

UNIVERSITY OF KWAZULU-NATAL

**Effects of *Momordica balsamina* methanolic extract
on cardiovascular and haematological function in
streptozotocin-induced diabetic rats: effects on
selected markers**

Asiphaphola Ludidi

2018

**EFFECTS OF *MOMORDICA BALSAMINA* METHANOLIC EXTRACT
ON CARDIOVASCULAR AND HAEMATOLOGICAL FUNCTION IN
STREPTOZOTOCIN-INDUCED DIABETIC RATS: EFFECTS ON
SELECTED MARKERS**

by

Asiphaphola Ludidi

216017652

Supervisor: Dr P.S Ngubane

Co-Supervisor: Dr A Khathi

Discipline of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

Date of submission: 16 January 2018

*Submitted in fulfilment for the degree of Master of Medical Science in the
School of Laboratory Medicine and Medical Sciences, University of KwaZulu-
Natal*



**UNIVERSITY OF
KWAZULU-NATAL**

**INYUVESI
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Preface

Chronic hyperglycaemia observed in diabetic patients has been associated with the development of cardiovascular complications, which increases the risk of heart failure. In addition, hyperglycaemia induces haematological changes that compromise red blood cell function thus decreasing the oxygen-carrying capacity of the blood to the cardiovascular system, further aggravating cardiac disorders. The use of anti-diabetic agents have been associated with the progression of the pathology of haematological and cardiac function disorders. The World Health Organization however, has proposed the use of medicinal plants as an alternative as some of these plants possess anti-hyperglycaemic and cardio-protective properties which is of benefit in alleviating hyperglycaemia-induced cardiovascular complications. The goal therefore, is to investigate the effects of *Momordica balsamina* methanolic extract on haematological and cardiovascular function which may bring to light, the mechanisms by which this plant may use to ameliorate hyperglycaemia-induced cardiovascular complications in a streptozotocin-induced diabetic rat model.

Declaration

I, **Asiphaphola Ludidi** (student number: **216017652**), hereby declare that the dissertation entitled (**Effects of *Momordica balsamina* methanolic extract on cardiovascular and haematological function in streptozotocin-induced diabetic rats: effects on selected markers**) is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of others work, it has been duly acknowledged. The research done in this study was carried out under the supervision of Dr P.S. Ngubane and Dr A. Khathi.

Student: Asiphaphola Ludidi (216017652)

Supervisor: Dr P.S. Ngubane

Co-Supervisor: Dr A. Khathi

Date: _____

Plagiarism declaration

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

MASTER'S DEGREE IN MEDICAL SCIENCES 2018

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2. Each contribution to, and quotation in, this thesis from the works of other people has been attributed and has been cited and referenced.
3. This thesis is my own work.
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Acknowledgements

I would like to firstly thank my God who has carried me through the duration of my master's degree and keeping me safe at all times as I walked alone in the middle of the night to and from campus, in preparation to submit my thesis. The following people have greatly contributed to the success of my project:

- Dr Phikelelani Ngubane who has been a great mentor to me and his constant smile even after missing submission dates. I am truly blessed to have a mentor that is patient and constantly believing in me even when I did not believe in myself. You have honestly helped me find my true passion for research.
- Dr Andile Khathi who has always kept a smile on his face, even when he was correcting me. Your mentorship and constant strife to make me the best researcher has truly made you more than a mentor to me, but an older brother. I will forever be grateful for your advice, jokes and warmth in your ways of imparting knowledge.
- Dr Ntethelelo Sibiya, Ms Anelisiwe Siboto, Mixo Charirty Baloyi, Lindokuhle Mabuza, Mr Mlindeli Gamede, Mluleki Luvuno and the rest of the UKZN endocrinology group, I appreciate your inputs and constant encouragement to do better. I would not have survived the unbearable long hours during experiments if you all had not been there to support me. May you continue giving and availing yourselves, selflessly to others as you did for me. In Xhosa we say “Umntu ngumntu ngabantu” meaning that nobody is an island, we all need someone. May God bless you all.
- My family and friends who have been praying for me and constantly encouraging me to not give up. Your love, consistent support and patience during all these academic years has motivated me to follow my true passion.
- Mr Dennis Makhubela, Bab'Dennis (as we call you), has been my father figure here in Durban. I know that seeing us mature and soar to greater heights brings you joy. I now understand why you have constantly availed yourself to assist me in any way you could. I hope to always make you proud. You are truly God sent to us.
- The academic and technical staff of the Human Physiology Department for their willingness to assist me in every way possible. Thank you.
- The UKZN Biomedical Resource Centre staff for the exceptional training, assistance and support during the time of conducting my animal studies. Thank you.
- The National Research Foundation and UKZN College of Health Science for the financial support towards my research project. Thank you.

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Abbreviations list

AMPK	activated protein kinase
AGE	advanced glycation end product
ATP	adenosine triphosphate
ANOVA	one-way analysis of variance
AR	aldose reductase
α	alpha
APLT	aminophospholipid translocases
Ang-II	angiotensin-II
AREC	animal research ethics committee
β	beta
BRU	biomedical research unit
1, 3 BPG	1, 3 biphosphoglycerate
BHT	butylated hydroxytoluene
Ca ²⁺	calcium ion
CML	carboxymethyllysine
CT-I	cardiotrophin-I
°C	celcius
CLIA	chemiluminescence immunoassay
CCK	cholecystokinin
CoBNeST	Conference of Biomedical and Natural Sciences and Therapeutics
CHS	College of Health Sciences
CRP	C-reactive protein
3-DG	3-deoxyglucosone
DNA	deoxyribonucleic acid
DC	diabetic control
DM	diabetes mellitus
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
DPX	distyrene plasticizer xylene
eNOS	endothelial cell NO synthase
ET-I	endothelin-I
EDHF	endothelium-derived hyperpolarizing factor

ELISA	enzyme-linked immunosorbent assay
EGCG	epigallocatechin-gallate
EPO	erythropoietin
FS	forward scatter
FITC	fluorescein isothiocyanate
FACS	Fluorescence-activated cell sorter
FFA	free fatty acids
GPx	glutathione peroxidase
G3P	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Hb	haemoglobin
H & E	haematoxylin and eosin
H/B	heart to body weight ratio
HRP	horseradish peroxidase
h	hour
HCl	hydrochloric acid
H ₂ O ₂	hydrogen peroxide
OH ⁻²	hydroperoxyl
OH [·]	hydroxyl radical
INS	insulin
i.p.	intraperitoneally
JAK	Janus kinase
JNK	c-Jun-N terminal kinase pathway
Kg	kilogram
λ	lambda
L	litre
LDL	low-density lipoprotein
Ltd	limited
MA	masilinic acid
MDA	malondialdehyde
MAP	mean arterial pressure
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume

MTF	metformin
μ	micro
μg	micrograms
μl	microlitre
MV	microvesicle
m	milli
mg	milligram
ml	millilitre
mmHg	millimeters of mercury
MAPK	mitogen activated protein kinase
MB	<i>Momordica balsamina</i> methanolic extract
NAD ⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NO ₂	nitrogen dioxide
NO	nitric oxide
NC	non-diabetic control
NF-κβ	nuclear factor kappa-beta
PYY	peptide YY
p.o.	per os (orally)
PPAR	peroxisome proliferator-activated receptor
PBS	Phosphate buffered saline
PI3K	phosphatidylinositol-4, 5-bisphosphate
PS	phosphatidylserine
3-PK	3-phosphokinase
PLSCR	phospholipid scramblases
PUFA	polyunsaturated fatty acids
PKC	protein kinase C
ROS	reactive oxygen species
RAGE	receptor for advanced glycation end product
RBC	red blood cell
RLU	relative light unit
SS	Side scatter
STAT	signal transducer and activator of transcription
SD	Sprague-Dawley

SEM	standard error of means
SOD	superoxide dismutase
STZ	streptozocin
s.c.	subcutaneously
TBA	thiobarbituric acid
TBARs	thiobarbituric acid reactive substances
TNF α	tumour necrosis factor α
T1D	type one diabetes
USA	United States of America
UKZN	University of KwaZulu-Natal

Study outline

The current dissertation is presented in manuscript format. It consists of 7 sections viz. dissertation abstract, chapter 1: introduction/literature review, chapter 2: manuscript 1, chapter 3: manuscript 2, chapter 4: synthesis, conclusions, and appendices. The dissertation abstract states the purpose of the study and summarizes the findings of the study. Chapter 1 is a brief background and a germane literature review to highlight the gaps that exist in literature and how the current study aims to fill these gaps. Chapter 2 is the first novel research paper that seeks to evaluate the effects of a medicinal plant, *Momordica balsamina* methanolic extract on cardiovascular dysfunction. A.Ludidi, A Khathi, N.H Sibiya and P.S Ngubane are authors to this paper. The manuscript is currently under review in the journal of Chemico-Biological Interaction. Chapter 3 entails the second research study manuscript, which sought to investigate the effects of *Momordica balsamina* methanolic extract on haematological function, which may unveil some of the mechanisms by which this plant may exert its cardio-protective effects. A Ludidi, M.C Baloyi, A Khathi, N.H Sibiya and P.S Ngubane are authors to this paper. The manuscript is prepared for submission to the journal of Biomedicine and pharmacotherapy. Chapter 4 is the synthesis, which discusses the link between the two studies and highlights the main findings for the specific aims of the current project. Appendices include the letter of ethical clearance, abstract and certificate of presentations to various conferences and journal's guideline to authors for both research papers.

Abstract

Background

The hyperglycaemia-induced haemodynamic changes reduces the oxygen-carrying capacity of erythrocytes, thus aggravating cardiovascular disorders in diabetic patients. The conventional therapies have been shown to be associated with the progression of haematological and cardiovascular dysfunction, which may not be favorable for patients with congestive heart failure. We have previously shown the anti-hyperglycaemic and antioxidant properties of *Momordica balsamina* (MB) methanolic extract which may be of benefit in alleviating cardiovascular disorders, thus providing an effective alternative therapy. The current study therefore, investigated the short-term effects of MB methanolic extract on cardiovascular and haematological function in streptozotocin-induced diabetic rats.

Methods

Briefly, air-dried MB leaves were extracted with methanol to yield a methanolic extract. STZ-induced diabetic rats were divided into untreated and treated groups with insulin ($170\text{ }\mu\text{g kg}^{-1}$ s.c.) and metformin (500 mg kg^{-1} p.o.) as standard drugs. MB (250 mg kg^{-1} p.o.) was administered twice daily for 5 weeks. Blood glucose concentration, body weight and blood pressure were monitored weekly for 5 weeks. Terminally, animals were sacrificed after which blood, heart and kidneys were collected for haematological and biochemical analysis. Histological analysis was also performed on the hearts.

Results

MB significantly decreased blood glucose concentration from week 3-5 by comparison with diabetic untreated animals. Treatment with MB reduced oxidative stress in the plasma, kidney and heart while improving their antioxidant status compared with untreated diabetic animals. This was associated with increased EPO secretion by the kidneys thus improving RBC production and haemoglobin concentrations. MB moderately increased erythrocyte indices: mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) by comparison with untreated diabetic animals. MB ameliorated heart hypertrophy and decreased CRP, CT-I and Ang-II concentrations by comparison with untreated diabetic animals. MB also decreased MAP by comparison with untreated diabetic animals.

Conclusion

MB administration protects against hyperglycaemia-induced cardiovascular and haematological changes by attenuating hyperglycaemia, oxidative stress in both the kidney and heart tissues of STZ-induced diabetic rats, which may reduce the risks of cardiac myopathology complications in diabetes mellitus.

Chapter 1: Literature review

1. Background

Sustained hyperglycaemia is the pathogenic feature of DM which has been shown to result in microvascular and macrovascular complications (1, 2). Macrovascular complications have been found to be the most common cause of deaths amongst diabetic patients with cardiovascular disorders accounting for 52% of deaths in type 2 diabetes mellitus (T2DM) and 44% in type 1 diabetes mellitus (T1DM) (3, 4). Studies indicate that hyperglycaemia-induced haematological changes such as altered red blood cell function play a significant role in the progression of cardiac dysfunction (5). Although conventional treatment decreases hyperglycaemia, their use is associated with undesirable side effects which amongst many include increased blood viscosity which is of concern in patients with congestive heart failure (6). As a result, the progression of macrovascular complications intensifies even during treatment (7, 8). There is therefore, a need for alternative treatment, which will lower blood glucose concentration and alleviate complications associated with diabetes. Medicinal plants have been shown to possess, with minimal side effects, anti-hyperglycaemic and cardio-protective effects in STZ-induced diabetic animal models and therefore could potentially serve as an effective alternative (9-11). The mechanisms by which these medicinal plants exert their hypoglycaemic, anti-hyperglycaemic effects and ameliorate hyperglycaemia-induced cardiovascular disorders, are still to be fully understood. We have previously reported on the anti-hyperglycaemic and reno-protective properties of *Mormodica balsamina*, which may be beneficial in alleviating hyperglycaemia-induced cardiac dysfunction. This study therefore, investigated whether *Mormodica balsamina* can avert the decline of cardiac function often seen in diabetic humans and animal models. The following section discusses the effects of hyperglycaemia on cardiac function.

1.1 Effects of hyperglycaemia on cardiovascular function

Hyperglycaemia has been shown to increase reactive oxygen species (ROS) including superoxide resulting in oxidative stress, which induces mitochondrial dysfunction through caspase 3 activation, which induces apoptosis in cardiomyocyte (12, 13). Furthermore, ROS activates scramblases in red blood cell (RBC) membrane to expose phosphatidylserine (PS) to the extracellular space, where annexin-V, a marker for RBC undergoing apoptosis, which targets it for engulfment by macrophages (14, 15). The latter decreases RBC count which decreases the oxygen carrying capacity of blood, further aggravating cardiac dysfunction due to decreased oxygen supply to the cardiomyocytes. Studies however, have shown that some medicinal plants have the ability to decrease caspase 3 activation which may delay apoptosis in cardiomyocyte (16).

Other mechanisms that induce cardiac cell death include the increased activation of the polyol and protein kinase C (PKC) pathways and advanced glycation end products (AGEs) formation which are further discussed below (17-21).

1.1.1 The role of the polyol pathway in cardiac dysfunction

The polyol pathway is a metabolic pathway where aldose reductase (AR) uses nicotinamide adenine dinucleotide phosphate (NADPH) as its cofactor to reduce excess glucose to sorbitol. In addition, NADPH is required as a cofactor during the catalytic regeneration of glutathione by glutathione reductase which is an antioxidant enzyme (22). The accumulation of sorbitol as seen in cardiac cells induces osmotic stress which has been proposed to promote apoptosis, however the mechanism is still unknown (23). Sorbitol is oxidized by sorbitol dehydrogenase to fructose with nicotinamide adenine dinucleotide (NAD^+) being reduced to NADH (24). Hyperglycaemia therefore decreases the NAD^+/NADH ratio (25). However medicinal plants have been shown to possess phytochemicals that inhibit AR which may protect the heart from ischemic injury (26, 27). Furthermore, the up regulation of the polyol pathway has been shown to promote ROS production thus activating other cellular damaging mechanisms such as the protein kinase C (PKC) pathway which has been shown to induce haematological changes that shorten the life span of erythrocytes, further promoting cardiovascular dysfunction (28).

1.1.2 The role of the protein kinase C (PKC) pathway in cardiac dysfunction

As mentioned previously, the up regulation of the polyol pathway decreases the NAD^+/NADH ratio, which blocks NAD^+ -dependant glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from converting glyceraldehyde 3-phosphate (G3P) to 1,3 biphosphoglycerate (1,3 BPG) (29). As a result, there is an increased amount of G3P, which

is a substrate for the synthesis of α -glycerol phosphate (29, 30). Furthermore, α -glycerol phosphate is a precursor of diacylglycerol (DAG) and DAG is known to activate some protein kinase C (PKC) isoforms (31). In the hearts of diabetic patients, both PKC- α and PKC- β isoforms are both upregulated which is associated with an irregular contractility progressing towards heart failure (32). The activation of the PKC has also been linked with the decreased RBC deformability thus inducing eryptosis (33). Medicinal plants such as *Centaurium erythraea* however, have been shown to decrease the activation of PKC in erythrocytes thereby restoring RBC membrane integrity and reducing the rate of agglutination (34, 35).

