α-glucosidase (%)	28.35±1.70	61.06±1.36	7.60 ± 0.78^{a}	8.02 ± 2.48^{a}	3.61±0.51	68.88±2.64	80.73±1.86
IC50 (mg/ml)	7.725		5.455		7.681	5.87	0.221*

ASAQ = *Albuca setosa* aqueous extract; ASME = *Albuca setosa* methanol extract; ASACT = *Albuca setosa* acetone extract; ABAQ = *Albuca bracteata* aqueous extract; ABME = *Albuca bracteata* methanol extract; ABACT = *Albuca bracteata* acetone extract. *acarbose (20mM)

All the values with the same superscripts are not significantly different at ($P < 0.01$)

5.3.5 Glucose utilisation in L6 Cells

The effect of *Albuca setosa* and *Albuca bracteata* extracts on myocytes (L6) glucose utilisation is shown in Figure 5.9. The cells treated with berberine showed the highest glucose utilisation with 302.74% of the control, which is higher than insulin-treated cells. Of the extracts treated, the *Albuca setosa* aqueous extract showed the highest glucose utilisation, which is not significantly different from the insulin-treated cells. Higher glucose utilisation was also observed in the *Albuca setosa* acetone and *Albuca bracteata* methanol extracts but this was significantly lower than that of insulin. The *Albuca bracteata* aqueous and acetone extracts and *Albuca setosa* methanol extract glucose utilisations did not differ significantly with the control treated.

The effect of the treatments on the cell density of L6 determined by the MTT assay showed no signs of toxicity as shown in Figure 5.10. It was observed that the treatments were not significantly different from the control except for the *Albuca setosa* aqueous and *Albuca bracteata* aqueous extracts, which were both significantly higher than the control respectively.

5.3.6 Glucose utilisation in 3T3-L1 Cells

The effect of *Albuca setosa* and *Albuca bracteata* extracts on adipocytes (3T3-L1) glucose utilisation is shown in Figure 5.11.All the treatments showed glucose uptake in 3T3-L1 cells compared to the control. The insulin treated cells showed the highest glucose uptake with 150.94% of the control followed by the aqueous extract of *Albuca bracteata* with 131.56% and *Albuca setosa* with 123.21%.

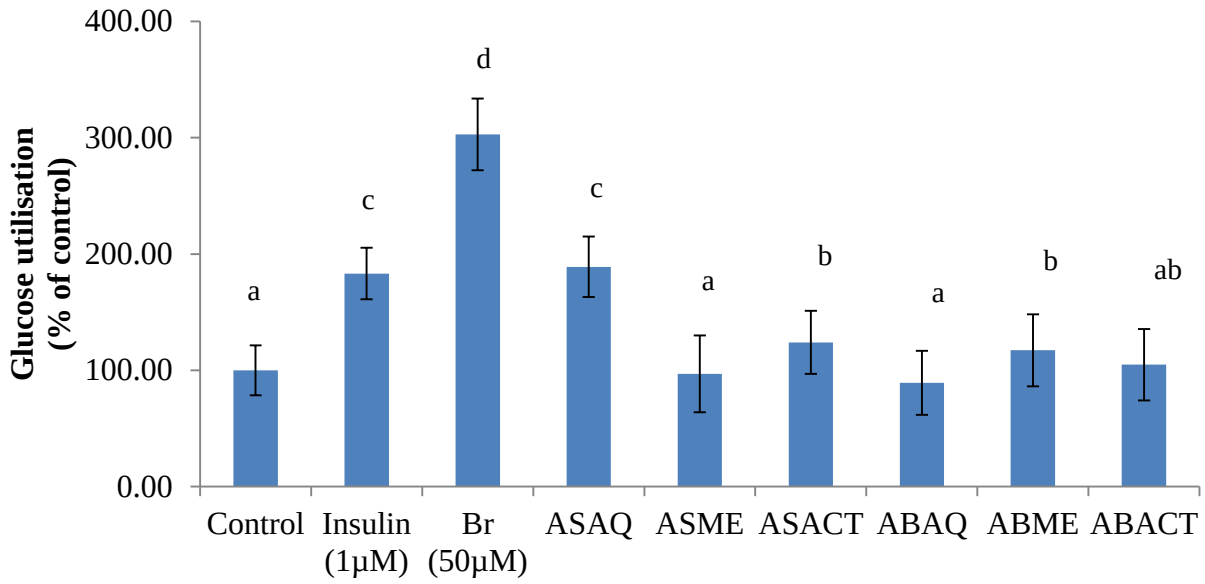

Figure 5.9: Effect of *Albuca setosa* and *Albuca bracteata* bulb extracts on L6 cells
Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).Br: Berberine; ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract; ABAQ: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract

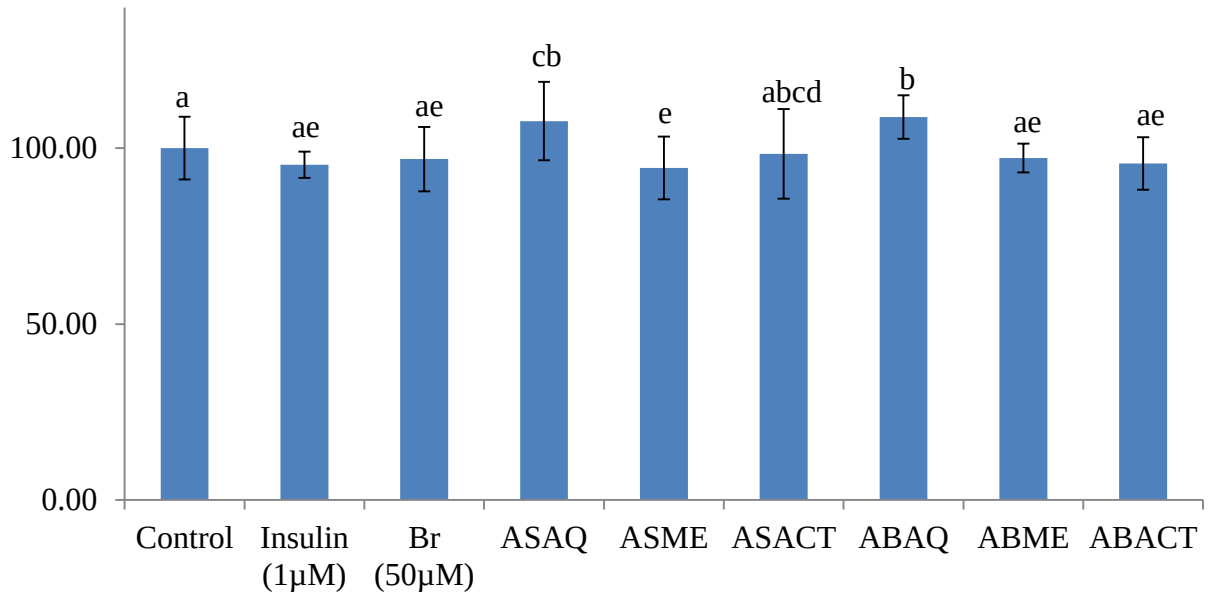

Figure 5.10: Cell density of the effect of *Albuca setosa* and *Albuca bracteata* bulb extracts on L6 cells
Data expressed as mean ± SD, n= 6. All the bars with the same letters are not significantly different at (P < 0.01).Br: Berberine; ASAQ: *Albuca setosa* aqueous extract; ASME: *Albuca setosa* methanol extract; ASACT: *Albuca setosa* acetone extract; ABAQ: *Albuca bracteata* aqueous

extract; ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract

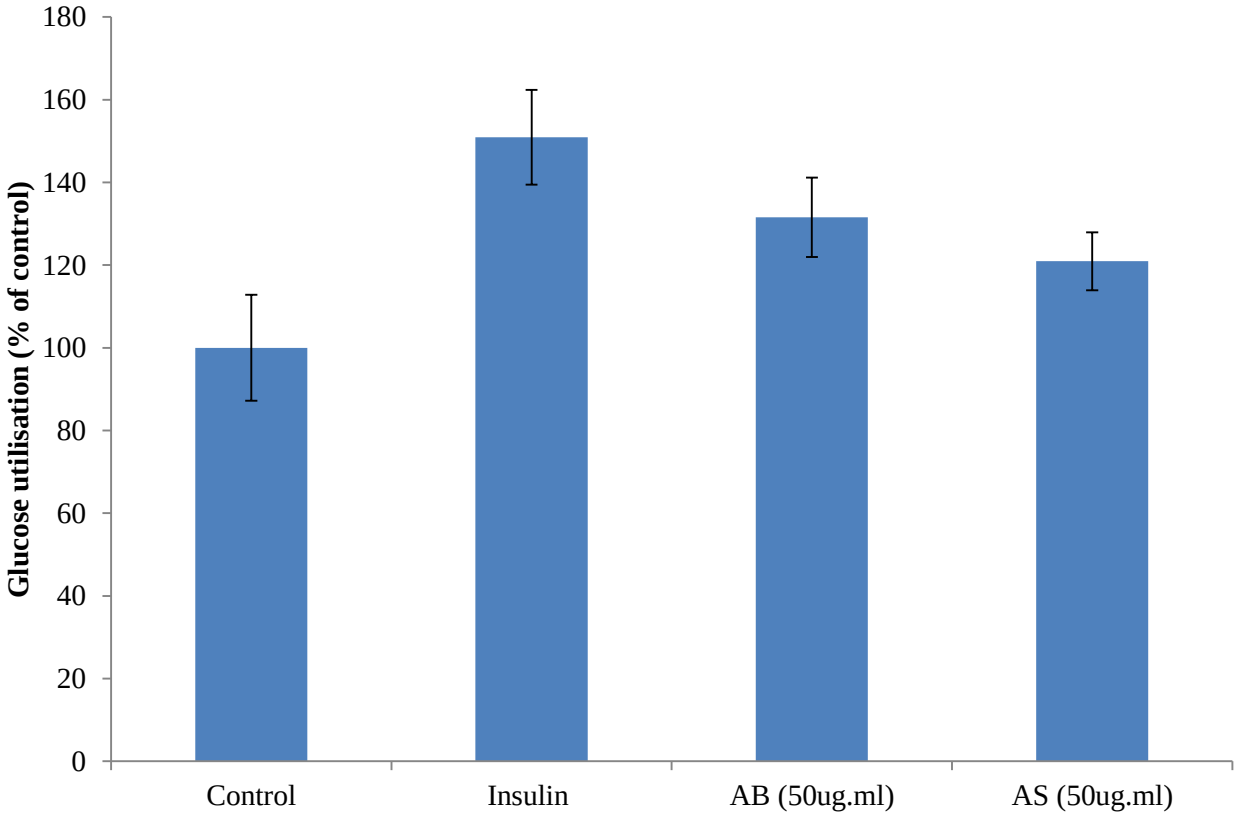

Figure 5.11: Effect of aqueous extracts of *Albuca setosa* and *Albuca bracteata* bulb extracts on 3T3-L1 cells

Data expressed as mean ± SD, n= 6, P < 0.01.AS: *Albuca setosa*; AB: *Albuca bracteata* aqueous extract; ABME: *Albuca bracteata* methanol extract; ABACT: *Albuca bracteata* acetone extract

5.4 Discussion

Diabetes mellitus is a complex metabolic disorder that has been associated with defects in insulin secretion or action in cells (Wild et al., 2004). Although the underlying mechanisms of this disease and various associated complications are unclear, earlier reports have suggested that the activation of certain proteins in the insulin-signalling pathway could be responsible (Giugliano, et al., 1996). Both type 1 and 2 diabetes mellitus are characterised by hyperglycaemia, and most drugs work by targeting a specific pathway to either inhibit gluconeogenesis or enhance glucose uptake in certain tissues with ultimate conversion into storage form. For example, postprandial hyperglycemia is a major contributing factor in the development of diabetes, which is why drugs that are able to inhibit either α- amylase or α-glucosidase slow down the absorption of glucose and ultimately reduce high blood glucose resulting from the digestion of carbohydrates. The results of α-amylase enzyme reported in this present study show a weak inhibition by both *Albuca setosa* and *Albuca bracteata* extracts when compared with acarbose. There was, however, an observable inhibition of α-glucosidase by both extracts. Interestingly, the *Albuca setosa* methanol extract showed a higher inhibition of α-glucosidase than the aqueous extract used in traditional medicine, and was observed not to be toxic (Figure 5.5). The weak inhibition of α-amylase observed here could be desirable, as total inhibition has been reported to provoke intestinal disorders (Cho, et al., 2011) which could be as a result of gas production by gut micro flora from the undigested starch. The total inhibition of α-amylase enzyme by acarbose has been associated with several undesirable side effects such as abdominal distension, flatulence, and diarrhoea (Horii et al., 1986). The weak inhibition observed against α-amylase and strong inhibition against α-glucosidase agrees with previous reports which suggested that phytochemicals are stronger inhibitors of α-glucosidase than α-amylase (Kwon, et al., 2007).

