

Investigating the Modulating effects of Fermented Rooibos (*Aspalathus linearis*) and Fermented Honeybush (*Cyclopia intermedia*) on aortic endothelial function and antioxidant status in Streptozotocin (STZ) – induced diabetic Wistar rats

By

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Declaration

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Abstract

Introduction: Diabetes mellitus is a metabolic disorder that causes chronic hyperglycaemia due to insulin resistance and deficiency. The implications that chronic hyperglycaemia has on vascular function have established diabetes mellitus as a risk factor for the development of endothelial dysfunction and the associated cardiovascular effects such as hypertension. *Aspalathus linearis* and the *Cyclopia* species are medicinal plants that have antioxidant, anti-inflammatory, anti-mutagenic and anti-diabetic properties. However, not enough research has been done to determine the modulating effects of fermented rooibos and fermented honeybush on vascular function, hypertension and antioxidant status in the context of diabetes mellitus.

Aim: To investigate the modulating capacity of 2% fermented rooibos (RB) (*Aspalathus linearis*) and 4% fermented honeybush (HB) (*Cyclopia intermedia*) on vascular function, hypertension and antioxidant status in streptozotocin-induced diabetic rats.

Methods: Adult male Wistar rats were divided into 7 groups and acclimatized to human handling and the blood pressure apparatus for 1 week. Subsequently, diabetic rats treated with 2% RB or 4% HB were acclimatized to the taste of the infusions for 1 week prior to receiving an intraperitoneal injection of 45mg/kg bw streptozotocin. Animals were then treated with 2% RB or 4% HB infusions for 6 weeks. Thereafter biometric measurements; body weight, fluid intake, food intake, blood glucose, relative kidney organ weight and intraperitoneal glucose tolerance test (IPGTT) were performed. Aortic tissue was used to assess the endothelial proteins eNOS and PKB/Akt involved in vascular function and to conduct vascular contraction/relaxation studies. Furthermore, antioxidant status was assessed in the kidney tissue by means of the superoxide dismutase (SOD) and catalase (CAT) antioxidant enzyme assays. Additionally, the TBARS assay was conducted in kidney tissue to assess lipid peroxidation, a biomarker of oxidative stress.

Results: At the end of the 6-week treatment period, the diabetic animals developed hyperglycaemia, impaired glucose tolerance, weight loss, increased fluid and food intake and increased kidney weight that was not ameliorated by the 2% RB and 4% HB treatment. Furthermore, the streptozotocin injury model exerted a pro-contractile and an anti-relaxation effect on aortic vascular reactivity. Treatment with 2% RB or 4% HB significantly reduced the vasoconstriction caused by streptozotocin and restored the vasorelaxation response of the aorta, with the RB exhibiting a greater restorative effect than HB. Furthermore, in the aortic tissue, the STZ injury model significantly reduced total and phosphorylated eNOS and

PKB/Akt expression which was not ameliorated by treatment with 2% RB and 4% HB. Additionally, no significant changes were observed in the blood pressure, antioxidant enzyme and TBARS measurements of all treatment groups.

Conclusion: Streptozotocin-induced diabetic animals developed hyperglycaemia, polydipsia, polyphagia, weight loss and impaired glucose tolerance. Furthermore, the fermented RB and fermented HB infusions ameliorated the streptozotocin-induced vascular injury of the aorta, thus improving aortic vascular reactivity and endothelial function. Furthermore, treatment with RB and HB did not alleviate hyperglycaemia or affect blood pressure and the antioxidant status of the diabetic rats as measured in the kidney in the 6-week treatment duration.

Opsomming

Inleiding: Diabetes mellitus is 'n metaboliese afwyking wat chroniese hiperglikemie as gevolg van insulien-weerstandigheid en tekort veroorsaak. Die gevolge wat chroniese hiperglikemie op die vaskulêre funksie het, het daartoe gelei dat diabetes mellitus as 'n risikofaktor vir die ontwikkeling van endoteel disfunksie en gepaardgaande kardiovaskulêre effekte soos hipertensie beskou word. *Aspalathus linearis* en die *Cyclopia*-spesie is medisinale plante wat antioksidant, anti-inflammatoriese, antimutageniese en anti-diabetiese eienskappe het. Daar is egter nog nie genoeg navorsing gedoen om die modulerende effekte van gefermenteerde rooibos en gefermenteerde heuningbos op vaskulêre funksie, hipertensie en antioksidantstatus in die konteks van diabetes mellitus te bepaal nie.

Doel: Om die modulasievermoë van 2% gefermenteerde rooibos (RB) (*Aspalathus linearis*) en 4% gefermenteerde heuningbos (HB) (*Cyclopia intermedia*) op vaskulêre funksie, hipertensie en antioksidantstatus in streptosotosien (STZ)-geïnduseerde diabetiese rotte te ondersoek.

Metodes: Volwasse mannetjies Wistar-rotte is in 7 groepe verdeel en vir 'n week aan menslike hantering en die bloeddrukapparaat blootgestel. Daarna is diabetiese rotte vir 'n week behandel met 2% RB of 4% HB om sodoende aan die smaak van die infusies gewoond te raak, voordat 'n intraperitoneale inspuiting van 45 mg / kg liggaamsgewig streptosotosien gegee is. Diere is daarna behandel met 2% RB of 4% HB-infusies vir 6 weke. Daarna is biometriese metings; liggaamsgewig, vloeistofinname, voedselinname, bloedglukose, relatiewe gewig van die nierorgaan en intraperitoneale glukosetoleransietoetsing (IPGTT) uitgevoer. Aortaweefsel is gebruik om die endoteelproteïene eNOS en PKB / Akt, wat by vaskulêre funksie betrokke is, te ondersoek en om vaskulêre kontraksie / ontspanningsstudies te doen. Verder is die antioksidantstatus in nierweefsel aan die hand van die superoksieddismutase (SOD) en katalase (CAT) antioksidant ensiem analises geassesseer. Daarbenewens is TBARS-toetsing in die nierweefsel uitgevoer om lipiedperoksidase, 'n biomerker van oksidatiewe spanning, te bepaal.

Resultate: Aan die einde van die behandelingsperiode van 6 weke het die diabetiese diere hiperglisemie ontwikkel, tesame met ingekorte glukosetoleransie, gewigsverlies, verhoogde vloeistof- en voedselinname sowel as verhoogde niergewigte, wat nie deur die 2% RB of 4% HB-behandeling verbeter is nie. Verder het die STZ-beseringsmodel 'n pro-kontraktiliteit en 'n anti-ontspanningseffek op die vaskulêre reaktiwiteit van die aorta uitgeoefen.

Behandeling met 2% RB of 4% HB het die vasokonstriksie wat deur streptosotosien veroorsaak is, aansienlik verminder en die vaatverslappings-respons van die aorta herstel, met die gefermenteerde RB wat 'n groter herstellende effek as HB ontlok het. Verder, in die aortaweefsel, het die STZ-beseringsmodel die totale en gefosforileerde eNOS- en PKB / Akt-uitdrukking aansienlik verminder, wat nie verbeter het na behandeling met 2% RB of 4% HB nie. Boonop is geen noemenswaardige veranderinge in die bloeddruk-, antioksidant-ensiem- en TBARS-metings van alle behandelingsgroepe waargeneem nie.

Gevolgtrekking: Streptosotosien-geïnduseerde diabetiese diere het hiperglisemie, polidipsie, polifagie, gewigsverlies en verswakte glukosetoleransie ontwikkel. Verder, het die gefermenteerde rooibos en gefermenteerde heuningbos infusies die streptosotosien-geïnduseerde vaskulêre letsels van die aorta verlig. Dus het dit 'n verbetering in aortiese vaskulêre reaktiwiteit en endoteel funksie meegebring. Verder het die behandeling met RB en HB nie die hiperglukemie verlig nie en ook nie die bloeddruk of die antioksidantstatus van diabetiese rotte, soos gemeet in die nier, gedurende die 6 weke behandelingsperiode beïnvloed nie.

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Units of Measurement

g	-	Gram
kg	-	Kilogram
L	-	Litre
mg	-	Milligram
ml	-	Millilitre
mm	-	Millimetres
mmol	-	Millimole
min	-	Minutes
mmHg	-	Millimetres mercury
m ²	-	Square meter
nmol	-	Nano molar
µg	-	Microgram
µl	-	Microliter
µmol	-	Micro molar
µM	-	Micro molar
°C	-	Degrees Celsius
%	-	Percentage

Symbols

α -	alpha
β -	beta
γ -	gamma

Abbreviations

AAF	-	2-Acetylaminofluorene
ACE	-	Angiotensin converting enzyme
ACh	-	Acetylcholine
ADMA	-	Asymmetric dimethylarginine
AFB ₁	-	Aflatoxin B ₁
AGEs	-	Advanced glycation end products
AOX	-	Antioxidant (s)
AOC	-	Antioxidant capacity
AOPP	-	Advanced oxidation protein product
APCs	-	Antigen presenting cells
AMPK	-	AMP-activated kinase
Ang II	-	Angiotensin II
ANOVA	-	Analysis of variance
Asp	-	Aspalathin
BCA	-	Bicinchoninic acid
BCL2	-	B-cell lymphoma 2
BH ₄	-	Tetrahydrobiopterin
BFGF	-	Basic fibroblast growth factor
BHT	-	Butylated hydroxytoluene
BSA	-	Bovine serum albumin
bw	-	Body weight
Ca ²⁺	-	Calcium
CO ₂	-	Carbon dioxide
COX-1	-	Cyclooxygenase-1
CAT	-	Catalase
cyclic-AMP	-	Cyclic adenosine monophosphate

cyclic-GMP	-	Cyclic guanosine monophosphate
CRP	-	C-reactive protein
Cu	-	Copper
CVD	-	Cardiovascular disease
<i>db/db</i>	-	Diabetic homozygous mice
deiH ₂ O	-	Deionised water
DETAPAC	-	Diethylenetriaminepentaacetic acid
DHB	-	Diabetic honeybush
DRF	-	Diabetic rooibos
dH ₂ O	-	Distilled water
ECL	-	Enhanced chemiluminescence
ED	-	Endothelial dysfunction
EDHF	-	Endothelial hyperpolarization factor
EDRF	-	Endothelial-derived relaxation factor
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	Ethylene-bis (oxyethylenitrilo) tetra acetic acid
ET	-	Endothelin
EtOH	-	Ethanol
eNOS	-	Endothelial Nitric oxide synthase
FAD	-	Flavin adenine dinucleotide
Fe	-	Iron
FRE	-	Fermented rooibos extract
FFA	-	Free fatty acid
FPG	-	Fasting plasma glucose
GA	-	Gallic acid
GADA/GAA	-	Glutamic acid decarboxylase antigen
GMP	-	Guanosine monophosphate

GPx	-	Glutathione peroxidase
GRE	-	Green rooibos extract
G6P	-	Glucose-6-phosphatase
HB	-	Honeybush
HBA1c	-	Glycated haemoglobin
HClO ₄	-	Perchloric acid
H ₂ O	-	Water
H ₂ O ₂	-	Hydrogen peroxide
HRP	-	Horseradish peroxidase
HLA	-	Human leukocyte antigen
HUVECs	-	Human umbilical vein endothelial cells
IA2-2AA	-	Tyrosine phosphatase IA2 antigen
ICAM	-	Intracellular adhesion molecule
IDDM	-	Insulin-dependent diabetes mellitus
IDF	-	International diabetes federation
IGF	-	Insulin-like growth factor
IgG	-	Immunoglobulin gene
IL	-	Interleukin
iNOS	-	Inducible nitric oxide synthase
IPGTT	-	Intraperitoneal glucose tolerance test
IR	-	Insulin resistance
K ⁺	-	Potassium channels
KHB	-	Krebs henseleit buffer
KPi	-	Potassium phosphate
LDL	-	Low density lipoprotein
LMICs	-	Low and middle income countries
L-NAME	-	N (gamma)-nitro-L-arginine methyl ester

MAP	-	Mean arterial blood pressure
MAPK	-	Mitogen-activated protein kinase
MDA	-	Malondialdehyde
MHC	-	Major histocompatibility complex
Mn	-	Manganese
mRNA	-	Messenger RNA
NaCl	-	Sodium chloride
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NaF	-	Sodium fluoride
Na ₃ VO ₄	-	Sodium orthovanadate
NF- κ B	-	Nuclear factor kappa-light-chain-enhancer
Ni	-	Nickel
NIDDM	-	Non-insulin-dependent diabetes mellitus
nNOS	-	Neural nitric oxide synthase
NO	-	Nitric oxide
Nrf2	-	Nuclear factor erythroid- 2-related factor 2
O ₂	-	Oxygen
O ₂ ⁻	-	Superoxide anion
OBIR	-	Obese insulin-resistant
OGTT	-	Oral glucose tolerance test
OH ⁻	-	Hydroxide
6-OHD	-	6-Hydroxydopamine
OONO ⁻	-	Peroxynitrite anion
ORAC	-	Oxygen radical absorbance capacity
OS	-	Oxidative stress
PAI-1	-	Plasminogen activator inhibitor
Phe	-	Phenylephrine

PGI ₂	-	Prostacyclin
P-eNOS	-	Phosphorylated-endothelial nitric oxide synthase
PEPCK	-	Phosphoenolpyruvate carboxykinase
PKC	-	Protein kinase C
PPAG	-	Phenylpyruvic acid glycoside
P-PKB	-	Phosphorylated -Protein Kinase B
PKB/Akt	-	Protein kinase B
PMSF	-	Phenylmethylsulfonyl fluoride
PVDF	-	Polyvinylidene fluoride
RAAS	-	Renin–angiotensin–aldosterone system
RAGE	-	Receptor for AGEs
RB	-	Rooibos
RF	-	Fermented rooibos
RSNA	-	Renal sympathetic nervous system
ROS	-	Reactive oxygen species
ROW	-	Relative organ weight
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	-	Standard error of the mean
SGLT	-	Sodium glucose co-transporter
SHR	-	Spontaneously hypertensive rats
SHRSP	-	Stroke-prone-spontaneously hypertensive rats
SNPs	-	Single nucleotide polymorphisms
SNS	-	Sympathetic nervous system
SOD	-	Superoxide dismutase
STZ	-	Streptozotocin
TCA	-	Trichloroacetic acid

TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TBS	-	Tris-buffered saline
TEAC	-	Trolox equivalent antioxidant capacity
TEMED	-	1, 2-Bis (dimethylamino) ethane
T-eNOS	-	Total-endothelial nitric oxide synthase
TNF- α	-	Tumor necrosis factor alpha
Tris-HCL	-	Hydroxymethylaminomethane hydrochloride
T-PKB	-	Total-protein kinase B
T1DM	-	Type 1 diabetes mellitus
T2DM	-	Type 2 diabetes mellitus
TSS	-	Total soluble solids
VCAM	-	Vascular cell adhesion molecule
VEH	-	Vehicle
VPR	-	Volume-pressure recording
WB	-	Western blot
WHO	-	World Health Organization
UV	-	Ultraviolet
Zn	-	Zinc
ZnT8a	-	Zinc transporter 8

CHAPTER 1 Literature Review

1.1. Overview and Diabetes Mellitus

The prevalence of diabetes mellitus is rapidly increasing and becoming a major public health concern globally. Furthermore, this chronic metabolic disorder has become a widespread epidemic, affecting an estimated 108 million people in 1980, 285 million in 2010, 415 million in 2015 and reaching 425 million people in 2017 (Guariguata *et al.*, 2014; Mutyambizi *et al.*, 2017; Ogurtsova *et al.*, 2017; Shaw, Sicree and Zimmet, 2010). It is estimated by the International Diabetes Federation (IDF) and the World Health Organisation (WHO) that the rapidly increasing prevalence of diabetes mellitus will grow to affect 629 million people by the year 2045 (**Figure 1:1**) (Guariguata *et al.*, 2014; Mutyambizi *et al.*, 2017; Ogurtsova *et al.*, 2017; Shaw, Sicree and Zimmet, 2010).

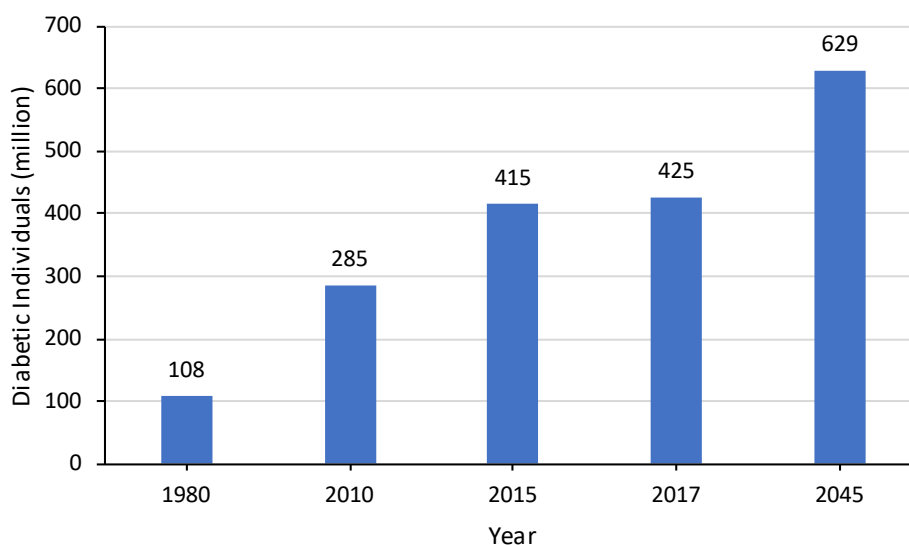


Figure 1:1 The estimated growth and prevalence of diabetes mellitus worldwide (Guariguata *et al.*, 2014; Mutyambizi *et al.*, 2017; Ogurtsova *et al.*, 2017; Shaw, Sicree and Zimmet, 2010).

Globally, the spread of diabetes mellitus varies across different regions, income and age groups. The regional prevalence of diabetes mellitus is highest in North America, the Middle East, the Caribbean and in South Asia (Guariguata *et al.*, 2014; Shaw, Sicree and Zimmet, 2010). Although the African region presents with the lowest regional prevalence, it is estimated that the largest number of adults affected by

diabetes mellitus by 2035 will be from Africa (Guariguata *et al.*, 2014). This is likely due to the growing rate of urbanisation and obesity in low and middle income countries across the continent. Moreover, statistics retrieved from the World Bank income group show that diabetes mellitus largely affects adults living in low and middle income countries (LMICs) (Guariguata *et al.*, 2014). The lack of well-developed health systems, health policies, health resources and diagnostic tools in LMIC countries are some of the contributing factors that serve as an explanation of the high global estimates of diabetes mellitus (Guariguata *et al.*, 2014; Ogurtsova *et al.*, 2017; Shaw, Sicree and Zimmet, 2010). Generally, diabetes mellitus predominantly affects adults who are between the ages of 20 and 79 years of age in LMIC countries (Ogurtsova *et al.*, 2017). In high income countries, diabetes mellitus predominantly affects the elderly or adults who are 50 to 60 years of age and older (Guariguata *et al.*, 2014; Shaw, Sicree and Zimmet, 2010).

In South Africa, diabetes mellitus was reported by the South African National Burden of Disease Study in the year 2000 as the 10th leading cause of death in people of all ages and the 7th leading cause of death in adults who are 30 years of age and older (Bradshaw *et al.*, 2007). Fourteen years later, diabetes mellitus has become the 3rd leading cause of mortality in South Africa (Mutymbizi *et al.*, 2017). Furthermore, diabetes mellitus is classified into type 1 diabetes mellitus [insulin dependent diabetes mellitus (IDDM)] and type 2 diabetes mellitus [non-insulin dependent diabetes mellitus (NIDDM)] (Guideline and Prevention, 2012). Globally, type 2 diabetes mellitus is the most common type of diabetes mellitus (Bradshaw *et al.*, 2007; Mutymbizi *et al.*, 2017). Moreover in South Africa, NIDDM accounts for more than 90% of all diagnoses made (Bradshaw *et al.*, 2007). Furthermore, patients with NIDDM and IDDM often present with micro- and macrovascular complications that exist as comorbidities. Heart disease, renal disease, and cardiovascular diseases (CVDs) including stroke, heart attack and heart failure are some of the micro- and macrovascular complications that affect patients with diabetes mellitus (Mutymbizi *et al.*, 2017). In comparison to non-diabetics, CVDs account for the highest mortality rates of diabetes mellitus patients worldwide. Heart disease affects almost 21% of diabetes mellitus patients in the Sub-Saharan region of Africa (Mutymbizi *et al.*, 2017). In South Africa and the Sub-Saharan region, it is noted that hypertension followed by heart disease and stroke are

the most commonly occurring CVD comorbidities in diabetes mellitus (Mutymbizi *et al.*, 2017).

1.1.1. Type 2 Diabetes Mellitus

NIDDM is primarily caused by insulin insensitivity or resistance that occurs as a result of lifestyle modifications such as excessive consumption of alcohol, a diet rich in saturated fats and refined sugars as well as a sedentary lifestyle (Olokoba *et al.*, 2012). Genetic predispositions, environmental toxins, and medical conditions such as the metabolic syndrome, hypertension, aging and obesity are some of the risk factors that lead to the progression of insulin resistance and the development of NIDDM (Olokoba *et al.*, 2012).

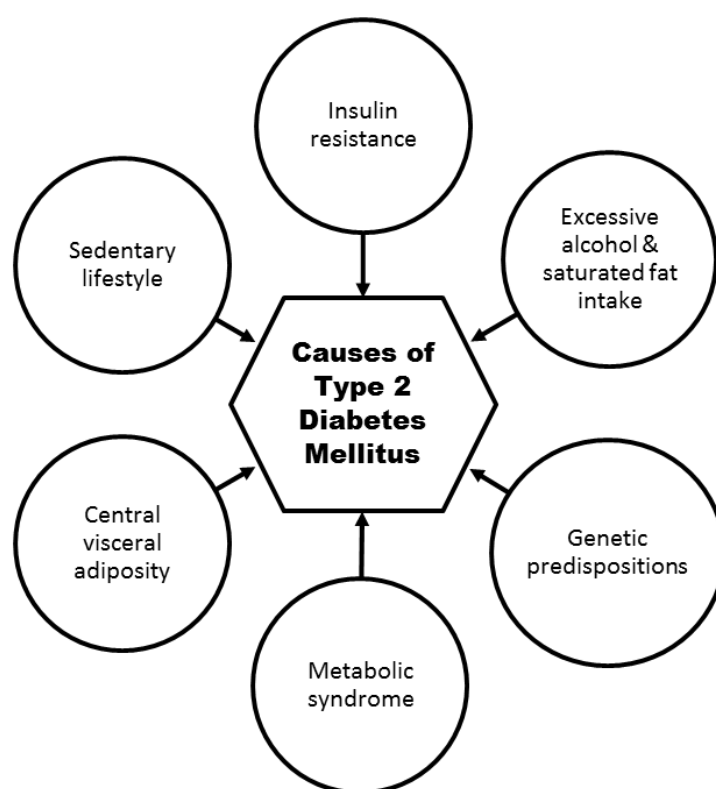


Figure 1:2 Causes of type 2 diabetes mellitus (Olokoba *et al.*, 2012).

Furthermore, insulin resistance and compensatory hyperinsulinemia are the main risk factors that precede the development of NIDDM. Insulin resistance is the reduced sensitivity of insulin to cells in peripheral tissues such as skeletal muscle, liver and adipose tissue (Caballero, 2003). NIDDM patients often present with obesity or increased weight gain. Thus, central to the development of insulin resistance in NIDDM patients is abdominal obesity or central visceral adiposity. Generally, central

visceral adiposity induces insulin resistance through the accumulation of fat-derived metabolic products, cytokines and associated hormones (Caballero, 2003). Excess adipose tissue stimulates the continuous production of fat-derived metabolites such as free fatty acids (FFAs), tumour necrosis factor alpha (TNF- α) and plasminogen activator inhibitor (PAI-1) which altogether contribute to insulin resistance and the development of vascular abnormalities (Caballero, 2003). Furthermore, in symptomatic NIDDM patients, compensatory hyperinsulinemia or the increased secretion of insulin occurs in order to restore normal glucose levels and repair the loss of insulin sensitivity experienced by insulin resistant cells (Caballero, 2003; Reaven, 1995). However, it is hypothesized by various researchers that by inducing continuous activation of the sympathetic nervous system and sodium retention, hyperinsulinemia contributes to the progression of high blood pressure (Reaven, 1995). Moreover, hyperinsulinemia primarily stimulates fat cells as opposed to liver and skeletal muscle cells, thus promoting lipogenesis and increased weight gain in diabetes mellitus (Caballero, 2003). Furthermore, in the symptomatic stage of NIDDM, patients present with overt hyperglycaemia and increased weight gain that is often characterized by central visceral adiposity. Moreover, the onset of NIDDM is more prevalent in adults than it is in children (Olokoba *et al.*, 2012).

1.1.2. Type 1 Diabetes Mellitus

IDDM, on the other hand, is a metabolic disorder characterized by overt hyperglycaemia and insulin deficiency (Alberti and Zimmet, 1998; Sena, Pereira and Seíça, 2013). A distinct difference between IDDM and NIDDM is that the reduced sensitivity of insulin to peripheral tissues is primarily caused by insulin deficiency in IDDM and by insulin resistance in NIDDM (**Figure 1:3**) (Alberti and Zimmet, 1998; Sena, Pereira and Seíça, 2013).

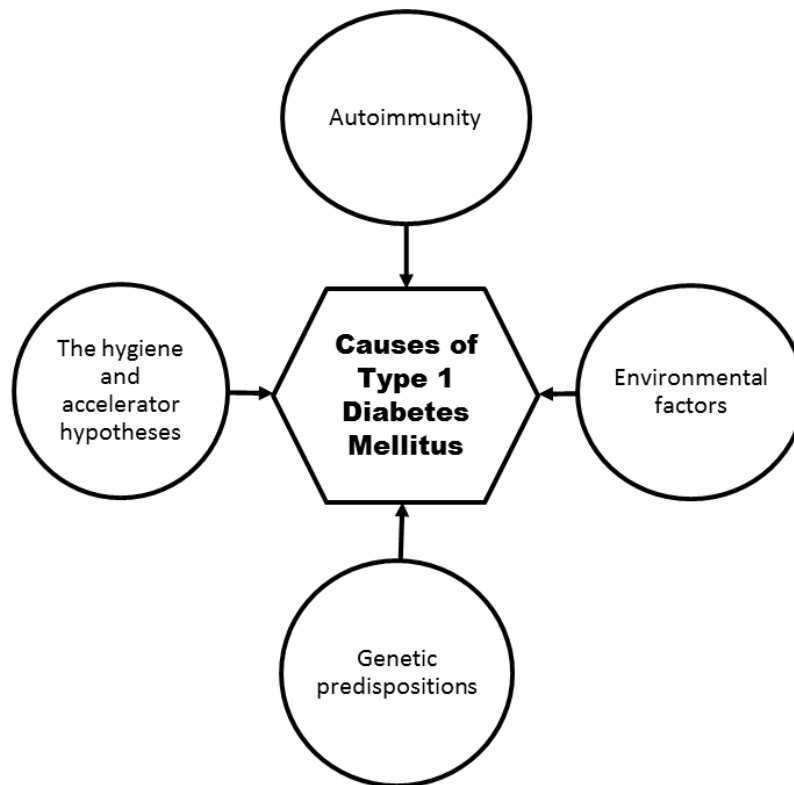


Figure 1:3 Causes of type 1 diabetes mellitus (Abbas, Lichtman and Pillai, 2014; Daneman, 2006; Guideline and Prevention, 2012).

In people with IDDM, adipose-tissue derived intracellular triglycerides and skeletal muscle tissue-derived intracellular proteins breakdown is significantly increased even in the presence of adequate blood glucose molecules (Alberti and Zimmet, 1998; Sena, Pereira and Seïça, 2013). As a result, IDDM patients often present with overt hyperglycaemia, continuous weight loss, high blood pressure, high cholesterol, atherosclerosis, CVDs and associated microvascular complications (Alberti and Zimmet, 1998; Sena, Pereira and Seïça, 2013). Furthermore, in the symptomatic stage of IDDM, patients, usually children and adults younger than 30 years, present with polydipsia, polyuria, weight loss and severe ketoacidosis (Guideline and Prevention, 2012). Microvascular complications such as retinopathy, neuropathy, nephropathy and various cardiovascular diseases including peripheral arterial and cerebrovascular disease are some of the many long-term effects that develop in IDDM individuals (Guideline and Prevention, 2012).

1.1.2.1. Autoimmunity

Insulin deficiency in IDDM is primarily caused by an autoimmune or immune-associated destruction of pancreatic islet β -cells. Autoimmunity is defined as “an immune response against self (autologous) antigens”. Furthermore, autoimmunity is an immunological pathology that primarily develops through the inheritance of susceptibility genes and environmental triggers or a combination of both (Abbas, Lichtman and Pillai, 2014; Daneman, 2006).

The first line of defence of the immune system against infections or microbes is innate immunity, while adaptive immunological responses to foreign microbes and infections after innate immunity has been established (Abbas, Lichtman and Pillai, 2014). Central to an effective immune response against disease-causing pathogens are the T-lymphocytes and B-lymphocytes involved in the adaptive immunological response of the body against foreign microbes. These adaptive immune cells provide immune responses against foreign microbes by binding to microbial antigens that are present on major histocompatibility complex (MHC) molecules of various antigen-presenting cells (APCs) (Abbas, Lichtman and Pillai, 2014). Under normal physiological conditions, both the T- and B-lymphocytes remain unresponsive to self-antigens or antigens originating from one’s tissues and cells. This unresponsiveness is called immunological tolerance and is acquired in the generative lymphoid organs and peripheral tissues (Abbas, Lichtman and Pillai, 2014).

Furthermore, immunological tolerance is acquired when immature lymphocytes strongly interact with self-antigens in a process called negative selection. As a result, immature lymphocytes fail to complete their maturation and are deleted from the immune system via apoptosis or programmed cell (Abbas, Lichtman and Pillai, 2014; Daneman, 2006). Similarly, in peripheral tissues, immunological tolerance is acquired when those mature T and B-lymphocytes, (also called self-reactive lymphocytes) that are most likely to react with self-antigens are deleted via apoptosis while others become functionally inactivated in a process called anergy (Abbas, Lichtman and Pillai, 2014; Daneman, 2006). However, when self-reactive lymphocytes fail to remain unresponsive to self-antigens or fail to develop immunological tolerance, autoimmunity develops (Abbas, Lichtman and Pillai, 2014; Daneman, 2006).