1.1.3 The role of advanced glycation end products (AGEs) in cardiac dysfunction

Hyperglycaemia has been shown to be associated with increased advanced glycation end products (AGEs) (36). AGEs develop when a reducing sugar such as glucose reacts non-enzymatically with an amino group of a protein to form Amadori products (37, 38). Then after days to weeks, the Amadori products undergo reactions that further rearrange to form irreversibly cross-linked senescent macroprotein derivatives which are called AGEs (37). When an AGE interacts with its receptor (RAGE), it generates oxidative stress in various cell types including cardiomyocytes and evoke inflammatory and thrombogenic reactions (39). AGEs thus play a significant role in the development and progression of cardiovascular complications in diabetic patients thus their formation and accumulation progress at accelerated rates in diabetic patients (40). Furthermore, AGEs have also been shown to induce the cross-linking of integral membrane proteins such as the sodium-potassium pump (Na^+/K^+ ATPase) of erythrocytes, resulting in the reduced activity of this pump (41, 42). Medicinal plants however, have been shown to possess flavonoids which decrease the AGEs-induced ROS production, this way restoring the function of glycosylated enzymes (43). The increased formation of AGEs, enhanced production of reactive oxygen species (ROS) and stimulation of protein kinase C (PKC), have been proposed to contribute to the endothelial dysfunction in diabetic patients which is further discussed below (44, 45).

1.2. Endothelial dysfunction

Free radicals also target and modify major extra-cellular and long lived proteins such as elastin, laminin and collagen to form glycoproteins (46). The presence of these glycoproteins in the vascular wall is associated with the development of endothelial dysfunction and cardiovascular complications including atherosclerosis (47). In addition, hyperglycaemia contributes to the progression of cardiovascular dysfunction, however, dislipidaemia, is another factor that contributes to endothelial dysfunction resulting in the development of cardiac dysfunction (48).

Endothelial dysfunction is defined as the inability of the endothelium to maintain vascular homeostasis, which is also associated with the decreased bioavailability of nitric oxide (NO) (49). This decrease is caused by the decreased expression of endothelial cell NO synthase (eNOS), inefficient eNOS activation, insufficient substrate or cofactors for eNOS and lastly, the accelerated degradation of NO₂ by ROS (50, 51). As a result, endothelial dysfunction is accompanied by increased vasoconstrictors including endothelin-1 (ET-1) and angiotensin II (49, 52). Additionally, there is increased thrombosis, cell growth in the vascular walls and poor regulation of inflammation (52). The endothelium orchestrates the production of vasodilators including NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) in order to maintain arterial tone and blood flow (53). Both vasoconstrictors and vasodilators work in synergy to maintain a homeostatic arterial patency and compliance (52). The pathology of endothelial dysfunction therefore results in increased arterial tone, which promotes vasospasm and increased arterial stiffness (54). Shear stress is the frictional force between blood flow and the endothelial wall (55). An increase in blood flow, increases shear stress however the diameter of the vessel is inversely proportional to shear stress (56). In healthy arteries, an increase in arterial flow which alters the diameter stimulates a “flow-mediated dilation”, therefore decreasing shear stress, thus maintaining homeostasis (57). Additionally, the endothelium plays a role in maintaining the chronic changes in the structure of the arterial lumen because of chronic changes in blood flow (58). Endothelial dysfunction therefore contributes to the clinical expression of atherosclerosis leading to cardiovascular dysfunction (59). Atherosclerosis is the narrowing of the arteries due to endothelial injury and inflammation (60). Additionally, the accumulation of the oxidized low-density lipoprotein (LDL) particles in the arterial walls may be promoted by a vasoconstrictor known as angiotensin II (Ang- II), resulting in vascular blockage (61). The inhibition of vascular blood flow eventually leads to the bursting of the vascular wall (62). Furthermore, oxidized LDL accumulation, results in monocytes infiltrating the arterial wall and differentiate into macrophages in order to accumulate oxidized lipids to form foam cells (63). These foam cells stimulate macrophages proliferation and attract T-lymphocytes. T-lymphocytes induce smooth muscle proliferation in the arterial walls and collagen accumulation resulting in the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Vascular occlusion is increased thereby increasing the risk of rupture, which further contributes to cardiovascular dysfunction. ROS has also been shown to also induce the nuclear factor-kappa B (NF-κB) to transcriptional activation of many transcriptional genes relevant to inflammation, immunity and atherosclerosis (64). As a result, there is elevated tumour necrosis factor α (TNF α), plasma

cardiotropin-I and C-reactive protein concentration in the circulation (65). These pro-inflammatory cytokines serve as experimental markers for the progression of cardiovascular diseases (65).

In addition, hyperglycaemia increases the accumulation of sorbitol in the cells, thus, osmotic stress has been postulated to underly the mechanism involved in the development of diabetic microvascular complications (66). Medicinal plants such as *Portulca oleracea* L. have been shown to improve endothelial dysfunction by promoting the activity of eNOS thereby restoring vasodilation and vasoconstriction which may improve the increased blood pressure of diabetic patients which may further improve cardiac dysfunction (67, 68).

Hyperglycaemia has also been shown to induce haemetic changes including RBC dysfunction associated with an increased risk of developing cardiac dysfunction and diabetic anaemia if left untreated. These haemetic changes are further discussed below.

1.3. Effects of hyperglycaemia on red blood cell function

1.3.1 Effects of hyperglycaemia-induced ROS on red blood cell function

Amongst the several factors that contribute to the haematological changes in diabetic patients, hyperglycaemia increases free radical accumulation resulting in oxidative stress, which target and damage cellular molecules namely: protein and lipids eventually altering cellular function (69-71). The up regulation of the polyol pathway as mentioned in the above section, increases the accumulation of sorbitol which results in osmotic stress (72). Osmotic stress has been shown to induce red blood cell (RBC) shrinkage as observed by a decrease in mean corpuscular volume (MCV) in diabetic patients (73, 74). The shrinkage of RBCs disturbs membrane proteins such calcium (Ca^{2+}) channels resulting in RBC death, a process known as eryptosis (75). Activated Ca^{2+} channels increase intracellular influx of Ca^{2+} , which activate aminophospholipid translocases (APLT) that activate phospholipid scramblases (PLSCR) thus inducing the exposure of phosphatidylserine (PS) to the extracellular fluid (76). In addition, conversion of sorbitol to fructose, which is further metabolized to fructose-3-phosphate by 3-phosphokinase (3-PK), results in the generation of 3-deoxyglucosone (3-DG) (77). 3-DG is the central precursor of an array of AGEs, particularly carboxymethyllysine (CML)–protein adducts in erythrocytes (78). CML adducts have been shown to increase thiobarbituric acid reactive substances (TBARs) including malondialdehyde (MDA) which increases oxidative stress thus increasing RBC membrane rigidity, decreasing the life-span of RBCs and promoting eryptosis (79). Cardiovascular function is therefore compromised; however, there are

scavenging systems or antioxidants such as superoxide dismutase (SOD), which neutralizes ROS. Furthermore, the imbalance of these protective antioxidant mechanisms due to hyperglycaemia as seen in DM, is associated with other complications such as increased lipid peroxidation (80). Medicinal plants such as *Ferulga angulata* have been shown to contain bioactive compounds that improve the antioxidant status including decreasing lipid peroxidation levels of experimental diabetic animals (81, 82). This could be of benefit in red blood cell function since lipid peroxidation induces hemolysis of RBC, thus increasing the risk of cardiovascular complications (83).

1.3.2 Effects of lipid peroxidation on RBC function

Lipid peroxidation is a result of increased ROS, with the hydroxyl radical (OH^\cdot) and hydroperoxyl (OH^\cdot_2) profoundly affecting lipids. The OH^\cdot radical is the most chemically reactive species of the ROS and produced from O_2 in cell metabolism. Lipid peroxidation is described as the process of oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond (s), that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides. Glycolipids, phospholipids and cholesterol and polyunsaturated fatty acids (PUFA) are targets for damaging and peroxidative modification (84). PUFA arachidonic acid, which is a component of cell membrane phospholipids, is peroxidized to finally form malondialdehyde (MDA) which is used as a biomarker for lipid peroxidation. The accumulation of MDA increases intracellular fluid viscosity, which disturbs haemoglobin (Hb) function, ultimately altering the function of RBC indices as observed by the decreased mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). As a result of prolonged decreased Hb concentrations, anaemia ensues due to the decreased Hb transporting oxygen to cells. The changes in RBC indices also result in decreased RBC fragility which is associated with a disrupting in the asymmetry of membrane phospholipids where cell membrane scrambling occurs accompanied with phosphatidylserine (PS) translocation to the external cell surface (85). PS exposure signals for eryptosis through phagocytosing cells engulfing targeted RBCs resulting in the rapid clearance of the affected erythrocytes (86). The increased rate of eryptosis, which invariably leads to the development of anaemia thus aggravating cardiovascular dysfunction (87, 88). However, the ability of some medicinal plants such as *Nigella sativa*, to stabilise the fragility of RBC thus decrease the rate of rupturing, which prolongs the life span of erythrocytes therefore, may improve diabetic myopathy (89, 90).

1.4 Effects of hyperglycaemia on erythropoietin secretion

Hyperglycaemia-induced microvascular complications including nephropathy, have been shown to be associated with a decrease in red blood cell formation since the kidney is the primary stimulus for erythropoiesis via erythropoietin (EPO) secretion (91, 92). Diabetic patients, as a result, develop anaemia if left untreated (93, 94). However, diabetic anaemia arises from a number of factors such as increased reactive oxygen (ROS) species and lipid peroxidation (95, 96). Some isolated derivatives of medicinal plants such as chlorogenic, caffeine and ferulic acid have been shown to improve EPO secretion thus erythropoiesis in experimental animals however (97, 98).

It is clear therefore that treatment strategies, should aim to lower blood glucose and alleviate hyperglycaemic-induced micro and macrovascular complications (99).

1.5. Management strategies

The management of hyperglycaemia and DM associated complications relies on various treatment strategies, which involves the use of anti-hyperglycaemic agents such as biguanides, sulphonylureas, alpha glucosidase inhibitors, thiozolidinediones and insulin injections (100). Metformin and insulin however, have been shown to affect cardiac and RBC function as highlighted below.

1.5.1 Metformin

Biguanides such as metformin, decreases hepatic glucose output and decreases fasting glycaemia (101). However, it may lead to renal dysfunction as it may increase lactic acidosis which is extremely rare (less than 1 case per 100,000 treated patients) but potentially fatal (102). Although metformin has been shown to have cardio-protective effects, it has been shown to contribute to the progression of anaemia through the malabsorption of vitamin B₁₂ which is important for erythropoiesis and RBC maturation (103).

1.5.2 Insulin

Insulin is currently the primary means of treatment for DM however, the administration of insulin as a bolus is associated with hyperinsulinaemia which increases blood volume thus inducing cardiac failure (104). Increased blood volume/ venous return over prolonged periods, induces cardiac hypertrophy (105). Insulin has also been shown to promote fibrosis in cardiac muscle which disturbs cardiac contractility. Furthermore, increased insulin has also been shown to increase sodium retention and hence increases plasma volume and ultimately blood pressure (106). An increase in plasma volume is of concern in patients with congestive heart failure (107). Insulin has been shown to promote RBC agglutination which increases blood

viscosity and mean arterial pressure (MAP) which further aggravates cardiac dysfunction (107).

Studies have also indicated that conventional drugs are still not accessible in some parts of our rural communities (108-110). There is therefore a need for alternative treatment strategies that will lower blood glucose and alleviate hyperglycaemia-induced micro and macrovascular complications (111). Medicinal plants have been shown to possess, with minimal side effects, anti-hyperglycaemic, reno and cardio-protective effects in STZ-induced diabetic animal models and therefore could potentially serve as an effective alternative (112-116). The mechanisms by which these medicinal plants exert their hypoglycaemic, anti-hyperglycaemic and ameliorate hyperglycaemic induced complications such as cardiovascular disorders, are still to be fully understood. In this study, the focus is on *Momordica Balsamina*'s effect on selected markers for cardiovascular and haematological function.

1.5.3 Medicinal plants and their effects on haematological and cardiovascular function

Various medicinal plants such as *Parkia biglobosa*, *Cyclopia maculata* (honeybush) *Momordica charantia*, *Syzygium cordatum* (Hochst.) have been shown to possess anti-hyperglycaemic, reno-protective, cardiovascular effects and haemoprotective activities (117-120). These are amongst several thousands of medicinal plants used to manage diabetes in Africa (121, 122). *Parkia biglobosa* called by different local names such as 'afitin' in Benin City, Nigeria, has been shown to initiate insulin secretion from the pancreatic beta cells, however the seeds are often fermented and consumed for nutritional condiment (123). In addition, *Parkia biglobosa* has been shown to improve red blood cell count and haemoglobin concentrations of streptozotocin-induced diabetic rats (124). *Cyclopia maculata* (honeybush) which is rich in hesperidin has been shown to possess cardio-protective effects via the PPAR-c pathway in an ischemic heart disease model in diabetic rats (125). In addition, pre-treatment with hesperidin was shown to significantly improve mean arterial pressure, reduce left ventricular end-diastolic pressure, and improve both inotropic and lusitropic function of the heart as compared to controls (125). A plant derived flavanol with antioxidant properties, epigallocatechin-gallate (EGCG), has been shown to protect ROS induced cellular DNA, with a much higher potency in reducing lipid peroxidation, as a result, RBC membrane integrity may be improved (108, 126, 127). *Momordica charantia*; Karela (Hindi) and Bitter Gourd (English) is a common folklore remedy for DM as it has demonstrated anti-hyperglycaemic effects in various animal models (128). Extracts from the leaves, fruit pulp, seeds and the whole plant are used to treat diabetes in various animal models (128). Shibib and

colleagues, have previously shown an ethanolic extract of *Momordica charantia* to be anti-hyperglycaemic and stimulate erythropoiesis in STZ-induced diabetic rats (129). Razif, et al. have also shown the cardio-protective effects of *Momordica charantia* through improving the disturbed metabolism of the vasodilator nitric oxide, thereby decreasing blood pressure (130). However, the study focused on the function of the aorta and vascular function and did not highlight the effects of *Momordica charantia* on the cardiac muscle. Furthermore, studies conducted on *Momordica charantia* have not fully explored the effects of this plant on RBC structure and function (131). In our laboratory, we have previously shown anti-hyperglycaemic effects of leaf extracts from *Syzygium cordatum* (Hochst.) [Myrtaceae] (132). This plant has been shown to possess bioactive compounds such as masilinic acid (MA) which possesses reno-protective and cardio-protective effects through a number of mechanisms including decreasing oxidative stress while improving the antioxidant status which may be of benefit in alleviating haematological changes induced by oxidative stress as a result of hyperglycaemia (133). However, the structure and markers for cardiac damage such as hypertrophy which is marked by an increase in cardiotropin-I, was not evaluated to further establish the cardio-protective effects of MA in these animals. Furthermore, cardiac damage is further aggravated by RBC dysfunction which has also not been assessed in studies that use *Syzygium cordatum* (Hochst.) [Myrtaceae] as treatment. The effects of *Syzygium cordatum* on haematological parameters are therefore not fully understood.

Medicinal plants evidently improve cardiac function and erythropoiesis; however, further investigations are warranted on their effects on red blood cell structure and function. Compromising RBC structure increases the risk of RBC haemolysis and clearance, which exacerbates cardiac dysfunction in diabetic patients. Studying RBC function provides a tool to understand the mechanism/s by which these medicinal plants exert their cardio-protective effects.