These inhibitory activities of the *Albuca setosa* and *Albuca bracteata* extracts could be beneficial and a property that confers an advantage over synthetic drugs. Flavonoids, alkaloids and terpenoids have been reported to possess α-glucosidase inhibitory activities (Ankita et al., 2011); therefore the inhibitory activity reported here could be a result of the presence of flavonoids and alkaloids in these plants. The highest inhibition observed by the acetone extract of *Albuca bracteata* indicates that the active principle is soluble in acetone; however, the toxicity effect of the acetone extract of these plants must be considered. The inhibition observed with the aqueous extract of *Albuca setosa* shows the potential inhibitory activity of *Albuca setosa* over *Albuca bracteata* in the traditional usage of these plants.

The incretin hormones, GIP (gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide) and GLP-1 (glucagon-like peptide-1) are peptidic hormones released by intestinal enteroendocrine cells into the bloodstream in response to the ingestion of nutrients, where they stimulate insulin secretion by the pancreatic cells, β-cell growth and differentiation, and the inhibition of glucagon secretion (Nauck, 2011). GLP-1 has been reported to be responsible for over 50% of postprandial insulin secretion (Ahrén, 2011; Holst, et al., 2011; Kim & Egan, 2008). These hormones have a short half-life (< 2mins) and are very sensitive to the degradation by dipeptidyl peptidase-IV (DPP-IV). DPP-IV is a serine protease that inactivates incretins by cleaving polypeptides containing proline and alanine, and therefore these inhibitors increase the time of action of incretins(Conarello et al., 2003; Stephan et al., 2011). Yang et al.(2007) also reported that the increase in DPP-IV expression and activity may play a role in the development of diabetes.

There is weak inhibitory activity observed by the extracts against DPP-IV as compared with the diprotein A, which is a potent inhibitor of DPP-IV. The inhibitory activity observed has been reported to be due to its tripeptide specificity and purity, such as the DPP-IV in the

central nervous system, endocrine system and on the CD-26 immune system (Maes et al., 2007).

In addition to its role in hepatic glucose output, the liver also accounts for the clearance of up to one third of the postprandial blood glucose load. Glucose uptake is the rate-limiting step in glucose metabolism, and an impaired hepatic glucose utilisation is a common feature in diabetic patients, which contributes to diabetic postprandial hyperglycaemia. Therefore, compounds that stimulate hepatic glucose consumption may have potential anti-diabetic properties, which may be as a result of the stimulatory effect on GLUT-2. However, the high presence of GLUT-2 in the liver allows hepatocytes to take up glucose without the presence of insulin. The result of glucose uptake observed in this study using HepG2 suggests that both the *Albuca setosa* and *Albuca bracteata* extracts have weak glucose uptake in hepatocytes, as compared to metformin and berberine. Metformin, which is a standard drug, improves glycemic control primarily by inhibiting hepatic gluconeogenesis and glycogenesis (Gerich, 1989). However, comparing the cell density of the cells with the amount of glucose uptake, the *Albuca bracteata* acetone extract and *Albuca setosa* aqueous and methanol extracts showed higher percentages than the control. It was determined that the high percentage value obtained for the acetone extract of *Albuca bracteata* could not be considered as the toxicity assay determined by the MTT and crystal violet revealed that the acetone extract of *Albuca bracteata* is highly toxic, and this toxicity may be responsible for the high positive values obtained in the glucose uptake assay in HepG2. Therefore, the use of the acetone extract of *Albuca bracteata* should not be encouraged but the *Albuca setosa* aqueous and methanol extracts could be further explored and can also explain the mechanism of action of *Albuca setosa* in reducing blood glucose. This means that *Albuca setosa* has a property similar to metformin on the liver that is independent of insulin as hepatic glucose uptake occurs through

GLUT-2. The result of this study also shows the higher glucose uptake potential of *Albuca setosa* over *Albuca bracteata* in HepG2 cells.

Skeletal muscle is the main site of glucose disposal in humans, with approximately 80% of the total body glucose uptake occurring in skeletal muscle through insulin- and exercise-sensitive glucose transporters (Thiebaud et al., 1982; Wood & O'Neill, 2012). The effects of plant extracts on insulin action as well as insulinomimetic activity can thus be assessed by measuring basal- and insulin-stimulated glucose uptake in muscle and adipocytes cell lines such as L6 and 3T3-L1. These cell lines have been used as a model for insulin-regulated glucose transport. Once glucose is taken up by the myocytes and adipocytes, it can either be oxidized to carbon dioxide and water, stored as glycogen or fat. Glycogen storage is regulated by glycogen synthase and the impairment of glycogen synthase activity has been reported to be one of the earliest defects in skeletal muscle seen in T2DM (DeFronzo, 2004). The glucose utilisation observed in the aqueous extract of *Albuca setosa* was comparable to that of insulin, and therefore the result reported in this study using L6 and 3T3-L1 differentiated cells established that the *Albuca setosa* aqueous extract has the ability to improve glucose uptake in muscle and fat cells respectively. This increase in glucose utilisation in the L6 and 3T3-L1 differentiated cells suggests that the *Albuca setosa* aqueous extract possesses insulin mimetic activity but the underlying mechanism needs to be established.

Similarly, the acetone extract of *Albuca setosa* and methanol extract of *Albuca bracteata* also possess this insulin mimetic property but adequate measures need to be considered regarding the cytotoxicity effect. However, both the acetone extract of *Albuca setosa* and the methanol extract of *Albuca bracteata* are less cytotoxic at the concentrations investigated, and therefore the glucose uptake reported in this study could be promising.

Insulin has been established to regulate three major functions; metabolic, mitogenic and signal transduction (Biddinger & Kahn, 2006; Leclercq, et al., 2007; Reaven, 1995; Shulman, 2000; Taniguchi, et al., 2006; White, 2002). Insulin works by binding to the heterotetrameric receptor on the plasma membrane that uses docking proteins to mediate signalling. The activation of PI3K, an enzyme which phosphorylates phosphatidylinositol biphosphate into phosphatidylinositol triphosphate (Anderson et al., 2001; Bugianesi, et al., 2005; Hickman et al., 2002) is an important step in the insulin signalling to the transport of glucose and glycogen synthesis through the translocation of GLUT 4 to the plasma membrane. Thus, the increase in PI3K, PKB/Akt activity and the initiation of the signal transduction through the CAP/Cbl/Tc10 pathway controls the membrane translocation of GLUT4 and it has been suggested that the adipocytes and muscle cells use this pathway of insulin signalling to regulate glucose intake (Chiang et al., 2001; Leclercq et al., 2007). It follows that the glucose uptake reported in the L6 myocytes and 3T3-L1 adipocytes treated with extracts of both *Albuca setosa* and *Albuca bracteata* may be due to the effects of these extracts on PI3K, PKB/Akt activity or through the CAP/Cbl/Tc10 pathway signal transduction. Reports have implied the presence of polyphenolics such as phenols, flavonoids, flavonols and proanthocyanidins, to have an insulinmimetic action in plants and plant extracts (Gomes, et al., 1995), while elaeocyanidin, gallotannins and ourateaproanthocyanidin A have been isolated from plants to possess anti-diabetic properties (Gorelik, et al., 2008; Gruendel et al., 2007).

The mechanism of action of *Albuca setosa* may be similar to the reports that polyphenols can exert anti-inflammatory effects by directly blocking mitogen-activated protein kinase (MAPK) pathways, NFκB activity and the expression of inflammatory cytokines (Ahn, et al., 2007; Chung, et al., 2001; Kim et al., 2005; Park et al., 2004). Therefore, the glucose uptake observed in the *Albuca setosa* aqueous and acetonic extracts

and the *Albuca bracteata* methanolic extract in this study could be attributed to the presence of these polyphenolics, and could be linked to the activation of one of the various proteins involved in insulin signalling pathways or in the movement of GLUT-4 to the plasma membrane and eventual translocation of glucose into the cell. For example, phenolic compounds in *Psidium guajava* have been reported to increase glucose uptake in adipocytes by activating PPAR (Owen et al., 2008; Singh et al., 1993). Previous reports have also shown that alkaloids have an anti-diabetic property by which they modulate carbohydrate metabolic enzymes through a response to insulin secretion (García López et al., 2004; Hii & Howell, 1985). It is worthwhile to mentioning that the antioxidant and ferric ion reducing capacity of *Albuca setosa* and *Albuca bracteata* may indicate antidiabetic property of these plants as earlier reported (Singh et al., 1993).

It can be concluded from the report of this study that *Albuca setosa* and *Albuca bracteata* extracts can improve diabetic conditions and also that the aqueous extracts of these plants are not toxic to Chang cells (from Chapter 4), 3T3-L1, L6 and HepG2 cell lines as they are used locally. *Albuca setosa* exhibits its antidiabetic potential through the inhibition of carbohydrate digesting enzymes and enhancing glucose uptake in myocytes while *Albuca bracteata* exhibits its antidiabetic potential through the inhibition of the α-glucosidase enzyme. However, both plants are useful in the amelioration of complications arising from diabetes mellitus and therefore could be a potential source of antidiabetic compounds. Interestingly, the *Albuca setosa* methanol extract showed higher inhibition of α-glucosidase than the aqueous extract used in the traditional medicine and was observed not to be toxic.

References

Ahn, J., Lee, H., Kim, S., & Ha, T. (2007). Resveratrol inhibits TNF-alpha-induced changes of adipokines in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, *364*(4), 972–7. http://doi.org/10.1016/j.bbrc.2007.10.109

Ahrén, B. (2011). The future of incretin-based therapy: novel avenues--novel targets. *Diabetes, Obesity & Metabolism*, *13 Suppl 1*, 158–66. http://doi.org/10.1111/j.1463-1326.2011.01457.x

Al-masri, I. M., Mohammad, M. K., & Tahaa, M. O. (2009). Inhibition of dipeptidyl peptidase IV (DPP IV) is one of the mechanisms explaining the hypoglycemic effect of berberine. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *24*(5), 1061–6. http://doi.org/10.1080/14756360802610761

Anderson, P. J., Critchley, J. A., Chan, J. C., Cockram, C. S., Lee, Z. S., Thomas, G. N., & Tomlinson, B. (2001). Factor analysis of the metabolic syndrome: obesity vs insulin resistance as the central abnormality. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity*, *25*(12), 1782–8. http://doi.org/10.1038/sj.ijo.0801837

Ankita Bachhawat, J., Shihabudeen, M. S., & Thirumurugan, K. (2011). Screening of Fifteen Indian ayurvedic plants for alpha-glucosidase inhibitory activity and enzyme kinetics. *International Journal of Pharmacy and Pharmaceutical Sciences*, *3*(SUPPL. 4), 267–274.

Biddinger, S. B., & Kahn, C. R. (2006). From mice to men: insights into the insulin resistance syndromes. *Annual Review of Physiology*, *68*, 123–58. http://doi.org/10.1146/annurev.physiol.68.040104.124723

Bugianesi, E., McCullough, A. J., & Marchesini, G. (2005). Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology (Baltimore, Md.)*, *42*(5), 987–1000.

