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Modulatory and antidiabetic effects of vindoline and *Catharanthus roseus* in type 2 diabetes mellitus induced male Wistar rats and in RIN-5F cell line

By

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DECLARATION

I, Mediline Goboza declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

30 August 2019

Signed   **Date**

Abstract

Diabetes mellitus (DM) is a group of metabolic disorders characterised by persistent high blood glucose levels together with abnormal metabolism of macromolecules. If the hyperglycemia is not controlled, adverse metabolic changes could occur leading to the progressive development of severe complications. Formation of reactive oxygen/nitrogen species and inflammatory responses are principal mechanisms that have been implicated in the development of hyperglycemia-induced tissue damage. The commercially available drugs utilised in the treatment of diabetes have been linked to detrimental side effects hence the need to discover alternative medicines especially from medicinal plants. *Catharanthus roseus* is both a medicinal and ornamental plant that is traditionally used to treat various diseases. It has been reported to possess antidiabetic, anticancer, antimicrobial and antioxidant properties. The plant has been shown to possess more than 100 monotepernoid indole alkaloids which were linked to the plants' antihyperglycemic and antioxidant effects. Therefore, this study was carried out to investigate the effect of vindoline; a bioactive compound derived from *C. roseus* against type 2 diabetes–induced complications. The study also investigated the effects of *Catharanthus roseus* extracts in RIN-5F cell line.

The study was carried out in two parts: viz *in vitro* and the *in vivo* assessments. The *in vitro* study initially investigated the polyphenolic content and antioxidant activities of vindoline and the 3 extracts (methanolic, aqueous and the dichloromethane) of *C.roseus*. The assays used to evaluate the antioxidant capacity of the extracts include oxygen radical absorbance capacity (ORAC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibitory assay. Among the evaluated extracts, the methanolic extract demonstrated both high total polyphenolic content and antioxidant capacity. The HLPC analysis of the extracts was performed and showed highest concentrations of vindoline in the dichloromethane extract and the aqueous extract exhibited the least. The antioxidant activities of vindoline were determined and compared to a known antioxidant, ascorbic acid. Vindoline revealed stronger ORAC activity than ascorbic acid however the ferric reducing antioxidant power did not show any significant differences ($p < 0.05$).

Insulin secretion studies were performed in a β-cell insulinoma cell line- RIN-5F exposed to different concentrations of glucose (high, low and in the absence of glucose). The studies were carried out to compare the β-cell stimulatory effect of vindoline to the extracts. After performing cytotoxic experiments, concentrations that resulted in about 80% cell viability were used to determine the insulin secretory effects. In cells that exposed to glucotoxicity (50 mM glucose), vindoline showed the highest β-cell stimulatory effect ($p < 0.05$) when compared to the untreated controls and to the cells that were treated with the methanolic extract. In cells that were exposed to a low glucose concentration, vindoline additionally showed significant β-cell stimulatory effect at $p < 0.05$ when compared to the aqueous and the methanolic extracts.

Thereafter, the intracellular reactive oxygen species assay (ROSA) was performed in glucotoxicity-induced cells after treatment with vindoline and the respective extracts. The results were compared to the untreated control: vindoline, methanolic and the dichloromethane extracts indicated significant reduction in ROS generation ($p < 0.05$). Further measurement of the release of TNF-α, a pro-inflammatory cytokine in the cells following treatment, the results were not significant among the groups at $p < 0.05$.

The carbohydrate enzymes inhibitory activity of vindoline and extracts of *C.roseus* (50, 25, 12.5 and 6.125 mg/ml) were measured. The alpha glucosidase inhibitory activities of the extracts at 50 mg/ml resulted in < 30% enzyme inhibition with no significant differences among the groups at $p < 0.05$. At lower concentrations, the dichloromethane extract exhibited significantly lower inhibitory activities when compared to the methanolic and the aqueous extract ($p < 0.05$). The alpha amylase inhibitory activity of the methanolic extract was significantly increased at all concentrations; recording the highest enzyme inhibition of approximately 40% ($p < 0.5$). However, the dichloromethane extract did not show any enzyme inhibitory activity. The enzyme inhibitory activity of vindoline was compared to acarbose-a known standard drug, for both enzymes; vindoline did not show appreciable enzyme inhibition when compared to acarbose ($p < 0.05$).

In vivo studies were performed in a type 2 diabetes (T2DM) rat model in which T2DM was induced in 6 weeks old male Wistar rats by having them drink 10% fructose

solution *ad libitum* for 14 days followed by a single intraperitoneal injection of streptozotocin (STZ 40 mg/kg) in freshly prepared 0.1 M citrate buffer (pH 4.5). Animals were randomly divided into six groups (n=8) and received daily treatments for 6 weeks with the vehicle, vindoline (20 mg/kg) or glibenclamide (5 mg/kg) via oral gavage. The effects of the treatments on blood glucose, insulin, body weight, organ weight, serum biochemical parameters, oxidative status, inflammatory markers and tissue histology were assessed in diabetic and non-diabetic rats. Administration of vindoline significantly ($p < 0.05$) reduced the fasting blood glucose in diabetic rats by 15% and significantly increased serum insulin levels when compared to the diabetic controls. Vindoline and glibenclamide significantly ($p < 0.05$) reduced the levels of circulating hepatic enzymes in T2DM; the results were significant when compared to the diabetic controls. Treatment with vindoline significantly improved the hepatic antioxidant status as indicated by increased ORAC, superoxide dismutase and catalase activities, indicative of the protective effect of vindoline in diabetes-induced hepatic injury. Assessment of the levels of pro-inflammatory cytokines in the hepatic tissue indicated remarkable reduction of TNF-α by (-41%) and IL-6 (-28%) in diabetic rats treated with vindoline when compared to the diabetic controls ($p < 0.05$).

The serum lipid profile showed marked increases in the levels of serum lipids (triglycerides, low density lipoproteins, total cholesterol and very low density lipoproteins) in diabetic controls when compared to all treatment groups ($p < 0.05$). Therefore, vindoline and glibenclamide showed possible protective effects against diabetes-induced cardiovascular disease. Kidney function assessment revealed increased levels of urea and creatinine in the diabetic control group. Vindoline and glibenclamide significantly reduced the urea and creatinine levels in diabetic rats.

Vindoline additionally improved the FRAP in diabetic hearts. The SOD activity and ORAC were increased while lipid peroxidation was reduced in the kidneys of diabetic rats treated with vindoline when compared to the diabetic control ($p < 0.05$).

Histopathological assessment in diabetic rats showed severe damage of the liver, kidney and pancreas. Treatment of diabetic rats with vindoline restored the structure of these organs which was indicated by minimum structural changes. The expression of pro-apoptotic marker caspase 9 in response to glucose stress was significantly higher in the diabetic control group when compared to all the treatment groups.

Treatment with vindoline showed remarkable reduction of caspase 9 expression in the diabetic rats.

In conclusion, persistent high blood glucose levels resulted in free radical induced tissue damage in the type 2 diabetes rat model. Vindoline demonstrated protective effects against diabetes induced hepatic, cardiac, pancreatic and nephritic injuries. In addition, vindoline improved insulin secretion in both *in vitro* and *in vivo* setups hence the findings suggest that vindoline could be an important agent that can be considered in the treatment and management of diabetes and diabetic complications.

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I would to appreciate Dr S. Meyer for her kindness and support. Thank you.

Dedication

I dedicate this thesis to God Almighty and to my parents for their love and support.

Preface

The thesis is made up of six chapters written in article-based format according to the author guidelines of the journal where each manuscript has been submitted to.

Chapter one: Briefly introduces the study by giving relevant information on the meaning of diabetes, its aetiology, prevalence and its global burden. The chapter provides a summary of the mechanisms that are implicated in the development of diabetic complications. The aims/objectives, research questions and hypothesis of the study are stated in this chapter.

Chapter two: Provides the literature review in detail, explaining the links between hyperglycemia, insulin resistance, beta cell dysfunction, oxidative stress, inflammation and apoptosis. The role oxidative stress plays in the development of diabetic complications is highlighted in this chapter. Previously reported medicinal activities of *Catharanthus roseus* are included in this chapter. This chapter forms part of a book chapter in the book **Bioactive Compounds of Medicinal Plants Properties and Potential for Human Health (Apple Academy Press INC: ISBN: 9781771886482).**

Chapter 3: Focuses on the *in vitro* studies of vindoline and the extracts in which the antioxidant, beta cell stimulatory effect and alpha amylase/ alpha glucosidase inhibitory activities were assessed. The manuscript has been submitted for publication to **Saudi Journal of Biological Sciences.**

Chapter 4: Presents experimental findings of the *in vivo* study entitled 'Vindoline effectively ameliorated diabetes-induced hepatotoxicity by docking oxidative stress, inflammation and hypertriglyceridemia in type 2 diabetes-induced male Wistar rats'. The manuscript has been published in **Biomedicine and Pharmacotherapy Journal 2019**. The findings were also presented at an international conference: **3rd International Conference: Medicinal Plants in Healthcare. Society of Medicinal Plants and Economic Development, Johannesburg, South Africa, 2018**.

Chapter 5: Is the research article entitled, 'Vindoline—A Natural Product from Catharanthus Roseus Reduces Hyperlipidemia and Renal Pathophysiology in Experimental Type 2 Diabetes and was published in the **Biomedicines Journal**. The work was presented at an international conference: **6th International**

Conference: Research, Innovation and Technology for African Development, Cape Town South Africa, 2018.

Chapter 6: Presents the general discussion and conclusion of the entire study.

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Abbreviations

AAPH - Azobis (2-amidino-propane) dihydrochloride

ABTS - 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ADP - Adenosine diphosphate

AGEs - Advanced glycated end products

ALT - Alanine aminotransferase

ANOVA - Analysis of variance

AQ- Aqueous

AST - Aspartate aminotransferase

ATP - Adenosine triophosphate

AUC- Area under curve

BCL_2- B-cell lymphoma 2

CAT – Catalase

DC-Diabetic controls

DAG-Diacylglycerol

c-JNK c-Jun N-terminal kinase

DM - Diabetes mellitus

DMACA - p-Dimethylaminocinnamaldehyde

DN-Diabetic nephropathy

DNA - Deoxy ribonucleic acid

DNP-Diabetic neuropathy

DR-Diabetic retinopathy

CR-Aq- aqueous extract of Catharanthus roseus

CR-DCM-dichloromethane extract of Catharanthus roseus

CR-Meth-methanolic extract of Catharanthus roseus

CRP-C-reactive protein

CVDs - Cardiovascular diseases

DPPH- 2,2-diphenyl-1-picrylhydrazyl

EDTA - Ethylenediaminetetraacetic acid

EGF- Endothelia growth factor

ESRD- end stage renal diseases

$FeCl_3$ - Iron (III) chloride

FFA-Free fatty acids

FRAP- Ferric reducing antioxidant power

GPX- Glutathione peroxidise

GSH- Reduced glutathione

GSIS-Glucose stimulated insulin secretion

HbA_1-C-Haemoglobin A-1C

HOCl- Hydrogen oxychloride

H_2O_2 - Hydrogen peroxide

HDL- High density lipoprotein

HNO_2-Nitrous oxide

HOCl- Hydrochlorous acid

IDDM- Insulin-dependent diabetes mellitus

IHC- Immunohistochemistry

IL-1 β- Interleukin-1 beta

IL-6- Interleukin-6

IL-10 Interleukin-10

IL-18- Interleukin-18

Inos-Inducible nitric oxide synthase

IR-Insulin resistance

IRS-Insulin receptor substrate

LDH- Lactate dehydrogenase

LDL- Low density lipoprotein

MAPK- Mitogen-activated protein kinase

MCP-1- Monocyte chemotactic protein-1

MDA- Malondialdehyde

NADH- Nicotinamide adenine dinucleotide

NADPH- Nicotinamide adenine dinucleotide phosphate

NAFLD-Non-alcoholic fatty liver disease

NC-Normal controls

NCD-Non communicable diseases

NEFA- Non-esterified fatty acids

NFk-B Nuclear factor kappa-light-chain-enhancer of activated β- cells

NIDDM - Non-insulin-dependent diabetes mellitus

NO- Nitric oxide

NC- Normal control

O_2^-- Superoxide anion

OADs- oral antidiabetic drugs

OH- Hydroxyl anion

ONOO- Peroxynitrite

ORAC- Oxygen radical absorbance capacity

OS-Oxidative stress

PKC - Protein kinase C

RNS- Reactive nitrogen species

RONOO- peroxynitrates

ROS - Reactive nitrogen species

SEM-Standard error mean

SD - Standard deviation

SOD- Superoxide dismutase

SRC- Standard rat chow

SSB-Sugar sweetened beverages

STZ - Streptozotocin

T1DM-Type 1 diabetes mellitus

T2DM- Type 2 diabetes mellitus

TBA - Thiobarbituric acid

TBARS- Thiobarbituric acid reactive substances

TC- Total cholesterol

TCA-Tricarboxylic acid cycle

TG- Triglycerides

TNFα - Tumor necrosis factor alpha

TPTZ- Tripyridyl triazine

VLDL-Very low density lipoprotein

WHO- World Health Organization

Chapter 1

Introduction

1.1 Background

Diabetes mellitus (DM) is a complex metabolic disorder that is clinically characterised by high blood glucose levels ≥ 7.0 mmol (Afolayan and Sunmonu, 2010; International Diabetes Federation, 2015 and 2017). Diabetes was first described by a Greek physician named Areateus in the 2nd century, where he used the term *diabainein* referring to the excessive passing out of fluid that he constantly observed in diabetic patients (Eshaq *et al.*, 2017; Lakhtakia, 2013). The Latin word 'mellitus' which means 'sweet-like honey' was later added to the word diabetes both terms indicating the sweet taste of urine that was passed by diabetes patient (glucosuria) (Ahmed, 2002; Karamanou *et al.*, 2016). Another rare form of diabetes identified as diabetes insipidus is in existence and is characterised by excessive urine output and fluid intake as a compensatory reflex action against fluid loss. Diabetes insipidus emerge due to the attenuated production of vasopressin, a hormone that controls water retention by reducing urine output (Bockenhauer and Bichet, 2015). Although the two disorders exhibit the same symptoms, their diagnosis and treatment are not the same (Afolayan and Sunmonu, 2010; Palumbo *et al.*, 2018).

Diabetes mellitus is a heterogeneous disorder that is classified into four main categories; type 1 DM (T1DM), type 2 DM (T2DM), gestational DM and DM that develop secondary to other conditions. The different types of DM are classified on the basis of aetiology, pathogenesis, disease progression and phenotypic manifestations (American Diabetes Association, 2014). In general, chronic hyperglycemia referring to elevated blood glucose levels is a common finding in all types of DM (Kerner, 2014). Hyperglycemia has been implicated in the development of detrimental effects on various organs such as the heart, kidneys, eyes, nerves, liver and blood vessels (American Diabetes Association, 2014). This classical endocrine disease arises as a result of a number of detrimental processes that

include autoimmunity, genetic defects and unhealthy life style practices (Asmat *et al.*, 2016). Insulin is a peptide exocrine hormone secreted by the pancreas and regulates glucose homeostasis. Deficiencies or absolute lack of this hormone affect normal metabolism of macronutrients leading to the development of diabetes (Bugianesi *et al.*, 2005; Tangvarasittichai, 2015). DM may alternatively arise as a result of insulin resistance characterised by tissue insensitivity to synthesised insulin (Abo *et al.*, 2008; Esser *et al.*, 2014).

Diabetes mellitus has recently been reported as a growing global health crisis (IDF, 2017). Several studies predicted DM to reach pandemic levels affecting people in both developing and developed countries. Based on the extrapolations done by the World Health Organization (2016), high blood glucose was named to be the third highest risk factor for premature mortality after high blood pressure and tobacco abuse. The global report of WHO on diabetes (2016) also documented that 422 million adults were suffering from DM in 2014. Alarmingly, when this figure is compared to the number of adults that were diagnosed with DM in 1980 globally, the 2014 estimates increased by more than two times. The current global estimates of DM predict 425 million people (between the ages 20-79) have DM, and account for 8.8% of the global population (IDF, 2017). Moreover, drastic escalation of DM prevalence is expected to affect 629 million people by the year 2045 if no proper strategies are taken promptly (Cho *et al.*, 2018).
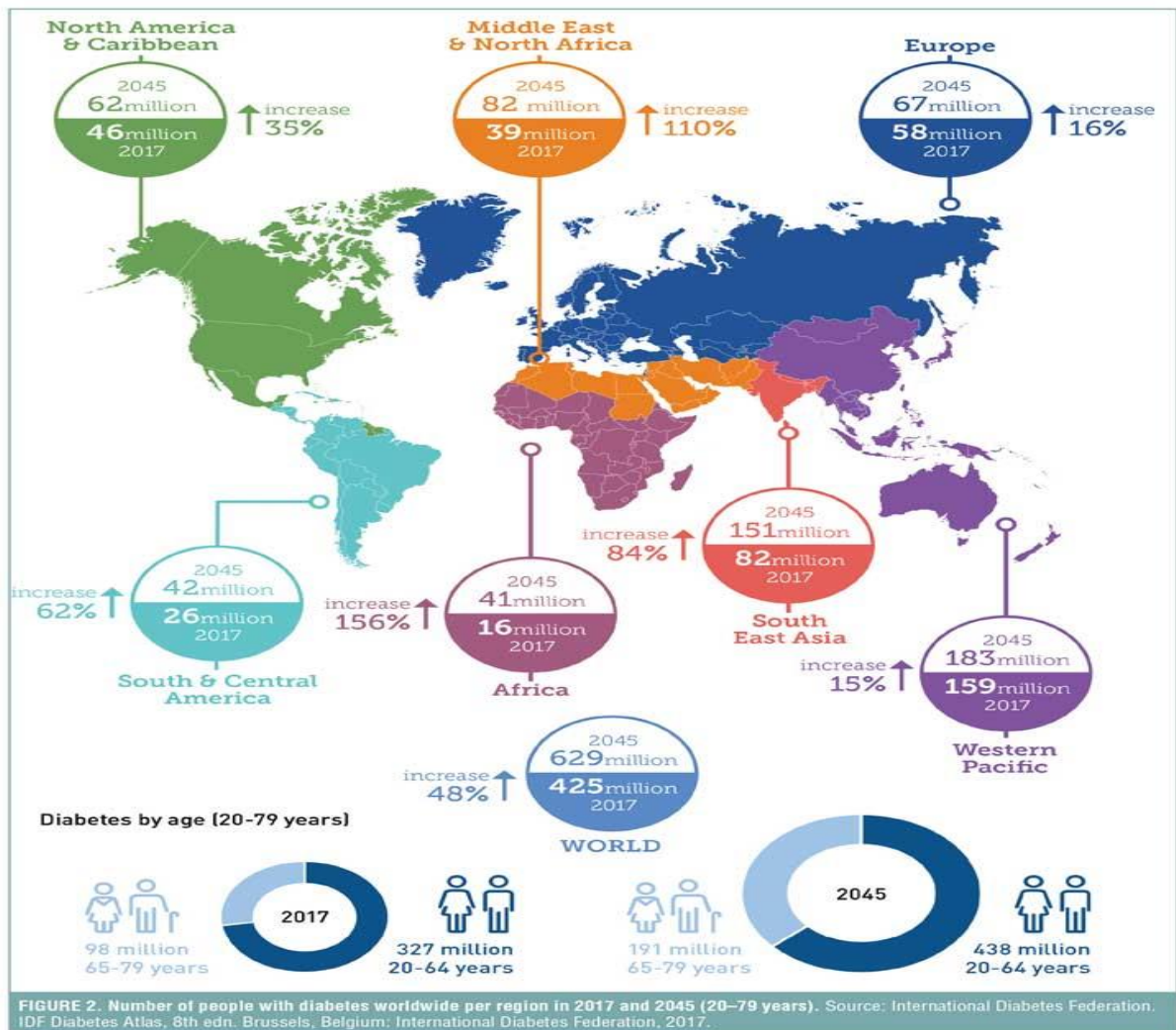
Figure 1: Regional estimates of diabetes (Adapted from International Diabetes Federation (IDF) 2017)

According to the global estimates of diabetes that were reported in 2015 by IDF:

- One in every 11 adults is reported to have diabetes

- One in every 2 adults with diabetes is undiagnosed

- 12% of the global health expenditure was spent on DM

- Globally; 1 in every 7 births was affected by gestational DM affecting about 25% of South East Asia births

- 522 000 juveniles were suffering from T1DM and the majority recorded in Europe

- More than 2/3 of people that had DM in Africa were not conscious of it.

- In North America and in the Caribbean region; 1 in every 8 adults had diabetes
- 37% of the adult population with diabetes lived in the Western Pacific

The same IDF report indicated that more than two thirds of people living in Africa have undiagnosed DM. An approximate of 14.2 million people in Sub-Saharan region had DM in 2014. More than 50% of them were documented to be residing in South Africa, Democratic Republic of Congo, Nigeria and Ethiopia (IDF, 2017). The management of DM and its complications in developing countries is difficult, therefore adequate health resources are required to offer proper health amenities.

1.2 The global economic burden of Diabetes mellitus

In 2012, it was estimated that DM and its secondary disorders were the root causes of the approximately 3.7 million deaths. About 43% of these deaths occurred in individuals that were ≤ 70 years old, under the demographic active group (World Health Organization, 2016). Based on the above reported estimates, it is reasonable to say that DM inflicts a substantial economic strain on household setups resulting into decreased productivity. Recent estimates on health costs incurred in 2017 as a result of diabetes were reported to have reached USD 727 billion. The burden of DM has an impact on health care systems owing to frequent hospitalisation, daily drug dependence and work absenteeism (Karuranga and Duke, 2018). If not adequately controlled, DM may lead to severe blindness, lower limp amputations, kidney failure and cardiovascular associated disorders. Other statistical reports showed that in 2010; DM was responsible for 4.5% of visual impairment and blindness cases (Nentwich and Ubig, 2015; Sayin *et al.*, 2015). A constellation data from 54 countries also revealed that 80% of the end-stage renal diseases (ESRD) were as a result of DM. These complications directly contribute to the regression of economic growth (IDF, 2017).

The rapid increase in the prevalence of DM is fuelled by the changes in the lifestyle patterns contributed by urbanisation, obesity, physical inactivity, smoking and sedentary practises (Hu, 2011; Guariguata *et al.*, 2014). Obesity and consumption of

foods high in glycaemic index have been labelled as major risk factor determinants of DM development. The detrimental consequence of diets high in calories is emergence of insulin resistance that further triggers the development of T2DM (Bugianesi *et al.*, 2005). Insulin resistance is a metabolic defect that predisposes to beta cell dysfunction and has been defined as the inability of target tissue to respond normally to the action of insulin. Beta cell dysfunction arises as a result of some compensatory responses that the cells undergo in trying to maintain normal glucose homeostasis (Canivell and Gomis, 2014; Zhang *et al.*, 2013). The release of insulin by pancreatic beta cells is considered to be a direct measure of beta cell function. Impairment of insulin secretion is therefore the earliest evidence of an individual's progression towards T2DM (Verma and Hussain, 2017). The metabolic effects of insulin resistance impel the onset of a cluster of metabolic comorbidities such as hypertension, hypertryglyceridemia and abdominal obesity. This constellation together increases the risk of co-morbidities such as cardiovascular related diseases (CVD), fatty liver disease, gallstones, polycystic ovary syndrome, obstructive sleep apnoea and gout (Jiamsripong *et al.*, 2008). The ever-rising prevalence and mortality rates of CVDs globally have been reported to be a major health concern. Moreover, CVDs were ranked to be the number one killer among the non-communicable diseases, with a prevalence rate of 31% according to the World Health Organisation (WHO, 2011). New therapeutic strategies should therefore aim at treating, preventing or correcting underlying disorders and complications (Kahn *et al.*, 2014).

It is well established that T2DM and IR play key roles in the progression of inflammatory processes (Feng *et al.*, 2016). Inflammatory cytokines such as TNF-α (tumour necrosis factor alpha), IL-6 (interleukin-6), C-reactive protein (CRP) and IL-18 (interleukin-18) are secreted by several tissues e.g. adipose tissue resulting in inflammation, oxidative tissue damage, recruitment of macrophages, stimulation of smooth muscle and fibroblast expansion (Navarro-González and Mora-Fernández, 2008; Elsawy and Emara, 2016). Extreme release of inflammatory cytokines directly enhances the development of metabolic disturbances and tissue damage. TNF- α in addition contributes to insulin resistance by inhibiting insulin receptor signalling (Agrawal and Kant, 2014).

Diabetic complications progress as a consequence of hyperglycaemia-induced oxidative stress (Tangvarasittichai, 2015). Previous studies reported increased levels

of oxidative stress biomarkers in the serum of diabetes mellitus patients (Rolo and Palmeira, 2006; Verdile *et al.*, 2015). Oxidative stress occurs following the overproduction of free radicals that negatively affect biological antioxidant systems. Several pathways have been implicated in the development of hyperglycaemic-oxidative tissue damage and includes, formation of advanced glycation end-products, increased glucose flux through the polyol pathway; activation of the protein kinase (PKC) pathway, glucose auto oxidation and increased activity of the hexosamine pathway (Giacco and Brownlee, 2010; Tangvarasittichai, 2015). Hyperglycemic milieu furthermore activates a transcription factor called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB). Activation of NF-KB favours the expression of inducible nitric oxide synthase (iNOS). The molecule iNOS is a key component required in the production of nitric oxide which has a high affinity of superoxide molecules, the reaction of the latter two results in the production of peroxynitrite. Peroxynitrite is a strong reactive oxygen species that increases lipid peroxidation and oxidative stress in cell membranes (Maritim *et al.*, 2003; Dias *et al.*, 2005; Ighodaro and Akinloye, 2017).

High blood glucose levels in diabetes mellitus promotes oxidative stress and dysregulated apoptosis leading to cell death especially hepatocytes and endothelial cells (Habib, 2013). Apoptosis is described as a process of physiological cell death that maintains tissue homeostasis and it is characterised by cellular shrinkage, chromatin condensation and DNA fragmentation (Ayepola *et al.*, 2013). A positive relationship between hypergylcemia-induced oxidative stress and apoptosis has been demonstrated and the two factors synergistically propel the development of diabetic complications (Oyenihi *et al.*, 2017). Production of free radicals in diabetes is also associated with the production of pro-apoptotic factors (Ayeleso *et al.*, 2014). It has been suggested that; in diabetic rats, renal tubular epithelial cells undergo ROS-dependent apoptosis which was indicated by Bax protein expression. In the above-mentioned cells, Bax protein expression increases mitochondrial permeability resulting in the release of cytochrome C (Tomita, 2017).
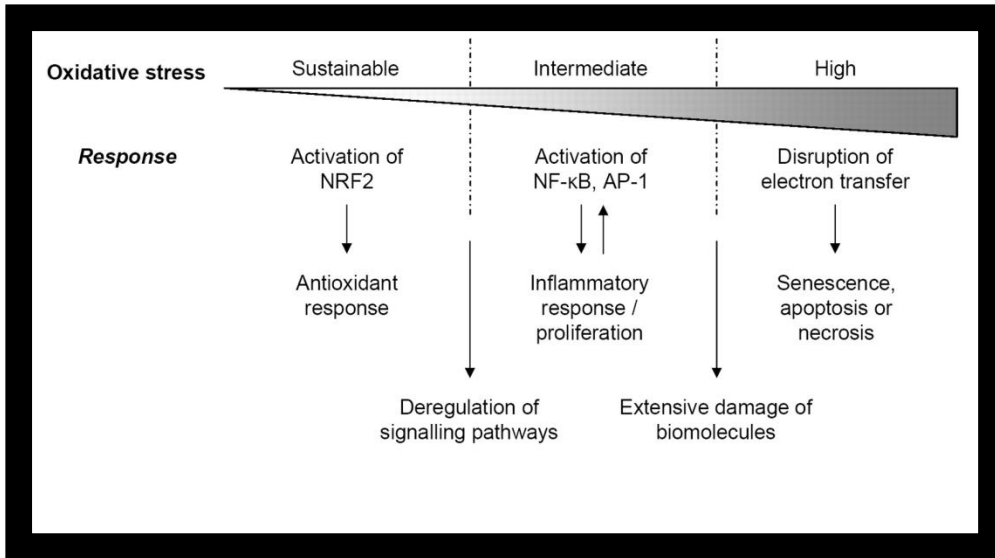
Figure 2: Represents the link between hyperglycemia-induced oxidative stress, inflammation and apoptosis. (Adapted from Sesti *et al.*, 2012).

1.3 Aims

Although the antidiabetic effect of vindoline was previously demonstrated in type 2 diabetic rats, no study to the best of our knowledge has explored the effects of vindoline on complications that arise as result of diabetes. Therefore, the study aimed at evaluating vindolines' effect on the pathways such as oxidative stress, inflammation and apoptosis that play principal roles in the development of diabetic complications. The study also aims to compare the effect of the vindoline on insulin secretion with effects of different extracts of *C.roseus*.

1.4 Objectives

1. To evaluate antihyperglycemic and hypolipidemic effects of vindoline in diabetic and non-diabetic rats.
2. To assess the antioxidant, anti-inflammatory and anti-apoptotic effects of vindoline in the kidney, liver and heart tissue lysates of normal and diabetic rats.
3. To measure and assess liver and kidney function biochemical parameters.

4. To evaluate the antioxidant effects of vindoline and the extracts of *C.roseus* n RIN-5F cells exposed to glucose toxicity.

5. To evaluate and compare the cytotoxic effects of vindoline and *C.roseus* extracts in RIN-5F cells.

6. To determine and compare the effect of vindoline and the extracts on insulin secretion in RIN-5F cells.

7. To assess the inhibitory effect of vindoline and the extracts on carbohydrate metabolising enzymes.

1.5 Research Questions

1. What are the possible effects vindoline have on serum lipid profile and glycemic parameters in T2DM and in normal rats?

2. What potential antioxidant effects do vindoline has in diabetic and in normal rats?

3. What are the effects of vindoline on inflammatory biomarkers in the liver, heart and kidney of normal and diabetic rats?

4. Does vindoline has any effects on the expression of apoptotic proteins in normal and diabetic rats?

5. What are the potential effects of vindoline on liver and kidney function in both diabetic and non-diabetic rats?

6. What are the possible effects of vindoline treatment on the release of insulin in RIN-5F cells?

7. Does vindoline possess better insulin secretory effects in RIN-5F cells when compared to the extracts of *C.roseus.*

8. Do vindoline and the extracts possess any inhibitory effects on the activities of carbohydrate metabolising enzymes

1.6 Hypothesis

H 0 'Vindoline would not effectively reverse hyperglycemia- induced tissue damage in i*n vivo.*' It was further hypothesized that vindoline would not reveal better insulin secretion in RIN-5F cells when compared to *C.roseus* extracts.

H 1 'Vindoline would reverse hyperglycemia–induced tissue damage *in vivo.*' We hypothesized that vindoline would exhibit appreciable insulin secretion in RIN-5F cells when compared to *C.roseus* extracts.

Chapter 2

Literature review

2.1 Classification of DM

Diabetes mellitus was originally classified into two classes: T1DM and T2DM (WHO, 1980). However, owing to the inconsistent patterns of DM pathogenesis; it was later categorised into four main classes based on certain distinct features such as aetiology, signs and symptoms presented by the patient at the time of diagnosis as well as patient history (American Diabetes Association) (ADA, 2014; IDF, 2017). These four types include T1DM, T2DM, gestational diabetes and the secondary class of DM. The secondary form of DM encompasses all types of diabetes that arise as a result of other causes: for instance, monogenic diabetes syndromes, diseases of the exocrine pancreas and drug/chemical and virus induced DM (IDF, 2015).

All forms of DM are characterised by an elevation of blood glucose, known as hyperglycemia. At the time of diagnosis, the patient should exhibit fasting blood glucose levels ≥ 7.0 mmol/L; or postprandial hyperglycemia: ≥11.1 mmol/L) due to either decrease in insulin secretion and/or insulin sensitivity of target tissues (ADA, 2014).

2.2 Type 1 diabetes mellitus

T1DM is a form of DM that predisposes patients to reliance on exogenous insulin in order to maintain a close to normal metabolism of glucose. It develops mainly in juveniles or young adults, accounting for 5-10% of reported cases of DM (IDF, 2015). T1DM abruptly develops after β-cell destruction leading to complete deficiency of insulin (Ozougwu, 2013). The onset of T1DM is associated with the presence of autoantibodies in most of these patients (Itariu and Stulnig, 2014). The autoantibodies isolated from the patients are against chief components that play

important roles in regulation of glucose metabolism e.g. insulin, β cells, tyrosinase phosphates and glutamine acid decarboxylase (Krishna and Srikanta, 2015).

The aetiology of T1DM still remains obscure, however genetic and environmental factors are strongly believed to take part in the production of autoantibodies that facilitates autoimmune destruction. Examples of these environmental factors include certain foods, viral infections caused by adenoviruses, cytomegalovirus and Cox B virus (Paschou *et al.*, 2018).

T1DM has been reported to occur with no known cause and this subclass of T1DM can be referred to as idiopathic DM (ADA, 2014). Idiopathic DM patients are mostly found in Africa and Asia. Idiopathic T1DM patients in these regions neither have autoantibodies nor abnormal immunologic evidence of T1DM (Piñero-Piloña and Raskin, 2001).

Besides hyperglycemia, insulinopenia and diabetic ketoacidosis are common characteristics of T1DM. Ketoacidosis is caused by diminished and altered glucose metabolism by peripheral tissues thus triggering lipid breakdown to act as the alternative source of energy (Krishna and Srikanta, 2015). Uncontrolled lipid catabolism results in increased levels of acetyl coenzyme A. Accumulation of acetyl coA favours ketogenesis which may lead to disturbances in maintaining normal body pH. In the case of T1DM, if ketoacidosis occur, death could result (Paschou *et al.*, 2018).

2.3 Type 2 DM.

Diabetes mellitus (T2DM) is a fast-growing global health threat that could reach pandemic proportions if no appropriate measures are taken urgently to prevent it (Bos and Agyemang, 2013). T2DM is the form of DM that outnumbers all of the known DM classes and described as non-insulin dependent diabetes mellitus (NIDDM). It is responsible for almost 90% of DM cases reported. Its progression has been observed in the adult population (>30 years old) with initial characteristics of high blood glucose levels, insulin resistance and/ deficiency (Olokoba *et al.*, 2012). Contrary to T1DM, T2DM is not caused by autoimmunity rather by lifestyle and genetic factors. Insulin resistance and deficiency are hallmarks of T2DM that eventually leads to hyperglycaemia (Palomer *et al.*, 2008). Patients who suffer from this type of DM do not solely depend on exogenous insulin, but hyperglycaemia is

controlled by lifestyle modifications and treatment with correct oral antidiabetic drugs (OADs) (Chatterjee *et al.*, 2017). In T2DM, the pancreas still retains its ability to produce insulin, however; it is the peripheral tissues that fail to respond appropriately to insulin, resulting into insulin resistance (IR) (Meier and Bonadonna, 2013). In addition; inadequate insulin secretion by β cells in T2DM individuals disturbs normal homeostatic functions of hepatic cells (gluconeogesis), myocytes (glucose uptake) and adipose (lipolysis) tissues (Gastaldelli, 2011; Esser *et al.*, 2014).

The risk factors of T2DM mainly revolve on behavioural practices and these include: physical inactivity, sedentary lifestyle, ethnicity, aging and obesity (Bos and Agyemang, 2013; Wu *et al.*, 2014).

The heterogeneous nature of T2DM has led to the emergence of various proposals and hypothesis regarding its pathogenesis. These encompass the following contingencies which will be discussed in detail:

- Obesity

- Reduction in β cell mass

- β cell dysfunction and exhaustion

- Insulin resistance (IR) (Akash *et al.*, 2013; Esser *et al.*, 2014; Tangvarasittichai, 2015; Yabe *et al.*, 2015).

2.4 Insulin action

The metabolism of macromolecules is tightly controlled by several hormones such as insulin and glucagon (Perry *et al.*, 2014). Insulin is an anabolic peptide hormone secreted by the pancreatic β-cells and is responsible for homeostatic utilisation and storage of dietary macromolecules thus maintaining correct circulating levels of glucose (Perry *et al.*, 2014; Karalliedde and Gnudi, 2016). After consumption of a meal loaded with carbohydrates, enzymes such as salivary amylase initiate the breakdown of polysaccharides into disaccharides. Disaccharides are further broken down to several monomers including glucose causing elevation of extracellular glucose levels (Mann and Bellin, 2016). When this happens, the β pancreatic cells become sensitised to oxidise glucose in turn stimulating adenosine triphosphate

(ATP) production in the mitochondria (Ripoll *et al.*, 2007). This reaction promotes an increase in ATP/ADP ratio resulting in closure of K+-$_{ATP}$ channels subsequently depolarizing the plasma membrane causing influx of Ca^{2+}, followed by insulin secretion (Fu *et al.*, 2012). The release of insulin enables the uptake of glucose in the myocytes and adipocytes while preventing hepatic glucose output by the inhibition of glycogenolysis and gluconeogenesis. In addition, insulin has the ability to inhibit lipolysis in adipocytes thus preventing the release of free fatty acids from adipocytes (Figure 3) (Bessesen, 2001; Meshkani and Adeli, 2009). In the event of prolonged fasting, the liver converts stored lipids, glycogen and even amino acids to glucose via the action of glucagon (Ripoll *et al.*, 2007).
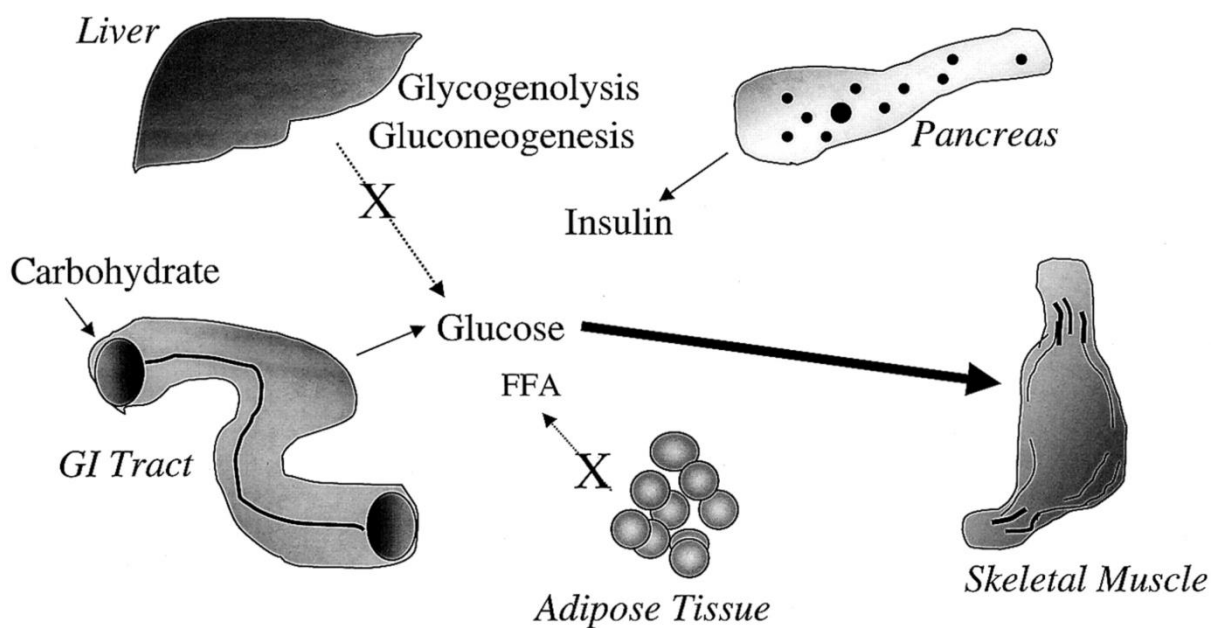
Figure 3: Insulin action. GI tract: gastrointestinal tract; FFA: free fatty acids; Adapted from (Bessesen, 2001).