1.6 Basis of the study

Of interest to our study is *Momordica balsamina*, a plant commonly known as “Intshungu” in isiZulu, South Africa, which is in the same family as *Momordica charantia*. *Momordica balsamina* is an African pumpkin that belongs to the cucurbitaceae family of plants widespread in Namibia, Botswana, Swaziland and all provinces of Southern Africa (134). The seeds, fruits, leaves and stems have been reported to contain nutritional and medicinal compounds that render them anti-hyperglycaemic (135). However, the effects of this plant on hyperglycaemic induced haematological changes and cardiovascular dysfunction have not been shown. In our

laboratory however, we have recently shown that MB possesses anti-hyperglycaemic and renoprotective effects and ameliorates kidney dysfunction in STZ-induced diabetic rats (136). Against this background, we investigated the effects of MB on cardiac dysfunction since renoprotection has been shown to be associated with improved EPO secretion, erythropoiesis and cardiovascular dysfunction in diabetic animal models (136). In addition, since we have previously shown the anti-hyperglycaemic effects of MB in STZ-induced diabetic rats, we sought to also investigate the effects of MB on hyperglycaemic induced red blood cell dysfunction in STZ-induced diabetic rats. This is in an effort to establish the mechanisms by which MB may improve cardiac dysfunction.

1.7 Aim

The aim of the study therefore is to evaluate the effects of *Momordica balsamina* methanolic extract on cardiovascular and haematological function in STZ-induced diabetic rats.

1.8 Objectives

The objectives of the study were to investigate the effects of *Momordica balsamina* methanolic extract on:

- blood glucose concentration of STZ-induced diabetic rats
- oxidative stress in the plasma, heart and kidney of STZ-induced diabetic rats
- mean arterial blood pressure in STZ-induced diabetic rats
- histological changes caused by hyperglycaemia on the cardiac muscle of STZ-induced diabetic rats
- endothelial function (plasma angiotensin-II concentration) in STZ-induced diabetic rats
- cardiac inflammation (heart cardiotrophin-I, heart and plasma CRP concentration) in STZ-induced diabetic rats
- red blood cell structure (annexin-V) and function (RBC profile) in STZ-induced diabetic rats

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Chapter 2

Prologue

Manuscript 1

Hyperglycaemia has been shown to induce cardiovascular complications observed in diabetic patients, which have been shown to progress cardiac failure. Furthermore, current anti-diabetic agents have been associated with the advancement of cardiac disorders. Alternative treatment strategies are therefore needed. We have previously shown the anti-hyperglycaemic and reno-protective effects of *Momordica balsamina*. Since improved renal function has been shown to be associated with improved cardiac function in diabetic animals, the current study, evaluated the effects of *Momordica balsamina* methanolic extract on selected cardiovascular function markers in a streptozotocin-induced diabetic rat model that has not been shown.

The effects of *Momordica balsamina* methanolic extract on cardiovascular function in STZ-induced diabetic rats: effects on selected markers.

The present manuscript was prepared for publication according to the **Chemico-Biological Interaction** journal's guidelines to authors. see (Appendix 2).

Chemico-Biological Interaction

The effects of *Momordica balsamina* methanolic extract on cardiovascular function in STZ-induced diabetic rats: effects on selected markers

A. Ludidi¹, A. Khathi¹, N. H. Sibiya², P. S. Ngubane¹

¹School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa

²Faculty of Pharmacy, P O Box 94, Rhodes University, Grahamstown, 6140, South Africa

Correspondence should be addressed to Asiphaphola Ludidi: asaludidi6@gmail.com

Abstract

Background: Renal dysfunction and cardiovascular disorders are amongst the leading causes of deaths in Diabetes Mellitus (DM). Insulin administration has been associated with the progression of cardiac disorders due to hyperinsulinemia however, medicinal plants have been shown to have antidiabetic effects. We have previously shown the ameliorative effects of *Momordica balsamina* methanolic extract (MB) on renal dysfunction in streptozotocin (STZ)-induced diabetic rats however, the cardiovascular effects have not yet been established. The study therefore, investigated the effects of MB on cardiovascular function in STZ-induced diabetic rats.

Methods: Air-dried *Momordica balsamina* leaves were sequentially extracted with methanol to yield a methanolic extract. Short-term (5 weeks) effects of MB on cardiovascular function were assessed in STZ-induced diabetic rats treated twice daily with MB (250 mg kg⁻¹ p.o.). Insulin (170 µg kg⁻¹ s.c.) and metformin (500 mg kg⁻¹ p.o.). Blood glucose concentration, body weight and blood pressure were monitored weekly for 5 weeks. Thereafter, the animals were sacrificed terminally, collecting blood and hearts for biochemical and histological analysis.

Results: MB significantly decreased blood glucose concentration from week 3-5 by comparison with diabetic untreated animals. Treatment with MB reduced oxidative stress while improving the antioxidant status compared with untreated diabetic animals. MB treatment decreased CRP, CT-I and Ang-II concentrations by comparison with untreated diabetic animals. MB decreased MAP by comparison with untreated diabetic animals. Histopathological hypertrophy was observed in untreated diabetic rats that was ameliorated by the administration of MB.

Conclusions: The administration of MB protects against hyperglycemia-induced cardiovascular changes, which may reduce the risks of cardiovascular complications in diabetes mellitus.

Key words: Cardiovascular disorder, C-reactive protein, oxidative stress, *Momordica balsamina*, cardiomyocyte, mean arterial pressure

1 Introduction

Diabetes mellitus (DM) is the leading non-communicable disease, which is associated with a significantly high morbidity and mortality rate and estimated to affect approximately 693 million people by 2045 (1-3). Cardiovascular complications have been shown to contribute significantly to the total number of deaths caused by DM (4, 5). Metabolic syndromes, including DM, doubles the risk of developing cardiovascular complications, therefore diabetic patients have a two to five-fold greater mortality rate due to cardiovascular disease (6). Chronic hyperglycemia contributes to the progression of cardiovascular dysfunction via increased oxidative stress and endothelial dysfunction (7, 8). Oxidative stress due to the overproduction of superoxide induces an alteration in the morphology and function of the cardiomyocyte as shown by cardiac hypertrophy which is marked by an increase in secretion of cardiotrophin-I (9). Furthermore, chronic inflammation is promoted and thus the increase in proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), C-reactive protein (CRP) and cardiotrophin-I (CT-I) which indicate cardiac damage (10, 11). In addition, hyperglycemia induced reactive oxygen species (ROS) production results in the reduction in the bioavailability of nitric oxide (NO) (12). NO decline is the result of the reduced expression of endothelial cell NO synthase (eNOS), inefficient eNOS activation, insufficient substrate or cofactors for eNOS and accelerated degradation of NO₂ by ROS (13, 14). Furthermore, endothelial dysfunction is accompanied by increased vasoconstrictors including angiotensin-II (15, 16). The pathology of endothelial dysfunction therefore, results in increased arterial stiffness and increased arterial pressure (17). In addition, dyslipidemia has been associated with an increase in atherosclerotic plaque formation, which narrows the vascular lumen, therefore raising blood pressure (18). The long-term increase in blood pressure is associated with cardiac myopathy, cardiac hypertrophy and eventually cardiac failure (19, 20).

There are however, conventional treatments to manage diabetes and associated complications with insulin being the mainstay treatment (21). The administration of insulin as a bolus however, has been shown to be associated with hyperinsulinemia, increased sodium retention

and therefore increased blood pressure (22). Moreover, insulin and oral hypoglycemic agents have been shown to deteriorate liver function and increase plasma volume which is of concern in patients with congestive heart failure (22, 23). These challenges have led to the investigation of alternative therapeutic interventions with less undesirable effects.

Traditionally, various plants have been shown to alleviate diabetes-associated complications. Of interest to our study is *Momordica balsamina*, commonly known as “Intshungu”, a plant widespread in Namibia, Botswana, Swaziland and all provinces of South Africa (24). The plant has been reported to possess hypoglycemic effects and improves renal function in streptozotocin (STZ)-induced diabetic rats (25, 26). Despite these developments, the cardiovascular effects of this plant remain elusive hence the interest in investigating the effects of MB on cardiovascular function in STZ-induced diabetic rat model. We envisaged that monitoring blood glucose concentration, mean arterial pressure (MAP), oxidative stress and inflammatory status may provide a holistic insight on the effects of cardiovascular function as a result of MB administration (25).

2 Materials and methods

2.1 Drugs and chemical reagents:

Streptozotocin (Sigma Aldrich Chemical Company, Missouri, St Louis, USA); glucose ($C_6H_{12}O_6$), calcium chloride ($CaCl_2$), citric acid, monosodium citrate (Merck chemicals (Pty) Ltd Wadeville, Johannesburg, South Africa); metformin, dimethyl sulphoxide (Sigma-Aldrich, St Louis, Missouri, United States of America); insulin (NovoRapid pen refill, Novordisk Pty Ltd, Sandton, South Africa).

2.2 Plant extraction

The leaves of *Momordica balsamina* were identified and authenticated by Professor H Baijnath, the former chief taxonomist/curator of the University of KwaZulu-Natal department of botany. The extraction of *Momordica balsamina* leaves were performed in the School of laboratory Medicine and Medical Sciences, at the University of KwaZulu-Natal, Westville Campus, using a previously validated protocol formerly reported by our laboratory (27). Briefly, the air-dried *Momordica balsamina* leaves (1.15kg) were sequentially extracted by cold percolation with methanol (95%, 6.9L) for 24 h. The methanolic extract was recovered from the mixture and methanol was added to the pulp for further extraction. To maximize the extraction process to increase the yield (609g), the process was repeated three times. The three extracts were

combined to yield a concentrated methanolic extract at a reduced pressure (22-26mmHg) and temperature of 45-60 °C.

2.3 Animals

Male Sprague-Dawley (SD) rats (250 -300 g, n=30) bred and housed in the Biomedical Research Unit (BRU) of University of KwaZulu-Natal were used in the present study. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO₂ content of <5000 p.p.m., relative humidity of 55±5% and illumination (12 h light/dark cycles) and the noise levels of less than 65 decibels and ad libitum access to food and water. Procedures involving animals and their care were conducted in conformity with the institutional guidelines of the University of KwaZulu-Natal (AREC/023/017M). Individual rats were housed in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) and were acclimatized in metabolic cages for 5 days before commencement of the study.

2.4 Induction of diabetes mellitus

The induction of type 1 diabetes mellitus by a single intraperitoneal injection of 60 mg kg⁻¹ streptozotocin, which was freshly prepared in 0.1 M citrate, buffer (pH 4.5). Control group received the vehicle, citrate buffer through the same route. Animals that presented glucosuria after 24 h, when tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days after the induction of diabetes, animals that had a blood glucose concentration greater than 20 mmol L⁻¹ were considered as having stable diabetes.

2.5 Experimental design

To study the short-term effects of *Momordica balsamina* methanolic extract (MB) on the cardiovascular system over 5 weeks of treatment, experimental rats were divided into separate groups of non-diabetic (group 1) and STZ-induced diabetic (group 2-5) male Sprague-Dawley rats, with six rats per group. Group 1 was treated with the drug vehicle DMSO to serve as a negative control. Group 2 was treated with *Momordica balsamina* methanolic extract (250 mg kg⁻¹, p.o.). Positive controls in Group 3 and 4 were treated with insulin (175 µg kg⁻¹, s.c.) and metformin (500 mg kg⁻¹, p.o.), respectively. Non-diabetic animals in Group 5 served as absolute control. MB was administered twice daily at 09h00 and 15h00. Food and water intake, body weight, mean arterial blood pressure (MAP) and blood glucose concentration were monitored over the 5-week period. MAP was measured using the non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) as previously described (28), while blood glucose

concentrations were measured via the tail pricking method. All parameters were assessed every 3rd day at 09:00 am for the duration of experimental period.

2.6 Tissue sample harvesting

At the end of the 5-week experimental period, all animals were sacrificed by exposing to halothane via a gas anesthetic chamber (100 mg kg^{-1}) for 3 minutes (Biomedical Resource Unit, UKZN, Durban, South Africa). Thereafter, blood was collected by cardiac puncture (RBCP) into individual pre-cooled heparinized container and centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C , 503 g for 15 minutes and separated plasma were stored at -80°C in a Bio Ultra freezer (Snijers Scientific, Holland) for hormonal analysis. The heart tissues were removed and weighed before snap freezing in liquid nitrogen and then stored in a BioUltra freezer (Snijers scientific, Tilburg, Netherlands) -80°C until use.

2.7 Biochemical analysis

2.7.1 C-reactive protein and angiotensin-II measurement

Heart and plasma C-reactive protein (CRP) and plasma angiotensin-II (Ang-II) were analysed using separate specific ELISA kits (Elabscience and Biotechnology, WuHan) that use the Sandwich-ELISA method. Kits included micro ELISA plates, which were coated with antibody specific to CRP and angiotensin-II, respectively. Standards and samples were pipetted into the appropriate wells of the micro ELISA plate and incubated for 90 minutes. The plate relevant biotinylated detection antibody ($100 \mu\text{L}$) was then added and incubated for 60 minutes. Avidin-Horseradish Peroxidase (HRP) conjugate ($100 \mu\text{L}$) was added to each micro-plate well and incubated for 30 minutes. Unbound components were washed out. Substrate solution ($100 \mu\text{L}$) was added to each micro-plate well. After incubating for a further 15 minutes, the stop solution ($50 \mu\text{L}$) was added. The optical density was measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm . The concentration of the samples was extrapolated from the respective standard curves.

2.7.2 Cardiotrophin-I measurement

Heart cardiotrophin-I (CT-I) was analysed using a separate specific CLIA kit (Elabscience and Biotechnology, WuHan) that uses the Sandwich- CLIA method. The kit included a micro CLIA plate, which was coated with an antibody specific to CT-I. Standards and samples were pipetted into the appropriate wells of the micro CLIA plate and incubated for 90 minutes. The plate relevant biotinylated detection antibody ($100 \mu\text{L}$) was then added and incubated for 60 minutes.

Avidin-Horseradish Peroxidase (HRP) conjugate (100 μ L) was added to each micro-plate well and incubated for 30 minutes. Unbound substances were washed out. Substrate mixture solution (100 μ L) was added to each micro-plate well. After incubating for not more than 5 minutes protecting from the light the relative light unit (RLU) was measured using the GloMax® 96 Microplate Luminometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany). The concentration of the samples were extrapolated from the respective standard curves.

2.7.3 Oxidative stress

To establish the effects of treatment on oxidative stress in the plasma and heart muscle, levels of malondialdehyde (MDA), a commonly known marker of oxidative stress, were measured in untreated and treated experimental animals using a biochemical assay as shown below. The antioxidant defense enzymes: superoxide dismutase (SOD) and glutathione peroxidase (GPx) were also measured in untreated and treated experimental animals (29).

2.7.3.1 Malondialdehyde measurement

Heart tissues (50 mg) were homogenized in 500 μ L of 0.2% phosphoric acid, respectively. The homogenate was centrifuged at 400 x g for 10 min. Thereafter, 400 μ L of the homogenate and 100 μ L plasma were supplemented with 400 μ L 2% phosphoric acid and then separated into three glass tubes, each receiving equal volumes of the solution. Subsequently, 200 μ L of 7% phosphoric acid was added into all glass tubes followed by the addition of 400 μ L of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into two glass tube (sample tests) and 400 μ L of 3 mM hydrochloric acid (HCl) into the third glass tube (blank). To ensure an acidic pH of 1.5, 200 μ L of 1 M, HCl was added to sample and blank test tubes. All solutions were heated at 100 °C for 15 min, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solutions; the sample tests were vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases are distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 x g for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek μ Quant spectrophotometer (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths were used to calculate the concentration of MDA using Beer's Law.