Chiang, S. H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., … Saltiel, A. R. (2001). Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature*, *410*(6831), 944–8. http://doi.org/10.1038/35073608

Cho, M., Han, J. H., & You, S. (2011). Inhibitory effects of fucan sulfates on enzymatic hydrolysis of starch. *LWT - Food Science and Technology*, *44*(4), 1164–1171. http://doi.org/10.1016/j.lwt.2010.09.019

Chung, J. Y., Park, J. O., Phyu, H., Dong, Z., & Yang, C. S. (2001). Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (-)-epigallocatechin-3-gallate and theaflavin-3,3'-digallate. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *15*(11), 2022–4. http://doi.org/10.1096/fj.01-0031fje

Conarello, S. L., Li, Z., Ronan, J., Roy, R. S., Zhu, L., Jiang, G., … Zhang, B. B. (2003). Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin

resistance. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6825–30. http://doi.org/10.1073/pnas.0631828100

DeFronzo, R. A. (2004). Pathogenesis of type 2 diabetes mellitus. *The Medical Clinics of North America*, *88*(4), 787–835, ix. http://doi.org/10.1016/j.mcna.2004.04.013

Ekwall, B., Silano, V., Paganuzzi-Stammati, A., & Zucco, F. (1990). Toxicity Tests with Mammalian Cell Cultures. In P. Bourdeau, E. Somers, G. M. Richardson, & J. R. Hickman (Eds.), *Short-term Toxicity Tests for Non-genotoxic Effects* (SCOPE 41, pp. 75–97). John Wiley & Sons Ltd.

García López, P. M., de la Mora, P. G., Wysocka, W., Maiztegui, B., Alzugaray, M. E., Del Zotto, H., & Borelli, M. I. (2004). Quinolizidine alkaloids isolated from Lupinus species enhance insulin secretion. *European Journal of Pharmacology*, *504*(1-2), 139–42. http://doi.org/10.1016/j.ejphar.2004.09.008

Gerich, J. E. (1989). Oral hypoglycemic agents. *The New England Journal of Medicine*, *321*(18), 1231–45. http://doi.org/10.1056/NEJM198911023211805

Giugliano, D., Ceriello, A., & Paolisso, G. (1996). Oxidative stress and diabetic vascular complications. *Diabetes Care*, *19*(3), 257–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8742574

Gomes, A., Vedasiromoni, J. R., Das, M., Sharma, R. M., & Ganguly, D. K. (1995). Anti-hyperglycemic effect of black tea (Camellia sinensis) in rat. *Journal of Ethnopharmacology*, *45*(3), 223–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7623488

Gorelik, S., Ligumsky, M., Kohen, R., & Kanner, J. (2008). A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *22*(1), 41–6. http://doi.org/10.1096/fj.07-9041com

Gruendel, S., Otto, B., Garcia, A. L., Wagner, K., Mueller, C., Weickert, M. O., … Koebnick, C. (2007). Carob pulp preparation rich in insoluble dietary fibre and polyphenols increases plasma glucose and serum insulin responses in combination with a glucose load in humans. *The British Journal of Nutrition*, *98*(1), 101–5. http://doi.org/10.1017/S0007114507701642

Hickman, I. J., Clouston, A. D., Macdonald, G. A., Purdie, D. M., Prins, J. B., Ash, S., … Powell, E. E. (2002). Effect of weight reduction on liver histology and biochemistry in patients with chronic hepatitis C. *Gut*, *51*(1), 89–94. http://doi.org/10.1136/gut.51.1.89

Hii, C. S., & Howell, S. L. (1985). Effects of flavonoids on insulin secretion and 45Ca2+ handling in rat islets of Langerhans. *The Journal of Endocrinology*, *107*(1), 1–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3900267

Holst, J. J., Knop, F. K., Vilsbøll, T., Krarup, T., & Madsbad, S. (2011). Loss of incretin effect is a specific, important, and early characteristic of type 2 diabetes. *Diabetes Care*, *34 Suppl 2*(Supplement_2), S251–7. http://doi.org/10.2337/dc11-s227

Horii, S., Fukase, H., Matsuo, T., Kameda, Y., Asano, N., & Matsui, K. (1986). Synthesis and alpha-D-glucosidase inhibitory activity of N-substituted valiolamine derivatives as potential oral antidiabetic agents. *Journal of Medicinal Chemistry*, *29*(6), 1038–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3519969

Kim, S. O., Kundu, J. K., Shin, Y. K., Park, J.-H., Cho, M.-H., Kim, T.-Y., & Surh, Y.-J. (2005). [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. *Oncogene*, *24*(15), 2558–67. http://doi.org/10.1038/sj.onc.1208446

Kim, W., & Egan, J. M. (2008). The role of incretins in glucose homeostasis and diabetes treatment. *Pharmacological Reviews*, *60*(4), 470–512. http://doi.org/10.1124/pr.108.000604

Koekemoer, T., Grierson, D., van de Venter, M., & Roux, S. (2009). In vitro anti-diabetic properties and cytotoxicity of four plants used to treat diabetes in South Africa. In *Federation of European Biochemical Societies (FEBS) Congress*. Prague.

Kwon, Y.-I., Apostolidis, E., Kim, Y.-C., & Shetty, K. (2007). Health benefits of traditional corn, beans, and pumpkin: in vitro studies for hyperglycemia and hypertension management. *Journal of Medicinal Food*, *10*(2), 266–75. http://doi.org/10.1089/jmf.2006.234

Leclercq, I. A., Da Silva Morais, A., Schroyen, B., Van Hul, N., & Geerts, A. (2007). Insulin resistance in hepatocytes and sinusoidal liver cells: mechanisms and consequences. *Journal of Hepatology*, *47*(1), 142–56. http://doi.org/10.1016/j.jhep.2007.04.002

Maes, M.-B., Dubois, V., Brandt, I., Lambeir, A.-M., Van der Veken, P., Augustyns, K., De Meester, I. (2007). Dipeptidyl peptidase 8/9-like activity in human leukocytes. *Journal of Leukocyte Biology*, *81*(5), 1252–7. http://doi.org/10.1189/jlb.0906546

Nauck, M. A. (2011). Incretin-based therapies for type 2 diabetes mellitus: properties, functions, and clinical implications. *The American Journal of Medicine*, *124*(1 Suppl), S3–18. http://doi.org/10.1016/j.amjmed.2010.11.002

Owen, P. L., Martineau, L. C., Caves, D., Haddad, P. S., Matainaho, T., & Johns, T. (2008). Consumption of guava (Psidium guajava L) and noni (Morinda citrifolia L) may protect betel quid-chewing Papua New Guineans against diabetes. *Asia Pacific Journal of Clinical Nutrition*, *17*(4), 635–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19114402

Park, J.-Y., Kawada, T., Han, I.-S., Kim, B.-S., Goto, T., Takahashi, N., … Yu, R. (2004). Capsaicin inhibits the production of tumor necrosis factor alpha by LPS-stimulated murine macrophages, RAW 264.7: a PPARgamma ligand-like action as a novel mechanism. *FEBS Letters*, *572*(1-3), 266–70. http://doi.org/10.1016/j.febslet.2004.06.084

Reaven, G. M. (1995). Pathophysiology of insulin resistance in human disease. *Physiological Reviews*, *75*(3), 473–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7624391

Singh, R. B., Rastogi, S. S., Singh, N. K., Ghosh, S., Gupta, S., & Niaz, M. A. (1993). Can guava fruit intake decrease blood pressure and blood lipids? *Journal of Human Hypertension*, *7*(1): 33–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8383769

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation.* 106(2): 171- 176

Stephan, M., Radicke, A., Leutloff, S., Schmiedl, A., Pabst, R., von Hörsten, S., … Nave, H. (2011). Dipeptidyl peptidase IV (DPP4)-deficiency attenuates diet-induced obesity in rats: possible implications for the hypothalamic neuropeptidergic system. *Behavioural Brain Research*, *216*(2), 712–8. http://doi.org/10.1016/j.bbr.2010.09.024

Taniguchi, C. M., Emanuelli, B., & Kahn, C. R. (2006). Critical nodes in signalling pathways: insights into insulin action. *Nature Reviews. Molecular Cell Biology*, *7*(2), 85–96. http://doi.org/10.1038/nrm1837

Thiebaud, D., Jacot, E., DeFronzo, R. A., Maeder, E., Jequier, E., & Felber, J. P. (1982). The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. *Diabetes*, *31*(11), 957–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6757014

Thorat, K., Patil, L., Limaye, D., & Kadam, V. (2012). Invitro Models for Antidiabetic Activity Assessment. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, *3*(2), 730–733.

White, M. F. (2002). IRS proteins and the common path to diabetes. *American Journal of Physiology. Endocrinology and Metabolism*, *283*(3), E413–22. http://doi.org/10.1152/ajpendo.00514.2001

Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, *27*(5), 1047–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15111519

Wood, R. J., & O'Neill, E. C. (2012). Resistance Training in Type II Diabetes Mellitus: Impact on Areas of Metabolic Dysfunction in Skeletal Muscle and Potential Impact on Bone. *Journal of Nutrition and Metabolism*, *2012*, 268197. http://doi.org/10.1155/2012/268197

Yang, J., Campitelli, J., Hu, G., Lin, Y., Luo, J., & Xue, C. (2007). Increase in DPP-IV in the intestine, liver and kidney of the rat treated with high fat diet and streptozotocin. *Life Sciences*, *81*(4), 272–9. http://doi.org/10.1016/j.lfs.2007.04.040

Yin, J., Gao, Z., Liu, D., Liu, Z., & Ye, J. (2008). Berberine improves glucose metabolism through induction of glycolysis. *American Journal of Physiology. Endocrinology and Metabolism*, *294*(1), E148–56. http://doi.org/10.1152/ajpendo.00211.2007

CHAPTER 6

INHIBITORYACTIVITY OF *ALBUCA SETOSA* AND *ALBUCA BRACTEATA* BULB EXTRACTS ON CYTOCHROME P450 ENZYMES AND PROTEIN GLYCATION

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INVITRO INHIBITIONOF CYTOCHROME P450 ENZYMES AND PROTEIN GLYCATION BY *ALBUCASETOSA* AND *ALBUCABRACTEATA* BULB EXTRACTS

6.1 Introduction

The human body is a complex system structured to recognise foreign compounds and respond to them in a well-organised manner. Cytochrome enzymes are involved in the metabolism of both exogenous and endogenous compounds in humans. Examples of exogenous compounds are drugs, alcohol, chemicals, and environmental pollutants while some of the endogenous compounds are steroids, prostaglandins, fatty acids and bile acids. These compounds are either detoxified or converted to inactive products that can be readily eliminated from the body through the action of cytochrome P450 present in the liver or intestine (Guengerich, 1992; Isin & Guengerich, 2007; Kerremans, 1996; Pan et al., 2010; Slaughter & Edwards, 1995). The metabolism of these substances occurs in two phases, phase I and phase II. Phase I metabolism involves hydrolysis and redox reactions which are catalysed majorly by cytochrome P450 and flavin-containing monooxygenases before being conjugated into a excretable product in phase II (Afifah, et al., 2010; Hanapi, et al., 2010; Hasler et al., 1999; Parkinson & Ogilvie, 2007; Rendic, 2002)by UDP-glucuronosyltransferase (UGT). Cytochrome P450 also plays active role in other reactions such as the biosynthesis of steroid hormones, oxidation of unsaturated fatty acids to intracellular messengers and stereospecific metabolism of fat-soluble vitamins (Hasler et al., 1999). The detoxification of drugs, toxicants and endogenous compounds of cytochrome enzymes into inert metabolites can be regarded as a protective mechanism, however, cytochrome enzymes can also cause toxicity by the activation of drugs into active metabolites (Kong et al., 2011). Understanding the metabolism of new compounds of plant origin to avoid plant-drug interactions that may lead to the activation of toxic metabolites or alter the rate of drug metabolism has been reported

and that most metabolic drug-drug interactions can be attributed to inhibition or induction of drug-metabolizing cytochrome P450 (CYP or P450) enzymes (Wienkers & Heath, 2005). The inhibition of cytochrome P450 enzymes may therefore result in a decrease in the rate of drug metabolism and consequently the clearance of toxic compounds. Some plants such as garlic (*Allium sativum*), *Gink gobiloba*, grapefruits, ginseng and St. John-wort (*Hypericum perforatum*) have been reported to be unlikely to interfere with CYP2D6 or CYP3A4 (Hanapi et al., 2010; Markowitz et al., 2000; Zhou, et al., 2004). Similarly, co-administration of ginseng extracts with other therapeutic agents (e.g. warfarin, digoxin and phenelzine) may lead to ginseng-drug interactions (GDIs) (Fugh-Berman, 2000; Izzo, 2005).