Autoimmunity in IDDM leads to an inflammatory response characterized by the development of an insulinitis (the infiltration of pancreatic islet β -cells with lymphocytes) and a humoral response characterised by the production of autoantibodies that are reactive to insulin antigens such as glutamic acid decarboxylase (GADA/GAA), tyrosine phosphatase IA2 (IA2-2AA) or zinc transporter 8 (ZnT8a) (Daneman, 2006; Abbas, Lichtman and Pillai, 2014). Consequently, the activated autoantibodies result in continuous activation of T-cell-mediated immunological responses to self-antigens or pancreatic islet β -cells. Thus, pancreatic islet beta cells are destroyed and insulin deficiency develops (Daneman, 2006; Abbas, Lichtman and Pillai, 2014).

1.1.2.2. Genetic Factors

In addition to autoimmunity, genetic susceptibility is a key role player in the development of type 1 diabetes mellitus. The inherited risk of most human autoimmune diseases including IDDM arises from the multiple gene loci of the human leukocyte antigens (HLA) or MHC genes. This inherited risk is multifactorial and heterogeneous (Abbas, Lichtman and Pillai, 2014). In other words, it is more than one genetic factor or common variants of the same gene (polymorphisms) that is responsible for disease causation. These polymorphisms are either commonly found in susceptible individuals or are rare when they are protective (Abbas, Lichtman and Pillai, 2014).

The HLA complex is used by APCs to present foreign protein antigens to lymphocytes of the immune system. (Baker and Steck, 2011). Furthermore, the HLA complex has over 200 genes that are situated on chromosome 6 and are subdivided into 3 classes namely: The MHC class I containing 3 genes (HLA-A, HLA-B and HLA-C), the MHC class II containing 6 genes (HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1) and the MHC class III which consist of genes that are involved in various immune responses such as the induction of inflammatory responses (Baker and Steck, 2011). According to Abbas, Lichtman and Pillai, 2014, IDDM could develop through the inheritance of the HLA-DRB1 MHC class II gene alleles in addition to various polymorphisms that arise from non-HLA genes (Abbas, Lichtman and Pillai, 2014). Similarly, Atkinson, Eisenbarth and Michels, 2014, found IDDM to be a polygenic disorder that is caused by a genetic susceptibility of the MHC class I and MHC class II loci. Furthermore, the DR and DQ genotypes of the MHC Class II loci

have been found to increase the genetic susceptibility of developing IDDM by 50% and the IDDM genes contributing to this inherited risk by 15% (Daneman, 2006). These IDDM genes are thought to be important role players when it comes to the regulation of any immune response (Daneman, 2006).

1.1.2.3. Environmental Factors

Various types of viruses such as the congenital rubella, coxsackie or enteroviruses and food constituents including proteins from cow's milk, gluten, cereals, vitamin D and vitamin D-pathway constituents have been identified as possible environmental triggers that lead to IDDM (Daneman, 2006; Atkinson, Eisenbarth and Michels, 2014). The above-mentioned environmental triggers are thought to initiate autoimmunity by triggering the T-cell mediated immune responses that lead to the removal of microbes and other foreign particles (Daneman, 2006). Under normal physiological conditions, mature T-lymphocytes that have acquired immunological tolerance are activated to facilitate T-cell mediated immune responses against foreign microbes and infections.

However, self-reactive T-lymphocytes that were inactivated through a process called clonal anergy, become activated and react with self-antigens thus breaking T-cell tolerance and mediating the destruction of pancreatic islet β -cells (Abbas, Lichtman and Pillai, 2014) This cross-reaction phenomenon where immune responses that are meant for foreign-antigens are initiated against self-antigens is termed molecular mimicry. It is a phenomenon that is explained by the fact that certain environmental triggers such as the previously-mentioned, may have antigens that are similar in structure to self-antigens (Abbas, Lichtman and Pillai, 2014).

Overall, although various environmental triggers have been identified as possible triggers for the development of autoimmune diseases, there is no evidence that confirms whether these environmental factors are indeed triggers or causes of the autoimmune response that leads to the pathogenesis of IDDM (Daneman, 2006).

1.1.2.4. The Hygiene and Accelerator Hypothesis

In addition to the Eisenbarth model or theory that describes the cause of IDDM, there is the hygiene and the accelerator hypotheses (Daneman, 2006). The hygiene hypothesis is based on the idea that infectious diseases such as asthma are more common in modernised societies than they are in traditional and conservative societies

(Daneman, 2006). Moreover, children who come from large families and attend nursery school are more likely to develop a strong immune system that makes them to become less susceptible to developing autoimmune diseases such as IDDM. However, the hygiene hypothesis lacks scientific support and credibility (Daneman, 2006). Additionally, in the accelerator hypothesis, Wilkin postulates that “diabetes mellitus is a single disease rather than two distinct entities, type 1 and type 2” that is caused by increased β -cell apoptosis (first accelerator), insulin resistance (second accelerator) and β -cell autoimmunity (third accelerator) (Daneman, 2006).

1.1.3. Diabetes Mellitus: Diagnostic Criteria

In addition to the clinical symptoms that NIDDM and IDDM patients present with, various laboratory diagnostic tests are used to deduce a diagnosis for diabetes mellitus (Guideline and Prevention, 2012). A fasting plasma glucose (FPG), an oral glucose tolerance test (OGTT), a glycosylated haemoglobin A1c (HbA1c) assay and a random plasma glucose test are the major tests used in South Africa in accordance to the standards of the WHO’s diagnostic criteria (Guideline and Prevention, 2012).

A fasting plasma glucose test is carried out when the patient has had no caloric or food intake for at least 8-hours prior to the test. A fasting glucose level of 6.2-6.9mmol/l is regarded as an impaired fasting glucose (IFG) and one that is higher than 7mmol/l is a symptom of impaired glucose tolerance and that of diabetes mellitus. An OGTT is a test that measures glucose tolerance or insulin sensitivity (Alberti and Zimmet, 1998; Guideline and Prevention, 2012). This test is carried out by administering 75g anhydrous glucose per kilogram (kg) body weight (bw) dissolved in water followed by the observation of the individual’s plasma glucose level or the rate of glucose disappearance in their blood over a period of two hours (Alberti and Zimmet, 1998; Guideline and Prevention, 2012). A patient who presents with a plasma glucose level higher than 11mmol/l following an OGTT is considered diabetic (Alberti and Zimmet, 1998; Guideline and Prevention, 2012). With regards to the glycosylated haemoglobin (HbA1c) assay, an occurrence of HbA1c molecules equivalent to or higher than 6.5% blood glucose concentration is diagnostic for diabetes mellitus (Alberti and Zimmet, 1998; Guideline and Prevention, 2012). Additionally, a random plasma glucose test, performed at any time of day that results in a plasma glucose level equivalent to or

higher than 11.1mmol/l is a classic symptom of diabetes mellitus (Alberti and Zimmet, 1998; Guideline and Prevention, 2012).

1.1.4. Serological Factors

In addition to glucose tests, the presence of a chronic inflammation infiltrate (insulinitis) and autoantibodies (GADA, IA2A, and ZnT8A) in blood serum serve as early markers of IDDM (Joslin and Kahn, 2005; Atkinson, Eisenbarth and Michels, 2014). These autoantibodies can be detected in blood serum in children who are as young as 6 months before the symptomatic stage of the disease (Atkinson, Eisenbarth and Michels, 2014). However, Daneman, 2006 argues that there is no evidence that suggests that the presence of these autoantibodies indicate the pathogenesis of IDDM. Furthermore, lipid and metabolite parameters such as decreased phosphatidylcholine, triglyceride and antioxidant ether phospholipids that are present in blood serum are additional markers that can be measured and included in the diagnostic criteria of IDDM (Atkinson, Eisenbarth and Michels, 2014).

1.1.5. Diabetes Mellitus: Animal Models

Streptozotocin (STZ) is an antibiotic that induces pancreatic destruction of insulin-producing islet β -cells (Furman, 2015). Relative to the amount of STZ used to induce glucotoxicity, STZ leads to rapid pancreatic B-cell mass loss through apoptosis and necrosis (Furman, 2015). In an *in vitro* analysis of rat-insulin producing cells (pancreatic beta INS-1E cells) that were incubated in 1mM STZ for 1 hour, it was observed that at high glucose concentrations (33 mM glucose), the preferred and dominate mode of death was apoptosis (40%) accompanied by 10% necrosis (Himpe *et al.*, 2016). At normal glucose concentrations (11 mM glucose), necrosis became the preferred and dominate mode of cell death (40% necrosis compared to 20% apoptosis) (Himpe *et al.*, 2016). Furthermore, as a nitric oxide (NO) donor, STZ mediates pancreatic β -cell DNA damage and repair by its alkylating activity (Himpe *et al.*, 2016). In a study where mice were injected with a single 200mg/kg dose of STZ, 1-5% of phosphorylated histones (a marker of DNA damage and repair) were detected in their insulin positive β -cells 11 days post STZ injection whereas, in their respective healthy controls, the phosphorylated histones were undetectable (Himpe *et al.*, 2016). Furthermore, STZ-induced diabetic rats and mic develop hyperglycaemia, polyuria and polydipsia. These symptoms are characteristic of type 1 diabetes mellitus (T1DM)

in humans. (Furman, 2015; Junod *et al.*, 1967). Furthermore, STZ diabetes mellitus is primarily induced in male rats and mice. Moreover, it has been documented previously that female mice and rats are less sensitive to STZ-induced islet β -cell toxicity when STZ is administered in low to moderate doses. For this reason, diabetes mellitus is primarily studied in male rats and mice (Furman, 2015).

1.1.5.1. Mice models of experimental diabetes

Various methods of experimental STZ diabetes have been used to induce diabetes mellitus in mice and rats (**Table 1:1**).

Table 1:1 Classification of rat and mice models of experimental STZ diabetes (Junod et al., 1967, 1969; Furman, 2015)

Animal model	STZ doses	Glucose levels (mmol/l)	Pathophysiology
Model 1 <i>Multiple low STZ doses in mice</i>	40 mg/kg 5 consecutive days	>16.7 to 33.3 measured 14 days post STZ injection	-insulin deficiency -delayed onset of hyperglycaemia -chronic β -cell inflammation -presence of Insulinitis -marked β -cell apoptosis -lymphocytic infiltration in β -cells
Model 2 <i>Single high STZ dose in mice</i>	200 mg/kg single STZ injection	>27.78 measured 10 days post STZ injection	-insulin deficiency - hyperglycaemia -severe β -cell necrosis
Model 3 <i>Single STZ dose in rats</i>	40-70 mg/kg single STZ injection	>13.9 to 33.3 measured 10 days post STZ injection	-insulin deficiency -hyperglycaemia - severe β -cell necrosis
Model 4 <i>Single STZ - Nicotinamide dose rat model</i>	40-70 mg/kg single STZ injection 60-230 mg/kg nicotinamide	>13.9 to 33.3 measured 10 days post STZ injection	-insulin deficiency - hyperglycaemia - β -cell necrosis
Model 5 <i>High-fat diet + single high STZ dose rat model</i>	40% to 60% fat content diet for 3 weeks 40mg/kg single STZ injection	>15 measured 10 days post STZ injection	-stable hyperglycaemia -insulin resistance -impaired insulin secretion -hyperinsulinemia

In a study by Junod *et al.*, 1969, the STZ method of inducing T1DM in mice was established by intraperitoneally injecting multiple low doses of STZ (40 mg/kg) over a period of 5 consecutive days (*Model 1*) (Furman, 2015). In this model of T1DM, it was noted that the mice developed a delayed onset of hyperglycaemia, accompanied by a minimal loss of β -cell function and morphological changes of the pancreatic islet cells such as chronic β -cell inflammation and insulinitis that are characteristic of human T1DM (Furman, 2015). However, in this animal model of T1DM, only about 50% of mice develop diabetes mellitus 3 weeks after STZ-injection (Furman, 2015). According to Junod *et al.*, 1969, this model of T1DM is more suited to investigating the underlying causes or pathogenesis of T1DM (Furman, 2015).

Alternatively, other investigators induce T1DM in mice by a single intraperitoneal injection of a high dose of STZ (200mg/kg) (*Model 2*) (Furman, 2015). In this model of T1DM, rapid necrosis-mediated β -cell destruction occurs and results in death of some of the mice within a period of 24 hours after STZ injection. According to Junod *et al.*, 1969, fatal hypoglycaemia occurs due to the sudden increase of insulin that is released from the damaged pancreatic islet cells. As a result, this mouse model of T1DM is more suited to test the antidiabetic effects of therapeutic agents (Furman, 2015).

1.1.5.2. Rat models of experimental diabetes

Rat models of diabetes mellitus have also been established (**Table 1:1**). A rat model of T1DM, induced by administering a single high dose STZ, is the rat model that is most frequently used by investigators to evaluate the pathogenesis of T1DM and the effectiveness of antidiabetic agents (*Model 3*) (Furman, 2015). In this rat model of T1DM, male Wistar or Sprague-Dawley rats are injected intraperitoneally with a single dose 40-70mg/kg STZ. According to Junod *et al.*, 1969, a single dose of 65 mg/kg STZ is the average dose used by most investigators. Moreover, it has been noted that an intravenous injection performed under anaesthesia has a higher success rate of inducing diabetes compared to the method of intraperitoneally administering a single dose of STZ to a rat model (Furman, 2015).

With respect to type 2 diabetes mellitus (T2DM), a rat model of T2DM has been previously established using the method of administering a single STZ dose administered concurrently with nicotinamide (*Model 4*) (Furman, 2015). Nicotinamide

is a vitamin B3 isoform that reduces the amount of β -cell loss and the diabetogenic effects caused by STZ (Furuman, 2015). In addition, nicotinamide is a compound used to induce insulin-deficient (and not insulin-resistant) T2DM (Furuman, 2015). In this rat model, about 60% of β -cell function is lost and the animals develop moderate hyperglycaemia. Furthermore, at 2-3 weeks after STZ injection, some of the rat models revert to normoglycaemia although they remain with an impaired glucose tolerance (Furuman, 2015). Alternatively, the method of administering a high fat diet in combination with an STZ dose is used by some investigators to establish a T2DM rat model that closely resembles human T2DM (*Model 5*) (Furuman, 2015). In this rat model of T2DM, a high fat diet that has a 40% to 60% fat caloric intake is consumed by the animals for 3 weeks prior to STZ injection. A diet rich in fat content induces insulin resistance, while a single (40mg/kg) dose of STZ induces impaired insulin secretion and hyperinsulinemia (Furuman, 2015). In another study, two lower STZ doses (30mg/kg) were administered intraperitoneally to the animals at a weekly interval in the place of a single STZ dose (Furuman, 2015). Compared to the STZ – nicotinamide rat model of insulin-deficient T2DM (*Model 4*), this high fat diet – STZ dose rat model of T2DM (*Model 5*) is an animal model that closely resembles the pathophysiology of human T2DM (Furuman, 2015).

1.2. Endothelial Dysfunction: A macrovascular and microvascular complication of diabetes mellitus

The impact of diabetes mellitus on the endothelium, through hyperglycaemia, increased intracellular free fatty acid levels, insulin resistance and deficiency, cause endothelial dysfunction (ED) which in turn leads to cardiovascular diseases such as hypertension, atherosclerosis and myocardial infarction (**Figure 1:4**) (Sena, Pereira and Seica, 2013).

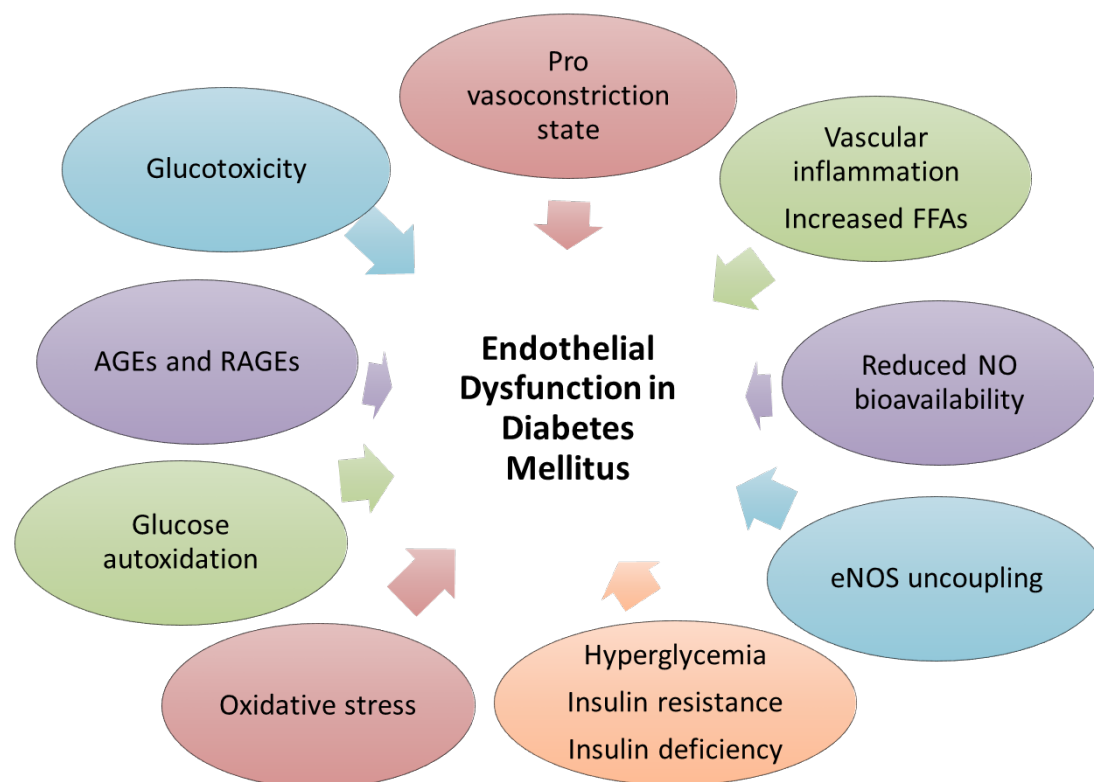


Figure 1:4 Causes of endothelial dysfunction in diabetes mellitus (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seíça, 2013).

Moreover, the vicious cycle of events that the metabolic milieu has on endothelial activity has qualified diabetes mellitus as a cardiovascular disease that leads to the progression of ED and associated micro- and macrovascular complications (Sena, Pereira and Seíça, 2013). Therefore, in order to develop treatments that may potentially ameliorate the effects that the metabolic milieu has on the vasculature, it is imperative to understand the endothelium and to investigate the mechanisms involved in mediating endothelial dysfunction in a diabetic setting (Sena, Pereira and Seíça, 2013).

Endothelial dysfunction (ED) is described as any functional impairment and irregular activity of the endothelium that is characterized by a dominant vasoconstrictive state of the endothelium (Deanfield, Halcox and Rabelink, 2007; Sena, Pereira and Seíça, 2013). In diabetes mellitus, this pathophysiological state of the endothelium is caused by several mechanisms including an imbalance in the production of vasodilative and vasoconstrictive molecules, vascular inflammation, reduced nitric oxide (NO) bioavailability and endothelial nitric oxide synthase (eNOS) uncoupling. In addition, it also includes interactions between hyperglycaemia and oxidative stress such as

glucose autoxidation, increased advanced glycation end products (AGEs) and receptor for AGEs (RAGEs) expression, glucotoxicity and other factors that are not well-established (**Figure 1:4**) (Flammer and Lüscher, 2010).

1.2.1. Endothelium

The endothelium is a thin layer of mononuclear cells that line the lumen of all blood vessels. The blood vessel wall is made up of three layers of tissue, the outmost tunica adventitia, the tunica media and the innermost endothelium intima (**Figure 1:5**) (Sena, Pereira and Seiça, 2013).

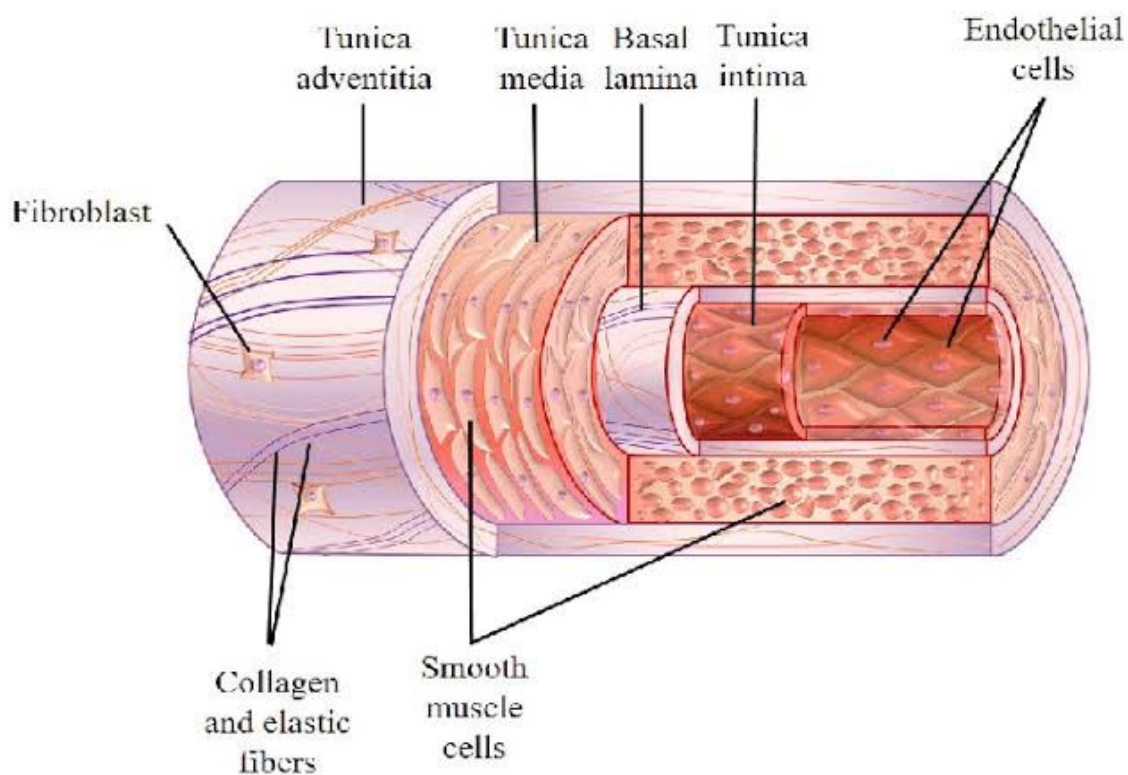


Figure 1:5 Basic structure of a blood vessel. (Ng et al., 2018).

The tunica adventitia primarily consists of connective tissues while the underlying tunica media consists of connective tissue, polysaccharide substances, elastic fibres and vascular smooth muscle cells (Sena, Pereira and Seiça, 2013). As the innermost layer, the endothelium serves as a physical barrier between the vessel lumen and the blood circulating within (Sena, Pereira and Seiça, 2013). For a very long time, it was believed that the endothelium only functioned merely as a metabolically inert physical barrier that inhibits blood from directly interfering with the underlying tissues of the

vasculature and the body (Deanfield, Halcox and Rabelink, 2007; Sena, Pereira and Seïça, 2013). However, in the 1980's Furchgott and Zawadzki discovered that without the endothelium vasorelaxation of the isolated rabbit aorta in response to acetylcholine was inefficient (Vallance and Chan, 2001). They also showed that the acetylcholine-mediated vasorelaxation of the isolated rabbit aorta was dependent on endothelial cells and an endothelium derived relaxation factor (EDRF) now known as nitric oxide (Desk, Williams and Health, 1983). Since then, it has become known that the endothelium is a metabolically active tissue that is necessary to perform various endocrine, paracrine and autocrine actions that are crucial for the maintenance of vascular homeostasis (Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seïça, 2013).

Because of the pioneering experiments on vascular function conducted by Furchgott and Zawadzki, the role of the endothelium in maintaining vascular homeostasis was the first of its multiple functions that was discovered (Deanfield, Halcox and Rabelink, 2007). The endothelium regulates vascular tone by secreting vasoactive molecules such as nitric oxide (NO), prostacyclin (PGI₂) and endothelium derived hyperpolarisation factors (EDHF) that stimulate vasorelaxation (Sena, Pereira and Seïça, 2013). In the same way, the endothelium secretes endothelin (ET), angiotensin II (ang-II) and reactive oxygen species (ROS) amongst others that stimulate the vasoconstriction action of blood vessels (Deanfield, Halcox and Rabelink, 2007). It is through a regulated interchange of vasorelaxation and vasocontraction that the endothelium facilitates blood flow and maintains vascular tone and homeostasis (Deanfield, Halcox and Rabelink, 2007; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seïça, 2013; Vallance and Chan, 2001).

1.2.2. Vascular Homeostasis

The vasorelaxation of blood vessels is mainly mediated by nitric oxide (NO). The endothelium secretes nitric oxide which diffuses to the vascular smooth muscle cells of the intima media in response to shear stress (a physical stimulus caused by circulating blood) and chemical stimuli, such as acetylcholine, bradykinin and serotonin (Sena, Pereira and Seïça, 2013). Once in the smooth muscle cells, NO activates soluble guanylate cyclase to secrete high levels of cyclic guanosine-3,5-monophosphate (cyclic GMP) that induces cyclic GMP-mediated vasodilation of the

blood vessel and increases blood flow (Deanfield, Halcox and Rabelink, 2007; Förstermann and Sessa, 2012; Sena, Pereira and Seïça, 2013). Independent of NO, the vasodilation of blood vessels particularly that of the microvasculature, is mediated by prostacyclin (PGI₂), an endothelium-derived relaxation factor, and by the hyperpolarisation of vascular smooth muscle cells (Sena, Pereira and Seïça, 2013). Prostacyclin (PGI₂) is a metabolite of arachidonic acid that is synthesized by cyclooxygenase-1 (COX-1) (Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Like nitric oxide, PGI₂ is secreted in response to shear stress and diffuses to the vascular smooth muscle layer where it stimulates the upregulation of cyclic adenosine monophosphate (cyclic AMP) and cyclic AMP-mediated vasodilation (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). In microcirculation, particularly the smaller arteries, endothelium derived hyperpolarisation factors (EDHF) such as potassium ions, AMP, C-type natriuretic peptide, cytochrome-derived products and reactive oxygen species amongst others, stimulate vasodilation by hyperpolarising the vascular smooth muscle cells (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). This mechanism of vasodilation persists in a state of endothelial dysfunction where PGI₂ and NO bioavailability is diminished (Sena, Pereira and Seïça, 2013).

The vasoconstricting action of blood vessels is mediated by vasoconstricting substances such as endothelin, Ang-II, inflammatory cytokines, ROS, prostanoids, thrombin and oxidised low-density lipoproteins amongst others (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seïça, 2013). These vasoconstrictors are produced and secreted by the endothelium in equilibrium to the vasodilating substances (NO, PGI₂, and EDHFs) (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seïça, 2013). When the equilibrium between vasorelaxation and vasoconstriction is lost, where the vasoconstriction state dominates, endothelial dysfunction occurs (Hadi, Carr and Al Suwaidi, 2005).

A disequilibrium in vasorelaxation and vasoconstriction has been documented in diabetes mellitus (Deanfield, Halcox and Rabelink, 2007). (**Figure 1:6**).

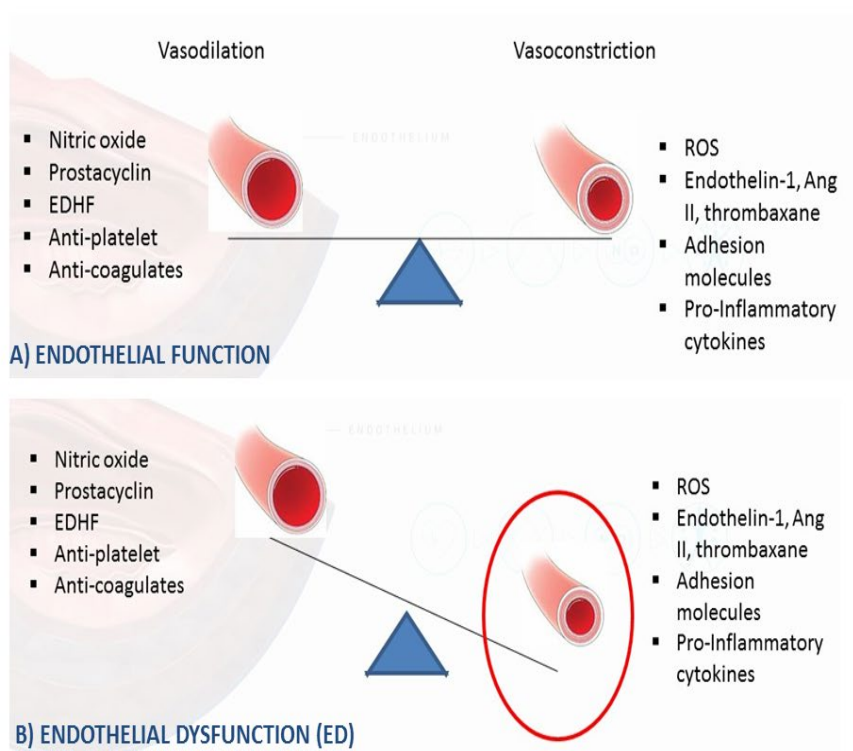


Figure 1:6 Schematic representation of vascular homeostasis in a healthy (A) and a pathophysiological state (B) of the endothelium (Flammer and Lüscher, 2010).

A reduction in acetylcholine (ACh) and NO-mediated vasodilation of thoracic aortas was evident in the streptozotocin-injured aortas of Sprague Dawley rats (Rodríguez-Mañas *et al.*, 1998). The rats were injected with streptozotocin (60mg/kg bw STZ) and vascular reactivity of the aorta was assessed 8 weeks after diabetes was induced (Rodríguez-Mañas *et al.*, 1998). Furthermore, the significant decrease in vasodilation was observed in those rats that presented with glycosylated haemoglobin levels higher than 7.5% (Rodríguez-Mañas *et al.*, 1998). Thus, these findings show that chronic hyperglycaemia (represented by high glycosylated HBA1c levels) is largely responsible for the impaired vascular reactivity that is evident in the vasculature of diabetic subjects (Rodríguez-Mañas *et al.*, 1998). In a similar 8-week observational study, ACh- and NO-mediated vasodilation was also impaired in the aortas of STZ-induced Sprague Dawley rats. Moreover, significantly high levels of the vasoconstrictor thromboxane B₂ were evident in these STZ-induced diabetic animals (Csanyi *et al.*, 2007).