Concentration of MDA (mM)

$$= \frac{\text{Average Absorbance}}{\text{Absorption coefficient (156 mmol}^{-1}\text{)}}$$

2.7.3.2 Superoxide dismutase and glutathione peroxidase measurement

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) in both the plasma and heart tissues were analysed using separate specific ELISA kits (Elabsience and Biotechnology, WuHan) that use the Sandwich-ELISA method. Kits included micro ELISA plates that were coated with antibody specific to SOD and GPx, respectively. Standards and samples were pipetted into the relevant wells of the micro ELISA plate and incubated for 90 minutes. The plate relevant biotinylated detection antibody (100 μ L) was then added and incubated for 60 minutes. Avidin-Horseradish Peroxidase (HRP) conjugate (100 μ L) was added to each micro-plate well and incubated for 30 minutes. Unbound components were washed out. Substrate solution (100 μ L) was added to each micro-plate well. After incubating for a further 15 minutes, the stop solution (50 μ L) was added. The optical density was measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

2.8 Histology of the heart

Heart tissues were fixed in formalin overnight, paraffin embedded and processed for sectioning. 0,5 μ M sections were made and stained with hematoxylin and eosin (H&E) to analyze the cardiomyocyte size and the arrangement of cardiomyocyte myofibres using Leica microsystems for analysis.

For the assessment of histological cardiovascular changes after treatment, the heart tissues of non-diabetic, untreated STZ-induced diabetic rats and treated STZ-induced diabetic rats were dissected out for fixation with 10% formaldehyde solution at the end of the 5-week experimental period. Excess formalin was removed from the heart tissues by rinsing with water. The heart tissues were then dehydrated in 70%, 80%, 90%, as well as 100% ethanol for 2, 2, 3 and 2 minutes respectively. To remove the alcohol, the tissues were then submerged in xylene rendering the tissue translucent. In preparation for sectioning, the tissues were embedded in wax blocks which were sectioned at a thickness of 3-5 μ m using a rotary microtome (Robert-Bosch-Straße, Walldorf, Baden-Württemberg, Germany). Sectioned tissues were then mounted onto clean slides and dried by placing them on a Ransom warming plate. 250 ml of xylene was then used to de-paraffinize the sections twice for 3 min each time. The rehydration of tissue sections in decreasing concentrations of 100%, 90%, 70% and 50% ethanol for 2 minutes each time in preparation for staining. The tissue slides were then flooded with hematoxylin, using a Pasteur pipette and allowed to stand for 5 minutes and the slide was tilted to remove the

hematoxylin stain. Using tap water, the slides were then gently flood with a Pasteur pipette three times or until the sections stained purple turned blue. Pasteur pipette was used over the tissue sections to drop the Eosin stain and left to stand for 3-5minutes. Tap water was used to rinse off the residual eosin on the tissue sections/slide. Tissue slides were then dehydrated by submerging the slides in increasing 90% and 100% ethanol. Sections were then cleared with then cleared with xylene in preparation for permanent mounting by dropping distyrene plasticizer xylene (DPX) mounting glue directly over the tissue section using an applicator, then covering with a cover slip. The sections were allowed to dry overnight. The processed tissue sections were then visualized and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany).

2.9 Statistical analysis

All data are expressed as means \pm standard error of means (SEM). GraphPad Prism Instat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used to perform statistical analysis. Blood glucose and MAP were analysed using analysis of variance (ANOVA) followed by Bonferroni post hoc test. ANOVA was used to analyze terminal parameters to compare the differences between control and experimental groups. Values of $p < 0.05$ were regarded statistical significant between the compared groups.

Results

3.1 Blood glucose concentration

Figure 1 shows blood glucose concentration of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹) metformin (MTF) and insulin (INS) over an experimental period of 5 weeks. The untreated STZ-induced diabetic animals sustained a significantly high blood glucose concentration over the period of 5 weeks by comparison with non-diabetic control ^α (DC vs NC, p<0.05, Figure 1). Treatment with MB, insulin and metformin showed a significant decrease in blood glucose from week 3,4 and 5 by comparison with the diabetic control animals * (MB vs DC, p<0.05, Figure 1). Blood glucose concentrations of animals treated with insulin significantly decreased at week 5 by comparison with animals treated with MB ^λ (INS vs MB, p<0.05, Figure 1).

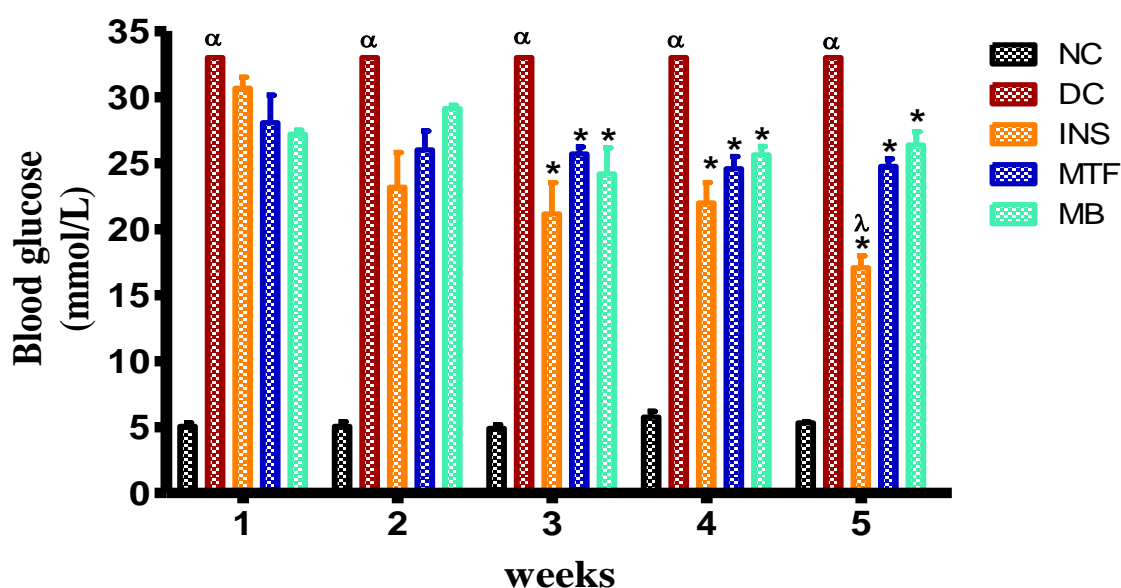


Figure 1: Comparison of blood glucose concentration in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) over the period of 5 weeks. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ^α p<0.05 by comparison with non-diabetic control animals. * p<0.05 by comparison with diabetic control animals. ^λ p<0.05 by comparison with MB treated animals.

3.2 Mean arterial blood pressure

Figure 2 shows the mean arterial pressure (MAP) of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) over an experimental period of 5 weeks, measured 24 hours after treatment. The diabetic control group showed a significantly high MAP throughout the 5-week experimental period by comparison with non-diabetic control ^α (DC vs NC, p<0.05, Figure 2). However, treatment with MB decreased MAP similarly to metformin and insulin from week 3 to 5 by comparison with the untreated STZ-induced diabetic rats ^{*}(MB vs DC, p<0.05, Figure 2), while insulin significantly increased MAP from week 3 to week 5 by comparison with metformin and MB ^λ (INS vs MTF and MB, p<0.05, Figure 2).

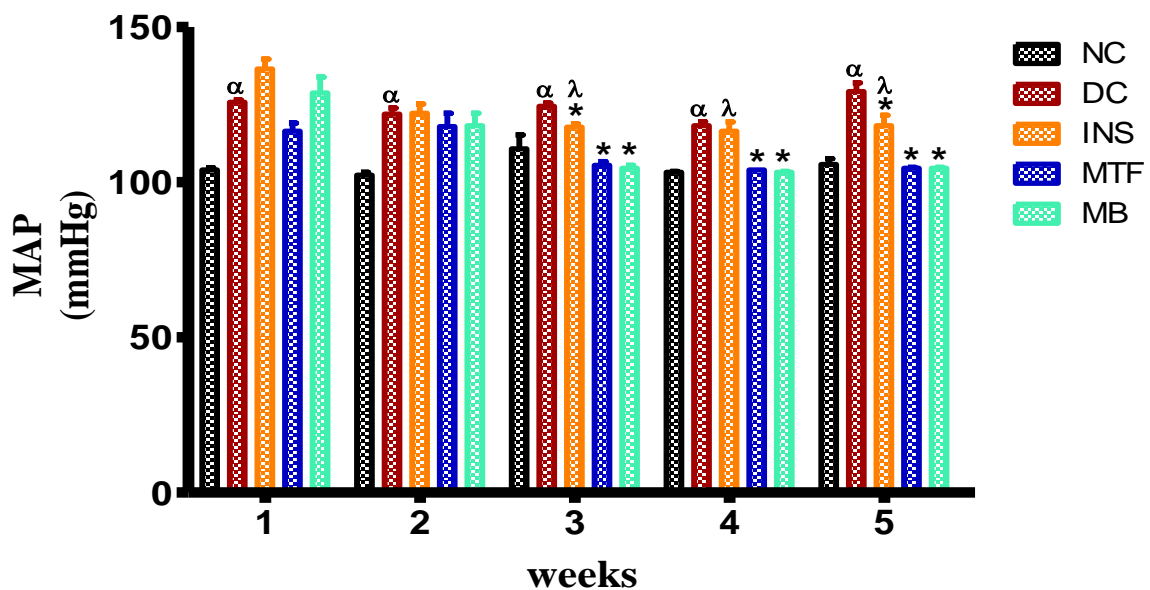


Figure 2: Comparison of MAP in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) over the period of 5 weeks. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ^α p<0.05 by comparison with non-diabetic control animals. ^{*} p<0.05 by comparison with diabetic control animals. ^λ p<0.05 by comparison with MB treated animals.

3.3 Heart to body weight ratio

Table 1 compares the heart to body weight ratios of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with MB (250 mg kg⁻¹), metformin (MTF) and insulin (INS) over a 5-week treatment period. Diabetic control rats exhibited an increase in heart to body weight ratio compared with non-diabetic control rats at the end of the 5-week experiment ^a (DC vs NC, p<0.05). MB administration similarly to standard drugs, decreased heart to body weight ratio compared with STZ-induced diabetic control rats at the end of the 5-week experimental period ^{*}(MB vs DC, p<0.05).

Table 1: Comparisons of the (H/B) ratios of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are expressed as mean \pm SEM (n=6 in each group).

Experimental groups	Final body weight (g)	Hearts weight (g)	H/B ratio (%)
NC	307.00 \pm 7.66	1.22 \pm 0.08	0.39 \pm 0.02
DC	169.17 \pm 4.75 ^a	1.22 \pm 0.06	0.72 \pm 0.04 ^a
INS	281.92 \pm 2.39 [*]	1.04 \pm 0.05	0.37 \pm 0.02 [*]
MTF	256.67 \pm 10.17 [*]	1.12 \pm 0.11	0.43 \pm 0.04 [*]
MB	243.83 \pm 5.39 [*]	1.08 \pm 0.03	0.44 \pm 0.02 [*]

^a p<0.05 by comparison with non-diabetic control animals. ^{*} p<0.05 by comparison with diabetic control.

3.4 MDA, SOD and GPx concentrations

Table 2 Depicts the comparisons of MDA, SOD and GPx concentrations in both the heart and plasma of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) after 5 weeks of treatment. Untreated STZ-diabetic heart and plasma tissues presented with increased concentrations of MDA by comparison to untreated non-diabetic rats ^a (DC vs NC, p<0.05). In addition, GPx concentrations in the plasma of untreated STZ-induced diabetic rats significantly decreased by comparison with non-diabetic animals ^a (DC vs NC, p<0.05). The plasma MDA concentrations of MB treated animals similarly to standard drugs decreased by comparison to

untreated STZ-induced diabetic rats *(MB vs DC, $p<0.05$). Furthermore, GPx concentrations in the plasma of MB treated animals similarly to standard drugs increased by comparison to untreated non-diabetic rats *(MB vs DC, $p<0.05$). However, heart GPx concentrations of MB treated animals significantly decreased by comparison with untreated STZ-diabetic rats *(MB vs DC, $p<0.05$). Furthermore, heart and plasma SOD concentrations of MB treated animals, significantly decreased by comparison with untreated diabetic animals *(MB vs DC, $p<0.05$).

Table 2: Comparison of MDA, SOD and GPx concentrations of both plasma and heart tissues of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are presented as means \pm SEM (n=6 in each group).

	Plasma		Heart
MDA ($\mu\text{mol/g protein}$)	NC	0.68 ± 0.12	1.21 ± 0.04
	DC	$9.37 \pm 1.19 \alpha$	1.38 ± 0.09
	INS	$0.54 \pm 0.03 *$	1.21 ± 0.02
	MTF	$0.35 \pm 0.06 *$	1.26 ± 0.12
	MB	$2.48 \pm 0.05 *$	$1.15 \pm 0.10 *$
GPx Concentration (ng/mL)	NC	1.44 ± 0.03	1.14 ± 0.01
	DC	$0.21 \pm 0.04 \alpha$	1.37 ± 0.09
	INS	$0.83 \pm 0.01 *$	1.39 ± 0.03
	MTF	$1.67 \pm 0.01 *$	1.23 ± 0.06
	MB	$1.06 \pm 0.04 *$	0.79 ± 0.05
SOD Concentration (ng/mL)	NC	35.39 ± 0.63	65.92 ± 0.98
	DC	33.55 ± 0.27	63.21 ± 1.92
	INS	31.68 ± 2.76	66.69 ± 1.11
	MTF	29.85 ± 2.44	65.17 ± 1.17
	MB	23.63 ± 0.87	33.55 ± 0.27

α $p<0.05$ by comparison with non-diabetic control animals. * $p<0.05$ by comparison with diabetic control.

3.5 CRP and CT-I concentrations

Table 3 shows the comparisons heart and plasma CRP and heart CT-I concentrations of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) after 5 weeks of treatment. Heart and plasma CRP with heart CT-I concentrations of untreated STZ-diabetic animals significantly increased by comparison with non-diabetic rats ^α (DC vs NC, p<0.05). Furthermore, insulin sustained a high heart and plasma CRP and heart CT-I concentration similarly to untreated STZ-diabetic animals *(INS vs DC, p<0.05). However, MB similarly to metformin, significantly decreased heart and plasma CRP and heart CT-I concentrations of untreated STZ-diabetic animals by comparison to untreated STZ-induced diabetic rats (MB vs DC, p<0.05).

Table 3: Comparison of heart and plasma CRP and heart CT-I concentrations in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are presented as means ± SEM (n=6 in each group).

Experimental groups	CRP (ng/mL)		CT-I (pg/mL)
	Plasma	Heart	Heart
ND	0.38 ± 0.01	5.61 ± 0.65	9.13 ± 0.85
DC	0.43 ± 0.01 ^α	14.31 ± 0.38 ^α	35.01 ± 0.26 ^α
INS	0.64 ± 0.03 *	15.66 ± 0.18 *	37.60 ± 1.62 *
MTF	0.42 ± 0.03 *	6.57 ± 0.21 *	11.14 ± 1.62 *
MB	0.32 ± 0.01 *	7.11 ± 0.72 *	9.73 ± 0.08 *

^α p<0.05 by comparison with non-diabetic control animals.* p<0.05 by comparison with diabetic control.

3.6 Ang-II concentrations

Table 4 displays the comparisons of plasma angiotensin-II (Ang-II) of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) after 5 weeks of treatment. Plasma angiotensin-II concentrations of untreated STZ-diabetic rats significantly increased by comparison with

non-diabetic animals ^α (DC vs NC, p<0.05). However, MB treated animals similarly to insulin and metformin treated animals exhibited a significantly decreased plasma Ang-II concentration*(MB vs DC, p<0.05).

Table 4: Comparison of plasma Ang-II concentrations of non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are presented as means ±SEM (n=6 in each group).

Experimental groups	Plasma Ang-II (ng/mL)
ND	3.98 ± 0.04
DC	4.34 ± 0.05 ^α
INS	4.01 ± 0.04 *
MTF	3.81 ± 0.06 *
MB	3.77 ± 0.09 *

^α p<0.05 by comparison with non-diabetic control animals. * p<0.05 by comparison with diabetic control.

