Glycemic control and pancreatic beta cells protective effect of a lanosteryl triterpene from *Protorhus longifolia* in hyperlipidemic and STZ-induced diabetic rats

UNIVERSITY OF ZULULAND

A dissertation submitted in fulfillment of the requirements for the degree of Master of Science in Biochemistry in the Faculty of Science and Agriculture at the University of Zululand, South Africa.

Candidate: **Sihle E. Mabhida**

Student number: 200902688

Supervisor: **Dr. R.A Mosa**

Co-Supervisor(s): **Prof A.R Opoku**

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DECLARATION

I **MABHIDA SIHLE EPHRAIM**, hereby affirm that the work entitled “Glycemic control and pancreatic beta cells protective effect of a lanosteryl triterpene from *Protorhus longifolia* in hyperlipidemic and STZ-induced diabetic rats” is my original work. I have not copied from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

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Signature of Supervisor **Date**

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General Abstract

Prevalence of diabetes mellitus (DM), a chronic metabolic disorder of carbohydrates and lipids that is characterized by persistent hyperglycemia, is rapidly reaching epidemic levels. Hyperglycemia-induced oxidative stress and accelerated inflammatory response may trigger various signaling pathways which further aggravates insult to insulin producing beta cells of the pancreas, thereby worsening the diabetic state. Current treatment regimen for DM comprises self-care and anti-diabetic drugs such as biguanides (metformin), thiazolidinediones and sulfonylureas. Metformin is currently the most commonly used antidiabetic drug. However, due to the lack of compliance to self-care recommendations and some undesirable side effects of metformin, there is an increasing need for alternative therapy. Drugs that cannot only decrease postprandial hyperglycemia, but also maintain pancreatic beta cells integrity for optimal function, could be vital in the management of DM. This study investigated the glycemic control and pancreatic beta cell protective effect of a lanosteryl triterpene (RA-3) from *Protorhus longifolia* stem bark in hyperlipidemic and streptozotocin-induced diabetic rats.

RA-3 was isolated from the chloroform extract of *P. longifolia* using chromatographic techniques and its chemical structure was confirmed based on spectral data analysis. The glycemic control and pancreatic beta cells protective effect of RA-3 were evaluated in the high fat diet (HFD) and streptozotocin (STZ) induced diabetes in rats. The rats were divided into two main groups; rats fed on normal diet and those fed on HFD for 28 days. The animals were then injected with STZ to induce diabetes and RA-3 (100 mg/kg) was orally administered to the diabetic rats daily for 28 days. At the end of the experimental period, animals were fasted overnight and oral glucose tolerance test was performed. The animals were then euthanized and blood, muscle, liver and pancreatic tissues were collected for analysis of biochemical parameters, protein expression and histopathology.

The effect of RA-3 on the pancreatic beta cell structure and function in HFD and STZ-induced diabetes in animals has been reported in Chapter Three. A significant decrease in serum levels of C-peptide and antioxidants (catalase-CAT, superoxide dismutase-SOD), accompanied by reduced glutathione (GSH), were observed in the untreated diabetic controls. Increases in serum levels of malondialdehyde (MDA), interleukin-6 (IL-

6), fasting blood glucose and total cholesterol levels, as well as damaged beta cell structure, were also noted. However, all these parameters were significantly reversed in the diabetic groups treated with RA-3.

Furthermore, the molecular mechanism through which the lanosteryl triterpene improves peripheral insulin signalling in skeletal muscle of STZ-induced diabetic animals was investigated. Treatment of the diabetic animals with the RA-3 showed marked reduction in fasting plasma glucose (67%), serum MDA and IL-6 levels, which were concomitant with the increased serum levels of antioxidants (SOD, CAT) and GSH, in comparison to the untreated diabetic group animals. An improved pancreatic beta cell structure along with increased serum C-peptide levels were also observed in the RA-3 treated diabetic animals. This was evidenced by the observed minimal histopathological changes when compared to the untreated diabetic groups in which major cell damage was evident. A decrease in IRS-1^{Ser307} expression along with increases in p-Akt, p-GSK-3β and GLUT 4 expression were observed in the RA-3 treated group vs untreated diabetic controls. Finally, the molecular basis of RA-3 in hyperlipidemic and STZ-induced type 2 diabetes in animals was studied. Diabetic animals treated with RA-3 effectively enhanced insulin signaling which was observed by a decrease in expression of IRS-1^{ser307} and higher expression levels of p-Akt, p-GSK-3β GLUT 2 and GLUT 4 in comparison to the diabetic control group.

The findings obtained from the current study on RA-3 treatment were similar and highly comparable to those of the metformin treated groups. It is apparent that lanosteryl triterpene, RA-3, improved glycemic control and possesses pancreatic beta cells protective properties. It is concluded that the molecular mechanism by which RA-3 improves glycemic control is based on its enhancement of the insulin signaling pathway, leading to increased recruitment of glucose transporters and thus increased cellular glucose uptake.

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LIST OF ABBREVIATIONS

AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	One way analysis of variance
BGL	Blood glucose levels
BW	Body weight
CAT	Catalase
CDC	Center of disease control and prevention
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
FPG	Fasting plasma glucose
GLUT 1	Glucose transporter 1
GLUT 2	Glucose transporter 2
GLUT 4	Glucose transporter 4
GLUTs	Glucose transporters
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione Reduced
GSK-3β	Glycogen synthase kinase 3 beta
HFD	High Fat Diet
IDF	International Diabetes Federation
IL-1	Interleukine-1
IL-6	Interleukin-6
iNOS	Inducible Nitric oxide synthase
IR	Insulin receptor

IRS-1	Insulin receptor substrate
JNK	Jun N-terminal Kinase
MDA	Malondialdehyde
NF-κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
Nrf2	Nuclear factor (erythroid derived 2)-like 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
SA-NRF	South African National Research Foundation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOCS	Suppressor of cytokines signaling
SOD	Superoxide dismutase
STAT-3	Signal transducer and activator of transcription 3
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes mellitus
TNF-α	Tumor necrosis factor alpha
UCPs	Uncoupling proteins
UZRC	University of Zululand Research Committee
WHO	World Health Organization

CHAPTER ONE:

1.0 INTRODUCTION

Diabetes mellitus (DM) is one of the leading causes of morbidity and mortality worldwide (WHO, 2017), thus a serious global health concern. DM is a group of metabolic disorders of carbohydrates and lipids characterized by chronic hyperglycaemia. It results from insulin secretion deficiency or cellular insulin insensitivity. Type 2 DM (T2DM) is responsible for approximately 90% of all diagnosed cases of diabetes while type 1 DM (T1DM) accounts for 10% (IDF, 2017). Several reports showed that the pancreatic beta cell dysfunction plays a major role in the development and progression of both T1DM and T2DM (Cnop *et al*., 2005; Cernea and Dobreanu, 2013; Swami *et al*., 2017). Although a T1DM state is already distinguished by beta cell dysfunction, T2DM patients also show up to 60% reduction in beta cell mass that is concomitant to decreased insulin secretion (Cerf, 2013). However, some reports have also shown that beta cells in a T2DM state are resilient and can cope with insulin demand despite reduced numbers (Costes *et al*., 2013).

Considering the central role of properly functioning pancreatic beta cells in maintaining glucose homeostasis, maintenance of the integrity of these cells could prove vital in the management of T1DM (WHO, 2017). Beta cell dysfunction may result from tissue insensitivity to glucose that subsequently results in the high accumulation of glucose (hyperglycaemia) and insulin (hyperinsulinaemia) in the blood (Kapitza *et al*., 2017). Both hyperglycaemia and hyperinsulinaemia, commonly observed in T2DM, are correlated with defective tissue insulin sensitivity and altered insulin signaling pathways. Therefore, modulation of insulin signaling in beta cells remains crucial in sustaining glucose homeostasis and improving beta cell function within a diabetic state.

Techniques used in the management of diabetes include regular exercise and proper diet (low fat and carbohydrate content). Various anti-diabetic drugs (meglitinides, α-glucosidase inhibitors, metformin etc.) are currently used by diabetic patients. Majority of the current anti-diabetic agents are only aimed at regulating and reducing blood glucose

to normal levels. The long-term use of these synthetic drugs has undesirable side effects particularly weight gain, bloating, diarrhea and hypoglycaemia. Thus, in trying to improve the health status of diabetic patients, there is a need to search for alternative treatment, preferably of natural origin.

Medicinal plants, as crude extracts or their pure active ingredients, play vital role in the maintenance of human health. A lanosteryl triterpene (RA-3) from stem bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae) has recently been reported to possess *in vivo* hypolipidemic (Machaba *et al*., 2014) and *in vivo* anti-hyperglycaemic (Mosa *et al*., 2015) activities. Based on these results, the present study is aimed at investigating the glycemic control and the possible pancreatic beta cell protective effect of the lanosteryl triterpene in hyperlipidemic and streptozotocin induced diabetic rats.

1.1 Structure of the Dissertation

The dissertation has been arranged in the following chapters

I. Chapter 1—Introduction—the chapter introduces the topic and gives a brief background of the study

II. Chapter 2—Literature Review—reviews literature relevant to the study and comprises of the following subtopics: (a) An overview of diabetes mellitus and beta cells dysfunction, (b) Mechanism involved in diabetes-induced pancreatic beta cell damage, (c) Insulin Signaling, (d) Importance of glucose transporters in reversing pancreatic beta cell damage, (e) Common anti-diabetic models,(f) Current approaches to diabetes treatment, (g) Medicinal plants and their derivatives as new anti-diabetic agents,(h) Triterpenes. The chapter ends with the scope (aim and objectives) of the study

Chapters 3, 4, and 5 report on the results obtained from the study. Each of the chapters is formatted in the form of a journal article and includes the abstract, introduction, materials and methods, results, discussion and conclusion. These chapters are structured in accordance with the specific journal format if the article was submitted to, published and prepared for submission.

III. Chapter 3—A Lanosteryl Triterpene from *Protorhus longifolia* Improves Glucose Tolerance and Pancreatic Beta Cell Ultrastructure in Type 2 Diabetic Rats

IV. Chapter 4—A Lanosteryl Triterpene from *Protorhus longifolia* Augments Insulin Signaling in Skeletal Muscle of Type 1 Diabetic Rats

V. Chapter 5— Molecular Basis of the Anti-hyperglycemic Activity of RA-3 in Hyperlipidemic and STZ-Induced Type 2 Diabetes in Rats.

VI. Chapter 6—General discussion—the results obtained from the study were generally discussed in this chapter in serving the major aim of the study.

VI. Chapter 7—General conclusions—the overall conclusions that could be drawn from the study are presented in this chapter

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CHAPTER TWO:

2.0 LITERATURE REVIEW

Glucose ($C_6H_{12}O_6$), derived from diet or gluconeogenic pathways, is an essential metabolic fuel for all body cells. Notwithstanding the significant physiological importance of this molecule, its blood levels need to be kept within narrow limits (4.5-6 mmol/L) (Rapsang and Shyam, 2014). Glucose homeostasis is hormonally regulated by insulin secreted by the pancreatic islet beta cells of Langerhans. Persistently high blood glucose levels (hyperglycemia) due to insulin deficiency, insulin action or both, characterises diabetes mellitus (DM).

2.1 An overview of diabetes mellitus and beta cells dysfunction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia and is rapidly reaching epidemic levels. World renowned organizations such as World Health Organization (WHO, 2016) and International Diabetes Federation (IDF, 2017) continuously report on the rising incidence of DM and its associated complications worldwide. These organizations have estimated that approximately 422 million people are already living with DM, while it is expected that over 642 million people will be diabetic by 2030. DM is no longer a threat to develop countries only, but to developing countries as well. This could partly be attributed to rapid urbanization accompanied by a shift from consumption of traditional foods towards more of the western diets characterized by high caloric values as well as adoption of sedentary life styles (Szkudelski, 2012).

Diabetes prevalence is constantly increasing due to factors such as autoimmune diseases, genetic background, obesity and physical inactivity (Fracchiolla *et al*., 2011). High blood glucose and lipid levels are the most common features of DM, usually diagnosed in diabetic patients. These factors are known to be the main cause of diabetic complications such as heart disease, kidney damage and retinopathy (Gutierrez, 2013). Literature has also shown that oxidative stress and inflammation play a vital role in the development and complications of DM (Ramachandran *et al*., 2012; Wu and Yan, 2015). The occurrence mechanism of these factors is quite complex involving many cell

signalling pathways such as the insulin signaling pathways. Exploration for compounds that control hyperglycemia, hyperlipidaemia and improve oxidative stress and inflammation is therefore a vital objective in protecting pancreatic beta cell destruction and preventing the DM-associated complications.

2.2 Mechanism involved in diabetes-induced pancreatic beta cell damage

Insulin is well known as one of the most important regulators required for the optimal maintenance of glucose levels in the blood system. This hormone is produced as pre-proinsulin by the rough endoplasmic reticulum of the beta cells. The translocation and cleavage of pre-proinsulin is facilitated by signal recognition particles and signal peptidase to yield proinsulin which is then cleaved in the Golgi apparatus to produce insulin and C-peptide (Shcherbina *et al*., 2017). Both insulin and C-peptide are subsequently released at equimolar concentrations into the circulation.

Insulin secretion is stimulated in response to pancreatic beta cell sensitivity to the increased plasma glucose levels and its metabolism. Thus, normal beta cell integrity is vital for the accurate response to the increased blood glucose levels and demand for insulin (Swisa *et al*., 2017). Lifestyle modifications such as lack of exercise and excessive intake of high fat diet and sugar are known to contribute to the increasing incidence of cellular insulin resistance (Moran *et al.*, 2010). As beta cells fail to meet increased demand for insulin, diabetes develops with hyperglycemia-induced oxidative stress or inflammation (Cernea and Dobreanu, 2013). Both oxidative stress and inflammation further contribute to more destruction and death of beta cells and thus development of diabetes-associated complications such as cardiovascular disorders and nephropathy.

Literature has reported that insulin level measurements alone cannot accurately be used to evaluate the beta cell function because of a huge and fluctuating uptake from the portal circulation into the liver (Shcherbina *et al*., 2017). However, C-peptide is minimally extracted by the liver and thus has the potential to reflect the proper function of beta cells more accurately than insulin (Leighton *et al*., 2017). C-peptide levels, in diabetic individuals, are reported to be elevated as the pancreas works harder to reverse insulin

resistance by increasing insulin secretion (Leighton *et al*., 2017). Thus, understanding of the mechanisms involved in insulin action within pancreatic beta cells remains important in the reversal of impairment of insulin action (insulin resistance).

2.2.1 Oxidative stress and inflammation as mediators of beta cell dysfunction and diabetes complications

Abnormally enhanced inflammation and over production of oxidants are the interrelated factors that are strongly linked with insulin resistance, DM and subsequent acceleration of pancreatic beta cell apoptosis. Inflammation and oxidative stress represent an important biological system for the optimal function of the human body. For example, a raised inflammatory response is required for tissue injury repair while oxidative stress can be important for certain signaling pathways. An appropriate regulation of these mechanisms is crucial to avoid the shift from tissue repair towards the damaging effect of organs such as pancreatic beta cells. Damaging effects of oxidative stress and aggravated inflammation can be induced by chronic hyperglycaemia and have been shown to trigger various signaling pathways resulting in deteriorated beta cell dysfunction (Cernea and Dobreanu, 2013).

Oxidative stress markers (e.g. malondialdehyde, MDA) are a very common feature in diabetic patients (Singh and Singh, 2017). Normally the body fights against oxidants using the endogenous antioxidant system which comprises non-enzymatic (GSH) and enzymatic antioxidants (CAT, SOD) (Figure 2.1). Hyperglycaemia in DM impairs glucose metabolic pathways by depleting the antioxidant defence and causing oxidative stress which further damages body cells (Drews *et al*., 2010). The ability of body cells to survive cellular stress is mostly reliant on the effectiveness of the antioxidant defense system (Drummond *et al*., 2017). Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcriptional factor that is normally activated in response to oxidative stress in many cell types, is attracting a lot of interest as it is targeted by various drug agents to protect against metabolic disease associated pancreatic beta cell damage (Arowojolu *et al*., 2017). Some of the downstream antioxidants that are targeted by Nrf2 include CAT, SOD, uncoupling proteins (UCPs) and glutathione reductase (Drummond *et al*., 2017).

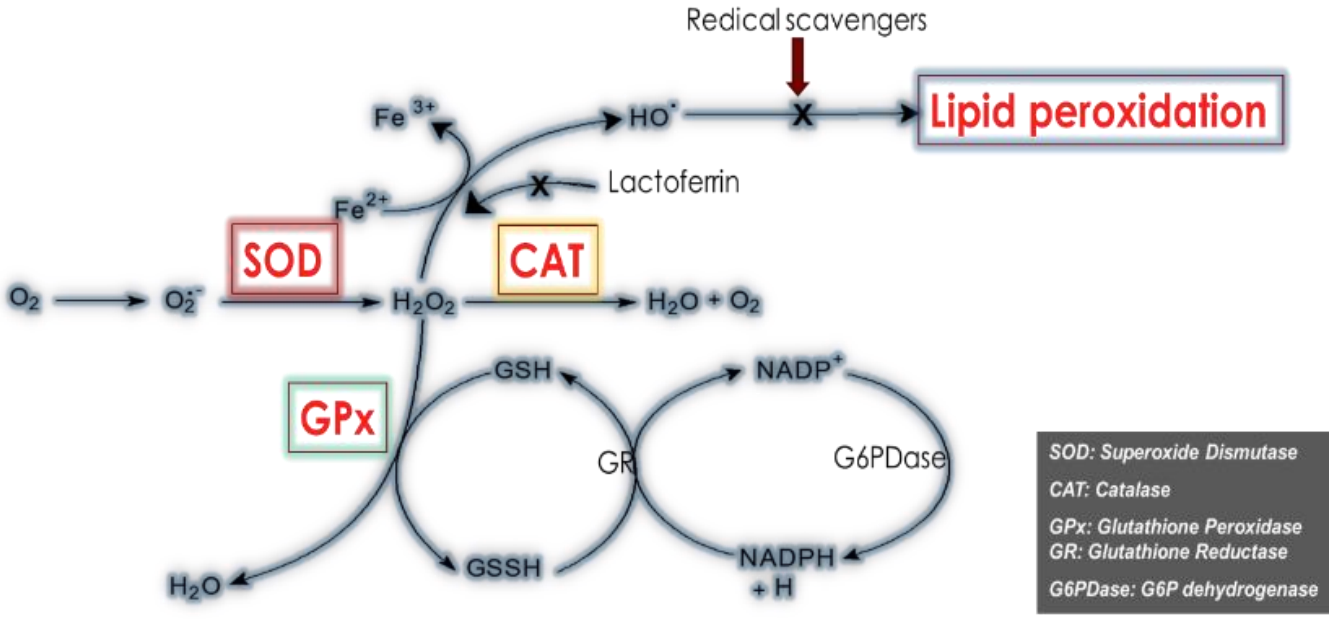

Figure 2.1: Schematic presentation showing antioxidant defence mechanism against damage by reactive oxygen species. SOD, CAT and GPx reduce and eliminate many damaging oxygen species (Zhang *et al*., 2013).

Although the body can improve intracellular antioxidant defense systems through activation of Nrf2, additional protective mechanisms such as modulation of a pro-inflammatory response is crucial to prevent or ameliorate tissue damage in diabetic patients. Pro-inflammatory cytokines [interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1)] are known to be closely linked with pancreatic beta cell destruction through the stimulation of the nuclear factor-kappa B (NF-κB) pathway (Figure 2.2). These inflammatory cytokines infiltrate vascular tissue and inhibit function and repair of the beta cells (Zeng *et al*., 2015).