1.2.3. Vascular Inflammation

In addition to maintaining vascular homeostasis, one of the crucial functions of the endothelium is its role in maintaining vessel integrity, preventing chronic vascular inflammation and atherothrombotic events that lead to the development of cardiovascular diseases such as atherosclerosis, myocardial infarction and hypertension amongst others (Deanfield, Halcox and Rabelink, 2007; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seica, 2013; Vallance and Chan, 2001). Vascular inflammation is evident in diabetes mellitus and is a condition that is a key role player in the development of micro and macrovascular complications (Sena, Pereira and Seica, 2013). High levels of plasma-soluble intracellular adhesion molecules, monocyte interleukin 6 (IL-6), sICAM, CD40 ligand (CD40L), monocyte interleukin 1 β and C-reactive protein (CRP) were detected in the fasting blood samples of IDDM patients when compared to the healthy controls (Devaraj *et al.*, 2006). Similarly, high levels of CRP and IL-6 were detected in the blood analyses of IDDM female participants who participated in the EURODIAB Prospective Complications Study (Chaturvedi *et al.*, 2001). Likewise, significant increases in CRP, sICAM-1 and soluble-vascular intracellular adhesion molecule (vICAM-1) were observed in the blood parameters of IDDM patients who participated in a 20-year longitudinal cohort study (Ferreira *et al.*, 2018). These findings show that an increased inflammatory activity is present in the vasculature of diabetic individuals. Thus, the high prevalence of low-grade vascular inflammation in diabetic patients confers IDDM as a high risk factor for atherothrombosis, atherosclerosis and the progression to ED (Chaturvedi *et al.*, 2001).

1.2.4. Nitric Oxide Synthase

The vasoactive molecule, nitric oxide (NO) plays a pivotal role in maintaining vascular homeostasis, suppressing vascular inflammation and ultimately preventing endothelial dysfunction in diabetes mellitus (Förstermann and Sessa, 2012). This endothelial derived relaxation factor (EDRF) is a very important messenger molecule that is synthesized by the dimeric enzyme Nitric Oxide Synthase (NOS) which exists in three isoforms; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Förstermann and Sessa, 2012).

1.2.4.1. Endothelial NOS

Endothelial NOS is the most important NO-producing enzyme in the vasculature and is essential in maintaining vascular homeostasis in diabetes mellitus. Though it has been identified in cardiomyocytes, nerve cells, syncytio-trophoblasts of the human placenta and tubular epithelial cells of the kidney, eNOS is primarily expressed in the caveolae of endothelial cells (Förstermann and Sessa, 2012). Moreover, eNOS is made up of two identical monomers that are kept in its dimeric form by a haem group. In the caveolae, eNOS is bound to caveolin-1 which keeps it in its inactive form (Sena, Pereira and Seiça, 2013). In the presence of elevated calcium levels, calmodulin binds to eNOS and causes caveolin-1 to dissociate and facilitate eNOS activation. Additionally, heat shock protein 90 (HSp90) binds to eNOS and acts as an allosteric modulator that mediates the displacement of its inhibitor caveolin-1 (Förstermann and Sessa, 2012). However, in the absence of calcium and the binding of calmodulin and HSp90, fluid shear stress against the endothelium serves as a mechanism of eNOS activation (Förstermann and Sessa, 2012). This mechanism is mediated by the phosphorylation of eNOS on specific serine, threonine and tyrosine residues by various protein kinases such as PKB/Akt. In humans, Akt1 phosphorylates eNOS on the phosphorylation site ser1177 (Förstermann and Sessa, 2012). Once activated, eNOS generates NO from the substrate L-arginine, and the co-substrates molecular oxygen and NADPH. In addition to this, FAD, FMD and BH4 act as co-factors that mediate the catalytic activity of eNOS (Förstermann and Sessa, 2012). Electron transfer from NADPH and the co-factors to the haem group of the enzyme leads to the reduction of oxygen to a hydroxyl group that hydroxylates and oxidises L-arginine. As a result, the oxidised L-arginine is catalysed to L-citrulline and NO is produced (Förstermann and Sessa, 2012).

Under normal physiological conditions, eNOS generates enough levels of NO. However, pathophysiological conditions such as atherosclerosis, hypertension, hyperglycaemia and oxidative stress results in the generation of high levels of reactive oxygen species (ROS), oxidative stress, eNOS uncoupling, reduced NO bioavailability and ultimately endothelial dysfunction (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seiça, 2013). eNOS uncoupling and reduced NO bioavailability is one of the mechanisms that lead to endothelial dysfunction in the vasculature of diabetic subjects (Deanfield, Halcox and Rabelink,

2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Correa and Alfieri, 2003, assessed plasmatic NO and urinary NO metabolites (nitrate and nitrite) in IDDM patients. A significant reduction in plasmatic NO levels was evident in the diabetic subjects when compared to their healthy non-diabetic counterparts (Correa and Alfieri, 2003). Moreover, the IDDM individuals who presented with retinopathy or nephropathy had significantly reduced plasmatic NO levels when compared to IDDM patients without microvascular complications (Correa and Alfieri, 2003).

1.2.5. eNOS Uncoupling

eNOS uncoupling is the conversion of eNOS from an NO-producing enzyme to one that generates superoxide anions (**Figure 1:7**) (Sena, Pereira and Seïça, 2013).

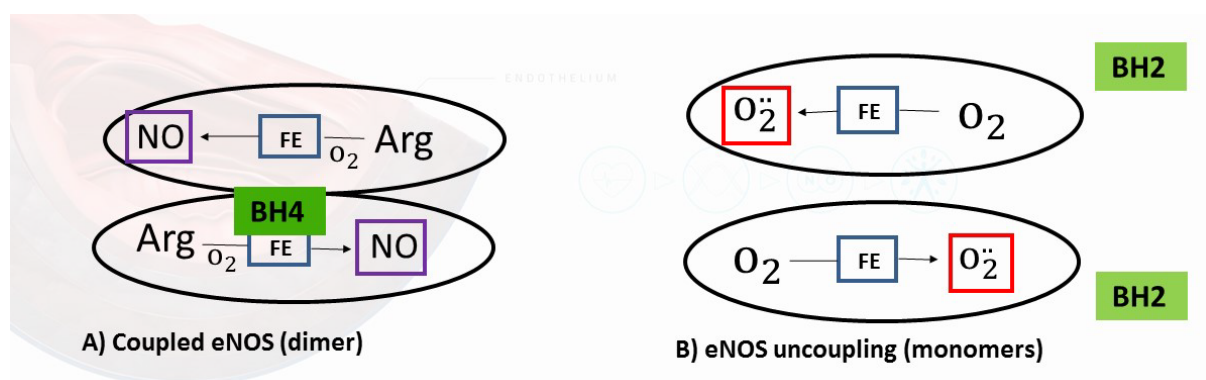


Figure 1:7 A) functional eNOS generates nitric oxide. B) Uncoupled eNOS generates superoxide anions and other types of ROS (Flammer and Lüscher, 2010).

The uncoupling of eNOS is caused by oxidative stress and high levels of circulating ROS in the vasculature. High amounts of circulating ROS lead to peroxynitrite-mediated oxidation and depletion of the cofactor BH4 (Sena, Pereira and Seïça, 2013). In the absence of the cofactor BH4, the dimeric structure of eNOS uncouples and uncoupled eNOS generates the harmful reactive oxygen species superoxide (Deanfield, Halcox and Rabelink, 2007; Förstermann and Sessa, 2012; Sena, Pereira and Seïça, 2013). Moreover, in a state where the substrate L-arginine is deficient, uncoupled eNOS generates another form of ROS termed hydrogen peroxynitrite (Deanfield, Halcox and Rabelink, 2007). Furthermore, during oxidative stress, increases in methylarginine or ADMA levels occur and its accumulation contributes to eNOS uncoupling (Sena, Pereira and Seïça, 2013). As a result, ROS production that is mediated by eNOS uncoupling increases and NO production by eNOS decreases.

With insufficient levels of circulating NO and functional eNOS, the endothelium is unable to effectively carry out its multiple functions that maintain vascular homeostasis and quiescence (Deanfield, Halcox and Rabelink, 2007). Thus, endothelial dysfunction prevails. eNOS uncoupling in the early stages of diabetes mellitus occurs as a result of oxidative stress (Joshi and Woodman, 2012). In Sprague Dawley rats that were induced with STZ diabetes, increments in superoxide anion production and NADPH-oxidase 2 expression were observed 6 weeks after STZ induction (Joshi and Woodman, 2012). A reduction in the dimer-monomer ratio of eNOS expression was observed as a consequent of increased superoxide anion production and oxidative stress (Joshi and Woodman, 2012).

1.2.6. Oxidative Stress

High amounts of circulating ROS and oxidative stress is a consequence of the impact that the diabetic metabolic milieu (hyperglycaemia, increased intracellular FFA metabolism, insulin resistance and deficiency) has on the endothelial wall and vasculature of diabetic subjects (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Moreover, increased oxidative stress and ROS production is a pivotal mediator of the mechanisms and signalling pathways that lead to endothelial dysfunction (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Superoxide anions, hydrogen peroxide, peroxy radicals as well as the highly reactive hydroxyl radical, amongst others, are some of the ROS molecules that are formed from the reduction of molecular oxygen and are highly reactive participants in the progression to ED in diabetes mellitus (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Under normal physiological conditions, various types of ROS function as important messengers or signalling molecules that facilitate cellular functions and regulate cell signalling pathways. One of the most noted functions of ROS is its role in mediating the cytotoxic effects that phagocytic cells induce against harmful foreign microorganisms and infections (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). Moreover, redox signalling is necessary for facilitating important cellular processes such as apoptosis, cell cycle growth control, mitogenic activity and the activation or inactivation of various enzymes (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). However, in the vasculature, diabetes mellitus exacerbates

ROS production through various mechanisms including eNOS uncoupling, glucose autoxidation, the formation of advanced glycation end-products (AGEs), increased expression of receptor for AGEs (RAGEs) and glucotoxicity (Förstermann and Sessa, 2012; Sena, Pereira and Seíça, 2013).

Increased ROS production and oxidative stress is detrimental to cells and impairs various cellular functions of the vasculature (Maritim, Sanders and Watkins, 2003; Sena, Pereira and Seíça, 2013). Lipid peroxidation of lipid membranes, DNA damage, and damage to cellular proteins and nucleic acids are some of the detrimental effects that high levels of ROS directly have on all cell types including endothelial cells (Maritim, Sanders and Watkins, 2003; Sena, Pereira and Seíça, 2013). With regards to lipid peroxidation, hydrogen peroxide (a form of ROS that is made up of two superoxide anions) facilitates direct damage of membrane lipids by interacting with iron and copper transition metals to form reactive malondialdehydes (Maritim, Sanders and Watkins, 2003). Similarly, unstable peroxy radicals mediate lipid peroxidation by scavenging electrons from membrane lipids (Maritim, Sanders and Watkins, 2003). Thus, the quantification of thiobarbituric acid reactive substances (TBARS), a by-product of lipid peroxidation, is an indicator of the rate of lipid peroxidation in cells. Moreover, elevations in TBARS are evident in streptozotocin-induced type 1 diabetes mellitus (Maritim, Sanders and Watkins, 2003).

1.2.7. Glucose Autoxidation

One of other the mechanisms by which oxidative stress leads to the development of endothelial dysfunction in diabetes mellitus is glucose autoxidation (**Figure 1:8**) (Maritim, Sanders and Watkins, 2003).

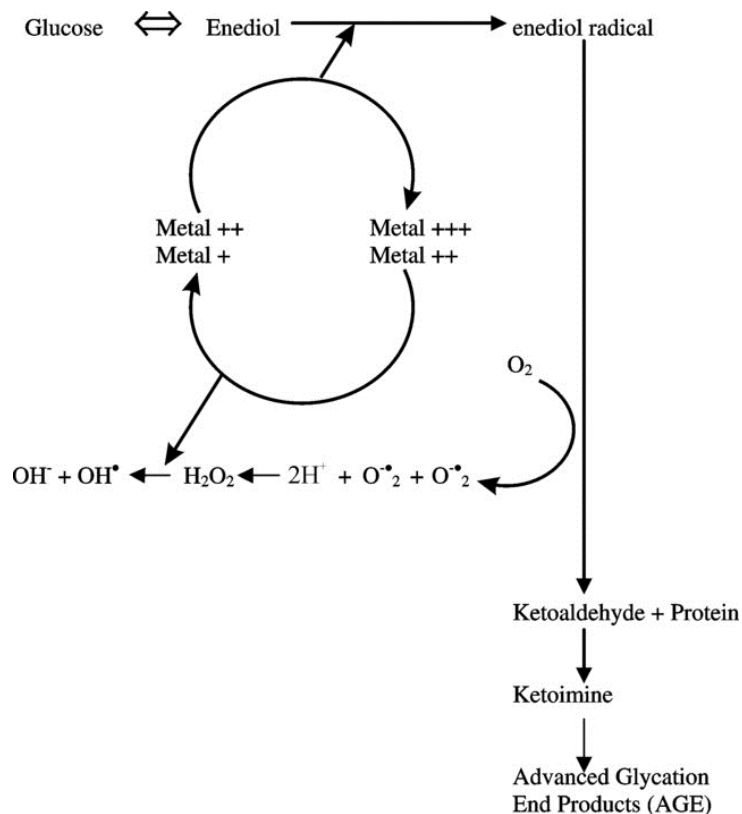


Figure 1:8 Glucose autoxidation: the spontaneous oxidation of glucose leads to the generation of ROS (Singh et al., 2009).

Glucose autoxidation is the spontaneous oxidation of glucose in the presence of molecular oxygen. This spontaneous biochemical reaction is thought to be the largest source of ROS in diabetes mellitus (Maritim, Sanders and Watkins, 2003). In the presence of transition ion metals, the enediol form of glucose is oxidised to an enediol radical anion. Moreover, the enediol radical anion is catalysed into highly reactive ketoaldehydes and superoxide anion molecules (Maritim, Sanders and Watkins, 2003). The resultant superoxide anions are further catalysed into hydrogen peroxide reactive molecules by the catalytic enzyme superoxide dismutase. When sufficient transition metal ions are present, hydrogen peroxide (H₂O₂) is spontaneously catalysed into highly reactive hydroxyl (OH⁻) radicals that damage endothelial cells (Maritim, Sanders and Watkins, 2003). Additionally, in the presence of excess amounts of NO, superoxide anions react with NO to form the ROS molecule peroxynitrite (Maritim, Sanders and Watkins, 2003; Förstermann and Sessa, 2012).

1.2.8. Advanced Glycation End-Products (AGEs)

Hyperglycaemia structurally impairs endothelial cells through the build-up of AGEs and their receptors (RAGE) in the endothelial wall. Advanced glycation end-products or AGEs are sugar-derived modifications or products that are produced through a biochemical reaction called protein glycation (Brownlee, M.D, 1995). Protein glycation is the nonenzymatic glycosylation or condensation of glucose molecules with the amine groups of nucleic acids and tissue proteins (Maritim, Sanders and Watkins, 2003). This biochemical reaction involves the formation of unstable Schiff bases that are rearranged into 1-amino-1deoxyketoses or Amadori products (Brownlee, M.D, 1995; Sena, Pereira and Seiça, 2013). Subsequently, these Amadori products undergo various dehydration reactions that lead to the formation of AGEs which are irreversibly attached to various tissue proteins through covalent bond interactions (Maritim, Sanders and Watkins, 2003). In diabetes mellitus, AGEs are formed from excess sugar molecules such as glucose, fructose and lactose that are ingested from postprandial activity and from intracellular sugars such as glucose-6-phosphate and glyceraldehyde-3-phosphate (Brownlee, M.D, 1995). Intracellular sugar-derived AGEs interact with many tissue proteins such as haemoglobin in erythrocytes, proteins of endothelial cells and basic fibroblast growth factor (bFGF); a protein that modulates mitogenic activity. The modification of haemoglobin and bFGF by AGEs leads to the reduced bioavailability of these target proteins in diabetes mellitus. Moreover, it has been noted that intracellular AGE formation increased by 13.8 folds in endothelial cells that were cultured in glucose-containing media (Brownlee, M.D, 1995).

An increment in glucose-derived AGE formation is caused by hyperglycaemia in diabetic patients. The build-up of glucose-derived AGEs in diabetes mellitus affects vascular function in various mechanisms such as the binding of AGEs to RAGEs, protein cross-linking, ROS formation, alterations in extracellular matrix compositions and in cell-matrix interactions (Brownlee, M.D, 1995; Sena, Pereira and Seiça, 2013). The protein crosslinking (mediated by AGEs) of collagen to plasma low-density lipoprotein (LDL) and immunoglobulin G results in vascular stiffness and loss of vascular elasticity (Brownlee, M.D, 1995). Similarly, the interaction of AGEs with various components of the extracellular matrix interferes with the functionality of the extracellular matrix and various matrix-cell mediated interactions (Brownlee, M.D, 1995; Sena, Pereira and Seiça, 2013). Such an alteration is evident in endothelial cells

where the interaction of adhesion molecules with collagen IV decreases as a result of an AGE-mediated alteration of the collagen's cell-binding domains (Brownlee, M.D, 1995). Moreover, in the extracellular matrix of large blood vessels of diabetic rats, it was discovered that the loss of blood vessel elasticity decreased as a result of structural and functionality changes caused by the presence of AGEs (Brownlee, M.D, 1995).

1.2.9. Receptor for AGE (RAGE)

Another mechanism of hyperglycaemia and oxidative stress mediated endothelial cell destruction and damage in diabetes mellitus is the increased expression of Receptor for AGEs (RAGE). RAGEs are AGE-binding receptors have been identified in macrophages, monocytes and endothelial cells (**Figure 1:9**) (Brownlee, M.D, 1995; Sena, Pereira and Seica, 2013).

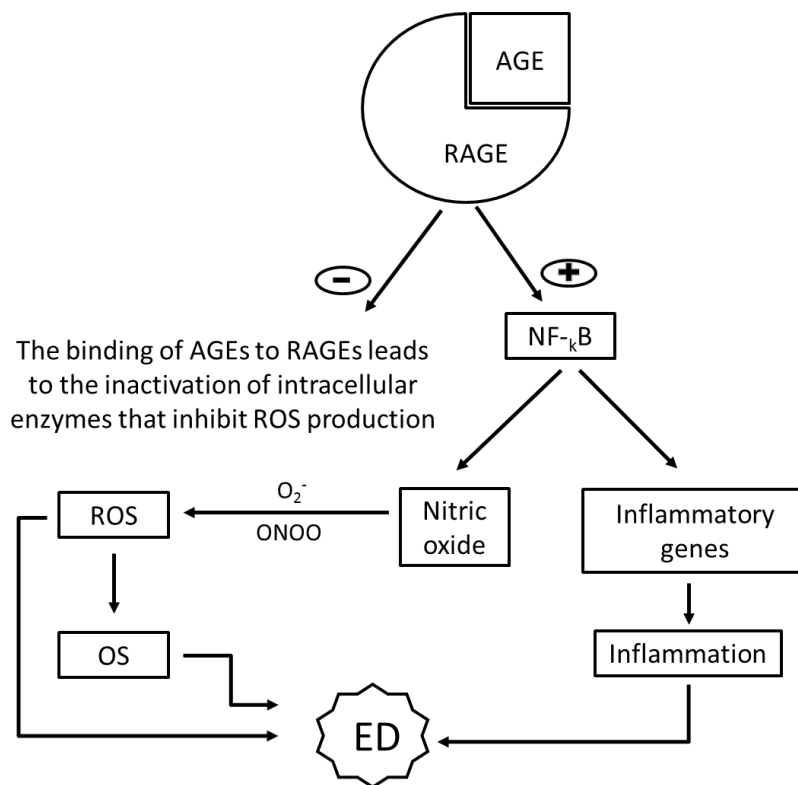


Figure 1:9 Schematic representation of the downstream mechanisms of RAGE-AGE interactions that contribute to endothelial dysfunction in a diabetic setting (Brownlee, M.D, 1995; Maritim, Sanders and Watkins, 2003).

In endothelial cells, the binding of AGEs to RAGEs causes the inactivation of important intracellular enzymes that prevent ROS production and oxidative stress (Maritim,

Sanders and Watkins, 2003). Thus, AGE-binding to RAGEs facilitates oxidative stress-mediated cell signalling. For example, the oxidative stress that results from the interaction of AGEs with their cellular receptors on endothelial cells interferes with the transcription factor NF κ B and its role in the regulation of the expression of genes such as thrombomodulin, tissue factor and endothelin-1 involved in cell-injury signalling (Brownlee, M.D, 1995). The activity of thrombomodulin, a protein involved in the prevention of blood coagulation, decreases in the presence of AGE-bound receptors (Brownlee, M.D, 1995). Moreover, the binding of AGEs to their RAGEs upregulate the expression of the vasoconstrictor endothelin-1 (Brownlee, M.D, 1995; Sena, Pereira and Seiça, 2013). Thus, RAGEs and their receptor-binding AGEs, impair vascular function by mediating, macrophage-mediated vascular inflammation, thrombosis and vasoconstriction in the vessel wall (Brownlee, M.D, 1995; Sena, Pereira and Seiça, 2013).

1.2.10. Glucotoxicity

Glucotoxicity refers to the detrimental effects that high blood glucose concentrations have on the structure and function of islet β -cells thus leading to the development of insulin resistance and the associated macro- and microvascular complications (Kaiser, Leibowitz and Nesher, 2003). In diabetes mellitus, glucotoxicity also plays a role in the development of chronic low-grade inflammation and atherosclerosis in the vasculature (Sena, Pereira and Seiça, 2013). The transcription factor NF κ B regulates the expression of genes that mediate inflammation and atherosclerotic events in macrophages, endothelial and vascular smooth muscle (Sena, Pereira and Seiça, 2013). Due to glucotoxicity, the expression of NF κ B in endothelial cells increases and contributes to the increased expression of adhesion molecules, chemo-attractants and inflammatory cytokines that collectively exacerbate the chronic inflammatory responses observed in endothelial dysfunction (Sena, Pereira and Seiça, 2013).

1.2.11. Endothelial Dysfunction in STZ Rat Model Diabetes Mellitus

A number of scientists have documented the effects that hyperglycaemia has on endothelial function in diabetic subjects (Joshi and Woodman, 2012; Rodríguez-Mañas *et al.*, 2003; Srinivasan *et al.*, 2004). Human aortic endothelial cells that were cultured in 25 mmol/l glucose-enriched media for more than 3 days exhibited increased activator protein-1 (AP-1) activity and reduced eNOS mRNA and eNOS

activity (Brownlee, M.D, 1995). Similarly, eNOS mRNA expression was down-regulated in aortic endothelial cells of 12-week old diabetic db/db mice (Srinivasan *et al.*, 2004). Srinivasan *et al.*, 2004, hypothesized that superoxide anions generated through mitochondrial respiration resulted in oxidative stress which in turn stimulated activator protein-1 (AP-1) expression and DNA-binding. Thus, these findings show that ROS is generated through the mitochondrial electron transport chain (mETC) and contributes to the oxidative stress that induces ED in diabetes mellitus (Srinivasan *et al.*, 2004). Moreover, NADPH oxidase (Nox 2) has also been identified as a major source of superoxide anion production in STZ-injured diabetic Sprague Dawley rats. Increased oxidative stress characterized by increases in NADPH oxidase (Nox 2) and superoxide anions was present in the 6-week STZ-injured aortas of diabetic rats (Joshi and Woodman, 2012). Likewise, oxidative stress was apparent in the aortic cylindrical segments of streptozotocin- induced diabetic Sprague-Dawley rats. Thus, treatment with superoxide dismutase (SOD) improved ACh-mediated vasodilation of the aortic segments (Rodríguez-Mañas *et al.*, 2003).

1.3. Hypertension in Diabetes Mellitus

Hypertension is a vascular complication that can either exist in isolation or as a comorbidity with diabetes mellitus (Mutiyambizi *et al.*, 2017). Hypertension frequently occurs with diabetes mellitus resulting in an overlap that exists between the cardiovascular implications that both these morbidities have on endothelial function and the vasculature (Cheung and Li, 2012). According to EURODIAB, hypertension is more prevalent (83%) in diabetes mellitus type 2 (NIDDM) than it is in diabetes mellitus type 1 (IDDM) (Perin, Maule and Quadri, 2001). In NIDDM, hypertension is usually present at diagnosis and is associated with insulin resistance and /or hyperinsulinemia, dyslipidaemia, impaired glucose tolerance and obesity. In contrast, in IDDM, hypertension mostly surfaces in the disease state of the metabolic disorder and is characterized by nephropathy or chronic renal failure. Of the 24% that develop hypertension in IDDM, 65% present with clinical nephropathy, 30% have microalbuminuria and about 19% have normal urinary albumin excretion and kidney function (Perin, Maule and Quadri, 2001).

1.3.1. The Renin-Angiotensin Aldosterone System (RAAS)

The renin-angiotensin aldosterone system (RAAS) is one of the mechanisms that play a significant role in the development of hypertension and ED in a diabetic setting (Cheung and Li, 2012).

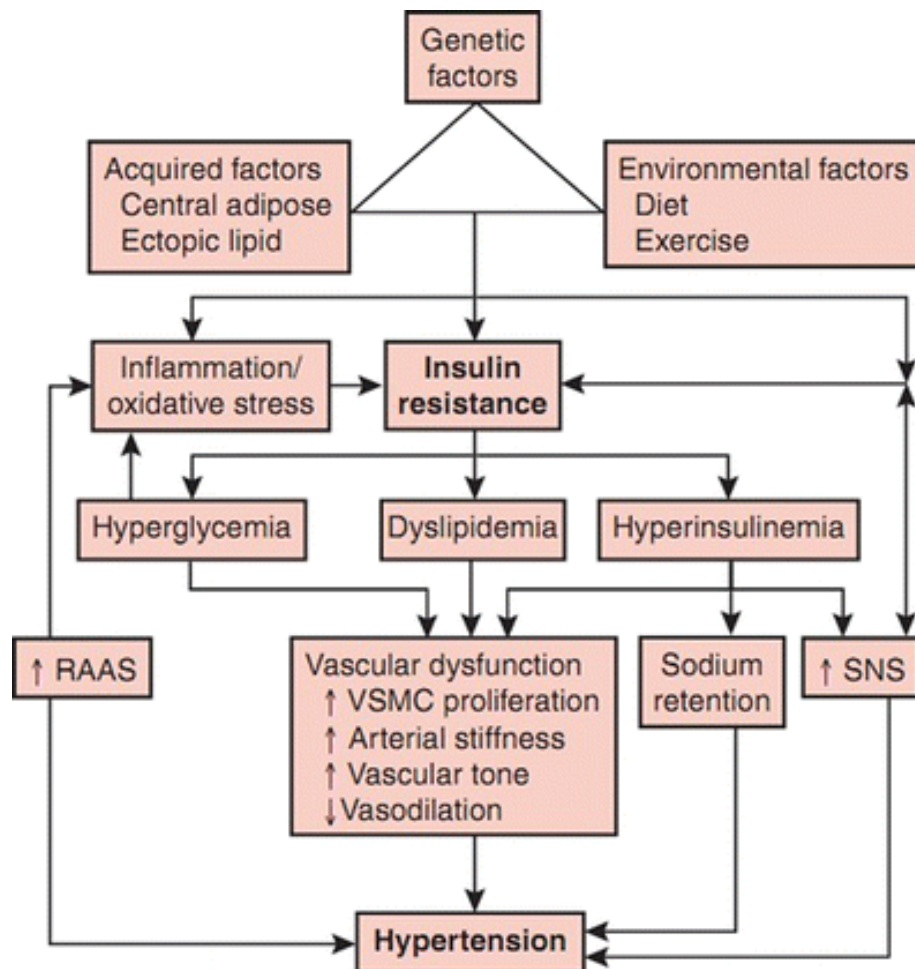


Figure 1:10 Pathophysiological pathways that cause hypertension in diabetes mellitus (Cheung and Li, 2012).

Angiotensin-converting enzyme (ACE) is an enzyme that facilitates the generation of the vasoconstrictor Angiotensin II (Ang-II) and is largely expressed in coronary arteries. High Ang-II activity in coronary arteries elicits high blood pressure and ED through various mechanisms (Cheung and Li, 2012). Angiotensin II (Ang-II) contributes to ED and vascular injury in coronary arteries by triggering vascular oxidative stress through the upregulation of NADPH oxidases and the downregulation of pro-inflammatory cytokines such as $\text{NF}\kappa\beta$, thus resulting in increased production of ROS (Cheung and Li, 2012). Furthermore, as a vasoconstrictor, Ang-II upregulates

blood pressure in coronary arteries in addition to down-regulating peroxisome proliferator-activated receptor (PPAR) which is responsible for lowering blood pressure (Cheung and Li, 2012).

In NIDDM, hypertension results as a consequence of insulin resistance induced hyperactivation of the SNS. High levels of insulin in the body (insulin resistance and/or hyperinsulinemia) activate the SNS to directly stimulate sodium retention and to concomitantly stimulate increased peripheral resistance via the baroflex activity of the central nervous system (Perin, Maule and Quadri, 2001). Consequently, hypertension develops as a consequence of the increased heart rate, cardiac minute output, sodium retention and peripheral resistance that results from SNS hyperactivation (Perin, Maule and Quadri, 2001). Likewise, in an STZ-induced rat model of IDDM, it was noted that glucose feeding or hyperglycaemia alone did not cause hypertension or alter basal superoxide production in the 4-week injured aortas (Midaoui, Wu and de Champlain, 2002). In contrast, increased blood pressure and basal superoxide production were observed in the insulin resistant (NIDDM) rats treated with exogenous insulin in addition to glucose feeding following 4 weeks of treatment (European Society of Hypertension and International Society of Hypertension, 1983).