3.7 Histology of the heart

Figure 3 are the H & E photomicrographs illustrating the effects of treatment on the morphology of the heart tissue in (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹) metformin (MTF) and insulin (INS) after an experimental period of 5weeks. Photomicrograph of the non-diabetic untreated group showed single, oval and centrally located nuclei (N) of the cardiomyocytes. The cardiac myofibres (MF) in the non-diabetic control group were arrangement in a regular pattern (A). Nuclei of the cardiomyocytes in the diabetic control group (B) showed deformation in sizes and shapes. Moreover, the cardiac myofibres in the diabetic untreated group (B) showed disarrayed patterns compared to the non-diabetic untreated group (A). Nuclei of the cardiomyocytes in the insulin treated group (C) showed deformation in size and shape. Additionally, the insulin treated group (C) showed disarrayed cardiac myofibres similarly to the diabetic control group (B). The nuclei of the cardiomyocytes in the metformin treated group (D) showed an improvement in size and shape by comparison to the diabetic control group (B). In particular, the nuclei were single, oval and centrally located similarly to the cardiomyocytes in the non-diabetic control group (A). The

arrangement of the cardiac myofibres in the metformin treated group improved to near normal compared to the non-diabetic control group (A). Nuclei of the cardiomyocytes in the MB treated group (E) showed an improvement in size and shape compared to the diabetic control group (B). Moreover, the nuclei were single, oval and centrally located similarly to the cardiomyocytes in the non-diabetic control group. Arrangement of cardiac myofibres in MB treated group (E) improved to a near normal pattern compared to the non-diabetic control group (A). Magnification 20X-100 μ m.

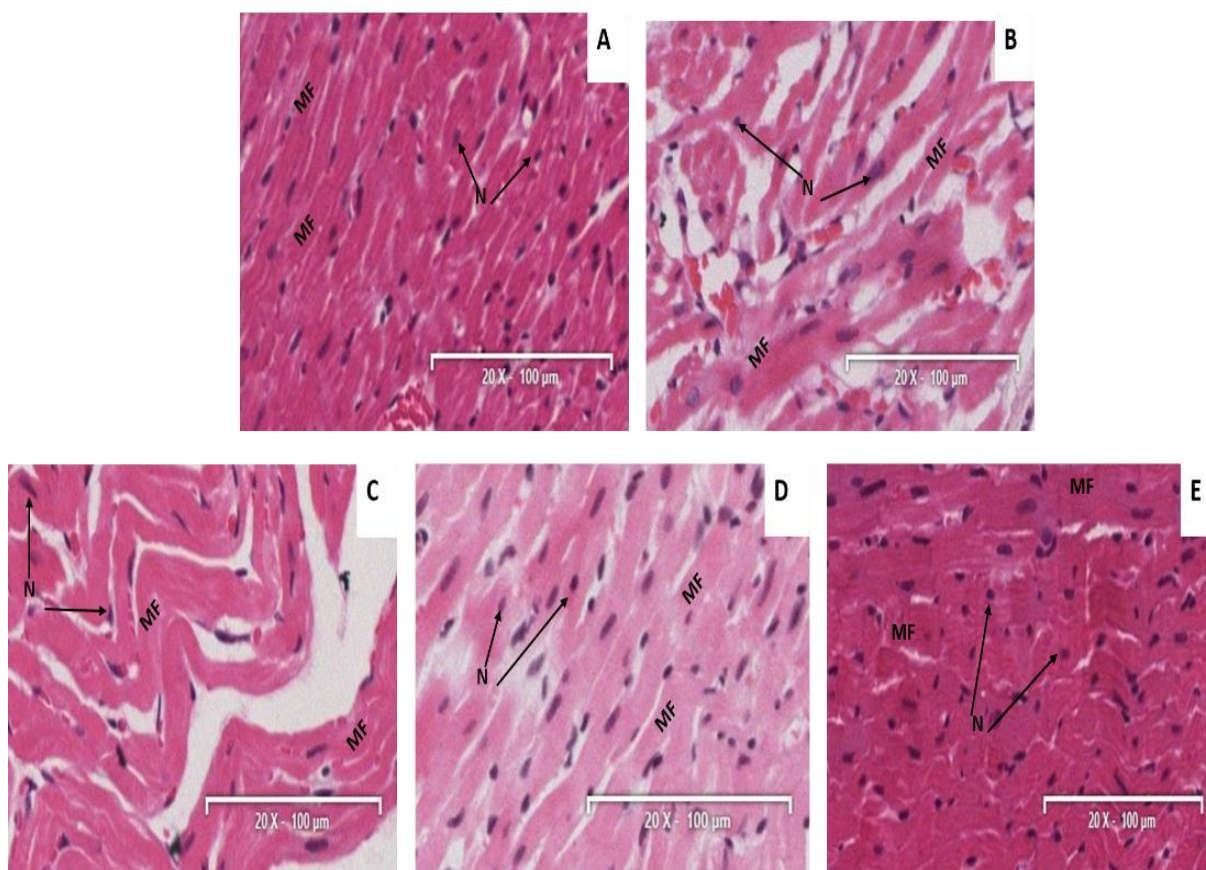


Figure 3: H & E photomicrographs illustrating cardiac tissue morphology in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) after 5 weeks of treatment. Magnification 20X-100 μ m.

4 Discussion

The present study investigated whether treatment with a methanolic extract of *Momordica balsamina* ameliorates cardiovascular dysfunction commonly observed in diabetic patients and modelled in streptozotocin (STZ)-induced diabetic rats. We have previously shown that *Momordica balsamina* possess antihyperglycemic properties and improves kidney function.

Here in this study, *Momordica balsamina* improves oxidative stress, cardiovascular structure and function in STZ-induced diabetic rats.

Chronic hyperglycemia which is a characteristic in DM alters cardiovascular function and indeed STZ administration resulted in sustained hyperglycemia throughout the study due to the obliteration of pancreatic β -cells as shown in previous studies (30, 31). Administration of MB however, decreased glycemia in STZ-diabetic rats. Medicinal plants such as *Momordica charantia* Linn which is in the same family as *Momordica balsamina* have been shown to possess antihyperglycemic phytochemicals such as flavonoids, cucurbitane triterpenoids and phytosterols (32). Flavonoids have been shown to inhibit the activity of α -amylase and α -glucosidase *in vitro*, delaying postprandial glucose absorption (33). In addition, flavonoids have been shown to inhibit glucose transport through the inhibition of intestinal sodium-glucose co-transporters (34, 35). Interestingly, *Momordica balsamina* has been reported in our laboratory to decrease food intake of STZ-induced diabetic rats (26). *Momordica balsamina* therefore may maintain glycemic control through its antihyperglycemic effects similarly to other medicinal plants such as *Tripterygium wilfordii* possibly via delaying postprandial glucose absorption, thus activating the release of appetite modulation hormones such as cholecystokinin (CCK) and peptide YY(PYY) to inhibit feeding thus decreasing ghrelin secretion (36, 37).

An improvement in glycemic control has been shown to attenuate oxidative stress-induced debilitating cardiovascular complications in diabetic patients and experimental animals (38). Evidently, STZ-diabetic rats in the present study maintained increased oxidative stress, which was associated with the dysregulation of the antioxidant defense system, inflammation and endothelial function. The antioxidant defense system is adapted to scavenge ROS to minimize alteration in cell structure and function (39-41). It has been documented that hyperglycemia-induced glycation of proteins including superoxide dismutase and glutathione peroxide alters their structure reducing their efficiency (42). In agreement with previous studies, the suppression of GPx which detoxifies a ROS product- hydrogen peroxide- to water, was observed in our STZ-induced diabetic rats (43). This may be correlated with a rise in hyperglycemia-induced enzyme glycation, increasing oxidative stress (44, 45). Interestingly, MB administration improved the antioxidant status possibly through increasing GPx that scavenges ROS although SOD was not improved possibly due to glycation of this enzyme augmenting loss of enzyme activity. In addition, an increase in oxidative stress has been shown to dysregulate inflammation evidenced in the increase in CRP, which is a marker for increased

cardiovascular dysfunction in the STZ-induced diabetic animal. Moreover, *Momordica balsamina* attenuated the rise in CRP associated with the morphological and functional alteration of cardiomyocytes.

The progression of cardiac dysfunction was further evidenced in the present STZ-induced rats which may be attributed to the increase in the polyol pathway, resulting in the accumulation of sorbitol which induces osmotic stress in cardiomyocytes (46, 47). Osmotic stress activates the extracellular signal-regulated protein kinase and protein kinase C which are associated with cell growth, hence hypertrophy which may account for alteration in cardiomyocyte structure (48). Interestingly, MB attenuated the metabolic injury specifically to the cardiac muscle as observed by the restoration in the histological architecture of cardiomyocyte that may be associated with the decrease in oxidative stress, marked by MDA (49-51). This is in harmony with previous findings showing that an improvement in oxidative stress improves cardiac function (52, 53).

Hyperglycemia has been associated with an increase in MAP, which may be attributed to kidney dysfunction due to the increase in renal sodium reabsorption increasing blood volume in synergy with increased vasoconstrictors including angiotensin-II that may have stimulated hyperglycemia-induced collagen synthesis in the vasculature, consequently narrowing and stiffening the blood vessels (54). Resistance of blood flow therefore may have ensued hence an alleviated MAP as observed in the present STZ-induced diabetic animals (55). The increase in MAP forces the cardiac muscle to stretch, activating the release of CT-I by cardiac fibroblasts via the stimulation of the JAK/STAT pathway (56). JAK/STAT pathway activation through CT-I signals the expression and transcription of genes involved in immunity, proliferation and differentiation in the cardiomyocytes nucleus as observed in our STZ-induced diabetic animals (57, 58). An elevation in CT-I of STZ-induced diabetic animals therefore exacerbates cardiac dysfunction. Additionally, the increase in collagen synthesis in the cardiac muscle dysregulates the synchronous contractility of the cardiomyocytes hence cardiac myopathy (54, 59). In the present study, elevated MAP of STZ-induced rats coincides with the increase in heart to body weight ratio, suggesting cardiac hypertrophy. However, MB attenuated the rise in MAP possibly through decreasing Ang-II decreasing cardiac overload and mechanical stretch of the cardiac muscle, hence a decrease in CT-I. Additionally, the ability of MB to improve heart to body weight ratio similarly to metformin, may have improved cardiac hypertrophy. In addition, we have previously reported that MB improves kidney function and a decrease in MAP may be attributed to the restoration of electrolyte handling (26). Our results are in line with literature as medicinal plants such as *Prosopis gradulosa* have been shown to possess hypotensive, anti-

ischemic effects in addition to an increased cardiomyocyte insulin sensitivity. Furthermore, plants such as *Hibiscus subdariffa* have been shown to possess lipid lowering activity and reverse cardiac hypertrophy (60). As predicted, treatment with insulin in this study also showed an increase in MAP as seen in STZ-induced diabetic rats, as insulin is mitogenic and promotes proliferation of cells, which was associated with the observed cardiac hypertrophy although the heart to body weight ratio was decreased. In addition, supraphysiological administration of insulin to diabetic patients has been shown to increase sodium reabsorption associated with hyperinsulinemia and edema consequently contributing to the alleviated MAP which is associated with cardiac hypertrophy, progressing cardiovascular complications (61).

The present study is in agreement with the medicinal use of MB and provides evidence that it may delay the onset of the progression of cardiovascular complications in diabetic patients. In addition, since *Momordica balsamina* is widely spread in developing countries such as South Africa, this study may provide evidence that MB may indeed be a beneficial and easily accessible management strategy for diabetic patients.

5 Conclusion

Momordica balsamina administration attenuates hyperglycemia accompanied by an improvement in the antioxidant status. Furthermore, the administration of the extract ameliorated an increase in MAP, cardiac hypertrophy and inflammatory markers in STZ-induced diabetic rats. The observations further highlight the cardiovascular risk associated with insulin administration. In conclusion, the observations from this study support the use of medicinal plants as an alternative for the management of diabetes related complications.

6 Conflicts of Interest

The authors declare no conflict of interest, and the work has not been published elsewhere.

7 Acknowledgments

The authors appreciate assistance and support from the Biomedical Research Unit, University of KwaZulu-Natal. The current study was partly funded by NRF South Africa and the University of KwaZulu-Natal, Research Division.

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Chapter 3

Prologue

Manuscript 2

The haematological changes associated with hyperglycaemia have been shown to aggravate cardiovascular complications in diabetic patients. In addition, conventional treatment has been shown to alter haematological function, which eventually, advances cardiovascular dysfunction in diabetic patients. Hence the need of alternative treatment strategies. We have previously shown the anti-hyperglycaemic and reno-protective effects of *Momordica balsamina* in STZ-induced diabetic rats which may benefit patients with hyperglycaemia-induced haematological changes associated with cardiovascular dysfunction. The effects of MB on haematological function in STZ-induced diabetic rats however, have not been established. The present study therefore investigated the effects of *Momordica balsamina* methanolic extract on haematological function in STZ-induced diabetic rats with specific interests on red blood cell function. This is in an effort to establish the mechanisms by which MB may alleviate cardiovascular dysfunction in diabetic animal models.

The effects of *Momordica balsamina* methanolic extract on haematological function in STZ-induced diabetic rats: Effects on selected markers.

The present manuscript was prepared for publication according to the **Biomedicine and pharmacotherapy** journal's guidelines to authors. see (Appendix 3).

Biomedicine and pharmacotherapy

The Effects of *Momordica balsamina* Methanolic Extract on Haematological Function in Streptozotocin-induced Diabetic Rats: Effects on Selected Markers.

A. Ludidi¹, M. C Baloyi¹, A. Khathi¹, N. H. Sibiyi², P. S Ngubane¹

¹Department of Human Physiology, University of KwaZulu-Natal, 4000, South Africa

²Department of Pharmacy, Rhodes University, 6140, South Africa

Correspondence should be addressed to Asiphaphola Ludidi; asaludidi6@gmail.com

Abstract

Background: Chronic hyperglycaemia-induced haemaneic changes increases the risk of cardiovascular complications in diabetic patients. The administration of Insulin injection as a bolus is accompanied with increased blood viscosity, which is not recommended for patients with congestive heart failure. *Momordica balsamina* (MB) methanolic extract has previously been shown to possess anti-hyperglycaemic and renal dysfunction ameliorative effects; however, the haematological effects of MB have not been shown. The current study therefore, investigated the short-term effects MB on selected haematological parameters in streptozotocin (STZ)-induced diabetic rats. **Methods:** Briefly, the air-dried *Momordica balsamina* leaves were sequentially extracted with methanol to yield a methanolic extract. STZ-induced diabetic rats were divided into untreated and treated groups with insulin (170 $\mu\text{g kg}^{-1}$ s.c.) and metformin (500 mg kg^{-1} p.o.) MB (250 mg kg^{-1} p.o.). MB was administered twice daily for the 5-week experimental period. Blood glucose concentration was monitored weekly. Animals were sacrificed terminally. Blood and kidneys were collected for haematological and biochemical analysis respectively. **Results:** Treatment with MB significantly decreased blood glucose concentration and improved erythropoietin secretion, thus significantly increasing red blood cell production in treated diabetic animals by comparison to untreated animals. MB also significantly improved haemoglobin concentrations and moderately increased erythrocyte indices specifically, mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) to no significance by comparison to untreated diabetic animals. MB treatment decreased the oxidative stress evoked by the induction of diabetes while improving the antioxidant status of treated animals by comparison to untreated animals respectively. **Conclusions:** Administration of *Momordica balsamina* methanolic extract protects against the injurious haematological changes induced by hyperglycaemia, which may reduce the risks of cardiovascular complications. **Key words:** Erythropoiesis, oxidative stress, *Momordica balsamina*, streptozotocin, hyperglycaemia

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia (1). Sustained hyperglycaemia has been shown to progress the pathology of cardiac damage through haemaneic changes which include haemolysis of erythrocytes, consequent decrease in red blood cell count and haemoglobin concentrations which is associated with the development of diabetic anaemia and increases the risk of cardiovascular complications if left untreated (2). Hyperglycaemia has been shown to increase the probability of the non-enzymatic glycosylation of red blood cell membrane proteins resulting in the non-specific aggregation of protein molecules and alters the protein-protein and protein-lipid interaction leading to the

modification and disrupts the integrity/and symmetry of the erythrocyte membrane (3, 4). A loss of membrane symmetry increases erythrocyte aggregation, decreases mobility of the red blood cells and increases blood viscosity, which consequently elevates arterial pressure, thus increasing cardiovascular complications (5-7). In diabetic patients, sustained hyperglycaemia results in an increase in reactive oxygen species (ROS) and lipid peroxidation (8). Furthermore, chronic hyperglycaemia induced ROS production such as H_2O_2 , crosses the erythrocyte membrane and oxidizes heme proteins, which have been shown to lead to the progressive loss of deformability and increased osmotic fragility of red blood cells (9). Consequently, diabetic patients with cardiovascular complications present with a decrease in haemoglobin (Hb) concentrations, mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) which are haematological parameters for erythrocyte function (10). In addition, these changes induce the rapid initiation of apoptosis in damaged erythrocytes, decreasing the oxygen-carrying capacity of erythrocytes due to the increased haemolysis rate thereby significantly decreasing their lifespan (11). Apoptosis is characterised by membrane morphological alteration such as the exposure of the phosphatidylserine (PS) to the outer erythrocyte membrane surface which can be detected by staining with fluorescein isothiocyanate (FITC)-conjugated annexin-V, consequent membrane blebbing and microvesicle (MV) formation is observed (12).