2.5 Insulin resistance

Obese and individuals who consume diets that are rich in fats and calories have been reported to have a degree of insulin resistance. Insulin resistance can be defined as a metabolic disorder often recognised by tissue insensitivity to circulating levels of insulin (Kendall and Harmel, 2014). Insulin insensitivity is observed in the peripheral organs involved in glucose storage such as skeletal muscle, adipose tissue and the liver (Björnholm and Zierath, 2005). In early stages of IR, the pancreatic β cells respond by releasing more insulin in order to compensate for deteriorating glucose uptake by the peripheral organs as shown in Figure 4. As a result of the insensitivity of these organs to insulin, the fasting insulin levels in blood continue to escalate up to a point where the β cells can no longer compensate for insulin resistance (Jain and Saraf, 2010). In due course, hyperinsulinemia, hyperglycemia and dysfunction and loss of pancreatic β-cells occur resulting in obvious T2DM (Björnholm and Zierath, 2005). In conjunction to glucose intolerance, patients with IR were further reported to present with metabolic abnormalities which include hypertension, dyslipidaemia and deranged blood clotting processes (Spence *et al.*, 2010).
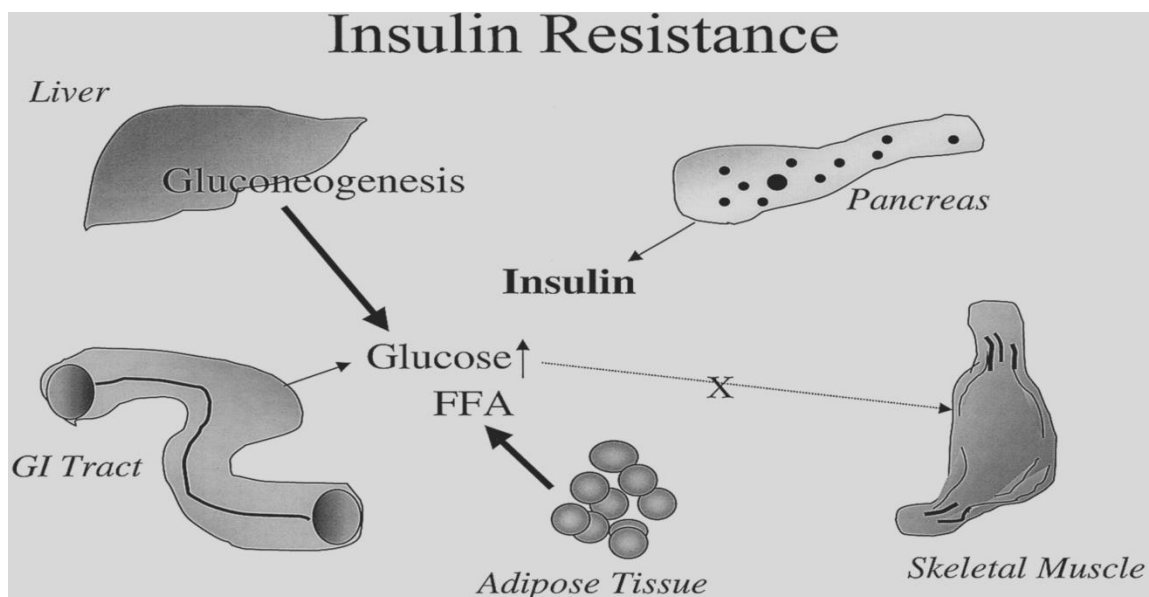
Figure 4: Insulin resistance. GI tract: gastrointestinal tract; FFA: free fatty acids; Adapted from (Bessesen, 2001)

2.6 Obesity and lipid induced IR

Obesity is a catastrophic disorder responsible for the health burden of non-communicable disease in both developed and developing countries with its prevalence uncontrollably escalating (Williams *et al.*, 2015). The terms 'overweight' and 'obesity' are defined as build-up of excess adipose tissue on and/ around body organs such that both physical and psychosocial well-being are affected (Al-Goblan *et al.*, 2014). It develops from uncontrolled processes involved in energy storage and expenditure following consumption of fructose and fat-rich foods. In the Southern African region; rapid transition to westernised lifestyles has led to a marked increase in obesity trends with females greatly affected (Cois and Day, 2015).

Obesity is regarded to be the chief factor responsible for the development of metabolic diseases (Esser *et al.*, 2014). Hypertrophy and hyperplasia of adipose tissue especially in area around the abdomen triggers over secretion of hormones, adipokines, glycerol and proinflammatory mediators, and non-esterified fatty acids (NEFAs). These substances are implicated in the modulation of abnormal insulin signals that promotes IR (Al-Goblan *et al.*, 2014).

Circulating NEFAs play significant roles in the development of IR especially in the skeletal muscle. In obese individuals there is increased transportation of NEFAs to muscle together with impaired intracellular metabolism of fatty acids (Kahn and Flier ,2000). Impaired fatty acid metabolism in myocytes and adipocytes results in the formation of products such as diacylglycerol, fatty acyl CoA and ceramides (Perry *et al.*, 2014). These products activate phosphorylation of serine/threonine sites located on insulin receptor substrates (IRS-1 and IRS-2). Consequently, the ability of IRS to activate PI3-kinase is affected resulting in deranged GLUT-4 activity. When this happens, uptake of glucose into skeletal muscle fails resulting in hyperglycemia and insulin resistance (Shulman, 2000).

2.7 Hepatic insulin resistance mechanism

2.7.1 Molecular mechanism behind hepatic IR

The liver is an important organ that coordinates and controls metabolism of carbohydrates, lipids and proteins. In the event of hepatic IR, the hepatocytes fail to respond to insulin's inhibitory effect on glycogenolysis and gluconeogenesis causing overwhelming glucose production (Meshkani and Adeli, 2009). Insulin resistance has been linked to defects in fatty acid delivery and uptake promoting excessive production of diacylglycerol (DAG) in the liver. DAG has been implicated in the activation and translocation of protein kinase Cε isoform (PKCε) to the cell membrane (Jornayvaz and Shulman, 2012). When this occurs; insulin receptor substrate-2 (IRS2) and PI(3)K fail to phosphorylate correctly leading to the suppression of insulin signalling. Defects in insulin signalling disrupt normal Akt2 activities, reducing activities of 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Perry *et al.*, 2014). Compromised activity of PDK1 in turn inhibits phosphorylation of glycogen synthase kinase-3 (GSK3) thus attenuating the activity of glycogen synthase (GS) which mediates glycogen synthesis. In addition, abnormal Akt2 signalling is linked to increased hepatic gluconeogenesis by amplifying the translocation of Fork head box protein O1 (FOXO1) to the nucleus. FOXO 1 movement into the nucleus has been shown to increase the expression pro-gluconeogenic targets (Samuel *et al.*, 2004; Perry *et al.*, 2014).

2.8 Beta cell dysfunction

For β cells to function correctly there must be an intact structural integrity in the parenchyma of the pancreas. Beta cell dysfunction is a critical complication of insulin resistance that leads to the development of T2DM and therefore potentiates its complications (Cerf, 2013). It is characterised by impaired or failure of β cells to secrete insulin following insulin resistant states (Kahn, 2003). Consumption of foods with high glycemic index such as sugar sweetened beverages (SSBs) and fatty or carbohydrates has been implicated in increased glucose stimulated insulin secretion

(GSIS) by the β cells. Excessive exposure of normal β-cells to glucose elicits liberation of copious amounts of insulin (hypersecretion) which induce increase in β-cells mass (hypertrophy and hyperplasia) to compensate for insulin resistance (Puddu *et al.*, 2013). Continuous exposure to glucose and FFA eventually desensitises normal β cells resulting in reduced GSIS. Reduced GSIS has been shown to disrupt the metabolism of glucose as well as the biosynthesis of insulin (Weir and Bonner-Weir, 2004). Erroneous expression of several genes that code for important enzymes (glucose-6-phosphatase, fructose-1,6- bisphosphatase, lactate dehydrogenase, and hexokinase) involved in glucose metabolism has been reported to accompany β cell dysfunction (Saisho, 2015). In due course, the progressive failure of normal glucose metabolism induces β cell apoptosis via inflammation, oxidative stress, mitochondrial dysfunction and endoplasmic reticulum stress (Poitout and Robertson, 2002).

2.9 Fructose: a major risk factor in T2DM development

Fructose is a monosaccharide that makes a large proportion of sucrose a natural sugar found in fruits, sugar cane and beets (Tappy and Lê, 2012). Dietary intake of refined fructose (high fructose corn syrup) has dramatically increased globally because of its incorporation in food and drinks (SSBs) as a sweetener (Wong *et al.*, 2016). Apprehensions concerning the safety of ingesting refined fructose are becoming apparent as it has been linked to serious health problems like dental caries, obesity, overweight, IR, metabolic syndrome and NAFLD (Berg, 2014; Balakumar *et al.*, 2016).

 Because fructose is a monosaccharide, when ingested, it is directly absorbed into the bloodstream from the small intestines. The intestinal epithelial cells found in the small intestines houses GLUT-2 and GLUT-5 transporters that are responsible for the transportation of monosaccharides (Klurfeld, 2015). Once absorbed, the metabolism of fructose immediately occurs in the liver where it is phosphorylated by the enzyme fructokinase into fructose-1- phosphate. Fructose-1-phosphate is then broken down by aldolase forming two triose intermediates: glyceraldehydes and dihydroxyacetone phosphate (Malik and Hu, 2015). The formed glyceraldehydes

subsequently undergo conversion to form glyceraldehyde-3-phosphate which can be metabolised to form acetyl-coenzyme A that is utilised in the tricarboxylic acid (TCA) cycle. It is acetyl-coenzyme A that is further oxidised to eventually form glucose, glycogen, lactate, pyruvate and fatty acids (Samuel, 2011; Softic *et al.*, 2016). The uncontrollable formation of these substances further promotes lipogenesis and overt accumulation of triglycerides (Basciano *et al.*, 2005).

Alarmingly, studies that were done in high fructose fed animals revealed increased plasma levels of very low-density lipoprotein (VLDL), decreased clearance of VLDL, reduced insulin sensitivity as well as impaired glucose metabolism (Basciano *et al.*, 2005). Significant accumulation of body and visceral fat in conjunction with IR were observed as early as 6 weeks following high fructose diet introduction in rats (Tappy and Lê, 2012). Mechanistic studies done to compare metabolic effects that manifest after high fat diet and high fructose diet showed similarities in the emergence of dyslipidemia, chronic inflammation and defects in insulin signalling (Basciano *et al.*, 2005). In addition, another study reported that animals that were fed with a high fructose diet exhibited extremely high levels of uric acid in the serum. Uric acid negatively impacts the production of endothelial nitric oxide (NO)- an essential component in insulin signalling. Moreover, NO plays a key role in vascular flow of blood to the skeletal tissue, hence increasing delivery and uptake of glucose by the myocytes. Diminished levels of endothelial NO promote the development of IR (Ang and Yu, 2018).

2.10 Oxidative stress in DM

Over the years, free radical build has been widely connected to the pathogenesis of diseases such as cancer, diabetes and neurodegenerative disorders. Free radicals are molecules that possess unpaired electrons that make them unstable and very reactive (Oguntibeju *et al.*, 2016). Free radicals exist as either reactive oxygen species (ROS) which include superoxide anion ($\bullet O_2^-$), hydroxyl ($\bullet OH$), peroxyl ($\bullet RO_2^-$), hydroperoxyl ($\bullet HRO_2$) or as reactive nitrogen species (RNS) such as nitric oxide ($\bullet NO^-$), nitrogen dioxide ($\bullet NO_2^-$) (Hurrle and Hsu, 2017). Molecules such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl), peroxynitrite (ONOO), nitrous oxide (HNO_2) and peroxynitrates (RONOO) have also been shown to take

part in oxidative tissue damage (Valko *et al.*, 2007; Verdile *et al.*, 2015). Metabolic disturbances that arise in diabetes shift the balance between free radicals and endogenous antioxidants towards free radicals ensuring oxidative stress. Moreover, these disturbances cripple the antioxidant defence systems rendering the tissue susceptible to damage (Niedowicz and Daleke, 2005). It is well accepted that the excess glucose levels in DM favours the formation of these unstable entities resulting in oxidative stress (Oguntibeju *et al.*, 2016). Because free radicals are highly reactive, they react with macromolecules such as carbohydrates, proteins and DNA altering their structures and functions (Stewart, 2010). Additionally, oxidative stress predicts the development of diabetic complications through activating other detrimental pathways such as inflammation, apoptosis, endothelial dysfunction and insulin resistance intensifying tissue damage (Verdile *et al.*, 2015).

Malignant transformations of tissues that arise following OS are predominantly observed in the eyes, nervous system, blood vessels and the kidneys (Pazdro and Burgess, 2010). In diabetes, it is believed that several pathways play critical roles that lead to oxidative stress and emergence of diabetic complication and are presented in Figure 5.

2.11 The mechanisms implicated in diabetes oxidative tissue damage

I. Increased glucose flux through the polyol pathway

Hyperglycemia has been reported to activate the polyol pathway through the action of aldose reductase (AR). In hyperglycemic conditions, AR catalyzes the conversion of glucose to sorbitol. Consequently, the formed sorbitol is metabolised to fructose by sorbitol dehydrogenase (Babizhayev *et al.*, 2015). Conversion of glucose to sorbitol depletes NADPH as a result of its oxidation in the reaction. Diminished levels of NADPH negatively affect the production glutathione- an important enzyme that plays major roles in antioxidant defence systems. When the antioxidant systems are attenuated, free radical attack on biological molecules will not be controlled resulting in tissue destruction (Brownlee, 2004).

II. Formation of advanced glycation end products (AGEs)

In the absence of catalytic enzymes, high levels of glucose if not controlled can covalently react with proteins, lipids and nucleic acids resulting in the formation of irreversible products (Chikezie *et al.*, 2015). The glycation process mainly targets or modifies structural and functional proteins like collagen, albumin and globulins. Modification of collagen by AGEs for instance disturbs the cell-cell matrix leading to impaired cellular adhesion/migration and apoptosis (Nowotny *et al.*, 2015). In diabetes, exaggerated glycation of molecules and accumulation of AGEs was reported to alter the expression of some genes, stimulate pro-inflammatory and ROS/RNS release hence contributing towards the pathogenesis of diabetic complications (Chikezie *et al.*, 2015; Tangvarasittichai, 2015).

III.   Activation of the protein kinase (PK) C pathway

The protein kinase C (PKC) enzyme belongs to the serine/threonine kinase family. These enzymes catalyze phosphorylation of proteins that take part in signal transduction (De Marchi *et al.*, 2013). The activity of PKC is significantly activated by diacylglycerol (DAG), which is severely expressed in hyperglycemia via *de novo* synthesis (Evcimen and King, 2007). Once activated by DAG or by high glucose concentrations, PKC increases the permeability of the endothelial cells worsening the progression of diabetic vascular complications (Rask-Madsen and King, 2013). Furthermore, activation of PKC in vascular tissue is implicated in abnormal cell growth and cytokine activation (Budhiraja and Singh, 2008). Another line of evidence suggests that increased activity of PKC could be triggered by ROS mechanisms that inhibit glyceraldehydes-3-phosphate dehydrogenase (Lazo-de-la-Vega-Monroy and Fernndez-Mej, 2013).

IV.   Increased activity of the hexosamine pathway.

Increased flux of intracellular glucose into the hexosamine pathway has been linked to the genesis of diabetic complications. This pathway involves the diversion of fructose-6-phosphate from glycolysis producing glucosamine-6-phosphate in a reaction catalysed by glutamine: fructose-6-phosphate amidotransferase (GFAT) yielding UDP-*N*-acetylglucosamine (Brownlee, 2004; Giacco and Brownlee, 2010). This pathway is essential for the synthesis of

glycolipids and glycoproteins glycosyl side chains. Increased hexosamine flux has been shown to decrease the expression of sarcoplasmic reticulum Ca 2+ - ATP-ase in cardiomyocytes, whereas in vascular smooth muscle cells it enhances the induction of TGF-β and plasminogen activator inhibitor–1 promoting the development of adverse changes in those tissue (Buse, 2006).
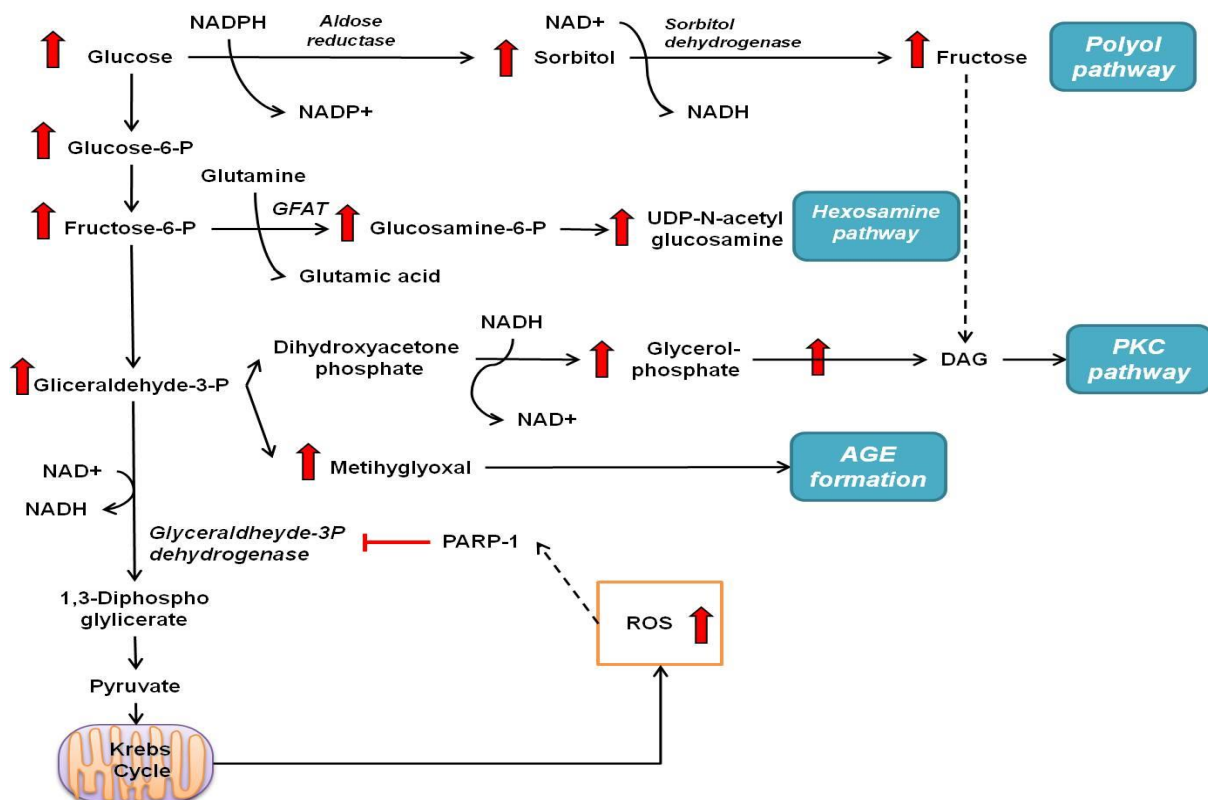
Figure 5: Metabolic pathways involved hyperlgycemia induced oxidative stress (adapted from (Lazo-de-la-Vega-Monroy and Fernndez-Mej, 2013).

2.12 Oxidative stress and insulin resistance

Oxidative stress is a major participant in pathways that lead to impaired glucose tolerance, insulin resistance, β-cell dysfunction and eventually, type 2 diabetes. Over nutrition is believed to be a link between oxidative stress and the earlier mentioned changes (Park *et al.*, 2009).

During normal metabolism, glucose is metabolised through glycolysis and the TCA cycle to produce ATP. However, in overweight and obese individuals, due to excess

glucose in circulation, more glucose is oxidized generating extra NADH and $FADH_2$ leading to increased mitochondrial superoxide anion generation which is a ROS (Park *et al.*, 2009; Verdile *et al.*, 2015). Furthermore; superoxide release in obesity is generated via FFA and acetyl coenzyme A (CoA) oxidation in TCA cycle that generates excess NADH and $FADH_2$ electron donors (Wolf, 2004; Tangvarasittichai, 2015). Pancreatic β cell is one of the tissues with high metabolic activity but with least concentrations of enzymatic antioxidants rendering them susceptible to adverse oxidative changes (Wang and Wang, 2017). Free radical build-up in β cells has additionally been reported to impair glucose-stimulated insulin responses, decrease β-cell gene expression and apoptosis. This impaired β-cell function decreases efficient insulin production contributing to hyperglycemia and eventually type 2 diabetes (Furukawa *et al.*, 2004). Taken together, oxidative stress actively damage pancreatic β-cells, thereby affecting its functionality, hence potential treatments of T2DM must aim to mitigate oxidative stress and/ improve the antioxidant responses (Verdile *et al.*, 2015).

2.13 Role of oxidative stress in inflammation responses in diabetes

It has been long suggested that inflammation plays a principal role in the pathogenesis of insulin resistance, impaired insulin secretion, β cell dysfunction, T2DM and diabetes-related complications (Pollack *et al.*, 2016). Inflammation is a physiologic response of the immune system that serves as a critical defence mechanism following the body's invasion with pathogens and foreign irritants; thus preventing tissue injury (Donath *et al*., 2013; Donath, 2014). Inflammatory responses involve the recognition of the molecular prototypes found on the surfaces of pathogens by pattern-recognition receptors located on cell membranes of immune cells (Oguntibeju, 2018). Recognition of these prototypes activates nuclear factor-kappa-B (NF-κB) signaling pathways, thus eliciting infiltration and activation of the immune cells resulting in subsequent release of inflammatory mediators like chemokines and cytokines to eliminate the pathogen/ irritant (Navarro-González and Mora-Fernández, 2008; Oguntibeju, 2018).

In diabetes, oxidative stress, high blood glucose and free fatty acids have been suggested to be key players that promote adverse inflammatory responses.

(Ambade and Mandrekar, 2012). Adverse inflammatory responses are characterized by a relative plasma increase of cytokines such as TNF-α, IL-6, IL-1β and CRP. (Navarro-González and Mora-Fernández, 2008). The toxic effects of glucose and lipids induce oxidative and endoplasmic reticulum stress through activation of thioredoxin-interacting protein (TXNIP) and the NLR family, pyrin domain containing 3 (NLRP3) inflammasome (Pollack *et al.*, 2016). Activation of these molecules in-turn triggers the release of active IL-1β. IL-1β is a pro-inflammatory cytokine that is capable of enhancing the release of other cytokines and chemokines thereby recruiting macrophages resulting in adverse inflammatory responses (King, 2008). It has also been shown that these adverse inflammatory responses exert fibrosis and destruction of tissues eventually leading to apoptosis (Calle and Fernandez, 2012). On the other hand, TNF-α has been implicated in a decline of normal insulin signalling mechanisms via decreasing the activities of insulin receptor substrate (IRS)-1 tyrosine phosphorylation, phosphatidylinositol 3-kinase (PI3K) and AKT (Domingueti *et al.*, 2016).

A mutual relationship between oxidative stress and chronic inflammation has been established in which both mechanisms work in synergy (Wang and Wang, 2017). The continuous accumulation of ROS/RNS in hyperglycaemic milleu has been shown to activate certain kinases such as ERK, JNK and p38 which are involved in pathways that drive inflammation (Calle and Fernandez, 2012). Furthermore, ROS can stimulate transcription factors, e.g. NF-kB and activator protein-1 (AP-1), to stimulate the transcription of genes that encode for pro-inflammatory cytokine. Peroxynitrite 'a ROS' has also been linked to the reduction of nitric oxide and increasing inflammation in vascular tissues (Elmarakby and Sullivan, 2012). On the other hand, some experimental studies demonstrated that TNF-α can activate the enzyme NADPH oxidase through the PKC/PI3 and mitogen-activated protein kinase (MAPK) pathways prompting the generation of ROS resulting in propagation of diabetic complications (Navarro-González and Mora-Fernández, 2008). It is therefore possible that immunomodulatory and antioxidant treatments in diabetes could significantly improve hyperglycemia, β-cell function, and/or insulin resistance. Moreover, targeting diabetes induced inflammation may ameliorate diabetic complications especially those that are vascular in nature.

2.14 Complications of diabetes mellitus

Diabetes is a complex disorder that is associated with devastating changes in body tissues ultimately leading to the formation of diabetic complications (Davey *et al.*, 2014). DM complications are grouped into two categories namely the microvascular and macrovascular complications shown in Figure 6 below. The former comprises of complications that affect small blood vessels of the kidney, eyes and neurons eventually leading to renal failure, vision impairment known as retinopathy and neuropathy. Stroke, cardiovascular and peripheral artery diseases on the other hand are regarded as macrovascular complications (Davey *et al.*, 2014; Sayin, 2015).

The extent of these complications and the risk of death increases with long-term failure in maintaining blood glucose levels that are close to normal (DeFronzo *et al.*, 2013). High morbidity and mortality rates are reported in DM patients owing to the effects of macrovascular complications e.g. myocardial infarction which occur 3 times more in DM patients when compared to non-diabetics (Bandeira *et al.*, 2013).

2.14.1 Diabetic Nephropathy

Diabetic nephropathy (DN) is a microvascular complication of DM and is the major cause of end stage renal disease in diabetic patients (Lin *et al.*, 2018). DN is characterised by both classic structural and functional changes, causing drastic alterations in glomerular permeability, filtration, thickness of the glomerular basement membrane, expansion of the mesangial matrix. Clinically, these changes manifests as hypertension, proteinurea and reduction in kidney function (Umanath and Lewis, 2018). The effect of changes finally predispose to development of glomerulosclerosis and interstitial fibrosis (Gallagher and Suckling, 2016). This kidney disease affects close to 40% of all diabetic patients worldwide and is also the main cause of mortality in T2DM as a result of the its link with cardiovascular events such as stroke, hypertension and atherosclerosis (Vinod, 2012).

 Hyperglycemia is implicated in the development of DN, the mechanisms by which it causes DN has been suggested to be through the effect of AGEs on structural

proteins. As previously discussed, glycation of proteins is known to produce modified protein products (Domingueti *et al.*, 2016). In diabetic kidneys; the extracellular proteins such as collagen are glycated resulting in the formation of Amadori products which form cross link with different molecules in cells, hence altering both the structure and function of the renal tissue (Soldatos and Cooper, 2008; Tangvarasittichai, 2015). Formation of AGEs has been shown to cause the formation and accumulation of immune complexes that amplifies the innate immune system to produce inflammatory changes (Lin *et al.*, 2018). The emergence of DN can be associated with factors like inflammation, oxidative stress, inflammation and haemodynamic and genetic factors. Haemodynamic events that occur in DN were significantly linked to inflammation. The association of renin and its receptor was reported to trigger the production of inflammatory cytokines (TNF-α, IL-1β, TGF-β1, vascular endothelial growth factor (VEGF) in the kidneys of diabetic patients (Donate-Correa *et al.*, 2015). Increased levels of TNF-α activate NF-κB; activation of NF-κB directly triggers the infiltration of renal interstitial inflammatory cells leading to inflammatory responses. In addition, unwarranted activation of NF-κB produces transcription signals for pro-inflammatory cytokines (Lin *et al.*, 2018). Furthermore, persistent uptake of glucose into renal cells level has been shown to trigger the production of oxidative stress especially through the mitochondrial superoxide anion formation (Wolf, 2004). Different pathways that favour oxidative stress in hyperglycemic conditions have been shown to cause renal fibrosis and dysfunction as well as renal cell death (Cao and Cooper, 2011).

2.14.2 Cardiomyopathy

A substantial percentage of T2DM patients are obese. Obesity has been recognised as an independent risk factor for cardiovascular disease (CVD) in diabetic patients (Paneni, 2014). CVD is regarded as the chief cause of morbidity and mortality in diabetes mellitus patients (Low Wang *et al.*, 2016). Compared to non-diabetic individuals, diabetic patients have a 3 fold risk of developing CVDs (Oktay *et al.*, 2018). Hyperglycemia, IR, oxidative stress and inflammation remain the hallmarks that kindle metabolic disturbances underlying atherosclerosis and heart failure in

T2DM patients (King and Grant, 2016). In T2DM, excess adipose tissue is a major source of cytokines, chemokines and adipokines which trigger chronic inflammatory events in blood vessels (Poirier *et al.*, 2006). It has been documented that elevated blood glucose levels down-regulate the expression of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO). Nitric oxide is an important vasodilator involved in the function of blood vessels. Therefore, low bioavailability of NO in the endothelium induces vasoconstriction and ultimately endothelial dysfunction (Low Wang *et al.*, 2016).

The superoxide radical and activation of PKC have also been implicated in the inactivation of NO and hence impelling endothelial damage and atherosclerosis in diabetic patients (Kasznicki and Drzewoski, 2014). It is suggested that IR cause dyslipidemia characterised by hypertriglyceridemia, low HDL cholesterol, increased LDL and VLDL. Dyslipidemia is an indicator of coronary artery risk because the circulating lipids can be easily oxidised thus activating the immune cells. These inflammatory cells lodge in the intima of the blood vessels in attempt to digest the oxidised lipids and the end result is formation of atherosclerotic plaques (Dokken, 2008; Paneni, 2014).

2.14.3 Diabetic neuropathy

Diabetic neuropathy (DNP) can be defined as the systemic loss of nerve fibres resulting in attenuated autonomic and somatic functions. Excruciating pain, constant tingling, burning, shooting, sharp-like and electric shock-like sensations are early features of DNP (Baron *et al.*, 2010; Schreiber, 2015). As the disease progresses, major modifications of the motor functions of limbs occur presenting as muscle wasting and weakness (Tesfaye *et al.*, 2013). These changes may further cause development of foot ulcers which are highly prone to complications such as infections, gangrene, amputation and death if not properly managed (Nasiri *et al.*, 2015).

It is widely recognised that oxidative stress is the principal mechanism involved in the generation of nerve damage in diabetes (Babizhayev *et al.*, 2015). Reduction in

antioxidant pools (particularly in the polyol pathway), formation of AGEs, activation of PKC, lipid peroxidation and damage to DNA have been reported to disrupt the lipids of the myelinated nerves hence obliterating the microvasculature of the peripheral nervous system (Negi *et al.*, 2011; Ko and Cha, 2012; Dewanjee *et al.*, 2018). It has also been proved that elevation of pro-inflammatory cytokines may take part in inflicting peripheral nerve damage. Therefore, new therapeutic approaches should target these mechanisms that drive development of DNP symptoms (Dewanjee *et al.*, 2018).

2.14.4 Diabetic Retinopathy

Diabetic retinopathy (DR) is a common microvascular complication of diabetes characterised by neovascularisation and oedema in the retina of the eye. It is a major cause of visual impairment and blindness in the middle-aged population (Tarr *et al.*, 2013). The risk of developing DR increases with poorly controlled blood glucose levels, high blood pressure and hyperlipidemia (Stewart, 2010). Several biochemical pathways that activate oxidative stress and inflammation in diabetes have been suggested to be potential participants in the aetiology DR (Eshaq *et al.*, 2017). Another line of evidence demonstrated the effect of leucocytes on the pathogenesis of DR. The hyperglycaemic environment in DM triggers the migration of leucocytes into the retinal area. There, they damage the endothelium of the vessels which eventually leads to retinal endothelial dysfunction, angiogenesis, increased retinal perfusion and vascular permeability (Ciulla *et al.*, 2003).
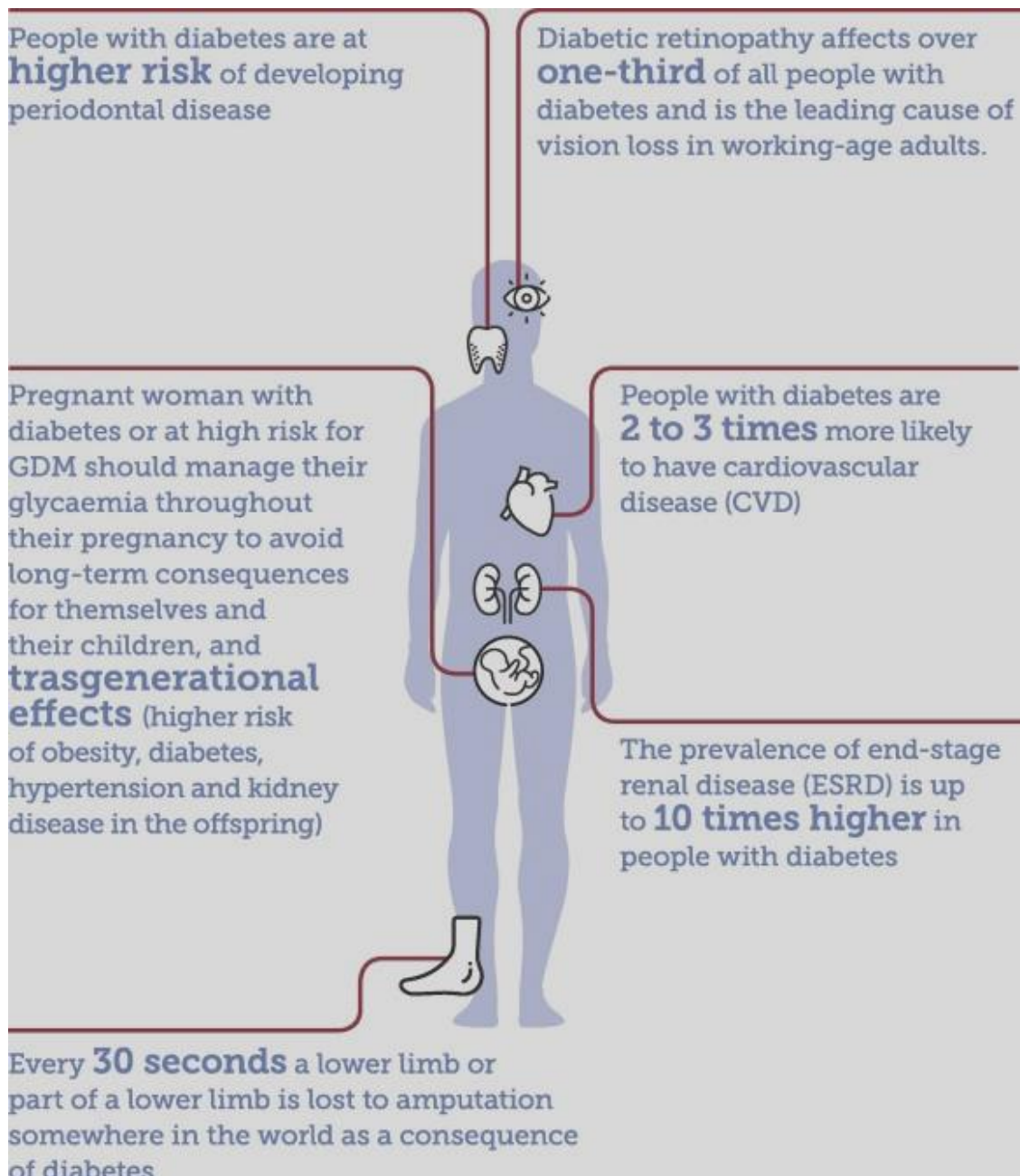
Figure 6: Complications of diabetes mellitus (adapted from IDF, 2017)

2.15 Treatment and management of T2DM

In T2DM therapy, sustaining controlled levels of glucose and HbA$_1$-C in the blood has been linked to the delay and prevention of the onset of macrovascular and microvascular complications (Marín-Peñalver *et al.*, 2016). Researchers and physicians worldwide are fully engaged in concerted efforts to discover different strategies that can effectively treat and possibly cure T2DM (Lind *et al.*, 2014).

Because T2DM is a heterogeneous condition, different pharmacological and non-pharmacological therapeutic strategies are currently used manage it. However, despite the availability of these well-tolerated pharmacological therapies, a significant number of diabetic patients fail to reach recommended glycemic targets in addition to the uncomfortable side effects they endure (Ahmed, 2010).

2.15.1 Non-pharmacological strategies

This category comprises of lifestyle interventions that can be employed by prediabetics and diabetics to prevent the onset of diabetes and diabetic complications respectively. Pursuing healthy dietary intake, weight loss and physical exercise is recommended to restore energy levels close to normal, thus contributing to the achievement of normoglycemia (Chaudhury *et al.*, 2017).