1.3.2. Genetic and Environmental Influence

Genetic factors is one of the mechanisms that gives rise to the development of hypertension in diabetes mellitus (Cheung and Li, 2012). It has been noted that genetic variants that occur in genes that encode for blood pressure regulation proteins such as angiotensinogen, α -adducin adrenomedullin and apolipoprotein give rise to a genetic predisposition for hypertension in susceptible individuals (Cheung and Li, 2012). Furthermore, various single nucleotide polymorphisms (SNPs) that are used as genetic markers for identifying individuals who are susceptible to developing hypertension are also found in individuals who are genetically susceptible to developing diabetes mellitus (Cheung and Li, 2012). Additionally, in NIDDM, obesity is an independent risk factor for the development of hypertension. In previous observational studies of the growing epidemic, obesity ranked highest amongst risk factors that predispose diabetic individuals to hypertension (Cheung and Li, 2012). In addition to physical stressors, mental stress is a major contributor and risk factor for hypertension. Though it is not fully understood how mental stress (e.g. work stress,

depression, anxiety) induces disturbances in physiological and psychological pathways, evidence shows that it plays a vital role in physical health (Cheung and Li, 2012). In a study conducted to assess the impact of mental stress on physical health, chronic stimulation of the renal sympathetic nerve activity (RSNA) in animals and chronic stimulation of the sympathetic nervous system (SNS) in humans increased blood pressure parameters (Cheung and Li, 2012). Moreover, chronic RSNA stimulation in animals resulted in disturbances of blood pressure control via the baroreflex function while hyperactivation of the SNS in humans stimulated high blood pressure through increased activation of RAAS (Cheung and Li, 2012).

1.3.3. The Influence of Diabetes Mellitus on Hypertension

Finally, diabetes mellitus itself is a high-risk factor for hypertension. The interplay of the metabolic milieu (hyperglycaemia, FFAs and oxidative stress) on the endothelium elicits low-grade vascular inflammation, arterial stiffness and consequent hypertension (Cheung and Li, 2012). In an inception cohort consisting of 227 newly diagnosed IDDM individuals, vascular inflammation markers [C-reactive Protein (CRP), soluble vascular cellular adhesion molecule-1 (sVCAM-1) and soluble intracellular adhesion molecule-1 (sICAM-1)] and blood pressure levels were monitored over the 20-year long longitudinal period of the study (Ferreira *et al.*, 2018). Significant increases in sVCAM-1, sICAM-1 and CRP were observed at the onset of the disease and throughout the 20-year period. Moreover, increases in expression of these vascular inflammation markers preceded the increases observed in blood pressure and pulse pressure measurements of the individuals (Ferreira *et al.*, 2018).

1.4. Antioxidant Defence Systems

Antioxidants are substances that remove or scavenge ROS, unstable free radicals and the subsequent oxidative damage that follows (Carocho and Ferreira, 2013). These defence substances delay or inactivate ROS production by upregulating the expression of various antioxidant defence systems and by reducing unstable free radicals to stable molecules (Carocho and Ferreira, 2013).

The body's antioxidant defence system is categorized into enzymatic and non-enzymatic antioxidants. Furthermore, enzymatic antioxidants are divided into primary enzymes (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx]

and secondary antioxidant enzymes (glutathione reductases and glucose-6-phosphate dehydrogenase) (**Figure 1:11**) (Carocho and Ferreira, 2013).

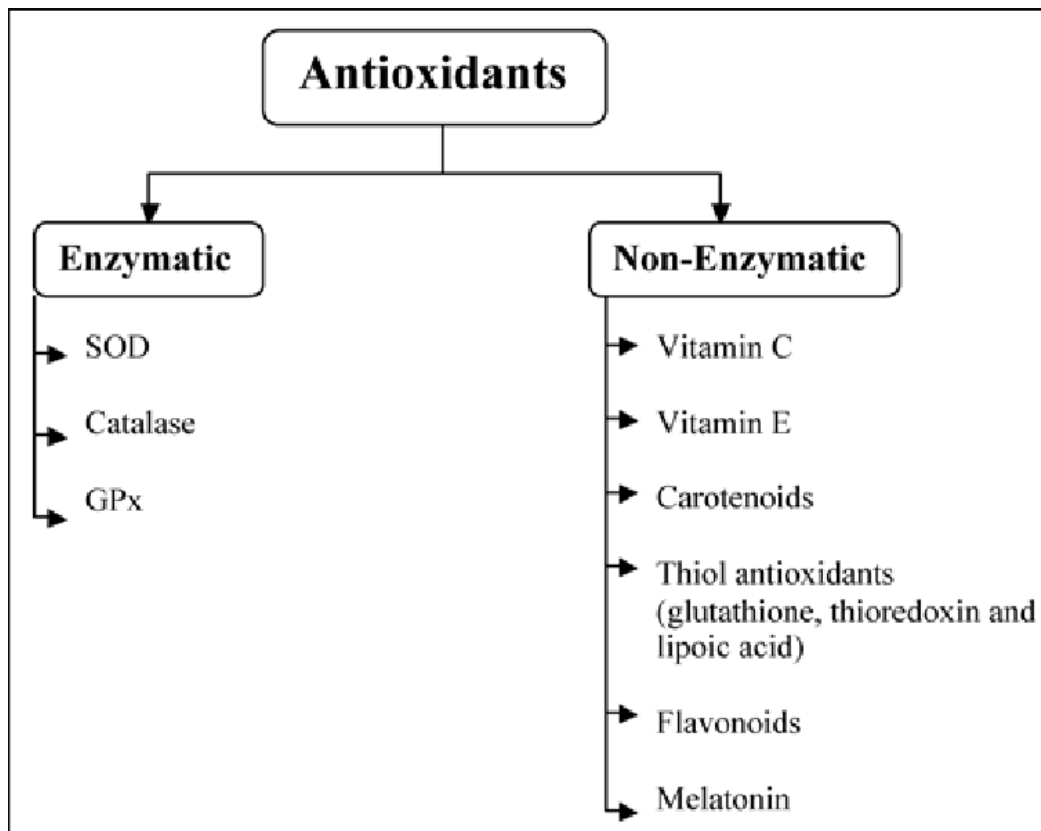


Figure 1:11 Classification of the enzymatic and non-enzymatic antioxidants present in the body's antioxidant defence system (Flora. 2009).

1.4.1. Enzymatic Antioxidants

Primary enzymatic antioxidants (SOD, CAT and GPx) play a pivotal role the body's defence system against oxidative stress (**Figure 1:12**) (Ajuwon, Marnewick and Davids, 2015).

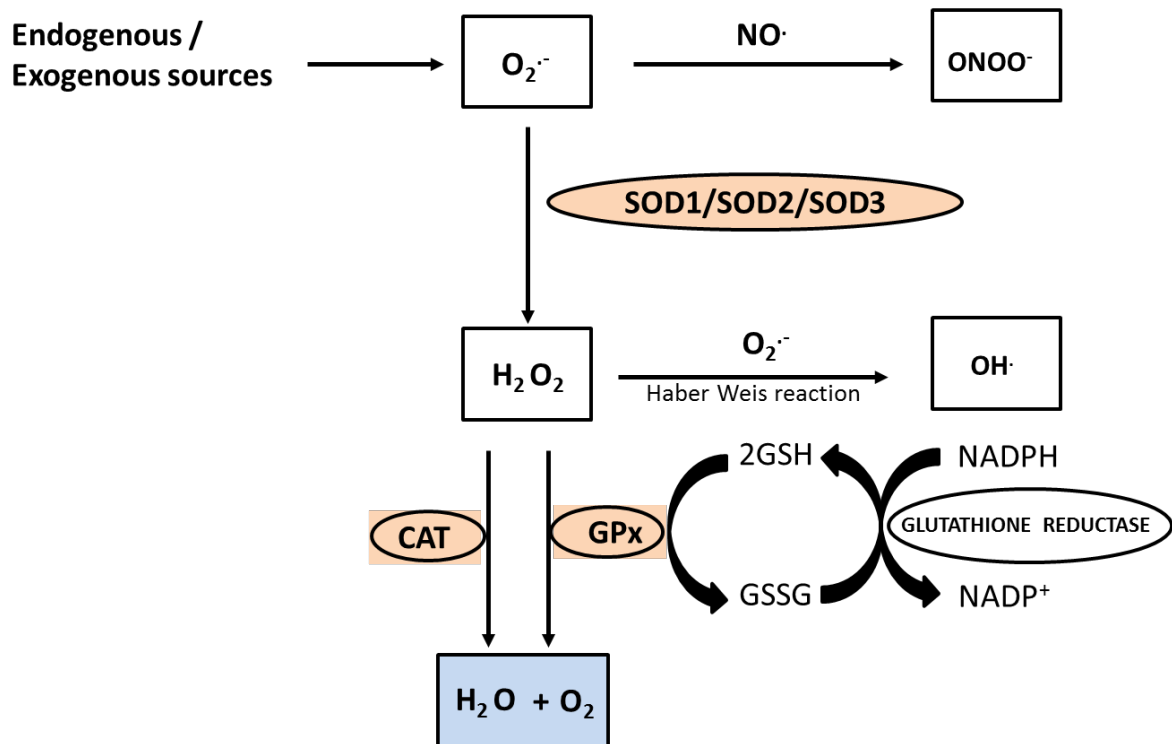


Figure 1:12 Schematic representation of the ROS scavenging pathways of the primary antioxidant defences superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Olawale R. Ajuwon, Marnewick and Davids, 2015).

Superoxide dismutase (SOD) is an enzyme that removes superoxide anions ($O_2^{\cdot-}$) by converting two superoxide anions ($2O_2^{\cdot-}$) at a time to hydrogen peroxide (H_2O_2) in a one-electron enzymatic dismutase manner (**Figure 1:12**) (Chaudière and Ferrari-Iliou, 1999; Maritim, Sanders and Watkins, 2003). Moreover, SOD is an enzymatic antioxidant that exists in three isoforms; copper-zinc superoxide dismutase (CnZnSOD), nickel superoxide dismutase (NiSOD) and manganese or iron superoxide dismutase (MnSOD or FeSOD). Furthermore, CnZnSOD is primarily found in the cytoplasm, the mitochondria and the nuclei while MnSOD is found in the mitochondria, the mitochondrial matrix and the surrounding extracellular space (Chaudière and Ferrari-Iliou, 1999; Maritim, Sanders and Watkins, 2003). On the other hand, the other primary enzymes, CAT and GPx both convert hydrogen peroxide (H_2O_2) molecules into water (H_2O) and molecular oxygen (O_2). However, the mechanism used by CAT and GPx to reduce H_2O_2 into H_2O and molecular O_2 differs from each other (**Figure 1:12**). CAT mediates the conversion of H_2O_2 into H_2O and molecular O_2 using a two-electron dismutation reaction (Chaudière and Ferrari-Iliou, 1999). Furthermore, CAT is a porphyrin-containing enzyme that is primarily found in peroxisomes but also exists

in non-peroxisomal regions of erythrocytes and the mitochondrial matrix (Chaudière and Ferrari-Iliou, 1999; Maritim, Sanders and Watkins, 2003).

On the other hand, glutathione peroxidases (GPx), a selenium-containing enzyme, catalyses the conversion of H_2O_2 into H_2O and molecular O_2 using a redox dependant reaction that is mediated by the formation of selenoles (Chaudière and Ferrari-Iliou, 1999; Maritim, Sanders and Watkins, 2003; Carocho and Ferreira, 2013). Furthermore, GPx reduces H_2O_2 to H_2O and molecular O_2 using the selenium substrates that are present in its active sites to form selenoles. Moreover, GPx uses the reduced form of glutathione (GSH) to facilitate the removal of H_2O_2 in a hydrogen donation dependant reaction manner (Maritim, Sanders and Watkins, 2003; Carocho and Ferreira, 2013). (**Figure 1:12**).

1.4.2. The Impact of Diabetes Mellitus on Antioxidant Status

The impact of diabetes mellitus on the activity and expression of antioxidants is contradictory (Maritim, Sanders and Watkins, 2003). Observational and experimental studies conducted on both diabetic patients and animal models present with conflicting reports. In some studies, the increased ROS and oxidative stress stimulated by diabetes mellitus upregulates antioxidant activity and expression whilst in other studies, a decreased antioxidant activity was observed (Asayama *et al.*, 1993; Maxwell *et al.*, 1997; Maritim, Sanders and Watkins, 2003; Moussa, 2008). In some of the human studies conducted, it is postulated that increases in antioxidant activity is a result of exogenous insulin therapy. Whereas, in other reports, a decrease in antioxidant activity is evident even in the presence of insulin therapy and a low glycaemic index dietary intake (Asayama *et al.*, 1993; Maxwell *et al.*, 1997; Maritim, Sanders and Watkins, 2003; Moussa, 2008). Moreover, some authors argue that the increases observed in antioxidant activity serve as indirect markers of increased free radical production and oxidative stress caused by diabetes mellitus (Asayama *et al.*, 1993; Maxwell *et al.*, 1997; Maritim, Sanders and Watkins, 2003; Moussa, 2008).

1.4.3. Non-enzymatic Antioxidants

In addition to enzymatic antioxidant defences, non-enzymatic antioxidants such as vitamin A, E and K, minerals such as zinc and selenium, the cofactor Coenzyme Q10, carotenoids, nitrogen non-protein compounds, various organosulfur compounds as well as flavonoids and phenolic acids are some of the many non-enzymatic

antioxidants that function in the human body (**Figure 1:12**) (Chaudière and Ferrari-Iliou, 1999; Maritim, Sanders and Watkins, 2003; Carocho and Ferreira, 2013).

Non-enzymatic antioxidants exert their effect of neutralizing free radicals and scavenging ROS in various mechanisms. Some non-enzymatic antioxidants are metal chelators that quench metal ions while other types of non-enzymatic antioxidants are reducing agents that act as electron donors to unstable free radicals (Maritim, Sanders and Watkins, 2003; Carocho and Ferreira, 2013). Furthermore, some non-enzymatic antioxidants quench ROS molecules and upregulate the gene expression of various enzymatic antioxidant defences while others are inhibitors of oxidation reactions and free lipid radical formation (Carocho and Ferreira, 2013).

1.5. *Aspalathus linearis* and the *Cyclopia* Species

1.5.1. Flavonoids and Phenolic Acids

Flavonoids are a dynamic group of diet-derived antioxidants that are non-enzymatic antioxidants and contain phenolic and polyphenolic phytochemical structures that are present in a variety of fruits and medicinal plants such as *Aspalathus linearis* (rooibos) and *Cyclopia intermedia* (honeybush) (**Figure 1:13**) (Carocho and Ferreira, 2013; Ajuwon, Marnewick and Davids, 2015).

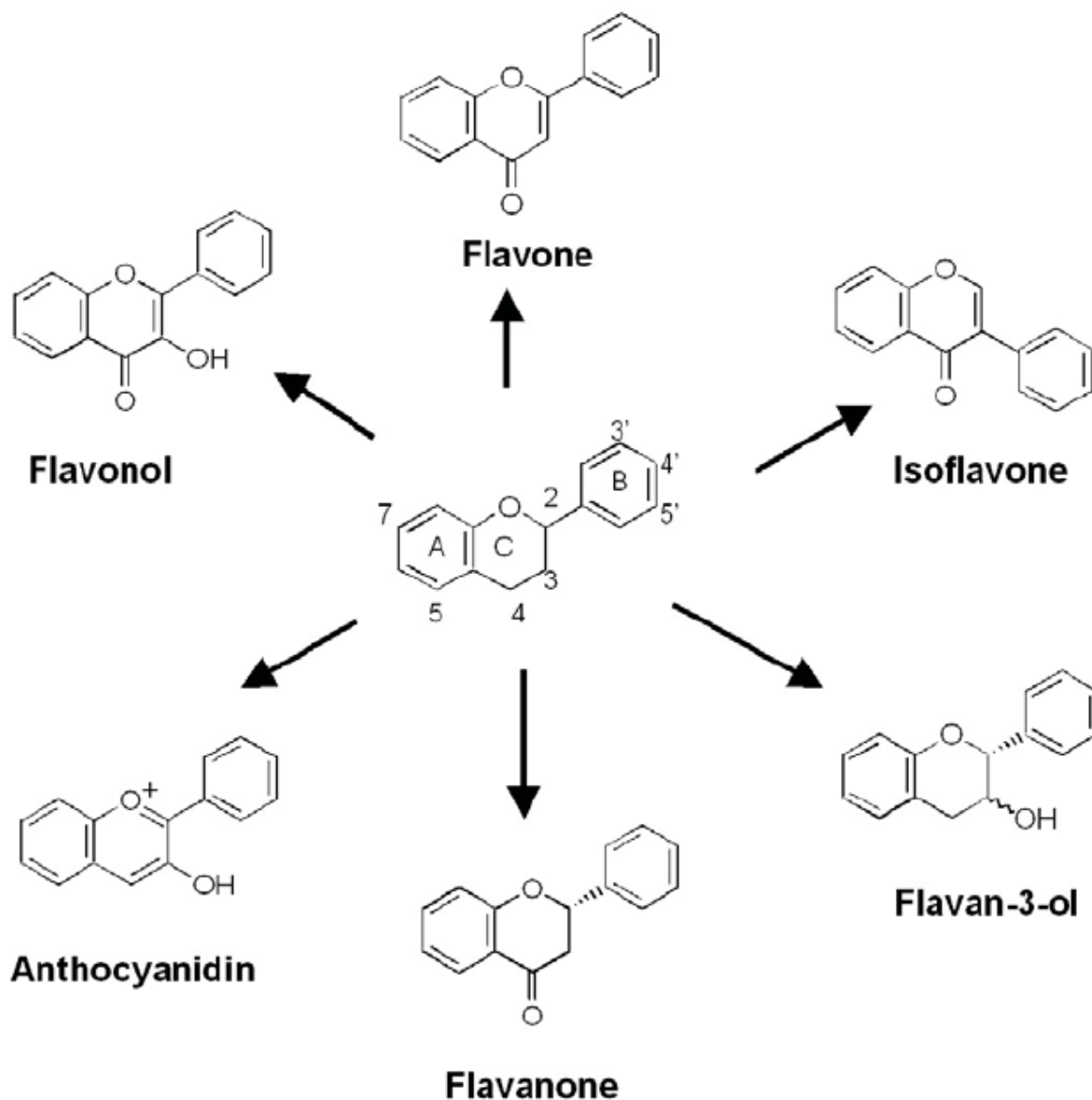


Figure 1:13 Types of flavonoids and their basic phytochemical structures (Nishiumi *et al.*, 2011).

Some flavonoids that have been studied intensively such as luteolin are present in both the medicinal plants whereas other flavonoids including aspalathin and mangiferin are unique to the plant species rooibos and honeybush respectively (McKay and Blumberg, 2007). Flavones, flavanols, flavonols and flavanones and their respective isoforms are some of the subclasses of flavonoids that contain antioxidant properties, anti-atherosclerotic, anti-inflammatory and antidiabetic properties (McKay and Blumberg, 2007; Muller *et al.*, 2011; Sena, Pereira and Seça, 2013; Kamakura *et al.*, 2015). Furthermore, the flavanones, flavones, flavonols, flavanols, phenolic acids and other polyphenol constituents such as anthocyanins which are present in rooibos

and honeybush, amongst others, are some of the sub-types of flavonoids that share a common diphenyl propane skeleton and a number of phenolic hydroxyl groups (Carocho and Ferreira, 2013). Due to the phenolic hydroxyl groups present in their chemical structures, flavonoids exist as highly potent non-enzymatic antioxidants that scavenge ROS and unstable free radicals (Ajuwon, Marnewick and Davids, 2015; Carocho and Ferreira, 2013). As a result, the polyphenolic components present in flavonoids act as secondary antioxidants that directly and non-enzymatically scavenge ROS by chelating with the transition metals involved in the generation of hydroxyl radicals (Joubert and Ferreira, 1996). Furthermore, flavonoids confer their antioxidant properties to ROS and unstable free radicals by activating and increasing the expression of the primary enzymatic antioxidants SOD, CAT and GPx (Carocho and Ferreira, 2013). Moreover, flavonoids act as electron donors, metal chelators and reducing agents that quench singlet oxygen and a number of unstable free radicals (Carocho and Ferreira, 2013). Additionally, phenolic acids, also found in medical plants such as *Aspalathus linearis* (rooibos) and *Cyclopia intermedia* (honeybush) are hydroxybenzoic and hydroxycinnamic acids that act as metal chelators and scavengers of free radical molecules such as superoxide anions and a number of hydroxyl and peroxy derived free radicals (Carocho and Ferreira, 2013).

1.6. *Aspalathus linearis*

Rooibos or *Aspalathus linearis* is a shrubby plant with green needle-shaped leaves and yellow flowers that grows up to 2 metres in height (**Figure 1:14**) (Ajuwon, Ayeleso and Adefolaju, 2018).



Figure 1:14 A) Fermented rooibos plant material and B) *Aspalathus linearis* plant (Rooibos Benefits and *Aspalathus linearis* Tea, 2016; Will SA government protect our indigenous medicine?, 2019).

Indigenous to South Africa, *Aspalathus linearis* grows in the Cederberg mountain area of Clanwilliam in the Western Cape (Ajuwon, Ayeleso and Adefolaju, 2018). Popular for its special taste as a tisane, fermented (red) rooibos is produced from the leaves and stems of *Aspalathus linearis* which are pounded and moistened before they are subjected to open-heap fermentation while unfermented green rooibos plant leaves and stems are quick-dried without fermentation. (McKay and Blumberg, 2007; Marnewick, 2009; Windvogel, 2019). Furthermore, *Aspalathus linearis*, is exported for medical use and for consumption as a herbal tisane to more than 37 countries including Japan, the United Kingdom, the United States of America, the Netherlands and Germany (Joubert and de Beer, 2011; Windvogel, 2019).

1.6.1. Flavonoids Present in *Aspalathus linearis*

The beneficial effects of *Aspalathus linearis* is mainly attributed to its polyphenol-enriched flavonoids (Joubert *et al.*, 2005). The flavonoids present in *Aspalathus linearis* are plant antioxidants that act as secondary or non-enzymatic antioxidants. Thus, as phytochemical structures enriched with phenolic hydroxyl groups, the flavonoids present in *Aspalathus linearis* act as scavengers of ROS and as metal chelators of metal transition ions involved in the generation of harmful free radical

substances such as hydroxyl ions (Joubert and Ferreira, 1996; Olawale R. Ajuwon, Marnewick and Davids, 2015; Windvogel, 2019). Furthermore, secondary antioxidants such as those present in *Aspalathus linearis*, play a crucial role in the upregulation of indigenous antioxidant enzymes including SOD and Catalase (Carocho and Ferreira, 2013; Windvogel, 2019).

Aspalathin, (a C-C linked dihydrochalone) and Aspalalinin (a cyclic dihydrochalcone) are monomeric flavonoids that have only been isolated in *Aspalathus linearis* (Ajuwon, Ayeleso and Adefolaju, 2018). The polyphenol-enriched flavonoids aspalathin, nothofagin (a 3-dehydrochalone glucoside) and phenylpyruvic acid are the 3 major polyphenols of rooibos that have been studied intensively and are largely responsible for the antioxidant ameliorative effects of rooibos in oxidative-stress and ROS-mediated pathological conditions (Marnewick, 2009) (**Figure 1:15**).

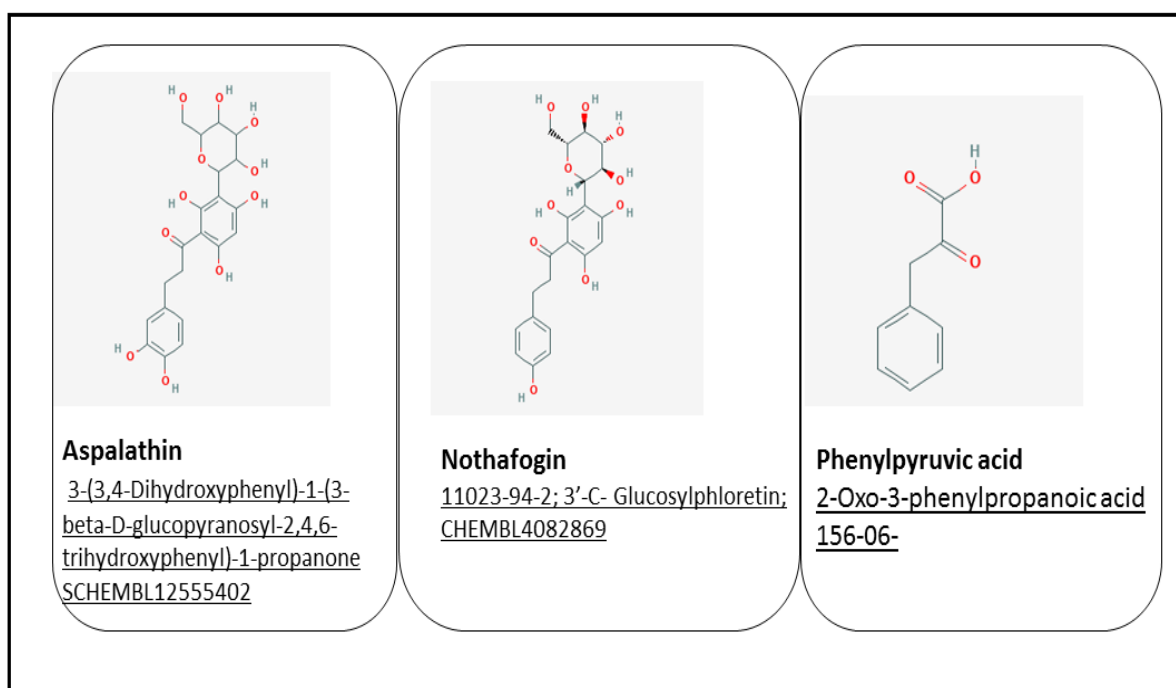


Figure 1:15 Phytochemical structures of Aspalathin, Nothofagin and Phenylpyruvic acid polyphenols found in *Aspalathus linearis* (Pubchem, 2019).

Furthermore, in most high-performance liquid chromatography - mass spectrometry (HPLC-MS) analyses, investigators have noted aspalathin followed by nothofagin and phenylpyruvic acid as the 3 major polyphenols responsible for the antioxidant and antidiabetic properties observed in experimental animal models of diabetes mellitus and obesity (Ajuwon, Ayeleso and Adefolaju, 2018; McKay and Blumberg, 2007).

Dludla *et al.*, 2014 discovered that isoorientin followed by phenylpyruvic acid, quercetin, aspalathin and phenylpropenoic acid glycoside were available in high concentrations in the fermented form of rooibos. Furthermore, in the same fermented extract, other polyphenols such as luteolin-7-O-glucoside, isoquercitrin, hyperoside, isovetexin, rutin vetexin and its isoform isovitexin were but at concentrations lower than 0.002g/100g (Dludla *et al.*, 2014). Additionally, a number of other bioactive compounds such phenolic acids are present in *Aspalathus linearis*. Phenolic acids such as catechin, coumarins, esculentin, esculin, lignans, protocatechuic acid, *p*-hydrobenzoic acid, caffeic acid, vanillic acid and ferulic acid are all present in *Aspalathus linearis*, but at concentrations lower than the major active compounds aspalathin, aspalalinin, nothofagin and phenylpyruvic acid (**Table 1:2**) (McKay and Blumberg, 2007; Ajuwon, Ayeleso and Adefolaju, 2018).

Table 1:2 Classification of polyphenol-enriched flavonoids present in *Aspalathus linearis* (McKay and Blumberg, 2007; Joubert *et al.*, 2008; Dlodla *et al.*, 2014; Ajuwon, Ayeleso and Adefolaju, 2018).

Flavonoids	<i>Aspalathus linearis</i>
Dihydrochalone	aspalathin, aspalalinin, nothofagin
Flavonone	dihydro-orientin, dihydro-iso-orientin hemiphlorin
Flavone	chrysoeriol, luteolin, luteolin-7-O-glucoside orientin, iso-orientin vitexin, iso-vitexin
Flavonol	hyperoside, iso-quercitrin quercetin, quercetin-3-roninobioside, rutin
Flavanol	catechins, procyanidin
Phenolic acid	caffeic acids, chlorogenic acids, ferulic acids gallic acids, gentistic acids hydroxybenzoic acids, hydroxycinnamic acids protocatechuic acids, salicylic acids sinapic acids, syringic acids, vanillic acids
Coumarin	esculetin, esculin
Chromone	5,7-dihydroxy-6-C-glucosyl-chromone
Lignan	glucopyranoside, secoisolariciresinol, vladinol
Inositol	- (+)-pinitol
Glycol derivative	- <i>p</i> -hydroxyphenylglycol, vanylglycol
Phenylpyruvic acid derivative	- phenylpyruvic acid phenylpyruvic acid glycoside (PPAG)

1.6.2. Fermentation Process of *Aspalathus linearis*

During open-heap fermentation, fermented rooibos, popularly consumed as a herbal tisane, acquires a deep red colour due to the chemical reactions that occur during the fermentation process. Moreover, various investigators have noted a decreased antioxidant capacity due to oxidation reactions and other chemical and enzymatic modifications during fermentation (McKay and Blumberg, 2007). As a result, aspalathin which was present in 9.3% and nothofagin in 1.03% of the unfermented

Aspalathus linearis dry plant material was reduced significantly in the fermented product (Joubert *et al.*, 2005). Similarly, in another study, about 7% of aspalathin was lost in the fermentation process of rooibos due to the oxidization reactions which convert aspalathin-dihydrochalcone to orientin, and dihydro-iso orientin (Joubert *et al.*, 2005; Marnewick, 2009; Joubert and de Beer, 2011). Thus, unfermented green rooibos has a higher antioxidant capacity and polyphenolic content than fermented red rooibos tisane (Ajuwon, Ayeleso and Adefolaju, 2018). In a study by McKay and Blumberg, 2007, the total phenolic content ratio in unfermented green rooibos extract decreased from 95.5 to 60.5 post-fermentation (McKay and Blumberg, 2007). Furthermore, the total antioxidant capacity determined in unfermented green rooibos was a 2.8 fold factor higher than in fermented red rooibos (Schulz, Joubert and Schütze, 2003; McKay and Blumberg, 2007). While fermented red rooibos is gaining popularity worldwide for its use as an antioxidant-rich tisane, unfermented green rooibos is in high demand for its potential use as an antioxidant-rich therapeutic agent in oxidative-stress / ROS mediated pathological conditions such as diabetes mellitus (Joubert *et al.*, 2005; Marnewick, 2009).