In addition, diabetic nephropathy arising from prolonged hyperglycaemia is associated with a decrease in erythropoietin (EPO) secretion which is the primary stimulus for erythropoiesis (13). Inadequate EPO secretion results in the decreased RBC production and thus diabetic anaemia (14). Insulin is currently the primary means of treatment for DM in addition to the hypoglycaemic and anti-hyperglycaemic drugs in the market (15). However, insulin has been shown to increase the rate of agglutination of erythrocyte, increasing the viscosity of blood and consequently increases arterial pressure, which augments cardiac myopathy particularly in high cardiovascular risk diabetic patients. Furthermore drugs such as metformin have been shown to contribute to the progression of anaemia through the malabsorption of vitamin B₁₂ (16). Vitamin B₁₂ is important for normal erythropoiesis and RBC maturation, from the digestive tract. There is therefore a need for economical, alternative treatment for DM patients who are at risk of developing cardiovascular complications (17).

Various medicinal plants such as *Momordica charantia* and *Syzygium cordatum* (Hochst.) have been shown to possess anti-hyperglycaemic and reno-protective effects (18, 19). *Hemidesmus indicus* Linn has been shown to possess antioxidant activity and inhibited lipid peroxidation which may improve erythrocyte fragility and improve RBC function (20). Furthermore, medicinal plants such as *Prosopis gradulosa* have previously demonstrated the ability to increase haemoglobin concentrations (21).

Of interest to our study is *Momordica balsamina* (MB), a plant widespread in Namibia, Botswana, Swaziland and all provinces of Southern Africa (22). In our laboratory, we have recently shown anti-hyperglycaemic and reno-protective effects of MB in STZ-induced diabetic rats. The reno-protective effects may have via its antioxidant properties, which may be of benefit to diabetic patients with hyperglycaemic-induced haematological changes. (23). Hence the need to evaluate the haematological effects of this plant which are not yet established (24). The aim of this study therefore, is to investigate the effects of *Momordica balsamina* on selected haematological parameters in experimental STZ-induced diabetic rats.

Materials and methods

Drugs and chemicals

Dimethyl sulphoxide (Sigma-Aldrich, St Louis, Missouri, United States of America); glucose ($C_6H_{12}O_6$), metformin, monosodium citrate, calcium chloride ($CaCl_2$), citric acid, (Merck chemicals (Pty) Ltd Wadeville, Johannesburg, South Africa); streptozotocin (Sigma Aldrich Chemical Company, Missouri, St Louis, USA); insulin (NovoRapid pen refill, Novordisk Pty Ltd, Sandton, South Africa); FITC annexin-V (clone 563), BD Falcon round-bottom tubes (BD Biosciences, San Jose, CA); phosphate buffered saline (PBS) (Sigma Aldrich Co., St. Louis, MO).

Plant extraction

Professor H Baijnath, the former chief taxonomist/curator of the University of KwaZulu-Natal department of botany identified and authenticated *Momordica balsamina* leaves. The extraction of *Momordica balsamina* leaves were executed in the School of laboratory Medicine and Medical Sciences, at the University of KwaZulu-Natal, Westville Campus, following a previously validated protocol which has been previously reported by our laboratory (25). Briefly, the air-dried *Momordica balsamina* leaves (1.15kg) were sequentially extracted for 24 hours by cold percolation with methanol (95%, 6.9L). The methanolic extract was recovered from the mixture and methanol was added to the pulp for further extraction. To maximise the extraction process to increase the yield (609g), the process was repeated three times. The three extracts were combined to yield a concentrated methanolic extract at a reduced pressure (22-26mmHg) and temperature of 45-60 °C.

Animals

In the present study, 30 male Sprague-Dawley rats (250-300 g) bred and housed in the Biomedical Research Unit (BRU) of University of KwaZulu-Natal. The rats were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO_2 content of <5000 p.p.m., relative humidity of $55\pm 5\%$ and illumination (12 h light/dark cycles) with the noise levels of less than 65 decibels. The animals were given standard rat chow daily and free access to water. Procedures performed on animal and their care were conducted in conformity with the institutional guidelines of the University of KwaZulu-Natal (AREC/023/017M). Before the study commenced, the animals were allowed to acclimatize for 5 days in metabolic cages.

Induction of diabetes mellitus

Type 1 diabetes mellitus were induced by a single intraperitoneal injection of 60 mg kg^{-1} STZ in freshly prepared 0.1 M citrate buffer (pH 4.5). Control group received the vehicle, citrate buffer via the same route. Animals that exhibited glucosuria after 24 h, when tested by urine strips were considered diabetic. Seven days following the induction of diabetes, the blood glucose concentration of STZ-induced diabetic animals above 20 mmol L^{-1} were regarded as stable diabetes.

Experimental design

The short-term effects of *Momordica balsamina* methanolic (MB) extract and standard drugs (insulin and metformin) were monitored over 5 weeks for haematological parameters in separate groups of non-diabetic (group 1) and STZ-induced diabetic (group 2-5) male Sprague-Dawley (SD) rats. Rats were individually housed in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa). Group 1 received the drug vehicle DMSO to serve as a negative control. *Momordica balsamina* methanolic extract (250 mg kg⁻¹, p.o.) were administered to Group 2. Group 3 and 4 served were treated with insulin (175 µg kg⁻¹, s.c.) and metformin (500 mg kg⁻¹, p.o.), respectively, serving as positive controls. Non-diabetic animals in Group 5 served as absolute. MB was administered twice daily at 09h00 and 15h00. Blood glucose concentrations were measured every 3rd day at 09:00 for the duration of experimental period via the tail pricking method using the Elite® glucometer (Elite (Pty) Ltd., Health Care Division, South Africa) assessed.). The weekly haematocrit was measured with a micro-haematocrit, with 75x 16 mm capillary tubes filled with blood also collected similarly and centrifuged for 5 min (Cheesbrough, 2004). The EDTA anti-coagulated blood was collected into the capillary tubes, which were then sealed at one end with plasticine, and centrifuged at 3000g for 5 min, after which red cell levels in the capillary tubes were read using the microhaematocrit reader.

Terminal studies

At the end of the 5-week experimental period, all animals were sacrificed by exposing to halothane via a gas anaesthetic chamber (100 mg kg⁻¹) for 3 minutes (Biomedical Resource Unit, UKZN, Durban, South Africa). Thereafter blood was collected by cardiac puncture (RBCP) into individual pre-cooled heparinized container and centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C, 503 g for 15 minutes and separated plasma was stored at -80 °C in a Bio Ultra freezer (Snijers Scientific, Holland) for hormonal analysis. In addition, the remaining RBCs were stored in separate Eppendorf tubes and stored at -80 °C in a Bio Ultra freezer until use for flow cytometry analysis. The kidneys were removed for biochemical analysis.

Haematological analysis

Whole blood was collected from groups of untreated and treated diabetic animals to measure red blood cell (RBC) count, Mean Cell Haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and mean cell volume (MCV) using an automated haematology analyser (Beckman Coulter, California, United States).

Oxidative stress and erythropoietin analysis

To establish the effects of treatment on oxidative stress, levels of MDA, a commonly known marker of lipid peroxidation, were measured in plasma and kidneys using a biochemical assay as described below compared (26). The antioxidant defence enzymes: Superoxide dismutase (SOD) and glutathione peroxide (GPx) in plasma and kidney tissues of experimental animals were also measured using ELISA kits. Erythropoietin (EPO) concentrations were also

measured in the plasma of experimental animals of untreated and treated animals using an ELISA kit.

Malondialdehyde measurement

Kidney tissues (50 mg) were homogenized. The homogenate was centrifuged at 400 x g for 10 min. 500 µL of 0.2% phosphoric acid was added to the kidney homogenate and 100 µL plasma, respectively. Thereafter, 400 µL of both the homogenate and plasma were each supplemented with 400 µL 2% phosphoric acid and then separated into three glass tubes, each receiving equal volumes of the solution. Subsequently, 200 µL of 7% phosphoric acid was added into all glass tubes followed by the addition of 400 µL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into two glass tube (sample tests) and 400 µL of 3 mM hydrochloric acid (HCl) into the third glass tube (blank). To ensure an acidic pH of 1.5, 200 µL of 1 M, HCl was added to sample and blank test tubes. All solutions were heated at 100 °C for 15 min, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases are distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 x g for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek µQuant spectrophotometer (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths were used to calculate the concentration of MDA using Beer's Law.

Concentration of MDA (mM)

$$= \frac{\text{Average Absorbance}}{\text{Absorption coefficient (156 mmol}^{-1}\text{)}}$$

Superoxide dismutase and glutathione peroxidase and erythropoietin concentration

SOD and GPx concentrations in plasma and kidney tissues, plasma EPO were analysed using a specific ELISA kit (Elabscience and Biotechnology, WuHan) that uses the Sandwich-ELISA method. Kits included micro ELISA plate which were coated with antibody specific to SOD, GPx and EPO. Standards and samples were pipetted into the appropriate wells of the micro ELISA plate and incubated for 90 minutes. The plate relevant biotinylated detection antibody (100 µL) was then added and incubated for 60 minutes. Avidin-Horseradish Peroxidase (HRP) conjugate (100 µL) was added to each micro-plate well and incubated for 30 minutes. Unbound components were washed out. Substrate solution (100 µL) was added to each micro-plate well. After incubating for a further 15 minutes, the stop solution (50 µL) was added. The optical density was measured using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany) at the wavelength of 450 nm. The concentration of the samples was extrapolated from the respective standard curves.

Flow cytometry

A comparison of the percentage of red blood cells (RBC) expressing annexin-V among untreated and treated STZ-induced diabetic rats.

Red blood cell membrane analysis

Instrument set up: BD FACS Canto-II flow cytometer and BD FACSDiva software (BD Biosciences, San Jose, CA) were used to acquire data. In order to report standardized results, BD cytometer setup and tracking beads (BD Biosciences, San Jose, CA) were used for flow check to verify the cytometer's optical path and laminar flow.

Detector settings: This In order to detect small particles, forward scatter (FS) and side scatter (SS) parameters, were set at a log-scale (Figure 1 A). Voltages for the FS/SS photomultiplier tubes were set using an unstained fresh blood sample (Figure 1B). Antibody titration assays were then performed to detect optimal antibody concentrations.

Measurement of apoptotic red blood cell levels: Annexin-V FITC was used (Figure 1 B) as this antibody binds to the translocated phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer leaflet consequently exposing PS to the external environment. Briefly, 50 μ L of heparinized RBCs were stained with annexin-V FITC (1:10) antibody and incubated in the dark for 20 minutes at room temperature; samples were then suspended in 500 μ L of PBS and analysed immediately.

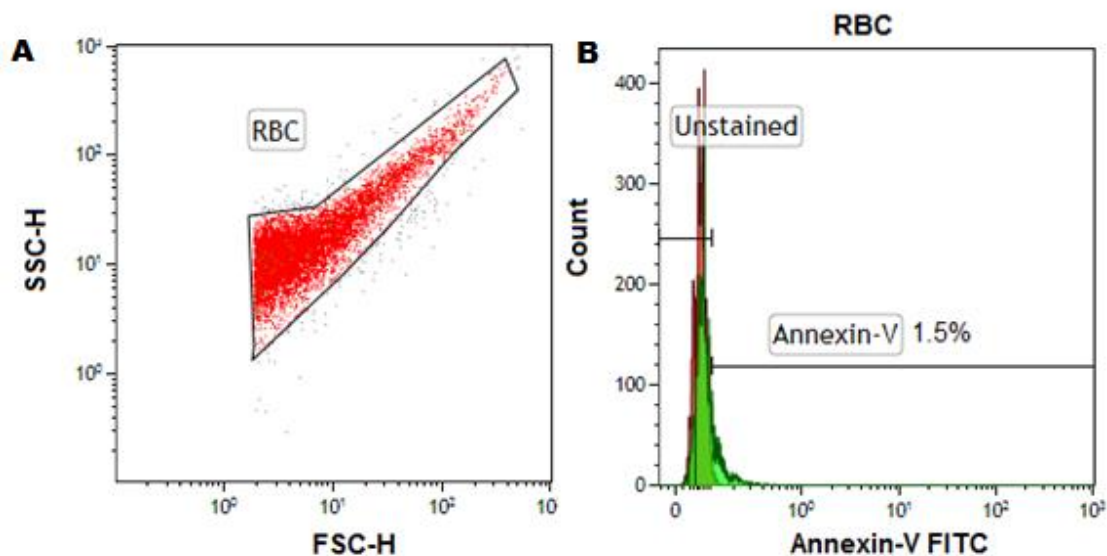


Figure 1: Gating strategy for annexin-V expression. The figure shows the gating strategy applied. (A) The colour dot plot depicts the red blood cells (RBCs) based on forward scatter (FSC) and side scatter (SS). (B) Demonstrates the expression of annexin-V.

Statistical analysis

All data are expressed as means \pm standard error of means (SEM). To perform statistical analysis, GraphPad Prism Instat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used. Blood glucose was analysed using analysis of variance (ANOVA) followed by Bonferroni post hoc test. ANOVA was used to analyse terminal parameters to analyse the differences between control and experimental groups. Values of $p < 0.05$ were considered statistical significant between the compared groups.

Results

Blood glucose concentration

Figure 2 illustrates the blood glucose concentrations in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) over an experimental period of 5 weeks. The untreated STZ-induced diabetic animals showed a significantly high blood glucose concentration throughout the period of 5 weeks by comparison with non-diabetic control ^α (DC vs NC, $p < 0.05$, Figure 2). Treatment with MB, insulin and metformin showed a significant decrease in blood glucose concentration from week three to week five by comparison with the diabetic control ^{*}(MB vs DC, $p < 0.05$, Figure 2). At week 5, blood glucose concentrations of animals treated with insulin significantly decreased by comparison with animals treated with MB ^λ (INS vs MB, $p < 0.05$, Figure 2).