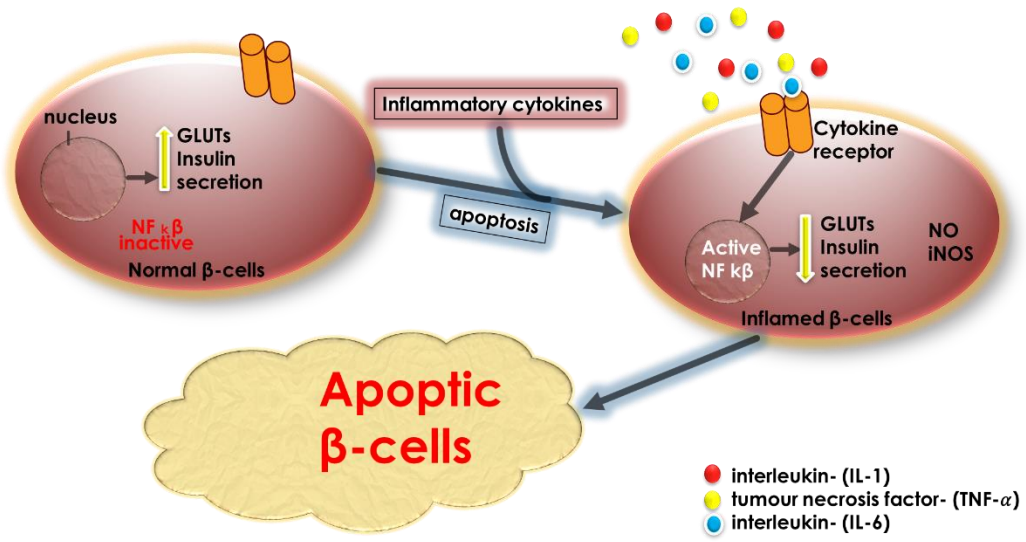

Figure 2.2: Schematic presentation showing the role of inflammatory system in pancreatic beta cell damage. In a disease state, cytokines such as IL-6 and IL-1 can upregulate the nitric oxide (NO) synthase expression and NO production, which damages the beta cell integrity and affects insulin secretion (Wang, 2010).

Elevated serum IL-6 levels have been linked with the impairment of insulin action in the skeletal muscles and the liver, and the increased risks of T2DM (Wang *et al.,* 2013). The high production of cytokines upregulates the expression of nitric oxide synthase leading to nitric oxide production, which impairs insulin action, causing T2DM and activating NF-$_k$B which contributes significantly to beta-cell dysfunction and death (Wang, 2010). The body system is capable of defending itself from inflammatory cytokines and oxidative stress. However when the body is overwhelmed by these factors, it necessitates the use of external sources such as drugs with a good anti-inflammatory and antioxidant activity. Hence, further therapeutic interventions and prevention should be modulated towards targeting oxidative stress-inflammatory cytokine signaling while boosting the metabolic pathways that promote enhanced cellular bioenergetics (Wang *et al.,* 2013). Experimental evidence has shown that an impaired beta cell function has been associated with increased oxidative stress, which is one of the major risk factors associated with disease progression (Karam, 2017).

2.2.2 Insulin Signaling

Insulin can achieve its task of controlling blood glucose levels in the body by using several systems such as intracellular signaling network and glycogenesis which are known to amplify signals. The main function of insulin is to reverse hyperglycemia through the uptake of glucose, conversion of glucose to glycogen and prevention of gluconeogenesis (Rui, 2014). There are different signaling pathways (IR-IRS-PI3K-Akt) present inside the cell which are responsible for controlling these different tasks (Figure 2.3). Signaling proteins are reported to play key roles in the cell because once activated, they activate more proteins to convert the blood glucose into glycogen and prevent gluconeogenesis (Boucher *et al.*, 2014).

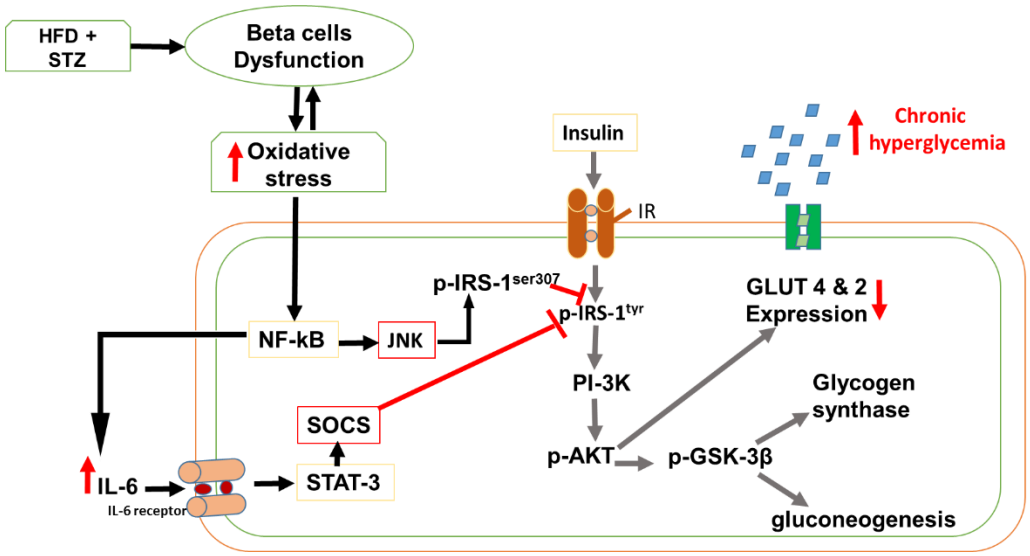

Figure 2.3: Overview of insulin regulation of major metabolic responses in the cells. Under physiological condition, insulin binds its receptor to enhance the positive tyrosine IRS1 phosphorylation promoting the activation of PI3K which in turn phosphorylates and activates p-Akt. High expression of Akt activates the translocation of glucose transporter 4 increasing glucose uptake by the cell and also further phosphorylates and inactivates GSK-3β promoting the activated glycogen synthase (GS) and later to an acceleration of glycogen synthesis (Saxton and Sabatini, 2017).

Generally, insulin-dependent glucose uptake can reverse insulin resistance and improve tissue function by modulating the intracellular signaling network. Studies (Manning and Toker, 2017; Saxton and Sabatini, 2017; Ruan and Kazlauskas, 2011) have demonstrated that a signaling pathway plays a dominant part in the metabolic actions of insulin in various cell types (Figure 2.3). It is well-established by Mukundwa *et al.,* (2016)

that optimal regulation of insulin signaling is crucial for the amelioration of DM and its associated complications, including pancreatic beta cell failure.

Under pathophysiological conditions, oxidative stress and pro-inflammatory cytokines (IL-6) have been reported to enhance the negative phosphorylation of IRS-1 serine (307) causing insulin resistance (Solinas and Becattini, 2017). IL-6 is reported to increase the suppression of cytokine signaling (SOCS) which interferes with insulin signaling by degrading insulin receptor substrate (IRS-1) (Solinas and Becattini, 2017). NF-κB also activates the expression of serine/threonine kinase such as jun n-terminal kinase (JNK) which is known to phosphorylate IRS-1 serine (307), inhibiting the insulin signaling pathway. The latter results in decreased expression of some insulin signaling proteins such as the serine kinases (PI3K/Akt/GSK) which are mediated in the insulin signaling cascade. Decreased expression of PI3K/Akt has been reported to be associated with poor recruitment of glucose transporter 4 (GLUT 4), preventing glucose from entering the cells and promoting gluconeogenesis (Rains and Jain, 2011). Thus, promotion of glucose uptake within many cell types including the pancreas, through optimal modulation of glucose transporters, remains essential for reversal of insulin resistance.

2.2.3 Importance of glucose transporters in reversing pancreatic beta cell damage

Glucose transporters (GLUTs) are a group of proteins located on the membranes or transport vesicles within cells, and these proteins are known to play an important part in glucose transport across the plasma membrane, either from cytoplasm to the membrane or in a reverse order (Shi, 2013). Plasma glucose is assimilated into cells when it binds to GLUTs which trigger a conformational change allowing glucose to be transported across the plasma membrane (Mueckler and Thorens, 2013). In a physiological state, the hormone insulin is known to stimulate cellular glucose uptake by facilitating the recruitment of cellular GLUTs (e.g. GLUT 1,GLUT 2,GLUT 4), and this process is established differently in different tissues and possesses varied biochemical properties (Mueckler and Thorens, 2013). GLUT 1 can be found in almost all cells and it regulates basal glucose and ensures stable entry of glucose into cells (Meireles *et al.*, 2017). GLUT 2 is a low-affinity glucose transporter expressed commonly in pancreatic beta cells, liver

and kidneys where it mediates serious features of glucose homeostasis. GLUT 2 expression is known to have a significant role in the ability of beta cells to react to increasing glucose levels by secreting insulin (Mather and Pollock, 2011).

GLUT 4 is reported to be mostly expressed in fat and skeletal muscle tissues (Shao and Tian 2015). Under pathophysiological conditions, loss or down-regulation of GLUT 2 and GLUT 4 in the liver and skeletal muscle tissues can result in persistent hyperglycemia (Alvim *et al.,* 2015). GLUT 4 is down-regulated in DM patient (Esteves *et al*., 2017). Literature has indicated that skeletal muscles are one of the main target tissues of insulin and account for 70-80% of body glucose metabolism (Kowalski and Bruce, 2014). Thus, it is significant to study the mechanism of the cells insensitivity to insulin. Moreover, improvement of insulin resistance is accompanied by up-regulation of GLUT 4 expression in skeletal muscles (Esteves *et al*., 2017). Therefore, it is vital to improve the expression GLUT 2 and GLUT 4 in order to improve insulin secretion and insulin resistance.

2.3 Common animal models of diabetes

Various *in vivo* animal models, such as rats with DM induced by streptozotocin (STZ) and alloxan, are commonly used in the search for new anti-diabetic agents. However, the STZ induced diabetes model is currently preferred over alloxan. STZ is known to cause partial destruction of beta cells and lower mortality rate in experimental diabetic animals as compared to alloxan that causes a complete destruction of the beta cells and consequent high mortality (King and Bowe, 2016). The mechanism of STZ has been reported to be through inhibition of aconitase activity, leading to DNA impairment (Eleazu *et al*., 2013). A high dose (60 mg/kg b.w) of STZ is commonly used to induce T1DM in rats (Ghorbani *et al.,* 2014). On the other hand, high fat diet (HFD) fed rats followed by a lower dose of STZ (30 mg/kg b.w), is a model commonly used to induce T2DM (Santos *et al*., 2012). This model is considered to be a clinically ideal alternative animal model for T2DM anti-diabetic drug evaluation (Suman *et al*., 2016). The oral glucose tolerance test is commonly used to determine the effectiveness of anti-diabetic agents. It detects how quickly the glucose is taken up from the blood into the cells (Figure 2.4), an indication of insulin sensitivity. Normally, in healthy individuals, an oral glucose load triggers a rise in

blood glucose levels which is quickly normalized (within 2 h) by insulin action. In diabetic individuals, due to either insulin deficiency or resistance, an oral glucose load abnormally increases blood glucose levels higher than the body's ability to normalise the glucose levels due to impaired insulin action. Thus, blood glucose levels remain abnormally high for a relatively longer period (>2h).

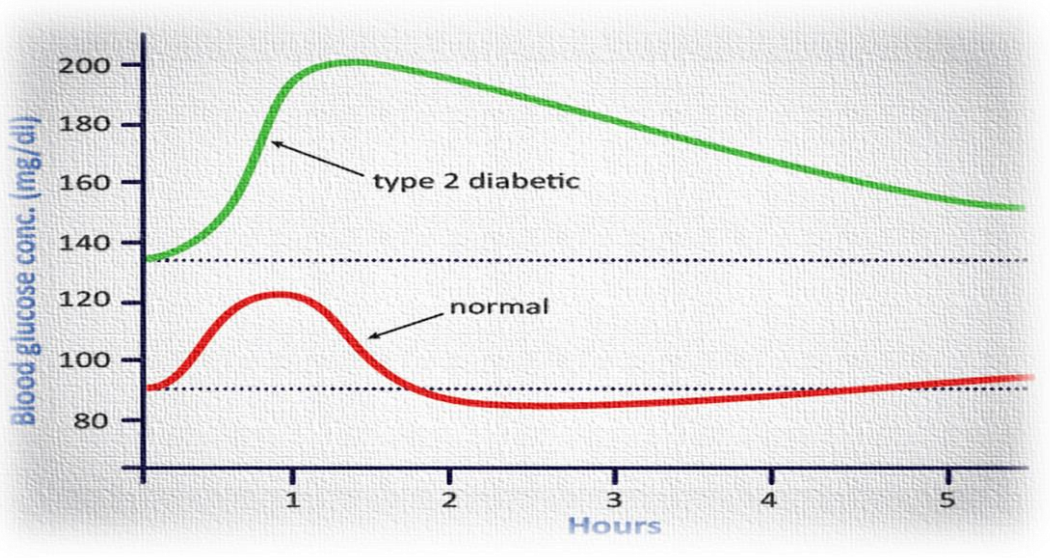

Figure 2.4: Schematic diagram showing oral glucose tolerance test (Nhanes, 2007).

2.4 Current approaches to diabetes treatment

The treatment of DM is still a big challenge worldwide. Regular exercise and maintaining a healthy diet are considered vital for those suffering from DM (Ly *et al.,* 2014). Physical exercise with a decrease in abdominal fat content in T2DM patients improves insulin sensitivity (White *et al.,* 2016). T1DM is managed with only insulin therapy. A number of conventional drugs available in the market but none of them are without side effect (weight gain, bloating, diarrhea and hypoglycaemia). These conventional drugs are also expensive and not affordable for the patients in developing nations. In table 2.1, a number of commonly used blood lowering therapies with a known effect in improving beta cell function such as metformin and glipizide are discussed. However, although such therapies can protect against hyperglycaemia-induced pancreatic beta cell damage, the increasing prevalence of DM warrants further investigation into alternative and/or

combinational approaches to protect pancreatic beta cells and contribute to prolonging the lives of diabetic patients (Thrasher, 2017).

Table 2.1: Some of the commonly used oral hypoglycemic and pancreatic beta cell therapies and their proposed mechanism of action

Drug (s)	Mechanism of Action (s)	Proposed protective effect against beta cell damage	Reference (s)
Metformin (Biguanide class)	Activates AMPK pathway	Protective effect of pancreatic beta cell Increase the production of hepatic glucose Increase insulin sensitivity and glucose uptake Cardioprotective effect	(Foretz *et al*., 2010) (An and He 2016) (Messaoudi *et al*., 2011)
Alogliptin (Dipeptidyl-peptidase-4 (DPP-4) inhibitors)	Inhibits DPP-4 activity	Stimulate the pancreatic insulin production Decrease glucagon secretion Increase Satiety	(Thrasher, 2017)
Glipizide (Sulfonylureas class)	Blocks $_K$ATP channels	Stimulate the pancreatic insulin production Decrease glucagon secretion Relatively higher HbA1c efficacy	(Thrasher, 2017)
Dulaglutide (Glucagon-like peptide-1 (GLP-1) agonist class)	Activates GLP-1 Receptors	Stimulate the pancreatic insulin production Decrease glucagon secretion Slows gastric emptying	(Papatheodorou *et al*., 2016)
Pioglitazone (Thiazolidinediones class)	Modulates nuclear transcription factors PPAR-y	Increase insulin sensitivity Reduce the production of glucose by the liver	(Hahr and Molitch, 2015)
Nateglinide (Meglitinides)	Blocks $_K$ATP channels	Stimulate the pancreas to produce more insulin	(Thrasher, 2017)

Metformin is currently one of the common and leading antidiabetic drugs. Some of the well-known mechanisms of action associated with the hypoglycaemic effect of metformin include enhancing insulin sensitivity in peripheral tissues while stimulating cellular glucose uptake and reducing hepatic gluconeogenesis via an AMPK-independent pathway. Metformin has also been suggested to have high antioxidant activity as demonstrated in erythrocytes of diabetic and normal rats (Rojas and Gomes 2013). However, this drug is known to cause loss of appetite and promote weight loss (Van Gaal and Scheen, 2015). Although hypoglycaemic drugs such as metformin remain active in

attenuating most of diabetes associated complications, the major concern has been the continued rise in diabetic cases. Thus, the increasing prevalence of DM warrants further investigation into alternative and/or combinational approaches, for example the protection of pancreatic beta cell and other vital organs such as the heart contribute to prolonging the lives of diabetic patients. Recent evidence has shown that plants and plant derived products such as polyphenols and triterpenes are generating a lot of interest for their medicinal benefits (Amuka *et al.,* 2017).

2.4.1 Medicinal plants and their derivatives as potential anti-diabetic agents

The use of medicinal plants to cure various ailments, including human metabolic disorders, has been a preference since the earliest of times. There is still a continuous rising interest in the use of medicinal plants either in their crude or pure form to fight diseases. A large percentage (80%) of the rural population relies mainly on medicinal plant-based traditional healing to meet their primary health care needs (Amuka *et al.,* 2017). The curative properties of these plants are attributed to their phytochemical (secondary metabolites) composition (e.g. terpenoids, saponins, tannins, flavonoids and alkaloids). Furthermore, about 25 to 50% of pharmaceuticals currently in clinical use have been derived from plants (Amuka *et al.,* 2017).

Various experimental evidences support the useful effects of plant therapy in managing DM (Moradabadi *et al*., 2013). Medicinal plants and their derivatives exert anti-hyperglycemic activity through different mechanisms. They act as insulin-like substances (Smirin *et al.*, 2010), increase glucose uptake by tissues (Sharma and Rhyu, 2014), insulinase (Nosiri *et al*., 2016), protect and regenerate pancreatic beta cells (Hosseini *et al*., 2015). Unlike the current synthetic anti-diabetic drugs which are single target, medicinal plants and their derivatives are multi-targeted. The root extracts of *Costus speciosus* significantly augment insulin, C-peptide, glucose tolerance levels, and protect liver and pancreatic tissues from diabetic damage in diabetic mice (Maiyah *et al*., 2016). *Lespedeza davurica* (Laxm.) Schindl methanol extract has been reported to have beta-cell protective effects against cytokine-induced beta-cell damage (Sharma and Rhyu, 2014).

Experimental evidence has demonstrated the potential effect of aqueous and methanolic extracts of *Anastatica hierochuntica* against hyperlipidaemia and hyperglycaemia in diabetic animals it also improved and increased beta cells mass in diabetic rats (Daisy and Saipriya, 2012). A number of medicinal plants extracts such as *Abroma augusta* (Hussain *et al*., 2013), *Mangiferin* (Wang *et al.*, 2012) and *Cassia alata* (Eliakim-Ikechukwu *et al*., 2013) have been reported to protect and regenerate beta cells. Abdul-Hamid and Moustafa (2013) documented that curcumin isolated from *Curcuma longa* has a protective effect in pancreatic islets**,** various other health-benefiting properties of this compound have been reported. Such properties include anti-diabetic (Zhang *et al.,* 2013), anti-inflammatory (Huang *et al*., 2016) and hypolipidemic effects (Swami *et al*., 2017). WHO (2007) has recommended further evaluation of plants traditionally used for the treatment of DM.

Protorhus longifolia (Figure 2.5) is one of the plants commonly used in traditional medicine for the treatment of various ailments, including blood-clotting related diseases (Mosa *et al.,* 2011). Several triterpenes with significant biological activities including anti-diabetic properties have been isolated from the stem bark of *P. longifolia.*

2.4.1.1 Protorhus longifolia (Benrh.) Engl.

Figure 2.5: A picture of *Protorhus longifolia* showing its bark and leaves.