Since the aqueous extracts of *Albuca bracteata* and *Albuca setosa* are used traditionally, this experiment was designed to mimic the folkloric usage as reported in the ethnobotanical survey (Oyedemi, et al., 2009). The aim of this study is to evaluate the inhibitory effect of *Albuca setosa* and *Albuca bracteata* aqueous extracts on the cytochrome P450.

6.2 Materials and methods

Vivid CYP3A4 red screening kit (Cat. No. P2856) and Vivid CYP2C9 red screening kit (Cat. No. P2859) were obtained from Life Technologies (Carlsbad, CA, United States of America), ketoconazole, sulfaphenazole, acetonitrile, dimethylsulfoxide (DMSO) were obtained from Merck Millipore (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade.

6.2.1 Preparation of plant extracts

The bulbs of *Albuca setosa* and *Albuca bracteata* were thinly sliced and oven-dried at 40^{o}C in the laboratory. The dried bulbs were further ground into powder using an electric blender (Hamilton Beach Commercial Blender type GB27 model HBF 400-CE). 80 g of the powdered plant material was extracted with 1000 ml of distilled water and shaken on a

mechanical shaker (Stuart Scientific Orbital Shaker, SOS1, Essex, UK) for 48 h at room temperature. The extracts were then vacuum filtered through Whatman No.1 filter paper in a vacuum pump. The aqueous filtrate was freeze dried (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 18.6 g and 15.2 g dry extract of *Albuca setosa* and *Albuca bracteata*, respectively.

6.2.2 CYP3A4 and CYP2C9 Inhibition assay

The inhibitory effects of the extracts on the activities of human P450 enzymes (CYP3A4 and CYP2C9) were determined using Vivid P450 screening kits (Vivid OOMR substrate CYP2C9 red and BOMR substrate CYP3A4 red) according to the manufacturer's instructions. Each kit contained P450 reaction buffer, P450 BACULOSOMES reagent, a fluorescent substrate, a fluorescent standard, the regeneration system (Part no. P2878; 333 mM glucose-6-phosphate and 30 U/mL glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 8.0), and 0.5 mL $NADP^+$(Part no.P2879; 10 mM $NADP^+$in 100 mM potassium phosphate, pH 8.0).The regeneration system and $NADP^+$ were stored at -80^oC.

6.2.2.1 Preparation of stock

All the reactant mixtures were allowed to thaw for 10 –15 min. The Vivid substrates were reconstituted using anhydrous acetonitrile while the fluorescent and red standards were reconstituted using DMSO and DMSO/water (1:1) respectively.

6.2.2.2 Kit validation

A standard curve for the Vivid fluorescent standard was prepared. Briefly, 100 µL of different concentrations of the Vivid fluorescent standards (0 – 500nM) were prepared in a 96 – well plate, in duplicate. Fluorescence was measured using a BioTek's Synergy™ 4 Multi-Mode Microplate Reader (Winooski, VT, USA) and used to plot the standard curve. 200 µl of 500 nM Vivid fluorescent standard was added into the first two wells of a 96 – well plate

while 100 µl of the reaction buffer was added into the other wells. 100 µl of the Vivid fluorescent standard was then transferred into the next well containing 100 µl of the reaction buffer making it a two – fold dilution. This dilution step was repeated until the last well contained only the reaction buffer without the Vivid fluorescent standard. The resulting concentrations of the fluorescent standard concentrations were 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0 nM.

6.2.2.3 Reaction procedure

The plant samples, positive inhibition control(ketoconazole and sulfaphenazole for CYP3A4 and CYP2C9 respectively) were prepared at 2.5X of different concentrations (0 – 200mg/ml) in CYP450 reaction buffer. 40 µL of the samples were then added to the desired wells of the black-wall 96-well plates. 50 µL of the master pre-mix was then added to each well and was incubated for 10 min. at room temperature to allow the compounds to react with the P450. After incubation, the reaction was started by adding 10 µL of the respective concentration of the Vivid substrate and $NADP^+$ mixture. This was then incubated for 20 min. at room temperature. The reaction was stopped by the addition of 50 µL of 0.5 M tris base. The fluorescence was measured at 550 nm (Excitation) and 590nm (Emission) using a Synergy Mx monochromator – based multi – mode microplate reader from BioTek. The same procedure was followed for the standards. A solvent control was also prepared; this contained only the reaction buffer instead of the sample. The percentage inhibition was calculated using the equation:

$$\% \text{ inhibition} = \left(1 - \frac{X-B}{A-B}\right) X\ 100$$

Where

X: the fluorescence intensity observed in the presence of the sample

A: the fluorescence intensity observed in the absence of the inhibitor (solvent control)

B: the fluorescence intensity observed in the presence of the positive inhibition control

For the determination of the IC_{50}, different concentrations of the samples were prepared.

6.2.3 Inhibition of protein glycation

The inhibition of protein glycation was carried out using a modified method of Vinson & Howard, (1996). Briefly, 250 µl of bovine serum albumin (BSA, 1.60 mg/ml) prepared in phosphate buffer (50 mM, pH 7.4) was added to 500 µl of phosphate buffer (50 mM, pH 7.4) in eppendorf tubes and pre-incubated at room temperature with plant extracts at various concentrations of 0, 5, 10, 50, 100, 200, 400, 500 mg/ml (distilled water for blank) while aminoguanidine was used as standard. 250 µl of glucose (400 mM) was then added to the reaction mixture and incubated at 60℃ for 30 h. Another set was also prepared as described above but was refrigerated at -20℃ for 30 h instead of been incubated at 60℃. 200 µl of each set was then transferred into black microplates. The fluorescence was read at 360 nm (Excitation); 460 nm (Emission). The experiments were performed in triplicate and the percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \left(\frac{A-B}{A}\right) \times 100$$

Where:

A = absorbance of blank – corresponding absorbance of refrigerated

B = absorbance of sample/standard 60℃– corresponding absorbance of sample/standard at -20℃

6.3 Results

6.3.1 CYP3A4 and CYP2C9 Inhibition

The inhibitory effect of *Albuca setosa* and *Albuca bracteata* aqueous extracts on CYP3A4 is shown in Table 6.1. The percentage inhibition of *Albuca setosa* and *Albuca bracteata* aqueous extracts are shown in Figure 6.2 while the standard curve is shown in Figure 6.12 confirmed that the kit was functional and the assay was linear in the range 0 – 500 nM. *Albuca setosa* aqueous extract has higher percentage inhibition at 200 mg/ml compared to *Albuca bracteata* aqueous extract with 77.08% and 55.76% respectively. The IC_{50} of *Albuca setosa* was observed to be lower with 84.85 than *Albuca bracteata* with 130.93. The inhibition on CYP2C9 was inconclusive with the aqueous extracts of both plants.

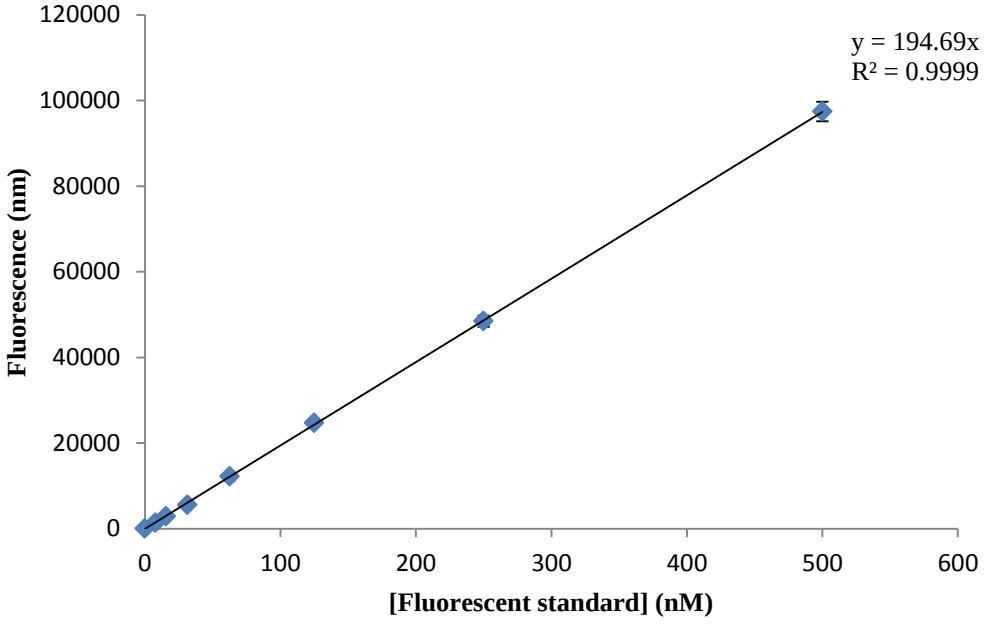

Figure 6.1: Vivid fluorescence standard curve, n = 3

Data expressed as mean ± SD, n= 3. y = 194.69x; R^2 = 0.9999

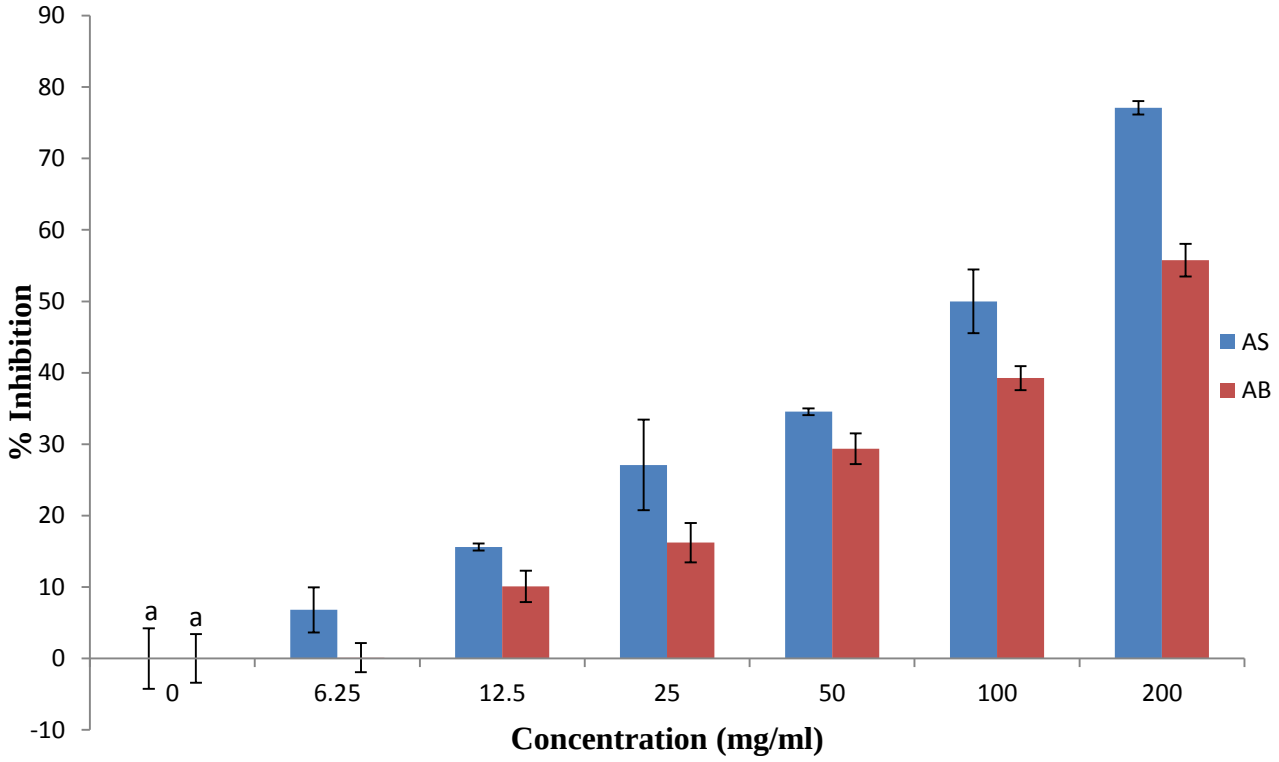

Figure 6.2: Inhibitory effect of *Albuca bracteata* and *Albuca setosa* bulb extracts on CYP3A4