1.6.3. The Uses of *Aspalathus linearis*

Due to the high antioxidant capacity and the caffeine-free and a low tannin content of this medical plant, *Aspalathus linearis* has been successfully used by traditional healers and the khoi-san to treat various ailments and illnesses (Marnewick, 2009; Ajuwon, Ayeleso and Adefolaju, 2018). In 1968, fermented rooibos tisane administered to a baby relieved restlessness, stomach cramps and nausea (Joubert and Ferreira, 1996). Moreover, the fermented rooibos tisane acted as an appetite enhancer that improved nervous complaints and cured insomnia in adults (Joubert and Ferreira, 1996). Nowadays, rooibos extract is used in the food industry as an antioxidant-rich agent, as a colorant and a flavouring agent added in various processed food products (Joubert *et al.*, 2005; Windvogel, 2019). In Japan, rooibos extract is added as an anti-ageing ingredient in natural medicinal products that aid in the treatment of various inflammatory diseases (Joubert and Ferreira, 1996). Furthermore, rooibos extracts are added in nutraceuticals, in cosmetic care ranges, pet food, pet skin care products and in food products such as jam, yoghurt, instant cappuccinos and iced-tea (Joubert and de Beer, 2011; Windvogel, 2019).

1.6.4. The Health Properties of *Aspalathus linearis*

Quercetin, a polyphenol-enriched flavonoid that has an antispasmodic effect, was found to be a key role player in the anti-allergy and anti-nausea effects that fermented rooibos tisane exhibits in infants (Joubert and Ferreira, 1996). However, in adults, the topical application of rooibos poultice to the forearm skin and the ingestion of rooibos tea by subjects with asthma or hay fever did not exhibit any antihistamine and anti-allergy effects (McKay and Blumberg, 2007). Aspalathin and Nothofagin that was administered for 12 and 50 hours in mice that had caecal ligation and puncture-induced sepsis exhibited anti-inflammatory properties by significantly reducing inflammatory and oxidative stress markers such as NF- κ B, TNF- α , IL-6, NO present in the blood parameters of the mice (Yang *et al.*, 2018). Additionally aspalathin and nothofagin exhibited antioxidant capacity by upregulating the activity of the indigenous antioxidant enzymes GPx, SOD and CAT in the kidney tissue of mice exposed to caecal ligation and puncture sepsis (Yang *et al.*, 2018). Moreover, 2% green and fermented rooibos tisane has potential anticancer properties which were evident in an *in vitro* analysis of the antimutagenic response of the extracts against aflatoxin-B₁ (AFB₁) and 2-acetylaminofluorene (2-AAF) induced mutagenesis and carcinogenesis (Marnewick, Gelderblom and Joubert, 2000).

1.6.5. The Effects of *Aspalathus linearis* on Antioxidant Status in Diabetes Mellitus

In experimental diabetes mellitus, 2.5% fermented rooibos tisane did not affect the blood glucose parameters of 45 mg/kg STZ-induced diabetic rats. However, a significant decrease in AGE's and advanced oxidation protein products (AOPP's) was apparent in the system of the diabetic rats that consumed the 2.5% rooibos for 8 weeks (Ulicna *et al.*, 2005). Furthermore, aspalathin (130 mg/kg/day) that was administered for 6 weeks to male homozygous (db/db) diabetic mice upregulated the expression of erythroid-derived nuclear factor-like 2 (Nrf2) and its downstream antioxidant genes and proved to be cardioprotective in high-glucose exposed H9c2 cardiomyocytes that were treated with 1 μ M Aspalathin (Dludla *et al.*, 2017). Furthermore, phenylpyruvic acid glucoside (PPAG), a flavanoid found in *Aspalathus linearis* proved to be cytoprotective by delaying the onset of hyperglycaemia (Himpe *et al.*, 2016). Moreover, at 11 days post-STZ injection, mice that received 10mg/kg body weight

PPAG daily starting 2 days before STZ injection (200mg/kg body weight) experienced 60% apoptotic beta cell mass loss compared to the untreated STZ mice that presented with a 80% apoptotic beta cell mass loss (Himpe *et al.*, 2016). Similarly, a 50-60% beta cell loss was observed *in vitro*, in rat-insulin producing cells (pancreatic beta INS-1E cells) that were pre-incubated in PPAG for a duration of 16 hours prior to being exposed to 1 mM STZ for an incubation period of 1 hour (Himpe *et al.*, 2016). Furthermore, at elevated glucose concentrations (33 mM glucose), PPAG protected the pancreatic beta INS-1E cells against β -cell apoptosis by preserving the expression of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) and by inhibiting caspase-9 and caspase-3 cleavage and activation (Himpe *et al.*, 2016).

1.6.6. The Effects of *Aspalathus linearis* on Endothelial Function in Diabetes Mellitus

The aspalathin and nothofagin flavonoids present in rooibos improved endothelial function by their inhibitory effects on glucose-mediated vascular permeability and the recruitment of monocytes and inactivation of the pro-inflammatory NF- κ B pathway (Ajuwon, Marnewick and Davids, 2015). Likewise, pre-treatment with cumulative concentrations of aspalathin and nothofagin inhibited vascular permeability-induced protein leakage in high glucose exposed mice endothelial cells (Ku *et al.*, 2015). In the same way, a decrease in membrane disruption was observed in glucose (25 mM) exposed primary human umbilical vein endothelial cells (HUVECs) that received pre-treatment with aspalathin or nothofagin following treatment (Ku *et al.*, 2015). Moreover, 50 μ M aspalathin and nothofagin inhibited ROS production (hydrogen peroxide) and the expression of various cell adhesion molecules (VCAM-1, ICAM-1, E-selectins) in primary HUVECs (Ku *et al.*, 2015).

1.6.7. The Effects of *Aspalathus linearis* on Hypertension in Diabetes Mellitus

The potential antihypertensive effects of *Aspalathus linearis* have been well-documented in various animal and human studies. The oral intake of 400 ml of rooibos tea prepared by steeping 10 g rooibos extract in 400 ml water had no significant effect on the blood pressure of seventeen healthy participants. However, consumption of this rooibos infusion significantly inhibited ACE activity at 30 minute and 60 minute time intervals respectively in the blood serum of the healthy participants (Persson *et al.*, 2010). In an *in vitro* analysis of the enzyme kinetics involved in rooibos tea-mediated

ACE inhibition, Persson, 2012 demonstrated that rooibos tea mediates ACE inhibition in human blood serum through a mixed inhibitor mechanism. In contrast, in HUVECs that were cultured in rooibos extracts, no significant effects on ACE activity was observed following a 10 minute incubation of the healthy HUVECs in rooibos tea (Persson *et al.*, 2006). On the other hand, in an experimental animal model, the intravenous injections of fermented rooibos infusions (10- 100 mg/kg bw) administered under anaesthesia to healthy rodents, resulted in significant decreases of the mean arterial blood pressure of the healthy rats as measured by means of the carotid artery cannulation method (Khan and Gilani, 2006).

1.7. *Cyclopia* Species

Honeybush or the *Cyclopia species*, is a group of shrubby plants that have pale-yellow flowers and green trifoliate leaves that grow up to 1.5 to 3 metres in height (**Figure 1:16**) (Ajuwon, Ayeleso and Adefolaju, 2018).



Figure 1:16 Fermented honeybush plant material and B) *Cyclopia intermedia* plant. (Mountain Honeybush (*Cyclopia intermedia*) iNaturalist, 2019; honeybush – GreenHeart Store, 2019).

Indigenous to South Africa, an estimated 23 -24 *Cyclopia species* have been identified in the Citrusdal area of the Western Cape and the Cederberg mountains of the western parts of South Africa (Ajuwon, Ayeleso and Adefolaju, 2018). Currently, it is only the *Cyclopia intermedia*, *Cyclopia genistoides* and *Cyclopia subternata* species that are available for commercial use and distribution (Joubert *et al.*, 2011; Ajuwon, Ayeleso and Adefolaju, 2018). Additionally, *Cyclopia maculata* is one of the species that is currently available for research investigations conducted in a laboratory setting.

Traditionally, fermented (brown-reddish) honeybush and unfermented (green) honeybush are both used for medicinal purposes and are enjoyed as herbal beverages and tisanes (Ajuwon, Ayeleso and Adefolaju, 2018). The sought-after sweet flavour and sweet-honey aroma of fermented (red-brownish) honeybush comes from the pale-yellow flowers of this medical plant (Ajuwon, Ayeleso and Adefolaju, 2018). Similar to *Aspalathus linearis*, fermented (brownish-red) honeybush is prepared by subjecting the previously pounded and moistened leaves, stems and flowers of the plant to open-heap or oven fermentation (McKay and Blumberg, 2007; Windvogel 2019). Furthermore, the *Cyclopia species* is exported globally and distributed in large quantities to more than 25 countries including Germany and the Netherlands (Joubert *et al.*, 2011; Windvogel, 2019).

1.7.1. Flavonoids Present in the *Cyclopia Species*

Similar to *Aspalathus linearis*, the bioactivity of the *Cyclopia species* and its antioxidant role in ameliorating oxidative stress and oxidative damage in pathophysiological conditions such as diabetes mellitus, cancer and CVDs is mainly attributed to the phytochemical polyphenols present in the plant material (McKay and Blumberg, 2007; Marnewick, 2009; Ajuwon, Ayeleso and Adefolaju, 2018; Windvogel, 2019). These polyphenolic flavonoids present in the plant material contain phenolic hydroxyl groups that chelate with metal ions that are involved in the production of harmful free radicals and ROS species. Moreover, the phenolic hydroxyl groups present in the *Cyclopia species* scavenge ROS and act as electron donors and reducing agents that reduce unstable free radicals to stable compounds (Joubert and Ferreira, 1996; McKay and Blumberg, 2007). Additionally, the polyphenolic flavonoids present in the *Cyclopia species* confer their antioxidant properties by increasing the activity and expression of indigenous antioxidant enzymes catalase, SOD and glutathione peroxidase (Carocho and Ferreira. 2012). Mangiferin (a xanthone C-glycoside), hesperidin and isokuranetin are the three major polyphenols of the *Cyclopia species* that have been studied intensively and have been reported by many investigators as the polyphenol-enriched flavonoids that are largely responsible for the antioxidant and antidiabetic ameliorative effects that *Cyclopia species* has in the context of diabetes mellitus and obesity (McKay and Blumberg, 2007).

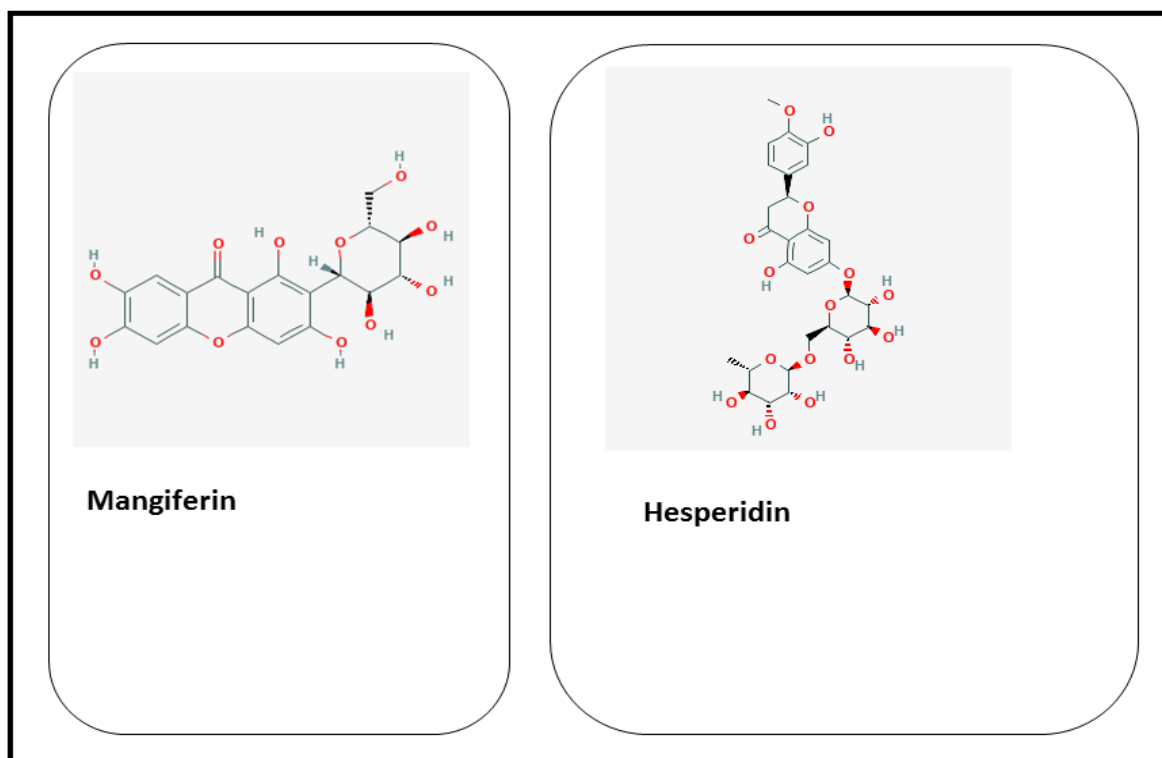


Figure 1:17 Phytochemical structures of Mangiferin and Hesperidin polyphenols found in *Cyclopia* species (Honeybush) (Pubchem, 2019).

In a study conducted by Muller *et al.*, 2011, the phenolic and polyphenolic compounds detected by high-performance liquid chromatography (HPLC) in *Cyclopia intermedia* were mangiferin, isomangiferin, hesperidin, hesperetin, luteolin, eriodictyol-glucoside and eriocitrin. Moreover, mangiferin and hesperidin were detected by high performance liquid chromatography (HPLC) and spectrophotometry using the folin-Ciocalteu technique as the two major flavanoids present in the methanol, ethyl acetate and chloroform extracts of the *Cyclopia intermedia* honeybush plant material (Dube *et al.*, 2016). In addition, scolymoside, mangiferin and its isoform were detectable at high concentrations in *Cyclopia subternata* (van der Merwe *et al.*, 2017). Additionally, various phenolic acids, coumestans, flavonols, flavanones, flavones and isoflavonones such as hesperetin are also present in the *Cyclopia* species (McKay and Blumberg, 2007; Ajuwon, Ayeleso and Adefolaju, 2018). (**Table 1:3**).

Table 1:3 Classification of flavonoids present in the *Cyclopia* species (Dube *et al.*, 2016; McKay and Blumberg, 2007; Van Der Merwe *et al.*, 2015; Ajuwon, Ayeleso and Adefolaju, 2018; Joubert *et al.*, 2008).

Flavonoids	<i>Cyclopia</i> species
Xanthone	mangiferin, isomangiferin
Flavonone	eriodictyol, encitrin, hesperitin, hesperidin isosakuranetin, maclurin-di-O-chexoside, narirutin, naringenin, prunin
Flavone	afromosin, calycosin, diosmetin formononetin, luteolin, orobol, scolymoside wistin
Flavonol	kaempferol glucosides
Flavanol	(-)-epigallocatechin gallate
Phenolic acid	coumaric acids, hydroxycinnamic acids shikimic acids
Inositol	(+)-pinitol
Coumestan	flemichapparin, medicagol sophoracoumestan
Methylendioxyisoflavone derivative	furikinetin, pseudobaptigen
Phenylethanol derivative	phenylethanol diglucoside, tyrosol
Benzaldehyde derivative	benzaldehyde diglucoside
Others	Iriflophenone-3-C- β -glucoside-4-O- β - glucoside Iriflophenone-3-C- β -glucoside, Vicenin-2 Phloretin-3',5'-di-C- β -glucoside 3-Hydroxyphloretin-di-C-hexoside

1.7.2. The Fermentation Process of *Cyclopia* Species

As a result of the chemical modifications and the enzymatic and oxidation reactions that takes place during curing heap or baking-oven fermentation, fermented honeybush attains a brown-reddish colour and a decreased antioxidant capacity compared to unfermented green honeybush (McKay and Blumberg, 2007; Ajuwon, Ayeleso and Adefolaju, 2018; Windvogel, 2019). Furthermore, fermented honeybush tisanes (20 g/500 ml) prepared from steeping the fermented plant material from five

Cyclopia species for 5 minutes respectively (*Cyclopia intermedia*, *Cyclopia subternata*, *Cyclopia maculata*, *Cyclopia sessiliflora* and *Cyclopia genistoides*) exhibited decreased superoxide anion scavenging capacity when compared to honeybush tisane prepared from unfermented green *Cyclopia* species (Hubbe and Joubert, 2000). Green/unfermented *Cyclopia intermedia* exhibited a higher antioxidant capacity when compared to fermented *Cyclopia intermedia*. Moreover, the higher antioxidant capacity present in green/unfermented *Cyclopia intermedia* was concentrated in the methanol extracts of the plant material when compared to the ethyl acetate and chloroform extracts (Dube *et al.*, 2016). This was the case in all antioxidant values detected by the oxygen radical absorbance capacity (ORAC) assay, the ferric reducing ability of plasma (FRAC) assay and the trolox equivalent antioxidant capacity (TEAC) assay (Dube *et al.*, 2016).

1.7.3. The Uses of the *Cyclopia* Species

Similar to *Aspalathus linearis*, the *Cyclopia* species is a low-tannin and caffeine free infusion that is suitable for consumption by babies and adults that suffer from various digestive problems and heart conditions (Joubert *et al.*, 2008; Ajuwon, Ayeleso and Adefolaju, 2018). Traditionally, honeybush has been used as an appetite enhancer and a breast milk stimulant in nursing mothers and in cows for the industrial production of cow milk. Moreover, as an expectorant, honeybush infusion has been used to treat chronic catarrh and pulmonary tuberculosis (Joubert *et al.*, 2011; Ajuwon, Ayeleso and Adefolaju, 2018). Furthermore, as a therapeutic agent, honeybush infusion provided relief to patients that suffer from arthritis and spastic colon (Joubert *et al.*, 2008). In folk medicine, honeybush extracts have been used as a sedative, as a laxative and as a suitable treatment of various skin rashes (McKay and Blumberg, 2007).

1.7.4. The Health Properties of the *Cyclopia* Species

In other accounts, honeybush has been reported to be a therapeutic agent in the treatment of cancers and as a medicinal plant that has anti-inflammatory, antioxidant and anti-mutagenic properties (Ajuwon, Ayeleso and Adefolaju, 2018). Furthermore, 4% fermented and unfermented honeybush tisane exhibited anti-cancer and anti-mutagenic activity in a dose-dependent manner by significantly reducing the mutagenic activity of 2-acetylaminofluorene (AAF) and aflatoxin B₁ (AFB₁) in a *Salmonella typhimurium* bacterial strain (Marnewick, Gelderblom and Joubert, 2000).

In Institute for Cancer Research (ICR) mice, the topical application of green and fermented honeybush extracts significantly reduced skin tumorigenesis (McKay and Blumberg, 2007).

1.7.5. The Effects of the *Cyclopia Species* on Antioxidant Status and Endothelial Function Diabetes Mellitus

Mangiferin, a potent flavonoid in honeybush, exhibited anti-diabetic and cardioprotective effects by improving hyperinsulinemia and lowering cholesterol levels in the blood of diabetic mice (McKay and Blumberg, 2007). Furthermore, in STZ-induced (35 mg/kg body weight) non-ketoacidosis diabetes, *Cyclopia intermedia* honeybush extract (50 mg/kg body weight) that was administered intramuscularly as a single dose to adult male Wistar rats significantly reduced blood glucose levels over a period of 3 to 6 hours. (Muller *et al.*, 2011). Similarly, *Cyclopia maculata* improved pancreatic β -cell proliferation, and glucose tolerance while significantly reducing fasting blood glucose levels and total serum triglyceride concentrations in STZ-induced (40 mg/kg body weight) diabetic Wistar rats were given a pre-treatment of *Cyclopia maculata* honeybush extract (300 mg/kg body weight) for 15 days before STZ exposure and again for 6 days after treatment with STZ (Chellan *et al.*, 2014). Similarly, in an obese insulin-resistant (OBIR) rat model of diabetes, *Cyclopia intermedia* honeybush ameliorated hyperglycaemia and significantly reduced total plasma cholesterol concentrations in the OBIR rats that received the *Cyclopia intermedia* honeybush extract as their drinking fluid over a period of 3 months (Muller *et al.*, 2011).

1.7.6. The Effects of the *Cyclopia Species* on Hypertension in Diabetes Mellitus

Scientific reports on the effects of honeybush on blood pressure measurements in humans and experimental animal models are few. However, there are a large number of publications available that report back on other indirect markers of high blood pressure such as such as high plasma cholesterol and high plasma triglycerides amongst other lipid profiles. In various research studies conducted, treatment with honeybush extracts significantly reduced lipid profiles in diabetes mellitus and obese experimental animal models. Furthermore, OBIR rats that consumed *Cyclopia intermedia* honeybush infusion for 3 months presented with a significant reduction in total plasma cholesterol levels (31.6 -39.1%) when compared to untreated OBIR rats

(Muller *et al.*, 2011). Furthermore, in an *in vitro* analysis of obesity, treatment with unfermented *Cyclopia maculata* and *Cyclopia subternata* inhibited fat accumulation, adipogenesis and intracellular triglyceride accumulation in 3T3-L1 mouse pre-adipocytes (Dudhia *et al.*, 2013). Similarly, hesperidin, a flavonoid present in honeybush, significantly reduced the total plasma cholesterol levels of type 2 diabetes mellitus patients following a 8 week treatment period in a randomized clinical trial study (Eghtesadi *et al.*, 2016). Furthermore, stroke-prone spontaneously hypertensive rats fed with a diet enriched with hesperidin, G-hesperidin and naringin exhibited significant decreases in the mean systolic blood pressure measurements following a 4 week treatment period (Ikemura *et al.*, 2012). On the other hand, diabetic nephropathy is a precursor to the development of hypertension in type 1 diabetes mellitus. In an animal model of type 1 diabetes mellitus, diabetic mellitus induced by a single intraperitoneal injection of a high STZ dose of 65 mg/kg in male Wistar rats, resulted in activation of the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signalling pathways involved in hyperglycaemia mediated oxidative stress. Treatment with 40 mg/kg bw mangiferin reversed the activation of PKC, MAPK, transforming growth factor-beta (TGF- β) and NF- κ B signalling pathways that are involved in the onset of diabetic nephropathy (Pal, Sinha and Sil, 2014). Thus, by significantly reducing increased lipid profiles and downregulating the expression of proteins involved in the progression to hypertension, various researchers have shown the potential antihypertensive and cardioprotective effects of the *Cyclopia species* in various experimental animal models.

1.8. Rationale and Motivation of the Study

Africa is a continent that has a rich diversity of plant species with an estimation of 30 000 plant species that have been identified. Furthermore, an estimated 3000 of the medical plants species indigenous to Africa are traditionally used as medicines in South Africa (Ajuwon, Ayeleso and Adefolaju, 2018). Out of the limited number of 500 species available in the informal market, only a few such as *Aspalathus linearis* rooibos and the *Cyclopia species* have been commercialized (Ajuwon, Ayeleso and Adefolaju, 2018).

Popular for their aroma and special taste, rooibos and honeybush are medicinal plants that are commercially produced and consumed as herbal teas or infusions prepared

by steeping and brewing (Ajuwon, Ayeleso and Adefolaju, 2018; McKay and Blumberg, 2007). Produced locally in the Cape fynbos region of the Western Cape Province, about 50% of the annual production of 7500 tons of rooibos and 90% of the 150 tons of honeybush is consumed globally as herbal teas (Marnewick *et al*, 2003). Rooibos and honeybush plants are both naturally caffeine free and contains low levels of tannins (Marnewick *et al*, 2011). Historically, both these medicinal plants have been used by traditional healers and the Khoisan to treat and alleviate various ailments and illnesses including cancer and diabetes mellitus. The polyphenolic-rich content of these medicinal plants has been demonstrated by various researchers to have high antioxidant capacity that have the potential to alleviate oxidative stress in various pathophysiological conditions including diabetes mellitus. Some flavonoids or polyphenols present in rooibos and honeybush including aspalathin from *Aspalathus linearis* and mangiferin and hesperidin from the *Cyclopia species* have been isolated and studied intensively and have been demonstrated to have antioxidant, anti-atherosclerotic, anti-inflammatory and antidiabetic properties (Ajuwon *et al.*, 2015; Joubert & Ferreira. 1996; Kamakura *et al.*, 2014; Marnewick *et al.*, 2003; McKay & Blumberg. 2007; Muller *et al.*, 2011; Sena, Pereira and Seïça, 2013). However, only a few and limited number of research studies have been conducted to explore the therapeutic effects that fermented *Aspalathus linearis* and fermented *Cyclopia species* have as an entire plant extract, containing all of its polyphenol-enriched flavonoids.

Furthermore, it is known that diabetes mellitus is a risk factor for the development of CVDs. In diabetes mellitus, the chronic hyperglycaemia that occurs due to insulin resistance and/or deficiency induces oxidative stress and ROS production through pathophysiological pathways such as eNOS uncoupling, glucose autoxidation, glucotoxicity and the production and binding of AGEs to their receptor RAGE amongst others (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013).

Furthermore, the endothelium plays a pivotal role in maintaining vascular homeostasis and in preventing vascular inflammation and the atherothrombogenic events that lead to the development of CVDs (Deanfield, Halcox and Rabelink, 2007; Flammer and Lüscher, 2010; Sena, Pereira and Seïça, 2013). However, in diabetes mellitus, hyperglycaemia, oxidative stress, increased ROS production and the associated pathophysiological pathways compromise the integrity of the endothelium and impair

endothelial function. Furthermore, when endothelial function is impaired or weakened, ED, CVDs, hypertension and other macro- and microvascular diabetic complications develop. (Deanfield, Halcox and Rabelink, 2007; Hadi, Carr and Al Suwaidi, 2005; Sena, Pereira and Seïça, 2013; Vallance and Chan, 2001). Thus, in the present study we hypothesize that by ameliorating oxidative stress, ROS production and hyperglycaemia, fermented rooibos and fermented honeybush (which contain polyphenol flavonoids that have the potential to scavenge ROS and upregulate intracellular antioxidant defences) may alleviate endothelial dysfunction and prevent the development of hypertension and CVDs in diabetes mellitus.

1.9. Research Question

Do fermented rooibos (*Aspalathus linearis*) and fermented honeybush (*Cyclopia intermedia*) alleviate hyperglycaemia, aortic endothelial dysfunction, elevated blood pressure and oxidative stress in STZ-induced diabetic Wistar rats?

1.10. Hypothesis

Streptozotocin (STZ)-induced diabetic adult male Wistar rats will develop hyperglycaemia, hypertension and oxidative stress that may impair aortic endothelial function and decrease their antioxidant status. Treatment with 2% fermented Rooibos (*Aspalathus linearis*) or 4% Honeybush (*Cyclopia intermedia*) infusion may ameliorate or prevent the development of aortic endothelial dysfunction, hyperglycaemia, hypertension, and oxidative stress in STZ-induced diabetic adult male Wistar rats.

1.11. Research Aim

To investigate the modulating capacity of 2% fermented rooibos (*Aspalathus linearis*) and 4% fermented honeybush (*Cyclopia intermedia*) infusions on aortic endothelial function, blood glucose levels, blood pressure and antioxidant status in STZ-induced diabetic male rats, following a 1-week pre-treatment and 6-week treatment period.

1.12. Research Objectives

- a) To establish an *in vivo* model of STZ-induced diabetes by determining an optimal STZ dose.
- b) To optimize the method of delivery of the infusions to the STZ-induced diabetic Wistar rats.

- c) To assess glucose homeostasis in STZ-rats using an intraperitoneal glucose tolerance test.
- d) To assess blood pressure in STZ-induced diabetic Wistar rats using a non-invasively tail-cuff method.
- e) To determine vascular reactivity in the thoracic aorta of STZ-induced diabetic Wistar rats and their age-matched controls through aortic contraction/relaxation studies.
- f) To elucidate the mechanisms in aortic vascular reactivity through western blot analysis of aortic tissue using selected proteins involved in endothelial health.
- g) To determine antioxidant status in kidney tissue of STZ-induced diabetic rats by measuring the activity of the primary antioxidant enzymes SOD and CAT and determining lipid peroxidation as an indicator of oxidative stress, by measuring TBARS.
- h) To determine whether fermented rooibos (*Aspalathus linearis*) and fermented honeybush (*Cyclopia intermedia*) infusions will ameliorate oxidative stress, blood glucose levels, endothelial dysfunction function and any effects on blood pressure in a model of STZ-induced diabetes in male Wistar rats.