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) is an ever-green plant with some yellow or red coloured leaves. This plant, known as unhlangothi in Zulu and red beech in English, is mostly found in Southern Africa (Mpumalanga, Eastern Cape, Northern Province and KwaZulu Natal). The traditional healers from KwaZulu Natal recommend the use of the stem bark of *Protorhus longifolia* in the treatment of several diseases such as heartwater and diarrhoea in cows (Dold and Cocks, 2001). Screening of phytochemical composition of the stem bark of *P. longifolia* has shown the presence of various phytochemicals such as terpenoids, saponins, tannins, flavonoids and alkaloids (Chalo *et al*., 2017). The stem bark of *Protorhus longifolia* has been reported to have 7% tannins and 10.2–18% tanning material (Mosa *et al*., 2011). Lupane triterpenoids and lanostane-type of triterpenes have been isolated from the leaves and stem bark of this plant, respectively (Ntuli, 2006; Mosa *et al*., 2011). Suleiman *et al* (2010) have reported the antimicrobial activity of the leaf extracts of this plant

2.4.1.2 Triterpenes as targets for new antidiabetic drugs development

Triterpenes, a class of chemical compounds unique for containing three terpene units, have demonstrated strong potential for the treatment of diabetes associated complications such as retinopathy (Thandavarayan *et al*., 2009), neuropathy (Kashyap *et al*., 2015), cardiomyopathy (Tan *et al*., 2015) and beta cell dysfunction (Castro *et al*., 2015). Several *in vitro* and *in vivo* studies have demonstrated the potential of natural triterpenoids to protect and regenerate pancreatic islets (Castellano *et al*., 2013). This class of compounds is also known for its inhibitory activity on alpha glucosidase and alpha amylase (Dong *et al*., 2012), protein tyrosine phosphatase 1B (Thareja *et al.* 2012) and inhibition of glycogen phosphorylase (Tahrani *et al*., 2011; Castellano *et al.,* 2013).

Santos *et al*, (2012) established that α, β-amyrin, improves hyperglycemia and dyslipidemia and reduces the atherogenic risk factor in diabetic mice. De-Almeida *et al.* (2015) demonstrated the anti-inflammatory effect of triterpenes (α, β-amyrin, acetylated α, β-amyrin, α, β-amyrone,) by inhibiting the production of inflammation markers (TNF-α, IL-6, IL-10) in murine J774 cells stimulated by lipopolysaccharide. The anti-diabetic properties of a lupane-type triterpene, bacosine, isolated from the herb of *Bacopa*

monnieri (L.) Wettst., has been linked to its antioxidant properties. Bacosine reduced the level of malondialdehyde while increasing the levels of antioxidants in diabetic animals (Ghosh *et al.,* 2011).

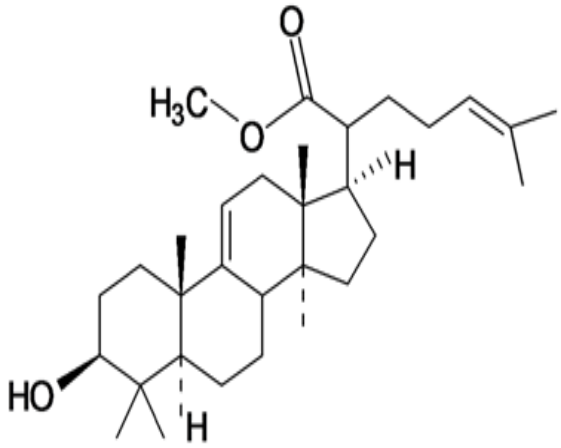

Figure 2 6: Chemical structure of **RA-3**, a lanosteryl triterpene.

Experimental data from our laboratory have demonstrated that RA-3 (Figure 2.6) possesses cardioprotective (Mosa *et al*., 2016), anti-hyperlipidaemic (Machaba *et al.*, 2014) and anti-hyperglycaemic activities (Mosa *et al.*, 2015). The anti-hyperglycemic potential of the compound was demonstrated in STZ-induced type 1 DM following only fourteen days treatment of the diabetic rats. Since T2DM is commonly characterized by elevated blood glucose and lipids levels, the potential dual effect of the triterpene (RA-3) in lowering both blood glucose and lipids could prove ideal in diabetes management. The current study focused on evaluating the long-term effect of RA-3 (isolated from *P longifolia*) on glycemic control and pancreatic beta cells integrity in diabetic rats.

2.5 Aim

This study was designed to evaluate the glycemic control and the pancreatic beta cell protective effect of the lanosteryl triterpene (RA3) from *Protorhus longifolia* stem bark in hyperlipidemic and STZ-induced diabetic rats.

2.5.1 Objectives

i. To isolate and characterize the triterpene from chloroform extract of the plant material
ii. To investigate the anti-hyperglycaemic effect of the RA-3 in HFD-STZ-induced diabetes in rats
iii. To study the effect of the isolated triterpene in the expression (Western blot analysis) of some proteins of interest in diabetes pathogenesis
iv. To evaluate histological changes of pancreatic tissues from experimental animals post the triterpene treatment

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CHAPTER THREE

The effect of the isolated triterpene on the structure and function of pancreatic cells in type 2 diabetic rats is reported here. The chapter, as presented here, has been published in **Molecules 2017**, 22, 1252; doi: 10.3390/molecules22081252. The details of the preparation of the reagents, including the HFD (Appendix A) and the details of the methods, including the isolation and characterization of the RA3 (Appendix B) appear as supplementary material at the end of the dissertation

My contribution:

Performed experiments

Analyzed data and interpretation

Wrote the paper

CHAPTER THREE: A Lanosteryl Triterpene from *Protorhus longifolia* Improves Glucose Tolerance and Pancreatic Beta Cell Ultrastructure in Type 2 Diabetic Rats

Sihle E. Mabhida [1], Rebamang A. Mosa [1,*], Dambudzo Penduka [1], Foluso O. Osunsanmi [1], Phiwayinkosi V. Dludla [2], Tryana G. Djarova [1]and Andy R. Opoku [1]

[1]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa; sihlemabhida@gmail.com (S.E.M.); propafadzo@gmail.com (D.P.); alafin21@yahoo.com (F.O.O.); drdjarova@yahoo.com (T.G.D); opokuA@unizulu.ac.za (A.R.O.)

[2]Biomedical Research and innovation Platform (BRIP), South African Medical Research Council, Tygerberg 7505, South Africa; pdludla@mrc.ac.za

*****Correspondence: rebamang@gmail.com; Tel.: +27-35-902-6824

Abstract

Type 2 diabetes remains one of the leading causes of death worldwide. Persistent hyperglycemia within a diabetic state is implicated in the generation of oxidative stress and aggravated inflammation that is responsible for accelerated modification of pancreatic beta cell structure. Here we investigated whether a lanosteryl triterpene, methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA-3), isolated from *Protorhus longifolia,* can improve glucose tolerance and pancreatic beta cell ultrastructure by reducing oxidative stress and inflammation in high fat diet and streptozotocin-induced type 2 diabetes in rats. In addition to impaired glucose tolerance, the untreated diabetic rats showed increased fasting plasma glucose and C-peptide levels. These untreated diabetic rats further demonstrated raised cholesterol, interleukin-6 (IL-6), and lipid peroxidation levels as well as a destroyed beta cell ultrastructure. Treatment with RA-3 was as effective as metformin in improving glucose tolerance and antioxidant effect on the diabetic rats. Interestingly, RA-3 displayed a slightly more enhanced effect than metformin in reducing elevated IL-6 levels and improving beta cell ultrastructure. Although the involved molecular mechanisms remain to be established, RA-3 demonstrates a strong potential to improve pancreatic beta cell ultrastructure by attenuating impaired glucose tolerance and reducing oxidative stress and inflammation.

Keywords: type 2 diabetes; hyperglycemia; hyperlipidemia; oxidative stress; inflammation; pancreatic beta cells; antioxidants; triterpenes; *Protorhus longifolia*

3.1. Introduction

Incidence of type 2 diabetes mellitus, characterized by insulin resistance, is increasing at an alarming rate and remains a serious global health concern [1,2]. Lifestyle modifications such as excessive food intake and lack of physical activity are some of the factors contributing to cellular insulin insensitivity and subsequent insulin resistance [1,2]. Insulin resistance results in the abnormally elevated levels of circulating blood lipids “hyperlipidemia” and glucose “hyperglycemia” observed in type 2 diabetic patients. Hyperlipidemia and hyperglycemia are considered to be the main contributors to type 2 diabetes and associated complications [1,2]. Such complications include accelerated oxidative injury through enhanced generation of free radical species and inflammatory response [3–5]. Increased oxidative stress as well as aggravated inflammatory response in type 2 diabetes are widely-reported phenomena, and are known to cause cellular damage to various organs, including the pancreatic beta cells [3,4]. Furthermore, it has been reported that the increase in pro-inflammatory cytokines such as interleukine-1 (IL-1), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in a type 2 diabetic state is commonly accompanied by a decrease in cellular antioxidant levels and increased apoptosis [5].

The ideal approaches in the management of type 2 diabetes and its associated complications should focus on the development of drugs that would improve cellular insulin sensitivity and antioxidant levels. This would subsequently help to control blood glucose and lipid levels and protect pancreatic beta cells from oxidative damage [6,7]. The majority of synthetic antidiabetic drugs currently on the market have long-term side effects and some are single target [8]. Literature indicates that plant derived products seem to be multi-target and can be effective in either their crude and/or pure forms [9–11]. Triterpenes are a group of plant-derived bioactive compounds that continue to display a wide array of significant potential bioactivities [12,13]. These plant-derived compounds have been demonstrated to have important bioactivity to ameliorate hyperlipidemia [14,15], inflammation [16,17], and diabetes [13,18–20]. In addition, these compounds have potential in protecting and enhancing the regeneration of pancreatic islets cells [21], improving glucose tolerance [22] and other diabetic complications [13,18–20]. Recently,

we have shown that a lanosteryl triterpene (Methyl-3β-hydroxylanosta-9,24-dien-21-oate; Figure 3.1) from the stem barks of *Protorhus longifolia* (Benrh.) Engl. (*Anacardiaceae*) possesses a broad spectrum of biological properties, including in vivo hypolipidemic and hypoglycemic properties [23–27]. We have already reported that RA-3 administration for 14 days suppressed glucose levels and increased hepatic glycogen content in streptozotocin (STZ)-induced diabetes rats [20]. Thus, based on these results, we aimed to investigate whether longer RA-3treatment can improve the ultrastructure of pancreatic beta cells through improving glucose tolerance in a high-fat diet and STZ-induced type 2 diabetes rat model.

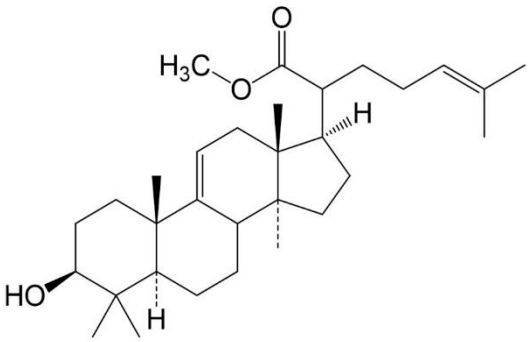


Figure 3.1: The chemical structure of methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA-3).

3.2. Results

3.2.1. RA-3 Displayed Hypoglycemic Effect in the Type 2 Diabetic Rats

The biological effect of RA-3 on blood glucose and C-peptide levels of diabetic rats was determined and results are shown in Table 3.1. As opposed to markedly higher fasting plasma glucose (FPG)levels after the 28 days treatment period, C-peptide levels were significantly lower in the diabetic control group (29.0 ± 1.09, $p \leq 0.0001$ and 0.2 ± 2.41, $p \leq 0.001$) when compared to non-diabetic controls (3.9 ± 0.04 and 0.8 ± 0.01, respectively). RA-3 treatment (4.3 ± 0.11, $p \leq 0.001$ and 0.4 ± 0.14, $p \leq 0.05$, respectively) showed a similar effect to metformin (4.5 ± 0.22, $p \leq 0.0001$ and 0.4 ± 0.12, $p \leq 0.05$, respectively) in reducing FPG and increasing C-peptide levels after the 28 days' treatment period.

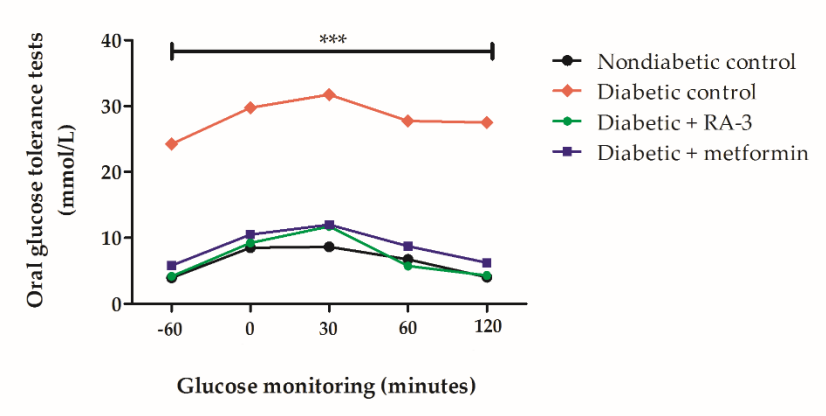
Table 3.1. The effect of RA-3 on fasting plasma glucose (FPG) and C-peptide levels after the 28 days treatment of the high fat diet and streptozotocin-induced type 2 diabetic rats.

Experimental Group	FPG Day 0 (mmol/L)	FPG Day 28 (mmol/L)	C-peptide Day 28 (µg/L)
Non-diabetic control	4.1 ± 0.22	3.9 ± 0.04	0.8 ± 0.01
Diabetic control	18.4 ± 0.78 ***	29.0 ± 1.09 ***	0.2 ± 2.41 ***
Diabetic + RA-3	11.5 ± 0.38 ***###	4.3 ± 0.11 ###	0.4 ± 0.14 *#
Diabetic + metformin	15.7 ± 0.66 ***#	4.5 ± 0.22 ###	0.4 ± 0.12 *#

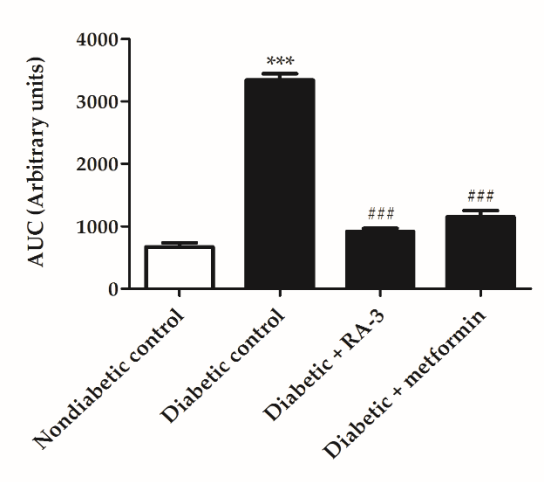
Day 0 was included to show that rats were already diabetic by the commencement of treatment (FPG ≥ 11 mmol/L). Results are expressed as the mean ± SEM and each treatment group contained at least five rats. * $p \leq 0.05$, *** $p \leq 0.0001$ vs. non-diabetic control, # $p \leq 0.05$, ### $p \leq 0.0001$ vs. diabetic control. One way analysis of variance (ANOVA), followed by a Tukey post-hoc test (Graph Pad Prism version 5.03) were used to determine statistical differences. The values were considered statistically significant where $p \leq 0.05$.

3.2.2. RA-3 Improved Glucose Tolerance in Type 2 Diabetic Rats

Non-diabetic and diabetic rats presented with increased levels of FPG levels from baseline (-60) to 30 min after administration of a 2 g/kg glucose bolus (Figure 3.2). However, these FPG levels were reduced in all animals after 30 min. Diabetic control animals displayed significantly elevated FPG levels ($p \leq 0.0001$) when compared to either non-diabetic controls or the diabetic animals treated with RA-3 and metformin (Figure 3.2A). RA-3 was effective in reducing increased FPG in diabetic animals back to levels similar to those of non-diabetic animals (Figure 3.2A) following the 28 days of the treatment period. Interestingly, the effect of RA-3 was similar to a commonly used anti-diabetic drug, metformin. The improvement of oral glucose tolerance with RA-3 and metformin treatment was confirmed by 'area under the curve' results (Figure 3.2B).



A



B

Figure 3.2. Oral glucose tolerance tests (**A**) and area under the curve (AUC) (**B**) in high fat diet and streptozotocin-induced type 2 diabetic rats treated with RA-3 and metformin (positive control). The untreated diabetic group presented with a significant increase in fasting plasma glucose levels (*** $p \leq 0.0001$) compared to the non-diabetic rats and diabetic rats treated with RA-3 and metformin.### $p \leq 0.001$ vs. diabetic control. Results are expressed as the mean ± SEM and each treatment group contained at least five rats. One way analysis of variance (ANOVA), followed by a Tukey post-hoc test (Graph Pad Prism version 5.03) were used to determine statistical differences. The values were considered statistically significant where $p \leq 0.05$.

3.2.3. RA-3 Prevented Lipid Peroxidation Through Enhancement of Endogenous Antioxidant Status in the Type 2 Diabetic Rats

The increased malondialdehyde (MDA) levels, as an indication of peroxidation, were significantly higher in the diabetic control group (1.31 ± 0.008, $p \leq 0.0001$) than the non-diabetic control (0.37 ± 0.004) (Table 3.2). Similarly, antioxidant markers such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were markedly reduced in the diabetic control group (2.38 ± 0.01, $p \leq 0.0001$; 30 ± 0.012,$p \leq 0.001$; and 0.08 ± 0.004, $p \leq 0.05$, respectively) when compared to non-diabetic control (7.33 ± 0.01, 56 ± 0.005, 0.12 ± 0.005, respectively) (Table 3.2).Treatment with RA-3 presented a comparable effect to metformin in enhancing GSH content (4.40 ± 0.006, $p \leq 0.05$ and 4.10 ± 0.003, respectively), CAT (0.13 ± 0.001, $p \leq 0.05$ and 0.11 ± 0.005, $p \leq 0.05$, respectively) and SOD (41 ± 0.004 and 39 ± 0.004, respectively) activity, though non-significantly for the latter.

Table 3.2. The effect of RA-3 on lipid peroxidation and antioxidant levels after the 28 days treatment of the high fat diet and streptozotocin-induced type 2 diabetic rats.

Experimental Group	**GSH Content (nmol/mL)**	**SOD Activity (Inhibition rate %)**	**CAT Activity (Units/mL)**	**MDA Levels (nmol/µL)**
Nondiabetic control	7.33 ± 0.01	56 ± 0.005	0.12 ± 0.005	0.37 ± 0.004
Diabetic control	2.38 ± 0.01 ***	30 ± 0.012 **	0.08 ± 0.004 *	1.31 ± 0.008 ***
Diabetic + RA-3	4.40 ± 0.006 *#	41 ± 0.004 *	0.13 ± 0.001 #	0.75 ± 0.005 *##
Diabetic + metformin	4.10 ± 0.003 *#	39 ± 0.004 *	0.11 ± 0.005 #	0.53 ± 0.003 *##

Results are expressed as the mean ± SEM and each treatment group contained at least five rats. * p ≤ 0.05, ** $p \leq 0.001$, *** $p \leq 0.0001$ vs. non-diabetic control, # $p \leq 0.05$, ## $p \leq 0.001$ vs. diabetic control. CAT: catalase, GSH: glutathione, MDA: malonaldehyde. One way analysis of variance (ANOVA), followed by an unpaired Student *t*-test (Graph Pad Prism version 5.03) were used to determine statistical differences. The values were considered statistically significant where $p \leq 0.05$.

3.2.4. RA-3 Reduced Cholesterol and Interleukin-6 Levels while it Improved the Pancreatic Beta Cell Ultrastructure of the Type 2 Diabetic Rats

The diabetic control group displayed significantly increased plasma cholesterol (183.4 ± 5.92, $p \leq 0.0001$) and serum IL-6 (145.5 ± 14.55, $p \leq 0.05$) levels in comparison to the non-diabetic rats (100.0± 4.21 and 100.0 ± 5.4, respectively) (Figure 3.3A, B). In addition, haematoxylin and eosin (H&E) stain demonstrated a condensed pancreatic beta cell ultrastructure in the diabetic control group (Figure 3.4). However, treatment with RA-3 was as effective as metformin in reducing cholesterol (114.2 ± 7.2, p ≤ 0.0001 and 106.5 ± 7.22, $p \leq 0.0001$, respectively) (Figure 3.3A), while it displayed a better effect than metformin in reducing IL-6 levels (115.5 ± 10.1, $p \leq 0.05$ and 135.0 ± 4.3, respectively) (Figure 3B). Interestingly, RA-3 treatment improved the pancreatic beta cell ultrastructure in comparison to diabetic control rats and diabetic rats treated with metformin (Figure 3.4).