Data expressed as mean ± SD, n= 3.All the bars with the same letters are not significantly different at ($P < 0.01$). AS = *Albuca setosa*; AB= *Albuca bracteata*

Table 6.1: Effect of *Albuca setosa* and *Albuca bracteata* aqueous extracts on CYP3A4

	% Inhibition at 200 mg/ml	**IC_{50}**
Albuca setosa	77.08	84.85
Albuca bracteata	55.76	130.93
Ketoconazole	100	-

6.3.2 Inhibition of protein glycation

The percentage inhibition of *Albuca setosa* and *Albuca bracteata* aqueous extracts on protein glycation is shown in Figure 6.3. The inhibitory effect was determined by the decrease in fluorescence intensity of the reaction mixtures containing the plant extracts as an indication of less AGEs formation from BSA. In comparison to the control, both plant extracts showed significant inhibition on AGEs formation ($P < 0.001$). The inhibitory activity of both plant extracts on AGEs formation was concentration-dependent. The highest concentration of the sample extracts exerts the highest inhibition on AGEs formation. Between the plant extracts, the *Albuca bracteata* showed the least $IC_{50}$of 740.74µg/ml compared to *Albuca setosa* with an IC_{50} 831.94 µg/ml as shown in Table 6.2. However, aminoguanidine has the least IC_{50} with 66.28 µg/ml.

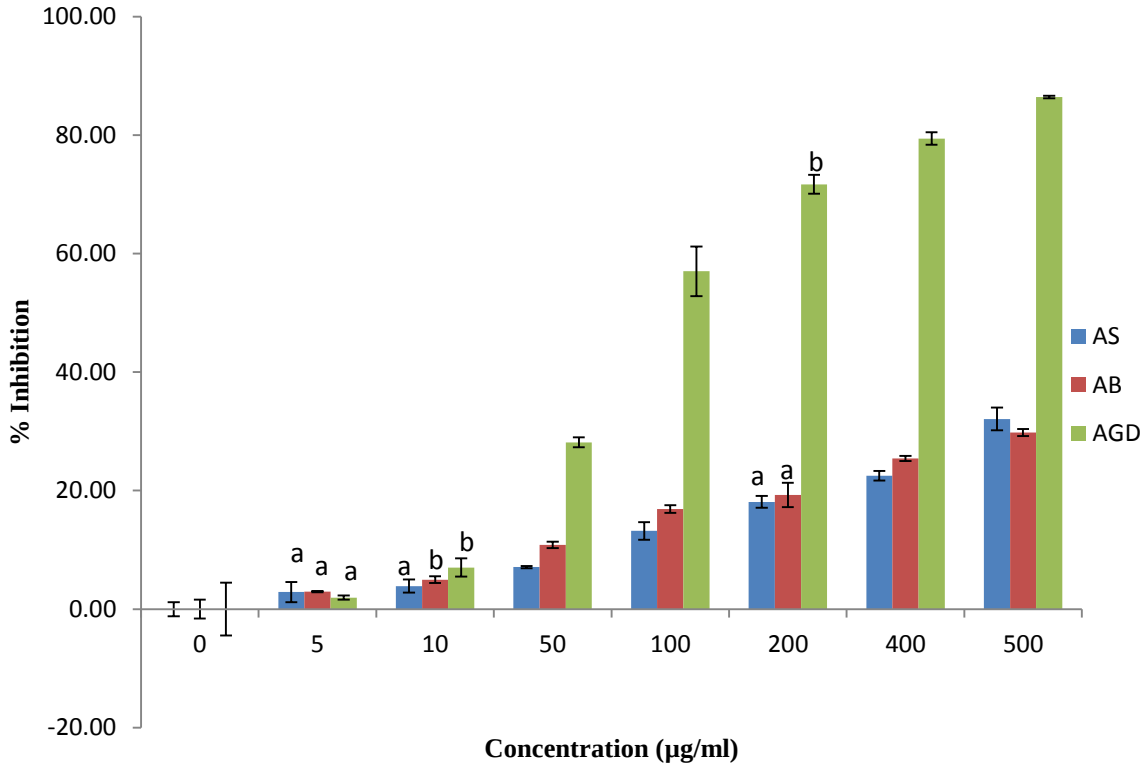

Figure 6.3: Inhibition of protein glycation by *Albuca bracteata* and *Albuca setosa* bulb extracts.

Data expressed as mean ± SD, n= 3. All the bars with the same letters are not significantly different at ($P < 0.01$). AB = *Albuca bracteata;* AS = *Albuca setosa;* AGD = Aminoguanidine

Table 6.2: Effect of *Albuca setosa* and *Albuca bracteata* aqueous extracts on protein glycation. n = 3

	IC50	**R^2**
Albuca bracteata	740.74	0.7148
Albuca setosa	831.94	0.9376
Aminoguanidine	66.28	0.9836

6.4 Discussion

The Cytochrome P450 (CYP450) is mainly involved in the phase 1 of drug metabolism that involves unmasking or inserting of polar functional groups such as –OH, –SH, $–NH_2$ leading to the synthesis of more polar (water soluble) active metabolites that are easily removed from the body. CYP450 enzymes, particularlyCYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4,areresponsible for the bulk of the metabolism of known drugs in humans (Wienkers & Heath, 2005). The CYP450 isozymes possess broad substrate specificity the reason why they are prone to inhibition from different types and structurally diverse drugs metabolites that can be metabolized by the same enzymes (Guengerich, 1997; Hollenberg, 2002; Wienkers & Heath, 2005).Inhibition or induction of these drug metabolising enzymes have been related to drug-drug or herb-drug interactions which can lead to increase or decrease in the concentrations of the drug in the blood (Takagi, et al., 2015). For instance the CYP3A4 isozyme has been reported in several clinically significant drug-drug interactions (Ogu & Maxa, 2000). Complex mixtures of herbal remedies and the presence of more than one active compound, may lead to interaction with conventional drugs if used concomitantly (Ernst, 2000). In addition to this, herbal medicines also follow the modern pharmacological principles. This means that herb-drug interactions are based on the same pharmacokinetic and pharmacodynamic mechanisms as drug-drug interactions (Izzo, et al., 2002). The lower percentage inhibitions of these extracts compared to ketoconazole indicate a low possibility of plant-drug interaction if the mechanism of metabolism is through CYP3A4 enzymes. Although *Albuca bracteata* aqueous extract has lower percentage inhibition compared to *Albuca setosa* aqueous extract, the lower IC_{50} observed in *Albuca setosa* aqueous extract suggests higher activity against CYP3A4 compared to *Albuca bracteata.* This result agrees with the cytotoxicity study of the aqueous extracts of these plants where the aqueous extract of *Albuca setosa* was observed to have higher percentage

cell viability compared to the aqueous extract of *Albuca bracteata* and the inhibition observed could be as a result of the flavonoid content of the plants (Bauer et al., 2002; Havsteen, 2002; Schmiedlin-Ren et al., 1997).Phytochemicals such as alkaloids, phenols, saponins and flavonoids have been specifically reported to be involved in plant-drug interactions (Hanapi et al., 2010). It is, therefore, expected that drugs metabolised through the CYP3A4 to show minimal adverse effects and the toxicity not amplified when concomitantly used with these herbs. This is in line with some reports on medicinal plants reported to have weak inhibition on CYP3A4 (Donovan, 2003; Donovan et al., 2004; Markowitz, et al., 2003). The inconclusiveness of CYP2C9 assay could be as a result of some reasons related to handling and storage. However, Hummel et al. (2006) suggested that several drug-drug interaction probes may be required to determine the inhibitory potential of new compounds that interact with CYP2C9 due to varied results of different probes of CYP2C9.

In the glycation assay, however, the higher percentage inhibition of aminoguanidine observed to be higher than both *Albuca bracteata* and *Albuca setosa* aqueous extracts suggest weak inhibition of protein glycation. Protein modification is one of the consequences of hyperglycemia seen in diabetes mellitus that is caused by the non - enzymatic reaction between reducing sugar and free amino group of proteins to form a Schiff base (Rahimi et al., 2005; Singh et al., 2001). The resultant Schiff base is readily reversible to an early glycosylation compound known as amadori product (AP) that can be further rearranged either by oxidation or reduction to irreversible protein compounds called advanced glycation end-products (AGEs). Protein glycation has been linked to oxidative stress. The glycation of proteins such as superoxide dismutase (SOD) during hyperglycaemia can result in oxidative stress even though the levels of reactive oxygen species (ROS) are within range. In this study, the percentage inhibition of both *Albuca setosa* and *Albuca bracteata* was determined by comparing the fluorescence of the plant extracts with the albumin without any inhibitory

compound. Both plants could inhibit protein glycation especially at higher concentration of 500 μg/ml but were not as efficient as aminoguanidine. The percentage inhibition of *Albuca bracteata* was higher compared to that of *Albuca setosa.* However, there was no significant difference between these two extracts at 5, 10, 200 μg/ml concentrations. The inhibition observed in this study can be attributed to the phenolics present in these plant extracts which has been reported to inhibit protein glycation formation in diabetic patients (Rice-Evans et al., 1995). AGEs may cause damage through the formation of cross – links in collagen that has been linked with vascular stiffness associated with hypertension found in diabetic patients (Cooper, et al., 2001).

Conclusion

The aqueous extracts of these plants showed weak inhibitory effects on both the plant drug interaction and the glycation assay. The weak inhibitory effect on CYP3A4 activity suggests that these plants may not likely inhibit the metabolism of the concurrent use of a given drug primarily dependent on the CYP3A4 pathways for elimination. The weak inhibition of protein glycation suggests that the primary mechanism of the antidiabetic property of these plants is not through the inhibition of protein glycation. However, this can contribute significantly to the antidiabetic properties of the plants.

References

Afifah, S. N., Darah, I., Fariza, S. S., Nordin, M. K. M. J., & Aili, Z. N. (2010). Antimicrobial activity of various extracts of a tropical chlorophyta macroalgae, Halimeda discoidea. *Journal of Applied Sciences*, *10*(23), 3007–3013. http://doi.org/10.3923/jas.2010.3007.3013

Bauer, S., Störmer, E., Kerb, R., Johne, A., Brockmöller, J., & Roots, I. (2002). Differential effects of Saint John's Wort (hypericum perforatum) on the urinary excretion of D-glucaric acid and 6beta-hydroxycortisol in healthy volunteers. *European Journal of Clinical Pharmacology*, *58*(9), 581–5. http://doi.org/10.1007/s00228-002-0527-5

Cooper, M., Bonnet, F., Oldfield, M., & Jandeleit-Dahm, K. (2001). Mechanisms of diabetic vasculopathy: an overview. *American Journal of Hypertension*, *14*(5), 475–486. http://doi.org/10.1016/S0895-7061(00)01323-6

Donovan, J. L. (2003). Siberian Ginseng (Eleutheroccus senticosus) Effects on CYP2D6 and CYP3A4 Activity in Normal Volunteers. *Drug Metabolism and Disposition*, *31*(5), 519–522. http://doi.org/10.1124/dmd.31.5.519

Donovan, J. L., Chavin, K. D., Devane, C. L., Taylor, R. M., Wang, J.-S., Ruan, Y., & Markowitz, J. S. (2004). Green tea (Camellia sinensis) extract does not alter cytochrome p450 3A4 or 2D6 activity in healthy volunteers. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *32*(9), 906–908. http://doi.org/10.1124/dmd.104.000083

Ernst, E. (2000). Herb-drug interactions: potentially important but woefully under-researched. *European Journal of Clinical Pharmacology*, *56*(8), 523–524. http://doi.org/10.1007/s002280000194

Fugh-Berman, A. (2000). Herb-drug interactions. *Lancet*, *355*(9198), 134–8. http://doi.org/10.1016/S0140-6736(99)06457-0

Guengerich, F. P. (1992). Characterization of human cytochrome P450 enzymes. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *6*(2), 745–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1537465

Guengerich, F. P. (1997). Role of cytochrome P450 enzymes in drug-drug interactions. *Advances in Pharmacology*, *43*, 7–35.