2.15.1.1 Diet

The majority of T2DM patients are either overweight or obese owing to genetic factors and unhealthy dietary intake to a larger extent (Sami *et al.*, 2017). Therefore, correct nutritional interventions in T2DM patients are essential to reverse deleterious metabolic changes which can lead to complications (Chaudhury *et al.*, 2017). Increased daily consumption of foods that contain lower glycemic indexes such as fruits, vegetables, poultry, legumes and whole grains has been associated with reduced oxidative stress, increased weight loss and insulin resistance (Asif, 2014).

2.15.1.2 Weight loss

Obesity appears to play a key role in the development of insulin resistance; for that reason, weight loss is essential in diabetics (Chaudhury *et al.*, 2017). It has been documented that weight loss in T2DM patients has successfully led to reduction in dyslipidemia, hyperglycemia and blood pressure thus decreasing the risk of cardiovascular complications. Increased physical activity is recommended in diabetic patients because it boosts energy expenditure thus improving insulin sensitivity

(Thent *et al.*, 2013; Asif, 2014). Additionally, long sessions of exercise facilitate the intracellular uptake of glucose by the skeletal muscles, as this happens, the intra-abdominal fat will be reduced resulting in decreased risk for IR (Sami *et al.*, 2017).

2.16 Behavioural practices

Unhealthy behavioural lifestyles are regarded as major causes of morbidity and mortality (Punjani *et al.*, 2018). Cigarette smoking is a well-known risk factor implicated in the development of diseases like cancer, diabetes, respiratory and cardiovascular diseases (Chang, 2012). Hazardous effects of smoking in T2DM have been linked to activation of chronic inflammatory responses, free radical formation, dyslipidemia, β cell and endothelial dysfunction (Chang, 2012; Prasad & Cucullo, 2015). It was also reported that smoking directly cause hyperglycemia, however, the exact mechanism on how it does so remains obscure. Other researchers believe that enhanced concentrations of epinephrine and norepinephrine released as a result of smoking could possibly stimulate hepatic gluconeogenesis and glycogenolysis (Sari *et al.*, 2018). Diabetic patients who smoke were shown to have a higher threat of cardiomyopathy development that contributes to increased mortality (Choi *et al.*, 2018). Patients with T2DM are therefore recommended to stop smoking in order to control and prevent diabetes and its complications.

2.16.1 Alcohol Consumption

Although moderate consumption of alcohol has been shown to prevent the development of T2DM, however; binge drinking of alcohol can lead to defective insulin secretion and sensitivity leading to increased risk of T2DM (Carlsson *et al.*, 2003; Nakanishi *et al.*, 2003).

2.16.2 Pharmacological drugs used in the treatment and management T2DM

Effective treatment and management of T2DM requires correct combinations of pharmacological drugs to successfully reverse the numerous pathophysiological abnormalities (DeFronzo *et al.,* 2013). Therapies presently used target 'the ominous octet' which refers to the eight core defects observed in T2DM pathophysiology (shown in Figure 7). These include:

i.   reduced insulin secretion

ii.   diminished incretin effect in the ileum

iii.   increased lipolysis

iv.   Enhanced renal glucose reabsorption

v.   decreased glucose uptake in the liver, skeletal and adipose tissues

vi.   neurotransmitter dysfunction in the brain tissue

vii.   increased hepatic glucose output

viii.   Increased glucagon secretion by the pancreatic α cells (Defronzo *et al.*, 2014; DeFronzo *et al.*, 2015; Thrasher, 2017).
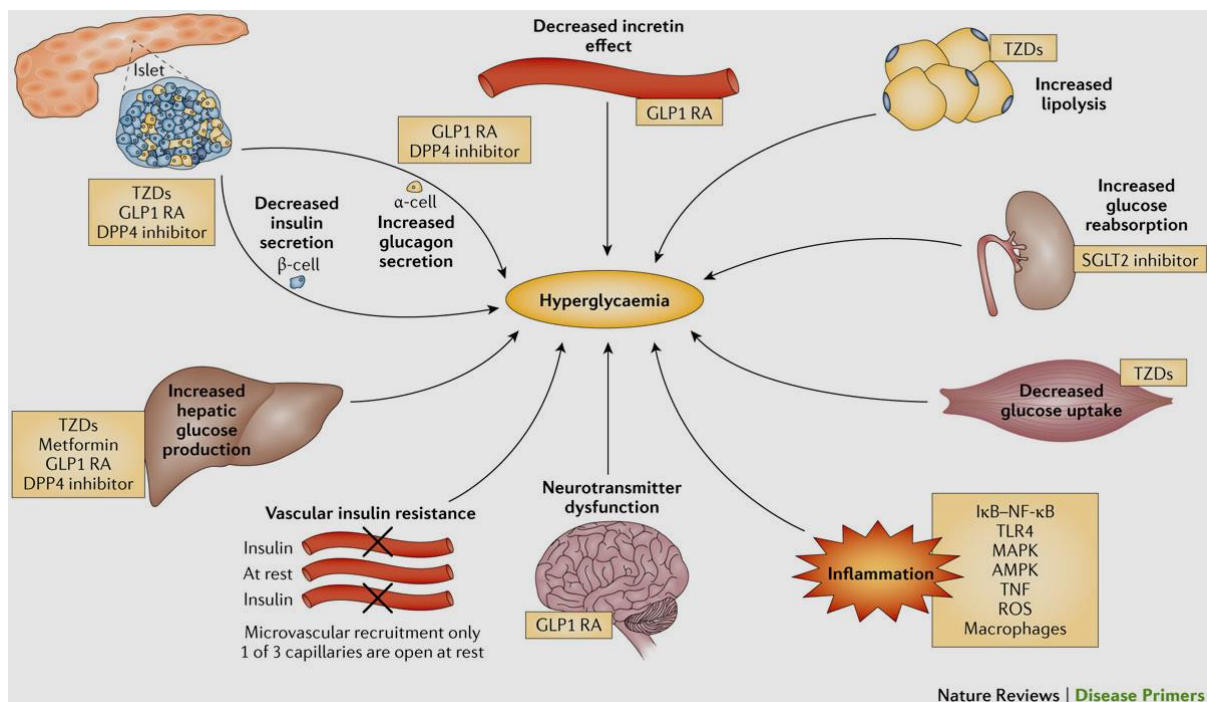
Figure 7: Pharmacological drugs used in the management to diabetes mellitus. Adapted from (DeFronzo *et al.*, 2015).

Early initiation of pharmacotherapy is important in delaying and in the prevention of the risk of irreversible complications. Currently, there are 6 classes of antidiabetic drugs used in the treatment of diabetes mellitus. These include thiazolidinediones (TZDs), sulfonylureas, sodium-glucose cotransporter (SGLT2) inhibitors, biguanides, meglitinides, dipeptidyl peptidase 4 (DPP-4) inhibitors and α-glucosidase inhibitors (Davis, 2004). Although these drugs are being used in the treatment regimens of DM, unwanted effects have been reported as indicated in Table 1.Therefore, safer, more effective alternative treatments especially from natural products are needed to control the progression diabetes (Medagama and Bandara, 2014).

Table 1: Examples of commonly used pharmacological drugs and their mechanisms of action

Class of drugs	Example	Mode of action	Disadvantages	Reference
Sulfonylurea	Glibenclamide	Stimulate β-cell to release insulin	Hypoglycaemia Weight gain Skin reactions Hyponatremia Lack of glycemic durability	(Bösenberg and van Zyl, 2008)
Thiazolidinediones	Pioglitazone	Increase insulin sensitivity of cells and decreases hepatic glucose output	Water retention Weight gain	(Prabhakar and Doble, 2011)
Biguanides	metformin	Prevents hepatic glucose out put	Nausea Metallic aftertaste Diarrhea Lactic acidosis vitamin B12 and folic acid deficiency	(Chaudhury *et al.*, 2017)
Alpha glucosidase inhibitors	Acarbose	Slows intestinal absorption of sugars	Bloating Excess gas passing	(Bösenberg and van Zyl, 2008)
Glucagon-like peptide-1 (GLP-1) agonist	Liraglutide	Enhance insulin secretion, supresses postparandial glucagon secretion	Vomitimg Nausea	(Nauck, 2016)
Amylin	Pramlintide	Delays gastric emptying, decreases glucose levels circulation	Hypoglycaemia	(Prabhakar and Doble, 2011)

2.17 Medicinal Plants in human health

Medicinal plants have been defined as naturally occurring substances that possess potent compounds that can be utilised for therapeutic reasons or synthetically modified to pharmacological drugs (Verma and Singh, 2008). Plants are fundamental to human and animal lives as both food and medicine. For thousands of years, various cultures relied on plants as the primary source of health care. It is estimated that almost 80% of the developing countries' and 40-60 % of the developed countries' populations still depend on medicinal plants for the management and treatment of ailments (Farnsworth *et al.*, 1985; Samy and Gopalakrishnakone, 2007; Verma and Singh, 2008). Africa is a continent where the use of medicinal plants is greatly practised for traditional and therapeutic purposes. The heavy dependence on medicinal plants is undeniably as a consequence of their accessibility, safety and affordability (Nasri *et al.*, 2015). It is documented that 122 pharmacological drugs have been developed from 94 plant species among the 250,000 plant species available globally, although more than 20,000 have been documented to have medicinal values (Fennell *et al.*, 2004; Srinivasan, 2005). Unfortunately, the validation and inclusion of medicinal plants in the orthodox treatment regimens are still lagging especially in Africa.

Throughout history, medicinal plants have been used to alleviate symptoms of various diseases such as cancer, diabetes, hypertension, infertility, gastrointestinal problems, infections, headaches etc (Joshi and Joshi, 2000). Globally, information pertaining to certain plants' medicinal activities has been passed from generation to generation, though some of the claims have been scientifically proven to be erroneous (Bailey and Day, 1989). Fortunately, scientific studies are able to confirm some of these claims and establish the importance of medicinal plants in health care. Plant-based diets composed of fruits, vegetables, spices, teas and seeds have been linked to the decreased risks of developing non-communicable diseases (NCDs) such as cancer and diabetes mellitus (Ramos, 2008; Torres-Avilez *et al.*, 2015). In the past decades, the aetiology of NCDs in Africa was generally low. However, due to rapid globalisation and behavioural changes; increased trends of NCDs are now common. Inclusion of genetically modified organisms was unusual in Africa, while

consumption of natural products such as plant materials was common; thus explaining prior lower trends of NCDs (Udenta *et al.*, 2014).

No doubt, scientific advances have played a major role in the management of human diseases while on the other hand, orthodox formulations have been reported to have unfavourable effects, in addition to the high costs that make it impossible for the populace in developing countries to rely on (Nasri *et al.*, 2015). This calls for comprehensive scientific investigations on medicinal plants for the development of cheap novel therapies. The non-nutritional chemical components of medicinal plants, also referred to as phytochemicals (including caretenoids, polyphenols, alkaloids, saponins, tannins, vitamins) possess established antioxidant activities against free radicals which are chief factors implicated in the development of non-communicable diseases (Shabbir *et al.*, 2013). Inclusion of exogenous antioxidants from dietary sources is important since there is consumption of endogenous antioxidants in biological systems in response to free radical attack (Nasri *et al.*, 2015).

In the 20th century, drugs such as ectoposide, metformin, vinblastine, vincristine, artemisinin e.t.c have been developed from medicinal plants. This indicates clearly how medicinal plants have made a significant contribution to modern therapeutics in the management and treatment of various diseases (Dias *et al.*, 2012). It is important to note that medicinal plants undisputedly play a key role in the management of human health. The goal for optimizing the usage of medicinal plants to prevent/ cure NCDs is thus imperative.

2.18 *Catharanthus roseus* in the treatment of DM

Catharanthus roseus is a popular ornamental shrub that has its origin in Madagascar, hence its alternative name 'Madagascar periwinkle'. It has been used traditionally in several countries to treat diseases such as malaria, Hodgkins diseases, skin diseases and diabetes (Natarajan *et al.*, 2012; Rasineni *et al.*, 2013). *C. roseus* grows vertically up to 100 cm and is easily identified with its white, dark pink or purple (Figure 8) petal like flowers and its oval leaves which are glossy and hairless (Muralidharan, 2014). The roots, leaves and flowers of this plant have been

used throughout ancient times in the treatment of various ailments. The medicinal values of *C. roseus* were ascribed to the presence of more than 100 alkaloids. Among these alkaloids, vincristine, vinblastine have been successfully isolated and used as anticancer agents because of the anti-mitotic properties they possess (Tiong *et al.*, 2013).

The antidiabetic activities of *C. roseus* have been studied (Ghosh and Suryawanshi, 2001; Singh *et al.*, 2001; Rasineni *et al.*, 2010; Al-Shaqha *et al.*, 2015; Tiong *et al.*, 2015). It was reported that its leaves is consumed as tea to treat DM (Rasineni *et al.*, 2010). Various scientific evidences documented the increase in glucose utilisation in both *in vivo* and *in vitro* setups following administration of *C. roseus* (Tiong *et al.*, 2015).

Figure 8: *Catharanthus roseus* **plant** *(***Adapted from Ken Fern, 2019)**

2.18.1 *C. roseus* increases the activities of enzymes of carbohydrate metabolism

Singh *et al* (2001) evaluated the effects of dichloromethane-methanol (DCMM) leaf and twig extract on the activities of the enzymes involved in the metabolism of carbohydrates in STZ-induced Sprague Dawley rats. From their findings; 500 mg/kg body weight (b.w) of DCMM leaf and twig extract of *C. roseus* reduced blood glucose

levels of diabetic rats by 57.6% and 48.6% in a treatment period of 15 and 7 days, respectively. They also reported increase in the activity of glucose-6-phosphate dehydrogenase enzyme in treated diabetic rats when compared to the untreated diabetic controls. Glucose-6-phosphatase dehydrogenase is a regulatory enzyme that plays an important role in the pentose phosphate pathway. The higher activity observed in the treated diabetic animals indicated improvement in glucose utilisation.

In the same study, the activity of glucokinase, the first regulatory enzyme in the glycolytic pathway, was assessed in both diabetic controls and the DCMM-treated diabetic group. It was reported that treatment of diabetic rats with the DCMM extract of *C. roseus* increased the activity of this enzyme.

The group additionally measured the levels of malate dehydrogenase, an enzyme that releases oxaloacetate required in the generation of citrate in the citric acid cycle. In this reaction, oxaloacetate has to react with acetyl-CoA to produce malate. Malate is a reactant needed in the cytosolic gluconeogenic pathway. The study showed significant increase in the levels of malate dehydrogenase in the liver and plasma of treated diabetic rats.

2.18.2 *C. roseus* enhances the expression of glucose transporter genes

In a study which assessed the effects of *C. roseus* on the expression of glucose transporter genes (GLUT-2 and GLUT-4), the investigators reported that the mechanism through which *C. roseus* mitigates hypoglycaemia is by increasing the expression of GLUT-4 and GLUT- 2 genes. GLU-4 and GLUT-2 molecules are insulin sensitive molecules that recruit glucose from the extracellular environment into the cytosol during insulin signalling. The liver and muscle cells are the main site of action.

In addition, the effect of two different concentrations of ethanolic extract of *C. roseus* leaves in STZ-induced rats was evaluated on the expression of GLUT genes. Treatment of diabetic rats with ethanolic extracts of *C. roseus* leaves was shown to enhance the expression of GLUT genes. Based on their findings, it was concluded that in untreated diabetic rats, insulin deficiency may contribute to the down regulation of GLUT gene expression (Al-Shaqha *et al.*, 2015).

2.18.3 *C. roseus* may prevent the development of CVDs

Administration of the methanolic extract of *C. roseus* leaves for 14 days in STZ-induced diabetic rats was found to significantly reduce the levels of serum lipids (Jesmin *et al.*, 2015). Improvement in the reduction of serum lipid parameters may contribute to the delay or prevention of macrovascular complications of DM. In the same study, a higher dose of 400mg/kg b.w significantly reduced the levels of other serum lipids while it elevated the levels of HDL. Investigations on the integrity of pancreatic β cells in treated diabetic rats *versus* the untreated diabetic controls showed that *C.roseus* can rejuvenate the loss of β cells. Loss of β cells in the pancreas is a pathologic feature observed in diabetes patients. The findings of this study (Jesmin *et al.*, 2015) strongly suggest a need for further evaluation of *C. roseus* in higher animals, or clinical trials. Similar findings were also reported by (Ghosh and Suryawanshi, 2001) where they measured the effects of the extracts of *C. roseus* leaf and flower in diabetic rats and normal controls for 7 days. The serum triglycerides, cholesterol and free fatty acids were reported to be elevated in diabetic controls, whereas treatment with either the flower or leaf extract reduced the serum lipids to near normal.

2.18.4 *C. roseus* may prevent diabetes-induced oxidative stress

As previously highlighted, the development of DM complications is attributed to the uncontrolled production of ROS. Zhang *et al* (2016) investigated the effect of *C. roseus* alkaloid mixture against DM in high fat diet- fed diabetic rats. Induction of DM in the Wistar rats resulted in reduction in the activities of antioxidant endogenous enzymes in different tissues. Treatment of diabetic rats with the alkaloid mixture significantly increased the activity of SOD in the heart, liver and kidney tissues by 130%, 108% and 71%, respectively when compared to the untreated diabetic controls. The activities of catalase (CAT) in the same tissues were reported to have increased by 82.52%, 65.95% and 25.79%, respectively. In addition, their findings showed the reversal of TBARS levels to near normal in treated diabetic group. The

increase in the activities of the endogenous antioxidant enzymes in diabetic rats after treatment indicated that the alkaloids derived from *C. roseus* may potentially reduce glycation of enzymes and/ decrease the levels of ROS. The results observed and reported from this study concluded that *C. roseus* might prevent diabetes-induced oxidative tissue damage and therefore the risk of diabetic complications.

2.18.5 Additional antidiabetic studies done on *C. roseus*.

Table 2: Summarised antidiabetic studies done on *C. roseus*

References	Extract of *C. roseus*	% blood glucose reduction	Other parameters improved	Comment
(Leena, 2014)	Aqueous leaf	51%	1.reduced serum lipids 2. increased HDL levels	1.*C. roseus* may reduce the risk of CVDs
(Islam *et al.*, 2009)	Ethyl acetate and petroleum ether extracts	48.34% reduction in petroleum ether fraction while 40.68% was observed at 24th hour for ethyl acetate	Significant reduction in the levels of total cholesterol and triglycerides	*C. roseus* may reduce the risk of CVDs
(Nammi *et al.*, 2003)	Leaf juice	1.0ml/kg caused 31.9% reduction (20 hr post treatment, p < 0.01)		*C. roseus* may serve as a good adjuvant in the present armamentarium of antidiabetic drugs

Recent experimental studies reported the *in vitro* hypoglycaemic activity of several known indole alkaloids from *C. roseus*, with vindoline (Figure 9) being one of the alkaloids exhibiting potential effects in protein-tyrosine phosphatase 1B (PTP-1B) inhibition which could serve as an "insulin sensitizer" in the management of T2DM (Tiong *et al.*, 2013; Tiong *et al.*, 2015). Furthermore, it has been reported that vindoline improved the uptake of glucose in β-TC6 and C2C12 cells in a dose dependant manner (Tiong *et al.*, 2013). In a study done by Yao *et al* (2013) in MIN6 cells and in *ex vivo* β pancreatic cells; the authors reported that vindoline may contribute to the anti-diabetic effects of *C.roseus.* In their study, it was reported that vindoline improves β pancreatic cell function confirmed by the enhanced glucose stimulated insulin secretion effect and also through inhibition of the Kv2.1 channel. Due to the reported β cell protective effects exhibited by vindoline, it will be therefore important to further investigate its potential in the treatment and management of T2DM
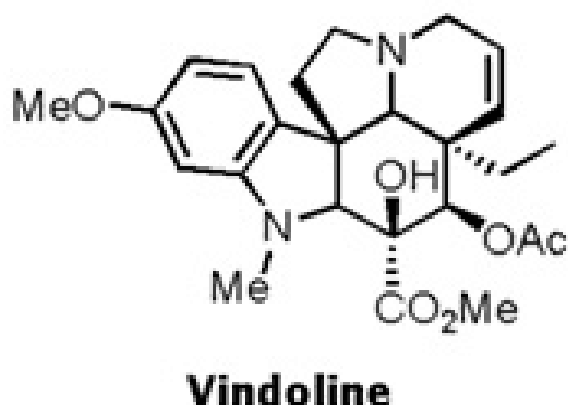
Vindoline

Figure 9: Molecular structure of vindoline (Adapted from Yao *et al* (2013)

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Chapter 3

In vitro antidiabetic and antioxidant effects of different extracts of *Catharanthus roseus* and its indole alkaloid, vindoline.

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Abstract

Catharanthus roseus plant has been used traditionally to treat several diseases, diabetes inclusive. Scientific evidence supporting the antidiabetic effects of this plant in Southern Africa has not been strongly evaluated. It has been shown that the medicinal properties of the same plant species tend to differ based on their geographical location. Vindoline is a bioactive compound derived from *C.roseus*. In this study, extracts of *C.roseus* were tested for antioxidant activities, alpha amylase, alpha glucosidase inhibitory activities and insulin secretory effects in pancreatic RIN-5F cell line cultured in the absence of glucose, at low and high glucose concentrations. The methanolic extract of the plant showed highest antioxidant activities in addition to the high total polyphenolic content ($p < 0.05$). The HPLC results exhibited increased concentration of vindoline in the dichloromethane and the ethylacetate extracts. Vindoline showed noticeable antioxidant activity when

compared to ascorbic acid at $p < 0.05$. When compared to the plant extracts, vindoline significantly improved the *in vitro* insulin secretion. The intracellular reactive oxygen species formation in glucotoxicity-induced cells was significantly reduced following treatment with vindoline, methanolic and the dichloromethane extract when compared to the high glucose untreated control ($p < 0.05$). Vindoline did not show significant inhibitory effects on the activities of carbohydrate metabolising enzymes when compared to acarbose which inhibited the activities of the enzymes by 80%. The plant extracts also exhibited weak alpha amylase and alpha glucosidase inhibitory effects. Results suggest that vindoline and the extracts may be of therapeutic importance in treatment of type 2 diabetes.

Key words: insulin secretion, glucotoxicity, reactive oxygen species, antioxidant, beta cells, alpha amylase, alpha glucosidase.

3.1 Introduction

Type 2 diabetes mellitus (T2DM) is a life-threatening disease of the endocrine system in which its prevalence is on the rise owing to the increasing trends of obesity and sedentary lifestyles (Tuomilehto *et al.*, 2001; Pheiffer *et al.*, 2018). These escalating trends may lead to the declaration of diabetes mellitus (DM) a global epidemic as its burden is evidently noticeable in both developed and developing regions (Olokoba *et al.*, 2012). T2DM is characterised by dysfunctional β pancreatic cells, insulin resistance and hyperglycemia, dyslipidemia and other metabolic disturbances like oxidative stress and low-grade inflammation collectively leading to deleterious complications (Giacco and Brownlee, 2010; Francini and Schinella, 2015).

Type 2 DM patients are vulnerable to complications that disrupt normal functions of the heart, kidneys, eyes, nerves and blood vessels (Yao *et al.*, 2013). These complications are thus responsible for the high mortality rates observed in these patients (Giacco and Brownlee, 2010). It is undeniable that the use of orthodox drugs improved the management of T2DM but is associated with adverse side-effects (Bahmani *et a*l., 2014). As a result of a wide array of metabolic abnormalities involved in the pathogenesis of T2DM and its complications, excellent treatment modalities should not only correct deranged glucose metabolism but also reverse or prevent the development of diabetic complications (David *et al.*, 2016). Efforts to improve the curative activities of pharmacological drugs have recently turned to assessing the properties of medicinal plants in different diabetic *in vitro* and *in vivo* models (Alarcon-Aguilar *et al.*, 2002; Fennell *et al.*, 2004; Bhogireddy *et al.*, 2013). Promising findings have been reported as a result of various compounds that are present in medicinal plants which work individually or in synergy to produce the desired antidiabetic, antioxidant, anti-inflammatory and antiapoptotic effects (Bhogireddy *et al.*, 2013; Salihu Shinkafi *et al.*, 2015).

Catharanthus roseus (*Linn. G. Don*) is both a medicinal and ornamental plant belonging to Apocyanaceaea family. It is a commercial plant that is grown in most parts of the world because of its medicinal uses (Jaleel *et al.*, 2008). *C. roseus'* water decoction has a long history of usage in the treatment of diseases such as cancer, diabetes, wounds, scurvy, hypertension and malaria (Mishra and Verma, 2017). Its

medicinal properties are attributed to the presence of a wide array of bioactive compounds. Besides, phenolic compounds, *C.roseus* is rich in alkaloids like vincristine, vinblastine, ajamalicine, serpentine, alstonine and reserpine. These alkaloids are popularly known to contribute significantly to the plant's medicinal properties (Mustafa and Verpoorte, 2007). In addition to the above properties, *C.roseus* has been shown to possess antifungal, antibacterial, antivirus and anti-inflammatory activities (Jaleel *et al.*, 2008; Ponarulselvam *et al.*, 2012; Almagro *et al.*, 2015). Vindoline is one of the alkaloids of *C. roseus* that is mainly found in its leaves. Previous studies demonstrated hypoglycaemic effect of vindoline which was suggested to be linked with stimulated insulin secretion (Yao *et al.*, 2013). This study aims at assessing and comparing the *in vitro* antidiabetic, antioxidant and anti-inflammatory effects of different crude extracts of *C. roseus* and vindoline in high glucose induced insulinoma cells and by evaluating their effect on glucose metabolising enzymes.

3.2 Materials and Methods

3.2.1 Reagents and Chemicals

Vindoline was purchased from Best of Chemicals Sciences (United States of America) (purity > 98%). The Rat insulin and TNF-α Elisa kits were obtained from Biocom BioAfrica while the diaminofluorescin-FM diacetate and dihhyroethidium fluorescence probes were purchased from Thermofisher Scientific group. The cell proliferation reagent WST-1, acarbose and all the solvents/ chemicals used for plant extraction and antioxidant analysis were obtained from the Merck-group.

3.2.2 Plant collection

Healthy green leaves of *Catharanthus roseus* were collected from a nature garden in Cape Town and the plant was identified by an experienced botanist in the Department of Horticultural Sciences, Cape Peninsula University of Technology and was given the following reference number (6597000). After collection, the leaves were washed, dried in the shade and crushed into a fine powder. To prepare the aqueous extract, 1 L of boiling water was added to 100 g of the powder and the mixture was left for 24 hours on magnetic stirrer. The organic extracts were prepared by adding 500 ml of solvents (100% methanol and dichlromethane) to 50 g of the plant powder and were left on a magnetic stirrer for 24 hours. The organic solvents were removed from the extracts by the use of a vacuum rotary evaporator at low pressure. The aqueous extract was filtered using the Whatman 1 filter paper, thereafter it was freeze dried and stored at -20 ℃.

3.2.3 High-Performance Liquid Chromatography (HPLC) Analysis of different extracts of *C. roseus*

The phenolic compounds present in different extracts of *C. roseus* were determined according to the method of Bramati *et al* (2002) using the high performance liquid chromatography-HPLC system (Agilent Technology 1200 series, Bellefonte, USA). On the HPLC system, a G1315C diode array detector and a C18 column of 5μm (4.6 mm x 150mm i.d) were used to separate the compounds. The chromatographic conditions were the following: twenty (20) μl sample injection volume, column set at

23 ℃, flow rate set at 1 mL/min for 15 minutes. Detection was performed at wavelengths of 220, 320 and 350 nm. Peaks were identified based on the retention time of vindoline, flavanoid and phenolic standards. The analytical signals were monitored at 2-20 mV potential applied. To determine the concentrations of the compounds present in the extracts, the following equation was used:

X mg/L = *(Area of sample / area of the standard) X 20mg/L.*

Results were reported as mg/g: *Xmg/L x Extraction volume (L)/weight (g)*

3.2.4 Total polyphenol measurement

The content of total polyphenols in plant extracts were measured spectrophotometrically using Folin-Ciocalteu phenol reagent and gallic acid was used as the standard. The experiment was performed according to the procedures described by Singleton *et al.*, (1999). Briefly, optimised concentrations of 25 µl of gallic acid standards and samples were mixed with 125 µl of Folin-Ciocalteu in the crystal clear 96-well plates. After 5 min incubation, 100 µl of sodium carbonate was added. The reaction mixtures were allowed to incubate for 2 hours at room temperature. Thereafter, the plates were read at 765 nm using a multiskan plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A). Total polyphenol concentrations in samples were extrapolated from a standard curve of gallic acid and were reported as milligram Gallic acid equivalents per gram of extract (mg GAE/g).

3.2.5 Determination of the Oxygen radical absorbance capacity (ORAC)

The ORAC is an assay that determines the rate at which biological or plant samples reduce the peroxyl radical. If a sample has high concentrations of antioxidants, there will be a delay in the decrease in fluorescence of the fluorescein reagent. In this study, the method of Ou *et al.*, (2001) was adapted to determine the antioxidant capacity of vindoline and different extracts of *C.roseus*. In brief, 12 µL of the trolox standard or samples was mixed with 138 µL of a 14 µM fluoresce reagent and were

incubated at 37 degrees Celsius for 30 mins. After the incubation step, 50 μL of 4.8 mM 2, 2'-azobis (2-methyl-propanamide) dihydrochloride (AAPH) was added introducing the free radical. The rate of fluorescence decrease was measured at 485 nm excitation and 538 nm emission readings recorded every 1 min for 2 hr using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as μM Trolox equivalents (TE)/L or μM Trolox equivalents (TE)/g.

3.2.6 DPPH Assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is a simple and accurate test that measures free radical scavenging activity of samples. The disappearance of the DPPH radical (purple in colour) to a DPPH molecule (yellow in colour) at 517nm signifies ample antioxidant amounts in sample. The method described by Sharma and Bhat (2009) was used to determine the disappearance of the DPPH radical in test samples.

3.2.7 *In vitro* cell line studies

The RIN-5F is an insulinoma cell line derived from the pancreatic β cells of rats. These cells were purchased from American Type Culture Collection (ATCC, USA). The cells were grown in Roswell Park Memorial Institute 1640 media (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were cultured in sterile cell culture flasks and were incubated at 37 ℃ in humidified atmosphere containing 5% CO_2. The media was changed after every 4 days to prevent starvation of cells. Upon reaching 80% confluence, cells were either sub-cultured or seeded at a cell count of 2 x 10^5 for further experiments.

3.2.8 Cell viability assay

The WST-1 is a colorimetric assay performed to quantify the count of viable cells per well after exposing cells to respective treatments. In this assay, the tetrazolium salt

(which is red in colour) in WST-1 reagent is broken down to form a yellow coloured formazan product catalysed by succinate-tetrazolium reductase enzyme found in metabolically active cells (Berridge and Tan, 1998). The intensity of the yellow formazan product measured by a plate reader at 620 nm is therefore directly proportional to the number of metabolically active cells in culture medium. The absorbance values of the formazan product are measured at a wavelength of 420 nm and reference wavelength of 620 nm.

After seeding cells at a concentration of 2×10^5 for 24 hours, they were treated with various concentrations of the stressor glucose: 50 mM glucose (to induce glucotoxicity), 6.25 mM (low glucose) and in the absence of glucose (0 mM). The influence of the different concentrations of glucose on cells was measured using the WST 1 reagent. Thereafter, cells received various treatments: vindoline, *Catharanthus roseus*- aqueous extract (CR-Aq), *Catharanthus roseus*- methanolic extract (CR-Meth), *Catharanthus roseus*-dichloromethane extract (CR-DCM) and cell viability was determined so as to acquire safe doses. 10% DMSO was used as the positive control in all viability assays.

Once the optimised concentrations were obtained, cells were seeded for 24 hours, exposed to the optimised concentrations of the glucose for further 24 hours. Thereafter, stressed cells were treated with the optimised concentrations of vindoline and CR extracts and the viability measurements were taken. Cell viability was calculated using the following formulae:

% cell viability = (Absorbance of sample- Absorbance of Blank) x 100

(Absorbance of negative control- Absorbance of Blank)

3.2.9 The Reactive Oxygen Species Assay (ROSA)

The formation of ROS inside the cells was determined using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CMH2DCFDA). In this reaction, when CM-H2DCFDA is added to the cells, it diffuses into the intracellular environment of the cells where it is converted by esterases to a non-fluorescent product CM-H2DCF (Venditti *et al.*, 1997). If the cell contains oxidants like ROS, CM-

H2DCF will consequently be oxidised to form CM-DCF-a highly fluorescent molecule hence making the intensity of the fluorescence to be proportional to the amount of ROS/RNS present inside the cells (Sarkar *et al.*, 2005). Measurement of ROS was performed as described by Taha *et al* (2014) using CM-H2DCFDA molecular probe with some modifications.

RIN-5F cells were seeded at a cell density of 2×10^5 cells per well in 24 well plates and were left to attach for 24 hours in the 37 ℃ incubator. After 24 hours, the attached cells were exposed to the stressors for another 24 hours and then eventually treated with vindoline, CR-Aq, CR-DCM and CR-Meth for 24 hours. The utilised media together with cells were trypsinised and spun to obtain a pellet. The cell pellets were then stained with 250 µl of 7.5 µM CM-H2DCFDA dye for 1 hour at 37 degrees Celsius. Stained cells were centrifuged, resuspended in media for 30 minutes and finally analysed using a flowcytometer set at 488 nm (excitation) and 520 nm (emission) at FL3 on a BD AccuriTM C6.

3.2.10 Insulin secretion assay in RIN-5F cells

The effect of vindoline and different extracts of *C.roseus* on the release of insulin in stressed and non-stressed cells was evaluated. The cells were seeded at a concentration of 2.0×10^5 cells per well in 24-well plates. After the 24 hour incubation time, the cells were exposed to the respective stressors for 24 hours. The next day, the media was removed from the wells and cells were washed with pre-warmed DPBS, thereafter, cells were exposed to a low concentration of glucose for 30 minutes. To determine the effect of plant treatments on the response of cells to high glucose, RIN-5F cells were treated with media containing a high concentration of glucose (50 mM) and the plant treatments. This was followed by a 2-hour incubation period; the media was then aspirated, centrifuged and stored at -80 ℃ until the concentration of insulin was determined by ELISA.

3.2.11 Determination of inflammation

The release of an inflammatory cytokine TNF-α was evaluated in RIN-5F cells (2 x 10^5 cells/well) that were exposed to glucotoxicity for 24 hours. The optimised concentrations of vindoline and of the extracts of *C. roseus* were added to the respective wells and were incubated for 24 hours. Following the 24 hour exposure to the plant treatments, the media in each well was aspirated, centrifuged and supernatants were then stored at -80 ℃ until the TNF-α concentration were measured by ELISA.

3.2.12 *In vitro* alpha amylase inhibitory activity

The inhibitory effects of vindoline and the extracts of *C.roseus* on the activity of α-amylase were assessed using the modified method described by Bhutkar and Bhise (2012). This assay determines the decrease in the units of maltose produced after the breakdown of starch by the enzyme alpha amylase. If an extract or compound possesses alpha amylase inhibitory activities, there will be a decrease in the amount of maltose formed which is signified by a yellowish colour instead of brick red. In this study, 200 µl of the test compounds was pre-incubated with 200 µl of the enzyme (1 U/ml) for 10 minutes. Soluble starch solution (1% w/v) was then added to the plant-enzyme solution and incubated for 30 minutes, the reaction was then stopped by adding 200 µl of 3, 5 dinitrosalicyclic acid at 96 Mm concentration (prepared 12 g of sodium potassium tartrate tetrahydrate in 8 mls of 2 M sodium hydroxide and 96 mM salicyclic acid. This was followed by a heating step (85°C for 15 minutes). Two blank samples were used in this experiment: one made without the enzyme and the other without the plant extract, 200 µl of 20 mM sodium phosphate buffer was added instead. The amount of maltose formed was measured at 540 nm using a spectrophotometer.

Percentage alpha amylase inhibition was calculated as follows:

$$\% \text{ inhibition} = \left\{ \frac{\text{Absorbance of control} - \text{Absorbance of test} \ \times \ 100}{\text{Absorbance of control}} \right\}$$

3.2.13 In vitro alpha glucosidase inhibitory assay

The alpha glucosidase inhibitory activities of *C.roseus* extracts and vindoline were tested by the method of Monteiro de Souza *et al* (2012). 200 µl of the plant samples were incubated with 1 U/ml alpha glucosidase enzyme for 5 minutes. To initiate the reaction, 200 µl of the substrate reagent pNpG (3 mM prepared in 10 mM phosphate buffer pH 6.9) was added and incubate at 37°C for 30 minutes. 100 mM sodium carbonate was added to stop the reaction and reaction was measured at an absorbance of 405 nm. Blanks were made by replacing enzyme/ plant with distilled water.

The percentage inhibition of alpha glucosidase was calculated as follows:

$$\% \text{ inhibition} = \left\{ \frac{\text{Absorbance of control} - \text{Absorbance of test X 100}}{\text{Absorbance of control}} \right\}$$

3.3 Results

3.3.1 Determination of and quantification of phenolic compounds and vindoline in *C. roseus* extracts.

HPLC determination of phenolic compounds and the alkaloid vindoline in CR-Aq, CR-Meth, CR-DCM extracts is represented in Table 3 below. The methanolic extract of *C. roseus* showed highest concentrations of chlorogenic acid (225.19 µg/g), quercetin (1.945 µg/g), coumaric (28.822 µg/g) and rutin (85.916 µg/g). At wavelength of 220 nm, vindoline was found to be predominant in the dichloro-methane and ethyl-acetate at concentrations of 57.891 µg/g and 57.323 µg/g respectively. The aqueous extract recorded the least concentration of vindoline (7.056 µg/g) in the plant extract-*C.roseus*.