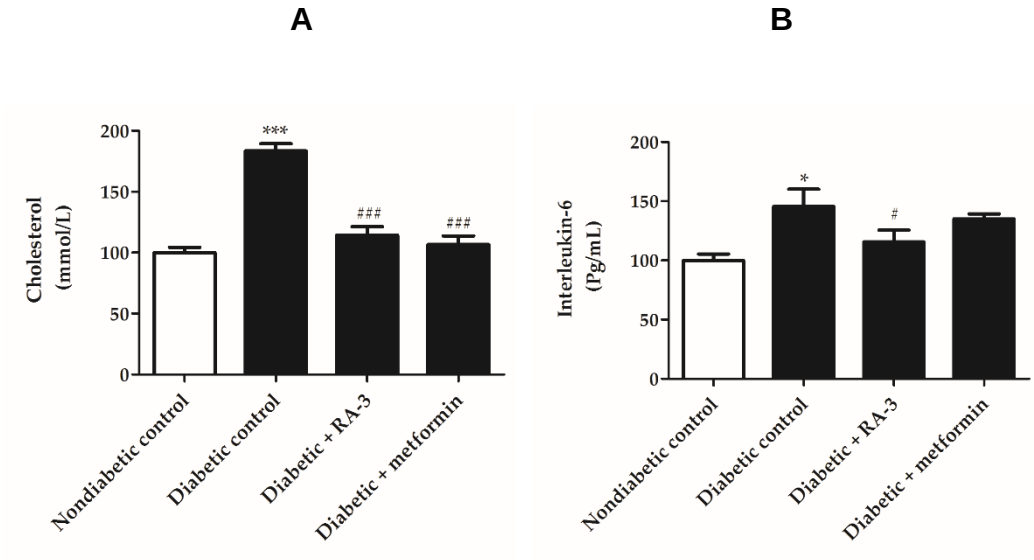


Figure 3.3. The effect of RA-3 on (**A**) plasma cholesterol and (**B**) serum interleukin-6 (IL-6) levels in the high fat diet and streptozotocin-induced type 2 diabetic rats. Results are expressed as the mean ± SEM and each treatment group contained at least five rats. * $p \leq 0.05$, *** $p \leq 0.0001$ vs. non-diabetic control, $^{\#}$ $p \leq 0.05$, $^{\#\#\#}$ $p \leq 0.001$ vs. diabetic control. One way analysis of variance (ANOVA), followed by a Tukey post-hoc test (Graph Pad Prism version 5.03) were used to determine statistical differences. The values were considered statistically significant where $p \leq 0.05$.

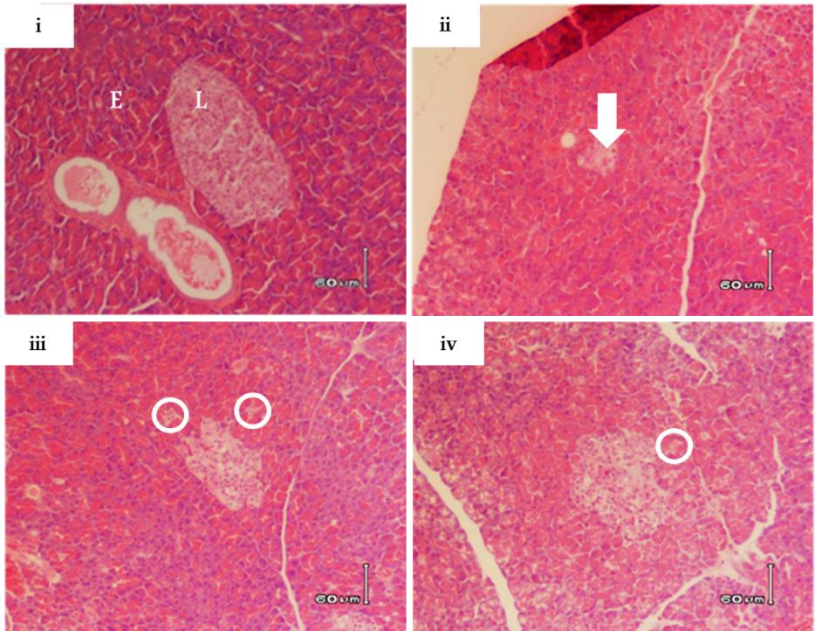


Figure 3.4. The effect of RA-3 on pancreatic beta cell ultrastructure in the high fat diet and streptozotocin-induced type 2 diabetic rats. (**i**) Normal structure of pancreatic islets from non-diabetic rats; (**ii**) A destructed structure as well as a reduced size of a pancreatic islets in untreated diabetic rats; (**iii**)An improved structure of pancreatic islets with some debris in diabetic rats treated with RA-3; (**iv**) A reduced size of pancreatic islets with debris of cells in diabetic rats treated with metformin. (L)- designates a normal structure of islets of Langerhans, (E) exocrine portion of pancreatic tissue, and an arrow shows destructed and condensed endocrine cells while a circle illustrates debris of destructed cells. NB: The indicator size for each image is 60 µm.

3.3. Discussion

Type 2 diabetes constitutes almost 90% of diabetes mellitus, and remains one of the leading causes of death worldwide [1,2]. Although commonly used antidiabetic and antilipidemic drugs such as metformin and atorvastatin prolong the lives of diabetic patients, the estimated rate of deaths due to diabetes continues to increase each year [1,2]. This has resulted in an increased exploration of natural products not just to lower elevated FPG levels but to improve the structure and function of pancreatic beta cells [21,28]. It is increasingly reported that persistent hyperglycemia through accelerated oxidative stress and inflammation may contribute to the loss of beta cell function [3,7]. Here we investigated the effect of RA-3 on improving glucose tolerance as well as associated markers of oxidative stress and inflammation in correlation with pancreatic beta cell ultrastructure in the high fat diet and STZ-induced type 2 diabetic rats.

A high fat diet and STZ-induced diabetic rat model is a well characterized system to study complications associated with type 2 diabetes [12,23]. In the present study, the type 2 diabetic rats displayed impaired glucose tolerance and hyperlipidemia which was evidenced by increased plasma cholesterol levels. This was concomitant to reduced serum C-peptide levels as an indication of low productivity of insulin by the beta cells. These rats further presented with reduced serum antioxidants, increased lipid peroxidation end product, MDA, and inflammatory marker, IL-6, while damage to the structure of pancreatic beta cells was also evident. It is hypothesized that hyperglycemia and fluctuations in blood glucose levels in the diabetic state result in excessive production of free radical species, which may lead to oxidative stress [4,29]. This complication is implicated in the progression of long-term diabetes consequences including damage to pancreatic beta cells [3,21,28]. Once hyperglycemia has developed, inflammation has been another factor linked to glucotoxicity and accelerated beta cell destruction, leading to phenotypic alterations and loss of beta cell mass through apoptosis [3,21,28]. Therefore, optimal islet beta cell function is vital for efficient insulin release and subsequent improved cellular glucose uptake.

The lanosteryl triterpene, RA-3, first identified from the stem bark of *Protorhus longifolia* [27] has already been established as possessing hypolipidemic properties by reducing total serum cholesterol and low density lipoprotein cholesterol in vivo [23], while its hypoglycemic potential was demonstrated in STZ-induced diabetic rats [20]. Results obtained from this study clearly demonstrated that RA-3 exerts a similar effect to that of metformin in improving glucose tolerance as well as FPG, cholesterol and C-peptide levels in the type 2 diabetic rats. In addition, RA-3 was more effective than metformin in reducing IL-6 levels. This is an interesting result since cytokines such as IL-6 are considered as the main regulators of inflammation during diabetic pathogenesis [30], and only a few compounds have displayed similar or better ameliorative effect of reducing diabetes associated complications than metformin. Supporting these results, Dudhgaonkar and colleagues have previously demonstrated that a triterpene extract from *Ganoderma lucidum* suppressed the secretion of IL-6 in lipopolysaccharide-stimulated macrophages [31]. A recent study showed that a novel triterpene (2α, 3β, 19α-trihydroxy-

24-oxo-olean-12-en-28-oic acid), isolated from Chinese acorns (*Quercusserrata var. brevipetiolata*) can inhibit tumor necrosis alpha (TNF-α)-induced IL-6 and IL-8 production in MH7A cells [17]. Although further studies are required to assess the synergistic use of RA-3 and metformin, the results obtained from this study support recent evidence promoting the combined use of natural products with current therapies to reduce the burden of type 2 diabetes and associated complications.

Furthermore, accumulating interest in the use of natural compounds such as RA-3 has been attributed to their strong antioxidant properties [20,24], which are essential in the prevention of hyperglycemia-induced inflammation and cellular damage. In agreement with previous findings [24], this study demonstrated that RA-3 can suppress lipid peroxidation, by reducing MDA levels, whereas this effect was parallel to raised antioxidant levels as measured by the assessment of serum GSH and CAT levels. GSH is one of the most important and abundant antioxidants in the body, while CAT remains essential in the detoxification of highly reactive hydrogen peroxide [29]. Antioxidant properties of RA-3 were consistent with the effect of improving beta cell ultrastructure in type 2 diabetic rats. Experimental data has already been presented that natural compounds such as resveratrol, a stilbenoid found in abundance in the skin of grapes and red wine, and aspalathin, a dihydrochalcone *C*-glucoside unique to *Aspalathus linearis,* present similar effects in reducing oxidative damage by preventing oxidative stress and inflammation in a diabetic state [29,32,33]. Moreover, these compounds, through their robust antioxidant effects, can improve the ultrastructure of the pancreatic beta cells and myocardium of type 2 diabetic mice [29,32,33]. The molecular mechanisms associated with the protective effect of resveratrol and aspalathin have been associated with modulation of intracellular energy homeostasis or antioxidant response through 5' adenosine monophosphate-activated protein kinase (AMPK) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), respectively [29,32,33]. Oleanolic acid, a naturally occurring pentacyclic triterpenoid, has been previously demonstrated to protect mice against acetaminophen hepatotoxicity through the activation of Nrf2 and its downstream target genes including those involved in GSH synthesis [34]. Indeed, increased levels of antioxidants such as GSH and CAT as well as other inflammatory markers have been

shown to be mainly mediated by Nrf2 activation in various disease models [34–36]. Thus, although additional studies are required, the strong antioxidant effects of RA-3 to combat diabetes-associated complications as well as improving altered pancreatic beta cell ultrastructure could be attributed to its capacity to upregulate Nrf2 expression.

In summary, results obtained from this study demonstrate that RA-3 improved glucose tolerance and pancreatic beta cell ultrastructure by inhibiting inflammation through the reduction of IL-6 levels and enhanced antioxidant status of the type 2 diabetic rats. The potential molecular mechanism by which RA-3 improves the ultrastructure of beta cells remains to be elucidated. However, recent research has highlighted that, similar to oleanolic acid, resveratrol and aspalathin, RA-3 may potentially induce its effect by modulating AMPK or Nrf2 [34,35,37]. Thus, future research directions, which are also important in addressing limitations of the current study, involve unravelling molecular mechanisms associated with the protective effect of RA-3, including its effect on regulating pancreatic beta cell function as well as insulin secretion in isolated islet and plasma insulin levels. These investigations will take into account both RA-3 as a monotherapy or in combination with metformin.

3.4. Materials and Methods

3.4.1. Reagents

Unless otherwise specified, all reagents, chemicals and assay kits used were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.4.2. Extraction and Compound Isolation

Protorhus longifolia fresh stem bark (specimen voucher number RAUZ01) was harvested from the KwaHlabisa area in KwaZulu-Natal, South Africa. The plant material was cleaned and routinely prepared for extraction. The triterpene was extracted and isolated from the powdered *Protorhus longifolia* stem bark as previously reported [27]. Briefly, n-hexane was used to defat (1:5 *w*/*v*) the powdered plant material and the defatted plant material was extracted with chloroform. The targeted lanosteryl triterpene (RA-3) was then isolated from the chloroform extract using silica gel chromatography (silica gel 60; 70–230 mesh ASTM; 0.063–0.2 mm; Merck, Billerica, MA, USA). The column was step-wisely eluted

with an n-hexane: ethyl acetate solvent system. The collected small fractions (20 mL) were analyzed with thin layer chromatography. RA-3 was obtained following its recrystallization in ethyl acetate (100%). The chemical structure of the compound (Figure 3.1) was confirmed using spectral techniques. The obtained physical and spectral data were in agreement with previous reports [27].

3.4.3. Animals

Ethical clearance (UZREC 171110–030 PGM 2016/329) for approval of procedures and use of laboratory animals was obtained from the University of Zululand Research Ethics Committee (UZREC). Sprague-Dawley rats (150–200 g) were obtained from the laboratory animal unit of Biochemistry and Microbiology Department, University of Zululand. The animals were maintained under standard conditions, in a controlled environment with a 12 h light/dark cycle in a temperature range of 23–25 °C (relative humidity ~50%), as outlined in the institutional and national guidelines for handling and caring of science laboratory animals. Animals were allowed five days acclimatization period with free access to enough normal rat feed and drinking water before subsequent experiments.

3.4.4. Establishment of a Type 2 Diabetic Rat Model

The method described by Machaba et al. [23] was followed with some modification to induce hyperlipidemia in rats. The Sprague Dawley rats of either sex were put on a high fat diet (pellets containing: commercial rat chow (79.3%), sunflower oil (15%), bile salt (0.5%), cholesterol (5%), and Thirmecil (0.2%) for a period of 28 days. The rats in the control group were fed a standard rodent diet. Hyperlipidemic condition in the animals was confirmed by measuring blood cholesterol levels (Accutrend cholesterol meter; Roche Diagnostics, Mannheim, Germany) from the rat's tail tip. Animals with the blood cholesterol level equal to or above 5.2 mmol/L were considered hyperlipidemic and used in the study.

After 28 days on high fat diet, the hyperlipidemic rats were fasted overnight. Thereafter, the rats were given intraperitoneal injection of a low single dose (30 mg/kg) of a freshly

prepared STZ solution. After five days of the STZ injection, blood glucose levels were measured from the blood collected from the tail tip with a glucometer (Accutrend glucometer; Roche Diagnostics, Mannheim, Germany). Animals with blood glucose levels equal to or above 11 mmol/L were considered diabetic and used in the study.

3.4.5. Treatment of High Fat Diet- Induced Diabetic Rats with RA-3

The diabetic rats were randomly divided into five groups of at least five rats per group. The rats in the experimental group received a daily single oral dose of 100 mg/kg of either RA-3 or metformin, a known antidiabetic drug, for 28 days. Animals in the non-diabetic and diabetic control groups received a daily single oral administration of distilled water and 2% Tween 20 (vehicle), respectively. RA-3 and metformin were dissolved in 2% Tween 20 and distilled water, respectively, before orally administered to the rats at the same time (08:00–09:00), and the dose used was based on a previously published study [20].

3.4.6. Oral Glucose Tolerance Test

At the end of the 28 days treatment period, the animals were fasted for 12 h and then received an oral glucose load (2 g/kg body weight). Changes in postprandial blood glucose levels were then monitored at baseline (-60), 0, 30, 60, 120 minute intervals. No visible side effects were observed in animals after the treatment period.

3.4.7. Determination of Fasting Plasma Glucose Levels

In rats fasted overnight, fasting plasma glucose levels were measured by tail prick using a handheld glucometer (Accutrend glucometer; Roche Diagnostics, Mannheim, Germany).

3.4.8. Biochemical Analysis

A day after oral glucose tolerance tests, rats were fasted for 4 hours before being weighed and anesthetized. Animals received the anaesthetic until no reaction could be recorded by pedal reflex before removal of blood. Blood was centrifuged at 4000 g at 4 °C for 15 min before the serum was removed for analysis of the levels of C-peptide, MDA, GSH,

SOD and CAT using respective commercial assay kits (Sigma-Aldrich, St. Louis, MO, USA), as per manufacturer's instructions. IL-6 was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, Steinheim, Germany).

3.4.9. Histopathological Studies

Following anesthezia and confirmation of no pedal reflex in the animals, pancreatic tissue was excised and preserved in 10% (*v*/*v*) neutral buffered formalin for histological studies. Tissue slides were prepared following standard procedures. Hematoxylin and eosin (H&E) were used to stain pancreatic tissues for histopathological analysis by photomicroscope (Vet Diagnostix Laboratories, Pietermaritzburg, South Africa). The slide examination was performed by a qualified pathologist with no prior knowledge of the respective animal groups.

3.4.10. Data Analysis

The experiments were replicated at least three times and reported as the mean ± standard error of mean (S.E.M). One way analysis of variance (ANOVA), followed by a Tukey post-hoc test or unpaired Student *t*-test where appropriate (Graph Pad Prism version 5.03) were used to determine statistical differences. The values were considered statistically significant where $p \leq 0.05$.

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Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

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38. **Sample Availability:** Samples of the compound (RA-3) are available from authors. Metformin is available through commercial sources.

CHAPTER FOUR

The molecular mechanism through which the lanosteryl triterpene (RA-3) from *Protorhus longifolia* improves peripheral insulin signalling in skeletal muscle of streptozotocin (STZ)-induced type 1 diabetic rats is reported here. The chapter, as presented here, is prepared for submission to the journal, **Phytomedicine**.

The details of the preparation of the reagents, (Appendix A), the details of the methods, including the isolation and characterization of the RA-3 (Appendix B) and the ethical clearance certificate for animal experiments (Appendix C) appear as supplementary material at the end of the dissertation

My contribution:

Performed experiments

Analyzed data and interpretation

Wrote the paper

CHAPTER FOUR: A Lanosteryl Triterpene from *Protorhus longifolia* Augments Insulin Signaling in Skeletal Muscle of Type 1 Diabetic Rats

Mabhida Sihle Ephraim[a], Johnson Rabia[bc], Ndlovu Musawenkosi[a], Louw Johan[b], Opoku Andy[a] and Mosa Rebamang Anthony[a*]

[a]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa.

[b]Biomedical Research and Innovation Platform (BRIP), South African Medical Research Council, Tygerberg 7505, South Africa.

[c]Division of Medical Physiology, Tygerberg, Stellenbosch University, Stellenbosch, South Africa.

Abstract

A substantial literature supports the antidiabetic properties of the lanosteryl triterpene (methyl-3 β -hydroxylanosta-9,24-dien-21-oate, RA-3) isolated from *Protorhus longifolia* stem bark. However, the molecular mechanism(s) associated with the antihyperglycemic properties of the triterpene remained to be explored. The current study aimed at investigating the molecular mechanism(s) through which RA-3 improves peripheral insulin signaling in skeletal muscle of streptozotocin-induced type 1 diabetic rats. The type 1 diabetic rats were treated daily with a single oral dose of RA-3 (100 mg/kg) for 28 days. The rats were then sacrificed, and blood, skeletal muscle and pancreases were removed for biochemical, protein expression and histological analysis, respectively. Persistently high blood glucose levels in the diabetic control rats significantly increased expression of IRS-1^{Ser307} while the expression of p-Akt ^{Ser473}, p-GSK-3β ^{Ser9} and GLUT 4 were decreased. However, enhanced muscle insulin sensitivity, which was indicated by a decrease in the expression of IRS-1^{ser307} with a concomitant increase in the p-Akt^{Ser473}, p-GSK-3β ^{Ser9} and GLUT 4 expression were observed in the diabetic rats treated with RA-3. The triterpene-treated animals also showed an improved pancreatic β-cells ultrastructure, along with increased C-peptide levels. An increase in the levels of serum antioxidants such as catalase, superoxide dismutase, and reduced glutathione was noted in the rats treated with the triterpene, while their serum levels of interleukin-6 and malondialdehyde were reduced. It is apparent that RA-3 is able to improve the insulin signaling in skeletal muscle of type 1 diabetic rats. Its beta (β)-cells protecting mechanism could be attributed to its ability to alleviate inflammation and oxidative stress in the cells.