Hanapi, N. A., Azizi, J., Ismail, S., & Mansor, S. M. (2010). Evaluation of selected Malaysian medicinal plants on phase I drug metabolizing enzymes, CYP2C9, CYP2D6 and CYP3A4 activities in vitro. *International Journal of Pharmacology*, *6*, 490–495. http://doi.org/10.3923/ijp.2010.494.499

Hasler, J. A., Estabrook, R., Murray, M., Pikuleva, I., Waterman, M., Capdevila, J., … Beaune, P. (1999). Human cytochromes P450. *Molecular Aspects of Medicine*, *20*(1-2), 1–137. http://doi.org/10.1016/S0098-2997(99)00005-9

Havsteen, B. H. The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics*, *96*(2-3): 67–202. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12453566

Hollenberg, P. F. (2002). Characteristics and common properties of inhibitors, inducers, and activators of CYP enzymes. *Drug Metabolism Reviews*, *34*(1-2), 17–35. http://doi.org/10.1081/DMR-120001387

Hummel, M. A., Tracy, T. S., Hutzler, J. M., Wahlstrom, J. L., Zhou, Y., & Rock, D. A. (2006). Influence of fluorescent probe size and cytochrome b5 on drug-drug interactions in CYP2C9. *Journal of Biomolecular Screening*, *11*(3), 303–9. http://doi.org/10.1177/1087057105285612

Isin, E. M., & Guengerich, F. P. (2007). Complex reactions catalyzed by cytochrome P450 enzymes. *Biochimica et Biophysica Acta*, *1770*(3), 314–29. http://doi.org/10.1016/j.bbagen.2006.07.003

Izzo, A. A. (2005). Herb-drug interactions: an overview of the clinical evidence. *Fundamental & Clinical Pharmacology*, *19*(1), 1–16. http://doi.org/10.1111/j.1472-8206.2004.00301.x

Izzo, A. A., Borrelli, F., & Capasso, R. (2002). Herbal medicine: the dangers of drug interaction. *Trends in Pharmacological Sciences*, *23*(8), 358–359. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12377575

Kerremans, A. (1996). Cytochrome P450 isoenzymes—importance for the internist. *The Netherlands Journal of Medicine*, *48*(6), 237–238. http://doi.org/10.1016/0300-2977(96)00002-2

Kong, W. M., Chik, Z., Ramachandra, M., Subramaniam, U., Raja Aziddin, R. E., & Mohamed, Z. (2011). Evaluation of the effects of mitragyna speciosa alkaloid extract on cytochrome P450 enzymes using a high throughput assay. *Molecules*, *16*, 7344–7356. http://doi.org/10.3390/molecules16097344

Markowitz, J. S., DeVane, C. L., Boulton, D. W., Carson, S. W., Nahas, Z., & Risch, S. C. (2000). Effect of St. John's wort (Hypericum perforatum) on cytochrome P-450 2D6 and 3A4 activity in healthy volunteers. *Life Sciences*, *66*(9), PL133–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10698361

Markowitz, J. S., Donovan, J. L., Lindsay DeVane, C., Sipkes, L., & Chavin, K. D. (2003). Multiple-dose administration of Ginkgo biloba did not affect cytochrome P-450 2D6 or 3A4 activity in normal volunteers. *Journal of Clinical Psychopharmacology*, *23*(6), 576–581. http://doi.org/10.1097/01.jcp.0000095340.32154.c6

Ogu, C. C., & Maxa, J. L. (2000). Drug interactions due to cytochrome P450. *Proceedings (Baylor University. Medical Center)*, *13*(4), 421–3. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1312247&tool=pmcentrez&rendertype=abstract

Oyedemi, S. ., Bradley, G., & Afolayan, A. J. (2009). Ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe municipality of South Africa. *Joournal of Medicinal Plants Research*, *3*(12), 1040–1044. Retrieved from http://www.academicjournals.org/JMPR

Pan, Y., Abd-Rashid, B. A., Ismail, Z., Ismail, R., Mak, J. W., Pook, P. C. K., … Ong, C. E. (2010). In vitro modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of Centella asiatica. *Journal of Ethnopharmacology*, *130*(2), 275–83. http://doi.org/10.1016/j.jep.2010.05.002

Parkinson, A., & Ogilvie, B. W. (2007). Biotransformation of xenobiotics. In C. D. Klaassen (Ed.), *Casarett and Doull's Toxicology, the basic science of poisons* (7th ed., pp. 161–304). New York: McGraw-Hill medical publishing division. http://doi.org/10.1036/0071470514

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomedicine & Pharmacotherapy = Biomédecine & Pharmacothérapie*, *59*(7), 365–73. http://doi.org/10.1016/j.biopha.2005.07.002

Rendic, S. (2002). Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metabolism Reviews*, *34*(1-2), 83–448. http://doi.org/10.1081/DMR-120001392

Rice-Evans, C. ., Miller, N. ., Bolwell, P. ., Bramley, P. ., & Pridham, J. . (1995). The relative activities of plant-derived polyphenolic flavonoid. *Free Radical Res.*, *22*, 375–383.

Schmiedlin-Ren, P., Edwards, D. J., Fitzsimmons, M. E., He, K., Lown, K. S., Woster, P. M., … Watkins, P. B. (1997). Mechanisms of enhanced oral availability of CYP3A4 substrates by grapefruit constituents. Decreased enterocyte CYP3A4 concentration and mechanism-based inactivation by furanocoumarins. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *25*(11), 1228–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9351897

Singh, S. N., Vats, P., Suri, S., Shyam, R., Kumria, M. M. L., Ranganathan, S., & Sridharan, K. (2001). Effect of an antidiabetic extract of Catharanthus roseus on enzymic activities in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology*, *76*(3), 269–277. http://doi.org/10.1016/S0378-8741(01)00254-9

Slaughter, R. L., & Edwards, D. J. (1995). Recent advances: the cytochrome P450 enzymes. *The Annals of Pharmacotherapy*, *29*(6), 619–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7663035

Takagi, M., Sakamoto, M., Itoh, T., & Fujiwara, R. (2015). Underlying mechanism of drug–drug interaction between pioglitazone and gemfibrozil: Gemfibrozil acyl-glucuronide is a mechanism-based inhibitor of CYP2C8. *Drug Metabolism and Pharmacokinetics*, *30*(4), 288–294. http://doi.org/10.1016/j.dmpk.2015.05.001

Vinson, J. A., & Howard, T. B. (1996). Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *Journal of*

Nutritional Biochemistry, *7*(12), 659–663. http://doi.org/10.1016/S0955-2863(96)00128-3

Wienkers, L. C., & Heath, T. G. (2005). Predicting in vivo drug interactions from in vitro drug discovery data. *Nature Reviews. Drug Discovery*, *4*(10), 825–33. http://doi.org/10.1038/nrd1851

Zhou, S., Koh, H.-L., Gao, Y., Gong, Z., & Lee, E. J. D. (2004). Herbal bioactivation: the good, the bad and the ugly. *Life Sciences*, *74*(8), 935–68. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14672753

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

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GENERAL DISCUSSION

7.1 General Discussion

Incidents of diabetes mellitus disorder are increasing at an alarming rate in the world (Lam & LeRoith, 2012), and because it is a metabolic disease it is difficult to manage. Diabetes is thought to be the fourth leading cause of death in developed countries, and complications arising from diabetes mellitus through connections with other life threatening diseases such as arteriosclerosis, renal failure, diabetic neuropathy, impotence, stroke, glaucoma and amputations lead to increase in disability, reduced life expectancy, loss of job and enormous health costs for the family and society.

There are several therapeutic targets with diverse mechanisms of actions. For example, TZDs are insulin-sensitizing drugs that target the nuclear hormone receptor, PPARγ, which is expressed mainly in the adipocytes tissue (Reginato & Lazar, 1999). Biguanides inhibit mitochondrial oxidative phosphorylation and respiration and inhibit glucose production in gluconeogenetic tissues and intestinal glucose uptake.

The burden of this disease necessitates constant monitoring of the glyceamic level of an individual and control with appropriate therapy. Different types of diabetes mellitus also require different therapeutic regimens and change of life style which may be difficult to diligently adhere to. Another challenge of diabetes treatments is adverse side effects of the available therapies such as worsening of heart diseases, gastrointestinal upset, lactic acid intoxication, diarrhoea and skin diseases (Boyda et al., 2010; Guénette, et al., 2013; Leung, et al., 2012; Li, et al., 2004; Wild et al., 2004). Phytomedicine, also known as alternative medicine, has become increasingly popular in the management of various diseases and believed by traditional healers to be safe and with little side effects.

The use of medicinal plants in the Eastern Cape province of South Africa has been the first line of traditional treatment but all these plants are yet to be verified and scientifically proven (Dold & Cocks, 2002). There is no documentation regarding safe dosages, and the long-term implications of taking these concoctions have not been established.

7.1.1. Antioxidant activity

Oxidative stress is the imbalance between the production of ROS and its neutralization by antioxidant defence mechanisms of the body and has been associated with the pathogenesis of several degenerative diseases such as Alzheimer's and Parkinson's diseases, cancer, hypertension, cardiovascular disease, diabetes, ischemia-reperfusion injury and aging (Olivares-Corichi, et al., 2005; Tokarz, et al., 2013; Touyz, 2004).The first line of defence by the body is endogenous antioxidants such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), ascorbate (Vitamin C) and catalase (CAT) that act by scavenging potentially damaging free radical moieties against oxidative stress (Harman, 2006).Although the underlying mechanisms of the pathogenesis of diabetes are yet to be well understood, oxidative stress has been shown to play an important role in the progression of the disease (Rösen et al., 2001), which can be explained by the increased oxidative damage to proteins, lipids, DNA and impaired antioxidative defence systems in people with diabetes (Bonnefont-Rousselot, et al., 2000).Oxidative stress has also been linked to pancreatic beta cell degradation, dysfunction and subsequent oxidative DNA damage in PBMCs (Song et al., 2007) and could in part explain the insufficient amount of insulin secreted by diabetic patients. The antioxidant potentials of both *Albuca setosa* and *Albuca bracteata* shown in this study revealed the presence of polyphenols, alkaloids tannins and saponins in these plants. These compounds could explain the high antioxidant and ferric ion-reducing potential of these plants. Previous reports have suggested that polyphenols can ameliorate the hallmark of type 2 diabetes in both preprandial and postprandial hyperglycaemia by inhibiting α-amylase

and α-glucosidase (Anhê et al., 2013; Hanhineva et al., 2010) which correlates with the findings in this study. The antioxidant potential of these plants could be of benefit in the amelioration of complications arising from diabetes.

7.1.2 Anti-inflammatory activity

Inflammation is the response of the immune system toward infection, irritation or a foreign body, initiated by cytokines such as TNF- α, IL-1β and nitric oxide (Kudo et al., 2009). Inflammatory response has been cited as key to the pathogenesis of several diseases. Inflammation plays an essential role in the progression of diabetes, and proinflammatory cytokines, c-reactive protein, TNF- α and IL-6 have been demonstrated to be increasingly expressed in diabetes (Festa et al., 2000; Ford, 1999). As inflammation persists, oxidative stress, hypoxia and advanced glycation end products AGEs/AGE receptor (RAGE) all come together to exacerbate the diabetic condition (Brownlee, 2005; Vincent, et al., 2011). Obesity is a major risk factor for diabetes type 2 and has been reported to induce inflammation by toll–like receptor activation (Kwon et al., 2007).