Table 3: HPLC analysis of different extracts of *Catharanthus roseus*

Extract	Chlorogenic acid (µg/g)	Caffeic acid (µg/g)	Quercetin (µg/g)	Coumaric acid (µg/g)	Vindoline (µg/g)	Rutin (µg/g)
CR-Aq	33.461	1.179	0.445	2.195	7.056	5.891
CR-Meth	225.19	0.614	1.945	28.822	13.597	85.916
CR-Eth	0.466	0.396	1.263	0.693	57.323	1.811
CR-DCM	2.308	0.017	0.253	0.197	57.891	4.506

3.3.2 Total polyphenolic and antioxidant assessment of *C.roseus* extracts

The total polyphenolic (TP) content and the *in vitro* antioxidant capacity of the aqueous, methanolic, dichloromethane and the ethyl acetate extracts of *C.roseus* are shown in Figure 10 below. The CR-Meth extract (10.913 $\pm$ 0.24 mg GAE/L) showed significantly high concentration of TP when compared to the other 3 extracts ($p < 0.05$). The CR-DCM (6.3 $\pm$ 0.0.123 mg GAE/L) extract showed higher TP when compared to the CR-Aq (4.06 $\pm$ 0.08 mg GAE/L) and CR-Ethyl (2.89 $\pm$ 0.107 mg GAE/L).The antioxidant determination measured as ORAC revealed high antioxidant capacity in the following order CR-Meth (64076.4 $\pm$ 1232 μmol TE/L), CR-DCM (27827.2 $\pm$ 1151μmol TE/L), CR-Ethyl (16808.8 $\pm$ 1646 μmol TE/L) and CR-Aq (13521.1 $\pm$ 290.5 μmol TE/L). The same trend was observed in the DPPH activities of these extracts however, the DPPH reading of the CR-Ethyl exhibited lower activity.
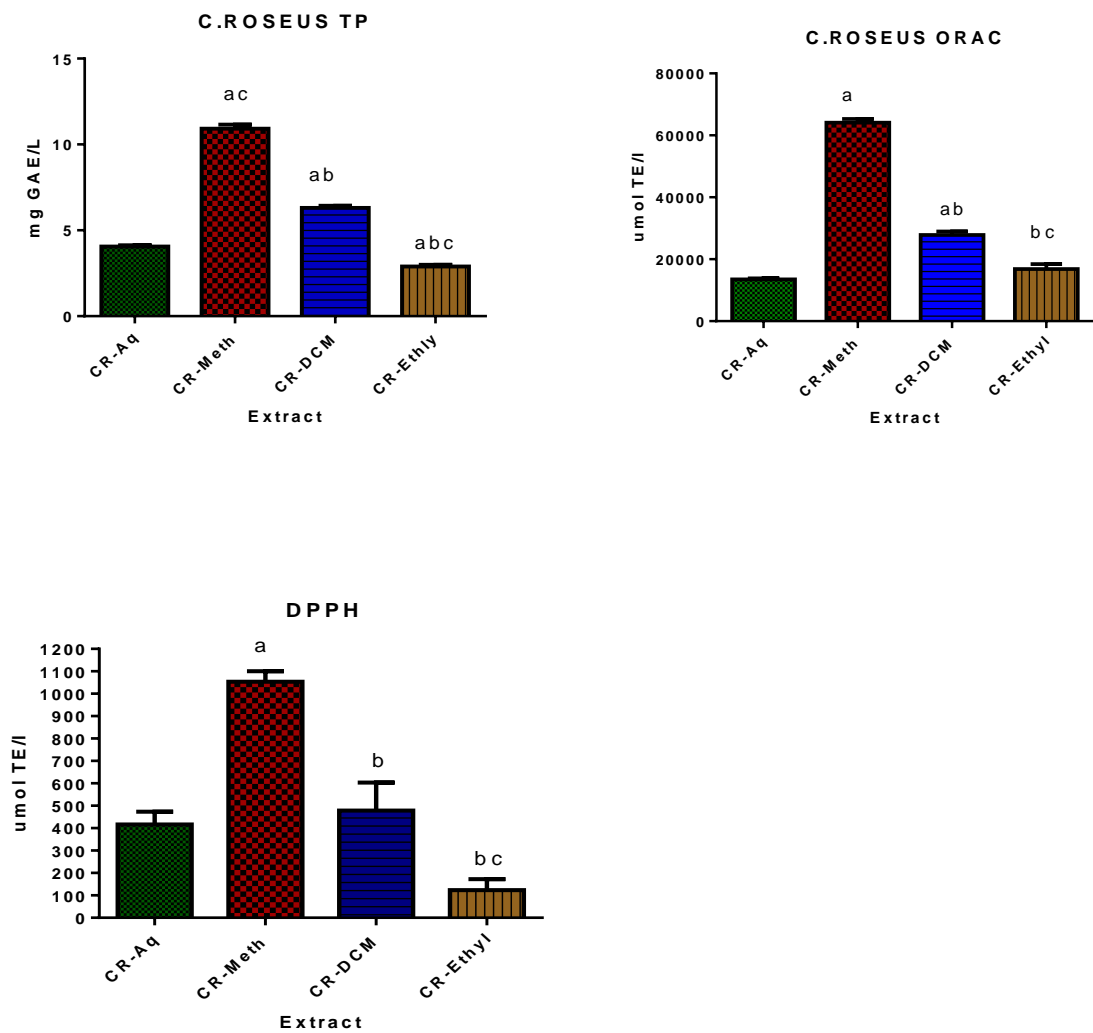
Figure 10: Antioxidant analysis and total polyphenol determination. [a] significant difference when compared to the CR-Aq; [b] significant difference when compared to CR-Meth; [c] significant difference when compared to CR-DCM at $p < 0.05$.

3.3.3 Determination of vindoline' antioxidant capacity

Table 4 below demonstrates the *in vitro* antioxidant assessment of vindoline and standard antioxidant ascorbic acid. The ferric reducing antioxidant power of vindoline was not significantly different to that of ascorbic acid at $p < 0.05$. However, vindoline exhibited stronger ORAC strength when compared to ascorbic acid at $p < 0.05$.

Table 4: Antioxidant activity of vindoline

	Concentration	Vindoline	Ascorbic acid
FRAP (µM)	0.05 µM	23842 ± 339.3^{ns}	24514 ± 95.7^{ns}
ORAC (µmol TE/L)	0.05 µM	56.0 ± 4.9^{a}	40.86 ± 3.8

Values are presented as (mean ± SEM); ns: non-significant; ^{a} value significantly different from ORAC value of ascorbic acid at $p < 0.05$.

3.3.4 The effect of high glucose concentration on the viability of RIN-5F cells

The result of glucose exposure on RIN-5F cells is presented in Figure 11. Glucose at a concentration of 50 mM resulted in the viability count of 106% of RIN-5F cells at $p < 0.05$ when compared to the other glucose concentrations. Maximum viability was observed in RIN-5F cells treated with 12.5 mM glucose solution (160%). At 6.25 and 3.125 Mm glucose concentrations, the number of viable cells decreased to 150 and 113% when compared to the cells treated with 12.5 Mm glucose ($p < 0.05$).
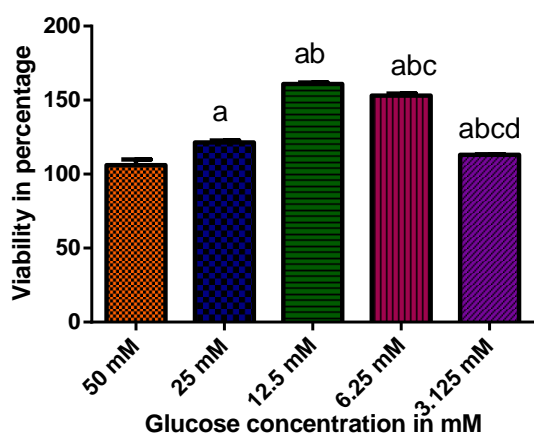
 Figure 11: Effect of glucose on cell viability. [a] **significant difference when compared to the 50 mM glucose concentration;** [b] **significant difference when compared to 25 mM glucose concentration;** [c] **significant difference when compared to 12.5 mM glucose concentration;** [d] **significant difference when compared to 6.25 mM glucose concentration; all at p < 0.05.**

3.3.5 Effect of different extracts of *C.roseus* on the cell viability of RIN-5F cells

To evaluate the effect of exposing RIN-5F cells to different concentrations extracts of *C.roseus,* cell viability was assessed using the WST-1 method and presented in Figure 12. Cells were treated for 24 hours with various concentrations of extracts ranging from 1 mg/ml to 0.03125 mg/ml. The CR-Aq extract at 1 and 0.5 mg/ml significantly increased the number of viable cells to approximately 150% when compared to the CR-Meth and CR-DCM which had less than 80% viable cells at p < 0.05. Concentrations that resulted in about 85% viability were used in further experiments and these included the 0.03125 mg/ml for CR-Aq and 0.0625 mg/ml for both the CR-Meth and CR-DCM.
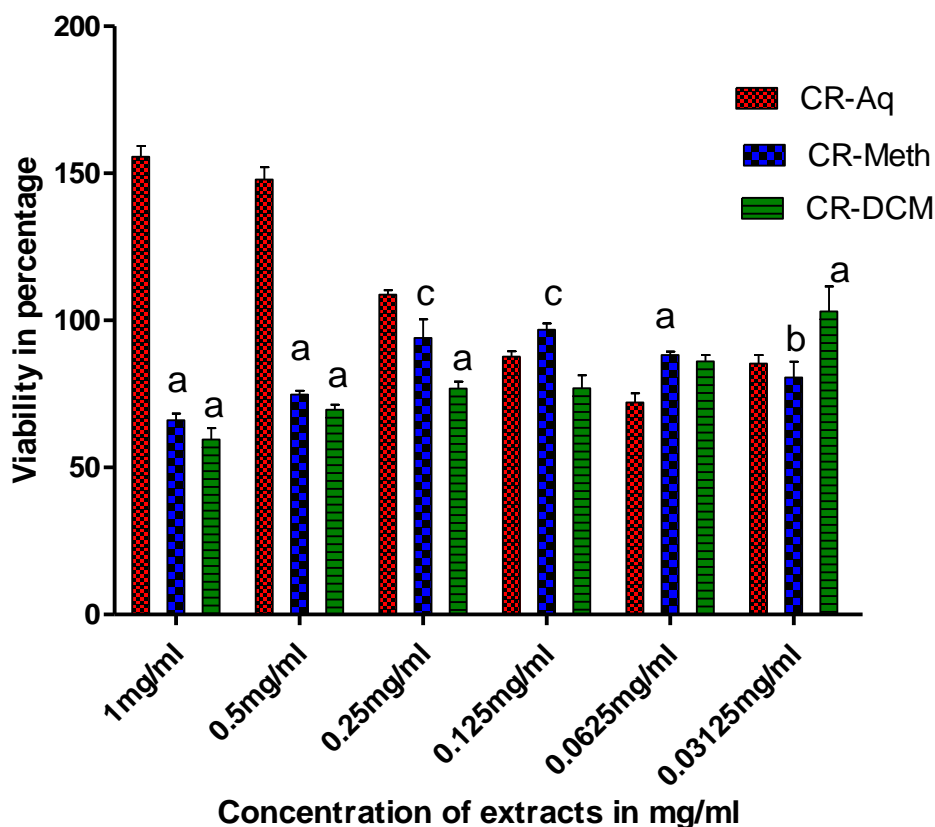
Figure 12: Effect of C.roseus extract on the viability of RIN-5F cells. [a] significant difference when compared to CR-Aq; [b] significant difference when compared to CR-Meth; [c] significant difference when compared to CR-DCM; at p < 0.05.

3.3.6 The effect of vindoline on cell viability of RIN-5F cells

Figure 13 below represents the effect of 24-hour exposure of various concentrations of vindoline in RIN-5F pancreatic cells. At a concentration of 1mM, vindoline resulted in significant cytotoxic effects indicated by cell viability of about 40% when compared to lower concentrations at $p < 0.05$. Weak cytotoxic effects were observed at 0.5 mM concentration with viability of nearly 65%. The concentration of vindoline at 0.125 mM with a viability of about 84% was further used to assess the insulotropic effect of vindoline in RIN-5F cells.
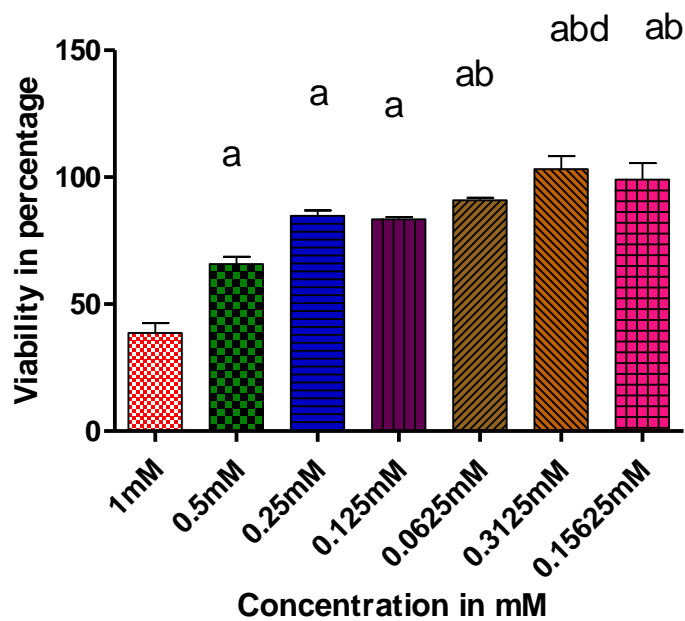
Figure 13: Effect of vindoline on cell viability. [a] significant difference when compared to 1 mM vindoline concentration ; [b] significant difference when compared to 0.5 Mm vindoline concentration; [c] significant difference when compared to 0.25 mM vindoline concentration; [d] significant difference when compared to 0.125 mM vindoline concentration; all at p < 0.05.

3.3.7 Effect of vindoline and the extracts of *C.roseus* on insulin secretion

The effect of the pure compound vindoline and *C.roseus'* extracts on the release of insulin by beta pancreatic cells that were previously exposed to high (50 mM), low (6.25 mM) and in the absence of glucose concentrations was investigated and presented in Figure 14 below. Following stimulation with high glucose with/ without treatments for 2 hours, the amount of insulin secreted was determined. In RIN-5F cells that were previously exposed to 50 Mm glucose, vindoline markedly increased the secretion of insulin (0.72 ng/ml) when compared to the cells that were treated with CR-Meth and untreated high glucose exposed cell with 0.43 and 0.3 ng/ml insulin levels respectively. Interestingly, no significant differences were observed in the amounts of secreted insulin in glucotoxicity-induced cells that were treated with vindoline, CR-Aq and CR-DCM at $p < 0.05$. In cells previously exposed to low glucose (6.25 mM), vindoline also exhibited improved insulin secretion when compared to the cells treated with CR-Aq, CR-Meth and the untreated controls. In normoglycemic (0 mM) glucose exposed cells, the secretion of insulin following treatment caused no significant changes among the groups, implying that the treatments in normal cells did not significantly alter insulin secretion.

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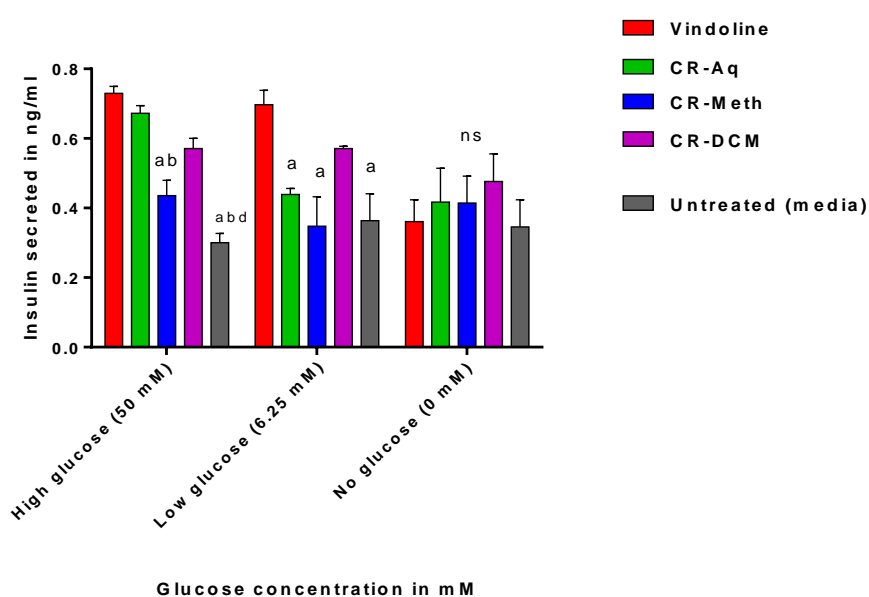
Figure 14: Effect of vindoline and the extracts of C.roseus on insulin secretion. [a] significant difference when compared to vindoline treated cells; [b] significant difference when compared to CR-Aq treated cells; [d] significant difference when compared to CR-DCM treated cells; all at p < 0.05.

3.3.8 Analysis of intracellular reactive oxygen species generation in RIN-5F cells

Effects of vindoline and the extracts of *C.roseus* on intracellular ROS production in RIN-5F cells exposed to high concentrations of glucose are presented in Figure 15 below. The shift to the left as shown in flow cytometer image represented cells that were negative for ROS, while a right shift indicated ROS positive cells. Histograms represent the percent of DCF positive cells and the results were compared to the untreated control of each experiment. Increased ROS production was observed in cells following high glucose treatment. Treating glucotoxic-induced RIN-5F cells with vindoline, CR-Meth and CR-DCM extracts resulted in significantly decreased ROS production (p < 0.05). Administration of CR-Aq extract did not significantly have an effect on ROS production when compared to the untreated high glucose control.
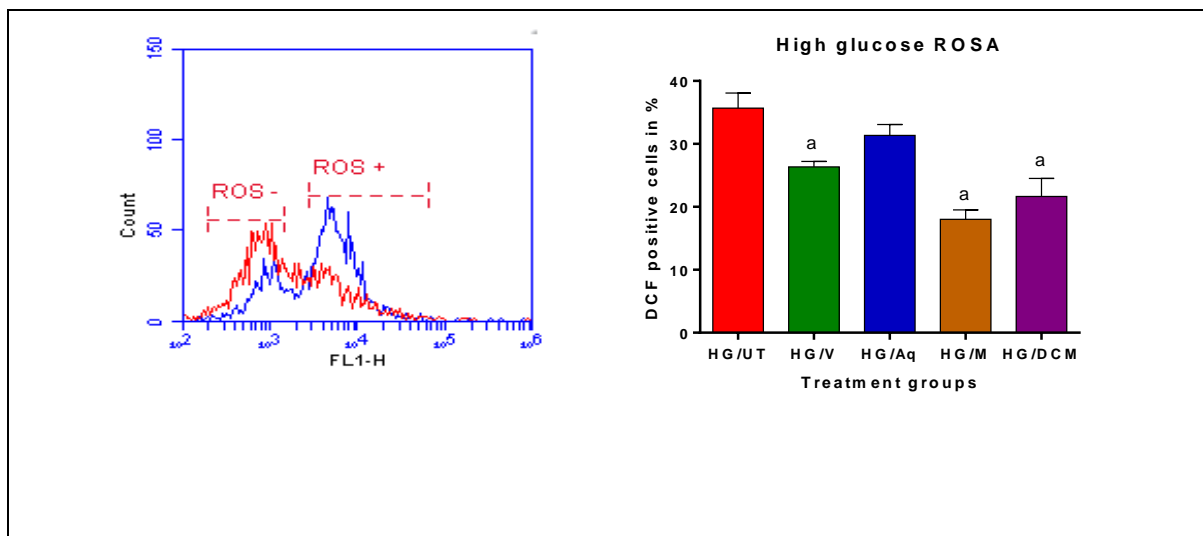
Figure 15: Effect of vindoline and the extracts on intracellular ROS. HG/UT: untreated high glucose exposed cell (control); HG/V: high glucose exposed cells treated with vindoline; HG/Aq: high glucose exposed cells treated with CR-Aq; HG/M: high glucose exposed cells treated with CR-Meth; HG/DCM: high glucose exposed cells treated with CR-DCM. p < 0.05 when compared to the control.

3.3.9 Effect of vindoline and the extracts on the levels of TNF-α levels

The levels of TNF-α were determined after treatment with respective treatments in high, low or no glucose exposed cells. Upon treatment, neither vindoline nor the extracts significantly altered ($p < 0.05$) the level of TNF-α in all treatment groups as shown in Figure 16.
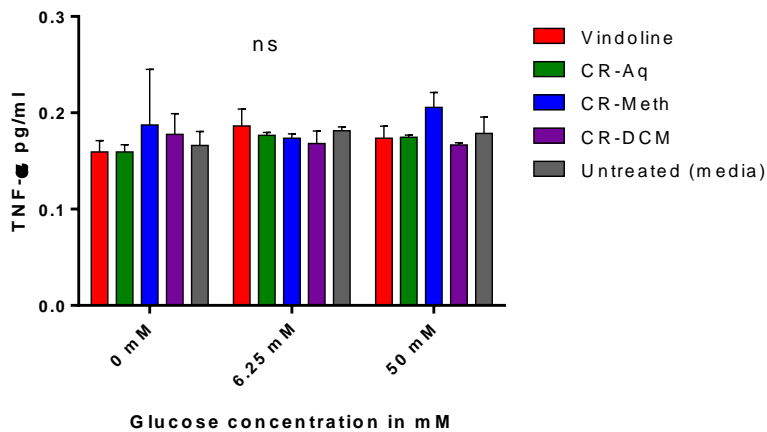
Figure 16: Effect of the treatments on the inflammation. [ns] indicates non- significant difference among the groups at $p < 0.05$

3.3.10   Alpha glucosidase inhibitory activity

The *in vitro* alpha glucosidase inhibitory activities of the different extracts of *C. roseus* are presented in Figure 17. Various concentrations of the plant extract (50, 25, 12.5 and 6.25 mg/ml) were assessed with the inhibitory activities of similar concentrations compared to each other. At a concentration of 50 mg/ml, the CR-Aq, CR-Meth and CR-DCM showed no significant differences in the percentage inhibition activity of 24%, 23% and 19% respectively.   However, at a concentration of 25 mg/ml, the CR-DCM (13%) showed significantly reduced inhibitory activity when compared to the CR-Aq which showed 22% (p < 0.05).   At 12.5 and 6.25 mg/ml concentrations, the CR-DCM showed the least inhibitory activities which were significantly lower when compared to similar concentrations of CR-Aq and CR-Meth at p < 0.05.
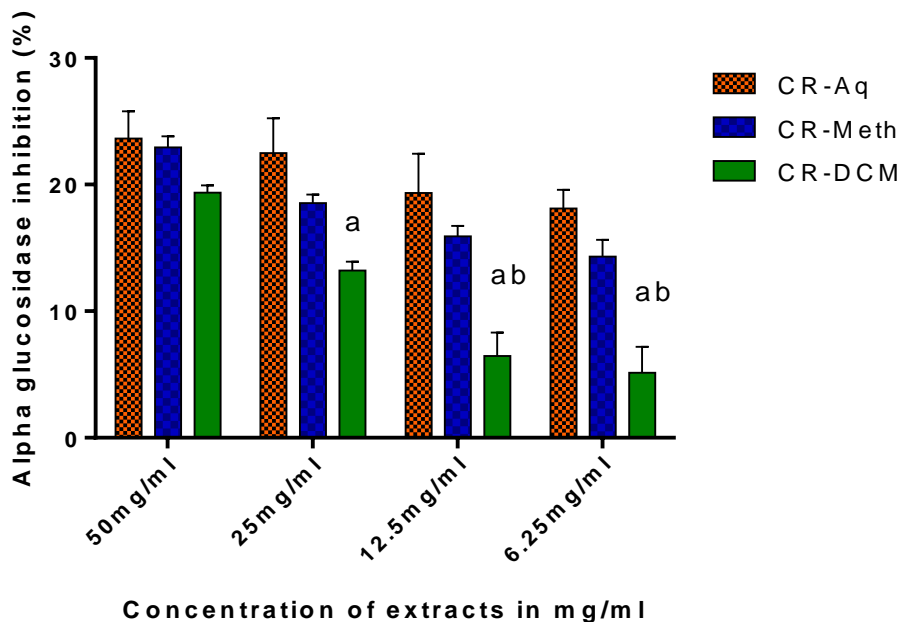
Figure 17: Effect of the extracts on the inhibition of alpha glucosidase. [a] significant difference when compared to CR-Aq; [b] significant difference when compared to CR-Meth; at p < 0.05.

3.3.11 Alpha amylase inhibitory effects of *C. roseus* extracts

The results represented in Figure 18 indicate the effect of different extracts of *C. roseus* on inhibition of alpha amylase activity. *The methanolic* extract exhibited high alpha amylase inhibitory activity when compared to counterpart concentrations of the aqueous and dichloromethane at p < 0.05. At 50 mg/ml, CR-Meth displayed appreciable alpha amylase inhibitory activity of about 40% while CR-Aq and CR-DCM showed 20% and -2% enzyme inhibitions respectively. The inhibitory effect of the methanolic extract at 25 and 12.5 mg/ml resulted in roughly 30% reduction of the enzyme activity whereas the aqueous extract showed relatively weaker activities of 11 and 9% respectively. In this study, the dichloromethane extracts of *C.roseus* failed to exert alpha amylase inhibitory effect.
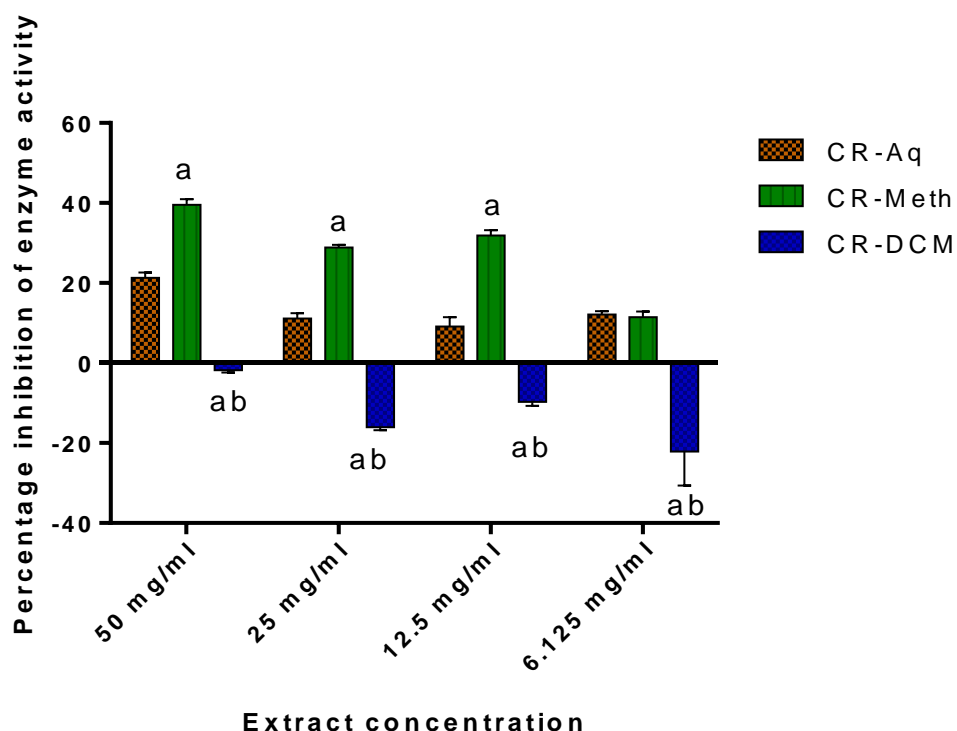
Figure 18: Alpha amylase inhibitory activities of the extracts. [a] significant difference when compared to CR-Aq; [b] significant difference when compared to CR-Meth; [c] significant difference when compared to CR-DCM; at p < 0. 05.

3.3.12 Alpha glucosidase and alpha amylase inhibitory activity of vindoline

The *in vitro* alpha amylase and alpha glucosidase inhibitory activities of vindoline was tested and compared to acarbose a standard drug. In Table 5, at a concentration of 0.375 mM, vindoline resulted in a weak alpha amylase activity of 28% whilst at the same concentration; acarbose showed significantly high enzyme inhibitory activity of approximately 80% at $p < 0.05$. In addition, vindoline recorded a significantly weak alpha glucosidase inhibitory activity of 11% when compared to acarbose which showed a strong inhibitory activity (83%) at $p < 0.05$.

Table 5: The alpha amylase and alpha glucosidase inhibitory activity of vindoline

Test	Concentration	Vindoline Inhibition (%)	Acarbose inhibition (%)
Alpha glucosidase	0.375 mM	11.31103± 2.351[a]	83.35±0.3
Alpha amylase	0.375 mM	18.57± 2.881[a]	79.50± 1.081

[a] **represents a significant difference when compared to the standard drug acarbose at $p < 0.05$.**

3.4 Discussion

The use of medicinal plants in the treatment of diabetes is motivated by the presence of chemical components that possess different characteristics which contribute to the plants' therapeutic effects (Anand *et al.*, 2017). The presence of phenolic compounds and alkaloids in *C.roseus* might be responsible for the previously reported therapeutic activities. Diabetes is a metabolic disorder where tissue damage arises from compromised antioxidant defence system and excessive build-up of ROS, hence, plant derived compounds might possess better characteristics that attribute to antidiabetic and antioxidant effects (Oguntibeju *et al.*, 2016).

In our present study, we measured the concentrations of vindoline and selected phenolic compounds in the aqueous, methanolic, dichloromethane and ethyl-acetate extracts of *C.roseus.* The HPLC chromatogram of the methanolic extract followed by the aqueous extract showed higher concentrations of the selected phenolic compounds. Polyphenolic compounds are present in plants and their inclusion in diet has been reported to have remarkable human health benefits. Polyphenolic compounds have the ability to hunt down ROS/RNS in biological systems. Their free radical scavenging power has been linked to the prevention or reduction of the risk of certain diseases like T2DM and cancer (Quideau *et al.*, 2011). In addition, modulatory effects of phenolic compounds like flavanoids (rutin and quercetin) in DM is linked to alterations of carbohydrate and lipid metabolism, attenuation of hyperglycemia, hyperlipidemia, inflammation and insulin resistance (Testa *et al.*, 2016). Chlorogenic acid was the most abundant phenolic compound in the methanolic extract. Previous studies reported the possible stimulated insulin secretory effect of chlorogenic acid from INS-1E cells; moreover it has been shown to enhance GLUT4 expression (Chellan *et al.*, 2012; Meng *et al.*, 2013). Results from total polyphenolic (TP) determination also showed high concentrations of TP in the (decreasing order) methanolic, dichloromethane, aqueous and ethyl acetate extracts. The quantitative polyphenolic assessment of the various extracts of *C.roseus* revealed the methanolic extract as the best solvent to extract the beneficial phenolic compounds. These findings are in agreement with results reported by (Rasool *et al* (2011) and Kabesh *et al* (2015) who observed high polyphenolic content and antioxidant activity in the methanolic extract.

Although the dichloromethane and the ethyl acetate extracts showed the least concentrations of the selected polyphenols, the HPLC quantification alternatively showed elevated concentrations of vindoline in these extracts while the aqueous extract recorded the least. It was previously reported that the ethyl acetate fraction of *C.roseus* reduced the fasting blood glucose levels by approximately 40% in DM rats. The observed reduction was attributed to the presence of hypoglycemic alkaloids such as vindoline. Increased glycogenesis, decreased glucose absorption and gluconeogenesis were assumed to be the underlying mechanisms behind the *in vivo* antidiabetic effect of the ethyl acetate extract (Islam *et al.*, 2009). To the best of our knowledge, this is the first study to investigate and compare the *in vitro* diabetic effects of vindoline to the aqueous, methanolic and dichloromethane extracts of *C.roseus* plant in South Africa.

Abnormally high levels of free radicals cause oxidative stress in tissue leading to pathophysiology of chronic diseases like diabetes. Antioxidants are molecules that prevent or slow down tissue damage by scavenging ROS/RNS or by obstructing free radical oxidation (Oguntibeju *et al.*, 2016). The antioxidant potential of various extracts of *C. roseus* was investigated by measuring the ORAC and DPPH radical scavenging activities. The methanolic extract exhibited significantly high ORAC and DPPH scavenging activities suggesting the potent antioxidant effect of this extract that could prevent oxidative tissue damage consequently ameliorating the progression of diseases such as DM and cancer (Zheng and Wang, 2001). The high antioxidant capacity observed in the methanolic extract may have been attributed to the high levels of polyphenolic compounds determined in this study. Polyphenols are bioactive compounds that act as antioxidants by donating hydrogen atoms to free radicals thereby getting rid of the unstable unpaired electrons (Quideau *et al.*, 2011). The results also confirmed that the dichloromethane extract was able to significantly reduce the DPPH and AAPH radicals in the DPPH and ORAC assays respectively when compared to the ethyl acetate extract. These findings propose that the compounds like polyphenols and alkaloids with high concentration in the CR-DCM extract contributed to the significant antioxidant potency. However, some studies in literature argued on the relationship between the phenolic content and antioxidant activities of plants, the reason being that plant extracts are composed of

complex combinations of many different compounds with distinct activities (Kaur, 2014).

The antioxidant potential of vindoline was measured using the FRAP and ORAC methods, with ascorbic acid as a standard antioxidant in these assays. The capacity of vindoline to reduce the ferric ion into the ferrous ion in the FRAP assay showed no significance changes when compared to standard ascorbic acid. Interestingly, vindoline showed high oxygen radical absorbance capacity with values that were significantly higher than a known antioxidant ascorbic acid. Our findings indicate that vindoline inhibited the fluorescence decay of fluorescein-peroxy radical complex through donating its hydrogen atom suggesting vindoline's antioxidant effectiveness. Augmentation of cellular antioxidant defence structures remains an approach in averting the progression of disease states in which oxidative stress has been implicated (Kaur *et al.,* 2016).

Glucotoxicity is a detrimental factor that contributes to advancement of beta cell dysfunction and/failure leading to development of diabetes (Alshatwi and Subash-Babu, 2016). The effect of 24 hour glucose exposure on the viability of RIN-5F cells was determined. We observed a significant decrease in the number of viable cells in cells treated with 50 mM glucose concentration when compared to lower concentrations. Therefore, the 50 mM glucose concentration was used to expose cells to hyperglycemia in order to compare the effect of vindoline and different extracts of *C.roseus* on the cells.

In order to assess the potential effect of *C.roseus* leaf extracts and vindoline on the RIN 5-F cell survival, cell viability was measured using the WST-1 assay. In our findings, the CR-Aq extract at high concentrations of 1 mg/ml and 0.5 mg/ml increased the number of viable cells signified by the cell viability of 150%. The enhanced viability observed may imply that the aqueous extract of *C.roseus* has the ability to increase the number of viable β cells in the pancreatic tissue (Tiong *et al.*, 2013). Increment of live β cells may be beneficial in diabetes patients since they have been reported to have a degree of beta cell loss and dysfunction (Weir and Bonner-Weir, 2004; Kaur *et al.*, 2016). Moreover, the improved cell viability attests to

the traditional consumption of the water decoction of C. *roseus* leaves in the treatment of DM despite the presence of cytotoxic constituents like alkaloids (Zheng and Wang, 2001). Contrarily, at 1 mg/ml and 0.5 mg/ml concentrations of the methanolic and dichloromethane extracts showed significant cytotoxicity against RIN-5F cells. The enhanced cytotoxicity may have been instigated by the presence of extreme amounts of indole alkaloids with pro-apoptotic effects thus restricting cell proliferation (Almagro *et al.*, 2015). However, evaluation of lower concentrations of the extracts refurbished the survival of the cells; concentrations resulting in approximately 80-85% viability were used in subsequent experiments.

We further investigated the effects of the extracts and vindoline on the functionality of RIN-5F cells after exposure to high/ low glucose concentrations or in the absence of glucose. Our results revealed that vindoline significantly enhances insulin secretion in response to the glucose concentrations; the result suggests that vindoline enhance the sensitivity of RIN-5F cells to exogenous glucose by increasing the release of insulin (Yao *et al.,* 2013; *Taha et al.,* 2014). The mechanism of action of vindoline is suggested to be identical to that of sulfonylureas which also promote insulin secretion in response to glucose levels. When compared to the methanolic extract, vindoline showed better insulin secretion in cells that were exposed to high glucose levels. This result may imply that the reported antidiabetic effect of *C.roseus* is not only associated with the phenolic compounds as previously reported but also to its alkaloid content (Tiong *et al.,* 2013). At low glucose concentration, vindoline also increased insulin release; the result was significantly higher when compared to the cells treated with the aqueous and the methanolic extract. Whereas no significant difference was observed in cells that were treated with the dichloromethane extract. Our findings were consistent with our vindoline HPLC quantification results which demonstrated high vindoline content in the dichloromethane extract; hence it is logical to suggest that vindoline might be the compound responsible for the antidiabetic activities of the dichloromethane extract. Interestingly, in cells that were not exposed to glucose, vindoline did not exhibit any stimulation on beta cells leading to the proposal that vindoline only stimulates the beta cells in hyperglycemic environments. This finding agrees with the results reported by Yao and colleagues (2013) where vindoline did not enhance insulin secretion in non-diabetic rats.

The beta cells of the pancreas are cells that are extremely susceptible to oxidative stress damage due to hyperglycemia-induced ROS/RNS generation. These cells contain relatively minute quantities of antioxidant enzymes hence worsening the risk of oxidative damage in hyperglycemia (Taha *et al.*, 2014). Glucotoxicity is the main culprit contributing to advancing beta cell dysfunction or apoptosis by instilling cellular stress. Prevention of ROS generation is a promising approach for impeding glucose-induced beta cell apoptosis (Yao *et al.*, 2013). Our data revealed decreased ROS production in RIN-5F cells that were treated with vindoline, methanolic and dichloromethane extract of *C.roseus*. It may be suggested that the antioxidant activities of vindoline and that of the extracts are associated with inhibition of ROS generation. The observed antioxidant effect of vindoline in beta cells was consistent with the reports demonstrated by Tiong *et al* (2013), where vindoline prevented ROS production in H_2O_2-induced β-TC6 cells. These findings highlight that vindoline may be a therapeutic product that can provide leads in the development of new antidiabetic drugs.