CHAPTER 2 Materials and Methods

2.1 Materials

AEC Amersham, Buckinghamshire, United Kingdom (UK) - Enhanced Chemiluminescence (ECL) Reagent, Horseradish peroxidase- conjugated Anti-Rabbit Immunoglobulin Gene secondary antibody

Afrinaturals, South Africa (SA) - fermented *Cyclopia intermedia* (Honeybush)

Bayer (Pty) Ltd, Isando, Gauteng, SA - Eutha-naze (C₁₁H₁₇N₂NaO₃)

Bio-Rad Inc. Hercules, California, USA - Criterion™ TGX (Tris Glycine eXtended) stain-Free™ precast gels

Carmien SA PTY LTD – fermented *Aspalathus linearis* (Rooibos)

Cell Signalling Technologies, Beverly, Massachusetts, USA - eNOS total, eNOS phosphorylated, PKB/AKT total, PKB/AKT phosphorylated

Cipla MedPro (Pty) Ltd. Bellville, Western Cape, SA - GlucoPlus™ glucose meter

Colorado Springs, USA- Trichloroacetic acid (TCA, C₂HCl₃O₂)

Dunedin, New Zealand - Lab Chart Pro 7 data capturing software

Fluka Chemie, Switzerland - Butylated hydroxytoluene (BHT)

Merck (Pty) Ltd. Darmstadt, Germany - Polyvinylidene Difluoride (PVDF) Membrane

Sigma St. Louis, MO, USA – Acetylcholine, Aprotinin (C₂₈₄H₄₃₂N₈₄O₇₉S₇), Bicinchoninic Acid (BCA) Protein Assay Kit, Bovine Serum Albumin (BSA), Bromophenol blue (C₁₉H₁₀Br₄O₅S), Coomassie Brilliant Blue, Diethylenetriaminepentaacetic Acid (DETAPAC; [(HOOCCH₂)₂NCH₂CH₂]₂NCH₂COOH; Cat D6518), Dipotassium Phosphate (K₂HPO₄), Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA, C₁₄H₂₄N₂O₁₀), Ethylenediaminetetraacetic Acid (EDTA, C₁₀H₁₆N₂O₈), Hamilton syringe, Hydrogen Peroxide (H₂O₂), 6-hydroxydopamine (6-OHD, C₈H₁₁NO₃), Leupeptin, Mercaptoethanol (C₂H₆OS), Monopotassium Phosphate (KH₂PO₄), Perchloric acid (HClO₄), Phenylephrine, Sodium Dodecyl Sulfate (SDS; C₁₂H₂₅NaO₄S), Sodium Orthovanadate (Na₃VO₄), Sodium Phosphate (Na₃PO₄), TBS (Tris-buffered saline), 1,2-Bis(dimethylamino) ethane (TEMED), 2-Thiobarbituric acid (TBA; C₄H₄N₂O₂S), Triton X-100 (C₁₈H₂₈O₅), Tween® 20 (C₅₈H₁₁₄O₂₆)

Thermo Scientific, Lithuania, European Union - Molecular Marker

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2.2 Pilot Study

Prior to commencement with the main study, two pilot studies (first pilot study and the second pilot study) were conducted to determine the lowest and most suitable concentration of streptozotocin (STZ) dose required to induce diabetes mellitus and to determine the most effective way of administering rooibos and honeybush treatments to the animals. Moreover, the pilot studies were conducted in order to determine the sample size necessary to conduct a full investigation of the modulating effects of 2% fermented rooibos (RB) and 4% fermented honeybush (HB) on vascular function and the antioxidant status of STZ-induced diabetic adult male Wistar rats. In compliance to the “Policy for Responsible Research Conduct at Stellenbosch University” (document reference number: BEL-001E-2013), ethical approval for the project (number: SU-ACUD16-00101) was granted by the Research Ethics Committee for Animal Care and Use of Stellenbosch University. The study was carried out under the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

2.2.1 Subjects

In both pilot studies, adult male Wistar rats with an average body weight of 250-300 g were obtained and housed under standard conditions (12-hour day and 12-hour night cycles at a room temperature of 23 °C ± 2 °C). Animals received *ad libitum* access to food (standard Epol™ rat chow) and their respective treatment [tap water, fermented rooibos (RB) or fermented honeybush (HB) infusions. Each STZ dose was dissolved in citrate buffer, pH 4.5 and injected intraperitoneally. Rats that presented with a fasting blood glucose level of 14 mmol/L at 72 hours after receiving the injection were considered diabetic.

Prior to treatment, rats were acclimatized to human handling and to the blood pressure equipment for a total duration of 1 week. In both pilot studies, 2% RB or 4% HB infusions was administered to the animals for a total duration of 6 weeks. Furthermore, biometric measurements (body weight, fluid and food intake) were measured thrice

weekly and the blood glucose levels of the animals were measured once a week by means of the tail prick method using a Glucoplus® glucometer. Furthermore, the blood pressure of the animals was monitored once a week using the CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific) that measures blood pressure non-invasively by means of a volume-pressure recording (VPR) method (**Figure 2:1**).

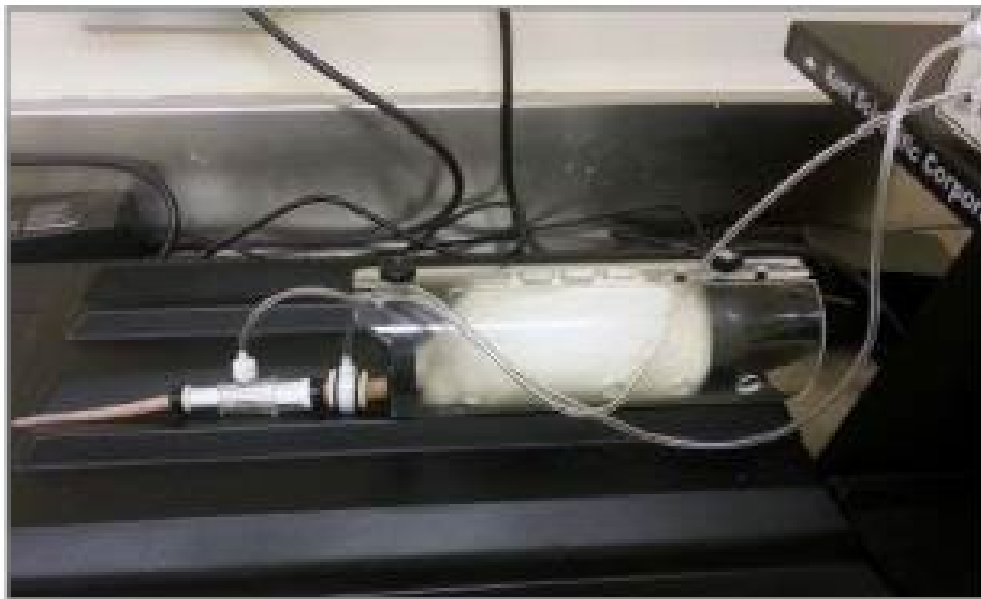


Figure 2:1 The CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific) showing a rat resting on a heating platform with its rat tail inserted through the blood pressure Occlusion and VPR-cuffs (Maqeda, 2018).

2.2.2 First Pilot Study

The aim of the first pilot study was to establish an optimal STZ dose that would successfully induce diabetes mellitus. A summary of the first pilot study and its outcomes are indicated in **Figure 2:2** below.

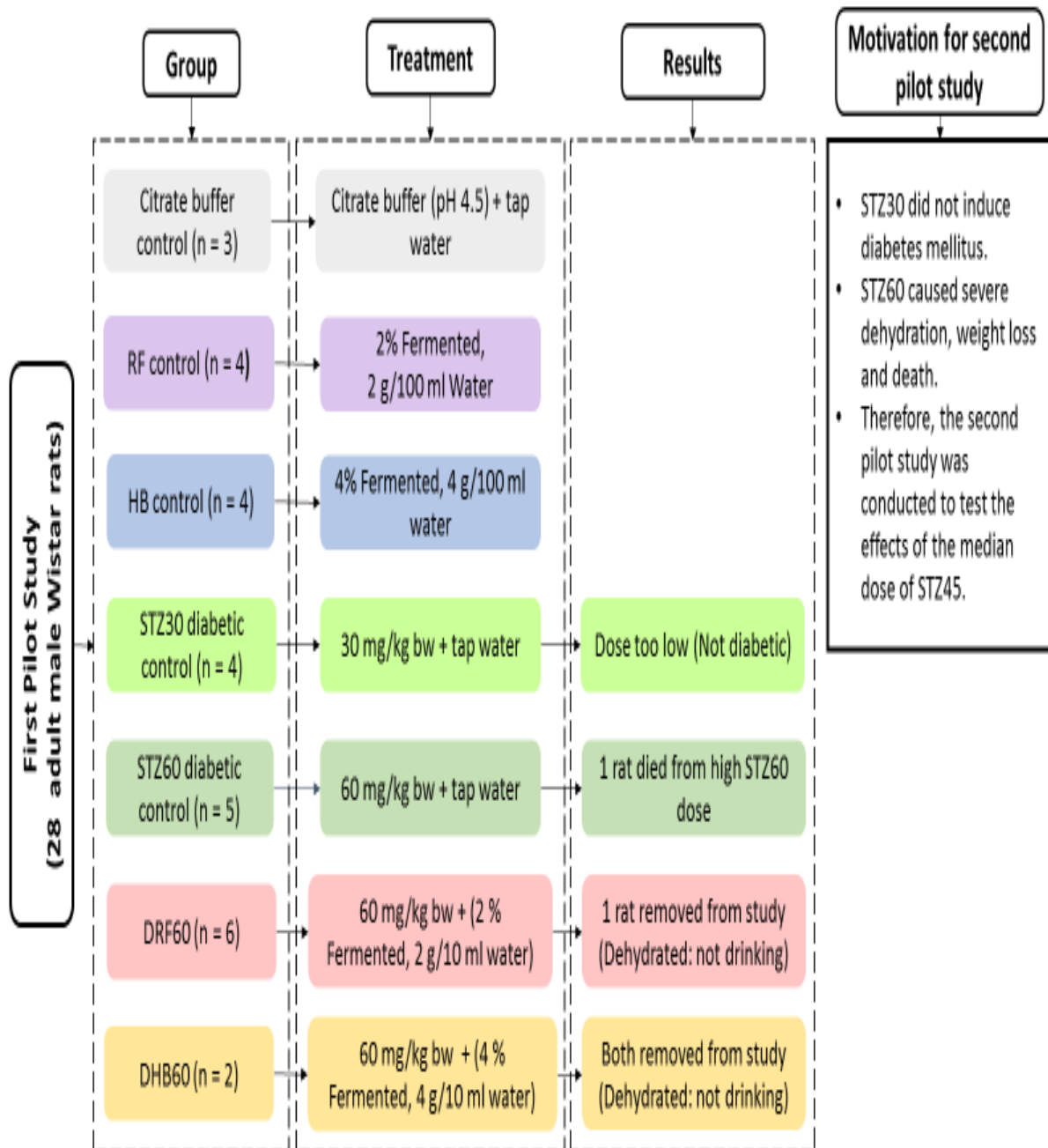


Figure 2:2 Summary of the first pilot study.

2.2.2.1 Methods

In the first pilot study, two STZ doses (STZ dose of 60 mg/ kg bw and 30 mg/kg bw) were used to induce diabetes mellitus (see **Figure 2:2**). A total of 28 animals were randomly assigned into 7 treatment groups (n=2-6 per group) as shown in **Table 2:1**.

Table 2:1 Treatment groups (n= 2-6 /group) of the first pilot study receiving treatment for 6 weeks.

Groups	Treatment	Number of animals per group
Group 1: Citrate buffer control	Citrate buffer (pH 4.5) (<i>STZ is dissolved in citrate buffer</i>) + tap water	3
Group 2: RF control	2% Fermented, 2 g/100 ml water	4
Group 3: HB control	4% Fermented, 4 g/100 ml water	4
Group 4: STZ30 Diabetic control	30 mg STZ/kg bw + tap water	5
Group 5: STZ60 Diabetic control	60 mg STZ/kg bw + tap water	6
Group 6: DRF60	(60 mg/kg bw) + (2% Fermented, 2 g/100 ml water)	3
Group 7: DHB60	(60 mg/kg bw) + (4% Fermented, 4 g/100 ml water)	2

Treatment was administered in the form of a drinking fluid. Rooibos (2% fermented, 2 g/100 ml water), and honeybush (4% fermented, 4 g/100 ml water) were prepared by steeping using standard laboratory herbal infusion preparation methods (see *section 2.5.1*) (**Figure 2:2: Table 2:1**). Furthermore, the concentrations used to prepare the herbal infusions were concentrations that are customarily found in a cup of fermented RB or HB tisane. (Du Toit and Joubert, 1999; Marnewick *et al.*, 2003). After the 6 week treatment period, animals were euthanized with an overdose intraperitoneal injection of Eutha-naze (160 mg/kg bw) and the aortas harvested and used to perform aortic contraction/ relaxation studies as described in *section 2.10*.

2.2.2.2 Results and Discussion

In the first pilot study, the 30 mg/kg bw STZ dose did not induce diabetes. However, the 60 mg/kg bw STZ successfully induced diabetes 72 hours after injection. As a result, Treatment (RB and HB) was only administered to the diabetic animals injected with the STZ dose 60 mg/kg bw. Furthermore, the 60 mg/kg bw STZ dose induced severe weight loss, severe diabetic hyperphagia, severe diabetic polydipsia and significant increases in the blood glucose levels in diabetic animals when compared to their age-matched controls (**Table 2:2**). The diabetic animals (DRF60 and DHB60) did not find either of the infusions palatable in the first 1-2 weeks of the treatment period. As a result, dehydration that resulted in loss of appetite and significant weight loss was observed in both diabetic groups (DRF60 and DHB60) in the first 1-2 weeks of the 6-week treatment period. However, the DRF60 group adapted to the taste of the RB infusion in the last 4-5 weeks of treatment. As a result, significant increases in their RB intake and food consumption was observed in the last 4-5 weeks of treatment (**Table 2:2**). Furthermore, a significant aortic pro-relaxation effect was observed in the aortic relaxation performance of the DRF60 group when compared to the untreated diabetic group (STZ60 mg/kg bw) drinking tap water (**Figure 2:3**). On the contrary, the DHB60 group failed to adapt to the taste of the HB infusion after the first 1-2 weeks of treatment. As a result, in the second or third week of treatment, all the animals in the DHB60 group were removed from the study due to physical signs of cachexia characterized by dehydration, asthenia and malnutrition (**Table 2:2**).

Table 2:2 Biometric measurement results and outcome of the first pilot study.

Group	Number of animals (before treatment)	Number of animals (after treatment)	Mean Body Weight (g)	Mean Fluid Intake (ml)	Mean Blood Glucose Levels (mmol)	Outcome (at the end of the 6-week treatment period)
Group 1: Citrate buffer control	3	3	365.8±16.10	30.92±1.98	6.57±0.088	All healthy
Group 2: RB control	4	4	362.7±9.66	48.16±1.47	6.70±0.091	All healthy
Group 3: HB control	4	4	367.3±8.37	41.97±2.31	6.53±0.21	All healthy
Group 4: STZ30 Diabetic control	5	5	391.3±13.60	32.88±1.71	6.76±0.12	Dose too low - not diabetic
Group 5: STZ60 diabetic control	7	6	233.5±12.22	186.5±8.13	26.10±0.41	1 died from the STZ
Group 6: DRF60	6	3	258.1±7.58	173.6±15.23	25.63±0.37	3 removed from study (Dehydrated: not drinking)
Group 7: DHB60	2	0	N/A	N/A	N/A	Both removed from study (Dehydrated: not drinking)

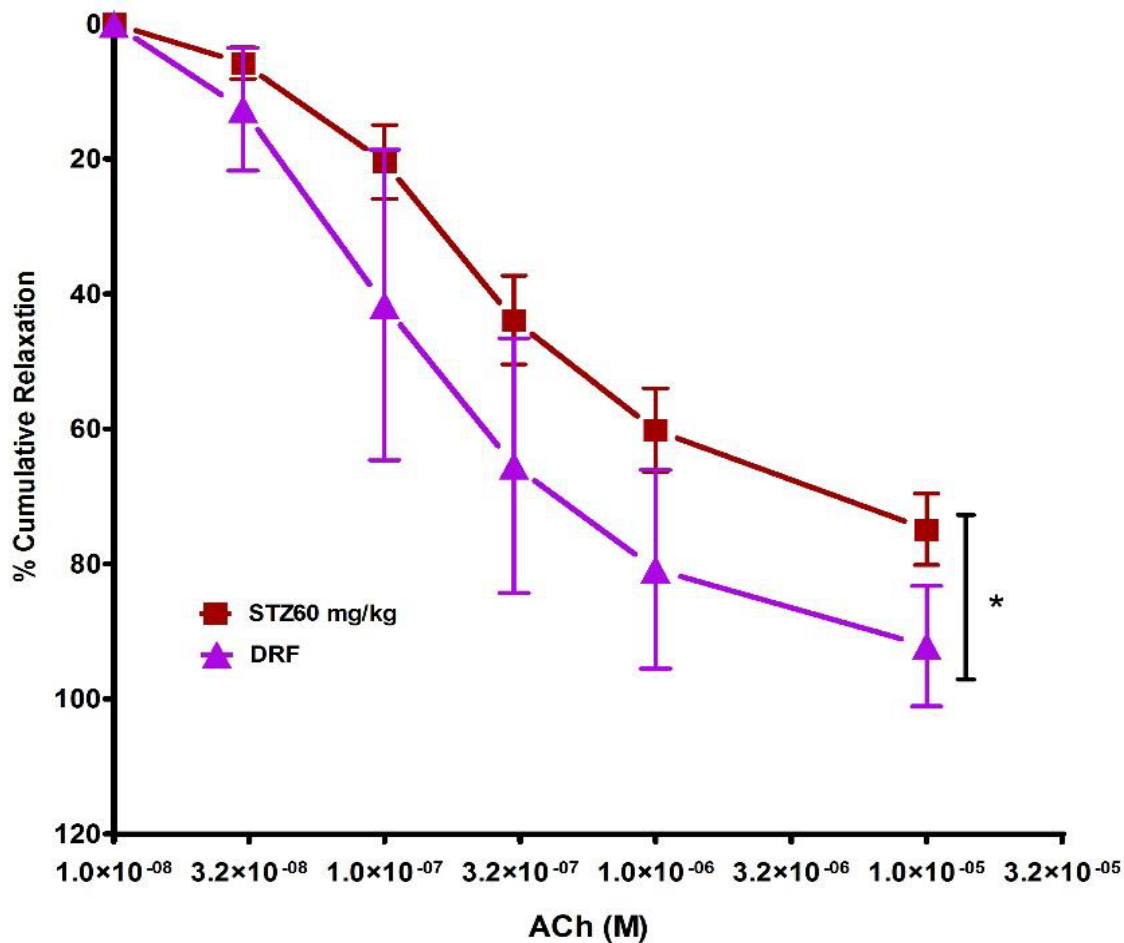


Figure 2:3 Diabetic rats (DRF60) treated with RB had significantly enhanced relaxation compared to the untreated diabetic group. (* $p < 0.05$) ($n = 3-5$ /group).

2.2.3 Second Pilot Study

In the first pilot study, the STZ dose of 30 mg/kg bw did not induce diabetic effects. Although the STZ dose of 60 mg/kg bw successfully induce diabetes, rats presented with physical signs of cachexia characterized by dehydration, asthenia, loss of appetite and severe weight loss due to the excessive cytotoxic effects that high dose has on the pancreatic islet β cells. As a result, the aim of the second pilot study was to determine whether a STZ dose of 45 mg/kg bw would induce a diabetic injury model that has less cytotoxic effects to the pancreatic islet β cells than the 60 mg/kg bw STZ dose (see **Figure 2:4**). Additionally, the second aim of the second pilot study was to determine the optimal method of delivery of the RB and HB extracts to the rats. As a result, the RB and HB extracts were administered to the animals in the form of jelly cubes or drinking fluids (infusions) (**Table 2:3**).

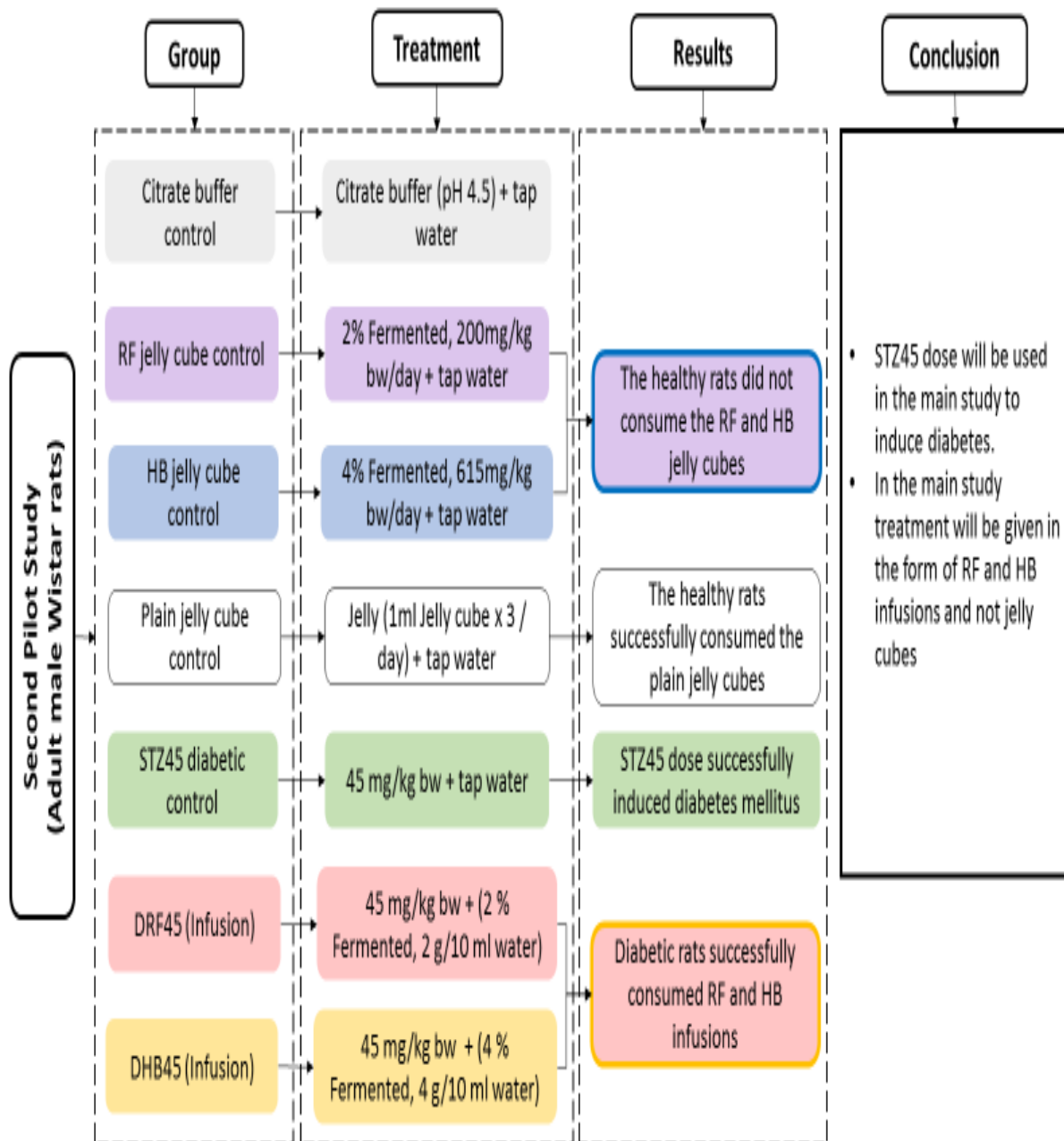


Figure 2:4 Summary of the second pilot study.

2.2.3.1 Methods

Table 2:3 Treatment groups of the second pilot study receiving treatment for 6 weeks.

Groups	Treatment
Group 1: Citrate buffer control	Citrate buffer (pH 4.5) (<i>STZ is dissolved in citrate buffer</i>) + tap water
Group 2: Jelly control	Jelly (1 ml Jelly cube x 3 /day) + tap water
Group 3: RB control	2% Fermented, 200 mg/kg bw/day + tap water
Group 4: HB control	4% Fermented, 615 mg/kg bw/day + tap water
Group 5: STZ45 diabetic control	45 mg/kg bw + tap water
Group 6: DRF45	STZ (45 mg/kg bw) + Rooibos 200 mg/kg bw/day + tap water
Group 7: DHB45	STZ (45 mg/kg bw) + Honeybush 615 mg/kg bw/day + tap water

In the second pilot study, healthy rats were initially given treatment in the form of jelly cubes (**Table 2:3**). Jelly cubes were prepared by freeze-drying the infusions (infusions prepared as described in *section 2.5.1*) over a period of 3-4 days using the FreeZone6 freeze drier (Labconco, Kansas City, MO, USA). Following this, the freeze-dried extracts [rooibos (200 mg/kg bw) and honeybush (615 mg/kg bw)] were dissolved in jelly that was prepared by dissolving 4 g of strawberry jelly and 4 g of gelatine in 25 ml hot tap water. The healthy control rats were given 3 x 1 ml Jelly cubes/day for 1 week. However, the healthy rats did not consume the jelly cubes infused with RB or HB. Thus, for the duration of the second pilot study, treatment was administered as drinking infusions for 6 weeks. To ensure that the animals acclimatize to the taste of the infusions, a 1-week pre-treatment of RB or HB was administered to the diabetic groups (DRF45 and DHB45) followed by a 6-week treatment.

2.2.3.2 Results and Discussion

In the second pilot study, the STZ dose of 45 mg/kg bw successfully induced diabetes characterized by weight loss, hyperphagia, polydipsia and increased glucose levels

when compared to the controls. Compared to the STZ60 dose of the first pilot study, the STZ45 dose did not induce asthenia, dehydration and loss of appetite in the diabetic groups (DRF45 and DHB45).

Based on the findings of the two pilot studies conducted, it was concluded that the STZ45 dose would be used to induce diabetes in the main study. Furthermore, the diabetic groups (DRF45 and DHB45) successfully consumed the treatment in the form of infusions after they were given the infusions as a pre-treatment for 1 week. The pre-treatment was consumed before the rats were injected STZ. Thus, for the main study, diabetic animals (DRF45 and DHB45) were placed on a 1-week pre-treatment to acclimatized them to the taste of the infusions prior to inducing STZ diabetes mellitus.

2.3 Main Study: Methods

A total of 70 adult male Wistar rats, weighing 220-300 g and divided into 7 groups (n=10), (see **Figure 2:6**) were used to achieve the aim and objectives of this study (**Table 2:4**). A summary of the main study and the experiments conducted are indicated in **Figure 2:5**.

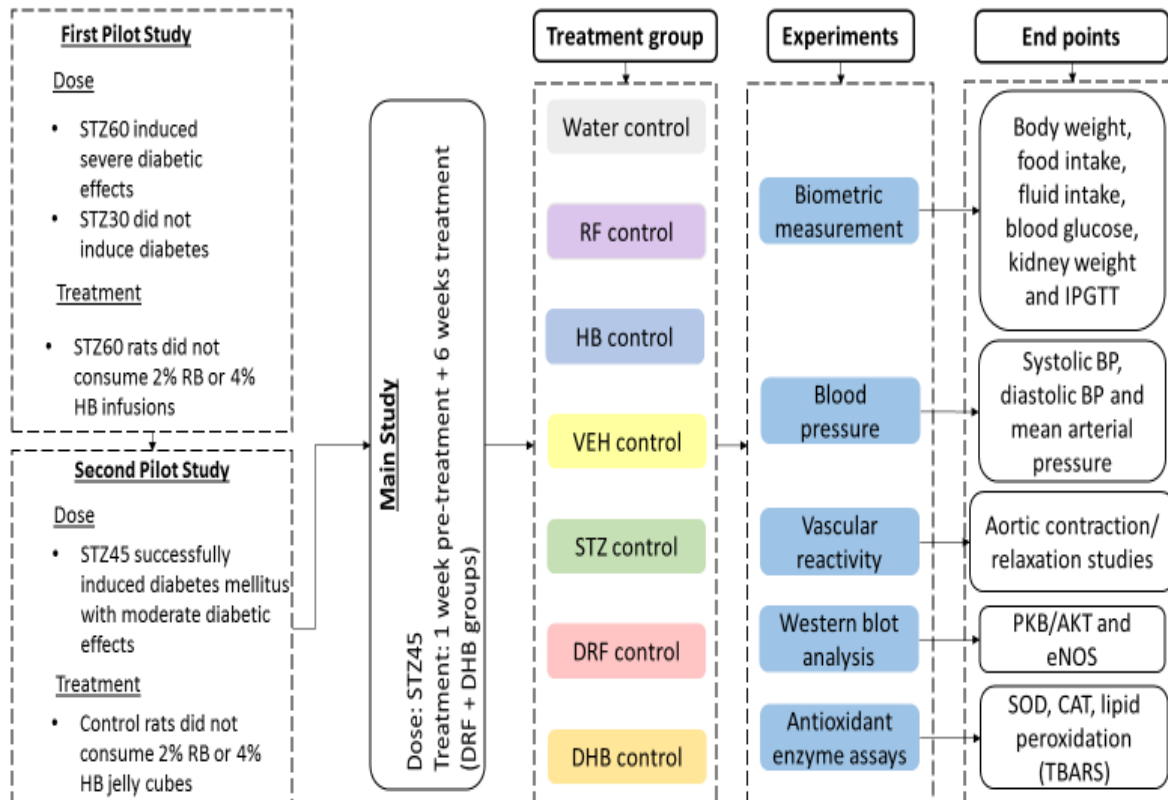


Figure 2:5 Summary of the measurements performed throughout the main study.

2.4 Animals

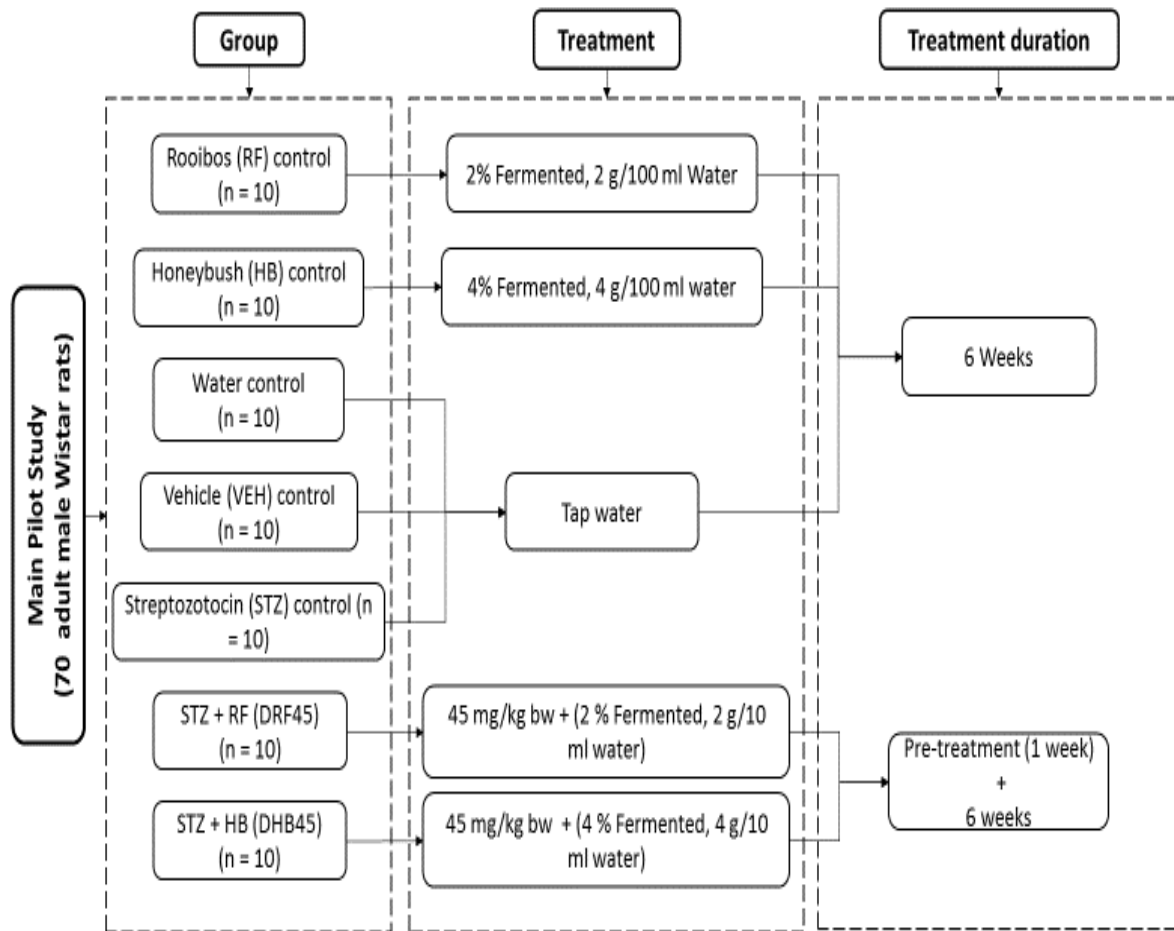


Figure 2:6 Treatment groups (n=10/group) for a total duration of 17 weeks.