It has also been proposed that polyphenols can protect against T2D through anti-inflammatory effects (Kostyuk, et al., 2011; Vitaglione et al., 2010) and can therefore reduce insulin resistance caused by inflammation (Kwon et al., 2012). From this study however, the anti-inflammatory activity of the crude extract and saponins isolated from *Albuca bracteata* correlates with previous findings (du Toit et al., 2005). It can therefore be suggested that the anti-inflammatory properties of *Albuca bracteata* could be as a result of the homoisoflavanone compounds present in the bulb. Homoisoflavanone compounds have been isolated from *Albuca bracteata* and have been reported to possess anti-inflammatory properties comparable to indomethacin in dermatitis induced on mouse ears (du Toit et al., 2005; Loggia, et al., 2007). The anti-inflammatory properties of the aqueous extract of

Albuca setosa leaves have been reported to inhibit the carrageenan-induced paw edema and limit the protein denaturation process during acute inflammation (Ndebia et al., 2011). The presence of serotonin, histamine, arachidonic acid and xylene have been suggested as responsible for the anti-inflammatory activity of *Albuca setosa* in aqueous leaf extracts (Ndebia et al., 2011)*Albuca setosa* has been reported to suppress the inflammation induced by serotonin which indicates that it may exhibit its anti-inflammatory action by means of inhibiting the synthesis or by the release of mediators that might be involved in inflammation, such as serotonin, histamine and prostaglandins (Ndebia et al., 2011). Extracts with membrane stabilizing properties are well known for their interfering activity with the early phase of the inflammatory mediators' release, namely, the prevention of phospholipases release that triggers the formation of inflammatory mediators (Aitdafoun et al., 1996).

7.1.3. Cytotoxicity

The toxicity of the *Albuca bracteata* bulb on rabbits has been reported (Watt & Breyer-Brandwijk, 1962) but the bulb has been found not to influence the mutagenic effect of mytomycin C and is not genotoxic in Ames and micronucleus tests. However, it has been reported to be among the most poisonous plants in south Africa (Van Wyk, Van Heerden, & Van Oudtshoorn, 2002; Verschaeve et al., 2004). This may be due to the presence of different steroids or glycosides. In this study we reported that the acetone extract of *Albuca bracteata* showed cytotoxicity in cell lines; this cytotoxicity may be as a result of the reported glycosides and steroids which were soluble in the acetone extracts. There is a paucity of data on the cytotoxicity of *Albuca setosa* but the findings of this study indicated that the aqueous extracts of both *Albuca setosa* and *Albuca bracteata* were not cytotoxic in cell lines used in this study, and instead enhanced the proliferation of these cell lines. The proliferation property could also be of serious concern although the study did not take into account the number of dead cells present in the medium.

7.1.4. Anti-diabetic screening and clinical relevance

Both *Albuca setosa* and *Albuca bracteata* inhibited α-glucosidase but with a mild inhibitory effect on α-amylase. The activity of these plants is beneficial and has an advantage over acarbose which is commonly used. The production of gases and diarrhoea associated with acarbose as a result of the actions of normal flora present in the gut on undigested carbohydrates will be avoided. From this study, however, it is important to note that *Albuca setosa* may possess properties similar to metformin. It is reported that metformin inhibits Complex I of the respiratory chain and consequently increases glycolysis to overcome the restricted ATP synthesis (Viollet et al., 2012). This suggests that the activation of glucose utilization is the primary effect of metformin in the liver, and that the suppression of gluconeogenesis through AMP-activated protein kinase may be secondary. Metformin may also exert some beneficial effect on incretins, as its effect on GLP-1 and the expression of the islet incretin receptor gene through the mechanism that depends on the peroxisome proliferator–activated receptor (PPAR-α) has been reported (Maida, et al., 2011). This suggests that *Albuca setosa* mimics the effect of metformin by enhancing glucose uptake in the liver and could suggest the mechanism of action of this plant, although no activity against DPP-IV was reported. Likewise the effect of *Albuca setosa* was comparable to that of insulin in myocytes. Insulin has been known to regulate a variety of metabolic and mitogenic events that involve the activation of intracellular signalling pathways (Zierath et al., 2002). The glucose uptake reported here may be as a result of the polyphenolic contents of *Albuca setosa.* Antidiabetic effects of polyphenols by improving glucose uptake in muscle and adipocytes have been reported (Hanhineva et al., 2010).The aqueous extracts of both *Albuca setosa* and *Albuca bracteata* these plants showed weak inhibitory effects on both the plant drug interaction and the glycation assay.

7.1.5. Probable mechanism of action

At present, based on previous reports, the mechanisms of action of these plants can be suggested. The results of this study are in correlation with the report of Li et al.(2004) that the multiple combinations of active compounds in plants used in Chinese medicine may not only lower blood glucose but also prevent complications associated with it. This suggests that crude extracts of medicinal plants may exhibit more than one mechanism of action to ameliorate or prevent the complications arising from diabetes. Increasing evidence has revealed different targets that may prevent or prolong the pathogenesis of diabetes (Raghav, et al., 2006). Results from the Chang liver toxicity assay revealed little evidence of change in the glucose consumption and lactate production rates, which suggests that there are no mitochondrial-driven alterations in glucose metabolism in response to the aqueous extract. There was also no significant indication of toxicity with *Albuca setosa*, this means that the positive effect observed on the L6 and 3T3-L1 cells must be independent of toxicity and changes in the mitochondrial metabolism. A possible mechanism for stimulation of glucose uptake may involve the insulin signalling pathway and subsequent Glut4 translocation, which would be absent from HepG2 cells and may be the reason why the positive effect was only observed on the differentiated L6 and 3T3-L1 cells. Insulin is well known as a regulator of a wide variety of metabolic and mitogenic events that involve activation of intracellular signalling pathways (Zierath et al., 2002). The use of *Albuca setosa* with insulin may probably have caused a greater hypoglycaemic effect considering the fact that the aqueous extract of *Albuca setosa* is not toxic to the liver and muscle cells. However, further studies can be done to ascertain this. Another possible alternative mechanism for the stimulation of glucose uptake may be due to PPARγ. The results of the Chang liver toxicity assay (increased MTT and CV relative to the untreated control) indicate that *Albuca setosa* stimulated cell growth. Given that the insulin signalling pathway is linked to cell

proliferation, this may be further evidence that *Albuca setosa* has an impact on the insulin signalling pathway.

The antioxidant properties of these plants can also contribute to the beneficial effect on the users, for example, the inhibition of protein glycation, inflammation and prevention of oxidative stress. The polyphenolic contents may also exert antidiabetic effects by increasing the hepatic glucokinase activity, which augments glucose utilization to promote energy storage in the form of glycogen, and by suppressing hepatic glucose output (i.e. gluconeogenesis) (Hanhineva et al., 2010). The effect of both *Albuca setosa* and *Albuca bracteata* aqueous extracts in the hepatocytes may not be reliable but the glucose uptake observed in the methanolic extract of *Albuca setosa* could be useful. This means that the methanolic extract enhances glucose uptake independent of insulin through GLUT-2 present in the hepatocytes.

The presence of this low affinity glucose transporter (GLUT-2) in the liver allows for rapid equilibration of the intracellular and extracellular glucose pools and implies that glucose uptake and release is essentially controlled by the intracellular glucose concentration.

7.2 Conclusion

From this study, there are no mitochondria driven alterations in glucose metabolism in response to the extracts and no significant indication of toxicity with *Albuca setosa aqueous* extract. This means that the positive effect (glucose uptake) observed in the L6 and 3T3-L1 cells is independent of toxicity and changes in the mitochondria metabolism. *Albuca setosa* and *Albuca bracteata* showed antidiabetic properties but the former showed more potent antidiabetic properties than the latter. *Albuca setosa* has an inhibitory activity on α-glucosidase that is similar to acarbose; its aqueous and methanolic extracts were not toxic in the cell lines investigated. *Albuca setosa* also exhibited glucose uptake in muscle cells but was weak in the liver cells while *Albuca bracteata* showed inhibition against α-glucosidase, but the ability to enhance glucose utilisation in liver and muscle cells was very weak compared to that of *Albuca setosa.*

The antioxidant and anti-inflammatory properties of both plants suggests that these plants can be useful in ameliorating the complications arising from diabetes and therefore justifies the folkloric usage of these plants. The presence of polyphenols, alkaloids and saponins in these plants contributes to the antidiabetic properties and free radical scavenging activities of these plants and may lessen or inhibit the pathogenesis of secondary complications arising from diabetes.

7.3 Recommendation

The possible alternative mechanism for stimulation of glucose uptake as reported here in this study involves the insulin signalling pathway and subsequent GLUT-4 translocation which is absent in HepG2 but present in L6. This also agrees with the cell proliferative effect of *Albuca setosa* on INS-1 cells that is similar to that of insulin and could be beneficial in wound healing and β- cell proliferation. Therefore further studies need to be carried out to ascertain these potentials and to differentiate between PPR-gamma effect and insulin. The actual mechanism of action of these plants in liver, adipocytes, the pancreas and myocytes also needs to be ascertained. Isolation and purification of the active compounds responsible for these antidiabetic properties needs to be done. The findings of this study is considered as the first line of investigations to validate the antidiabetic potentials of these plants, therefore further studies such as *in vivo* investigations will be of great importance to authenticate how these plants will interact in animal models. By doing so, the enzymes and hormones involved in glucose metabolism and insulin signalling pathway could be determined.

References

Aitdafoun, M., Mounier, C., Heymans, F., Binisti, C., Bon, C., & Godfroid, J.-J. (1996). 4-Alkoxybenzamidines as new potent phospholipase A2 inhibitors. *Biochemical Pharmacology*, *51*(6), 737–742. http://doi.org/10.1016/0006-2952(95)02172-8

Anhê, F. F., Desjardins, Y., Pilon, G., Dudonné, S., Genovese, M. I., Lajolo, F. M., & Marette, A. (2013). Polyphenols and type 2 diabetes: A prospective review. *PharmaNutrition*, *1*(4), 105–114. http://doi.org/10.1016/j.phanu.2013.07.004

Bonnefont-Rousselot, D., Bastard, J. P., Jaudon, M. C., & Delattre, J. (2000). Consequences of the diabetic status on the oxidant/antioxidant balance. *Diabetes & Metabolism*, *26*(3), 163–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10880889

Boyda, H. N., Tse, L., Procyshyn, R. M., Wong, D., Wu, T. K. Y., Pang, C. C., & Barr, A. M. (2010). A parametric study of the acute effects of antipsychotic drugs on glucose sensitivity in an animal model. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *34*(6), 945–54. http://doi.org/10.1016/j.pnpbp.2010.04.024

Brownlee, M. (2005). The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*, *54*(6), 1615–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15919781

Dold, A., & Cocks, M. (2002). The trade in medicinal plants in the Eastern Cape Province, South Africa. *South African Journal of Science*, *98*(December), 589–597. Retrieved from http://reference.sabinet.co.za/sa_epublication_article/sajsci_v98_n11_12_a19

Du Toit, K., Elgorashi, E. E., Malan, S. F., Drewes, S. E., van Staden, J., Crouch, N. R., & Mulholland, D. A. (2005). Anti-inflammatory activity and QSAR studies of compounds isolated from Hyacinthaceae species and Tachiadenus longiflorus Griseb. (Gentianaceae). *Bioorganic & Medicinal Chemistry*, *13*(7), 2561–8. http://doi.org/10.1016/j.bmc.2005.01.036

Festa, A., D'Agostino, R., Howard, G., Mykkänen, L., Tracy, R. P., & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation*, *102*(1), 42–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10880413

Ford, E. S. (1999). Body mass index, diabetes, and C-reactive protein among U.S. adults. *Diabetes Care*, *22*(12), 1971–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10587828

Guénette, L., Moisan, J., Breton, M.-C., Sirois, C., & Grégoire, J.-P. (2013). Difficulty adhering to antidiabetic treatment: factors associated with persistence and compliance. *Diabetes & Metabolism*, *39*(3), 250–7. http://doi.org/10.1016/j.diabet.2012.12.005

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H., & Poutanen, K. (2010). Impact of dietary polyphenols on carbohydrate metabolism. *International Journal of Molecular Sciences*, *11*(4), 1365–402. http://doi.org/10.3390/ijms11041365