Alpha amylase and alpha glucosidase are enzymes that play fundamental roles in carbohydrate metabolism. Alpha amylase catalyses the breakdown of polysaccharides such as starch into maltose via disruption of the alpha-1,4-glycosidic bonds (Wickramaratne *et al.*, 2016). In turn, alpha-glucosidase cleaves disaccharides into glucose which is absorbable into the intestinal lumen (Monteiro de Souza *et al.*, 2012). Inhibition of the activity of these enzymes is an effective therapeutic approach that has been shown to control postprandial hyperglycemia in diabetic patients. Inhibitors such as acarbose delay the cleavage of carbohydrates consequently diminishing the rate of glucose absorption thereby improving the glycemic index (Bhutkar and Bhise, 2012).

The alpha amylase and alpha glucosidase inhibitory activities of the vindoline, aqueous, methanolic and dichloromethane extracts of *C. roseus* were investigated. Our results demonstrated appreciable alpha glucosidase inhibitory activities in highest concentrations (50 mg/ml) of the plant extracts. However, at lower concentrations of 12.5 and 6.25 mg/ml, the dichloromethane demonstrated weaker inhibitory activities. On the other hand, the methanolic extract exerted significantly higher alpha amylase inhibitory activities at 50, 25, 12.5 mg/ml when compared to

the aqueous and the dichloromethane extracts. These findings are in agreement with results of Jyothi *et al (*2013) who reported increased alpha amylase inhibitory activity of the methanolic extract of *C.roseus*. Despite the reported *in vivo* antidiabetic effects of dichloromethane extract of *C.roseus* by previous researchers (Singh *et al.*, 2001; Jayanthi *et al.*, 2010), we did not observe any alpha amylase inhibitory effects of this extract, indicated by the negative inhibitory values. Our result proposed that the dichloromethane extract of *C.roseus* does not delay the breakdown of carbohydrates after a meal. The carbohydrate inhibitory activities observed in this study may be associated with presence of compounds such as phenolics and terpernoids which were previously reported to possess alpha glucosidase and alpha amylase inhibitory activities (Malathi *et al.*, 2010; Monteiro de Souza *et al.*, 2012).

The natural product vindoline demonstrated extremely lower enzyme inhibitory activities when compared to the standard drug acarbose. Vindoline exhibited an alpha glucosidase inhibitory activity of 11% whereas acarbose inhibited 83% of the enzyme activity. The *in vitro* alpha amylase activity of vindoline additionally showed a weak alpha amylase inhibitory activity of 18% whilst acarbose had significantly higher inhibitory activity of 79%. Our findings imply that vindoline's antidiabetic effects do not include inhibition of carbohydrate metabolising enzymes.

3.5 Conclusion and recommendations

Medicinal plants remain a good source of diverse, natural and biologically active compounds. The presence of these phytochemicals contributes to beneficial activities such as antioxidant and antihyperglycemia, making plants such as *Catharanthus roseus* an attractive alternative approach for treating diabetes and as well as its complications. On the basis of the results obtained in this study, vindoline and *C. roseus'* methanolic and aqueous leaf extract may be used in pharmaceutical applications especially in the management of diabetes.

3.6 Competing interests

Authors declare that there are no competing interests

3.7 Funding

3.8 Acknowledgements

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Chapter 4

Vindoline effectively ameliorated diabetes-induced hepatotoxicity by docking oxidative stress, inflammation and hypertriglyceridemia in type 2 diabetes-induced male Wistar rats.

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Abstract

Vindoline, an indole alkaloid present in the leaves of the *Catharanthus roseus* plant, has been recently reported to have insulotropic effects. This present study evaluated the possible hepatoprotective effects of vindoline in a type 2 diabetes mellitus rat model. Diabetes mellitus was induced by exposing rats to 10% fructose water for two weeks followed by a single intraperitoneal injection of 40mg/kg body weight of streptozotocin (STZ). Rats were randomly divided into six groups (n=8) and treated daily for 6 weeks with the vehicle via oral gavage, vindoline (20mg/kg) or glibenclamide (5mg/kg). Weekly fasting blood glucose (FBG) levels and body weight were measured and recorded. Administration of vindoline significantly ($p < 0.05$)

reduced FBG by 15% when compared to the diabetic controls. Vindoline significantly (p< 0.05) decreased diabetes-induced hepatic injury shown by decreased levels of serum alanine transferase (ALT) (-42%), aspartate aminotransferase (AST) (-42%) and alkaline phosphatase (-62%) compared to the diabetic controls. The oxygen radical absorbance capacity and the activities of superoxide dismutase (SOD) and catalase (CAT) were also improved following treatment with vindoline. The results also showed decreased levels of pro-inflammatory cytokines such as TNF-α by (-41%) and IL-6 (-28%) which may have also contributed to the reduction of serum triglycerides (-65%) in the diabetic group treated with vindoline. Histopathological findings showed improvement of both the hepatic and pancreatic tissues following vindoline treatment. Overall, these findings suggest that vindoline may protect the diabetic hepatic tissue from injury via antioxidant, anti-inflammatory and anti-hypertriglyceredemia mechanisms thereby retarding the development of diabetic complications.

Graphical Abstract
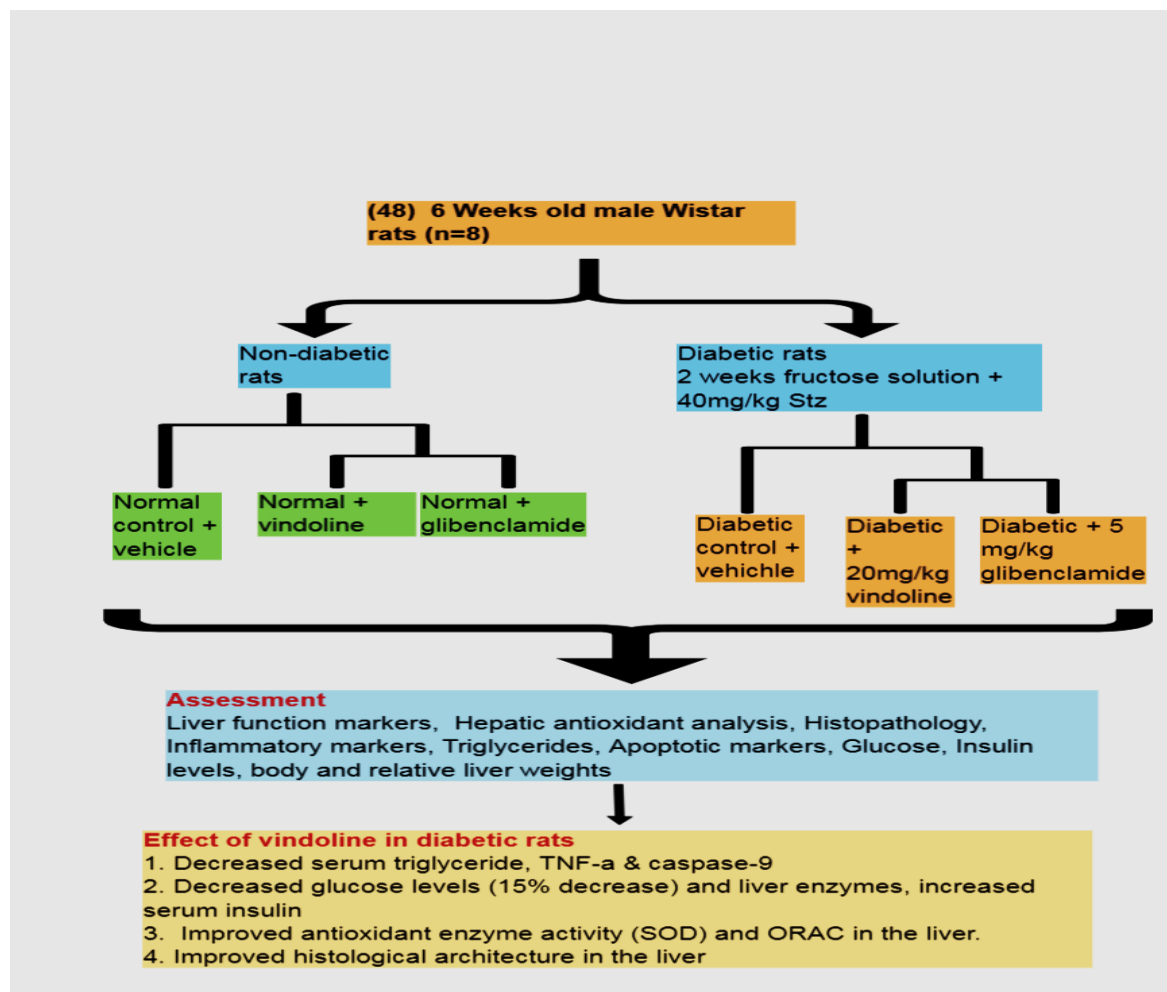
Figure 19: Graphical abstract

Keywords

Type 2 diabetes mellitus, oxidative stress, inflammatory cytokines, hepatic injury, vindoline,

4.1 Introduction

Diabetes mellitus (DM) has recently been labelled as one of the chronic diseases that have become a global health threat [1]. According to the World Health Organisation (WHO) 2016 report [2], an estimate of 422 million people globally were diagnosed with DM in 2014 while a 108 million had diabetes in 1980. DM is also one of the four most common non-communicable-diseases and currently the seventh leading cause of morbidity and mortality globally [3]. This high mortality rate is associated with complications such as diabetic liver disease (DLD) which progressively develop in these patients. Liver cirrhosis, steatohepatitis, non-alcoholic fatty liver disease (NAFLD) and hepatic carcinomas are examples of different spectrums of DLDs that are observed in T2DM patients with persistent hyperglycaemia [4,5]. DLDs primarily develop as a consequence of hyperglycaemia, hypertriglyceridemia and insulin resistance. In the absence of sufficient cytoprotective molecules, these cofactors induce inflammation, oxidative damage and finally necrosis or apoptosis [5,6]. In diabetes, chronic hyperglycaemia provides an environment that facilitates free radical formation, concomitantly depleting the endogenous antioxidant reserves ultimately leading to oxidative tissue damage [7,8]. Additionally, increased levels free radicals activate pro-inflammatory cytokines and the transcription of pro-apoptotic genes thus mediating chronic inflammatory responses and hepatocyte death respectively [9]. Abnormal high glucose levels have the ability to induce apoptosis by activating Bax–caspase proteases that alter mitochondrial function leading to apoptosis [10]. Therapeutic strategies which can efficiently prevent or reverse the adverse effects of prolonged oxidative stress and inflammation in DM may be highly beneficial in preventing diabetic complications such as DLDs.

From past decades, concerted efforts were made to address various issues in attempts to manage diabetes. These efforts evoked the discovery of different orthodox drugs currently used to achieve better glycaemic control and thus prevention of DM complications. A major drawback of oral antidiabetic agents is their failure to effectively cure diabetes; moreover, they have been associated with

increased side effects [11]. In this regard, medicinal plants appear as promising therapeutic avenues motivated by the presence of phytoconstituents that have numerous health benefits and may act as precursors in drug formulations [12–14].

Catharanthus roseus or *Vinca rosea* is a medicinal plant that has its origin in Madagascar hence the other name Madagascar periwinkle [15–18]. Traditionally, it was used to treat diseases like cancer, malaria, diabetes, insomnia and high blood pressure [18–21]. Different scientific studies reported the anti-hyperglycaemic nature of different extracts of *C. roseus* attributed to the presence of indole alkaloids, flavonoids, beta sitosterol, quercetin, catharanine [17,22–26]. Vindoline is an indole alkaloid extracted from *C.roseus* which was recently reported to possess protein-tyrosine phosphatase 1B (PTP-1B) inhibitory effects and therefore may serve as an "insulin sensitizer" in the management of T2DM [18,21]. From the best of our knowledge there is limited knowledge of the effects of vindoline in T2DM and associated complications. The present study was undertaken to evaluate the effect/s of vindoline against hyperglycaemia-induced oxidative tissue damage in the liver of T2DM- induced male Wistar rats.

4.2 Materials and Methods

4.2.1 Animal Care

Six weeks old male Wistar Rats were purchased from Charles River (Margate United Kingdom). Animals were housed at the South African Medical Research Council (PUDAC) according to the national standards and policies set out in the South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards SANS (10386:2008). Six rats per cage were housed under controlled standard environmental conditions (23±1 ºC, 55±5% humidity, 12:12 hour cycles of light to darkness. All the rats had free access to water and standard laboratory diet called Standard Rat Chow (SRC).

4.2.2 Ethical approval

This study was granted approval from the Ethics Committee for Research on Animals of the South African Medical Research Council (REF-01/17) and from the Faculty of Health and Wellness Research Ethics Committee of Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2016/A4).

4.2.3 Plant-derived chemical and standard drug

Vindoline was purchased from the manufacturer: Best of Chemicals (BOC) Sciences, USA). Specific guidelines, storage and preparation methods were followed as per suppliers' instructions. The standard drug glibenclamide was purchased from a local pharmacy and used in the experiment.

4.2.4 Induction of T2DM

T2DM was induced in rats by having them drink 10% fructose solution *ad libitum* for 14 days. Non-diabetic rats only drank normal tap water ad libitum. On day 15, rats received a single intraperitoneal injection of low dose of streptozotocin (STZ, 40 mg/kg body weight (b.w)) in freshly prepared 0.1M citrate buffer (pH 4.5) after an overnight fast. Diabetes was confirmed 72 hours post STZ administration, rats with 4hr fasting blood glucose level; between 15 mmol/ and 40 mmol/l were considered diabetic. Daily treatment (for 6 weeks) through oral gavage with respective compounds (vindoline/ glibenclamide) commenced after 5 days of STZ administration to ensure stable hyperglycaemia.

4.2.5 Experimental design

Forty-eight (48), 6 weeks old male Wistar rats (190-230g) were randomly divided into six (6) groups with a minimum of eight rats each (n=8). Rats in all the groups were fed *ad libitum* with standard rat chow (SRC) and tap water.

Group **N**: normal control administered with vehicle only;

 Group **NV**: normal treated group administered with vindoline (20mg/kg b.w);

 Group **NG:** normal treated group treated with glibenclamide (5mg/kg b.w).

 Group **DC:** diabetic control group administered with vehicle;

Group **DV:** diabetic treated with 20mg/kg b.w of vindoline;

 Group **DM:** diabetic treated with 5mg/kg b.w of glibenclamide;

Fasting blood glucose levels and weights were measured once a week using a glucometer and a balance respectively while blood samples were collected via the tail prick. After the 8 week study period, the rats were fasted overnight in preparation for sacrifice and sample collection. Final blood glucose levels and body weights were

recorded prior to termination of experiment. Rats were anaesthetised and euthanized using isoflurane gas at 2% with 1% oxygen during laparotomy.

4.2.6 Serum Preparation

Blood samples were collected from the abdominal vena cava into 10ml serum separator vacutainer tubes (anticoagulant contained). Tubes were allowed to stand at room temperature for 30 minutes prior to centrifugation. The samples were centrifuged at 3500 rpm for 15 minutes. Once centrifuged, the serum and plasma were aliquoted into cryo-tubes, snap-frozen in liquid nitrogen and stored at -80°C.

4.2.7 Organ preparation

Liver samples were collected, quickly washed in phosphate buffered saline (PBS) to remove blood and then weighed. Some portions to be used for antioxidant analysis were snap frozen in liquid nitrogen and stored at -80°C for future analysis. Remaining portions were preserved in 10% (v/v) neutral buffered formalin and embedded in paraffin wax for histological analysis. Later on, the snap frozen samples were homogenised in ice-cold respective buffers for different endogenous antioxidant activity determination. Homogenates were centrifuged for 15 minutes at 15000 rpm at 4 °C, aliquoted and stored at -80°C until analysis.

4.2.8 Determination of the relative liver weights

The relative liver weights were calculated using the following formula
Relative liver weight = (liver weight ÷ total body weight) 100g

4.2.9 Liver function enzymes activity

The activities of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured using ABX PENTRA 400 automated chemistry analyser machine.

4.2.10 Measurement of hepatic antioxidant markers enzymes

Catalase (CAT) activity was determined spectrophotometrically by assessing the exponential disappearance of H_2O_2 at 240 nm. The assay was carried out according to the method of Aebi (1984) [27]. The activity of superoxide dismutase (SOD) was measured following the procedure described by Crosti et al (1989) [28]. The level of reduced glutathione (GSH) was determined by the method of Jollow et al [29] and measured spectrophotometrically at 412nm.

4.2.11 Determination of lipid peroxidation

Lipid peroxidation (LPO) in the liver homogenates was assessed by measuring the amount of malondialdehyde (MDA) products that were reacted with the thiobarbituric acid. The coloured complex formed when MDA reacted with thiobarbituric acid (TBA) was measured at 532 nm according to the method used by Tug *et al* [30].

4.2.12 Hepatic antioxidant analysis

Oxygen radical absorbance capacity (ORAC) is an assay that measures the ability of antioxidants in a particular sample to scavenge radicals [31]. The principle of the assay is based on the scavenging and inhibition capacity of lipophilic antioxidants in the presence of a cyclodextrin water-based enhancer upon the free radical damages.

The free radical damage is evaluated by the loss of fluorescence of a fluorescent probe over time; therefore the decrease of fluorescence signifies the extent of free radical damage and as well as a direct proportional relationship with the free radical concentration [31,32].

4.2.13 Measurement of triglycerides

The concentration of triglycerides in the serum was determined using the ABX PENTRA 400 chemistry analyser. The kit was purchased from Scientific Group Company (South Africa).

4.2.14 Inflammatory cytokines

The amount of cytokines: interleukin-6 (IL-6), IL-10, IL-1b and tumor necrosis factor (TNF)-α in the homogenates were evaluated using the Bio-plex® platform (Bio-rad Laboratories, Hercules, CA (USA). The MILLIPLEX® MAP rat cytokine magnetic bead-based Luminex kit was purchased from Merck Millipore, Billerica, MA (USA).

4.2.15 Histopathological studies

Washed liver samples were fixed in 10% formalin, processed using routine histology techniques. The liver samples were then embedded in paraffin and were cut into sections of 5 μm thickness using the Leica RM2125 microtome (Leica Microsystems, Inc., Buffalo Grove, United States of America). The sections were deparaffinised and stained using haematoxylin and eosin (H & E) stain

4.2.16 Immunohistochemistry

Anti-Bcl2, anti-caspase 3 and anti-caspase 9 antibodies were obtained from Abcam. Formalin-fixed, paraffin-embedded liver sections were first pre-treated with heat-

induced epitope retrieval (HIER) for 20 minutes at 98°C so as to expose antigen sites. Respective antibodies at a dilution of 1:100 were added to the slides. Immuno labelling staining was performed using the Leica Bond autostainer (Leica Biosystems, SA). Slides were incubated with peroxidase block for 5 minutes using the Bond Polymer Refine Detection Kit. The sections were then incubated for 30 minutes in respective primary antibodies. Following incubation, post primary block incubation using post primary antibody was performed at room temperature for 30 minutes. Incubation of slides with 3,3' Diaminobenzidine (DAB) chromogen solution and DAB substrate buffer polymer was done to facilitate the production of a brown end-product. Finally, hematoxylin was used to counterstain the nuclei during a 5 minute incubation step. Liver tissue sections were dehydrated by moving slides in a series of graded alcohols. Slides were finally mounted using dibutyl phthalate xylene (DPX). Slides were viewed and images captured using the EVOS XL Cell imaging microscope. Positive intensities were analysed and quantified using ImageJ Immuno Profiler software (version 10.2 image analysis).

4.2.17 Statistical analysis

Results were analysed using GRAPH PAD Prism software package, Version 5.0. Data were expressed as mean ± standard error mean (SEM). The comparisons within groups were determined by using the one way analysis of variance (ANOVA) and Bonferonni's multiple test comparison. The values were considered to be statistically significant when the p value was < 0.05.

4.3 Results

4.3.1 Effect of vindoline or glibenclamide on body weight, liver weight and blood glucose levels.

The effect of vindoline or glibenclamide administration on blood glucose, serum insulin, and body and liver weights in T2DM-induced and normal rats as represented in Table 6 below. Treating diabetic rats with vindoline resulted in significant decrease of blood glucose (15% decrease) in comparison with the diabetic control (DC) ($p<0.05$) whereas glibenclamide did not show significant alterations of glucose levels when its effect on glucose levels was compared to both the diabetic controls as well as the diabetic group that received vindoline ($p<0.05$). In normal rats, vindoline and glibenclamide caused no major changes in the glucose levels when compared to the normal controls. The insulin level in diabetic rats treated with vindoline (DV) was elevated significantly ($p<0.05$) in comparison to the diabetic controls, in addition we did not observe any substantial differences in insulin levels between the diabetic-glibenclamide group and diabetic controls and diabetic rats treated with vindoline. Neither the percentage body weight change nor the liver weights (expressed as relative liver weights) were significantly modified by vindoline treatment in the diabetic and normal groups when compared to the diabetic controls and the normal untreated group, respectively.

Table 6: Effect of vindoline or glibenclamide administration on glucose, serum isulin, body and liver weights in T2DM-induced and normal rats

Parameters	NC	NV	NG	DC	DV	DG
FG (mmo/L	10.62 ± 0.34^b	10.04 ± 0.31^b	10.18 ± 0.29^b	31.94 ± 0.54^a	27.15 ± 1.47^a ^{b}	29.23 ± 1.33 ^{a}
Insulin U/ml	11.67 ± 1.03	7.43 ± 0.37^a	9.85 ± 0.92^b	5.13 ± 0.46^a	9.56 ± 1.32^b	8.47 ± 1.09
FBW(g)	293.1 ± 23.34 ^{b}	338.4 ± 37.05^a ^{b}	310.0 ± 29.45 ^{b}	243.1 ± 33.6	241.0 ± 26.64 ^{a}	255.8 ± 15.80
WC (%)	94.56	83.0^b	79.51^b	31.21^a	46.06^a	52.339^a
LW (g)	9.71 ± 1.18	11.31 ± 1.67	9.46 ± 1.07	11.97 ± 1.04	11.04 ± 1.54	12.02 ± 1.02
RLW (g)	3.32 ± 0.38^b	3.34 ± 0.27^b	3.05 ± 0.1^b	4.97 ± 0.51	4.59 ± 0.48^a	4.70 ± 0.28^a

Data represented as means ± SEM. FG: final blood glucose; FBW: final body weight; WC: percentage weight change; LW: liver weight; RLW: relative liver weight. [a] $p< 0.05$ vs normal control. [b] $p< 0.05$ vs diabetic control. [C] $p< 0.05$ vs normal rats treated with vindoline. [d] $p< 0.05$

vs normal rats treated with glibenclamide. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide.

4.3.2 Effects of Vindoline or glibenclamide on serum levels of hepatic enzymes (hepatic function) in T2DM-induced rats

Table 7 illustrates the effects of vindoline or glibenclamide on the activities of liver enzymes (liver function). Induction of T2DM in rats especially the diabetic controls resulted in substantial elevation of serum liver function enzymes (AST, ALT, ALP and LDH) when compared to the non-diabetic groups. Interestingly, vindoline and glibenclamide attenuated the levels of AST, ALT and ALP in diabetic rats in comparison with the diabetic control group ($p < 0.05$) but LDH remained unaltered. Normal rats that received vindoline showed marginally elevated AST levels although not significant when compared to the normal controls and normal rats treated with glibenclamide.

The levels of total protein (TP) and albumin in the diabetic controls were significantly higher than the diabetic group treated with glibenclamide ($p < 0.05$), vindoline did not significantly modify the levels of TP and albumin in T2DM rats when compared to the diabetic controls or the diabetic group treated with vindoline. Normal rats treated with vindoline showed significantly increased TP when compared to all the groups.

Table 7: Effect of vindoline or glibenclamide treatment in T2DM-induced and normal rats

Test	NC	NV	NG	DC	DV	DG
ALT	34.50±5.13	40.88±2.64[b]	27.88±1.49[b]	161.6±31.56[a]	94.43±17.68[ab]	73.67±13.33[b]
AST	88.13±5.45	103.9±9.05[b]	85.75±8.13[b]	180.9±37.35[a]	105.4±18.98[b]	84.29±13.61[b]
ALP	105.7±9.14[b]	106.3±7.73[b]	90.51±5.33[b]	861.4±132[a]	330.6±51.6[abc]	469.7±64.2[abc]
LDH	15.36±1.86[b]	19.18±3.14[b]	19.75±2.74[b]	78.81±8.55	73.03±8.85[a]	52.60±9.5[a]
TP(g/L)	50.75±5.55	61.16±8.37[ab]	48.16±3.1[c]	51.30±7.84	43.85±5.23[c]	39.48±5.80[abc]
Alb(g/L)	27.11±2.65	31.95±3.72[a]	27.40±1.35	28.40±3.07	25.85±2.48[c]	22.58±1.61[abc]
Glob	23.64±1.06	29.21±1.69	20.76±1.45[c]	22.90±2.05	18.00±1.21[c]	16.90±1.66[c]

Data represented as means ± SEM. [a] $p < 0.05$ vs normal control. [b] $p < 0.05$ vs diabetic control. [c] $p < 0.05$ vs normal rats treated with vindoline. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide

4.3.3 Antioxidant activity of liver homogenates

Presented in Figure 20 is the hepatic antioxidant status of non-diabetic and diabetic rats. After administration of vindoline and glibenclamide in T2DM, there was a significant improvement of ORAC and SOD enzyme activity while CAT, lipid peroxidation and reduced-GSH were not significantly altered ($p < 0.05$) in comparison with the diabetic control. Additionally, vindoline and glibenclamide administration in non-diabetic rats also improved the activity of SOD and CAT significantly when compared to the normal control group ($p < 0.05$).
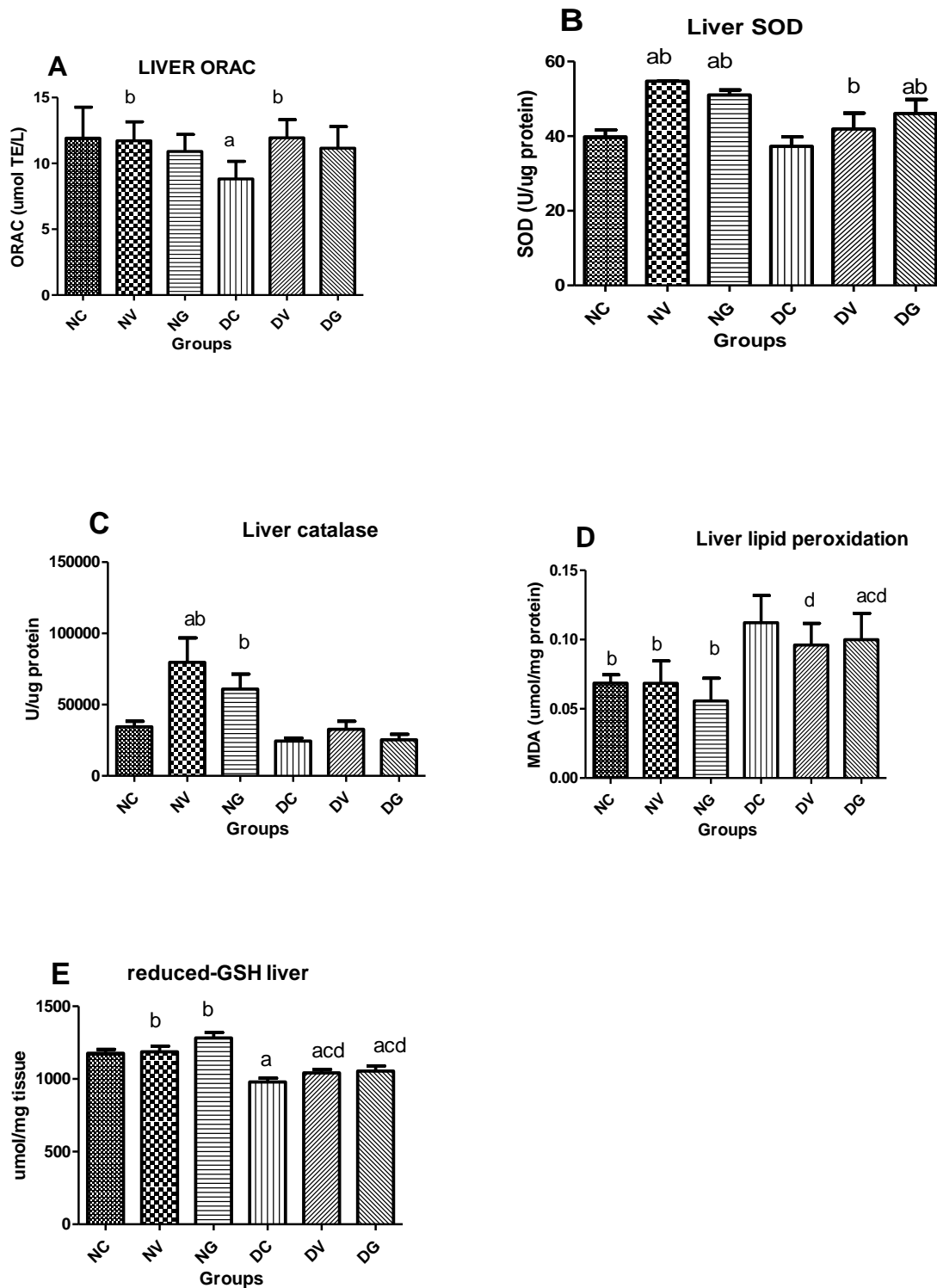
Figure 20: (A) Oxygen radical antioxidant capacity (ORAC), (B) Superoxide dismutase (SOD), (C) Catalase (CAT) measurements in groups, (D) Lipid peroxidation, (E) Reduced glutathione. Data represented as means ± SEM. [a] $p < 0.05$ vs normal control. [b] $p < 0.05$ vs diabetic control. [c] $P < 0.05$ vs normal rats treated with vindoline. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide

4.3.4 Effects of vindoline or glibenclamide on the levels of triglycerides, TNF-α, IL-10 and IL-6

Figure 21 shows the effect of vindoline administration on the levels of inflammatory biomarkers and triglycerides. An increase in TNF-α levels was observed in the diabetic controls when compared to the normal control group ($p < 0.05$). Subsequent treatment of T2DM rats with vindoline led to a significant down regulation ($p < 0.05$) of TNF-α levels when compared to the diabetic controls. IL-10 and IL-6 levels in diabetic groups treated with vindoline and glibenclamide were not significantly different from diabetic and normal control groups ($p < 0.05$).

In this study, a marked reduction of serum triglycerides in T2DM treated rats was noted in comparison with the diabetic controls ($p < 0.05$). In addition, the serum triglycerides in the diabetic group treated with vindoline and glibenclamide were restored to near normal levels with no significant differences to normal controls ($p < 0.05$).
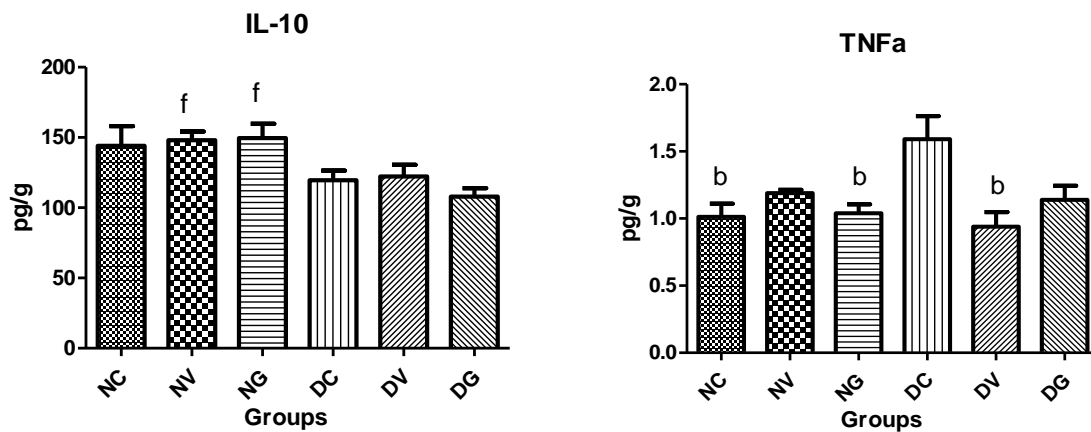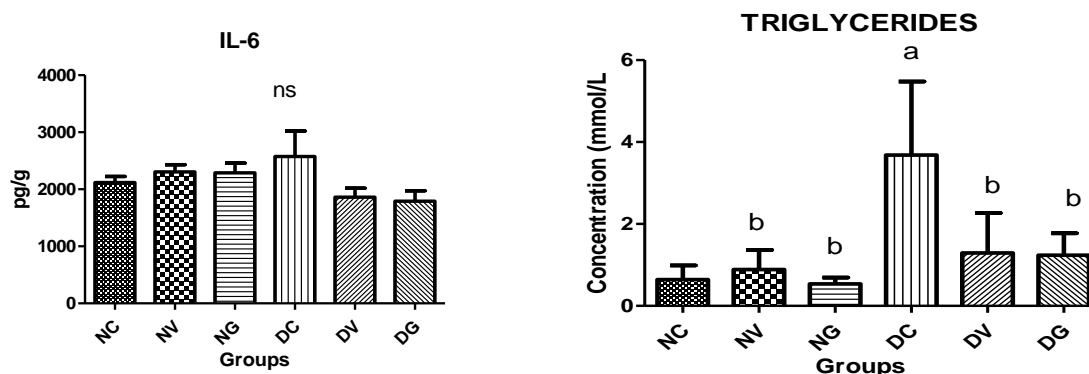
Figure 21: Effects of vindoline or glibenclamide on the levels of inflammatory cytokines: TNF-α, IL-10 and IL-6. Data represented as means ± SEM. [a] $p < 0.05$ vs normal control. [b] $p < 0.05$ vs diabetic control. [C] $P < 0.05$ vs normal rats treated with vindoline. [d] $p < 0.05$ vs normal rats treated with glibenclamide. [f] $p < 0.05$ vs diabetic rats treated with glibenclamide. [ns] $p < 0.5$ non-significant change among all groups. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide

4.3.5 Effect of vindoline or glibenclamide on the histological architecture of the hepatic tissue.

Histological assessment of the liver sections of experimental groups revealed the protective effect of vindoline against T2DM-induced tissue damage. As indicated in Table 8 and Figure 22; all the non-diabetic groups showed normal liver architecture. Induction of diabetes resulted in hepatocellular damage especially in the areas around the central vein, where the cells showed signs of degeneration. However, treatment of the diabetic groups with vindoline or glibenclamide resulted in minimised tissue damage according to the histopathological scoring presented in Table 3. Central vein congestion accompanied by sinusoidal congestion was prominent in the diabetic control group (Fig 3 (D)) but minimal in the vindoline treated diabetic group as shown in Figure 3. Dilation and disruption of normal sinusoidal architecture were evident in the diabetic control group and in the diabetic group treated with glibenclamide (arrows in Fig 3 D & F). Moderate infiltration of inflammatory cells was observed in the three diabetic groups.

Table 8: Histopathology score in hepatic tissue

	NC	NV	NG	DC	DV	DG
Liver						
Hepatocyte injury	0	0	0	2	1	1
Central vein congestion	0	0	0	2	1	1
Sinusoidal congestion	0	0	0	2	1	1
Sinusoidal dilation	0	0	0	2	0	1
Infiltration of inflammatory cells	0	0	0	1	1	1

Hepatic injury was scored as follows: o: absence of cell damage in > 80% of the tissue; 1: damage < 30% of the tissue; 2: damage between 30-50% of tissue; 3: necrosis > 50% of tissue. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide

Figure 22: represents the haematoxylin and eosin stained liver sections (X100). A: normal control; B: normal control treated with vindoline; C: normal control treated with glibenclamide; D: diabetic control; E: diabetic tr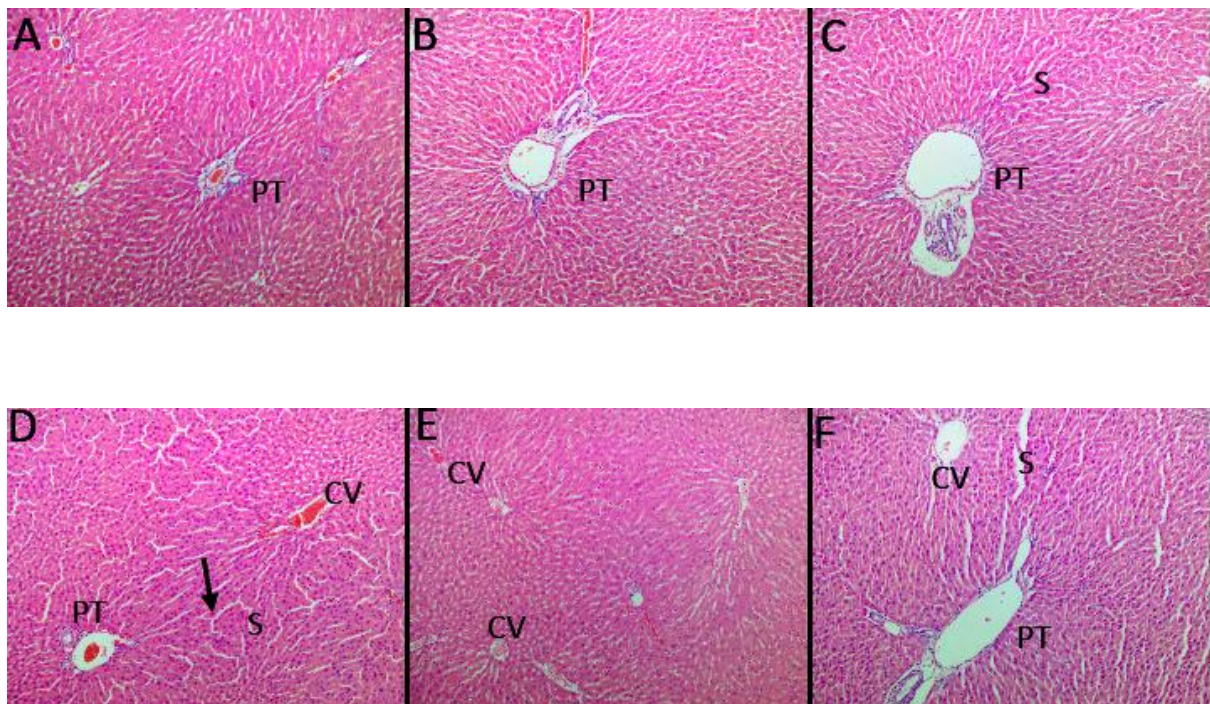eated with vindoline; F: diabetic treated with glibenclamide. PT: portal tract; CV: central vein; S: sinusoids; arrows indicate sinusoidal dilation and disordered arrangement of sinusoids.