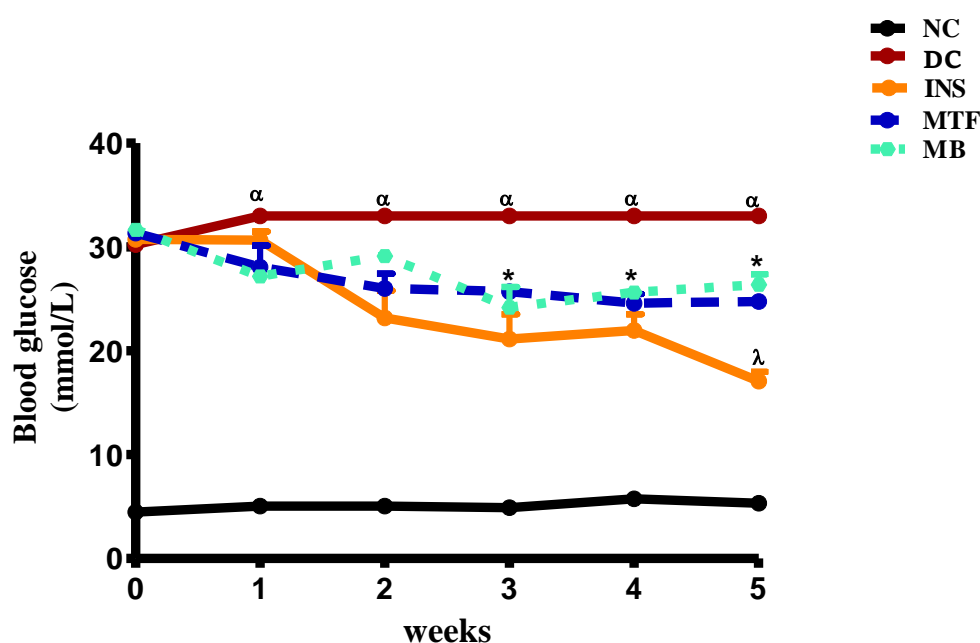


Figure 2: Blood glucose concentration in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB), metformin (MTF) and insulin (INS) over the period of 5 weeks. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ^α $p < 0.05$ by comparison with non-diabetic control animals. ^{*} $p < 0.05$ by comparison with diabetic control. ^λ $p < 0.05$ by comparison with MB treated animals.

Haematological parameters

Table 1 shows the comparisons of haematological parameters in non-diabetic control (NC), diabetic control (DC) and STZ-induced diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) at the end of the 5 weeks of treatment. Haematocrit and haemoglobin in untreated STZ-diabetic rats was significantly decreased in comparison to untreated non-diabetic rats. RBC in untreated STZ-diabetic rats were

significantly lower than untreated non-diabetic rats ^α (DC vs NC, p<0.05). Interestingly, MB significantly increased the haematocrit, RBC and haemoglobin similarly to insulin and metformin treated rats by comparison with untreated STZ-diabetic rats *(MB vs DC, p<0.05). Red blood cell indices including MCV, MCHC, MCH in untreated animals slightly decreased by comparison non-diabetic animal. In addition, MB, insulin and metformin treatment moderately improved RBC indices in by comparison to untreated animals although no significance was reached.

Table 1: Shows the comparison of haematological parameters in non-diabetic control (NC), diabetic control (DC) and STZ-induced diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are presented as means ±SEM (n=6 per group).

Parameters measured	Treatment				
	NC	DC	INS	MTF	MB
RBC (×10 ⁶ cells/μL)	06.66 ± 0.41	05.39 ± 0.06 ^α	08.60 ± 0.31 *	08.56 ± 0.09 *	07.31 ± 0.44 *
Hct (%)	38.58 ± 0.05	31.38 ± 0.57 ^α	43.41 ± 2.34 *	44.15 ± 1.59 *	45.93 ± 0.43 *
Hb (g/dL)	13.07 ± 0.61	09.16 ± 0.18 ^α	15.23 ± 0.81 *	16.05 ± 0.47 *	15.82 ± 0.22 *
MCHC (g/dL)	37.18 ± 0.63	36.10 ± 0.20	35.60 ± 0.58	34.87 ± 0.81	36.15 ± 0.80
MCV (fL/cell)	51.83 ± 0.75	50.66 ± 0.21	52.83 ± 0.47	53.00 ± 0.58	51.33 ± 1.05
MCH (pg/cell)	19.21 ± 0.52	17.00 ± 0.29	19.33 ± 0.59	19.45 ± 0.67	18.98 ± 0.16

^α p<0.05 by comparison with non-diabetic control animals. * p<0.05 by comparison with diabetic control.

MDA, SOD and GPx concentrations

Table 2 shows the comparisons of MDA, SOD and GPx concentrations in both plasma and kidney tissues in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) at the end of the 5 weeks of treatment. Plasma and kidney tissues of untreated STZ-diabetic animals presented with increased concentrations in MDA by comparison to untreated non-diabetic rats ^α (DC vs NC, p<0.05). Furthermore, there was a significant decrease in both plasma and kidney

SOD and GPx concentrations in untreated STZ-diabetic animals, respectively, by comparison with untreated non-diabetic animals ^α (DC vs NC, p<0.05). Both plasma and kidney tissues of MB treated STZ-induced diabetic rats, similarly to insulin and metformin treated rats, presented with a significant decrease in MDA concentrations by comparison to both plasma and kidney tissues in untreated diabetic rats *(MB vs DC, p<0.05). In addition, plasma and kidney GPx concentrations improved while plasma SOD concentrations deteriorated by comparison to untreated STZ-induced diabetic rats *(MB vs DC, p<0.05).

Table 2: Comparison of MDA, SOD and GPx concentrations of both the plasma and kidney tissues in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS). Values are presented as means ±SEM (n=6).

		Plasma	Kidney
MDA (μmol/g protein)	NC	0.68 ± 0.12	0.99 ± 0.02
	DC	9.37 ± 1.19 ^α	4.21 ± 0.12 ^α
	INS	0.54 ± 0.03 *	1.10 ± 0.01 *
	MTF	0.35 ± 0.06 *	1.19 ± 0.01 *
	MB	2.48 ± 0.05 *	1.14 ± 0.01 *
GPx Concentration (ng/mL)	NC	1.44 ± 0.03	2137.31 ± 0.03
	DC	0.21 ± 0.04 ^α	1703.491 ± 0.03 ^α
	INS	0.83 ± 0.01 *	2062.21 ± 0.02 *
	MTF	1.67 ± 0.01 *	2125.75 ± 0.01 *
	MB	1.06 ± 0.04 *	21370.37 ± 0.03 *
SOD Concentration (ng/mL)	NC	35.39 ± 0.63	13.43 ± 0.76
	DC	33.55 ± 0.27	06.01 ± 0.09 ^α
	INS	31.68 ± 2.76	09.89 ± 0.10 *
	MTF	29.85 ± 2.44	10.11 ± 0.02 *
	MB	23.63 ± 0.87 *	10.08 ± 0.05 *

^α p<0.05 by comparison with non-diabetic control animals. * p<0.05 by comparison with diabetic control.

Plasma erythropoietin concentrations

Figure 3 visualises the comparisons of plasma erythropoietin (EPO) concentration in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg⁻¹), metformin (MTF) and insulin (INS) after 5 weeks of the experimental period. Diabetic controls showed a significant decrease in plasma EPO

concentration by comparison to the non-diabetic control group ^α (DC vs NC, $p < 0.05$, Figure 3). Interestingly, the administration of MB, similarly to insulin, and metformin significantly increased plasma erythropoietin concentrations after the 5-week experimental period ^{*}(MB vs DC, $p < 0.05$, Figure 3).

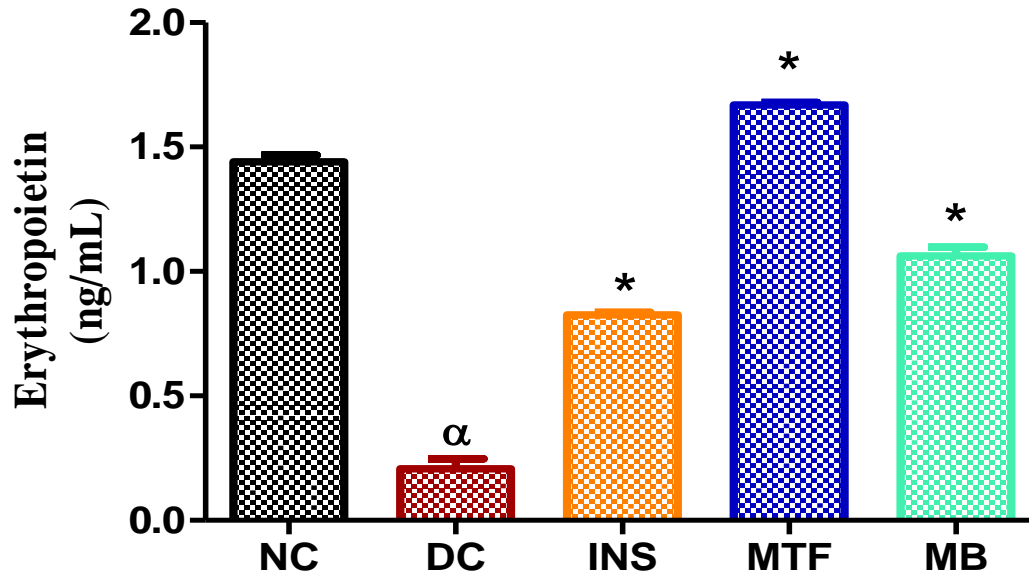


Figure 3: Plasma erythropoietin concentration in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB), metformin (MTF) and insulin (INS) after the period of 5 weeks. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ^α $p < 0.05$ by comparison with non-diabetic control animals. ^{*} $p < 0.05$ by comparison with diabetic control.

Percentage of annexin-V on red blood cell membrane

Figure 4 visualizes the comparisons percentage of annexin-V expressed on red blood cell membrane in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB) (250 mg kg^{-1}), metformin (MTF) and insulin (INS) measured upon termination of the 5-week experimental period. Diabetic controls a significantly increased red blood cell annexin-V expression by comparison to the non-diabetic control group ^α (DC vs NC, $p < 0.05$, Figure 4). Insulin and metformin treated animals exhibited a significant decrease in the percentage of annexin-V expressed on red blood cell membrane by comparison with untreated STZ-induced diabetic animals ^{*}(INS vs DC, $p < 0.05$, Figure 4), ^{*}(MTF vs DC, $p < 0.05$, Figure 4). However, the administration of MB increased the percentage of annexin-V expressed on red blood cell membrane significantly by comparison to both untreated STZ-induced diabetic animals after the 5-week experimental period ^{*}(MB vs DC, $p < 0.05$, Figure 4).

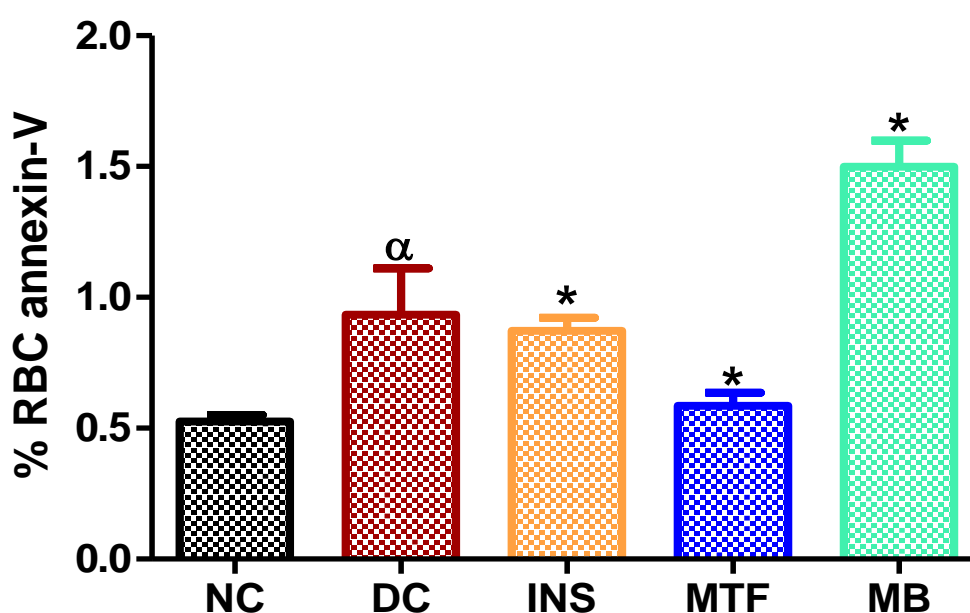


Figure 4: The percentage of red blood cell membranes expressing annexin-V in non-diabetic control (NC), diabetic control (DC) and diabetic animals treated with *Momordica balsamina* (MB), metformin (MTF) and insulin (INS) after the experimental period of 5 weeks. Values are presented as means and vertical bars indicate SEM (n=6 in each group). α $p < 0.05$ by comparison with non-diabetic control animals. * $p < 0.05$ by comparison with diabetic control.

Discussion

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia which is associated with haematological changes that progress cardiovascular pathology (27, 28). The current study assessed the effects of treatment with *Momordica balsamina* methanolic extract on selected haematological parameters which have been shown to be altered under diabetic conditions, increasing cardiovascular dysfunction (29). In our laboratory, we have shown *Momordica balsamina* possesses anti-hyperglycaemic effects and ameliorates kidney dysfunction (23).

The red blood cells (RBC) are adapted to readily undergo cellular deformation without rupturing as it navigates through the vascular system for gaseous exchange between blood and tissues (30). Furthermore, hyperglycaemia has been shown to impair erythrocyte deformability through increasing lipid peroxidation and oxidative stress, which increases their risk of rupturing which may account for the decrease in red blood cell count in the STZ-induced diabetic animals. The decrease in the number of RBCs was accompanied by a significant decline in haemoglobin (Hb) among the STZ-induced diabetic animals (31). The severe deterioration in Hb observed in the STZ diabetic animals suggest that the animals were anaemic since the decline was well below that of normal expected hemoglobin range (32). The Hb molecule contains iron atoms which bind oxygen atoms to be transported throughout the system (33). The increased risk of ischemia would consequently correlate with the development of cardiac myopathy in STZ-induced diabetic animals (34). In addition, as hyperglycaemia increases the glycation rate of Hb, thereby raising extracorporeal Hb that has been shown to

decrease RBC fragility thus shortening the life span of RBCs. However, treatment with insulin, metformin and MB improved Hb concentrations to within a normal expected range, possibly via an improvement of hyperglycaemic state which was also observed by Komolafe and et al. when treating STZ-induced diabetic rats with *Momordica charantia* (35). Notably, the homeostatic shift of a decline in Hb concentrations had no statistical significant impact on RBC indices such as MCV, MCH and MCHC. However, there was a physiological significance as RBCs can present with normal mean corpuscular volume, a condition known as normocytic anaemia which may be associated with kidney dysfunction (36). In harmony with a study conducted by Keskin and et al. , MCV, MCH and MCHC of untreated STZ-induced diabetic rats, showed no significant change at the end of the experimental period (37). It has also been extensively shown in literature that STZ-induced diabetic animals have an increase in mean arterial blood pressure, which may further increase the rupturing rate of erythrocytes (38). However, the antidiabetic properties of *Momordica balsamina* may have buffered the MDA concentrations, attenuating lipid peroxidation as the RBC membrane is rich in lipids to maintain its fluidity to facilitate deformability without rupturing which was in agreement with previous studies (39). In addition, antioxidant status was improved through increasing GPx, which detoxifies ROS product, hydrogen peroxide, to water thereby partially restoring membrane integrity (5, 40). However, hyperglycaemia results in disturbances in protein function due increased production of ROS and non-enzymatic glycation of many proteins including superoxide dismutase rendering it inefficient as observed in our experimental animals. A plant with antidiabetic properties such as *Allium sativum* has been shown to inhibit alanine production in ROS exposed erythrocytes thereby protecting erythrocytes from protein degradation, loss of deformability and increased osmotic fragility (8). Surprisingly, administration with *Momordica balsamina* did not improve the exposure of phosphatidylserine (PS) to the extracellular fluid, which may mark the erythrocytes for engulfment by local macrophages. A study conducted by Parminder K, et al showed that the seeds of *Momordica balsamina* are rich in balsamin, which is a 28 kDa protein that has been shown to promote apoptosis in breast cancer cells (41). The increase in the apoptotic activity of *Momordica balsamina* treated animals may be therefore attributed to some proapoptotic properties of this plant. Further studies on other antidiabetic compounds of *Momordica balsamina*, such as flavonoids warrant investigation to further understand the mechanism by which medicinal plants may restore RBC membrane integrity in STZ-induced diabetic rats. In addition, due to ethical constraints, the experimental period was five weeks; therefore increasing the treatment period may have shown an improvement on some haematological function markers.