Key words: Hyperglycemia, glucose uptake, oxidative stress, inflammation and lanosteryl triterpene.

4.1 Introduction

Type 1 diabetes mellitus (T1DM), a severe form of diabetes common in children and young adults, is characterized by the inability of pancreatic beta cells to produce insulin. T1DM accounts for 5-10% of all diabetes cases world-wide (Wherrett *et al*., 2013; WHO, 2017; IDF, 2015; Maahs *et al.,* 2011). According to the latest statistical report from the Center of Disease Control and Prevention (CDC), T1DM affects 80 000 children annually and this number is expected to increase by 1.4% each year (CDC, 2017). Due to early onset and longer duration, affected individuals are at increased risk of developing cardiac failure at a young age (IDF, 2017). This does not only put financial burden on individual households but also on a nation's health system and national budget.

Insulin secretion and action are crucial in maintaining glucose homeostasis. Insulin stimulates cellular glucose transport via a family of facilitative membrane proteins referred to as glucose transporters (GLUT). Skeletal muscle is responsible for 75% of insulin-mediated glucose disposal (Wei *et al*., 2008). Insulin stimulates muscle glucose uptake by promoting translocation of GLUT 4 via activation of the phosphatidylinositol 3-kinase pathway (PI3-K) and Akt2 (Li *et al*., 2017). Persistent hyperglycemia or glucotoxicity observed in diabetic patients, as a result of insulin signaling impairment, is the underlying cause of various diabetic complications. This impairment is concomitant to oxidative stress and an augmented pro-inflammatory response (Solinas and Becattini, 2017).

To date, the exact causes of T1DM remain unknown, however its onset is linked to an autoimmune attack on the body's own pancreatic beta cells as a result of environmental stimuli on genetically predisposed individuals. Though regular intravenous insulin injection is used to manage T1DM, this management strategy neither cures nor prevents onset of diabetes-induced complications. Thus, more effective treatment regimens are required in the quest to either prevent, delay or more effectively treat the disease. There is a growing interest in plant-derived bioactive compounds as candidates in the development of drug formulations with multiple targets to combat diabetes and its associated complications.

A large body of literature supports plant-derived triterpenes, due to their diverse bioactivities, as current targets in the development of new antidiabetic drugs (Santos *et al*., 2012, Castellano *et al*., 2013; Castro *et al*., 2015). Their hypoglycemic effect has been associated with their ability to decrease intestinal glucose absorption (Hou *et al*., 2009) and stimulate insulin secretion and cellular glucose uptake in peripheral tissues (Lee and Thuong, 2010; Callahan *et al*., 2015). A lanosteryl triterpene (RA-3, Figure 4.1) isolated from *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae) stem bark has been reported to possess hypoglycemic properties (Mosa *et al.,* 2014; Mosa *et al.,* 2015; Mabhida *et al*., 2017). However, the molecular mechanism associated with this improved cellular glucose uptake remains to be elucidated. Therefore, this study was set out to further explore the molecular mechanism through which RA-3 improves peripheral insulin signaling in skeletal muscle of STZ-induced type 1 diabetic rats.

4.2 Materials and methods

4.2.1 Extraction and isolation of RA-3

Fresh stem bark of *Protorhus longifolia* (specimen voucher number RAUZ01) was collected from KwaHlabisi, KwaZulu-Natal (KZN), South Africa. The targeted lanosteryl triterpene, RA-3 (Figure 4.1), was extracted and isolated from the chloroform extract of *P. longifolia* using chromatographic techniques as previously reported (Machaba *et al*., 2014, Mosa *et al*., 2014). Spectroscopic (Infra-red, NMR) data analysis was used to confirm the chemical structure of the compound.

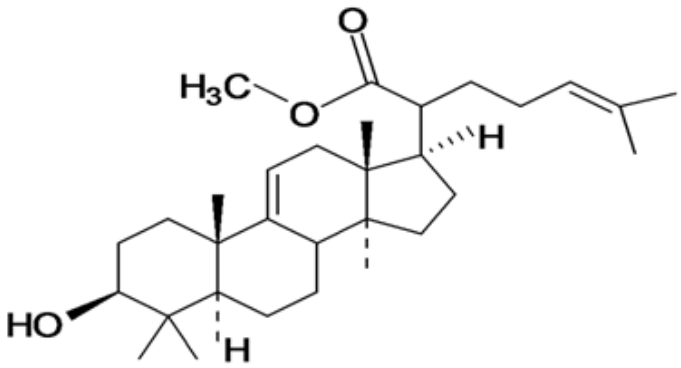

Figure 4.1: Methyl-3β-hydroxylanosta-9, 24-dien-21-oate (**RA-3**) chemical structure

4.2.2 STZ-induction of type 1 diabetes

Approval for procedures and use of laboratory animals (ethical clearance number: UZREC 171110–030 PGM 2016/329) was granted by the University of Zululand research ethics committee (UZREC). *Sprague- Dawley* rats (150-200 g) were obtained from the Biochemistry and Microbiology animal unit, University of Zululand. The animals were maintained under standard conditions (12-hour light/dark cycle, relative humidity, ~50%, and temperature, 23–25 °C). The rats were allowed five days of acclimatization, with free access to water and pelleted rat feed *ad libitum* period, before commencement of experimental procedures. After acclimatization, the rats were divided into two major groups (normal control and STZ-induced diabetic groups). T1DM was induced by a freshly prepared single intraperitoneal injection of STZ solution (60 mg/kg) into the overnight fasted rats. Five days after the STZ injection, the fasting blood glucose was measured by the tail prick method, using the accutrend glucometer (Roche Diagnostics, Mannheim, Germany). Blood glucose level ranges higher than or equal to 11 mmol/L confirmed the diabetic state of the animals to be used in the study.

4.2.3 Preparation of RA-3 solution

The lanosteryl triterpene (RA-3) was dissolved in Tween 20 (2%) to prepare a working solution of 100 mg/kg body weight of the rat. The prepared dosage was based on the previous studies performed in our laboratory (Machaba *et al.,* 2014; Mosa *et al.*, 2015).

4.2.4 Treatment of diabetic animals with triterpene

Type 1 diabetic rats were randomly divided into three groups (n=5) and orally administered with the drugs or carrier solvent as follows: (I) diabetic control group animals were given Tween 20 (2%); (II) diabetic treated with RA-3 (100 mg/kg); and (III) diabetic treated with metformin (100 mg/kg). The normal control group were given an equivalent volume of distilled water. The animals received an oral single dose of the RA-3 and the standard drug metformin daily for 28 days. The tail prick method, using the accutrend glucometer (Roche Diagnostics, Mannheim, Germany), measured fasting blood glucose concentrations weekly. The animals were overnight fasted, then euthanized under anaesthesia. Blood, skeletal muscle and pancreatic tissues were collected for subsequent biochemical, histological and protein expression analysis.

4.2.4.1 Biochemical Analysis of serum antioxidants, malondialdehyde, interleukin-6 and C-peptide levels

The blood samples from different groups were centrifuged for 10 min at 1 200 rpm at 4 °C in a micro centrifuge. The supernatant (serum) was then removed and transferred to a fresh tube and stored at -80 °C until required. The serum levels of CAT, SOD, GSH and MDA were measured using commercially available assay kits from Sigma-Aldrich (St. Louis, MO, USA.). An enzyme-linked immunosorbent assay (ELISA) kit obtained from Sigma-Aldrich Co. Ltd (Steinheim, Germany) was used to evaluate the serum IL-6. The serum C-peptide levels were measured using standard clinical laboratory procedures (Global Clinical & Viral Laboratory, Richards Bay, SA).

4.2.4.2 Hematoxylin and Eosin (H&E) stain of pancreas

The excised pancreas was preserved in neutral buffered formalin (10%), before being processed by a Leica TP 1020 automated processor (Leica Biosystems, Buffalo Grove, IL, USA) and embedded in paraffin wax. Tissues were sectioned and attached to aminopropyltriethoxysilane coated glass slides, after which they were stained with H&E as previously described (Abunasef *et al*., 2014). Stained pancreatic tissues were

analyzed by an independent Veterinary Pathologist without prior knowledge of the experimental groups (Vet Diagnostix Laboratories, Pietermaritzburg, South Africa).

4.2.4.3 Western blot analysis

To investigate the effect of RA-3 on the insulin signaling pathway, immunoblots against some proteins known to be involved in the insulin signaling pathway were performed. Snap frozen skeletal muscle tissue (100 mg) was defrosted on ice and lysed in lysis buffer (Pierce Biotechnologies, Rockford, CA, USA) using a Tissue lyser. Thereafter, the samples were centrifuged for 20 min at 12,000 rpm at 4 °C in a micro centrifuge. Supernatant was collected and stored at -20 °C until required. Protein (30 µg) was mixed with an equal volume of 2x Laemmli sample buffer before it was denatured at 95°C. The denatured protein samples (30 µg) were loaded on the 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and run for 1h at 150 V in Tris/Glycine/SDS-PAGE Buffer Buffer 1X. The proteins from the gel were then transferred to the polyvinylidene fluoride membrane (PVDF) (Bio-Rad, Hercules, CA, USA) (Johnson *et al.,* 2017). Membranes containing the proteins were incubated at 4 °C for 16 h with the following primary antibodies: anti- IRS-1^{Ser307} (1:500), phospho-Akt$^{Ser\ 473}$ (1:1000), phospho-GSK-3β^{Ser9} (1:1000) (Cell signaling, Danvers, MA, USA), and anti-GLUT 4 (1:000) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The membranes were washed and incubated with the appropriate horseradish peroxidase conjugated secondary antibody at room temperature for 90 min. All proteins were normalized to a loading control (β-Actin) (1:500) (Santa Cruz Biotechnology, Dallas, TX, USA). Chemidoc-XRS imager and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) was used to Detect and quantify the proteins.

4.2.5 Data Analysis

Data were analyzed statistically using Graph Pad Prism (Graph Pad Prism version 5.03). Experiments were performed in triplicates and data were expressed as mean ± SEM. Statistical differences between groups was determine by One way analysis of variance (ANOVA), followed by Tukey post-hoc test. The values were considered statistically significant where $p \leq 0.05$.

4.3 Results

4.3.1 Effect of RA-3 on blood glucose levels (BGL) of STZ-induced diabetic animals

Table 4.1 shows the results of the effect of RA-3 on the BGL of STZ-induced diabetic animals following the experimental period of 28 days. Persistently higher fasting plasma glucose levels were observed in the diabetic control rats. However, the 28 days treatment of the diabetic animals with RA-3 effectively lowered (67%) the blood glucose levels. The observed effect was similar and comparable to the metformin treated group (69%), which served as the positive control group.

Table 4.1: The effect of RA-3 on blood glucose levels (BGL) of the STZ-induced type 1 diabetic animals.

Group	**BGL Day 0 (mmol/L)**	**BGL Day 28 (mmol/L)**	**∆BGL (%)**
Non-diabetic control	4.2 ± 0.22	4.3 ± 0.04	
Diabetic control	14.0 ± 0.58****	27.0 ± 1.14****	
Diabetic + RA-3	13.3 ± 0.58****	4.4 ± 0.44####	67
Diabetic + metformin	13.8 ± 0.4****	4.3 ± 0.44####	69

Results are expressed as the mean ± SEM, n=5. **** $p \leq 0.0001$ versus non-diabetic control, #### $p \leq 0.0001$ versus diabetic control.

4.3.2 Effect of RA-3 on serum antioxidants and C-peptide levels

The results of the activity of RA-3 on serum antioxidants and C-peptide levels in the STZ-induced diabetic animals are presented in Table 4.2. Increased GSH, CAT, SOD)and C-peptide levels were observed in the diabetic animals treated with RA-3 when compared to the untreated diabetic control group following the 28 days of experimental period. The effect of RA-3 on the tested serum parameters was highly comparable to that of the metformin treated group.

Table 4.2: Effect of RA-3 on serum glutathione, catalase, superoxide dismutase and C-peptide levels of the STZ-induced type 1 diabetic animals.

Group	Glutathione (nmol/mL)	Superoxide dismutase (Inhibition rate %)	Catalase (Units/mL)	C-peptide (µg/L)
Non-diabetic control	7.33 ± 0.01	56 ± 0.005	0.12 ± 0.005	0.8± 0.01
Diabetic control	4.31 ± 0.15***	29 ± 0.040**	0.05 ± 0.006*	0.5± 0.22****
Diabetic + RA-3	6.05 ± 0.13*#	55 ± 0.011#	0.10 ± 0.004#	0.8± 1.02####
Diabetic + metformin	6.40 ± 0.14*#	54 ± 0.012#	0.10 ± 0.006#	0.8± 0.41####

Results are expressed as the mean ± SEM, n=5. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 versus non-diabetic control, # p ≤ 0.05, #### p ≤ 0.0001 versus diabetic control.

4.3.3 Effect *of RA-3 on serum MDA and IL-6*

Consistent with the increase in antioxidant levels in the RA-3 treated group, decrease in MDA (121 ± 12.5%, p ≤ 0.0001) and IL-6 (135 ± 10.9%) levels were observed in the triterpene treated group (Figure 4.2). A similar trend was noted in the metformin treated group. However, relatively higher serum MDA (270 ± 13.4%, p ≤ 0.0001) and IL-6 (153 ± 2.70%, p ≤ 0.01) levels were observed in the untreated diabetic group.

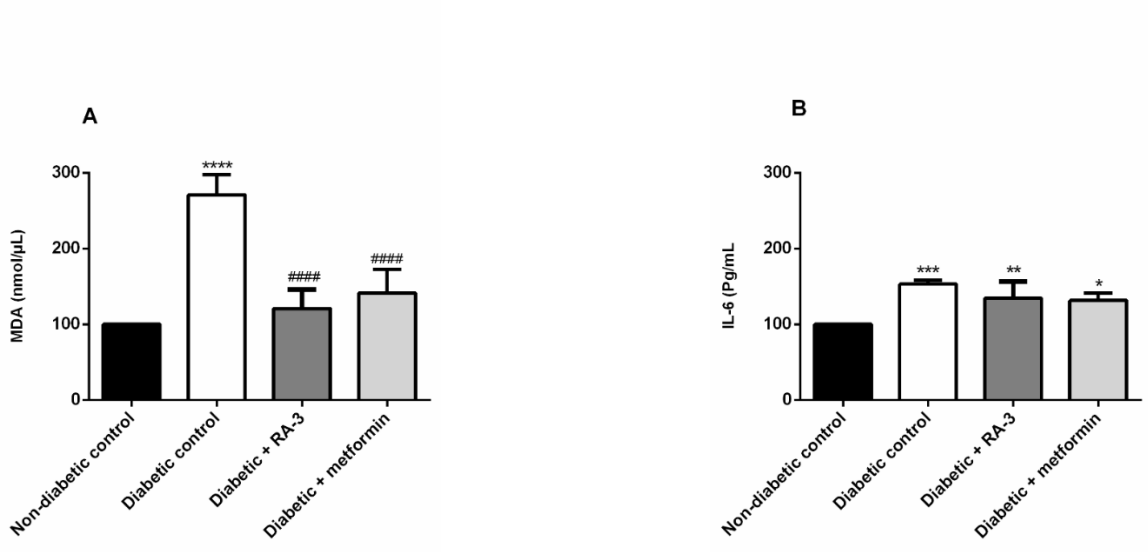

Figure 4.2: Effect of RA-3 on serum (A) MDA and (B) IL-6 levels in STZ-induced type 1 diabetic animals. Results are expressed as the mean ± SEM, n=5. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ vs. non-diabetic control, #### $p \leq 0.0001$ vs. diabetic control.

4.3.4 Effect of RA-3 on the ultrastructure of beta cells of STZ-induced diabetic animals.

Histological analysis of the pancreatic tissues from the different experimental groups was performed. The results of the histological analysis are shown in Figure 4.3. The pancreas of the normal rats (Figure3 A) showed normal architecture of islets without any histological alterations. Whereas the ultrastructure of the pancreatic tissues from the untreated diabetic group (Figure3 B) exhibited shrinkage of the islets cells mass, missing cells and cells with diminished size. However, treatment of the diabetic animals with RA-3 as well as metformin improved and preserved islet architecture with slight histopathological indications such as few debris of destroyed cells and intact islet architecture as shown in Figure 3 C and D, respectively.

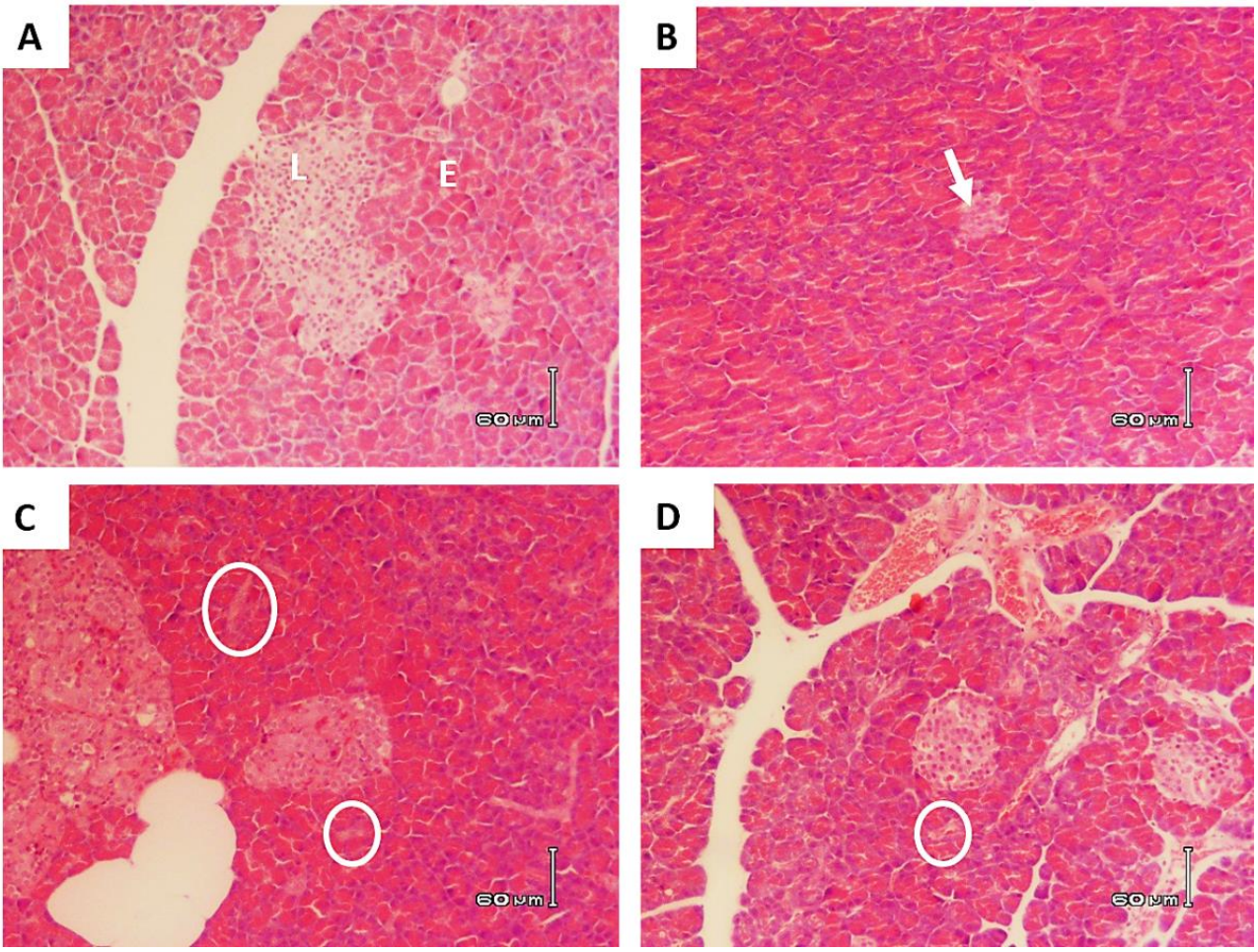

Figure 4.3: The effect of RA-3 on pancreatic β-cell ultrastructure of STZ-induced type 1 diabetic rats. (A) Normal control group, (B) Untreated diabetic group, (C) RA-3 treated group, and (D) Metformin treated group. (L) designates a normal architecture of islets without any histological alterations, (E) exocrine portion of pancreatic tissue and an arrow illustrates destruction and shrinkage of endocrine cells, while a circle shows debris of destroyed cells. NB: For each image the magnification (200X) size indicator is 60 µm.