Harman, D. (2006). Free radical theory of aging: an update: increasing the functional life span. *Annals of the New York Academy of Sciences*, *1067*, 10–21. http://doi.org/10.1196/annals.1354.003

Kostyuk, V. A., Potapovich, A. I., Suhan, T. O., de Luca, C., & Korkina, L. G. (2011). Antioxidant and signal modulation properties of plant polyphenols in controlling vascular inflammation. *European Journal of Pharmacology*, *658*(2-3), 248–56. http://doi.org/10.1016/j.ejphar.2011.02.022

Kudo, H., Takahara, T., Yata, Y., Kawai, K., Zhang, W., & Sugiyama, T. (2009). Lipopolysaccharide triggered TNF-alpha-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. *Journal of Hepatology*, *51*(1), 168–75. http://doi.org/10.1016/j.jhep.2009.02.032

Kwon, Y.-I., Apostolidis, E., Kim, Y.-C., & Shetty, K. (2007). Health benefits of traditional corn, beans, and pumpkin: in vitro studies for hyperglycemia and hypertension management. *Journal of Medicinal Food*, *10*(2), 266–75. http://doi.org/10.1089/jmf.2006.234

Lam, D. W., & LeRoith, D. (2012). The worldwide diabetes epidemic. *Current Opinion in Endocrinology, Diabetes, and Obesity*, *19*(2), 93–6. http://doi.org/10.1097/MED.0b013e328350583a

Leung, J. Y. T., Barr, A. M., Procyshyn, R. M., Honer, W. G., & Pang, C. C. Y. (2012). Cardiovascular side-effects of antipsychotic drugs: the role of the autonomic nervous system. *Pharmacology & Therapeutics*, *135*(2), 113–22. http://doi.org/10.1016/j.pharmthera.2012.04.003

Li, W. L., Zheng, H. C., Bukuru, J., & De Kimpe, N. (2004). Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. *Journal of Ethnopharmacology*, *92*(1), 1–21. http://doi.org/10.1016/j.jep.2003.12.031

Loggia, R., Del Negro, P., Tubaro, A., Barone, G., & Parrilli, M. (2007). Homoisoflavanones as Anti-inflammatory Principles of Muscari comosum. *Planta Medica*, *55*(07), 587–588. http://doi.org/10.1055/s-2006-962120

Maida, A., Lamont, B. J., Cao, X., & Drucker, D. J. (2011). Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-α in mice. *Diabetologia*, *54*(2), 339–49. http://doi.org/10.1007/s00125-010-1937-z

Ndebia, E., Umapathy, E., Nkeh-Chungag BN, I., & Puto, J. (2011). Anti-inflammatory properties of Albuca setosa and its possible mechanism of action. *J. Med. Plant Res.*, *5*(18 4658-4664), 4658–4664. Retrieved from http://www.academicjournals.org/article/article1380730621_Ndebia et al.pdf

Olivares-Corichi, I. M., Ceballos, G., Ortega-Camarillo, C., Guzman-Grenfell, A. M., & Hicks, J. J. (2005). Reactive oxygen species (ROS) induce chemical and structural changes on human insulin in vitro, including alterations in its immunoreactivity. *Frontiers in Bioscience : A Journal and Virtual Library*, *10*, 838–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15569593

Raghav, S. K., Gupta, B., Agrawal, C., Goswami, K., & Das, H. R. (2006). Anti-inflammatory effect of Ruta graveolens L. in murine macrophage cells. *Journal of Ethnopharmacology*, *104*(1-2), 234–9. http://doi.org/10.1016/j.jep.2005.09.008

Reginato, M. J., & Lazar, M. A. (1999). Mechanisms by which Thiazolidinediones Enhance Insulin Action. *Trends in Endocrinology & Metabolism*, *10*(1), 9–13. http://doi.org/10.1016/S1043-2760(98)00110-6

Rösen, P., Nawroth, P. P., King, G., Möller, W., Tritschler, H. J., & Packer, L. (2001). The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes/metabolism Research and Reviews*, *17*(3), 189–212. http://doi.org/10.1002/dmrr.196

Song, F., Jia, W., Yao, Y., Hu, Y., Lei, L., Lin, J., … Liu, L. (2007). Oxidative stress, antioxidant status and DNA damage in patients with impaired glucose regulation and newly diagnosed Type 2 diabetes. *Clinical Science (London, England : 1979)*, *112*(12), 599–606. http://doi.org/10.1042/CS20060323

Tokarz, P., Kaarniranta, K., & Blasiak, J. (2013). Role of antioxidant enzymes and small molecular weight antioxidants in the pathogenesis of age-related macular degeneration (AMD). *Biogerontology*, *14*(5), 461–82. http://doi.org/10.1007/s10522-013-9463-2

Touyz, R. M. (2004). Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Brazilian Journal of Medical and Biological Research = Revista Brasileira de Pesquisas Médicas E Biológicas / Sociedade Brasileira de Biofísica ... [*et al.*]*, *37*(8), 1263–73. http://doi.org//S0100-879X2004000800018

Van Wyk, B.-E., Van Heerden, F. R., & Van Oudtshoorn, B. (2002). *Poisonous Plants of South Africa*. Pretoria, South Africa: Briza Publications.

Verschaeve, L., Kestens, V., Taylor, J. L. S., Elgorashi, E. E., Maes, A., Van Puyvelde, L., … Van Staden, J. (2004). Investigation of the antimutagenic effects of selected South African medicinal plant extracts. *Toxicology in Vitro : An International Journal Published in Association with BIBRA*, *18*(1), 29–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14630059

Vincent, A. M., Callaghan, B. C., Smith, A. L., & Feldman, E. L. (2011). Diabetic neuropathy: cellular mechanisms as therapeutic targets. *Nature Reviews. Neurology*, *7*(10), 573–83. http://doi.org/10.1038/nrneurol.2011.137

Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M., & Andreelli, F. (2012). Cellular and molecular mechanisms of metformin: an overview. *Clinical Science (London, England : 1979)*, *122*(6), 253–70. http://doi.org/10.1042/CS20110386

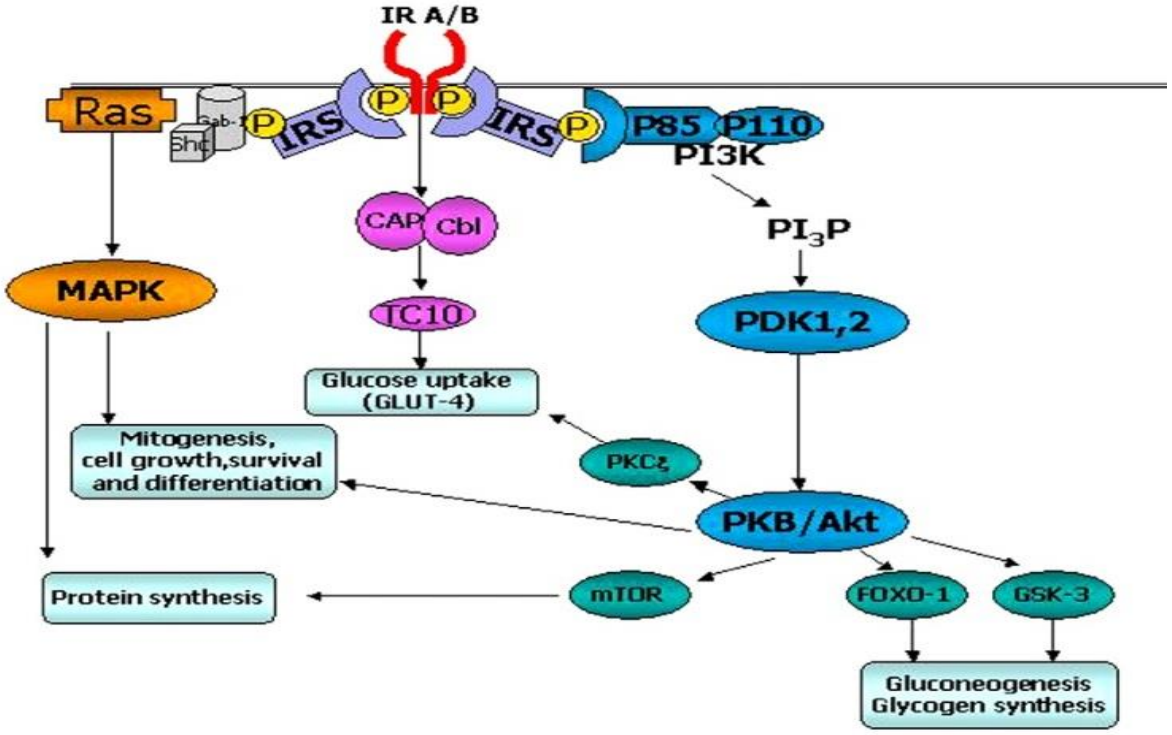
Vitaglione, P., Morisco, F., Mazzone, G., Amoruso, D. C., Ribecco, M. T., Romano, A., … D'Argenio, G. (2010). Coffee reduces liver damage in a rat model of steatohepatitis: the underlying mechanisms and the role of polyphenols and melanoidins. *Hepatology (Baltimore, Md.)*, *52*(5), 1652–61. http://doi.org/10.1002/hep.23902

Watt, J. M., & Breyer-Brandwijk, M. G. (1962). *The medicinal and poisonous plants of Southern and Eastern Africa* (2nd ed.). Edinburgh, Great Brittain: E & S Livingstone Ltd. http://doi.org/10.1177/030098586500200308

Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, *27*(5), 1047–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15111519

Zierath, J. R., &Wallberg-Henriksson, H. A. R. R. I. E. T. (2002). From receptor to effector: insulin signal transduction in skeletal muscle from type II diabetic patients. *Annals of the New York Academy of Sciences.* 967(1), 120-134.

Appendices



Schematic representation of intracellular insulin signaling

(Leclercqet al., 2007)

List of articles for publication from this Thesis

- **Samuel Odeyemi, Anthony Afolayan and Graeme Bradley:** *The use of medicinal plants in the traditional management of diabetes in Eastern Cape South Africa: Considering the pharmacology and toxicology*. (To be submitted to South African Journal of botany).
- **Samuel Odeyemi, Anthony Afolayan and Graeme Bradley:** *In vitro anti-inflammatory and free radical scavenging activities of crude saponins extracted from Albuca bracteata Jacq. Bulb.* (Published in African Journal of Traditional, Complementary and Alternative Medicine)
- **Samuel Odeyemi, Anthony Afolayan and Graeme Bradley:** *Phytochemical analysis and anti-oxidant activities of Albuca bracteata Jacq. Bulb extracts found in the Eastern Cape, South Africa*. (To be submitted to BMC- Complementary and Alternative medicine)
- **Samuel Wale Odeyemi, Trevor Koekemoer, Maryna van de Venter, Anthony Jide Afolayan and Graeme Bradley:** *In vitro anti-diabetic screening of Albuca setosa and Albuca bracteata bulb extracts, commonly used in the Eastern Cape, South Africa* (To be submitted to Journal of ethnopharmacology)

Conference abstracts

- **Samuel Odeyemi, Anthony Afolayan and Graeme Bradley:** *Qualitative and quantitative anti-oxidant activities of dried and fresh bulb extracts of Albuca bracteata Jacq. used in the management of diabetes mellitus in Eastern Cape, South Africa*. 24th Biennial congress of South African Biochemistry and Molecular Biology (SASBMB). Cape Town, South Africa. 6^{th} – 9^{th} July, 2014
- **Samuel Wale Odeyemi, Trevor Koekemoer, Maryna van de Venter, Anthony Jide Afolayan and Graeme Bradley:** *Cytotoxicity of two medicinal plants commonly used in the management of diabetes mellitus in Eastern Cape South Africa using Chang liver cell lines*. 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). Budapest, Hungary. 23^{rd} – 27^{th} August, 2015
- **Samuel Odeyemi, Anthony Afolayan and Graeme Bradley**: *In vitro anti-inflammatory and free radical scavenging activities of crude saponins extracted from Albuca bracteata Jacq. Bulb*. 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). Budapest, Hungary. 23^{rd} – 27^{th} August, 2015