4.3.6 Histological examination of the pancreas

Effect of vindoline or glibenclamide on the histological architecture of the pancreatic tissue as shown Figure 23. Plate A, B and C represents normal pancreas with intact islets tissue with no visible signs of injury. The diabetic untreated controls had small islets with vacuolated cells as indicated in plate D1 below. The diabetic rats that were treated with vindoline (plate E) had islets that were larger in size with minimum destruction when compared to the diabetic controls. Administration of glibenclamide to diabetic rats resulted in minimum pancreatic tissue injury when compared to the diabetic controls (plate F).
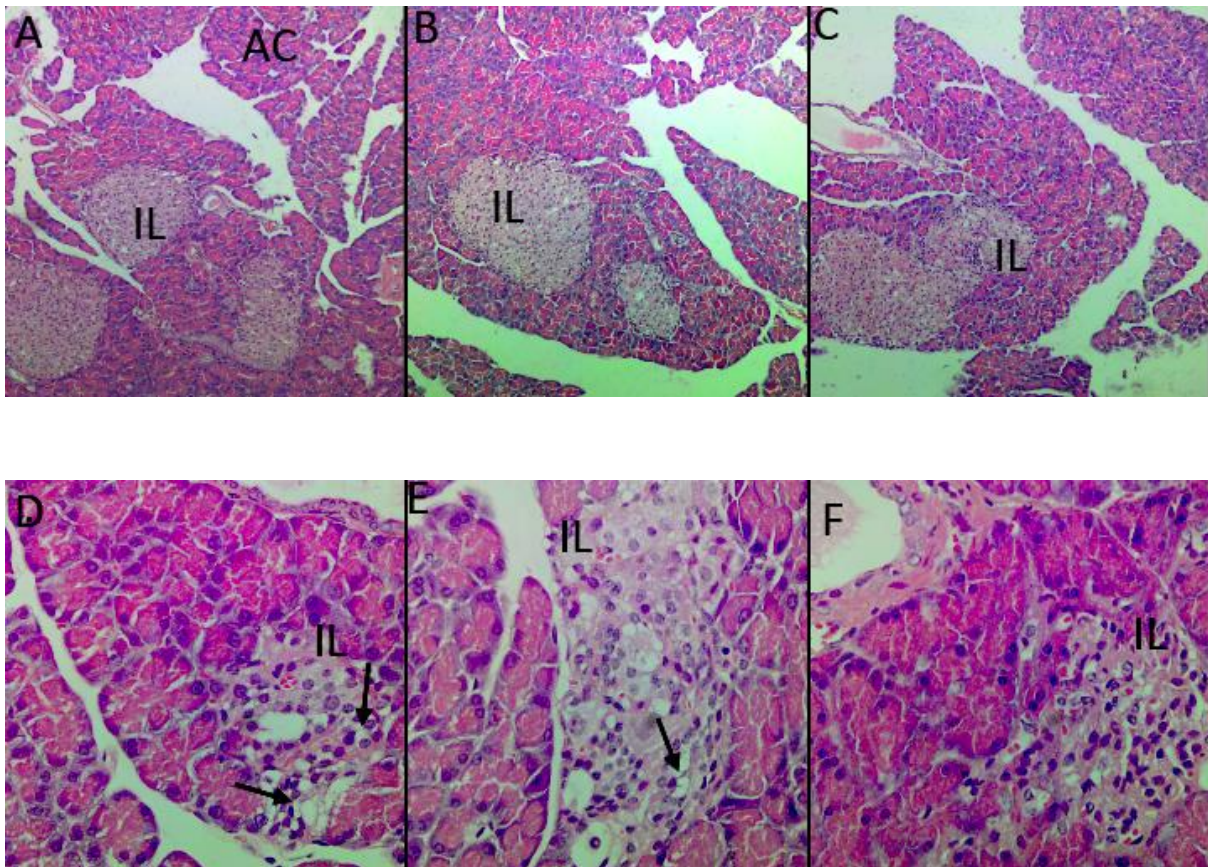
Figure 23: Histopathological changes in the pancreatic tissue of treated and non-treated diabetic and non-diabetic rats. Plate A (normal control) shows normal islets tissue (IL) with well-defined borders, surrounded by the exocrine acinus cell (AC). Plates B (normal-treated with vindoline) and C (normal treated with glibenclamide) show normal islets (IL). Plate D (diabetic control) shows loss of the normal size and compactness of the islets, vacuolated degenerated islets (black arrows). Treated islets with vindoline were smaller in size when compared to the normal but relatively larger in size than the diabetic controls. The Islets

showed a degree of vacuolation (arrows in Plate E) with slight regeneration. Glibenclamide treatment in diabetic rats (Plate F) showed few vacuolated islets and minimum loss of islet architecture.

4.3.7 Quantitative immunohistochemical findings

Figure 24 illustrates the effect of vindoline and glibenclamide on the expression of apoptotic markers in hepatic tissue of normal and T2DM rats. Quantitative evaluation of images based on different nuclear colour intensities was performed using imageJ software. Caspase 9 was found to be significantly expressed in the diabetic control group at ($p<0.05$) when compared to all treatment groups. In addition; administration of vindoline and glibenclamide in normal rats resulted in increased expression of caspase 9 ($p<0.05$) when compared to the normal controls. With respect to the expression of BCL-2, treating T2DM rats with vindoline or glibenclamide did not significantly alter the expression of BCL-2 when compared to the diabetic control group ($p<0.05$). However the expression of BCL-2 in normal controls was significantly increased when compared to the diabetic control, diabetic groups treated with vindoline and glibenclamide ($p<0.05$).
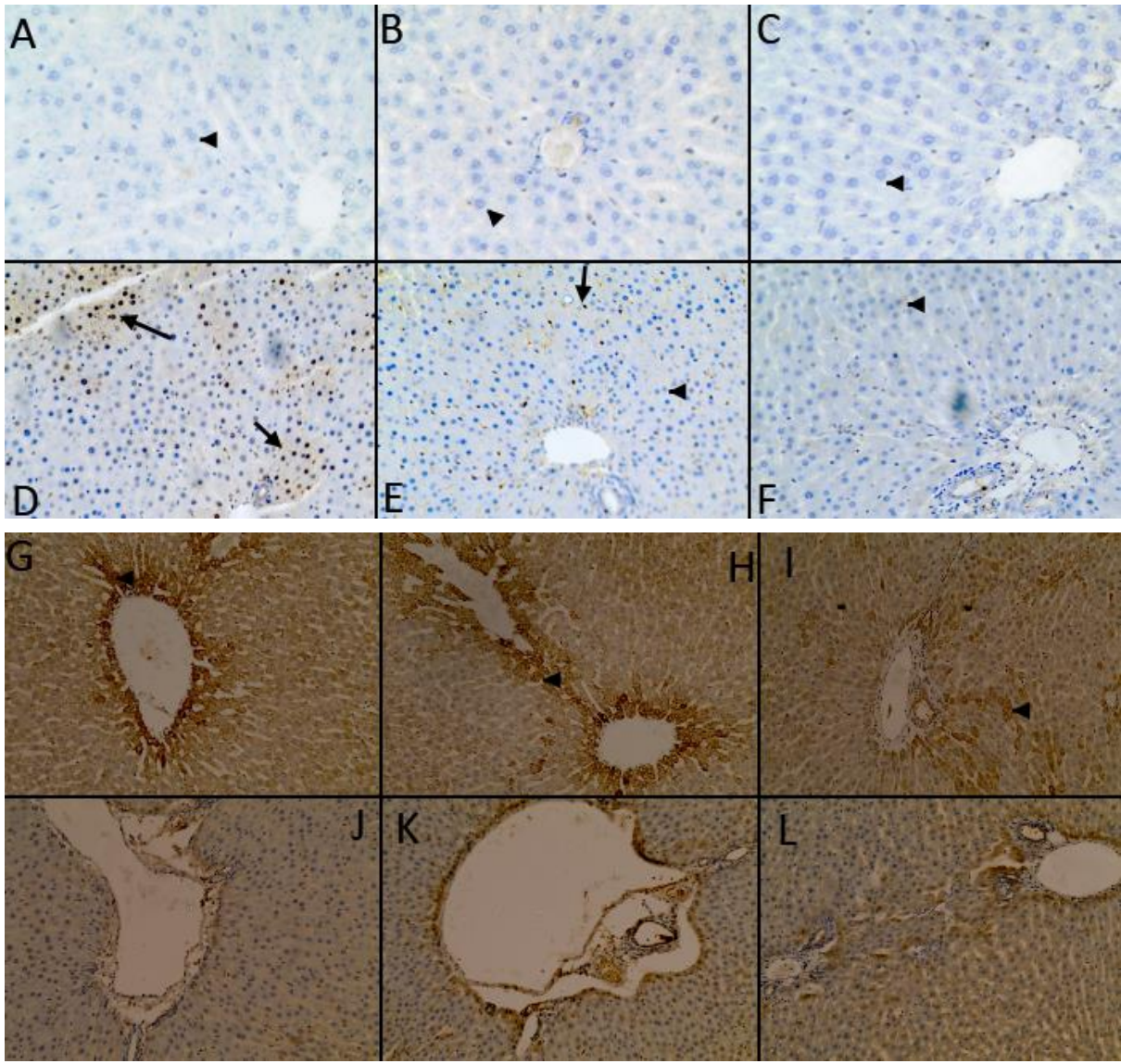
(a)
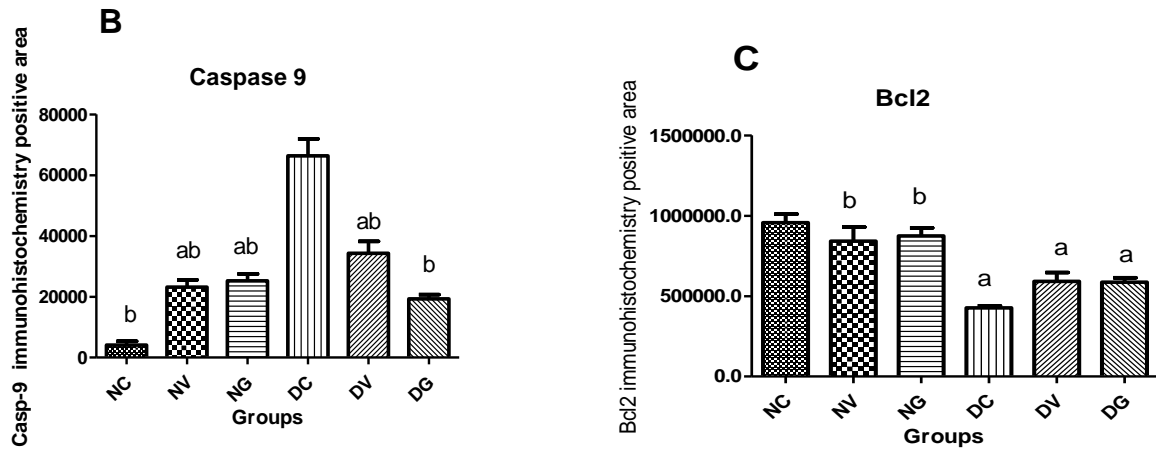
Figure 24: Pictorial and quantitative immunohistochemical representation of apoptotic markers of the liver sections labelled with anti-Bcl-2 and anti-caspase 9. Data represented as means ± SEM. ^{a} p< 0.05 vs normal control. ^{b} p< 0.05 vs diabetic control. ^{C} p< 0.05 vs normal rats treated with vindoline. ^{f} p< 0.05 vs diabetic rats treated with glibenclamide. Plate A-F represents caspase 9 marker intensities; Plate G-L represents Bcl-2 marker intensities. A/G= (NC): normal control; B/H= (NV)= normal control treated with vindoline; C/I= (NG)= normal control treated with glibenclamide; D/J=DC=diabetic control; E/K=DV= diabetic treated with vindoline; F/L=DG= diabetic treated with glibenclamide; arrows indicate high immunoreactive cells in anti-caspase 9 pictographs, arrow heads represents negative immunoreactivity in anti-caspase 9 pictographs. In Bcl-2 pictographs; arrow heads indicate high immunoreactivity.

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4.4 Discussion

The present study aimed to evaluate the effect of vindoline in hepatotoxicity induced by T2DM conditions using glibenclamide as reference drug. Uncontrolled hyperglycemia in DM is the hallmark of free radical formation implicated in the modification of structures and functions of macromolecules contributing to early development of diabetic complications [33,34]. Antidiabetic therapy must therefore aim to maintain normoglycemia in addition to delay or prevention of tissue injury [35]. Natural products are promising avenues that could be effective and safer in ameliorating diabetic complications [36].   Vindoline is an indole alkaloid derived from the leaves of *C.roseus* and has been reported to stimulate the release of insulin from pancreatic beta cells [37].

The results of this study showed a significant reduction in fasting blood glucose levels in diabetic rats following treatment with vindoline.   It appeared that administration of glibenclamide in diabetic rats exhibited lesser effects on fasting glucose level than vindoline which suggests that vindoline may possess a better insulin secretory effect than glibenclamide. We presumed that vindoline's mechanism of action was through stimulation of existing pancreatic β cells to secrete insulin. Our findings are in agreement with the results reported by Yao *et al* (2013) [37] who observed a marked reduction in blood glucose levels in T2DM obese model. One of the side-effects of compounds that enhance insulin excretory effect is hypoglycaemia. Based on our current findings; administration of vindoline in non-diabetic rats did not induce hypoglycaemia probably because the dosage administered was lower and tolerable [37,38].

Insulin deregulation is predictive of abnormal cofactors like hepatic insulin resistance, hyperglycemia and β-cell dysfunction which modulate metabolic derangements [10]. Vindoline administration in T2DM rats led to the elevation of insulin, the levels were higher than those observed in the diabetic group treated with glibenclamide. Increased insulin levels may have been due to its previously reported Kv2.1 inhibitory effect which reduces the voltage-dependent outward potassium current resulting in insulin secretion by pancreatic β-cells [37]. Despite the increased

insulin level in the serum of both diabetic treated rats, hyperglycemia was still present thus indicating resistance to the action of insulin. In normal rats, vindoline administration did not stimulate secretion of insulin, suggesting that vindoline may not have insulotropic effects in normoglyecemic condition which is also in agreement with Yao *et al* [37] findings.

It has been shown that DM patients suffer from severe weight loss as a result of increased catabolism of fats and proteins in the skeletal muscle in response to the deranged carbohydrate metabolism [39]. The current study showed diminished body weight gain in all diabetic groups in which vindoline and glibenclamide both failed to ameliorate weight loss. Such result may be an indication that glycogenolysis, lipolysis and proteolysis were still going on in order to compensate for the lack of glucose in the cells [40]. Liver hypertrophy is a common observation in diabetic animals attributed to increased free fatty acids pools in the hepatocytes as a consequence of abnormal insulin metabolism [32]. All diabetic groups in our study had increased relative liver weight when compared to the normal groups. Treatment with vindoline and glibenclamide to diabetic rats did not significantly reverse liver hypertrophy.

Hepatic enzymes which include amino transferases (AST and ALT) and alkaline phosphatase (ALP) are the first line of markers used to determine hepatic injury. In a state of hepatocellular damage, these enzymes leak into the serum resulting in elevated levels. Elevated serum transaminases are commonly observed in diabetics as a result of functional disturbances of hepatocyte membranes [41–43]. Upon oral administration of vindoline and glibenclamide in T2DM rats, the serum levels of ALT, ALP and AST reduced significantly while LDH remained unchanged when compared to the diabetic untreated group. These results may indicate comparable hepatoprotective effect of vindoline and glibenclamide probably brought about by increased hepatocyte membranal stability and hepatocellular regeneration. No significant alterations were seen in normal rats treated with vindoline when compared to the normal controls and normal rats treated with glibenclamide.

It is known that hyperglycaemia stimulates the breakdown of structural proteins directly affecting the synthesis and secretion of vital proteins like albumin [36,44]. Albumin is a major transporter protein that carries analytes during various metabolic

processes [45]. In the present study, total protein concentration was lower in the diabetic groups treated with vindoline (not significantly) and glibenclamide (significantly) when compared to the untreated diabetic control. This decrease could have been attributed to the increased binding of the vindoline and glibenclamide components to serum albumins. This result is in agreement with the results obtained by Iweala and Okeke (2005) [46] who reported low levels of total protein in rats treated with *C.roseus* and chlorpropamide. Unexpectedly, the total protein levels were high in the diabetic controls without any significant difference when compared to the normal controls. It is logical to argue that the high total protein levels in the diabetic controls could have been falsely increased as a result of dehydration due to polyuria [47,48]. In the vindoline treated normal controls, a marked significant increase in the total protein was seen when compared with the normal control and the glibenclamide treated normal rats.

It is well established that persistent hyperglycemia is closely linked to oxidative stress [49]. Oxidative stress is the underlying mechanism in the development of diabetic complications via the excessive formation of ROS and/ insufficient production of antioxidants [11,50]. In order to evaluate if vindoline could decrease/ prevent oxidative damage, we measured the oxygen radical absorbance capacity (ORAC), SOD and CAT activities, lipid peroxidation and reduced-GSH levels in the liver homogenates. The ORAC assay is performed to assess the ability of biological samples to withstand the deleterious effects of excess free radicals. The ability of the homogenates to scavenge oxygen radical was augmented significantly to near normal in the T2DM rats treated with vindoline more than the diabetic controls. Glibenclamide's effect on ORAC in diabetic rats was lower than that of vindoline suggesting that vindoline may prevent cellular damage through its strong free radical reduction capacity [50].

Superoxide dismutase, CAT and GSH are endogenous antioxidants that work hand in hand to combat free radical toxicity in tissues. SOD is an antioxidant enzyme responsible for the dismutation of the unstable superoxide radicals into H_2O_2, while catalase catalyses the decomposition of H_2O_2 into inert water and oxygen [45]. GSH is an antioxidant defence molecule with powerful scavenging and detoxifying potential against hydroxyl anion and oxygen radical [51]. Excessive free radical build-

up has been implicated in the initiation peroxidation of cell membrane lipids. Peroxidation of membranal lipids destroys the structural and functional integrity of the cell causing faulty permeability which is harmful to cellular organelles [52].

In our findings; we observed obvious decrease in the activities of these enzymes as well as in the levels of GSH in the diabetic controls. Interestingly, administration of vindoline and glibenclamide in diabetic rats significantly improved the hepatic antioxidant status as evidenced by elevated activities of SOD thus increasing protection against oxidative injury. The same increase was observed in non-diabetic groups that received glibenclamide and vindoline in comparison with the non-treated controls. On the other hand; the CAT activity and reduced-GSH level in the vindoline treated diabetic group was increased but not significantly when compared to the diabetic controls and diabetic-glibenclamide treated group. Treatment of diabetic rats with glibenclamide or vindoline did not significantly reduce the formation of by-products of lipid peroxidation suggesting that their antioxidant effect only boosts the first line of free radical defence antioxidants [52]. The antioxidant effect exhibited by vindoline in this study shows an alternative therapeutic effect of vindoline that could be useful in the treatment of the diabetes mellitus [49,53].

Various studies have indicated the important role inflammation plays in the pathogenesis of insulin resistance, T2DM and its complications [54]. Inflammation is an adaptive response elicited by the body following tissue injury in attempts to reverse injury [55]. The increased release of inflammatory cytokines such as TNF-a, IL-6 and decreased production of IL-10 in DM has been documented in diabetic models [34]. We observed that the levels of IL-10-an anti-inflammatory cytokine were significantly higher in the normal rats treated with vindoline or glibenclamide when compared to the diabetic group treated with glibenclamide. Vindoline administration in diabetic rats did not significantly affect the levels of IL-10.

Depending on the tissue and metabolic state; IL-6 exists as both a pro and anti-inflammatory cytokine [56]. According to our result, IL-6 levels in the hepatic homogenates were decreased in diabetic rats treated with vindoline and glibenclamide although the results were not significant when compared to the diabetic control. This result may indicate that vindoline and glibenclamide could possibly safeguard the hepatic tissue by suppressing the release of IL-6 by kupffer

cells in hyperglycaemic milleu. Due to the complexity of IL-6 metabolism, the non-diabetic groups' IL-6 levels were not significantly different from the diabetic control. This finding in the normal rats may be due to the beneficial roles (anti-inflammatory) exerted by IL-6 [57]. A positive correlation between TNF-α and hepatic injury in diabetes has been documented mainly because TNF-α promotes insulin resistance that leads to severe hyperglycemia and finally oxidative injury [58]. Assessment of TNF-α demonstrated elevated levels in the diabetic control group while, oral administration of vindoline evidently decreased TNF-α levels. This result suggests better potential immunomodulatory effect of vindoline in comparison to glibenclamide which may be of therapeutic relevance in preventing and/ retarding the onset of hepatic tissue injury elicited by adverse inflammatory responses [59].

Abnormal fatty acid metabolism in diabetes mellitus is associated with hypertriglyceridemia which is a risk factor for the development of cardiovascular related diseases (CVDs) and NAFLD [60,61]. Lipoprotein lipase is an enzyme that normally acts by hydrolysing triglycerides in the presence of insulin. However in DM, its activation is denied due to insulin deficiency resulting in hypertriglyceridemia [61,62]. Based on our results, it is evident that treatment of the diabetic rats with vindoline decreased serum triglyceride levels when compared to the diabetic controls. Vindoline and glibenclamide administration in T2DM rats restored the triglyceride levels to near normal levels as the non-diabetic controls. The reduction of the serum triglycerides in the diabetic treated groups may have been due the increased activity lipoprotein lipase thus increasing the hydrolysis of triglycerides [40]. This observation implies that as glibenclamide, vindoline may reduce the cardiovascular and NAFLD risks associated with diabetes [63].

Our histopathological findings revealed that induction of T2DM resulted in hepatic tissue damage which was confirmed by changes in the tissue architecture. Liver section in diabetic rats demonstrated severe mild leukocyte infiltration, central vein congestion, sinusoidal dilation and congestion and hepatocyte vaculoation. These findings were in agreement with the findings of Aldahmash *et al* (2016) [64] who reported similar changes in DM mice. Administration of vindoline showed appreciable improvements to these abnormal alterations revealed by close to normal sinusoidal pattern and reduced congestion of the central vein. Administration of

glibenclamide in diabetic rats could not reverse the dilation of sinusoids, which indicated vindoline's more robust effect in restoring diabetic liver pathology. Consequently, it was clearly observed that induction of STZ in conjunction with the 10 % fructose water resulted in the irreversible destruction of insulin secreting β cells of the pancreas as shown in Figure 23 in plate D1 and D2. The pancreas of vindoline treated rats showed improvement in the size and islet morphology when compared to the diabetic control and the diabetic-glibenclamide treated groups. Our results largely highlight vindoline may not only stimulate insulin secretion but also protect the intact functional β cells from STZ-induced destruction [37].

Oxidative stress together with adverse cytokine responses elicits the activation of apoptosis in biological systems [65]. Bcl-2 is an important protein that regulates apoptosis by preventing cell death [66]. According to the hepatic immunohistochemical reports, there was downregulation of Bcl-2 protein expression in the diabetic control rats. Oral administration of vindoline in diabetic rats did not significantly increase the expression of Bcl-2 when compared to the diabetic control group. However, there was a marked downregulation of caspase 9 in the hepatic tissue of diabetic rats treated with vindoline when compared to the diabetic controls. Unlike Bcl-2, caspase 9 is a pro-apoptotic protein which activates executioner-caspase 3 thus decreasing survival chances of the cell. Enhanced expression of caspase 9 in diabetic control rats in this study could indicate failed control of apoptosis in the mitochondrial pathway [65,67]. Therefore, vindoline may be a valuable therapeutic approach in the prevention of hepatic cell death in diabetes mellitus.

4.5 Conclusion

In conclusion, our experimental findings suggest that vindoline as a plant-derived product may be of great relevance in the treatment of diabetes mellitus and its complications owing to its anti-hyperglycemic, antihyperlipidemic, anti-inflammatory and antioxidant activities. Further experimental studies are recommended to improve vindoline's delivery and absorption so as to increase its therapeutic effect.

4.6 Competing interest

The authors declare no competing interest.

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Chapter 5

Antihyperlipidemic and nephroprotective effects of vindoline-a natural product from *Catharanthus roseus* in type 2 diabetes-induced male Wistar rats

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Abstract

Cardiovascular (CVD) and kidney diseases in diabetes are linked to increased mortality and morbidity. The aim of this study was to evaluate the effect of vindoline derived from *Catharanthus roseus* on diabetes-induced CVD and kidney disease through assessing inflammation, oxidative stress, hyperlipidemic and kidney function parameters. Type 2 diabetes was induced in male Wistar rats by 10% fructose water intake for two weeks followed by a single intraperitoneal injection of 40mg/kg body weight of streptozotocin (STZ). Six groups (n=8) of randomly divided rats received, vindoline (20mg/kg) or glibenclamide (5mg/kg) daily for 6 weeks via oral gavage. Lipid profile markers and markers of athrogenic index were decreased in diabetic rats after treatment with vindoline and glibenclamide. The levels of urea were significantly increased in the diabetic control group (13.66±0.9) as compared to the diabetic groups treated with vindoline and glibenclamide (10.62±0.6 and 10.82±0.8) respectively. Vindoline did not significantly alter the levels of inflammatory cytokines; however glibenclamide lowered the levels of TNF-α in kidney and heart tissues. Vindoline improved the ferric reducing antioxidant power in diabetic hearts while superoxide dismutase (SOD), oxygen radical absorbance capacity were increased in

the kidneys. Lipid peroxidation was reduced when compared to the diabetic controls. Vindoline restored the structure of the renal parenchyma and was accompanied by significant decrease in the expression of caspase 9 in diabetic rats when compared to the diabetic controls.

Graphical Abstract
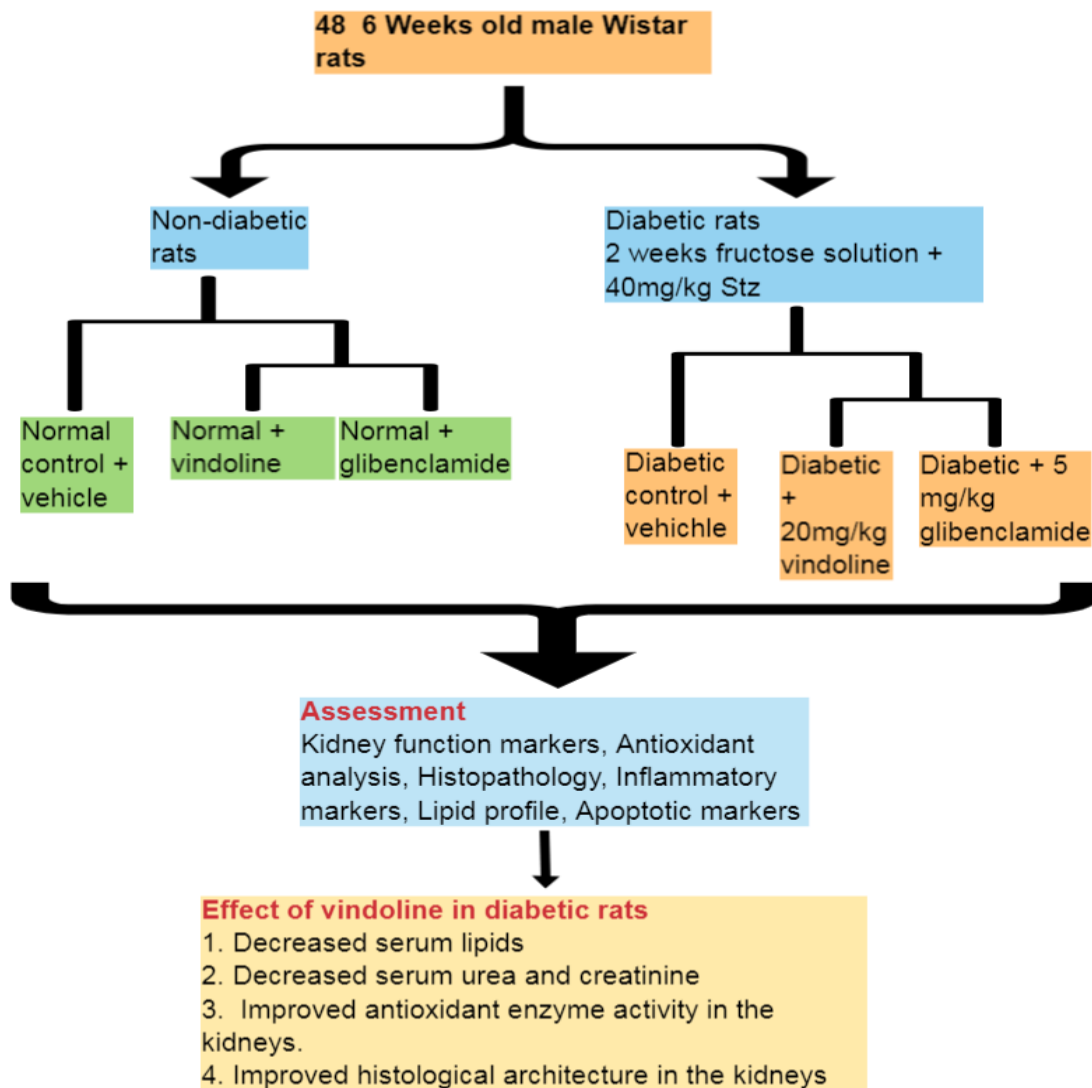
Figure 25: Graphical abstract

Keywords: cardiovascular related diseases, diabetic kidney disease, oxidative stress, inflammation, apoptosis

5.1 Introduction

The global prevalence of cardiovascular diseases (CVDs) is a major health concern that is rising substantially [1]. Recent statistical projections reported about 17 million deaths (31% of all global deaths) in 2015 that are related to CVDs. It is disturbing to note that the prevalence of CVDs is mostly predominant in low and middle-income countries where more than 75% of CVDs-related deaths are currently documented [2]. It is well ascertained that unhealthy behavioural and lifestyle factors greatly influence the pathogenesis of CVDs [3]. Several studies have shown the negative roles played by unhealthy dietary practices in the development of a constellation of metabolic diseases such as diabetes mellitus. Therefore a relationship between CVDs and type 2 diabetes mellitus has been established owing to the common risk factors shared [4]. Subsequently, patients with uncontrolled hyperglycemia are 2-3 times at risk of developing CVD [5]. This is because diabetic patients exhibit dyslipidaemia. Dyslipidaemia is metabolic condition characterised by hypercholesteremia, hyperlipidaemia and hypertriglyceridemia [3]. In due course, these abnormal lipid parameters accumulate in blood vessel walls causing the formation of plaque deposits that compromise the flow of blood [6].

Furthermore, the increased blood glucose level in DM influences the pathogenesis of microvascular and macrovascular complications. Pathological effects of DM deter normal functions of kidneys, heart, blood vessels, nerves and the eyes and may result in death [3]. The hyperglycaemic environment encourages persistent build-up of reactive oxygen/ nitrogen species (ROS/RNS) at the expense of antioxidant pools [7]. This imbalance leads to the occurrence of oxidative stress (OS) which propagates the formation of diabetic complications [5]. In addition to OS, inflammation and insulin resistance play major roles in cellular events that drive the development of the complications [8]. Enhanced concentration of ROS accelerates oxidation and glycation of low density lipoproteins (LDL). As a result, the modified LDL activates pro-inflammatory processes [9].This results in the reduction of nitric

oxide (NO) production and increased vascular lipid deposition thus initiating endothelial dysfunction and overt atherosclerotic injury [10].

Cardiovascular disease and diabetic nephropathy (DN) are comorbidities that occur in T2DM [11]. Scientific studies have documented the role lipids play in the induction of glomerular and tubulointerstitial injury [12]. It is believed that the interrelationship observed among mechanisms that drive dyslipidaemia, OS and inflammation are well involved in the pathogenesis of DN [13]. For that reason, lipid lowering treatments could be important in the prevention and treatment of diabetic nephropathy.

In order to control T2DM, different conventional treatment regimens have been put in place. These comprise drugs that supress postprandial hyperglycemia, possess insulotropic effects, lower hepatic glucose output and increase tissue sensitivity to insulin [14,15]. Concomitantly; as these drugs effectively lower blood glucose level; undesired effects are however observed [16]. Besides the available of these orthodox drugs and associated side effects, there is a continuous quest for alternative therapies that are safer and effective in the mitigation of T2DM and its complications. Medicinal plants may therefore serve as leads to the discovery of novel, safer and cheaper therapies for T2DM [4]. This may be attributed to the presence of multiple biomolecules that work singly or synergistically to ameliorate hyperglycemia, hyperlipidaemia, oxidative stress, inflammation and insulin resistance [17].

Catharanthus roseus is a plant that has been utilised since ancient times to treat and manage various ailments such as DM [18]. *C. roseus* is a rich source of indole alkaloids. Vincristine and vinblastine are indole alkaloids isolated from *C. roseus* that have been successfully incorporated into anticancer therapy [18]. Vindoline is one of the alkaloids that is highly concentrated in the leaves or twigs of the *C. roseus* [19]. Upon administration of this alkaloid in rats, hypoglycemic effects were observed by Svoboda et al (1964). It was then hypothesised that vindoline could be one of the major compounds that is responsible for the antidiabetic activities of *C. roseus* [20]. Yao and colleagues (2013) [19] recently reported vindoline's mode of antidiabetic action to be linked to β-cell stimulation. However, the antioxidant and anti-inflammatory mechanisms of vindoline in T2DM-induced dyslipidaemia and

nephropathy have not yet been reported. Therefore, we investigated the effects of vindoline on possible markers of CVD, oxidative stress and inflammation in a T2DM rat model that exhibits both insulin resistance and insufficiency.

5.2 Materials and Methods

5.2.1 Chemicals

Chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich (Johannesburg, South Africa). These include streptozotocin (STZ), 6-hydroxydopamine, 2-thiobabituric acid (TBA), malondialdehyde, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), -6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ) and ascorbic acid.

5.2.2 Bioactive Compound

The alkaloid, vindoline was purchased from the company Best of Chemicals (BOC) Sciences, USA). Specific guidelines, storage and preparation methods were followed as per suppliers' instructions.

5.2.3 Animal Handling and Ethics Statement

The animal ethical and experimental approval was sorted and granted by two committees: Ethics Committee for Research on Animals of the South African Medical Research Council (REF-01/17) and from the Faculty of Health and Wellness Research Ethics Committee of Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2016/A4).

The experimental animals used in this study were six weeks old (190-230g) male Wistar rats procured from Charles River (Margate United Kingdom). Two rats were housed per cage in temperature and humidity controlled standard environmental conditions with 12 hour light-darkness cycle. All the rats were allowed to have free access to water and standard laboratory diet *ad libitum* throughout the entire study period.

5.2.4 Animal Grouping

A total of forty eight (48) animals were randomly divided into 6 groups of 8 rats named the NC: non-treated normal control; NV: non-diabetic control treated with vindoline (20mg/kg) body weight (b.w); NG: non-diabetic control treated with the standard antidiabetic drug glibenclamide (5mg/kg b.w); DC: diabetic non-treated control; DV: diabetic treated with vindoline (20mg/kg b.w); DG: diabetic treated with glibenclamide (5mg/kg b.w).

5.2.5 Induction of type 2 diabetes mellitus (T2DM)

To induce insulin resistance, diabetic groups were exposed to 10% fructose water solution *ad libitum* for 2 weeks instead of drinking normal tap water. In contrast, non-diabetic groups were supplied with normal tap water *ad libitum*. After this period, a single low dose of STZ (40mg/kg b.w) prepared freshly in 0.1M citrate buffer (pH 4.5) was injected intraperitoneally after an overnight fast into the DC; DV and DG groups. A low dose of STZ induces partial β cell destruction. The non-diabetic groups NC; NV and NG were injected only with the same volume of 0.1M citrate buffer. Diabetes was confirmed 3 days after STZ induction. Rats were fasted for 4 hours and thereafter fasting blood glucose was measured. Rats that had blood glucose concentrations greater than 15mmol/l were considered diabetic.

5.2.6 Treatment

Daily treatment (for 6 weeks) through oral gavage with respective compounds (vindoline/ glibenclamide) commenced after 5 days of STZ administration to ensure stable hyperglycaemia.

5.2.7 Oral glucose tolerance test (OGTT)

An OGTT was conducted on day 28 in fasted rats, where rats receive their daily treatments. After 30 minutes a single dose of D-glucose (50%) solution (0.5 g/kg b.w) was administered orally to each animal. subsequent levels of blood glucose were measured at 0 (just before treatment with vindoline/glibenclamide), 30, 60, 90 and 120 minutes after the ingestion of glucose. Blood (10 microlitres) was collected through the tail of the rat by the tail prick method and glucose levels were measured using a glucometer.

5.2.8 Collection of heart, kidney and blood samples

At the end of the experiment, rats were fasted for 4 hours, anaesthetised and euthanized using isoflurane gas at 2% with 1% oxygen during laparotomy. Blood was immediately drawn through the abdominal vena cava and collected into respective tubes. Tubes were allowed to stand for an hour at room temperature before centrifugation. The kidneys and hearts were removed and washed in cold phosphate buffered saline (PBS). Thereafter; the organs were weighed and dried using a paper towel. The left kidneys and hearts were used for antioxidant analysis were snap frozen in liquid nitrogen and stored at -80°C for future analysis. The right kidneys were fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin wax for histological and immunohistological analysis. Later, the snap frozen samples were homogenised in ice-cold respective buffers for different endogenous antioxidant activity determination. Homogenates were centrifuged for 15 minutes at 15000 rpm at 4 °C, aliquoted and stored at -80°C until analysis.