4.3.5 Western blot analysis

Figure 4.4 presents the findings on the activity of RA-3 on some proteins (IRS-1^{ser307}, Akt, p-GSK-3β and GLUT 4) involved in the insulin-dependent signaling pathway in skeletal muscle cells. The results revealed that high blood glucose levels in the diabetic animals significantly increased the expression of IRS-1^{Ser307} (183 ± 4.06, $p \leq 0.0001$) while decreasing the expression of p-Akt^{Ser473} (41 ± 0.91%, $p \leq 0.0001$) and p-GSK-3β ^{Ser9} (10 ± 2.71%, $p \leq 0.0001$) as compared to the non-diabetic group. The observed effects of high blood glucose levels were reversed following the 28 days of RA-3 treatment of the

diabetic rats (96 ± 2.61%, p ≤ 0.0001; 109 ± 3.14%, p≤ 0.0001 and 94 ± 3.67%, p ≤ 0.0001, respectively). Significantly increased expression of GLUT 4 (80 ± 8.16%, p ≤ 0.01) from the skeletal muscle was also observed in the diabetic rats treated with RA-3 in comparison to the untreated diabetic group (30 ± 3.97%, p ≤ 0.01, respectively) (Figure 4.4D).

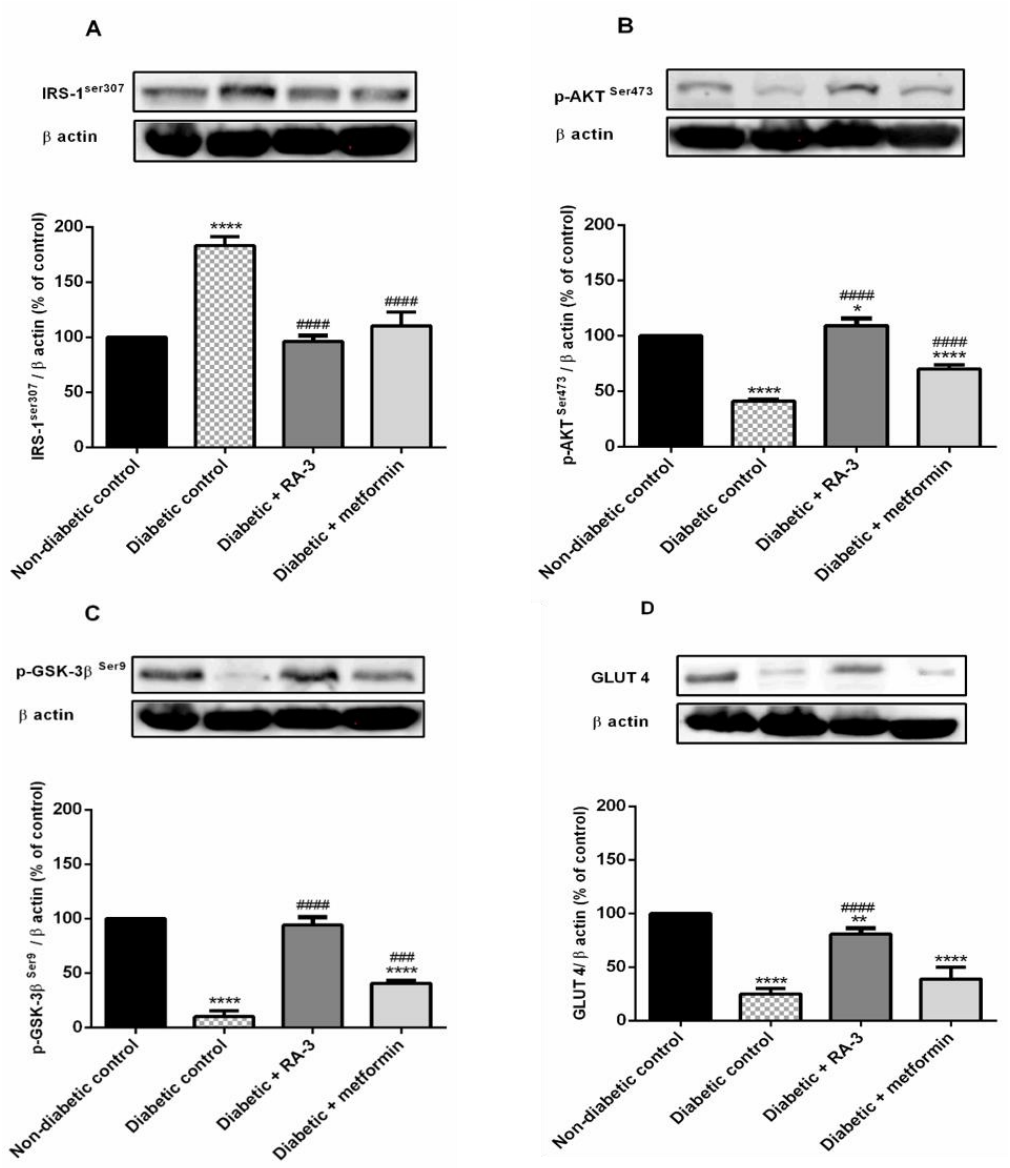

Figure 4.4: Effect of RA-3 on IRS-1^{ser307} (A), p-Akt^{Ser473} (B), p-GSK-3β ^{Ser9} (C) and GLUT 4 (D) protein expression in in STZ-induced type 1 diabetic animals. Results are expressed as the mean ± SEM, n=5. * p ≤ 0.05, **** p ≤ 0.0001 vs. non-diabetic control, ### p ≤ 0.001, #### p ≤ 0.0001 vs. diabetic control.

4.4 Discussion

Understanding the molecular mechanism that leads to the development and progression of T1DM is an important objective in the management of the disease. The anti-hyperglycemic property of the lanosteryl triterpene (RA-3) from *P. longifolia* has previously been demonstrated in the STZ-induced type 1 diabetic animals (Mosa *et al.,* 2015). The molecular mechanisms through which the compound exerts its hypoglycemic effect in T1DM model has, however, not been elucidated. Thus, this study investigated the ability of RA-3 to augment insulin signalling in skeletal muscle of the STZ- induced type 1 diabetic rats.

STZ is known to selectively destroy the insulin-producing beta cells by inducing oxidative stress and DNA damage mediated cell death and thus inhibiting insulin production by the pancreatic cells (Mirmira *et al.*, 2016). Oxidative stress has recently been shown to be responsible, at least in part, for pancreatic beta cell dysfunction as a result of glucose toxicity in hyperglycemia (Eleazu *et al*., 2013). The results obtained in this study showed the potential of RA-3 to improve the ultrastructure of the beta cells (Figure 4.3 C). The potential beta cell protective effect of the triterpene could be attributed to its ability to increase tissue antioxidant status which is evidenced by increases in GSH, CAT and SOD in the RA-3 treated group (Table 4.2). A similar effect of the triterpene on tissue antioxidant status has previously been reported (Mosa *et al*., 2015). The decrease in serum MDA levels, a marker of lipid peroxidation, and IL-6 (Figure 4.2) further supported the antioxidant potential of the compound. Both oxidative stress and inflammation are known to contribute to the destruction and death of beta cells and to the development of other diabetes-associated complications. The results indicate the potential of RA-3 to prevent pancreatic beta cell damage and dysfunction as well as other related complications.

The measurement of C-peptide levels is known to play a significant role as an indicator of the pancreatic beta cell's ability to secrete insulin (Gabr *et al*, 2015). This may be due to fact that C-peptide can be minimally extracted by the liver, and thus have potential to reflect the proper function of beta cells more accurately than insulin (Leighton *et al*., 2017).

Increased serum C-peptide levels observed in the RA-3 treated animals (Table 4.2) further supported the potential beta cell protective effect of the triterpene. The obtained results show a similar pattern to those reported by Mabhida *et al.* (2017) in which the lanosteryl improved pancreatic beta cell ultrastructure along with increased serum C-peptide levels in type 2 diabetic animals.

Insulin stimulates muscle glucose uptake by promoting recruitment of GLUT 4 via activation of the phosphatidylinositol 3-kinase pathway (PI3-K) and Akt2 (Li *et al*., 2017). Under physiological conditions, expression of the proteins (IR,IRS,PI3K,Akt) involved in the insulin signalling cascade is upregulated. However, the expression of these proteins is down regulated in the diabetic state. RA-3 has previously been reported to stimulate cellular glucose uptake in C2C12 myocytes and 3T3-L1 adipocytes (Mosa *et al*., 2014) and improve glucose tolerance in both type 1 (Mosa *et al.*, 2015) and type 2 (Mabhida *et al*., 2017) diabetic rats. However, the molecular mechanisms through which it exerts its hypoglycemic effect remained to be explored.

The results from the current study show that the hypoglycemic effect of RA-3 could partly be linked to its ability to augment insulin signaling. This is evidenced by the observed decrease in IRS-1^{Ser307} expression and increase in p-Akt and p-GSK-3β expression, which was well correlated with the increased expression of GLUT 4 (Figure 4.4) in the RA-3 treated group. The increased expression of GLUT 4 was further supported by the lower blood glucose levels in RA-3 treated diabetic animals (Table 4.1). The ability of RA-3 to increase the expression of p-GSK-3β shows the potential of this compound to control glucose homeostasis by maintaining the balance between glucose storage and glycogen breakdown, while preventing chronic gluconeogenesis. A pentacyclic triterpene, oleanolic acid, has also been reported to enhance the insulin signaling pathway in the skeletal muscle of STZ-induced diabetic rats (Mukundwa *et al*., 2016). Furthermore, since oxidative stress and pro-inflammatory cytokines such as IL-6 are implicated in the insulin signaling impairment and beta cell dysfunction, the antioxidant and anti-inflammatory effects mediated by RA-3 further supports its stimulation of muscle cell glucose uptake.

4.5 Conclusion

The present study provides evidence that the anti-hyperglycaemic activity of RA-3 could be linked to its ability to improve the insulin signaling pathway in skeletal muscle of type 1 diabetic rats. This is evidenced by increased expression of proteins involved in insulin signaling and eventual increased expression of GLUT 4 in the muscle of diabetic animals treated with the compound. The triterpene also reduced oxidative stress and inflammation which are implicated in insulin resistance, pancreatic beta cell dysfunction and tissue damage in the diabetic animals. It is also recommended to further investigate the effect of the triterpene on insulin signalling in a type 2 diabetes model (characterized by insulin resistance). Furthermore, the consistent similar results exhibited by RA-3 and metformin, a standard anti-diabetic drug, suggest a need to evaluate the anti-diabetic effects of a combination of the two drugs.

4.6 Authors' contribution

R.A.M. and A.R.O. conceived and designed the experiments; S.E.M. performed the experiments; R.J. and L.J. the experimental design and interpretation of western blot analysis; S.E.M and M.N. analysed the data; A.R.O., S.E.M., R.J. and R.A.M. wrote the manuscript.

4.7 Conflict of interest

There is no conflict of interest.

4.8 Acknowledgments

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CHAPTER FIVE

While chapters 3 and 4 presented the findings of the RA-3 effect on type 2 and type 1 diabetes, respectively, chapter 5 represents the study on the molecular basis of the anti-hyperglycemic activity of RA-3 in hyperlipidemic and STZ-induced type 2 diabetes in rats. The chapter, as presented here, is in preparation to be submitted to a specific accredited journal (**PLOS One**).

The details of the preparation of the reagents, including the HFD (Appendix A) and the details of the methods, including the isolation and characterization of the RA-3 (Appendix B) appear as supplementary material at the end of the dissertation

My contribution:

Performed experiments

Analyzed data and interpretation

Wrote the paper

CHAPTER FIVE: Molecular Basis of the Anti-hyperglycemic Activity of RA-3 in Hyperlipidemic and Streptozotocin-Induced Type 2 Diabetes in Rats

Mabhida Sihle Ephraim[a], Mosa Rebamang Anthony[a*], Johnson Rabia[bc], Ndlovu Musawenkosi[a], and Opoku Andy[a]

[a]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa.

[b]Biomedical Research and Innovation Platform (BRIP), South African Medical Research Council, Tygerberg 7505, South Africa.

[c]Division of Medical Physiology, Tygerberg, Stellenbosch University, Stellenbosch, South Africa.

Abstract

Insulin resistance is a hallmark of type 2 diabetes mellitus (T2DM) and the underlying cause of various metabolic derangement observed in type 2 diabetic patients. This study investigated the molecular basis of the anti-hyperglycemic activity of the lanosteryl triterpene (RA-3), from *Protorhus longifolia* stem bark, in hyperlipidemic and streptozotocin (STZ)-induced type 2 diabetes in rats. The high-fat diet fed (HFD) and STZ-induced type 2 diabetes in rat model was used to evaluate the antihyperglycemic activity of RA-3. The hyperlipidemic rats received a single intraperitoneal injection of STZ (35 mg/kg body weight) to induce diabetes. The experimental animals received a daily oral single dose of RA-3 (100 mg/kg body) for a period of 28 days. The animals were euthanized and liver as well as skeletal muscle were collected for protein (IRS-1, Akt, GSK) expression analysis. Western blot confirmed expression of the proteins. Treatment of the diabetic animals with RA-3 showed marked reduction in fasting plasma glucose (63%) levels in comparison to the untreated diabetic group animals. Relatively low p-GSK-3β and p-Akt expression and increased expression of IRS-1^{ser307} were observed in the diabetic control group. However, high expression of both p-GSK-3β and p-Akt and low expression of IRS-1^{ser307} was observed in the RA-3 treated diabetic animals. An increase in the expression of GLUT 2 and GLUT 4 was also observed in the tissues from diabetic animals treated with RA-3 when compared to the untreated diabetic animals. The results obtained in the present study indicated that the antihyperglycemic effect of RA-3 could partly be associated with its ability to improve the insulin signaling pathway in T2DM.

Keywords: Streptozotocin; hyperglycemia; Glucose uptake; inflammation; lanosteryl triterpene; *Protorhus longifolia,* oxidative stress

5.1 Introduction

The use of medicinal plants and their derived bioactive compounds to cure various ailments, including metabolic disorders, has been a preference since the earliest of times. There is still a continuous rising interest in the use of medicinal plants either in their crude or pure form to fight diseases such as diabetes mellitus (DM). Triterpenes, a unique class of plant-derived chemicals containing three terpene units, have recently gained much attention as new targets towards development of new pharmacologically active drugs. This is due to their wide range of significant biological activities including anti-diabetic properties (Kuo *et al*., 2015, Mukundwa *et al*., 2016, Castro *et al*., 2015).

Their hypoglycemic effect has been associated with their ability to decrease intestinal glucose absorption (Hou *et al*., 2009) stimulate insulin secretion and cellular glucose uptake in peripheral tissues (Lee and Thuong, 2010; He *et al*., 2014). Several *in vitro* and *in vivo* studies have demonstrated the potential of natural triterpenoids to enhance the insulin signaling pathway as well as protect and regenerate pancreatic islets (Mukundwa *et al*., 2016; Castellano *et al*., 2013). This class of compounds is also known for its inhibitory activity against alpha glucosidase and alpha amylase (Telagari and Hullatti, 2015), protein tyrosine phosphatase 1B (Thareja *et al*. 2012) and glycogen phosphorylase (Tahrani *et al*., 2011). They have also demonstrated a strong potential for the treatment of DM associated complications such as retinopathy (Thandavarayan *et al*., 2009), neuropathy (Kashyap *et al*., 2016), cardiomyopathy (Tan *et al*., 2015) and beta cell dysfunction (Castro *et al*., 2015).

Experimental data in our laboratory have demonstrated that methyl-3β-hydroxylanosta-9,24-dien-21-oate, RA-3 (Figure 5.1), a lanosteryl triterpene from stem bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae), possesses cardioprotective effect (Mosa *et al*., 2016), anti-hyperlipidaemic (Machaba *et al.*, 2014), and anti-hyperglycaemic activities (Mosa *et al.*, 2015). This compound has been reported to improve glycemic control and pancreatic beta cell ultrastructure in HFD-STZ-induced type 2 diabetic animals (Mabhida *et al*., 2017). However, the molecular mechanism(s) through which the compound improves the glucose tolerance in the type 2 diabetic animals remains to be explored.

Therefore, this study is aimed at evaluating the molecular mechanism by which RA-3 improves glucose tolerance in the hyperlipidemic and STZ-induced type 2 diabetic rats.

5.2 Materials and methods

5.2.1 The lanosteryl triterpene extraction and isolation

The fresh stem bark of *Protorhus longifolia* (specimen voucher number RAUZ01) was collected from KwaZulu-Natal, SA and routinely prepared for extraction. The chromatographic techniques previously described by Machaba *et al*. (2014) and Mosa *et al*. (2014) were adapted to successfully isolate and purify the lanosteryl triterpene (RA-3) from the chloroform extract of *P. longifolia* stem bark. Chemical structure of the triterpene was confirmed based on the spectral data analysis.

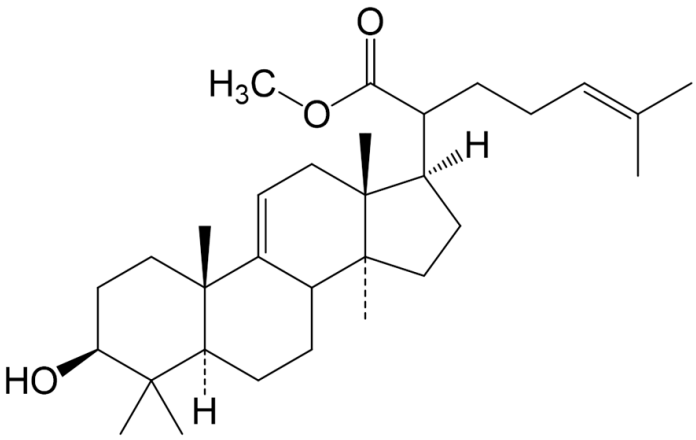

Figure 5.1: The lanosteryl triterpene (methyl-3β-hydroxylanosta-9,24-dien-21-oate, RA-3) Chemical structure.

5.2.3. Animals

Sprague-Dawley rats (150-200 g) of either sex were collected from the Biochemistry and Microbiology departmental animal unit, University of Zululand. Rats were housed and maintained under standard conditions [12-hour light/dark cycle, humidity (~50%), temperature (23–25°C)]. Before commencement of experimental procedures, the rats were acclimatized for a period of five days with free access to water and pelleted rat feed, *ad libitum*. After acclimatization, the rats were divided into a normal control and an experimental group. The University of Zululand Research Ethics Committee (UZREC)

granted the approval for procedures and use of laboratory animals (UZREC 171110–030 PGM 2016/329).

5.2.4 Induction of hyperlipidemia

The method previously described by Machaba *et al*. (2014) was adapted to induce hyperlipidemia in rats. The animals in the experimental group were put on high fat diet (HFD) for 28 days. The blood cholesterol levels (BCL) were measured using accutrend cholesterol strips (Roche Products) from the rat's tail tip as to confirm the hyperlipidemic state. BCL range above or equal to 5.2 mmol/L were used to confirm the hyperlipidemic state of the animals to be used in the study.

5.2.5 Type 2 diabetes induction

The hyperlipidemic rats were fasted overnight and then intraperitoneally injected with a freshly prepared low dose of STZ solution (30 mg/kg) to induce diabetes (T2DM). The blood glucose levels were measured after five days using a glucometer (accutrend glucose strips, Roche Products) from the rat's tail tip to confirm the hyperglyceamic state of the animals. Blood glucose levels above or equal to 11 mmol/L were used to confirm the diabetic state of the animals to be used in the study.