The administration of STZ to mimic a type-1 diabetes rodent model has been shown to induce renal dysfunction which was previously observed in our laboratory (23). In addition, kidneys of STZ-induced diabetic rats in our study showed a decrease in the antioxidant status, which was associated with an insufficient erythropoietin secretion and RBC function. However, *Momordica balsamina* improved EPO secretion by the kidneys possibly through preventing the metabolic injury to peritubular interstitial fibroblast-like cells by advanced glycation end products (AGEs) formation. The increase in EPO, may account for the increase in RBC count and improvement in RBC indices in *Momordica balsamina* treated animals. Improving RBC function also restores the blood's oxygen-carrying capacity, which may improve cardiovascular dysfunction associated with diabetes.

Conclusion

As the foregoing discussion on the use of medicinal plants and their potential therapeutic effects on hyperglycaemia-induced haematological changes is underway, the current study enlightens some beneficial properties of *Momordica balsamina* administration. Results show improvement of the antioxidant status stemming from *Momordica balsamina*'s antidiabetic properties. This was associated with an improvement in RBC function possibly through increased EPO secretion although RBC membrane exposure of PS to the surface was not improved. Taken together, *Momordica balsamina* methanolic extract administration improves some haematological alterations associated with hyperglycaemia. Further investigations are envisaged to expand the indigenous knowledge in an effort to widen the spectrum range of easily accessible remedies for diabetes management.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

The authors appreciate assistance and support from the haematology group under the supervision of Dr B.B Nkambule and Biomedical Research Unit, University of KwaZulu-Natal. The current study was partly funded by NRF South Africa and the University of KwaZulu-Natal, Research Division.

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Chapter 4: Synthesis

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia which has been associated with micro and macroangiopathies that contribute to the increased mortality and morbidity rate of diabetic patients (1). Chronic hyperglycaemia increases the risk of developing cardiovascular complications and induces haematological changes that may aggravate cardiac dysfunction (2). In this study, we investigated the short-term effects of *Momordica balsamina* (MB) methanolic extract administration on selected cardiovascular and haematological function markers in streptozotocin (STZ)-induced diabetic rats.

The induction of diabetes mellitus with STZ, an antibiotic that selectively destroys pancreatic β -cells, achieved a hyperglycaemic rat model as evidenced by the sustained hyperglycaemia of untreated animals. Studies have shown haematological and cardiovascular dysfunction in STZ-induced hyperglycaemic animal models. In addition, literature has shown a link between alleviating hyperglycaemia-induced haematological changes and improved cardiac myopathies. Medicinal plants have shown the ability to lower blood glucose and improve haematological and cardiovascular function although the mechanisms have not been fully established. In particular, the effects of these medicinal plants on RBC structure and function. Hence, the study investigated the effects of *Momordica balsamina* methanolic extract on haematological and cardiovascular function of STZ-induced diabetic rats. In this present study, the selective destruction of pancreatic β cell resulted in insufficient insulin secretion thereby resulting in sustained hyperglycaemia in untreated animals. Furthermore, there was a deterioration of cardiac function as observed by an increase in proinflammatory cytokines such as cardiotrophin-I and C-reactive protein. The deterioration of cardiac function may have been associated with the elevated oxidative stress as marked by an increase in malondialdehyde (MDA) of untreated STZ-induced diabetic animals in this study. The increased oxidative stress was also associated with endothelial dysfunction as evidenced by an increase in a vasoconstrictor Ang-II. Furthermore, the cardiac dysfunction may have also been due to hyperglycaemia-induced haematological changes via increased lipid peroxidation as shown by increased MDA concentrations, which have been shown to decrease RBC function as marked by decreased MCV, MCH and MCHC observed in untreated diabetic animals. Furthermore, an alteration in RBC indices shows a compromised red blood cell profile that was further shown by a decline in haemoglobin concentrations, may progress to anaemia if left untreated. Haemoglobin is a protein which binds oxygen in RBCs for transport to cells, therefore

assessing haemoglobin may enlighten researchers on drug-target for the management of DM related haematological complications (178). An improvement in hyperglycaemia, oxidative stress upon treatment with MB was observed, suggesting that MB may possess bioactive compounds, which improve the antioxidant status and promote RBC production thus improving both haematological and cardiac function in diabetic animals. Since there was an improvement in the RBC production, we further assessed the quality of the RBCs by investigating the integrity of the RBC membrane. The integrity of the membrane of RBCs must be maintained in order for it to deform with ease through the vasculature without rupturing. However, when the RBC membrane is compromised as marked by the exposure of phosphatidylserine (PS) to the exterior, it is marked for engulfment by macrophages. The administration of *Momordica balsamina* therefore improved some cardiovascular and haematological parameters.

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Conclusion

Our results taken together show that the administration of *Momordica balsamina* methanolic extract has the potential to alleviate the haematological changes induced by hyperglycaemia by attenuating hyperglycaemia, oxidative stress and promoting erythropoietin secretion thus improving red blood cell profile. Furthermore, *Momordica balsamina* methanolic extract also improved cardiac function as observed by a decrease in mean arterial pressure, inflammation and cardiac hypertrophy. The ability of medicinal plants such as *Momordica balsamina* to improve red blood cell function may therefore be a therapeutic target for alleviating cardiovascular dysfunction often observed in diabetic patients.

Recommendations

Further studies on the isolation of the bioactive compounds of *Momordica balsamina* need to be conducted in order to further understand the mechanisms by which this plant improves haematological and cardiac function. Furthermore, studying the effects of bioactive compounds on other haematological parameters such as profiling red blood cell development in the bone marrow, white blood cell activation and platelet regulation, which are impaired in hyperglycaemia-induced cardiovascular complications, of treated experimental animals will further unveil the mechanisms by which medicinal plants may improve cardiac function.

Appendices

Appendix 1 - AREC ethics approval letter



12 June 2017

Ms Asiphaphola Ludidi (216017652)
School of Health Sciences
Westville Campus

Dear Ms Ludidi,

Protocol reference number: AREC/023/017M

Project title: The effects of *Momordica balsamina* on glucose metabolism in STZ diabetic rats: Effects on selected metabolic markers

Full Approval – Research Application

With regards to your revised application received on 07 June 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 June 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Prof S. Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Phikelelani S Ngubane
Cc Academic Leader Research: Professor J van Heerden
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Stephanie Keulder
Cc BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)

Ms Mariette Snyman (Administrator)

Westville Campus, Govan Mbeki Building

Postal Address: Private Bag X54001, Durban 4000

Telephone: +27 (0) 31 260 8350 Facsimile: +27 (0) 31 260 4809 Email: animalethics@ukzn.ac.za

Website: <http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx>



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Appendix 3 – Manuscript 2 Journal guide

Journal Title

Concise and Informative Article Title

Firstname M. I. Lastname,¹ Firstname A. Lastname,² and Firstname B. Lastname^{1,2}

¹ Department, Institute, City ZIP/Post code, Country.

² Department, Institute, City ZIP/Post code, Country.

Correspondence should be addressed to Firstname B. Lastname; lastname@institution.edu

Abstract

The abstract should be a single, self-contained paragraph which summarises the manuscript. Ideally, it will provide a brief context for the study, before describing the scientific approach and some key results in a qualitative manner. It should finish with a sentence to describe the implications for the field. The abstract must not include references, figures or tables.

Introduction

The introduction should be succinct, with no subheadings. Limited figures may be included only if they are truly introductory, and contain no new results.

Materials and Methods

The materials and methods section should contain sufficient detail so that all procedures can be repeated. It may be divided into headed subsections if several methods are described.

Results and Discussion

Subheadings

The results and discussion may be presented separately, or in one combined section, and may optionally be divided into headed subsections.

Advice on Equations

Equations should be provided in a text format, rather than as an image. Microsoft Word's equation tool is acceptable. Equations should be numbered consecutively, in round brackets, on the right-hand side of the page. They should be referred to as Equation 1, etc. in the main text.

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (1)$$

Advice on Figures

At the point of submission, authors may provide all figures embedded within the manuscript at a convenient break near to where they are first referenced or, alternatively, they may be provided as separate files. All figures should be cited in the paper in a consecutive order.

Where possible, figures should be displayed on a white background. When preparing figures, consider that they can occupy either a single column (half page width) or two columns (full page width), and should be sized accordingly. All figures must have an accompanying caption which includes a title and, preferably, a brief description (see Figure 1).

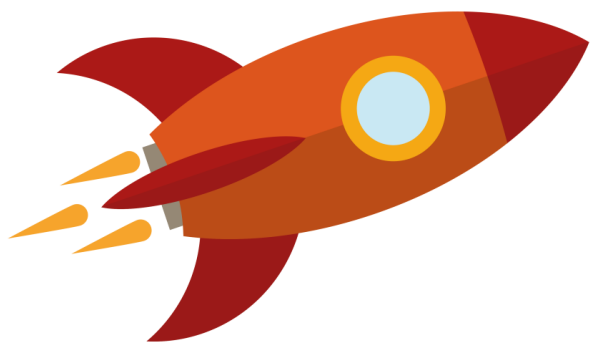


Figure 1: Basic rocket ship design. The rocket ship is propelled with three thrusters and features a single viewing window. The nose cone is detachable upon impact.

The caption can also be used to explain any acronyms used in the figure, as well as providing information on scale bar sizes or other information that cannot be included in the figure itself. Plots that show error bars should include in the caption a description of how the error was calculated and the sample size (see Figure 2).

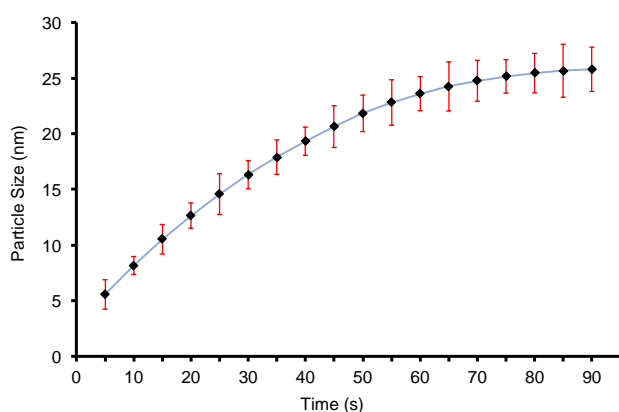


Figure 2: Plot of nanoparticle size with respect to time, recorded over a 90 s period. The error bars represent the standard deviation of measurements for 20 particles in five separate sample runs ($n = 100$).

If a figure consists of multiple panels, they should be ordered logically and labelled with lower case roman letters (i.e., a, b, c, etc.). If it is necessary to mark individual features within a panel (e.g., in Figure 3a), this may be done with lowercase Roman numerals, i, ii, iii, iv, etc. All labels should be explained in the caption. Panels should not be contained within boxes unless strictly necessary.

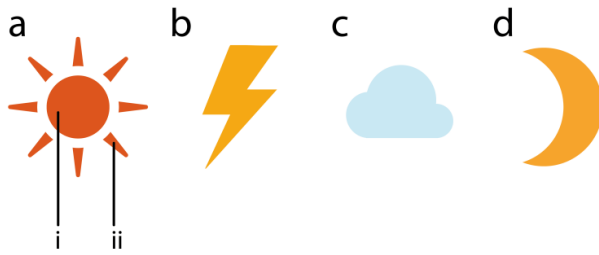


Figure 3: Representations of some common weather symbols. (a) The sun with (i) core, and (ii) rays. (b) Thunder bolt. (c) Cloud. (d) Moon.

Upon acceptance, authors will be asked to provide the figures as separate electronic files. At that stage, figures should be supplied in either vector art formats (Illustrator, EPS, WMF, FreeHand, CorelDraw, PowerPoint, Excel, etc.) or bitmap formats (Photoshop, TIFF, GIF, JPEG, etc.). Bitmap images should be of at least 300 dpi resolution, unless due to the limited resolution of a scientific instrument. If a bitmap image has labels, the image and labels should be embedded in separate layers.

Advice on Tables

Every table must have a descriptive title and, if numerical measurements are given, the units should be included in the column heading. Vertical rules should not be used (see Table 1).

Tables should be cited consecutively in the text.

Table 1: Temperature and wildlife count in the three areas covered by the study.

Location	T [° C]	Turtles	Sharks	Octopuses	Starfish
Blue Lagoon	21.2	5	3	4	543
Regent's Canal	5.2	8	0	24	312
Shark Bay	12.8	4	7	9	122

Conclusions

The Conclusions section should clearly explain the main findings and implications of the work, highlighting its importance and relevance.

Data Availability

A data availability statement is compulsory for research articles and clinical trials. Here, authors must describe how readers can access the data underlying the findings of the study, giving links to online repositories and providing deposition codes where applicable. For more information on how to compose a data availability statement, including template examples, please visit: <https://www.hindawi.com/research.data/#statement>.

Conflicts of Interest

This section is compulsory. A competing interest exists when professional judgment concerning the validity of research is influenced by a secondary interest, such as financial

gain. We require that our authors reveal any possible conflict of interest in their submitted manuscripts. If there is no conflict of interest, authors should state that “The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.”

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Funding Statement

Authors should state how the research and publication of their article was funded, by naming financially supporting bodies followed by any associated grant numbers in square brackets.

Acknowledgments

An Acknowledgements section is optional and may recognise those individuals who provided help during the research and preparation of the manuscript.

Supplementary Materials

If Supplementary Materials are provided (e.g., audio files, video clips or datasets) they should be described here. Note that authors are responsible for providing the final Supplementary Materials files that will be published along with the article, which are not modified by our production team. You should remember to reference the Supplementary Materials’ contents at appropriate points within the manuscript. We recommend citing specific items, rather than referring to the Supplementary Materials in general, for example: “See Figures S1-S10 in the Supplementary Material for comprehensive image analysis.”

References

References will be reformatted in house, there is no need to adhere to a specific style at the point of submission. Authors are responsible for ensuring that the information in each reference is complete and accurate. All citations in the text must be numbered consecutively in square brackets, before any punctuation, for example, “as discussed by Smith [1],” and “as discussed elsewhere [2,3].” All uncited references will be automatically removed. The references should not contain footnotes. For your information, our citation style is:

[x] Author initials and surname, “Title in sentence style,” Journal title, vol. (volume number), no. (issue number), pp. (page numbers separated by an en-dash), Year.

For example:

[1] J. D. Watson and F. H. C. Crick, “A structure for deoxyribose nucleic acid,” *Nature*, vol. 171, no. 4356, pp. 737–738, 1953.

For articles with six or more authors, the first three authors are listed followed by ‘et al.’.

When journals use only article numbers, no page numbers are necessary. For example:

[2] B. P. Abbott, R. Abbott, T. D. Abbott et al., “Observation of Gravitational Waves from a Binary Black Hole Merger,” *Physical Review Letters*, vol. 116, no. 6, Article ID 061102, 2016.



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Appendix 5 – Abstract of CHS symposium 2018 poster presentation

Chronic hyperglycaemia as observed in Diabetes Mellitus (DM) has been shown to increase the probability of the non-enzymatic glycosylation of red blood cell membrane proteins resulting in the non-specific aggregation of protein molecules and alters the protein-protein and protein-lipid interaction leading to the modification and damage of the erythrocyte membrane (1). The modifications increase erythrocyte aggregation, decreased mobility of the red blood cells and increased blood viscosity which consequently elevates arterial pressure, this way inducing cardiac myopathy (2). However, the administration of insulin and other conventional drugs have been associated with the increased rate of agglutination of erythrocyte, increasing the viscosity of blood and consequently increases arterial pressure and disruption of erythrocyte function, which augments cardiac myopathy particularly in high cardiovascular risk diabetic patients (3). There is therefore a need for economical, alternative treatment such as medicinal plants for DM patients who are at risk of developing cardiovascular complications. Traditionally various plants have been shown to alleviate diabetes associated complications. Of interest to our study is *Momordica balsamina*. (MB) commonly known as “Intshungu” is a plant widespread in provinces of Southern Africa (4). In our laboratory, we have recently shown anti-hyperglycaemic and reno-protective effects of MB. However, the haemantic effects of this plant are not yet known (5, 6).