5.2.9 Relative kidney and heart weights

The relative weights of the organs were calculated using the following formula:
Relative organ weight = (organ weight ÷ total body weight) 100g

5.2.10 Serum lipid profile measurement

Serum lipids such as total cholesterol (TC), triglycerides (TG) and high-density lipoprotein-cholesterol (HDL-c) were analysed by an Automated Chemistry Analyzer ABX PENTRA 400. Low density lipoprotein-c (LDL-c) LDL-c was calculated from TC, HDL-c and TG levels using the Friedewald's equation: *TC-HDL-C-TG/5*

Very low-density lipoprotein-c (VLDL-c) using the formula: *TG/5*

5.2.11 Atherogenic Indices

The extent of atherosclerotic plaques in the blood vessels was determined by assessment of the atherogenic indices serum (AIS). The atherogenic index is calculated using the formulas:

  i.   *AIS =TC-HDL /HDL* [21]

  ii. *AIS= TC/HDL* [22]

5.2.12 Endogenous antioxidant analysis

The antioxidant enzyme activities of superoxide dismutase (SOD) and catalase (CAT) were determined in clear 96-well plate using the Multiskan plate reader (Thermo Fisher Scientific, USA). Spectrophotometric determinations of SOD and CAT activity were measured in tissue homogenates following the modified method of [23].

5.2.13 Lipid peroxidation

Lipid peroxidation (LPO) in the kidney tissue was assessed by measuring the amount of malondialdehyde (MDA) a product. MDA levels were determined by the thiobarbituric acid reactive substance (TBARS) method. The coloured complex formed when MDA reacted with thiobarbituric acid (TBA) was measured at 532 nm according to the method of [24].

5.2.14 The oxygen radical absorbance capacity (ORAC)

The ability of biological samples to prevent oxidation of a fluorescein reagent by the peroxly radical of 2,2 azobis-2-methyl propanimiddamide, dihydrochloride (AAPH) is analysed using the ORAC assay. In the presence of antioxidants, hydrogen atoms are donated to the peroxyl radical hence inhibiting degradation of the fluorescein reagent. The net area under the curve depicts the fluorescence intensity and therefore the amount of antioxidant present in the sample. Fluorescence intensity was measured by means of a fluoroskan at every minute over 120 minutes by excitation and emission at 485nm and 538nm respectively [25].

5.2.15 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power of tissue samples was measured using the method that was developed by Benzi and Strain in 1996 [26]. FRAP is a concentration independent colorimetric assay that assesses the strength of samples to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). Reduction of the Fe^{3+} ion present in the (TPTZ) complex to a ferrous form (tripyridyltriazine complex) results in blue coloured product. Absorbances were determined using a spectrophotometer at a wavelength of 593 nm.

5.2.16 Inflammatory cytokines measurement

The levels of both pro-inflammatory (interleukin-10) and anti-inflammatory cytokines (TNF-α, IL-1β,) were detected using a Bio-plex Pro-magnetic bead-based Luminex kit (Merck) on the Bio-Plex platform. 200mg of the tissue was homogenised in phosphate buffer (pH 7.4), centrifuged at 14,000g for 15 min at a temperature of 4 °C. The undiluted supernatants were mixed with dyed magnetic beads coated with primary anti-cytokine antibodies. The primary antibodies then bind to its corresponding cytokine. This results in the formation of complex which in turn binds to biotinylated anti-cytokine secondary antibody in a sandwich manner. The reaction mixture is detected by the addition of fluorescent streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies allowing visualization and analysis by Bio-Plex Manager software.

5.2.17 Histological assessment of the kidney using the haematoxylin and eosin stain

After sacrificing, the right kidneys were fixed in 10% (v/v) neutral buffered formalin and dehydrated by passing through increasing concentrations of alcohol, cleared and embedded in paraffin blocks. Blocks were cut to produce 5 µm thick sections that were mounted on a slide. The sections were deparaffinised by passing them through xylene, decreasing concentrations of alcohol and finally in water. The sections were then stained in haematoxylin and eosin stains.

5.2.18 Immunohistochemistry analysis

Apoptotic markers such as caspase 3, caspase 9 and Bcl-2 were immunohistochemically assessed using (Leica Bond autostainer (Leica Biosystems, SA). Kidney sections that were fixed in formalin and embedded in paraffin were first deparaffinised before the antigen retrieval process. Specific antibodies of the

selected apoptotic markers were added to the slides with exposed antigen sites. The peroxidase block was added to the slides in order to avoid non-specific binding. Thereafter, primary antibodies were added to the slides, incubated using the post primary antibody at room temperature for 30 minutes. 3, 3' Diaminobenzidine (DAB) a chromogen solution and DAB substrate buffer polymer were introduced to the slides to facilitate the production of a brown end-product. Slides were then counterstained using haematoxylin in a 5 minute incubation step. Kidney tissue sections were dehydrated by moving slides in a series of graded alcohols followed by a mounting step using dibutyl phthalate xylene (DPX). Slides were viewed and images captured using the EVOS XL Cell imaging microscope. Positive intensities were analysed and quantified using ImageJ Immuno Profiler software (version 10.2 image analysis).

5.2.19 Statistical analysis

Results were analysed using GRAPH PAD Prism software package, Version 5.0. Data were expressed as mean ± standard error mean (SEM). The comparisons within groups were determined by using the one way analysis of variance (ANOVA) and Bonferonni's multiple test comparison. The values were considered to be statistically significant when the p value was < 0.05.

5.3 Results

5.3.1 Effect of vindoline on the 2 hr OGTT in non-diabetic and diabetic rats

The blood glucose levels of non-diabetic rats, diabetic control rats, and diabetic rats treated with vindoline or glibenclamide is shown in Table 9 and Figure 26 below. All the diabetic groups had significantly elevated blood glucose levels when compared to the normal control group. However, all the diabetic groups showed lower final (T=2 hr) glucose levels when compared to the initial value (0 min level). The 2 hr OGTT results clearly indicated that vindoline significantly reduced the blood glucose levels in the diabetic treated rats (from 29.05±2.41 to 23.39±2.4 mmol/l) at 1.5 hr post glucose load when compared to the non-treated diabetic controls (from 31.89±0.75 to 30.96±0.81 mmol/l). Pre-treatment of diabetic rats with vindoline or glibenclamide reduced the AUC relative to the diabetic control group by 15% and 9%, respectively. No significant differences in glucose levels were observed in the non-diabetic treated groups when compared to the non-diabetic non-treated control group.

Table 9: Effect of vindoline in non-diabetic and diabetic rats on 2hr oral glucose tolerance test

Groups	T=0hr	T=0.5hr	T=1hr	T=1.5hr	T=2hr	AUC
NC	5.21±0.14	5.31±0.19	5.25±0.13	5.16±0.19	4.76±0.07	621.4
NV	5.28±0.11[b]	5.56±0.22[b]	5.09±0.19[b]	5.06±0.11[b]	4.74±0.18[b]	622.3
NG	5.68±0.17[b]	5.775±0.39[b]	4.74±0.36[b]	3.86±0.45[b]	3.99±0.31[b]	576.2
DC	31.89±0.75[acd]	31.95±0.72[a]	30.31±0.75[a]	30.96±0.81[a]	27.98±1.30[a]	3695
DV	29.05±2.41[acd]	28.11±2.19[a]	26.48±2.42[a]	23.39±2.4[ab]	26.08±2.27[a]	3166
DG	27.59±1.65[acd]	31.48±0.8[a]	27.98±1.26[a]	26.66±1.97[a]	25.39±1.62[a]	3375

Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide. AUC: area
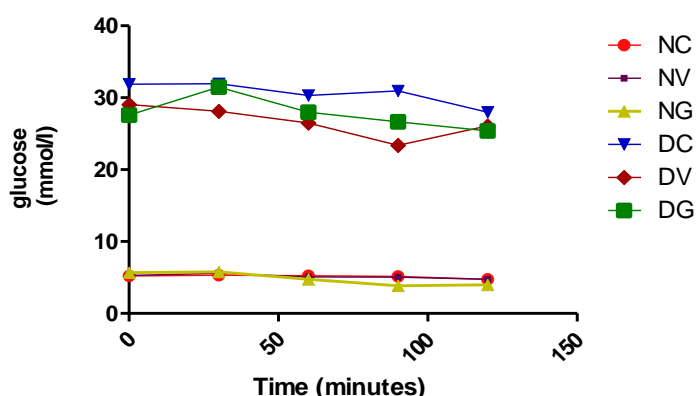
Area under curve

Figure 26: Graphical presentation of the effect of vindoline in non-diabetic and diabetic rats on 2hr oral glucose tolerance test. Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide.

5.3.2 Effect of vindoline administration on the kidney and heart weights and kidney function parameters in non-diabetic and diabetic rats.

The changes in heart and kidney weights of the controls and experimental groups are represented in Table 10 below. The results indicated significant organ hypertrophy in all T2DM-induced rats when compared to the non-diabetic rats. Neither administration of vindoline nor glibenclamide in the diabetic DV (0.30 g) and DG (0.30 g) groups respectively significantly prevented heart hypertrophy when compared to the diabetic control group DC (0.34 g). Similarly, the relative kidney weights in diabetic treated groups DV (1.07±0.04 g) and DG (1.07±0.04 g) were not significantly reduced when compared to the diabetic control (1.19±0.03 g p<0.05). On the other hand, normal rats treated with vindoline (NV) group showed no significant changes in both organs when compared to the normal control group NC. In comparison to the diabetic control group, diabetic rats that were treated with vindoline and glibenclamide showed significant reduced levels of serum urea, however, the recorded concentrations were still significantly raised when compared to all non-diabetic groups. The serum creatinine concentration level was not significantly reduced in the diabetic groups that were treated with vindoline and

glibenclamide (37.24±1.6mg/dl and 37.49±2.3mg/dl; p<0.05) respectively when compared to the diabetic controls (47.59±4.3mg/dl). When compared to the normal control group (33.72±1.5mg/dl), only the diabetic control group recorded significantly elevated serum creatinine levels (p<0.05).

Table 10: Relative organ weights and kidney function parameters following treatment

Groups	RHW(g)	RKW(g)	Urea(g/L)	Creatinine(mg/dl)
NC	0.28±0.009	0.64±0.02	7.471±0.34	33.72±1.5
NV	0.25±0.005[b]	0.61±0.01[b]	7.97±0.5[b]	37.47±2.5
NG	0.25±0.004[b]	0.66±0.02[b]	6.974±0.5[b]	31.14±1.5[b]
DC	0.34±0.008[a]	1.19±0.03[a]	13.66±0.9[a]	47.59±4.3[a]
DV	0.30±0.005[a]	1.07±0.04[a]	10.62±0.6[ab]	37.24 ±1.6
DG	0.30±0.006[a]	1.07±0.04[a]	10.82±0.8[ab]	37.49±2.3

Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide. RHW: relative heart weight in grams; RKW; relative kidney weight in grams.

5.3.3 Serum lipid levels in normal and T2DM-induced rats after receiving respective treatments for 6 weeks.

Table 11 below indicates the level of serum lipids in non-diabetic and T2DM-induced rats after the respective treatment interventions. Induction of T2DM in rats evidently contributed to the elevation of 'bad' lipids (TC, TG, LDL, and VLDL) in the serum of the diabetic control group while the percentage HDL of the total cholesterol was significantly decreased. Significantly higher levels of lipids were observed in the diabetic control group when compared to all the groups (p<0.05). Both treatment of diabetic rats with vindoline or glibenclamide reinstated the serum lipid levels to near normal as shown in Table 11 below since there were no significant differences in the levels of 'bad lipid' when compared to the normal control group. Moreover, administration of vindoline to diabetic rats (DV) significantly increased the levels of the 'good' cholesterol: HDL which was 65.58±1.02% of the total cholesterol, whereas

the diabetic control group had the lowest percentage of the HDL (51.85±3.86%, p< 0.05).

Table 11: Serum lipid profile for T2DM-induced rats after 6 weeks treatment with vindoline

Groups	TC(g/L)	HDL (%)	TG(g/L)	LDL(g/L)	VLDL(g/L)
NC	0.93±0.07	68.72±2.0	0.54±0.08	0.17±0.04	0.13±0.02
NV	1.3±0.08[b]	61.04±1.11[bd]	1±0.16[b]	0.32±0.04[b]	0.18±0.03[b]
NG	0.83±0.08[b]	74.11±3.54[b]	0.54±0.05[b]	0.13±0.05[b]	0.11±0.01[b]
DC	2.83±0.48[a]	51.85±3.86[a]	2.87±0.6[a]	0.75±0.13[a]	0.78±0.15[a]
DV	1.43±0.12[b]	65.58±1.02[b]	1±0.2[b]	0.3±0.03[b]	0.2±0.04[b]
DG	1.48±0.18[b]	63.10±2.2[b]	1.24±0.2[b]	0.25±0.04[b]	0.24±0.05[b]

Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide; g/L: grams per litre.

The athrogenic index was determined to evaluate the potential risk of CVD development in the diabetic rats. As shown in the information presented in Figure 27 graphs, the intake of vindoline in diabetic rats reduced the risk of future CVD development as there was no significant difference in the values observed between the diabetic group treated with vindoline (DV), the normal controls (NC) and the non-diabetic rats administered with glibenclamide (NG). The diabetic control group exhibited increased values of athrogenic index when compared to all treatment groups (p<0.05). Likewise, the serum total cholesterol/HDL ratio in the diabetic control group was increased significantly when compared to the normal control group (p<0.05). The diabetic groups that was treated with vindoline and glibenclamide showed no significant differences of total cholesterol/HDL ratio when compared to the normal control group (p<0.05).
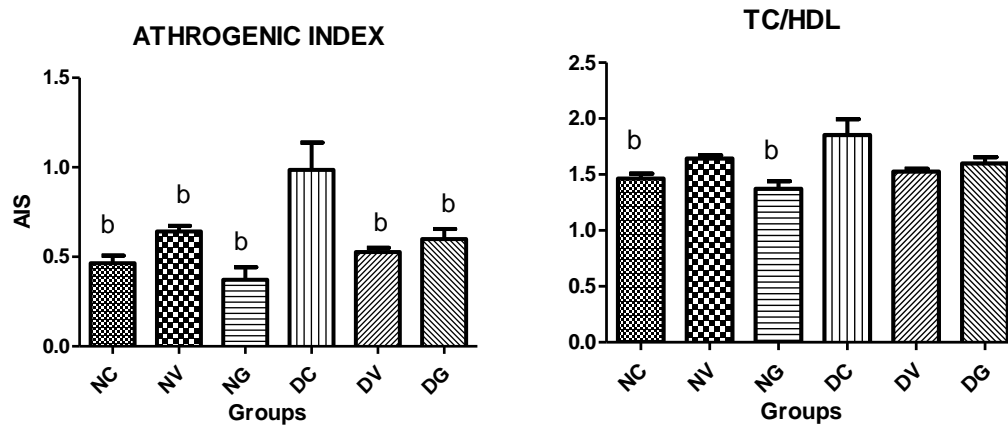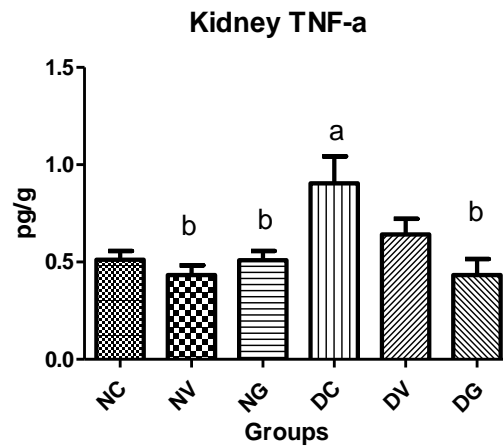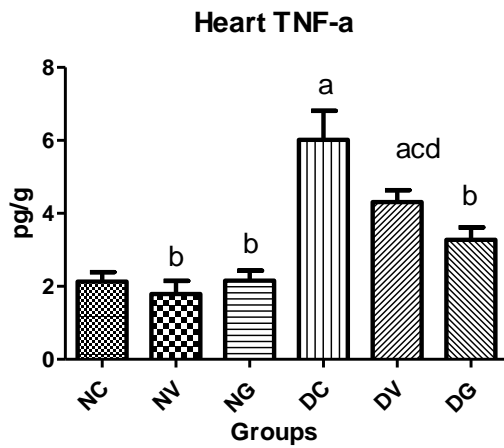
Figure 27: Athrogenic index assessment after 6 weeks treatment period. Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide.

5.3.4 Effect of vindoline on levels of inflammatory cytokines in the heart and kidney tissues.

In the present study, induction of diabetes significantly increased ($p < 0.05$) levels of pro-inflammatory cytokines TNF-a, IL-1b and IL-6 in rats (Figure 28(a–d)). The levels of TNF-α in the hearts (Fig 28a) of diabetic control and the diabetic group treated with vindoline were elevated significantly when compared to the normal control group. Vindoline in diabetic rats did not significantly reduce the level of TNF-α in the heart tissue when compared to the diabetic control group; however glibenclamide administration in diabetic rats showed significant reduction of TNF-α. Daily treatment of diabetic rats with vindoline also resulted in no significant changes in the concentrations of TNF-α (Fig 28b) in the kidneys when compared to the normal untreated and diabetic controls. Administration of glibenclamide in diabetic rats effectively decreased levels of TNF-a in the kidneys (Fig 28b), the decrease was significant ($p<0.05$) when compared to the diabetic control group. The levels of IL-1b and IL-6 (Fig 28c and 28d) in the diabetic controls were elevated significantly when compared to the normal controls. Although the levels of pro-inflammatory cytokines in both organs were lower in the diabetic group treated with vindoline than in the diabetic control group, the reduction was not significant at ($p<0.05$). Rats treated with

glibenclamide and vindoline displayed significantly increased ($p< 0.05$) cardiac levels of IL-6 (Fig 28d) when compared to the normal controls; treatment with either vindoline or glibenclamide failed to alter the levels of IL-6 in the hearts when compared to the diabetic control group.

In addition, treatment of normal and diabetic rats with vindoline or glibenclamide did not significantly alter the levels of the anti-inflammatory cytokine IL-10 in the heart tissue when compared with normal control rats at ($p<0.05$).

(a)   (b)

(c)   (d)
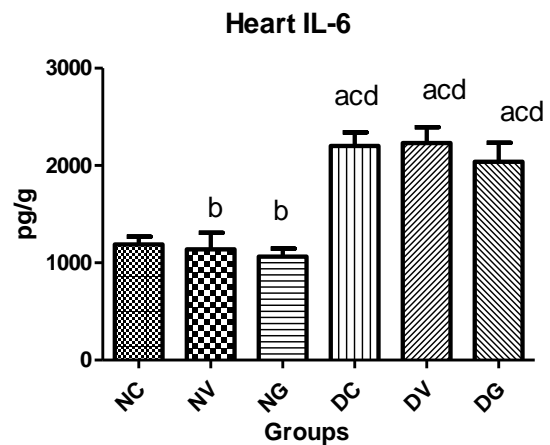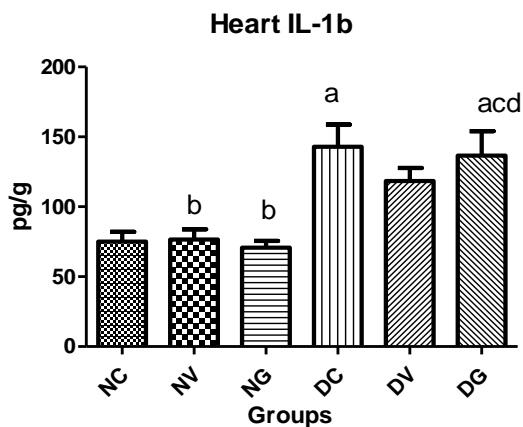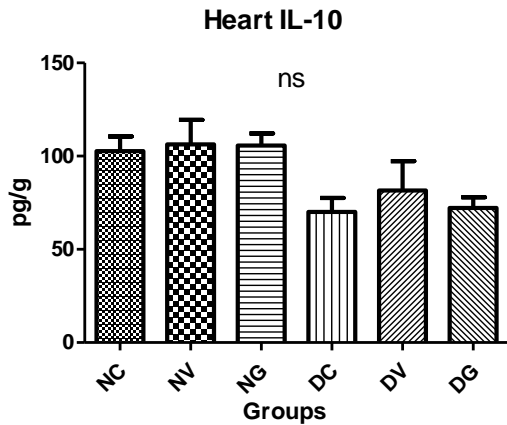
Figure 28: Effect of vindoline on levels of inflammatory cytokines in the heart and kidney tissues after 6 weeks treatment period. Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide; ns: non-significant vs all groups.

5.3.5 Effect of vindoline on oxidative stress markers in the cardiac and nephron tissues

Figure 29 indicates the antioxidant status in the hearts and kidneys of normal and diabetic rats after treatment with vindoline and glibenclamide for 6 weeks. The ferric reducing antioxidant power was significantly lower in the cardiac tissue of diabetic control group when compared to all treatment groups (p<0.05). Treating diabetic rats with vindoline and glibenclamide improved the ferric reducing antioxidant power in the cardiac tissue with no significant differences when compared to all the non-diabetic groups (p<0.05). The activity of catalase in the hearts was determined, the results showed significantly increased activity in the diabetic control group when compared to all non-diabetic groups (p<0.05). Vindoline and glibenclamide administration did not significantly change the activity of catalase when compared to the diabetic control group (p< 0.05). The activity of SOD in the kidneys was significantly elevated in all treatment groups when compared to the diabetic control (p<0.05). Treating diabetic rats with vindoline and glibenclamide significantly improved SOD activity when compared to the diabetic control however the activity was significantly lower when compared to all non-diabetic groups (p<0.05). The oxygen radical absorbance capacity measured in the kidneys was found to be significantly decreased in the diabetic controls when compared to all treatment

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groups. In the diabetic groups treated with vindoline and glibenclamide; the oxygen radical absorbance capacity was significantly improved when compared to the diabetic control group (p<0.05). On the other hand, catalase activity in the kidney tissue of diabetic controls increased significantly when compared to the normal non-treated control group. In addition, no significant changes in the activity of catalase in the kidneys were displayed after treating diabetic rats with vindoline when compared to the normal non-treated and diabetic control groups (p<0.05). Interestingly, treating diabetic rats with vindoline significantly reduced the degree of lipid peroxidation in the kidneys when compared to the diabetic control group; nevertheless, the result was still significantly high when compared to the normal untreated control (p<0.05).
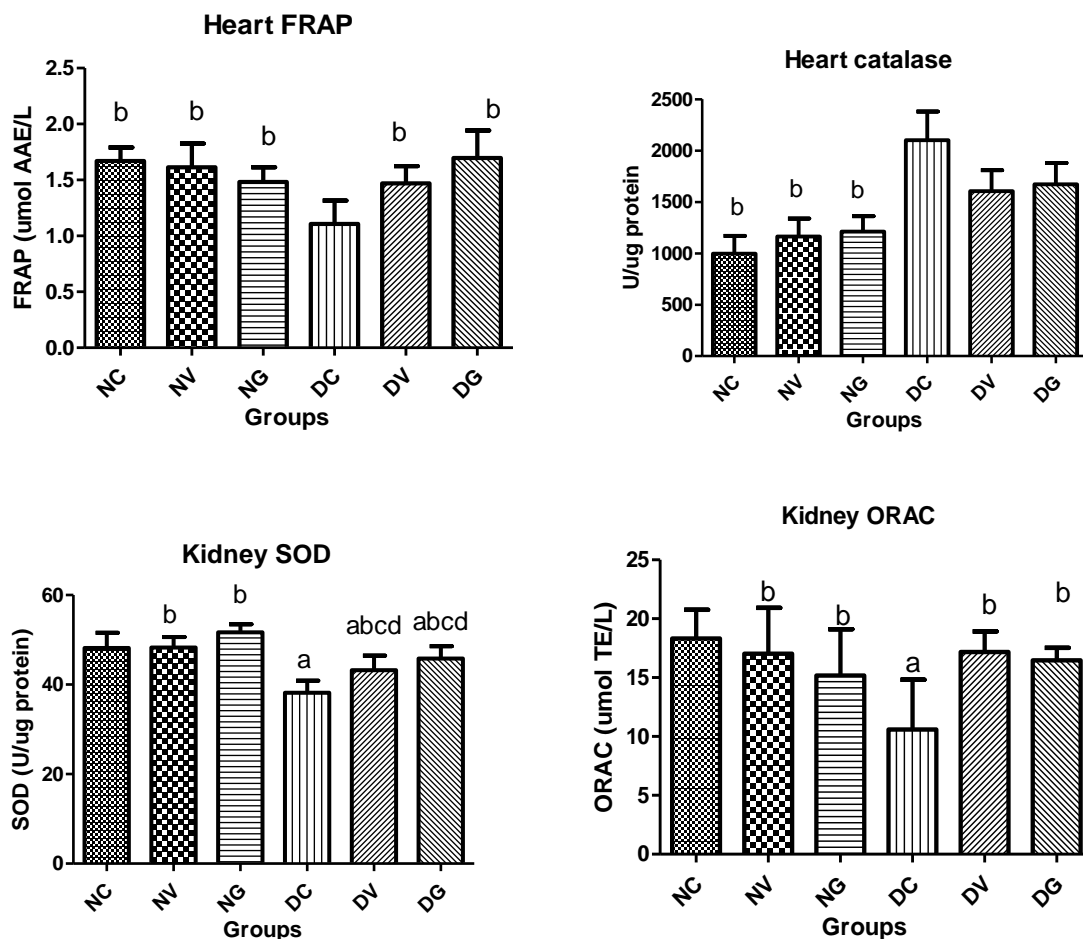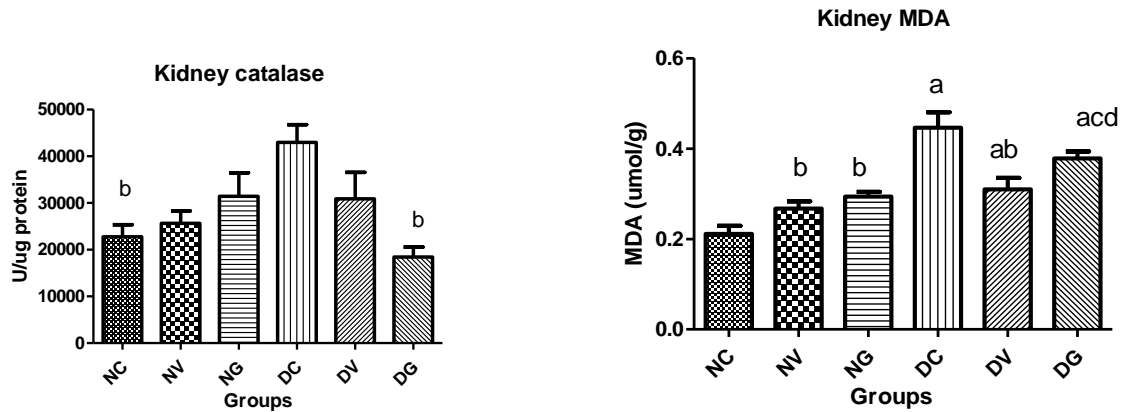
Figure 29: Shows the markers oxidative stress in the hearts and kidneys of normal and diabetic rats after treatment with vindoline and glibenclamide. Data represented as means ± SEM. [a] p< 0.05 vs normal control. [b] p< 0.05 vs diabetic control. [c] p< 0.05 vs normal rats treated with vindoline. [d] p< 0.05 vs normal rats treated with glibenclamide. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide.

5.3.6 Histopathology

The sections of the non-diabetic rats represented in Figure 30 (A, B and C) showed normal kidney histological architecture with no visible signs of lesions. However, the diabetic control group showed severe glomeruli capillary distortion surrounded with a significantly extended capsular space (D1). The renal cortical tubular cells revealed a degree of vacuolation as well as presence of hyaline casts (D2) indicating tubular degeneration. Treatment of diabetic rats with vindoline and glibenclamide resulted in progressive restoration features which include narrowing of the capsular space and abundant glomerular capillaries (E1 and F). Hyaline casts were also observed in the diabetic rats treated with vindoline (E2).

Figure 31 below represents glomerular space morphometric analysis in the normal and diabetic groups. Significant increase in the capsular space was noted in the diabetic control group when compared to all non-diabetic groups and the diabetic group that was treated with vindoline (p<0.05). No significant changes were seen between the diabetic control and the diabetic group treated with glibenclamide (p<0.05).
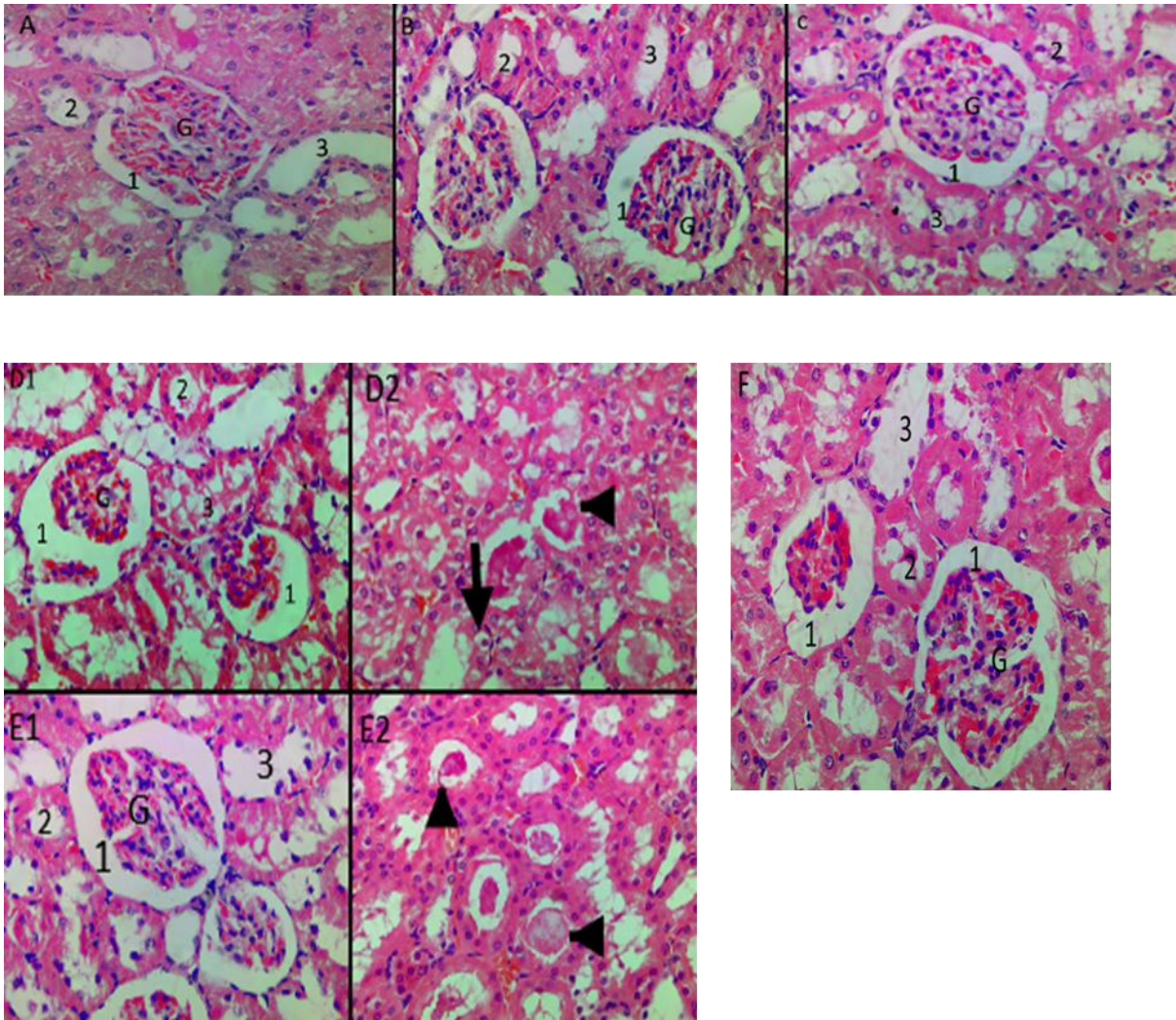
Figure 30: Haematoxylin and eosin stained kidney parenchyma photo micro graphs. 'A' normal control kidney showing the renal cortex with normal glomerulus (G) surrounded by capsule with normal capsular space (1) and normal tubular system distal convoluted tubules (DCT) (2) and proximal convoluted tubules (PCT) (3). 'B and C' photo micrographs of normal rats treated with vindoline and glibenclamide respectively showing normal renal parenchyma. D1 represents the diabetic control kidney section showing shrunken glomeruli (G) with distorted capillaries surrounded by excessive widened Bowman's space (1), the PCT cells appear to be vacuolated cytoplasm (3). 'D2' indicates cortical tubules with acidophilic hyaline casts (arrow head). 'E1' diabetic group treated with vindoline with glomerulus that are surrounded by narrower Bowman's space (1), hyaline casts (arrow heads) noted (E2). 'F' diabetic group treated with glibenclamide showing restored glomerulus (G) with prominent glomerular capillaries, capsular space looks narrow (1).

Glomerular space area
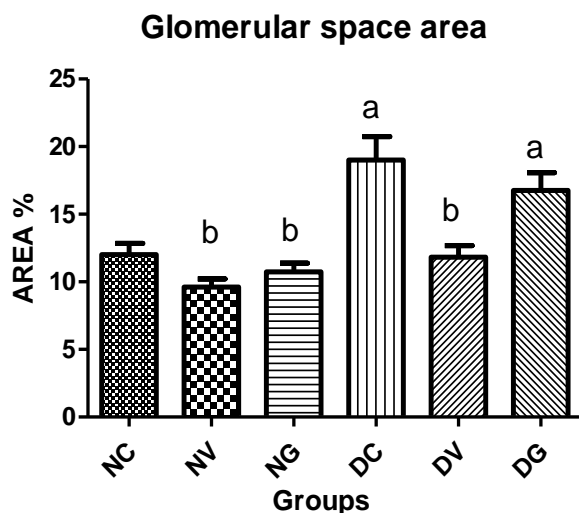
Figure 31: Represents the area occupied by the glomerular space per glomerulus. . Data represented as means ± SEM. [a] $p < 0.05$ vs normal control. [b] $p < 0.05$ vs diabetic control. [C] $p < 0.05$ vs normal rats treated with vindoline. [d] $p < 0.05$ vs normal rats treated with glibenclamide. NC: normal control; NV: normal control treated with vindoline; NG: normal control treated with glibenclamide; DC: diabetic control; DV: diabetic treated with vindoline; DG: diabetic treated with glibenclamide

5.3.7 Effect of vindoline on apoptosis markers on the kidney tissues

Figure 32 below shows immunohistochemical quantification and picto graphs of caspase 3, caspase 9 and BCL-2 in the kidney sections of diabetic and normal rats. Caspase 3 was significantly expressed in all diabetic groups when compared to the normal non-treated control group. Vindoline and glibenclamide administration to diabetic rats did not significantly change the expression of caspase 3 when compared to the diabetic control group. On the other hand, normal rats that were treated with glibenclamide showed significant elevated levels of caspase 3 when compared to the normal non-treated controls. The levels of caspase 9 were found to be significantly increased in the diabetic control group when compared to all the groups. Treating diabetic rats with vindoline or glibenclamide prevented the overexpression of caspase 9 when compared to the diabetic control group. However the levels of BCL-2 in diabetic treated groups were not significantly changed following treatment when compared to the diabetic control group.