Table 2:4 Main study treatment groups (n= 10 /group) receiving treatment for 6 weeks.

Treatment group	Pre-treatment (1 week)	Treatment (6 weeks)
Group 1: Water control	-	Tap water
Group 2: RB control	-	2% Rooibos fermented, 2 g/100 ml water
Group 3: HB control	-	4% Honeybush fermented, 4 g/100 ml water
Group 4: VEH control	-	Citrate buffer (pH 4.5) (<i>STZ is dissolved in citrate buffer</i>) + tap water
Group 5: STZ45 control	-	Streptozotocin 45 mg/kg bw + tap water
Group 6: DRF45	Streptozotocin 45 mg/kg bw) + (2% Rooibos Fermented, 2 g/100 ml water)	Streptozotocin 45 mg/kg bw) + (2% Rooibos Fermented, 2 g/100 ml water)
Group 7: DHB45	Streptozotocin 45 mg/kg bw) + (4% Honeybush Fermented, 4 g/100 ml water)	Streptozotocin 45 mg/kg bw) + (4% Honeybush Fermented, 4 g/100 ml water)

On arrival, animals were acclimatized to human handling and the blood pressure equipment [CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific)] for 1 week. In the first pilot study, the RB and HB infusions were found unpalatable by the diabetic animals (see section 2.2.2). As a result, in the second pilot study, a 1 week pre-treatment of the infusions was administered to the animals in order to acclimatize them to the taste of the infusions (see section 2.2.3). Furthermore, in the second pilot study, the pre-treatment was given only to the group of animals in which

diabetes was induced. The pre-treatment was not administered to healthy controls as these controls successfully consumed the RB or HB infusions (see section 2.2.2). Therefore, in the main study, a 1 week pre-treatment of the infusions was given prior to STZ induction in animals (DRF45 and DHB45) that were to receive STZ injections (see **Table 2:4; Figure 2:6**) After the 1 week pre-treatment, diabetes was induced by a single intraperitoneal injection of STZ (45 mg/kg bw dissolved in citrate buffer, pH 4.5) in all diabetic groups (**Table 2:4**). Animals that presented with a blood glucose level of 14 mmol/L a week following injection were considered diabetic. During the first 2 days of the 6-week treatment period, animals were given the 2% fermented RB or 4% fermented HB infusions supplemented with a 10% glucose solution to prevent fatal hypoglycaemia. Severe hypoglycaemia occurs in experimental streptozotocin-induced diabetes mellitus due to the sudden surge in insulin release that occurs as a result of STZ-induced pancreatic β -cell necrosis (Furman, 2015). Thereafter, two groups of healthy non-diabetic animals and two groups of diabetic animals were given RB and HB infusions respectively as their drinking fluid for 6 weeks (**Figure 2:6**). A vehicle (VEH) control receiving normal tap water as its drinking fluid, was also established as the age-matched control group for the streptozotocin solution (**Figure 2:6**). This group of animals received a single intraperitoneal injection of citrate buffer; the solvent used to dissolve streptozotocin. Lastly, a group of animals receiving normal tap water (H₂O) was established as the absolute or baseline age-matched control group of the study (**Table 2:4; Figure 2:6**).

Animals were bred and cared for in the Animal Research Facility of the Faculty of Medicine and Health Sciences, Stellenbosch University. The animals were housed under a 12-hour day and night cycle, at a room temperature of $\pm 22-23$ °C. Animals were kept individually in cages where each one had *ad libitum* access to food (standard Epol™ rat chow) and their respective drinking fluid.

2.5 Treatment

2.5.1 Preparation

Rooibos (2% fermented, 2 g/100 ml water) [Carmien, South Africa (SA) PTY LTD] and honeybush (4% fermented, 4 g/100 ml water) (Afrinaturals, SA) infusions were prepared by steeping according to standard laboratory tea preparation methods (Du Toit and Joubert, 1999; Marnewick *et al.*, 2003). Rooibos (20 g) or honeybush (40 g)

plant material was added to 1 L boiling water and allowed to steep for 30 minutes whilst stirring the infusion every 5 to 10 minutes. Subsequently, rooibos or honeybush plant material was removed from the infusion by filtration using a single layer of cheese cloth. Furthermore, the infusions were filtered through two filter papers (pore size 3 HW 185 mm 65 g/m² and 288 HW 185 mm 85 g/m² - Whatman, England). Thereafter, infusions were transferred into dark plastic bottles and stored at 4 °C and used within 7 days.

2.5.2 Chemical Analyses

2.5.2.1 Total Soluble Solids

Soluble solids refer to contents such as sugars, acids, vitamins, minerals, proteins, phenolics and pigments that are present in plants. The total soluble solid (TSS) content analysis is an important indicator of the soluble solid concentration (mg/ml) present in the plant material used to prepare the RB and HB infusions. Moreover, TSS is an indicator of the quality of the plant materials used to prepare the infusions. The soluble solid content of RB and HB was determined gravimetrically. Small empty beakers were allowed to stand overnight for ±12 hours in an oven (Hotbox oven size two, Gallenkamp, England) at 80 °C. Thereafter, the empty beakers were removed and placed in a desiccator overnight for ±12 hours. Subsequently, the weight of the empty beakers was measured before adding 1 ml of RB or HB infusion (in triplicate) into the beakers. Thereafter, the beakers containing 1 ml RB or HB were placed a second time in the oven (HOTBOX OVEN size two, Gallenkamp, England) at 80 °C for ±12 hours. Following this, the beakers were placed in the desiccator a second time for ±12 hours. The soluble solid content was calculated by subtracting the weight of the empty beaker from the weight of the beaker containing 1 ml RB or HB.

2.5.2.2 Total Flavonol Content

Flavonols are polyphenolic phytochemical structures present in RB and HB that confer antioxidant and free-radical scavenging properties. The total flavonol content of RB and HB was evaluated spectrophotometrically using the Spectronic® 20 Genesys™ spectrophotometer (Spectronic Instruments, Leeds, UK).

Table 2:5 Preparation of quercetin standards in triplicates.

Quercetin concentration (mg/l)	0	8	4	2	1
Quercetin (ml)	0	0.8	0.4	0.2	0.1
+ EtOH (ml)	4	3.2	3.6	3.8	3.9

Table 2:6 Spectrophotometric determination of total flavanol content in triplicates.

	Dilution factor	Infusion (ml)	95% EtOH (ml)
Fermented Rooibos	65x	0.08	4.92
	100x	0.05	4.95
	125x	0.04	4.96
Fermented Honeybush	100x	0.05	4.95
	120x	0.04	4.96
	150x	0.03	4.97

Quercetin, a flavonoid of known concentration [1 mg 0.004% quercetin in 25 ml 95% ethanol (EtOH)] was used to set up the standard curve used to calculate the unknown total flavanol content present in RB and HB infusion respectively. The quercetin standards were prepared to a final volume of 4 ml as indicated in **Table 2:5**. Thereafter, RB and HB infusions were diluted in 95% EtOH to a final volume of 5 ml according to various dilution factors (

Table 2:6) Following this, quercetin standards and samples were transferred into 1ml cuvettes and absorbance was measured at a wavelength of 362 nm using the Spectronic® 20 Genesys™ photo spectrometer (Spectronic Instruments, Leeds, UK).

2.5.2.3 Total Polyphenol Content

Total polyphenol content was determined using the Folin-Ciocalteu microplate method (Arthur *et al.*, 2011). Gallic acid (GA), a phenolic acid of known concentration (10 mg 0.1% GA in 10 ml deiH₂O) was used to set up the standard curve used to calculate the unknown total polyphenol content present in RB and HB infusions respectively. The GA standards were prepared to a final volume of 4 ml as indicated in **Table 2:7**. Samples (RB and HB infusions) were prepared according to appropriate dilutions using deiH₂O as the solvent (**Table 2:7** and **Table 2:8**).

Table 2:7 Preparation of Gallic acid (GA) standards.

GA concentration (mg/l)	0	10	20	40	60	80	100
GA (µl)	0	5	10	20	30	40	50
+ deiH ₂ O (µl)	500	495	490	480	470	460	450

Table 2:8 Total polyphenol content determination in RB and HB infusions.

Dilution factor	RB or HB (µl)	deiH ₂ O (µl)
25x	10	240
50x	10	490
75x	10	740
100x	10	90
125x	5	620
150x	5	740

Thereafter, in a 96 well plate, 20 µl of the standards and samples were loaded in triplicate. Subsequently, 100 µl Folin-Ciocalteu reagent (2 ml 10% Folin-Ciocalteu reagent in 20 ml deiH₂O) was added to all wells and mixed gently on a vortex using a microplate attachment. Thereafter, the plate was left to rest at room temperature for 5 minutes before adding 80 µl 7.5% Na₂CO₃ (7.5 g in 100 ml deiH₂O) to all wells. The

plate was mixed gently on a vortex using a microplate attachment, covered with foil and allowed to stand in the dark for 2 hours at room temperature. Thereafter, absorbance was measured at 765 nm using the FLUOstar Omega® microplate reader (BMG Labtech, Offenburg, Germany).

2.6 Biometric Measurements

The body weights of animals and their fluid intake was measured thrice weekly. Animals were given fresh infusions or tap water thrice weekly. Furthermore, animals were restrained in a rodent holder and their blood glucose measurements were taken once a week by means of the tail-prick method using a Glucoplus™ glucometer (Ayeleso, Brooks and Oguntibeju, 2014; Dlodla *et al.*, 2014). Additionally, blood pressure was monitored non-invasively and measured once weekly, every second week using the CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific) (**Figure 2:1**). Furthermore, in the last week of treatment, intraperitoneal glucose tolerance tests (IPGTT) were performed (see **Figure 2:5**).

2.7 Blood Pressure

The blood pressure measurement of each animal was recorded once every second week following acclimatisation for one week. Blood pressure was measured with the CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific) (**Figure 2:1**). To measure blood pressure, the system uses VPR to detect the blood flow volume in the tail using a sensor and an occlusion tail cuff (Hem *et al.*, 2019). The animals were placed in a rodent holder on a heating platform with their tail inserted through a volume pressure recording (VPR)-cuff and an occlusion tail-cuff (**Figure 2:1**). Animals thermoregulated over period of 10-40 minutes in order to stimulate an increased tail blood flow volume so that a blood pressure signal could be detected. Each session was set to record diastolic blood pressure, systolic blood pressure and the mean arterial pressure (MAP) 6 times. The 6 blood pressure readings generated with each session were used to calculate the mean systolic and diastolic blood pressure as well as the mean MAP.

2.8 Intraperitoneal Glucose Tolerance Test (IPGTT)

The glucose tolerance test is a method used to assess glucose tolerance and insulin sensitivity *in vivo*. This is achieved by measuring or monitoring the rate at which

glucose disappears over time (Matsuda, and DeFronzo, 1999). IPGTT was measured in the last week of the 6-week treatment period. Animals were fasted overnight while receiving *ad libitum* access to their respective drinking fluid. Thereafter, animals were restrained in a rodent holder and their baseline or fasting blood glucose level was measured by means of the tail-prick method using a Glucoplus™ glucometer (Ayeleso, Brooks and Oguntibeju, 2014; Dlodla *et al.*, 2014). Subsequently, animals were injected intraperitoneally with 10 µl glucose/g bw and their blood glucose level was monitored for 2 hours and measured with a Glucoplus™ glucometer at time points; 3 minutes (min), 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 45 min, 60 min, 90 min and 120 min. Thereafter, animals were left to recover from this metabolic insult for 5-7 days before sacrifice.

2.9 Sample Collection

Prior to sacrifice, the body weight of each animal was recorded, and they were fasted overnight while receiving *ad libitum* access to their respective drinking fluid. Subsequently, animals were euthanized by an overdose intraperitoneal injection of Eutha-naze [(sodium pentobarbital (160 mg/kg bw)]. To confirm that the animal was fully anaesthetized, a corneal reflex was performed, and a pair of tweezers was used to test for a pedal reflex by means of a foot-pinch test. Thereafter, the fasting blood glucose level of the animal was measured by means of the tail-prick method using a Glucoplus™ glucometer (Ayeleso, Brooks and Oguntibeju, 2014; Dlodla *et al.*, 2014). Organs were harvested by opening the chest cavity of the animals when there was no response to the pedal and eye-blink reflex test. All organs such as the heart, liver, lung, pancreas and blood were collected and stored for future experimental purposes. The aorta and kidneys were harvested for use in this study. The aorta was excised and kept in cold Krebs buffer to preserve its biological function. Thereafter, the aorta was cleaned of excess tissue and perivascular fat before a small segment of it was mounted onto the aortic ring system used to conduct aortic vascular contraction /relaxation tension studies. The organs were harvested, rinsed in saline and weighed. Thereafter, a small segment of each organ was preserved in formalin for histological analyses in future studies. The remaining portions of each organ, including the aorta, was flash frozen in liquid nitrogen and stored at -80 °C for further analysis.

Furthermore, blood was left to clot on ice for 30 minutes. Thereafter, blood serum was collected after the blood was centrifuged at 1200 g for 10 minutes at 4 °C and stored away in aliquots at -80 °C for future experimental purposes.

2.10 Vascular Aortic Contraction/Relaxation Studies

Vascular aortic contraction/relaxation studies were conducted *ex vivo* to evaluate aortic endothelial function. The aortic contraction/relaxation studies were conducted according to a modified version of the isometric tension measurement protocol adapted from Privett *et al.*, 2004. The aortic ring system used consists of a computer installed with Lab Chart Pro 7 data capturing software, a Power Lab 4/35 data acquisition system, a 25 ml organ bath (AD Instruments, Bella Vista, New South Wales, Australia), an isometric force transducer (TRI202PAD, Panlab, ICornellà, BCN, Spain), an oxygen tank and a warm bath (**Figure 2:7**) (Privett *et al.*, 2004).

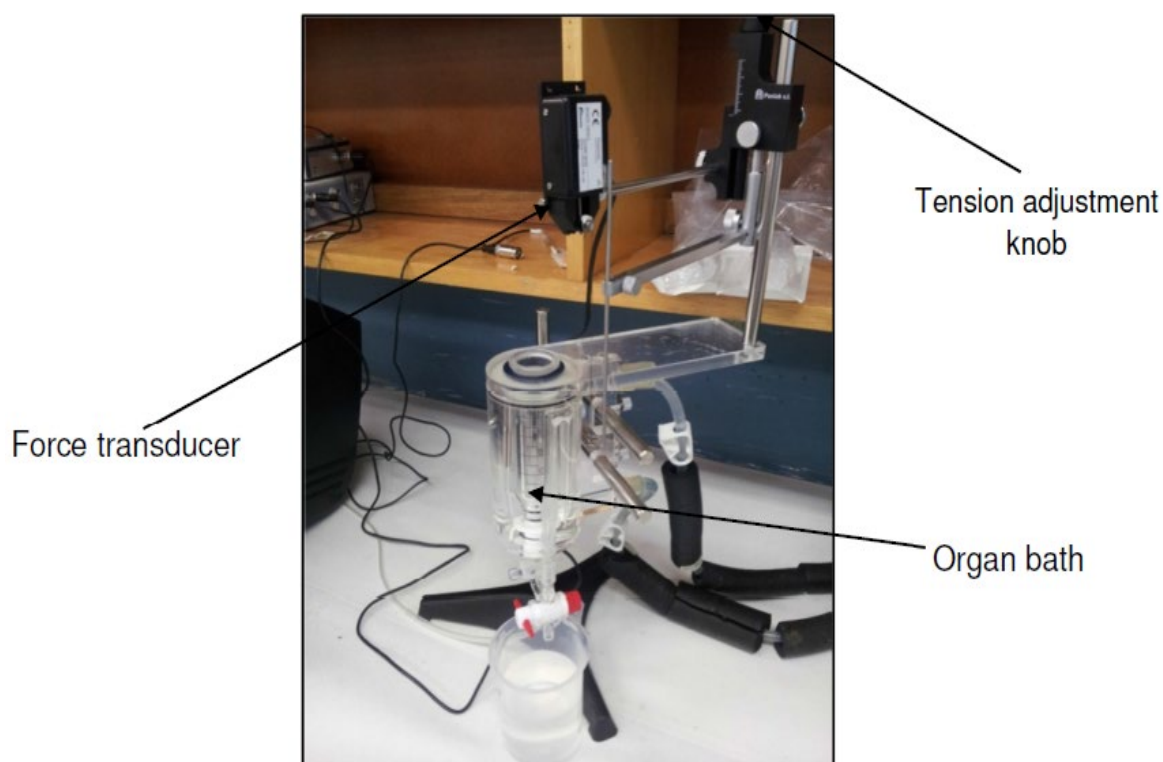


Figure 2:7 Aortic ring system (Loubser and Strijdom, 2014)

Prior to conducting the experiment, the Power Lab 4/35 data acquisition system as well as the computer were switched on and set to the appropriate data acquisition settings using Lab Chart Pro 7 data capturing software. Thereafter, the oxygen tank was switched on and set to buffer the organ bath with 95% O₂ and 5% CO₂.

Subsequently, the organ bath was rinsed (3 times) and filled with 25 ml Krebs-Henseleit (KHB) buffer (composed of 10 mM glucose, 1.25 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 4.75 mM KCl, 1.2 mM KH_2PO_4 , 0.6 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 19 mM NaCl, 25 mM NaHCO_3 and 0.6 mM Na_2SO_4). Thereafter, the water bath was set to $\pm 36\text{-}37^\circ\text{C}$ to keep KHB buffer at a temperature of $\pm 36\text{-}37^\circ\text{C}$ throughout the experiment.

2.10.1 Preparation of Drugs

Phenylephrine (Phe) was used to induce aortic relaxation and acetylcholine (ACh) was used to induce contraction in a stepwise manner or cumulatively. Phe stock (1 mM) was prepared by dissolving 0.002 g Phe in 10 ml 0.9% saline. ACh stock (10 mM) was prepared by dissolving 0.0182 g ACh in 10 ml 0.9% saline (Stock A). Thereafter, a serial dilution of stock A was done to make up 1 mM ACh by dissolving 1 ml of Stock A in 9 ml 0.9% saline (Stock B). Finally, 1 ml of Stock B was dissolved in 9 ml 0.9% saline to make 100 μM ACh (Stock C).

2.10.2 Aortic Ring Segment Preparation

The thoracic aorta was carefully excised from the chest cavity of the rat and submerged in cold KHB buffer inside a (100 mm) petri dish. Under magnification, the aorta was carefully cleaned from all perivascular fat, blood and surrounding tissue using a pair of scissors and thin tweezers (**Figure 2:8**).

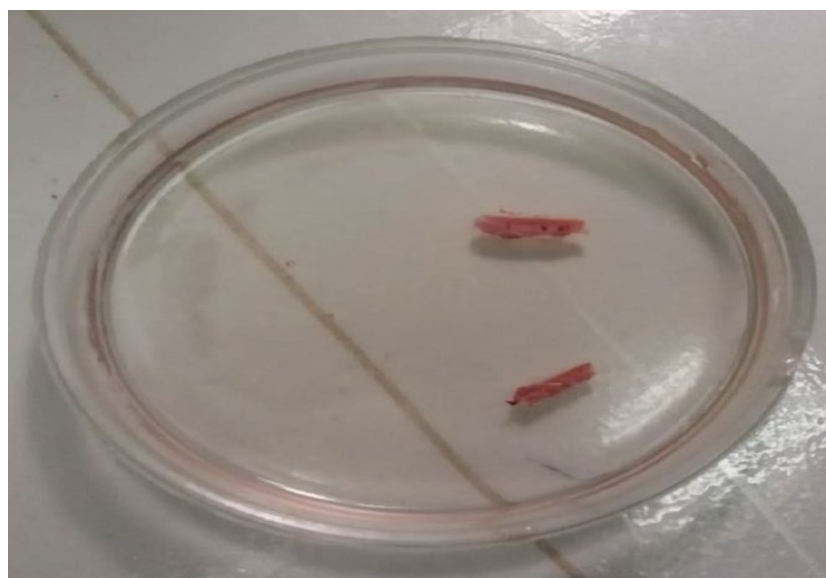


Figure 2:8 Thoracic aortic ring segments (3-4 mm) (Maqeda, 2018).

Thereafter, two 3-4 mm sections of the aorta were cut from the side of the aorta that was handled with scissors or tweezers the least and mounted onto the hook of the organ bath containing pre-warmed ($\pm 36-37$ °C) KHB buffer (**Figure 2:9**).

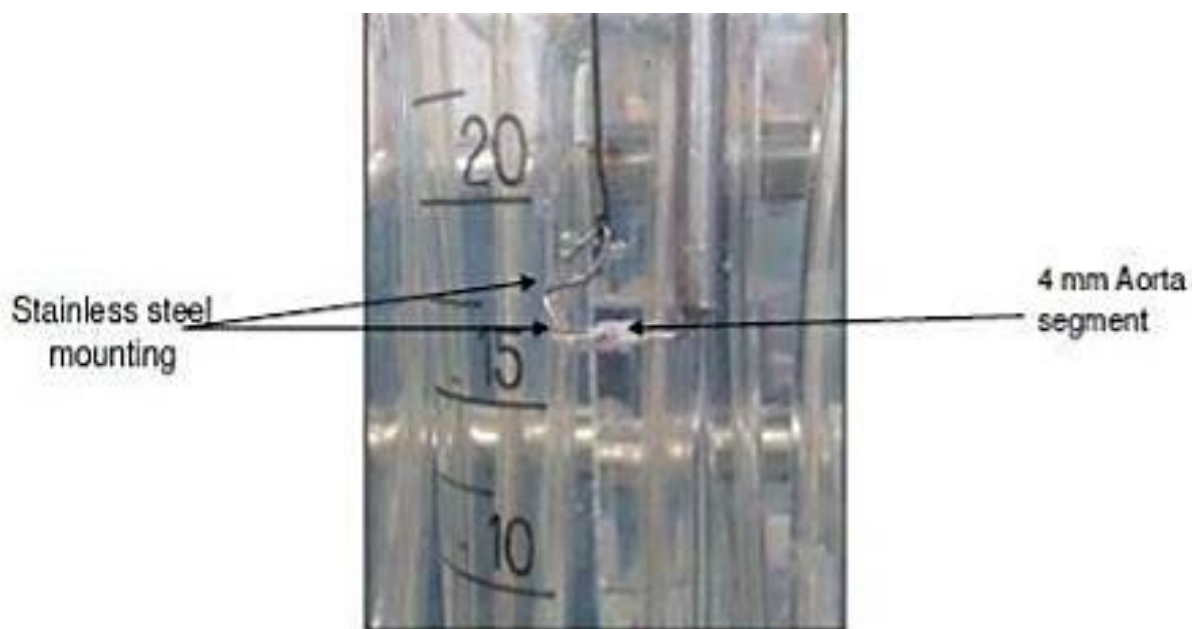


Figure 2:9 A Clean aortic ring segment mounted between two stainless steel hooks in an organ bath (Maqeda, 2018).

2.10.3 Cumulative Aortic Contraction and Relaxation

Once the aortic ring segment was mounted inside the organ bath and submerged in oxygenated KHB buffer, aortic contraction/relaxation studies were performed as described in **Figure 2:10**. Aortic tension was stabilized for 30 minutes by gradually increasing the resting tension to 1.5 (grams of tension). During this initial stabilisation, KHB buffer was replaced with fresh buffer at 10 minutes and 20 minutes time points respectively. Following this, a single dose of 25 μ l Phe (1 mM) for aortic contraction followed by a single dose of 25 μ l ACh [(10 μ M) stock A] for aortic relaxation was added in order to test for aortic activity. Functional aortic activity is characterized by $\pm 70\%$ aortic relaxation which is an indicator of an intact endothelium and vascular smooth muscle layer. Following this, the organ bath was rinsed (3 times) with KHB buffer and the aortic ring was stabilised a second time to 1.5 (grams of tension) for 30 minutes with KHB buffer being replaced with fresh buffer at 10 minutes and 20 minutes time points respectively. At the end of the second stabilisation, cumulative

concentrations of Phe (100 nM, 300 nM, 500 nM, 800 nM, 1 μ M) were added followed by the addition of the cumulative concentrations of ACh (30 nM, 100 nM, 300 nM, 1 μ M, 100 μ M) (see **Table 2:9** and **Figure 2:10**).

Table 2:9 Cumulative concentrations of Phe and ACh.

Phenylephrine (Phe)		Acetylcholine (ACh)	
Concentration	Volume	Concentration	Volume
100 nM	2.5 μ l stock	30 nM	7.5 μ l stock C
300 nM	5.0 μ l stock	100 nM	17.5 μ l stock C
500 nM	5.0 μ l stock	300 nM	42.5 μ l stock C
800 nM	7.5 μ l stock	1 μ M	14.3 μ l stock B
1 μ M	5.0 μ l stock	100 μ M	220.0 μ l stock B

A) Phenylephrine - Acetylcholine

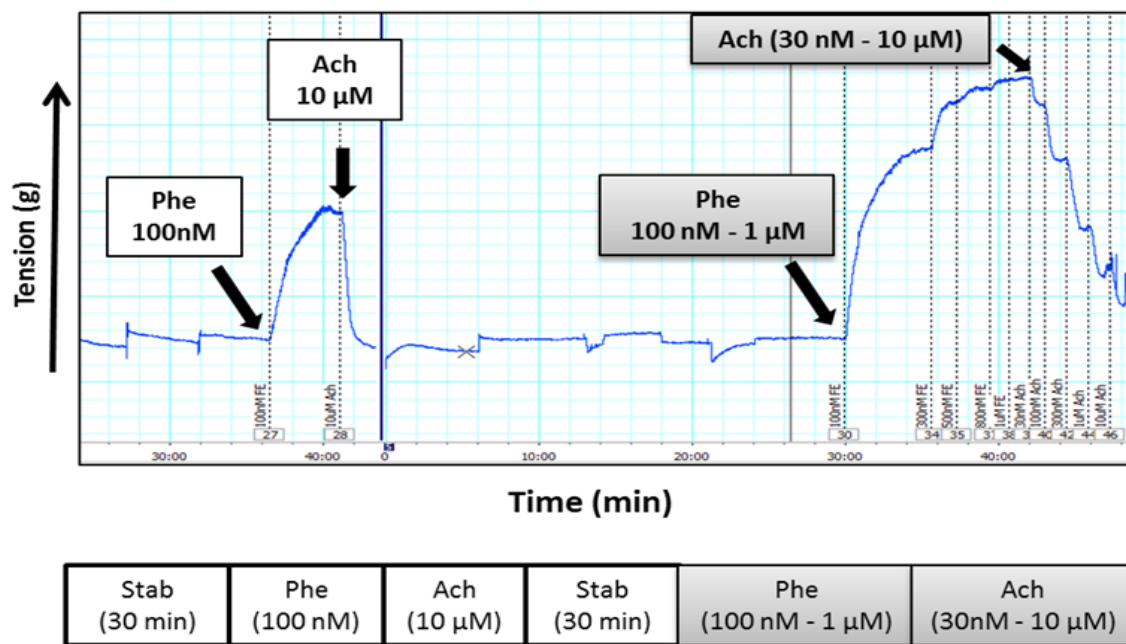


Figure 2:10 Cumulative aortic contraction/relaxation studies experimental protocol (Vascular Reactivity: Aortic ring protocol, Laboratory Manual, Stellenbosch University, Westcott, 2015).

At the end of each aortic contraction/relaxation experiment, data was analysed with Lab Chart Pro 7 data capturing software and aortic activity was expressed as % contraction or % relaxation from the baseline tension of 1.5 grams of tension.

2.11 Western Blot Analysis

Western blotting is a technique used to study the expression of proteins. It is an analytical technique that involves the process of extracting proteins from a given tissue in order to determine the size of a specific protein against a size marker as well as the amount of protein expressed in the treated tissue sample in comparison to an untreated sample or control group.

Western blot analysis was performed on the aortic tissue for the analysis of endothelial proteins [(eNOS total, catalogue number: 9572 or eNOS phosphorylated, catalogue number: 9571 or PKB/AKT total, catalogue number: 9272 or PKB/AKT phosphorylated, catalogue number: 9271), Cell Signalling Technologies (Beverly, Massachusetts, USA)].

Proteins were extracted from the aortic tissue by means of a lysis buffer and primary antibodies were used for the detection of the endothelial proteins of interest. Moreover, a secondary antibody, Horseradish peroxidase (HRP) -conjugated Anti-Rabbit Immunoglobulin G (IgG), catalogue number: 7074 (Amersham life science, Buckinghamshire, UK) was added to each primary antibody to enhance its detection signal.