5.2.6 In vivo anti-hyperglycemic activity

The hypoglycemic activity of RA-3 was assessed in the type 2 diabetic animals following the method described by Kumar *et al*. (2012). The type 2 diabetic animals were randomly divided into four groups (n=5). The treated groups received a single dose of RA-3 or metformin orally for 28 days, both at a dosage of 100 mg/kg. Untreated groups (HFD-diabetic control and non-diabetic control) received 2% Tween 20 (vehicle) and distilled water, respectively as presented below.

Group I: Non-diabetic control, received distilled water only
Group II: HFD-Diabetic control, received vehicle (2% Tween 20)
Group III: HFD-D + RA-3 (100 mg/kg)
Group IV: HFD-D + Metformin (100 mg/kg)

All the animals continued on their respective treatments for 28 days. The animals were allowed free access to water and pelleted rat feed throughout the experimental period. The amount of food consumed was measured at two days intervals. Weight changes and blood glucose levels were monitored (seven day intervals). After 28 days, the animals were fasted overnight and euthanized under anesthesia. Liver and skeletal muscles were immediately collected and store at -80°C for western blotting analysis.

5.2.7 Western blot analysis of some proteins involved in insulin signaling pathway

The frozen skeletal muscle or liver (100 mg each) was weighed, defrosted on ice and lysed in lysis buffer (800 µL) (Pierce Biotechnologies, Rockford, CA, USA) using a tissue lyser at 25 Hrtz. Thereafter, the sample was centrifuged for 20 min at 12 000 rpm at 4°C in a micro centrifuge. The supernatant was collected and kept in a fresh tube at -20°C until required. Protein (30 µg) was mixed with an equal volume of 2x Laemmli sample buffer (containing β-mercaptoethanol), before it was denatured at 95°C. The denatured protein sample (30 µg) was loaded onto a 12% Mini-protein TGX Precast Gels, 10 well comb, 50ul/well (BioRad), along with 12 µL BioRad-Western C molecular weight marker. The gel was run for 1h at 150 V in Tris/Glycine/SDS-PAGE Buffer 1X and then transferred to a polyvinylidene fluoride membrane (PVDF, Bio-Rad, Hercules, CA, USA) (Jonson *et al*., 2017). Membranes were incubated at 4°C for 16 h with different primary antibodies which include IRS-1^{Ser307}, p-Akt and p-GSK-β, [Cell Signaling Technology, Danvers, MA, USA; (1:500), (1:1000), (1:1000) respectively], GLUT 4 [Sigma-Aldrich Chemical Co., St. Louis, MO, USA, (1:1000)] and GLUT 2 [Abcam plc, USA; (1:500)]. β-Actin [Cell Signaling Technology, Danvers, MA, USA, (1:4000)] antibody was added as a loading control. The membrane was incubated with an appropriate horseradish peroxidase conjugated secondary antibody (1: 4000) in blocking buffer (2.5% milk) at room temperature for 90 min, and then placed on a plastic sheet and 2ml chemiluminescent substrate (1:1 of luminal solution and reaction buffer) was added to the membrane for signal development. The proteins were detected using BioRad ChemiDoc.

5.2.8 Calculations

$$\text{Food conversion(FC)} = \frac{Food\ intake(g)}{wt.gain(g)}$$

$$\text{Food efficiency ratio(FER)} = \frac{wt.gain(g)}{Food\ intake(g)}$$

5.2.9 Data Analysis

Data were analyzed statistically using Graph Pad Prism (Graph Pad Prism version 5.03). Experiments were performed in triplicates and data were expressed as mean ± SEM. Statistical differences between groups was determine by One way analysis of variance (ANOVA), followed by Tukey post-hoc test. The values were considered statistically significant where $p \leq 0.05$.

5.3 Results

5.3.1 The effect of RA-3 on the food intake and change in body weight (∆BW) of the HFD-STZ-induced type 2 diabetic animals.

The results of the of RA-3 effect on the food intake and ∆BW in the diabetic rats after the 28 days of treatment with RA-3 are presented in Table 5.1. The results revealed a significant difference in food consumption in the diabetic group vs the normal control group. The diabetic animals consumed more food than the non-diabetic animals. However, a significant reduction in food intake was observed in RA-3 treated diabetic animals when compare to the untreated group. The non-diabetic control rats, subjected to a normal diet, displayed 15% increase in body weight. A similar effect on body weight was observed in the RA-3 treated rats group with an increase of 2% as compared to the untreated diabetic group which displayed a decrease (-3%).

Table 5.1: The RA-3 effect on the food intake and ΔBW of the type 2 diabetic animals.

	Food Intake (g)	**Change in Body Weight (ΔBW) (g)**		
Group	(g/day)	Initial	Final	ΔBW (%)
Normal control	71.07 ± 0.39	179.50±6.98	195.65±6.25	+15
HFD-Diabetic control	108.43 ± 0.24$^{\#\#}$	192.62 ± 6.26	180.32 ± 5.49	-12***
HFD-D + RA-3	75.46 ± 0.30$^{\#\#}$	187.40 ± 5.05	189.42 ± 5.65	+2$^{\#\#}$
HFD-D + Metformin	93.03 ± 0.07$^{*\#}$	194.61 ± 6.88	191.10 ± 6.14	-3*$^{\#}$

Values are expressed as the mean ± SEM (*n*=5). * p ≤ 0.05, *** p ≤ 0.001 vs. non-diabetic control, $^{\#}$ p ≤ 0.05, $^{\#\#}$ p ≤ 0.001 vs. diabetic control. D-Diabetic.

5.3.2 Effect of RA-3 on food conversion and food efficiency ratio of the HFD-STZ-induced type 2 diabetic animals.

The RA-3 effect on food conversion and food efficiency ratio in the diabetic rats was determined following the 28 days of treatment. The results are presented in Figure 5.2. While a lower food conversion and higher food efficiency ratio was witnessed in the normal control group, the complete opposite was observed in the untreated control group. However, treatment of the diabetic rats with RA-3 decreased the food conversion and improved the food efficiency ratio, a trend similarly observed in the metformin treated group.

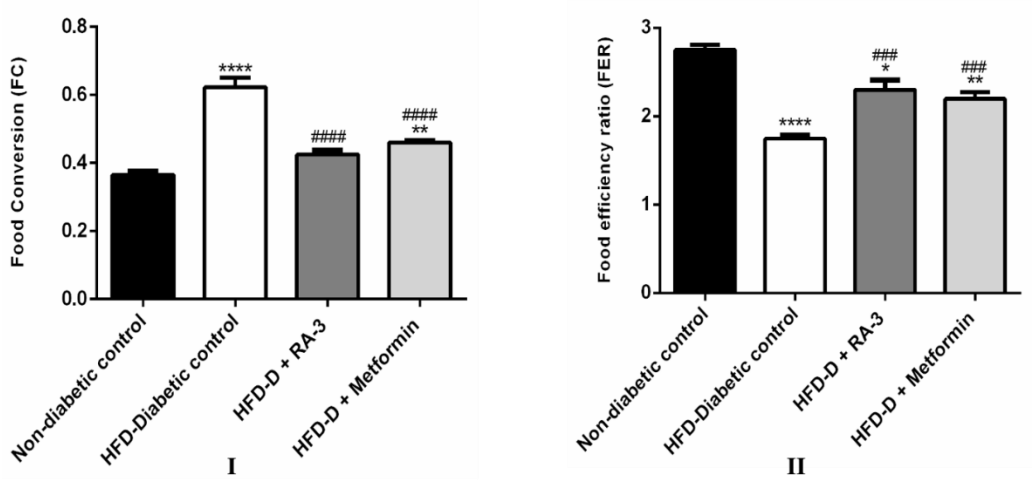

Figure 5.2: The RA-3 effect on food conversion (I) and food efficiency ratio (II) after the 28 days treatment of the type 2 diabetic rats. Values are expressed as the mean ± SEM (*n*=5). * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001 vs. non-diabetic control, $^{\#\#\#}$ p ≤ 0.001 $^{\#\#\#\#}$ p ≤ 0.0001 vs. diabetic control. D-Diabetic

5.3.3 RA-3 effect on blood glucose in the HFD-STZ-induced type 2 diabetic animals

Table 5.2 shows the biological activity of RA-3 on fasting blood glucose (FBG) levels of the type 2 diabetic rats. While a persistently higher fasting plasma level of glucose was observed in the diabetic control group, treatment of the diabetic animals with RA-3 effectively lowered their blood glucose (63%) levels. The observed effects were similar and comparable to those of the metformin treated group (positive control group).

Table 5.2: The effect of RA-3 on FBG after the 28 days treatment of the type 2 diabetic animals.

Group	**FBG Day 0 (mmol/L)**	**FBG Day 28 (mmol/L)**	**∆FBG (%)**
Type 2 diabetes			
Non-diabetic control	4.2 ± 0.22	4.3 ± 0.04	
HFD-Diabetic control	18.4 ± 0.78***	29.0 ± 1.09***	
HFD-D + RA-3	11.5 ± 0.38***###	4.3 ± 0.11###	63
HFD-D + Metformin	15.7 ± 0.66***#	4.5 ± 0.22###	71

Values are expressed as the mean ± SEM (*n*=5). * p ≤ 0.05, *** p ≤ 0.001, vs. non-diabetic control, # p ≤ 0.05, ### p ≤ 0.001, vs. diabetic control. D-Diabetic

5.3.4 Western blot analysis

5.3.4.1 Effect of RA-3 on some insulin signaling proteins (p-GSK-3β, p-Akt and IRS-1^{ser307})

Figure 5.3 shows the findings of the effect of RA-3 on some insulin signaling proteins. A relatively low expression of p-GSK-3β and p-Akt along with higher expression of IRS-1^{ser307} was observed in the diabetic control group vs non-diabetic control group. However, following treatment of the diabetic animals with RA-3, higher expression of both p-GSK-3β and p-Akt and lower expression of IRS-1^{ser307} were observed in comparism with the untreated diabetic group. The observed effects were similar and comparable to the metformin treated group.

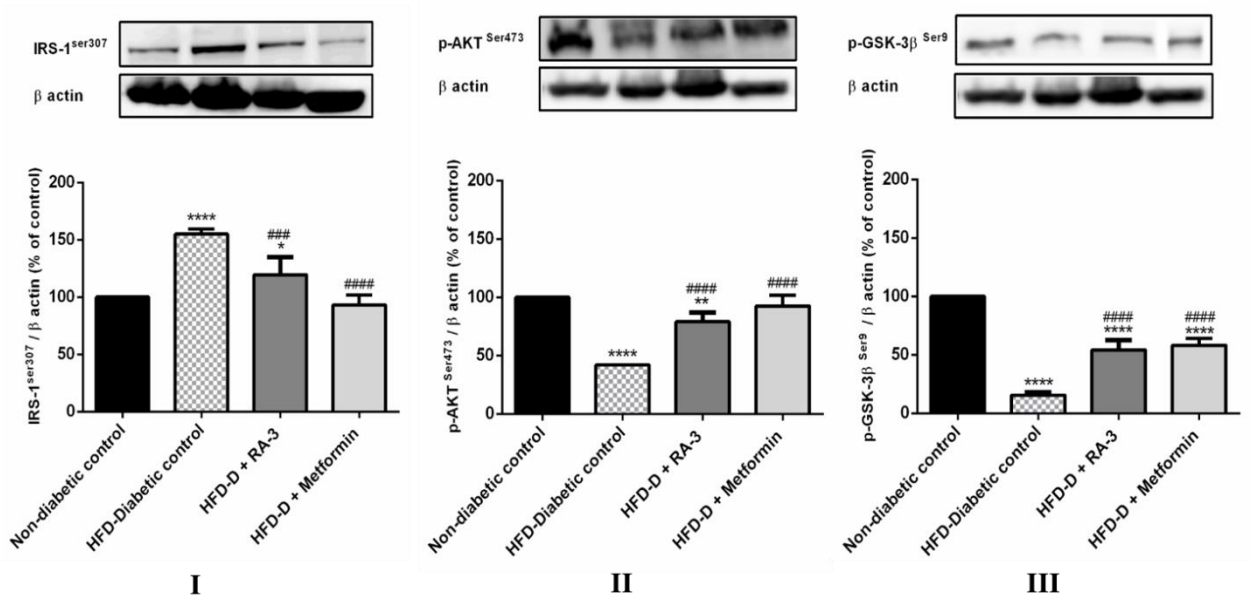

Figure 5.3: effect of RA-3 on IRS-1^{ser307} (**I**), p-Akt ^{Ser473} (**II**) and p-GSK-3β ^{Ser9} (**III**) protein expression in type 2 diabetic rats. Values are expressed as the mean ± SEM (*n*=5). * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001 vs. non-diabetic control, ### p ≤ 0.001, #### p ≤ 0.0001 vs. diabetic control. D-Diabetic

5.3.4.2 Effect of RA-3 on GLUT 2 and GLUT 4 in skeletal muscle and liver of the type 2 diabetic animals.

Figure 5.4 shows the expression of glucose transporters (GLUTs) (GLUT 4 and GLUT 2) following treatment of the diabetic rats with RA-3 for 28 days. The expression of both GLUT 4 and GLUT 2 were significantly decreased in the untreated diabetic animals vs normal control group. However, treatment of the diabetic animals with RA-3 or metformin for 28 days, significantly increased the expression of both proteins (GLUT 4 and GLUT

2). The expression of GLUT 2 was even higher than that of the GLUT 4 and favorably compared to the non-diabetic control.

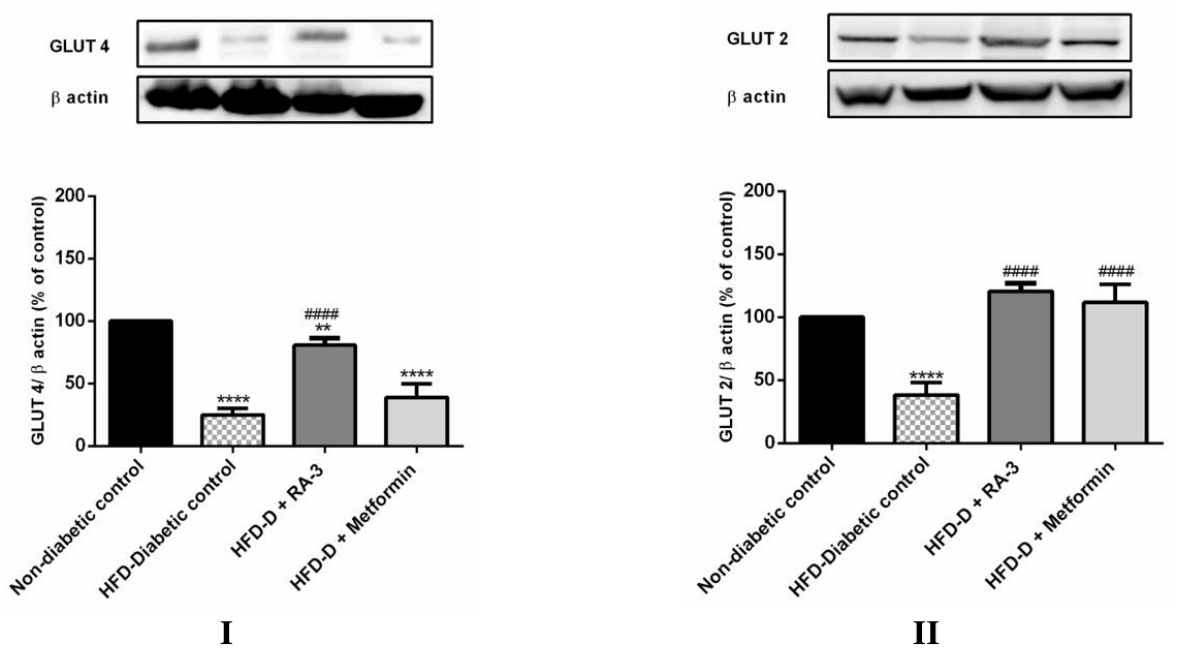

Figure 5.4: Effect of RA-3 on GLUT 4 (**I**) and GLUT 2 (**II**) protein expression in the skeletal muscle and liver, respectively, in type 2 diabetic animals. Values are expressed as the mean ± SEM (*n*=5). ** $p \leq 0.01$, **** $p \leq 0.0001$ vs. non-diabetic control, #### $p \leq 0.0001$ vs. diabetic control. D-Diabetic

5.4 Discussion

Experimental evidence has shown that oxidative stress and increase in pro-inflammatory cytokines is closely related to insulin resistance, through their negative activity on insulin signaling pathways and glucose transport into the cell. Thus, their effects have been suggested to explain the development of insulin resistance in T2DM (Ndisang *et al*., 2017). Lanosteryl triterpene (RA-3) has recently been established to improve glycaemic control and pancreactic beta cell ultrastructure in HFD-STZ-induced type 2 diabetic rats. The pancreatic beta cells protective effect of RA-3 has been linked to the ability to enhance antioxidant status and reduce the release of pro-inflammatory cytokines such as IL-6 (Mabhida *et al*., 2017). However, the molecular mechanism(s) through which the compound improves the glycaemic control in the type 2 diabetic animals remained to be explored. Thus, the current study evaluated the molecular mechanism through which RA-3 improves glycaemic control in the hyperlipidemic and STZ-induced type 2 diabetic

animals. Normally insulin stimulates cellular glucose uptake by activating a cascade of reactions leading to plasma membrane recruitment of glucose transporters (GLUT 2 and GLUT 4) in the liver and muscle, respectively. The translocation of these GLUTs is regulated by a series of proteins such as PI3-K, Akt and IRS-1. However, while PI3-K and Akt are known to be downregulated in T2DM, IRS-1^{Ser307} is highly expressed (Solinas and Becattini, 2017).

Binding of insulin to its receptor triggers a cascade of phosphorylation reactions leading to activation of PI3-K, which in turn activates p-Akt. High expression of Akt activates translocation of glucose transporters and thus increases glucose uptake by the cell. AKT also further phosphorylates GSK-3β, promoting the glucose storage as glycogen. On the contrary, increased Ser^{307} phosphorylation is known to decrease the IRS-PI3K-Akt-GSK3B pathway, causing reduced glucose transporter translocation, while promoting gluconeogenesis and inhibiting glycogen synthesis in liver and muscle (Rains and Jain, 2011).

Interestingly, the results from this study demonstrated that RA-3 was able to stimulate the insulin signaling pathway by reducing the expression of IRS-1^{Ser307} and increased p-Akt/p-GSK-3β expression (Figure 5.3) after 28 days of treatment. These findings were further evidenced by the upregulation of GLUT 2 and GLUT 4 and the subsequent antihyperglycemic effect indicated by an effective reduction of the fasting blood glucose levels (Table 5.2). Phosphorylated GSK-3β at ser9 by Akt is well-known to play a major role in insulin-induced stimulation of glycogen synthesis. The ability of RA-3 to increase the expression of p-GSK-3β shows the potential of this compound to control glucose homeostasis by maintaining the balance between glucose storage and glycogen breakdown, while preventing chronic gluconeogenesis. The results obtained from this study provides the molecular basis through which RA-3 improves glucose tolerance in HFD-STZ-induced type 2 diabetic animals, which has recently been established in our laboratory (Mabhida *et al*., 2017). The results are also similar and consistent with those observed in the T1DM model reported by Mabhida *et al*. (Unpublished data). The results from this study could also partly serve to explain the stimulatory effect of RA-3 on glucose uptake by C2C12 myocytes and 3T3-L1 adipocytes previously reported by Mosa *et al*.

(2014). Oleanolic acid has also been reported to exert its hypoglycemic effects through activation of the insulin signaling pathway in skeletal muscle of STZ-induced diabetic rats (Mukundwa *et al*., 2017).