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Kidney caspase 3

Kidney caspase 9

Kidney Bcl2
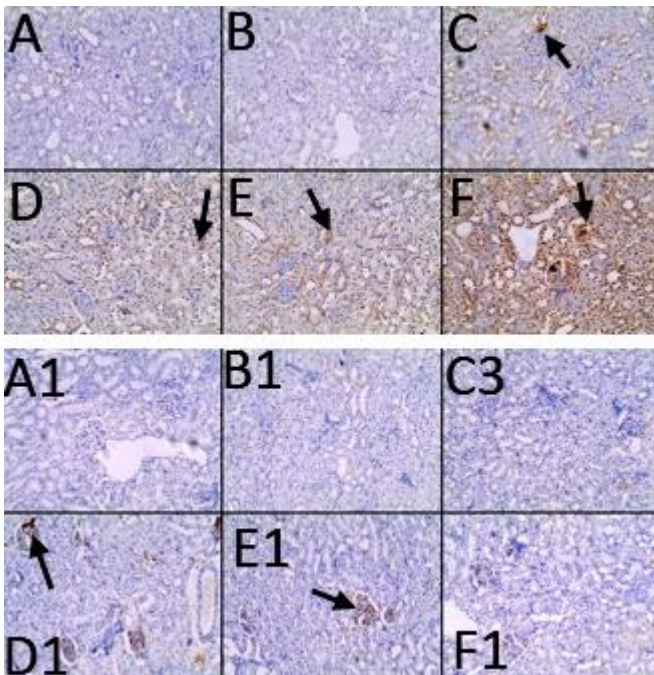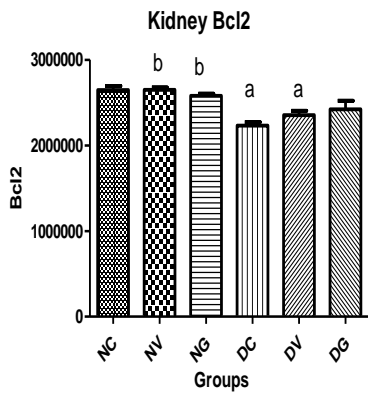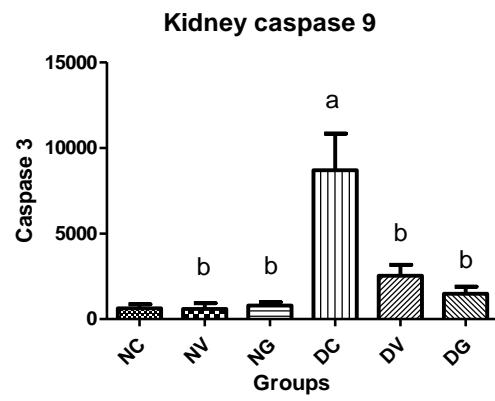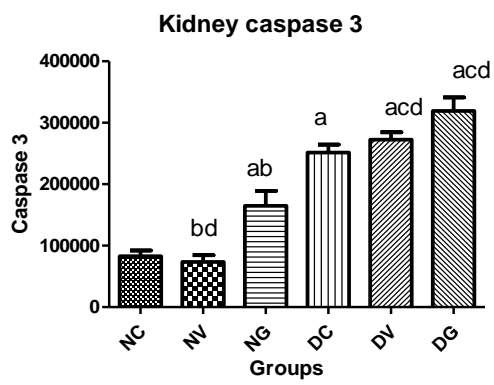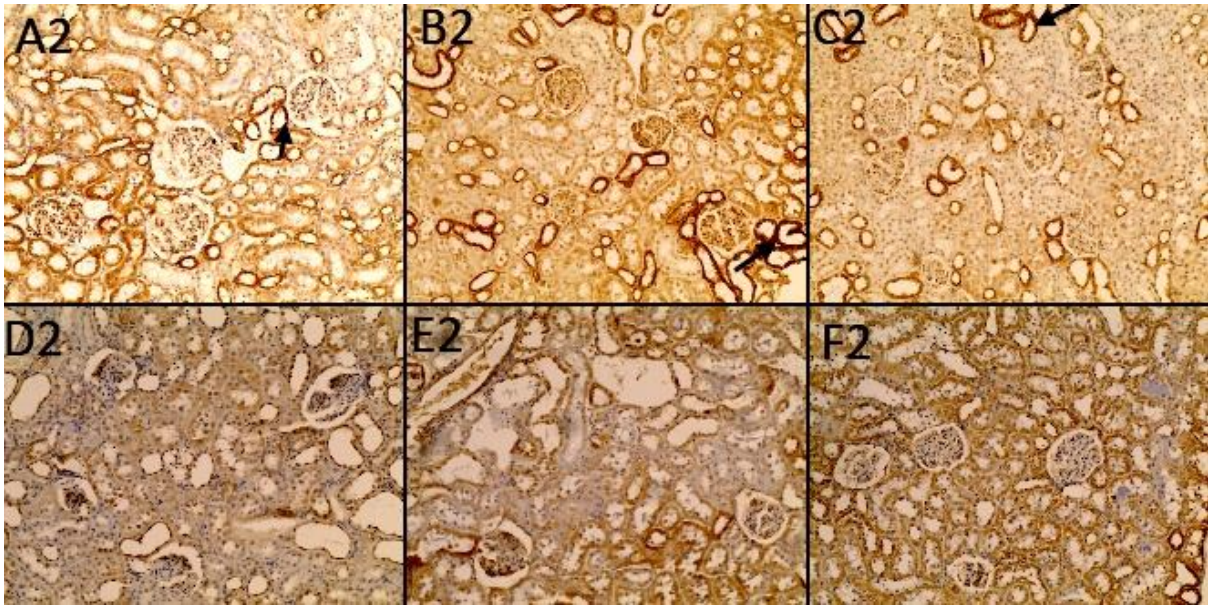
Figure 32: Represents immunohistochemical staining in intensies of apoptotic markers caspase 9 plates (A-F), caspase 3 (A1-F1) and BCL-2 (A2-F2). Arrows represent areas of hyper intensity in varying degrees of the brown stain. Data represented as means ± SEM. ^{a} p< 0.05 vs normal control. ^{b} p< 0.05 vs diabetic control. ^{C} p< 0.05 vs normal rats treated with vindoline. ^{d} p< 0.05 vs normal rats treated with glibenclamide. (NC; A; A1; A2): normal control; (NV; B; B1; B2): normal control treated with vindoline; (NG; C; C1; C2): normal control treated with glibenclamide; (DC; D; D1; D2): diabetic control; (DV; E; E1; E2): diabetic treated with vindoline; (DG; F; F1; F2): diabetic treated with glibenclamide.

5.4 Discussion

Diabetes mellitus is a strong risk factor that leads to the malfunction of the cardiovascular and renal systems resulting in increased mortality [27]. Over the years, there has been an increased research focus on the potentials of plant-derived products in the prevention of diabetes and its complications. This is because plant materials are rich in compounds that may have single or multiple effects in preventing the progression of diabetes and its associated complications [17].

The oral glucose tolerance test is a test that is used to evaluate the body's ability to utilise glucose [28]. In our present study, we observed a marked reduction in the blood glucose levels in diabetic rats that were treated with vindoline at 90 minutes post glucose load and treatment when compared to the diabetic control suggesting improved glucose tolerance. The decrease in blood glucose level may be attributed to the insulotropic effects of vindoline as previously reported [19].

Increase in organ weight (hypertrophy) relative to the total body weight is common in diabetes-induced rats [29]. The hyperglycaemic environment in diabetes triggers hyper function of the renal and cardiac tissues leading to adverse growth changes of these organs [30]. Alterations in the production of growth factors in the renal tubules have also been linked to kidney hypertrophy [29]. In this study, the heart and kidney weights in relation to the total body weight of all diabetic groups were found to be significantly increased when compared to the non-diabetic rats. Oral administration of vindoline and glibenclamide in diabetic rats did not significantly change the weights of the kidney and heart tissue when compared to the diabetic control group.

Diabetes leads to the progressive deterioration of renal function and eventually resulting in end stage renal disease [31]. Creatinine is a product of the breakdown of creatine phosphate found in the skeletal muscle [32]. Elevated levels of creatinine and urea in blood serum are considered good and sensitive markers of deranged glomerular filtration and are linked to the severity of kidney injury [33]. The current study observed significantly increased levels of serum creatinine in the diabetic untreated control group when compared to the normal untreated control. This increase evidently showed the extent of renal damage and destruction of functioning

tubules and nephrons related to DN [34]. Interestingly, the concentration of creatinine in diabetic rats that were treated with vindoline and glibenclamide did not differ significantly when compared to the normal untreated controls. Remarkably, treatment of diabetic rats with vindoline or glibenclamide treatments showed significant reduction of serum urea levels when compared to the diabetic control group. These kidney function results may suggest that there was an improvement in the elimination of waste products particularly urea by the kidneys. In addition to presumably improved kidney function, there might be also a decrease in the rate of muscle degradation in these diabetic treated rats [35]. However, the levels of serum urea in both the diabetic treated groups remained significantly higher than that of the normal untreated controls.

Dyslipidaemia is a common complication of diabetes mellitus; it includes increased levels of TC, LDL, VLDL, triglycerides and diminished levels of HDL. Abnormal lipid parameters in DM have been shown to predispose diabetic patients to cardiovascular complications such atherosclerosis, stroke, high blood pressure and coronary heart disease [31]. It has been documented that high circulating levels of LDL and cholesterol are risk factors of pathogenesis of diabetic micro and macrovascular complications. LDL acts by transporting cholesterol from the liver to the peripheral organs resulting in lodging of unwanted cholesterol plaques in the endothelium of blood vessels leading to atherosclerosis [36]. In our study, significantly lower levels of TC, LDL and VLDL were observed in the diabetic groups that were treated with vindoline and glibenclamide when compared to the diabetic untreated control group. The decrease in these lipid parameters may possibly indicate the hyperlipidaemic activities of vindoline and glibenclamide. When compared to the normal non-treated controls, there were no significant differences observed in the levels of TC, LDL and VLDL of diabetic treated groups. We additionally assessed the atherogenic index which is a sensitive marker of potential risk of CVDs development. The diabetic control group exhibited higher atherogenic index values when compared to all the groups. Our results indicate that administration of vindoline and glibenclamide to diabetic rats reduced the potential risk of CVD development when compared to the diabetic controls. No significant differences were noted in the diabetic groups treated with vindoline or glibenclamide when compared to the normal non-diabetic control group. The percentage of good

cholesterol HDL was determined and the results showed that administering vindoline and glibenclamide significantly restored HDL levels when compared to the diabetic controls therefore likely to reduce the risk of CVD development. Our results are in agreement with those reported by Yao *et al* [19] who observed decreased triglyceride levels in their T2DM model following treatment with vindoline. Islam *et al* [37] reported antihyperlipdemic effects of *C. roseus*; it is possible that vindoline could be one of the compounds that are responsible for this plant's reported antihyperlipdeamic effects.

Type 2 DM can be regarded as an immunometabolic disorder in which hyperglycemia and oxidative stress play pivotal roles in initiating systemic inflammatory responses [38]. Amplified inflammatory responses in DM aggravate endothelial dysfunction which generates vascular complications [39]. Patients with T2DM have been reported to have high levels of circulating inflammatory cytokines which include TNF-α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein, suggesting an increased risk of tissue injury [40,41]. In our findings, induction of diabetes in rats caused significant elevation in the levels of all the pro-inflammatory cytokines (Figure 27) in the diabetic control group when compared to the normal non-treated control group. Vindoline administration to diabetic rats did not significantly reduce the levels of the pro-inflammatory cytokines in the kidney and heart tissues when compared to the diabetic non-treated control group. However, when comparing the diabetic group that was treated with vindoline to the normal non-treated control group, we observed no significant differences as well. We therefore argue that vindoline might possess anti-inflammatory effects since there were no significant differences in the level of all pro-inflammatory cytokines when compared to the normal control group. On the contrary; glibenclamide treatment in diabetic rats caused significant decreases in the levels of TNF-α in both the heart and kidney tissues when compared to the diabetic controls.

Reactive oxygen species (ROS) are by-products of normal homeostasis; however in T2DM; hyperglycemia encourages excessive formation of ROS which ultimately overpowers the endogenous antioxidant machinery leading to oxidative stress [42]. In the presence of oxidative stress, damage to structures such as DNA, lipids, proteins and carbohydrates occurs leading to structural and functional anomalies

thus fostering the onset and progression of diabetic complications [43]. We assessed the ferric reducing antioxidant power (FRAP) of the heart homogenates. The results showed significantly decreased FRAP in the heart homogenates of the diabetic control group when compared to all the groups. This finding suggests the potent antioxidant power of vindoline and glibenclamide which is important in prevention of oxidative events that can lead to cardiac injury.

In biological systems, catalase and SOD are the first line of defence against free radicals. SOD acts by dismutating the unstable superoxide anion into oxygen and hydrogen peroxide while catalase converts the latter into water and oxygen [44]. Attenuated functions or production in these enzymes can result in the accumulation of ROS, thus making the tissue susceptible to oxidative damage [43]. Our findings revealed significantly decreased activities of SOD in the kidneys of the diabetic control group when compared to all the groups. Vindoline and glibenclamide treatment in diabetic rats interestingly improved the activity of SOD thereby confirming the possibility of delaying/ preventing DN development [45]. On the contrary, the activity of catalase in both organs of the diabetic control group was found to be elevated when compared to the normal non-treated controls. This finding indicates compensatory responsive mechanisms employed by the antioxidant defence system to overcome oxidative stress [46].

The ORAC assay is a test usually performed to determine the total antioxidant capacity in diabetic models [47]. The ORAC values tend to be low in T2DM signifying uncontrolled hyperglycaemia and oxidative tissue damage [48]. We measured the kidney ORAC in both normal and diabetic rats; we observed significantly low ORAC values in the diabetic control group when compared to the ORAC values of all the groups in this experiment. It was evident that vindoline and glibenclamide boosted the antioxidant defences in the kidneys of diabetic rats. Similar findings were reported by Ayeleso *et al* [47], where they observed diminished ORAC in the erythrocytes of diabetic rats but reinstated in diabetic treated groups.

Free radical build up in biological systems is implicated in the cytotoxic peroxidation of polyunsaturated fatty acids found in cell membranes. MDA are products of lipid peroxidation that are proportional to membrane destruction [41]. In this study, there was increased formation of MDA products in the kidney homogenates of the diabetic

control group. The significantly high MDA concentration observed in the diabetic control may have occurred as a result of the destruction of the glomerular and renal tubular cell membranes via glucose and lipid oxidation [49]. This finding substantiates deranged kidney function results observed in this study. On the other hand; oral administration of vindoline to diabetic rats protected the kidney tissue membranes from free radical damage due to the supressed MDA levels noted in comparison to the diabetic control group. However; the MDA levels in diabetic groups treated with vindoline remained significantly high when compared to the normal non-treated controls.

Histopathological examination of the kidney tissue in diabetic control group showed severe loss of the glomerular capillaries, tubular cell degeneration and presence of hyaline casts. These changes observed may have been possibly attributed to glomerular hyper filtration, disturbed glucose metabolism and oxidative tissue damage [50,51]. The diabetic group that was treated with vindoline showed gradual improvement in the structure of the glomerulus and the renal tubules although the renal tubules still had hyaline casts within their lumen. Less injury of the glomerulus and the renal tubules might have been enforced by the antioxidant defence mechanisms [34]. Furthermore, we observed significant dilation of the glomerular capsular space in the diabetic control group when compared to all groups confirming renal injury due to failed control of hyperglycemia.

Apoptosis is a normal process whereby cells are programmed by BCL-$_2$ family and caspase proteins to undergo suicide shedding off old, useless or damaged cells [52]. However, several stimuli including changes in DNA expression, low grade inflammation, oxidative, mitochondrial and endo-reticulum stress have been implicated in activating abnormal apoptosis signals [41]. BCL-$_2$ super family consists of proteins that are either positive or negative regulators of apoptosis. Bcl-$_2$ has been shown to inhibit mitochondrial apoptosis via inhibiting the insertion of BAX (pro-apoptotic) into the mitochondrial outer membrane thus preventing the release of cytochrome C [53]. In diminished concentrations of BCL-$_2$; there is successful release of cytochrome C which plays a role in the activation of caspase 9. Caspase 9 in turn initiates apoptosis by activating the effector caspase 3 which executes downstream events of apoptosis which result in cell death [54,55].

Immunohistopathological measurement on the markers of apoptosis in the kidneys of normal and diabetic rats was performed. We observed significantly increased expression of caspase 9 in the diabetic control rats when compared to all treatment groups. This result is in agreement with the findings of Mishra *et al* [56] who reported elevated caspase 9 in their type 2 diabetes rat model. High levels of caspase 9 expression suggested the initiation of apoptosis in the glomerulus and renal tubules in response to hyperglycemia induced oxidative stress. Interestingly, diabetic rats that received daily treatments of vindoline and glibenclamide showed significantly low levels of caspase 9 suggesting that vindoline may prevent initiation of apoptosis. However, we did not observe similar findings in caspase 3 expression in both the diabetic controls and diabetic treated groups. No significant changes in the expression of caspase 3 in both diabetic treated groups were found when compared to the diabetic controls; hence vindoline and glibenclamide mechanisms of action may not prevent the execution stages of apoptosis.

5.5 Conclusions

Administration of vindoline at a dose of 20 mg/kg body weight could potentially delay the progression of diabetes-related cardiovascular and kidney diseases via improving the antioxidant defence system and delay the initiation of apoptosis. Moreover, vindoline exhibited excellent antihyperlipidemic activities and could be utilised in the management of microvascular and macrovascular complications associated with diabetes. It is worthwhile to perform detailed toxicity and long term studies to further substantiate these effects.

5.6 Declaration of Interest

Authors declare that there are no competing interests.

5.7 Funding

5.8 Acknowledgements

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Chapter 6

6.1 General Discussion

Diabetes mellitus is a complex heterogeneous group of metabolic disorders associated with impairments in the metabolism of glucose and macromolecules. It is characterised by severely elevated blood glucose levels (hyperglycemia) as a result of decreased insulin production by beta pancreatic cells and/ attenuated insulin action (Singh *et al.*, 2001; Lee and Pervaiz, 2007). Type 2 diabetes (T2DM) is defined as weakened responses to insulin (known as insulin resistance) and progressive beta cell dysfunction. It is the most common type of diabetes that arises due to poor lifestyle practices (Samuel and Shulman, 2016). With the rising prevalence and burden of T2DM, it is imperative to pursue drug development research in order to eradicate this burden. Several *in vitro* and i*n vivo* experimental studies are widely used in diabetes drug development to predict the outcome of tissue response to drugs.

This present study was divided into two main sections: *in vitro* and *in vivo* assessments. The *in vitro* section focussed on the quantification of polyphenolic compounds, antioxidant and carbohydrate enzyme inhibitory activities of different extracts of *Catharanthus roseus* and the pure compound vindoline. Insulin secretory studies were additionally performed in RIN-5F pancreatic cell line to evaluate the effect of vindoline and the plant extracts on insulin release. Furthermore, inflammatory response and oxidative stress effects were evaluated in this cell line following high glucose and respective treatment.

The *in vivo* part of the study focused on analysing the effect of vindoline in T2DM-induced male Wistar rats. To the best of our knowledge, this is the first *in vivo* study to investigate the effect of vindoline on the inflammatory, anti-oxidative and apoptotic parameters in diabetic nephropathy, hepatic, pancreatic and cardiac injury resulting from T2DM-induced tissue damage.

In the in vitro study, we used HPLC to quantify the polyphenolic compounds (chlorogenic acid, rutin, caffeic acid, quercetin and coumaric acid in the methanolic, aqueous, dichloromethane and ethylacetate extracts of *C.roseus*. Polyphenolic compounds have been shown to possess disease preventive effects thus contributing to the medicinal activities observed in plants. Polyphenols have been reported to possess antidiabetic, anticancer and anti-inflammatory activities. Additionally, the structure of these polyphenolic compounds have been linked to free radical scavenging mechanisms (Testa *et al.*, 2016). In our findings the methanolic extract of *C.roseus* showed the highest concentrations of the measured polyphenolic compounds while the aqueous extract exhibited appreciable amounts. On the other hand, the dichloromethane and the ethylacatate extracts had the least concentrations of polyphenols. The results of the total polyphenolic (TP) content in the extracts are in agreement with the HPLC findings we observed; in which the methanolic extract exhibited the highest total polyphenolic content and additionally showed highest concentrations of the selected polyphenols via HPLC quantification.

However, quantification of the alkaloid vindoline in these extracts revealed the highest concentrations in the dichloromethane and the ethylacatate extracts. These results obtained may ascertain that the methanolic extract of *C.roseus* may be the best solvent to extract the beneficial phenolic compounds.

Oxidative stress is a major disorder implicated in the development of detrimental complications of diseases like diabetes, cancer and degenerative pathologies (Oguntibeju *et al.*, 2016). Accumulation of reactive oxygen or nitrogen free radicals can overpower the biological antioxidant enzymes, if this happens oxidative tissue destruction occur leading to adverse organ damage. Plants are a good source of exogenous antioxidants and hence consumptions of plant materials has been shown to protect/ prevent the pathogenesis of diseases like DM (Giacco and Brownlee, 2010; Ighodaro and Akinloye, 2017).

The antioxidant activities of the extracts and vindoline were determined by using oxygen radical absorbance capacity (ORAC) and the DPPH activity. The results elucidated strong antioxidant activities in the methanolic extract followed by the dichloromethane, aqueous while ethylacetate extract has the least antioxidant power. These findings show that the extracts of *C.roseus,* especially the methanolic

extract can be utilised in disease states where oxidative stress inflict unfavourable complications by scavenging and trapping of radicals.

The antioxidant activity of vindoline was measured using the ORAC and the ferric reducing antioxidant power (FRAP). Its antioxidant activity was compared to that of a known antioxidant ascorbic acid. No significant differences were observed in the FRAP activity between vindoline and ascorbic acid, while the ORAC results showed better oxygen radical absorbance capacity. This implies that vindoline's antioxidant effect is through the release of the hydrogen atom to the radicals therefore stabilising the singlet oxygen radicals. Vindoline might therefore be a promising candidate in the prevention of diabetes related complications as it can also target oxygen derived free radicals.

RIN -5F is an insulinoma cell line derived from the pancreatic beta cells of rats. The cell line has been extensively utilised in insulin secretory studies. Severe hyperglycemia induces toxic metabolic, functional and structural changes in pancreatic beta cells. The ultimate effect of persistent high glucose level is induction of apoptosis in beta cells as consequence of increased formation of destructive free radicals (Bathina *et al.*, 2017).

In this study, RIN-5F cells were initially exposed to different concentrations of glucose in order to determine the effect of high glucose on the viability of the cells using the WST 1 assay. At 50 mM and 6.25 mM glucose concentrations, no signs of cytotoxicity were observed after 24 hours and were then used in subsequent experiments 50 mM as the highest concentration whilst 6.25 mM as lower concentration. Cell viability studies on the effect of different extracts of *C. roseus* and vindoline were performed. At a concentration of 1 mg/ml, the aqueous extract stimulated the proliferation of RIN-5F cells, confirmed by cell viability count of 150%. This result may attest to the traditional utilisation of water decoction of *C.roseus* to manage the symptoms of diabetes (Iweala and Okeke, 2005). At same concentration, the methanolic and the dichloromethanic extracts exhibited significant decreases in the viability of the cells indicating the possible cytotoxic consequences of these extracts using organic solvents. Concentrations that resulted in approximately 80% viability of cell were chosen for further assessments.

On the other hand, vindoline produced cytotoxic changes in cells at 1 mM, showing significant reduction in the number of viable cells. Vindoline is an alkaloid that forms vinblastine (a known anti-cancer drug derived from *C.roseus)* via a condensation reaction with catharanthine. It is possible that vindoline on its own possesses antiproliferation activities (Tiong *et al.,* 2015). In this study, the concentration of 0.125 mM resulted in the about 80% and was hence used in functional experiments.

In the present study, we further investigated the effect of vindoline and the extracts of *C.roseus* on the protection of RIN-5F cells from glucotoxicity and restoration of insulin secretory activities following a 24-hr exposure of cells to glucotoxicity (50 mM glucose), lower glucose concentration (6.25 mM) and in the absence of glucose. Our results revealed that vindoline enhances beta cell sensitivity in response to glucotoxicity through increased insulin secretion. Vindoline displayed the strongest beta cell stimulatory effect when compared to the plant extracts in glucotoxic-induced cells.

The aqueous extract demonstrated appreciable beta cell stimulatory activity whereas the methanolic extract exhibited the lowest insulin secretory effect despite high polyphenolic content. This result indicates that the beta cell stimulatory effect of *C.roseus* does not correlate with the amount of polyphenolic content. In low glucose exposed cells, vindoline and the dichloromethane extract additionally showed increased insulin secretion raising the possibility that vindoline might be one of the compounds responsible for the previously reported antihyperglycemic effect of the dichloromethane extract of *C.roseus.* Interestingly, insulin secretion was not significantly altered in non-glucose (0 Mm) exposed cells following treatment with vindoline and the respective plant extracts.

The control of hyperglycemia in diabetes patients is a critical approach in the prevention of diabetes complications. Inhibition of enzymes that catalyse the breakdown of carbohydrates following consumption of a meal has been shown to prevent the upsurge of blood glucose levels in diabetics (Mousinho *et al.,* 2013; Sagbo *et al.,* 2018). Drugs such as acarbose are presently being used to control glucose levels by inhibiting the activities of alpha amylase and alpha glucosidase enzymes. Plants are a rich source of different compounds hence exploration of

materials with carbohydrate metabolising inhibitory activities is imperative (Jarald *et al.*, 2008).

In this study, we evaluated the alpha amylase and alpha glucosidase inhibitory activities of vindoline and different extracts of *C.roseus.* Our results indicated significant higher alpha amylase inhibition (approximately 20%) in the extracts at 50 mg/ml. Lower concentrations of the dichloromethane extract revealed diminished inhibitory effects. These findings are in contrast to those of previous authors who reported higher inhibitory effect (68%) in lower concentrations (9 mg/ml) of the aqueous extract of *C roseus.* Differences in geographic and environmental locations in which the plants were collected may have attributed to the difference in inhibitory activity.

On the contrary, the methanolic extract (50 mg/ml) demonstrated increased inhibition of alpha glucosidase enzyme indicated by a 40% inhibitory effect whilst the aqueous showed decreased inhibitory activities when compared to the methanolic extract. We did not observe any inhibition of the alpha glucosidase enzyme by the dichloromethane extract.

Vindoline's enzyme inhibitory activities were compared to a known standard drug acarbose. We observed poor inhibition of both enzyme activity by vindoline (< 20% inhibition) when compared to acarbose which inhibited close to 80% of the enzyme activity. These results suggest that inhibition of carbohydrate metabolising enzymes may not be one of the mechanisms of vindoline's antidiabetic effect.

The in vivo part of the study focused on analysing the effect of vindoline in T2DM-induced male Wistar rats. From the best of our knowledge, this study was the first *in vivo* study to investigate the effect of vindoline on the inflammatory, anti-oxidative and apoptotic parameters in diabetic nephropathy, hepatic, pancreatic and cardiac injury resulting from T2DM-induced tissue damage.

 Forty-eight (48), 6 weeks old male Wistar rats (190-230g) were randomly divided into six (6) groups with a minimum of eight rats each (n=8); the groups are summarised in the table below.

Table 12: In vivo study design

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GROUP (n=8)	TREATMENT
N	Rats + vehicle (Normal control)
NV	Rats + vindoline (20 mg/kg b.w) (normal treated control)
NG	Rats + glibenclamide (5mg/kg b.w) (normal treated control)
D	Rats + Fructose 10% + streptozotocin, 40 mg/kg body weight (b.w) (diabetic control)
DV	Rats + Fructose 10% + streptozotocin, 40 mg/kg body weight (b.w) + vindoline (20 mg/kg b.w) (diabetic treated)
DG	Rats + Fructose 10% + streptozotocin, 40 mg/kg body weight (b.w) + glibenclamide (5mg/kg b.w) (diabetic treated)

Type 2 diabetes mellitus was induced by having rats consume 10% fructose water *ad libitum* for 2 weeks. Thereafter a single low dose of streptozotocin (40 mg/kg) was injected intraperitoneally. Rats that had blood glucose levels ≥18 mmol/l were considered to be diabetic and continued with respective treatments for 6 weeks. After the treatment period, rats were anaesthetised and euthanized using isoflurane gas at 2% with 1% oxygen, then blood and organs were collected, stored appropriately and used for analysis.

Failure of a sustained control of blood glucose levels in diabetic patients propagates the pathogenesis of detrimental complication that can lead to fatality. Excess glucose has been shown to drive the formation of reactive oxygen/ nitrogen species which are implicated in diabetes-related disease states (Giacco and Brownlee, 2010). In this study, assessments on the effect of vindoline on glucose metabolism showed significant decrease in blood glucose levels when compared to the diabetic non-treated control, whilst the results were not significant when compared to the diabetic group that was treated with glibenclamide. Moreover, our results exhibited elevated insulin concentration in the diabetic group that was treated with vindoline whereas in the non-diabetic treated controls, vindoline did not influence insulin secretion. This finding agreed with the results we observed in RIN-5F cells, in which vindoline enhanced insulin secretion from the glucotoxic- exposed beta cells. These outcomes tend to show that vindoline has insulotropic and antihyperglycemic effects that are beneficial in lowering the blood glucose levels in hyperglycaemic conditions (Tiong *et al.*, 2013).

Induction of T2DM resulted in significant loss of total body weight of diabetic groups when compared to non-diabetic groups. Reduction in body weight in diabetics has

been linked to progressive degradation of protein stores (gluconeogenesis in order to generate energy) as a compensatory response to decreased influx of glucose into the cells where it is needed in the production of ATP (Kataya and Hamza, 2008). Vindoline and glibenclamide did not prevent the loss of body weight in diabetic rats in this study.

We additionally observed increase in sizes of organs such as hearts, kidneys and the livers of all diabetic animals in comparison to the non-diabetic rats. Organ hypertrophy and hyperplasia occur in disease states in order to compensate for decreased functionality or due to inflammatory responses (Mestry *et al.*, 2017).

Liver function integrity is assessed by determining the serum levels of hepatic enzymes (AST, ALP, ALT and LDH). The activities of these enzymes were elevated significantly in the hyperglycaemic rats confirming hyperglycemia-mediated hepatocellular damage (Aldahmash *et al.*, 2016). Administration of vindoline and glibenclamide resulted in significant reduction of these enzyme activities suggesting the protective effect of vindoline and glibenclamide on the hepatic tissue.

The serum total protein levels were found to be severely elevated in the diabetic control group, indicative of pseudo-proteinemia as a result of excessive fluid loss due to polyuria. In T2DM rats, vindoline did not significantly change the serum total protein levels when compared to the normal control group, but significantly increased the protein levels in vindoline treated normal rats.

Diabetic nephropathy (DN) is a microvascular complication of diabetes and it is the leading cause of end-stage renal disease. Severe hyperglycemia accelerates the development of DN-a major predictive marker of cardiovascular disease. Several biochemical markers are used to determine the kidney dysfunction (Musabayane, 2012). Urea, a by-product of protein breakdown is measured in serum to study the filtration and excretory functions of the kidneys. Accumulation of excess urea signifies abnormal renal function (Krishnakumari and Bhuvaneswari, 2012; Li *et al.*, 2014; Hassanalilou *et al.*, 2017). In this study, vindoline and glibenclamide administration in T2DM lowered the serum levels of urea. The serum creatinine levels were additionally reduced in T2DM groups that were treated with vindoline and glibenclamide. These findings are suggestive of the nephroprotective effect of vindoline in T2DM.

T2DM is associated with disturbed metabolism of lipids hence increasing the risk of developing cardiovascular diseases. Excess lipids/cholesterol (LDL, triglycerides, total cholesterol, VLDL) tends to accumulate in the endothelium of arteries forming plaques that impede the flow of blood (Patel *et al.*, 2016). Atherosclerotic plaques increase the risk of stroke as enough blood does not reach the brain. HDL is beneficial cholesterol that transports "bad" cholesterol to the liver where it is modified and excreted (Lafta, 2014). Vindoline supplementation reduced the serum levels of atherogenic lipids; moreover, it enhanced the level of good cholesterol HDL. Based on these observations, administration of vindoline in T2DM could potentially reduce the risk of cardiovascular events (Yao *et al.*, 2013).

Biological systems have endogenous antioxidant defence systems in place to curb free radical build-up. Endogenous antioxidants comprise of catalase, SOD and glutathione peroxidases/reductases (GPX) (Rolo and Palmeira, 2006). The metabolic deviations that occur in diabetic states trigger overproduction of mitochondrial superoxide anion in endothelial cells of vessels (Kim *et al.*, 2016). Excess superoxide production overpowers the ability of the enzymes SOD and GPX to reduce the superoxide anion to hydrogen peroxide and water. On the other hand, the removal of hydrogen peroxide by catalase will be compromised and ultimately leading to increased polyol pathway flux, protein glycation, overactivity of the hexosamine pathway and activation of protein kinase C (PKC) isoforms (Giacco and Brownlee, 2010). Activation of these pathways result in the damage of macromolecules including nucleic acids, proteins and lipids thus leading to pathogenesis of diabetes complications (Tiwari *et al.*, 2013).

This study established that vindoline significantly enhanced the activity of SOD in the liver and kidney tissues of T2DM rats. The decreased activity of SOD in diabetic controls may be due to the imbalance between excess ROS and antioxidants. The catalase activity in the liver of diabetes rats was not significantly changed following treatment with vindoline and glibenclamide. On the contrary, the catalase activity in the kidney and cardiac tissues of the diabetic control group were significantly high when compared to the normal controls. The increase in the catalase activities observed in diabetic rats could have been due to compensatory responses to

increased concentrations of free radicals (Qujeq and Rezvani, 2007). The ORAC assay was conducted in the liver and kidney tissues, the results showed increased oxygen radical absorbance capacity in rats that were treated with vindoline and glibenclamide. In the cardiac tissue, vindoline administration in diabetic rats significantly improved the ferric reducing antioxidant power (FRAP) in comparison to the diabetic control group. The diabetic control group exhibited significant elevated levels of MDA (peroxidation status) while T2DM rats that were treated with vindoline recorded lower levels of MDA. Increased levels of MDA in tissue are indicative of disrupted membranal lipid bilayers which has a negative impact on the fluidity of the cell membranes (Tug *et al.*, 2005).

The synergy between oxidative stress and inflammation has been established (Ayepola *et al.*, 2013; Ayeleso *et al.*, 2014). Oxidative stress has been shown to increase the expression of genes that code for pro-inflammatory cytokines (Dang *et al.*, 2010; Ayepola *et al.*, 2013; Oguntibeju, 2018). In T2DM, insulin resistance has been linked to the activation of pro-inflammatory cytokines. Abnormal activation of pro-inflammatory cytokines in diabetes triggers adverse inflammatory responses that cause tissue fibrosis and eventually organ damage (Akash *et al.*, 2013; Piya *et al.*, 2013). A significant reduction of TNF-α level in the liver and cardiac tissues of diabetic rats treated with vindoline was seen in this study. Lower levels of TNF-α in the kidney tissue of T2DM rats treated with vindoline was observed (though the reduction was not significant). The observed decrease in the levels of TNF-α in diabetic rats after treatment with vindoline shows the probable retardation of DN onset. Administration of vindoline in diabetic rat did not significantly affect the expression of IL-10, IL-6 and IL-1.

Apoptosis is a well-coordinated programmed execution of cell death. It is a principal mechanism in maintenance of normal tissue homeostasis. Aberrations in normal regulatory signals of apoptosis leads to the pathogenesis of diseases like cancer, neuro-degenerative disorders and diabetes (Lee and Pervaiz, 2007). Increased expression of pro- apoptotic proteins known as caspases in T2DM humans and rats has been reported. In this study, we determined the expression of pro- apoptotic (caspase 3 and caspase 9) and anti-apoptotic (BCL-$_2$) caspases using immunohistochemical staining technique. This study revealed a significant increased

expression of caspase 9 in the hepatic and kidney tissues of diabetic controls, treatment with vindoline in diabetic rats resulted in significant suppression of the pro-apoptotic protein caspase-9. Suppression of caspase-9 might have been associated with increased antioxidant responses in conjunction with the anti-apoptotic effects of vindoline.

Expressions of BCL-2 and caspase-3 in the liver and kidney tissues were not significantly altered by vindoline administration in diabetic rats. Furthermore, histological evaluation of the liver, pancreas and kidney sections of both diabetic and non-diabetic rats was performed to compare the extent of tissue damage. Liver sections of the diabetic control rats showed severe disruption and dilation of sinusoids, both the sinusoids and the central vein showed signs of congestion. When the liver sections of diabetic rats that were treated with vindoline/ glibenclamide were compared to the diabetic controls, mild central vein and sinusoidal congestion were reported. These findings suggest that vindoline retarded the events that lead to the development of diabetic liver disease.

Kidney sections of diabetic controls showed severe distortion of the glomeruli, dilation of glomerular space, increased vacuolation of cells as well as expanded tubular degeneration. These changes were found to be minimal in diabetic rats treated with glibenclamide and vindoline and it is most likely that vindoline delayed the emergence of DN.

In the pancreatic sections diabetic controls, the islets appeared to be smaller in size without demarcated borders. Islet cells showed degenerative changes which led to β-cell apoptosis. Vindoline rejuvenated the islet integrity although degenerative features were visible. Vindoline showed potential islet repairing effects which offer protection from hyperglycemia-induced free radical damage.

6.2 Conclusion

The antidiabetic activity of *Catharanthus roseus* has been reported previously, hence antidiabetic studies of its bioactive compounds is imperative. The results obtained in this study revealed the antioxidant effect of this plant, especially in the methanolic

extract possibly due to the ample amount of polyphenols. The results of this study further substantiates the insulotropic effects of the natural product-vindoline, the dichlromethane and the aqueous extract of *C roseus* as exhibited in glucotoxicity-induced assay in pancreatic RIN-5F cells. Vindoline additionally induced insulin secretion in the beta cells of type 2 diabetes mellitus rat model used in this study. The insulotropic effects resulted in the decrease in blood glucose levels of diabetic rats. The decreased blood glucose levels minimised the formation of free radicals in the liver, heart and kidney which in turn hampered the activation of oxidative stress tissue damage. Moreover, vindoline demonstrated excellent hypolipidemic activities that can offer the much needed protection from diabetes-related cardiovascular events. As a result of *in vitro* and *in vivo* antioxidant and anti-inflammatory effects demonstrated by vindoline in our study, this natural product could therefore be considered as a medicinal candidate for the management of diabetes and ultimately, prevention of its complications.

6.3 Recommendations

Although our 8 week study was able to highlight the beneficial effect of vindoline in the management of type 2 diabetes, longer studies are recommended to effectively monitor and observe the effects it has in chronic studies. More detailed molecular studies that involve gene expression are highly recommended to efficiently establish the pathways that are involved.

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ADDENDUM

RESEARCH OUTPUT

PUBLISHED MANUSCRIPTS, BOOK CHAPTERS AND ABSTRACTS

1. Mediline Goboza, Prisca Kachepe, Yapo Guillaume Aboua, Oluwafemi Omoniyi Oguntibeju. Potential of *Catharanthus Roseus* and *Punica Granatum* in the Management and Treatment of Diabetes Mellitus and Its Complications: Chapter 11: In **Bioactive Compounds of Medicinal Plants Properties and Potential for Human Health**, Ed: *Megh R. Goyal, Ademola O. Ayeleso* 2018.

2. Mediline Goboza, Yapo G. Aboua, Novel Chegou, Oluwafemi O. Oguntibeju. Vindoline effectively ameliorated diabetes-induced hepatotoxicity by docking oxidative stress, inflammation and hypertriglyceridemia in type 2 diabetes-induced male Wistar rats. **Abstract presented at 3rd International Conference: Medicinal Plants in Healthcare. Society of Medicinal Plants and Economic Development,** Johannesburg, South Africa, 2018.

3. Mediline Goboza, Yapo G. Aboua, Novel Chegou , Oluwafemi O. Oguntibeju. Antihyperlipidemic, antioxidant and anti-inflammatory effects of vindoline in type 2 diabetic rats. **Abstract presented at 6th International Conference: Research, Innovation and Technology for African Development** and published in the abstract book. 4-6 September 2018.

4. Mediline Goboza, Yapo G. Aboua, Novel Chegou, Oluwafemi O. Oguntibeju. Vindoline effectively ameliorated diabetes-induced hepatotoxicity by docking oxidative stress, inflammation and hypertriglyceridemia in type 2 diabetes-induced male Wistar rats. **Published in Biomedicine and Pharmacotherapy 2019.**

5. Oluwafemi Omoniyi Oguntibeju, Yapo Aboua and Mediline Goboza. Vindoline—A Natural Product from Catharanthus Roseus Reduces Hyperlipidemia and Renal Pathophysiology in Experimental Type 2 Diabetes. **Published in Biomedicines 2019.**