In normal body metabolism, consumption of excessive amount of food results in weight gain. However, in the diabetic state, patients consume more food which is less utilized by the body and this food is converted into fats rather than muscle building and body mass (Han and Lean, 2016). In this study, RA-3 treated rats consumed less food but converted more of the food into body mass (Figure 5.2), this was concomitant with the 2% increase in body weight (Table 5.1) in comparison to the untreated diabetic group that lost weight. Diabetic rats consumed more food, but less was converted into body weight and as a result, decrease in body weight was observed. This is one of the characteristics of STZ-induced diabetes (Howarth *et al*., 2017). Furthermore, the results from this study suggest that RA-3 has the potential to improve and regulate metabolic homeostasis. . These findings are also consistent with the results obtained by Machaba *et al*. (2014) who established the *in vivo* lipid-lowering activity of the RA-3 in the hyperlipidemic rats.

5.5 Conclusion

The findings obtained in the present study confirm that the hypoglycemic activity of RA-3 is through its apparent ability to enhance the insulin signaling pathway and consequently increase the expression of GLUTs in the skeletal muscles of diabetic animals. These findings suggest that RA-3 could be a potent therapeutic candidate in the development of new anti-diabetic drugs. However, further studies in which RA-3 regulates the functioning of pancreatic beta cells are still required.

5.6 Authors' contribution

R.A.M. and A.R.O. conceived and designed the experiments; S.E.M., performed the experiments; R.J., experimental designed and interpretation of western blot analysis. S.E.M and M.N., analysed the data; A.R.O., S.E.M., R.J., L.J. and R.A.M. wrote the manuscript.

5.7 Conflict of interest

There is no conflict of interest.

5.8 Acknowledgments

South African National Research Foundation (SA-NRF) for studentship awarded to SE Mabhida, the University of Zululand Research Committee (UZRC) and South African Medical Research Council's Biomedical Research & Innovation Platform baseline funding for their financial support to the success of this work.

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CHAPTER SIX:

6.0 General discussion

Hyperglycemia-induced inflammation and oxidative stress is accountable for augmented alteration of pancreatic beta cell structure. Several *in vitro* and *in vivo* studies have demonstrated the potential of natural triterpenoids to enhance the insulin signaling pathway as well as protect and regenerate pancreatic islets (Mukundwa *et al*., 2016; Castellano *et al*., 2013). In this study the glycemic control and pancreatic beta cell protective effect of the lanosteryl triterpene (RA-3) from *P. longifolia* in hyperlipidemic and streptozotocin-induced diabetic rats were evaluated.

Streptozotocin (STZ), a drug commonly used to chemically induce diabetes mellitus in rodents, directly targets pancreatic beta cells via oxidative stress resulting in increased hyperglycemia (Eleazu *et al*., 2013). The destruction of beta cells increases the stimulation of the inflammatory response which has a negative effect on the insulin signaling pathway (Lin *et al.,* 2017) (Figure 6.1). It is apparent from the findings obtained in the present study that RA-3 has the potential to improve the pancreatic beta cell's ultrastructure and function. The protective effect of RA-3 is indicated by the increased levels of C-peptide in the serum of diabetic rats treated with RA-3. The serum C-peptide levels have been considered an important marker of functional beta cells and insulin secretion (Wang *et al*., 2009; Gabr *et al.*, 2015). Experimental evidence has also shown that an increase in pro-inflammatory cytokines, such as IL-6, in the diabetic state is usually concomitant with the reduction of antioxidant levels and an increase in lipid peroxidation (MDA) (Elmarakby and Sullivan, 2012). The pancreatic beta cell protective effect of the triterpene could be attributed to the observed increase in antioxidant levels with concomitant decrease in lipid peroxidation (MDA) in the diabetic rats treated with the triterpene. These observations correlated with the decreased levels of IL-6, an inflammatory marker known to interfere with insulin signaling (Solinas and Becattini, 2017).

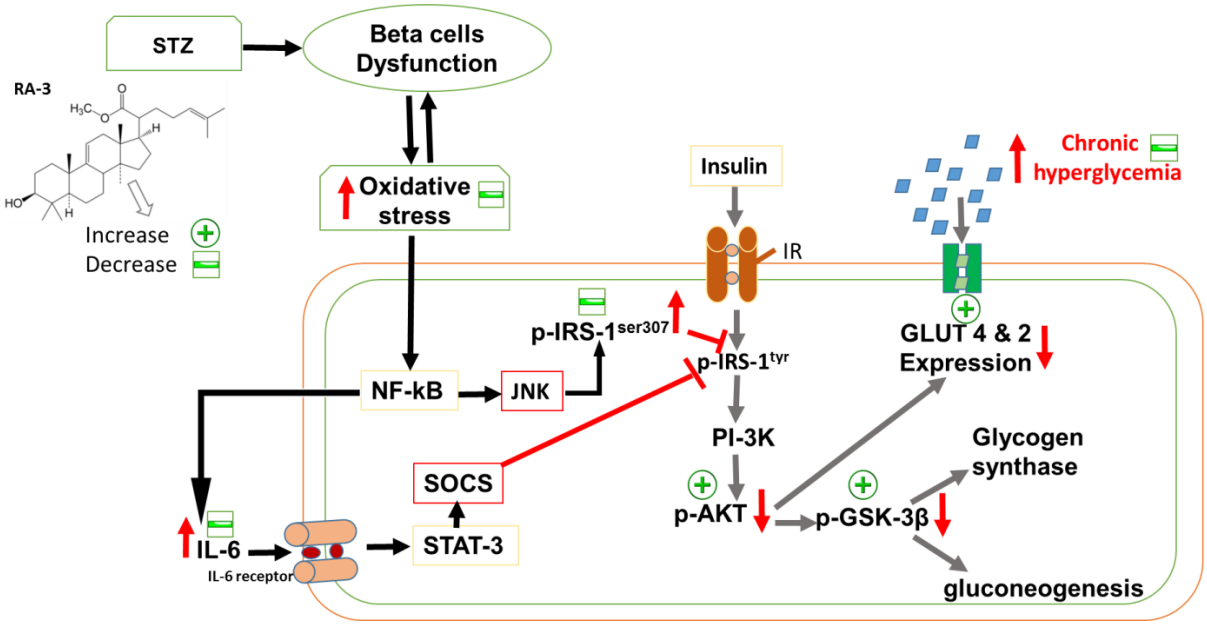

Figure 6.1: Overview of insulin regulation of major metabolic responses in the cells. Under physiological conditions, insulin binds to its receptor (IR) to enhance positive tyrosine IRS-1 phosphorylation, promoting the activation of PI3K which in turn phosphorylates and activates p-Akt. High expression of Akt activates translocation of glucose transporter 4 and thus increasing glucose uptake by the cell. Akt also further phosphorylates GSK-3β, promoting the glucose storage as glycogen. Under pathophysiological conditions oxidative stress and pro-inflammatory cytokines such as IL-6 enhance negative phosphorylation of IRS-1 $^{\text{serine (307)}}$, causing insulin resistance. Activated NF-$_{\kappa}$B activates serine/threonine kinases (JNK) which phosphorylates IRS-1 and also works via other pathways such as SOCS expression to inhibit the insulin signaling pathway.

Furthermore, high blood cholesterol and glucose, commonly observed in diabetics, contributes to development of insulin resistance in diabetic patients (Fung and Berger, 2016). The ability of the triterpene to reduce the levels of fasting blood glucose and cholesterol, as well as improving glucose tolerance in HFD-STZ induced diabetic animals, indicates the potential of RA-3 to improve insulin resistance and thus stimulate cellular glucose uptake. The cellular glucose uptake stimulating effect of RA-3 has previously been reported by Mosa *et al.* (2014). However, its mechanism of action remained to be elucidated.

It is apparent from the results of this study that RA-3 improves glycemic control through activation of the insulin signaling pathway. Inhibition of insulin signaling has a negative effect on the recruitment of glucose transporters (GLUT 4 and GLUT 2) translocation due to the down-regulation of some proteins (PI3K/Akt/GSK) involved in the insulin signaling pathway. The translocation of the glucose transporters, upon insulin activation, is

regulated by a series of proteins such as PI3-K, Akt and IRS-1 (Figure 6.1). Insulin, binding to its receptor, activates a cascade of phosphorylation reactions leading to the activation of PI3-K, which in turn activates p-Akt. High expression of Akt activates the translocation of glucose transporters and thus increases glucose uptake by the cell. In this study, it has been observed that RA-3 was able to decrease IRS-1^{Ser307} expression and increase p-Akt and p-GSK-3β expression. This correlated with the increased expression of GLUT 4 and GLUT 2 in the muscle and liver, respectively, of the treated diabetic animals. The overall effect of RA-3 in the expression of these proteins was confirmed by a decrease in fasting blood glucose levels in the treated diabetic rats.

Since oxidative stress together with the inflammation are well known to contribute to the dysfunction of beta cells and the development of insulin resistance, the apparent ability of RA-3 to reduce these contributing factors, whilst activating insulin signaling pathways, confirms its glycemic control and ability to protect the pancreatic beta cells. A study conducted by Lin *et al.* (2017) reported on the increased expression of GLUT 4 through the activation of insulin dependent (IRS1/Akt) and insulin independent (AMPK) pathways in an *in vitro* C2C12 myotube cell model. Similarly, Mukundwa *et al.* (2016) demonstrated the ability of oleanoic acid, a triterpenoid, to ameliorate insulin sensitivity in the skeletal muscles of STZ-induced diabetic animals. Furthermore, Reisman *et al.* (2009) and Loboda *et al.* (2012) attributed improved insulin signaling in a diabetic model to enhanced levels of endogenous antioxidants driven by the increased expression of Nrf2. In accordance with literature, the findings in the present study elaborated on the robust antioxidant activities of RA-3 to fight DM and its associated complications. Thus, these results suggest that RA-3 can be a potent agent in protecting and attenuating damaged pancreatic beta cell ultrastructure through the upregulation of Nrf2.

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CHAPTER SEVEN:

7.0 General conclusion

Failure of pancreatic beta cells to secrete insulin in amounts sufficient for glycemic control and also the development of cellular insulin resistance in the peripheral tissues are crucial determinants of DM (Fung and Berger 2016). The relationship between beta cell dysfunction and insulin resistance remains highly complex and can be exacerbated by the onset of hyperglycemia. Hyperglycemia-induced oxidative stress and accelerated inflammatory response may trigger various signaling pathways which further aggravates the insult to the beta cells, thereby worsening the disease state. In an effort to discover an alternative therapeutic candidates to manage the increased prevalence of DM, our research group has previously reported the potential antidiabetic activity of RA-3 (Machaba *et al*., 2014, Mosa *et al*., 2015). However, its apparent mechanism(s) of action still remained to be explored. As such, in this study we investigated the glycemic control of RA-3 and its potential ability to protect the pancreatic beta cells in hyperlipidemic and STZ induced diabetes in rat model.

Data from the present study confirmed the hypoglycemic properties of RA-3 as it improved glucose tolerance in diabetic rats. Its apparent mechanism has been associated with its ability to enhance insulin signaling via the inhibition IRS-1^{Ser307} and the subsequent activation of p-Akt, p-GSK-3β and GLUT 2 and 4. Furthermore, histological analysis of the pancreatic tissue from the diabetic animals displayed significant improvement in pancreatic beta cell ultra-structure following treatment with RA-3. These observations were further supported by the ability of RA-3 to increase the tissue antioxidant levels with concomitant decrease in IL-6 and thus attenuate oxidative stress and inflammation-induced beta cell damage.

In conclusion, the results obtained from this study indicate that RA-3 from *P. longifolia* stem bark improved glycemic control and the pancreatic beta cell protective effect. The potential molecular mechanism through which RA-3 improves glycemic control is based on its enhancement of the insulin signaling pathway, leading to increased recruitment of glucose transporters and thus increased cellular glucose uptake. In addition to the ability

of the triterpene to enhance insulin signaling, its pancreatic beta cell protective effect could also be linked to its reduction of IL-6, an inflammatory marker known to interfere with insulin signaling, and enhancement of antioxidant status of hyperlipidemic and STZ-induced diabetic animals.

7.1a Limitations in the current study

Due to time and limited funding, we could not explore the molecular basis through which RA-3 regulates the functioning of the pancreatic beta cells. However, the histological analysis conducted in this study was indicative of RA-3's beta cells protective potential.

7.1b Future studies

- Assessment of the pharmacokinetics and efficacy of the combinatory treatment of RA-3 and metformin in an *in vivo* diabetic model is recommended.
- Evaluation of protein and gene expression to validate the mechanism in which RA-3 regulates beta cell function is also necessary for future study.

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APPENDIX A

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-030)

RESEARCH & INNOVATION
Website: http://www.unizulu.ac.za
Private Bag X1001
KwaDlangezwa 3886
Tel: 035 902 6887
Fax: 035 902 6222
Email: ManqeleS@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030 PGM 2016/329**							
Project Title	**Glycaemic control and pancreatic beta cells protective effect of a lanosteryl triterpene from Protorhus longifolia in hyperlipidemic and STZ-induced diabetic rats**							
Principal Researcher/ Investigator	SE Mabhida							
Supervisor and Co-supervisor	Dr RA Mosa				Prof AR Opoku			
Department	Biochemistry & Biochemistry							
Nature of Project	Honours/4th Year		Master's	x	Doctoral		Departmental	

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions:

(1) This certificate is valid for 2 years from the date of issue.
(2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-31 October 2017]
(3) Principal researcher must submit a report at the end of project in respect of ethical compliance.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
X	X				
Low Risk		Medium Risk		High Risk	
				X	

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation	X		
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants	X		
Informed consent from parent/guardian			X
Permission for access to sites/information/participants	X		
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire	X		
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research

Professor Gideon De Wet
Chairperson: University Research Ethics Committee
Deputy Vice-Chancellor: Research & Innovation
22 November 2016

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH
ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
22-11-2016
RESEARCH & INNOVATION OFFICE

SE Mabhida - PGM 2016/329

APPENDIX B

B. Additional data

B 1 The effect of RA-3 on body weight of the STZ-induced type 1 diabetic rats.

Table 1. The effect of RA-3 on the Change in body weight (∆BW) after the 28 days treatment of the STZ-induced type 1 diabetic rats.

Experimental group	Initial (g)	Final (g)	∆BW (%)
Type 1 diabetes			
Non-diabetic control	179.50±6.98	195.65±6.25	+15
Diabetic control	184.62±7.17	152.32±5.49	-32****
Diabetic + RA-3	180.40±6.01	195.42±5.65	+15####
Diabetic + metformin	181.61±4.98	193.10±6.14	+14####

Results are expressed as the mean ± SEM and each treatment group contained at least five rats. *** p ≤ 0.001 **** p ≤ 0.0001 vs. non-diabetic control, #### p ≤ 0.0001 vs. diabetic control.

B 2 The effect of RA-3 on food conversion and food efficiency ratio STZ-induced type 1 diabetic rats.

Table 2. The effect of RA-3 on food conversion and food efficiency ratio after the 28 days treatment of the STZ-induced type 1 diabetic rats.

Experimental group	**Food Conversion**	**Food Efficiency Ratio**
Type 1 diabetes		
Non-diabetic control	0.36 ± 0.01	2.75 ± 0.47
Diabetic control	0.72 ± 0.01***	1.40 ± 0.37***
Diabetic + RA-3	0.40 ± 0.03###	2.58 ± 0.12***###
Diabetic + metformin	0.49 ± 0.02**###	2.08 ± 0.28***###

Results are expressed as the mean ± SEM and each treatment group contained at least five rats., *** p ≤ 0.0001 vs. non-diabetic control, ### p ≤ 0.0001 vs. diabetic control.

B 3 Effect of RA-3 on GLUT 2 and liver of the type 1 diabetic rats

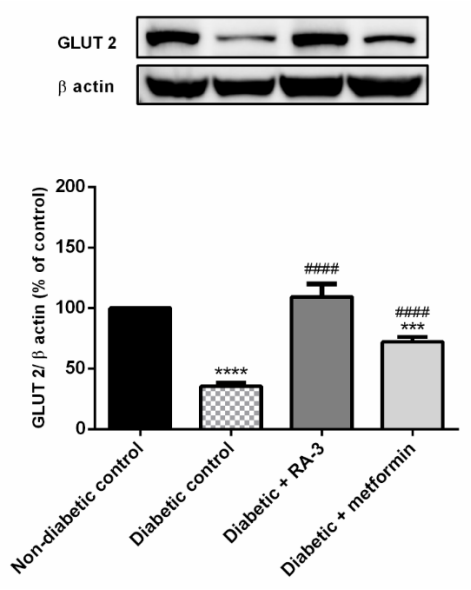

Figure D 1: Effect of RA-3 on GLUT 2 protein expression in the liver in type 1 diabetic rats. Values are expressed as the mean ± SEM (*n*=5). **** $p \leq 0.0001$ vs. non-diabetic control, #### $p \leq 0.0001$ vs. diabetic control. D-Diabetic

PPENDIX C

molecules

MDPI

Article

A Lanosteryl Triterpene from *Protorhus longifolia* Improves Glucose Tolerance and Pancreatic Beta Cell Ultrastructure in Type 2 Diabetic Rats

Sihle E. Mabhida [1], Rebamang A. Mosa [1,*], Dambudzo Penduka [1], Foluso O. Osunsanmi [1], Phiwayinkosi V. Dludla [2], Tryana G. Djarova [1] and Andy R. Opoku [1]

[1] Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa; sihlemabhida@gmail.com (S.E.M.); propafadzo@gmail.com (D.P.); alafin21@yahoo.com (F.O.O.); drdjarova@yahoo.com (T.G.D.); opokuA@unizulu.ac.za (A.R.O.)

[2] Biomedical Research and Innovation Platform (BRIP), South African Medical Research Council, Tygerberg 7505, South Africa; pdludla@mrc.ac.za

* Correspondence: rebamang@gmail.com; Tel: +27-35-902-6824

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Abstract: Type 2 diabetes remains one of the leading causes of death worldwide. Persistent hyperglycemia within a diabetic state is implicated in the generation of oxidative stress and aggravated inflammation that is responsible for accelerated modification of pancreatic beta cell structure. Here we investigated whether a lanosteryl triterpene, methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA-3), isolated from *Protorhus longifolia* can improve glucose tolerance and pancreatic beta cell ultrastructure by reducing oxidative stress and inflammation in high fat diet and streptozotocin-induced type 2 diabetes in rats. In addition to impaired glucose tolerance, the untreated diabetic rats showed increased fasting plasma glucose and C-peptide levels. These untreated diabetic rats further demonstrated raised cholesterol, interleukin-6 (IL-6), and lipid peroxidation levels as well as a destroyed beta cell ultrastructure. Treatment with RA-3 was as effective as metformin in improving glucose tolerance and antioxidant effect in the diabetic rats. Interestingly, RA-3 displayed a slightly more enhanced effect than metformin in reducing elevated IL-6 levels and in improving beta cell ultrastructure. Although the involved molecular mechanisms remain to be established, RA-3 demonstrates a strong potential to improve pancreatic beta cell ultrastructure by attenuating impaired glucose tolerance, reducing oxidative stress and inflammation.

Keywords: type 2 diabetes; hyperglycemia; hyperlipidemia; oxidative stress; inflammation; pancreatic beta cells; antioxidants; triterpenes; *Protorhus longifolia*

1. Introduction

Incidence of type 2 diabetes mellitus, characterized by insulin resistance, is increasing at an alarming rate and remains a serious global health concern [1,2]. Lifestyle modifications such as excessive food intake and lack of physical activity are some of the factors contributing to cellular insulin insensitivity and subsequent insulin resistance [1,2]. Insulin resistance results in the abnormally elevated levels of circulating blood lipids "hyperlipidemia" and glucose "hyperglycemia" observed in type 2 diabetic patients. Hyperlipidemia and hyperglycemia are considered to be the main contributors to type 2 diabetes and associated complications [1,2]. Such complications include accelerated oxidative injury through enhanced generation of free radical species and inflammatory response [3–5]. Increased oxidative stress as well as aggravated inflammatory response in type 2 diabetes are widely-reported phenomena, and are known to cause cellular damage to various organs, including the pancreatic beta cells [3,4]. Furthermore, it has been reported that the increase in pro-inflammatory cytokines such as