2.11.1 Lysate Preparation (Aortic Tissue)

Aortic tissue (30-101 mg) was pre-cooled in liquid nitrogen and pulverized with a mortar and pestle. The pulverised tissue was placed in 600 µl lysis buffer in an Eppendorf tube. The composition of the lysis buffer is described in **Table 2:10**. To homogenise the tissue, 1.6 mm stainless still beads (7-10 beads/tissue) were added to the tissue prior to homogenisation by means of a Bullet blender® 24 (Next Advance, Inc. New York) for 5 minutes at speed 8 with 1-minute rest intervals at a temperature of 4 °C (repeat three times).

The homogenate was allowed to rest on ice for 15 minutes and subsequently centrifuged for 20 minutes at speed 15 000 rpm at 4 °C. The resultant supernatant

was transferred to a clean Eppendorf tube to be used for Bradford protein and western blotting analysis.

Table 2:10 Composition of lysis buffer used for aortic tissue lysate preparation (Adapted from Eldieb, 2017).

Reagent	Stock	FINAL Content	Amount for 10 ml
Triton X	10%	1%	1 ml
Tris-Hydrochloride with EGTA (pH 7.5)	200 mM	20.0 mM	1 ml
Ethylenediaminetetraacetic acid (EDTA)	100 mM	1 mM	100 μ l
Sodium chloride (NaCl)	1 M	150 mM	1.5 ml
Beta-glycerophosphate	-	1 mM	0.002 g
Tetra-sodium-pyrophosphate	-	2.5 mM	0.01 g
NaF	-	50 nM	0.0213 g
Na ₃ VO ₄ [0.018 g/10 ml]	10 mM	1.0 mM	1 ml
Leupeptin	10 μ g/ μ l	10 μ g/ μ l	10 μ l
Aprotinin	10 μ g/ μ l	10 μ g/ μ l	10 μ l
Sodium dodecyl sulphate (SDS)	10%	0.1%	10 μ l
Phenylmethylsulfonyl fluoride (PMSF)	100 mM	50 μ g/ml	30 μ l
Distilled water			Fill up to 10 ml

2.11.2 Bradford Protein Determination

The Bradford technique was used to determine protein concentration in each lysate (Bradford, 1976). Samples (supernatants) were prepared in two dilution steps. In the

first dilution step (A), 20 μl sample was diluted in 80 μl of dH_2O . In the second dilution step (B), 80 μl of dH_2O was added to 20 μl (A).

Bovine serum albumin (BSA) [100 μl (5 mg/ml) BSA stock in 400 μl dH_2O] was used to set up the standard curve required to calculate the unknown protein concentration of the aortic lysates. The BSA standards with final volume of 100 μl were prepared as indicated in **Table 2:11**. Bradford reagent containing 500 mg Coomassie Brilliant Blue G-250 protein stain, 250 ml 95% ethanol and 500 ml phosphoric acid was diluted in a 1:5 ratio (20 ml Bradford reagent in 80 ml dH_2O) and filtered through a double layer of Whatman filter papers (pore size; 0.4 μm). Subsequently, 900 μl of Bradford reagent was added to 100 μl BSA standards and samples (B). Thereafter, BSA standards and samples (B) were vortexed and allowed to rest for 15 minutes to allow for the colour change to blue. Thereafter, BSA standards and samples were transferred into a 1 ml cuvette and absorbance was read at 595 nm using the GENESYS™ 20 Visible Spectrophotometer (Thermo Fisher Scientific).

Table 2:11 Preparation of BSA standards for Bradford protein determination in aortic tissue (Adapted from Eldieb, 2017).

BSA concentration ($\mu\text{g/ml}$)	BSA volume (μl)	Distilled H_2O (μl)	Bradford reagent (μl)
0	0	100	900
1.25	5	95	900
2.5	10	90	900
5	20	80	900
10	40	60	900
15	60	40	900

After determining the absorbance of the BSA standards and samples, protein concentration was calculated using the BSA standard curve generated in Microsoft™ Excel and by plotting the BSA protein concentration values shown in **Table 2:11** (x-axis) against the absorbance reading detected at 595 nm (y-axis) using the GENESYS™ 20 Visible Spectrophotometer (Thermo Fisher Scientific). The aortas or

samples yielded a protein concentration of 0.05-0.42 µg/µl. Furthermore, samples were diluted with lysis buffer and Laemmli sample buffer (content: 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 10% Glycerol, 0.03% Bromophenol Blue and 5% β-mercaptoethanol) in a 1:2 ratio. This yielded 0.5-3 µg/12 µl protein (to be loaded in the gels). Subsequently samples were boiled for 5 minutes and stored overnight at -80 °C.

2.11.3 Protein Separation

Samples that were stored overnight (±12 hours) at -80 °C were boiled again for 4 minutes and centrifuged at 5000 rpm for a total duration of 5 minutes. Following this, a 25 µl Hamilton® syringe (Sigma-Aldrich, St. Louis, MO, USA) was used to load samples (0.5-3 µg/12 µl) and to load the molecular marker (5 µl) used to detect the sizes of the proteins of interest) into the wells of the 26-well Bio-Rad TGX stain-free™ precast polyacrylamide gels (4-12%). The molecular marker was loaded in lane 1 and the samples were loaded sequentially in the other lanes. The total number of samples (35 samples, n=5/group) exceeded the number of wells of the 26-well Bio-Rad TGX stain-free™ precast polyacrylamide gels (4-12%). As a result, samples were loaded onto two separate 26-well Bio-Rad TGX stain-free™ precast polyacrylamide gels (4-12%) (see **Figure 2:11**). Following the loading of samples, the 26-well Bio-Rad TGX stain-free™ precast polyacrylamide gels (4-12%) were placed inside the Bio-Rad Mini Gel Protean System and running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3) was poured into the outer and middle compartments of the system. Subsequently, the system was set to facilitate protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V, 200 mA for 10 minutes followed by the second run at 200 V, 200 mA for 50 minutes. Subsequently, the separated proteins were activated using the Bio-Rad ChemiDoc™ MP imager system.

MARKER	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
GEL 1	H 2 0 1	H2O 2	H2O 3	RF 1	RF 2	RF 3	HB 1	HB 2	HB 3	Veh 1	Veh 2	Veh 3	STZ 1	STZ 2	STZ 3	DRF 1	DRF 2	DRF 3	DHB 1	DHB 2	DHB 3
MARKER	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
GEL 2	H 2 0 1	H2O 2	H2O 3	RF 1	RF 4	RF 5	HB 1	HB 4	HB 5	Veh 1	Veh 2	Veh 3	STZ 1	STZ 4	STZ 5	DRF 1	DRF 4	DRF 5	DHB 1	DHB 4	DHB 5

Figure 2:11 Diagrammatic representation of samples loaded into 26-well Bio-Rad TGX stain-free™ precast polyacrylamide gels

2.11.4 Protein transfer

The separated proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) microporous membrane. Prior to transfer, the membrane was equilibrated by submerging it in methanol for 30 seconds and placing it in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol, pH 8.3) at 4°C for 15 minutes. Subsequently, the transfer of proteins was done at 200 V, 200 mA for 60 minutes using the Bio-Rad Midi-transfer system. Following protein transfer, membranes were submerged in methanol for 30 seconds and air dried for 15 minutes at room temperature. Thereafter, membranes were assessed visually for successful protein transfer in the Bio-Rad ChemiDoc™ MP imager system.

2.11.5 Immunoblot Analysis

Following successful protein transfer, membranes were incubated in primary antibody that binds to the specific protein of interest. Prior to incubating the membranes in their respective primary antibodies, it was mandatory to block non-specific binding sites on the membrane to ensure that the primary antibody binds to its target protein and not to non-specific sites on the membrane. To block non-specific sites, membranes were incubated in long life fat free milk (5%) diluted in TBS-Tween (Tris-buffered saline and 0.1% Tween® 20) at room temperature for 1-2 hours at 25 °C on a shaker (Digi system laboratory instrument, Inc). Thereafter, membranes were washed in TBS-Tween (3x 5 minutes) for 15 minutes on the shaker before leaving the membranes to incubate overnight at 4 °C in their respective primary antibody prepared by diluting 5 µl primary antibody in 5 ml TBS-Tween. [(eNOS total, catalogue number: 9572 or eNOS

phosphorylated, catalogue number: 9571 or PKB/AKT total, catalogue number: 9272 or PKB/AKT phosphorylated, catalogue number: 9271), Cell Signalling Technologies (Beverly, Massachusetts, USA)]. Following incubation, membranes were washed again in TBS-Tween (3 x 5 minutes) for 15 minutes on the shaker. Thereafter, membranes were incubated a second time for 1 hour at 4 °C in 1.25 µl HRP-conjugated Anti-Rabbit Immunoglobulin G (IgG) secondary antibody, catalogue number: 7074 (Amersham life science, Buckinghamshire, UK) diluted in 5 ml TBS-Tween. Subsequently, membranes were washed in TBS-Tween (3x 5 minutes) for 15 minutes on the shaker. Finally, 2 ml of enhanced chemiluminescence (ECL) detection agent (1 ml black and 1 ml white ECL) was added to the membranes for 5 minutes where after, membranes were exposed in the Bio-Rad ChemiDoc™MP system and analysis performed using Image Lab™ 6.0 software.

2.12 Antioxidant Activity Analysis

The antioxidant activity of tissue samples is measured by conducting various antioxidant assays. In this study, antioxidant activity was assessed by performing catalase, superoxide dismutase and the thiobarbituric acid reactive substances assays in the kidney tissues of the diabetic rats treated with rooibos and honeybush as well as their age-matched controls.

2.12.1 Lysate Preparation (Kidney Tissue)

Small thin sections of the kidney tissue (± 100 mg) were cut, transferred into Eppendorf tubes and homogenised in a Bullet blender® 24 (Next Advance, Inc. New York) for 4 minutes at speed 8 and for 5 minutes at speed 9 with a 1-minute rest interval between each homogenisation period. Zirconium oxide beads (0.5 mm) (± 100 mg), as well as ± 200 µl of 50 mM potassium phosphate (KPi) buffer, pH 7.5 containing Triton X-100 (50 µl in 10 ml KPi buffer) were added to the kidney tissue to facilitate homogenisation. The homogenates were allowed to rest on ice for 30 minutes. Thereafter, an additional volume (± 800 µl) of KPi buffer, pH 7.5, was added to the kidney homogenates and the homogenates were centrifuged for 20 minutes at 15000 rpm and at a temperature of 4 °C. Following this, the supernatants (samples) were transferred into clean Eppendorf tubes and small aliquots were stored away at -80°C.

2.12.2 Bicinchoninic Acid (BCA) Protein Determination

The Bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich) was used to determine the protein concentration of the kidney lysates. This assay measures protein quantity by reducing copper (II) ions to copper (Cu) in an alkaline environment (pH 7.5). Subsequently, two BCA molecules bind to the Cu ions of the proteins and this association results in a colour change from green to purple in the indicator of the BCA reagent. The intensity of the purple colour change is detected at an absorbance of 562 nm.

A known concentration of BSA (1.088 mg/ml) or (10.088 mg BSA in 10 ml dH₂O) was used to set the standard curve required to calculate the unknown protein concentration in the kidney lysates. The BSA standards with a final volume of 100 µl were prepared as indicated in **Table 2:12**. Thereafter, 12.5 µl of sample (kidney lysates) were diluted in 487.5 µl dH₂O according to a 40x dilution factor. Subsequently samples and BSA standards were vortex and 25 µl sample and BSA standards were assayed in triplicates on a 96-well plate. A mixture of bicinchoninic acid solution (reagent A) and copper (II) sulphate pentahydrate 4% solution (Reagent B) was prepared according to a 50:1 ratio (50 reagent A: 1 reagent B) and vortex. Subsequently, 200 µl of the mixture was added to all wells containing samples and BSA standards. Thereafter, the plate was placed inside a microplate attachment that was attached to a vortex (Lab net International, Inc) to mix gently. The plate was incubated for 30 minutes in a heating block that was pre-heated to 37 °C and subsequently allowed to cool to room temperature for 10 minutes. Thereafter, absorbance was determined at 562 nm using the FLUOstar Omega® microplate reader.

Table 2:12 Preparation of BSA standards for BCA protein determination in kidney tissue.

BSA concentration	0	0.2	0.4	0.6	0.8
BSA (µl)	0	20	40	60	80
dH ₂ O (µl)	100	80	60	40	10

2.13 Catalase Assay

Catalase (CAT) is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide (H_2O_2) to H_2O and O_2 . The catalase assay is a highly sensitive fluorometric measurement of catalase activity in tissue. It measures catalase activity by monitoring the rate at which H_2O_2 is decomposed to H_2O and O_2 over a given period of time (Ellerby and Bredesen, 2000).

Kidney lysates (samples) were diluted to a protein concentration of $0.1 \mu\text{g}/\mu\text{l}$ in 50 mM KPi buffer, 0.5% Triton X-100, pH 7.0 (CAT buffer) using the protein concentration calculations determined by BCA assay (see *section 2.12.2*). Thereafter, $5 \mu\text{l}$ of samples and a blank (CAT buffer) were loaded in triplicate on a 96-well ultraviolet (UV) plate. Subsequently, $170 \mu\text{l}$ CAT buffer was added to all wells containing samples and the blank. Thereafter, H_2O_2 stock solution was prepared by adding $34 \mu\text{l}$ of 30% H_2O_2 to 10 ml CAT buffer. Subsequently, $50 \mu\text{l}$ of the H_2O_2 stock solution was immediately added to all wells and CAT activity was measured by monitoring the linear decrease of H_2O_2 or the rate at which H_2O_2 is consumed for 5 minutes at 30 second intervals at 240 nm using the FLUOstar Omega® microplate reader. Results were expressed as $\mu\text{mol H}_2\text{O}_2$ consumed/min/ μg protein with the molar extinction coefficient ($43.6 \text{ M}^{-1} \text{ cm}^{-1}$) used for the normalization of the path length of the well.

2.14 Superoxide Dismutase Assay

Superoxide dismutases (SOD) are antioxidant metalloenzymes that dismutase reactive superoxide anions (O_2^-) to either molecular O_2 or H_2O_2 . There are three isoforms of SOD metalloenzymes namely; Copper/Zinc (Cu/Zn) SOD, Manganese (Mn) SOD and Iron (Fe) SOD. The SOD assay conducted in this study measures Cu/Zn SOD activity according to the method of Ellerby and Bredesen, 2000 which records the auto-oxidation of 6-hydroxydopamine (6-OHD). 6-OHD generates ROS upon its oxidation. Furthermore, 50% inhibition of auto-oxidation of 6-OHD is defined as one unit of SOD activity and the total SOD activity was determined in the cytosolic fraction of the kidney tissue.

Kidney lysates (samples) prepared as described in *section 2.12.1* were used to determine SOD activity. Samples were diluted to a protein concentration of $0.1 \mu\text{g}/\mu\text{l}$ in 50 mM KPi buffer, 0.5% Triton X-100, pH 7.4 (SOD buffer) using the protein concentration calculations determined by BCA assay (*section 2.12.2*). Thereafter,

10 μ l of samples and a blank (plain SOD buffer) was assayed in triplicate on a 96-well plate. Diethylenetriaminepentaacetic acid (DETAPAC), used for sequestering metal ions, was prepared by diluting 1 mg of 0.1 mM DETAPAC in 25 ml SOD buffer. Thereafter, 170 μ l of DETAPAC was added to all wells containing samples and the blank. 6-hydroxydopamine (6-OHD) was prepared to a concentration of 1.6 mM by diluting 50 μ l of 70% perchloric acid (HClO₄) in 10 ml deionised water. This solution was nitrogen purged for 15 minutes before adding 4 mg 6-OHD. Subsequently, 15 μ l of the 6-OHD solution was added immediately to all wells containing samples and the blank. Auto-oxidation of 6-OHD was monitored for 4 minutes in 1-minute intervals at 490 nm using the FLUOstar Omega® microplate reader and results were expressed as units /mg protein.

2.15 Thiobarbituric Acid Reactive Substances Assay

Thiobarbituric Acid Reactive Substances (TBARS) are by-products of lipid peroxidation, a marker of oxidation stress. Lipid peroxidation is the oxidative degradation of the lipid constituents of cell membranes and a mechanism of cellular injury and damage. In the TBARS assay, malondialdehyde (MDA), an end-product of lipid peroxidation, attaches to thiobarbituric acid (TBA) thus forming an MDA-TBA adduct under acidic conditions at high temperatures of 95 °C. Furthermore, The MDA-TBA adduct is detected calorimetrically at an absorbance of 532 nm according to the modified TBARS assay method of Esterbauer and Cheeseman (1990).

2.15.1 Kidney Lysate Preparation

Small thin sections of the kidney tissue (\pm 100 mg) were cut and zirconium oxide beads (0.5 mm) (\pm 100 mg), as well as \pm 200 μ l of 50 mM KPi buffer at pH 7.4 (TBARS buffer) were added to the kidney tissue to facilitate homogenisation. The kidney tissue was homogenised in a Bullet blender® 24 (Next Advance, Inc. New York) at 4 °C for 4 minutes at speed 8 followed by 5 minutes at speed 9 with 1-minute rest interval between each homogenisation period. After homogenisation, an additional volume (\pm 800 μ l) of KPi buffer, pH 7.4 was added to the kidney homogenate and vortex. The kidney homogenates were aliquoted and stored away at -80 °C.

2.15.2 Protein Determination

The BCA protein assay kit (Sigma-Aldrich) was used for protein determination. This assay was conducted as described in *section 2.12.2* replacing deiH₂O with sodium dodecyl sulphate (2% SDS, [2 g in 100 ml deiH₂O]) as the solvent used for the preparation of BSA standards and samples.

2.15.3 Experimental Procedure

An MDA standard was prepared in order to set the standard curve required to calculate the amount of MDA-TBA adduct content present in the lipid membranes of the kidney tissues. The MDA stock solution was prepared by diluting 1.23 μ l commercial MDA in 10 ml deiH₂O. Subsequently, 500 μ l of the MDA stock solution was diluted in 1500 μ l deiH₂O. Thereafter, the MDA standards were prepared as described in **Table 2:13**. Following this, 100 μ l of MDA standards and 100 μ l of the samples were transferred to falcon glass tubes. Thereafter, 2 ml of trichloroacetic acid (10% TCA) solution containing butylated hydroxytoluene (BHT) prepared by adding 5 ml 10% TCA and 62.5 μ l BHT (12 mM) to 44.94 ml deiH₂O was added to the falcon glass tubes. Additionally, 2 ml thiobarbituric acid (TBA) (0.67% w/v) prepared by diluting 335 mg TBA in 50 ml deiH₂O and heating the solution for 15 minutes before allowing it to cool at room temperature was added to the falcon glass tubes. Following this, the falcon glass tubes were covered with marbles and incubated for 1 hour in a water bath pre-heated to 95 °C. Thereafter, tubes were cooled on ice to room temperature for 10 minutes and centrifuged at 3000 rpm for a total duration of 15 minutes. The supernatants were transferred into clean falcon glass tubes and vortex before loading 150 μ l of MDA standards and samples on a 96-well plate. Following this, the MDA-TBA adduct formation was measured at an absorbance of 530 nm using the FLUOstar Omega® microplate reader. Thereafter, the protein concentration values determined by BCA were used for the normalization of results. The results were expressed in μ mol MDA equivalent /mg protein.

Table 2:13 MDA standards. The preparation of MDA standards for MDA analysis in kidney tissue.

MDA (μ l)	DeiH20 (μ l)	Final concentration (nmol/ml MDA)
0	1000	0
2.5	997.5	0.3125
5	995	0.625
10	990	1.25
20	980	2.5
40	960	5
80	920	10
200	800	25
400	600	50

2.16 Statistical Analysis

The results were analysed using GraphPad Prism ® software (GraphPad Software, San Diego, CA, USA). Analysis of variance (one-way or two-way ANOVA) was performed followed by a Bonferroni post-test (to make a comparison between all groups). Results were expressed as the Mean \pm Standard Error of the Mean (SEM) and a probability value less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER 3 Results

3.1 Overview

The modulating effects of rooibos (RB) and honeybush (HB) on the aortic endothelial function and the antioxidant (AOX) status of healthy and diabetic adult male Wistar rats was investigated following a 6-week treatment period. Treatment (2% RB fermented, 2 g/100 ml water or 4% HB fermented, 4 g/100 ml water) was administered as infusions prepared by steeping according to standard protocols. Furthermore, the soluble solids, total polyphenols and flavonol content of the RB and HB infusions were determined.

In the animals, diabetes mellitus was induced by a single intraperitoneal injection of 45 mg/kg bw streptozotocin (STZ). Diabetic animals (DRF45 and DHB45) received a pre-treatment of the RB or HB infusion for a week prior to the induction of diabetes mellitus (see *section 2.4; Table 2:4*).

Throughout the 6-week treatment period, blood pressure (systolic, diastolic and MAP) and biometric measurements (body weight, fluid intake, food intake, blood glucose and IPGTT) were recorded. Following the 6-week treatment period, animals were euthanized and the aortas used to conduct aortic contraction/relaxation studies and western blot analysis of proteins (eNOS and PKB/AKT) involved in endothelial function. Furthermore, antioxidant assays (CAT, SOD and TBARS) were performed on the kidney tissue.

3.2 Treatment: Chemical Analyses

3.2.1 Soluble Solids

The 4% fermented HB infusion had a significantly higher soluble solid content than the 2% fermented RB infusion (see **Table 3:1**).

Table 3:1 Soluble solid content of RB and HB infusions.

	Soluble solids (mg/ml)
2% RB fermented	5.18 ± 0.18
4% HB fermented	13.40 ± 0.51 ^{***}

All data is expressed as mean ± SEM, (^{***} p < 0.0001 4% HB fermented vs 2% RF fermented), n = 15 per group.

3.2.2 Total polyphenol content

There were no significant differences in the total polyphenol content of fermented 2% RB and the fermented 4% HB infusion (**Table 3:2**). However, the HB drinking control and diabetic group had a significantly higher total polyphenol daily intake than that of the RF drinking control and diabetic groups (**Table 3:2**).

Table 3:2 Total polyphenol content of RB and HB infusions.

Total polyphenol content (mg gallic acids equivalents /mg soluble solids)	
2% RB fermented	0.174
4% HB fermented	0.171
Total polyphenol daily intake (mg gallic acid equivalents /day/100 g bw)	
RB control group	12.59 ± 0.67
HB control group	38.10 ± 3.54 ^{***}
DRF45 treatment group	55.30 ± 5.53
DHB45 treatment group	101.7 ± 14.53 ^{**}

All data is expressed as mean ± SEM, (^{***} p < 0.0001 4% HB control vs 2% RF control, ^{**} p < 0.001 DHB45 vs DRF45) n = 10 per group.

3.2.3 Total Flavonol Content

The HB drinking control had a significantly higher total flavonol daily intake than that of the RF drinking control group (**Table 3:3**).

Table 3:3 Total flavonol content of RB and HB infusions.

Total flavonol content (mg quercetin equivalents /mg soluble solids)	
2% RB fermented	0.0879
4% HB fermented	0.0633
Total flavonol daily intake (mg quercetin equivalents /day/100 g bw)	
RB control group	6.35 ± 0.34
HB control group	14.14 ± 1.31 ^{***}
DRF45 treatment group	27.92 ± 2.79
DHB45 treatment group	37.74 ± 5.34

All data is expressed as mean ± SEM, (***) $p < 0.0001$ 4% HB control vs 2% RF control) n = 10 per group.

3.3 Biometric Measurements: Body Weight, Food Intake, Fluid Intake, Blood Glucose and IPGTT

3.3.1 Body Weight

Body weight was measured thrice weekly throughout the 6-week treatment period. A significant decrease in the mean body weight of all diabetic animals (STZ45, DRF45 and DHB45) was observed at the end of the 6-week treatment when compared to the mean body weight of the water, rooibos (RF), honeybush (HB) and the vehicle (VEH) control groups respectively (**Table 3:4**).

Table 3:4 Mean body weight of the STZ, DRF45 and DHB45 treatment groups compared to the mean body weight of the control groups respectively.

Mean Body Weight (g)	
Water control vs STZ45	344.5 ± 6.580 vs 277.6 ± 6.135***
Water control vs DRF45	344.5 ± 6.580 vs 262.9 ± 10.53***
Water control vs DHB45	344.5 ± 6.580 vs 290.6 ± 12.33 **
RF control vs STZ45	347.4 ± 7.094 vs 277.6 ± 6.135 ***
RF control vs DRF45	347.4 ± 7.094 vs 262.9 ± 10.53 ***
RF control vs DHB45	347.4 ± 7.094 vs 290.6 ± 12.33***
HB control vs STZ45	348.3 ± 9.307 vs 277.6 ± 6.135***
HB control vs DRF45	348.3 ± 9.307 vs 262.9 ± 10.53***
HB control vs DHB45	348.3 ± 9.307 vs 290.6 ± 12.33***
VEH control vs STZ45	333.3 ± 9.181 vs 277.6 ± 6.135***
VEH control vs DRF45	333.3 ± 9.181 vs 262.9 ± 10.53***
VEH control vs DHB45	333.3 ± 9.181 vs 290.6 ± 12.33*
STZ45 vs DRF45	277.6 ± 6.135 vs 262.9 ± 10.53
STZ45 vs DHB45	277.6 ± 6.135 vs 290.6 ± 12.33
DRF45 vs DHB45	262.9 ± 10.53 vs 290.6 ± 12.33

All data is expressed as ± SEM, (***) p < 0.0001, ** p < 0.001, *p < 0.05), n = 10 per group.

3.3.2 Food Intake

Animals were given rat pellets as their food intake. The amount of food consumed daily was measured once a week throughout the 6-week treatment period. At the end of the 6 weeks, there was a significant increase in the mean food intake of all diabetic animals (STZ45, DRF45 and DHB45) when compared to the mean food intake of the water, rooibos (RF), honeybush (HB) and the vehicle (VEH) control groups respectively (**Table 3:5**).

Table 3:5 Mean food consumption of the STZ, DRF45 and DHB45 treatment groups compared to the mean food consumption of the control groups respectively.

Food Intake (g/day)	
Water control vs STZ45	19.72 ± 0.9780 vs 32.94 ± 2.088**
Water control vs DRF45	19.72 ± 0.9780 vs 40.38 ± 2.180***
Water control vs DHB45	19.72 ± 0.9780 vs 37.35 ± 3.527***
RF control vs STZ45	22.55 ± 1.531 vs 32.94 ± 2.088*
RF control vs DRF45	22.55 ± 1.531 vs 40.38 ± 2.180***
RF control vs DHB45	22.55 ± 1.531 vs 37.35 ± 3.527***
HB control vs STZ45	21.94 ± 0.8891 vs 32.94 ± 2.088*
HB control vs DRF45	21.94 ± 0.8891 vs 40.38 ± 2.180***
HB control vs DHB45	21.94 ± 0.8891 vs 37.35 ± 3.527***
VEH control vs STZ45	20.76 ± 2.569 vs 32.94 ± 2.088**
VEH control vs DRF45	20.76 ± 2.569 vs 40.38 ± 2.180***
VEH control vs DHB45	20.76 ± 2.569 vs 37.35 ± 3.527***
STZ45 vs DRF45	32.94 ± 2.088 vs 40.38 ± 2.180
STZ45 vs DHB45	32.94 ± 2.088 vs 37.35 ± 3.527
DRF45 vs DHB45	40.38 ± 2.180 vs 37.35 ± 3.527

All data is expressed as ± SEM, (***) p < 0.0001, ** p < 0.001, *p < 0.05), n = 10 per group.

3.3.3 Fluid Intake

Animals were given either tap water, rooibos or honeybush infusions as their daily drinking fluid. Throughout, the 6-week treatment, the amount of fluid consumed was measured 3 times a week. At the end of the 6 weeks, a significant increase in mean fluid intake of all diabetic animals (STZ45, DRF45 and DHB45) was observed when compared to the mean fluid intake of the water, rooibos (RB), honeybush (HB) and the vehicle (VEH) control groups respectively. Furthermore, the HB control animals consumed more fluids than the Water controls (**Table 3:6**).

Furthermore, the honeybush treatment group (DHB45) consumed less fluids when compared to fluid intake of the untreated diabetic (STZ45) (**Table 3:6**).

Table 3:6 Mean fluid intake of the STZ, DRF45 and DHB45 treatment groups compared to the mean fluid intake of the control groups respectively.

Fluid Intake (ml/day)	
Water control vs STZ45	40.14 ± 2.932 vs 186.4 ± 9.215***
Water control vs DRF45	40.14 ± 2.932 vs 156.7 ± 14.16***
Water control vs DHB45	40.14 ± 2.932 vs 124.1 ± 14.76***
RF control vs STZ45	48.41 ± 2.708 vs 186.4 ± 9.215***
RF control vs DRF45	48.41 ± 2.708 vs 156.7 ± 14.16***
RF control vs DHB45	48.41 ± 2.708 vs 124.1 ± 14.76***
HB control vs Water control	57.34 ± 4.379 vs 40.14 ± 2.932**
HB control vs STZ45	57.34 ± 4.379 vs 186.4 ± 9.215***
HB control vs DRF45	57.34 ± 4.379 vs 156.7 ± 14.16***
HB control vs DHB45	57.34 ± 4.379 vs 124.1 ± 14.76***
VEH control vs STZ45	48.23 ± 3.176 vs 186.4 ± 9.215***
VEH control vs DRF45	48.23 ± 3.176 vs 156.7 ± 14.16***
VEH control vs DHB45	48.23 ± 3.176 vs 124.1 ± 14.76***
STZ45 vs DRF45	186.4 ± 9.215 vs 156.7 ± 14.16
STZ45 vs DHB45	186.4 ± 9.215 vs 124.1 ± 14.76***
DRF45 vs DHB45	156.7 ± 14.16 vs 124.1 ± 14.76

All data is expressed as ± SEM, (***) p < 0.0001, (**) p < 0.001, (*) p < 0.05), n = 10 per group.

3.3.4 Blood Glucose Levels

The blood glucose level of the animals was measured once a week. A glucometer was used to obtain the blood glucose reading by means of the tail prick method. By the end of the 6-week treatment period, the final blood glucose measurements of the diabetic animals (STZ45, DRF45 and DHB45) treatment groups was significantly higher than that of the water, rooibos (RB), honeybush (HB) and the vehicle (VEH) control groups respectively (**Table 3:7**).

Table 3:7 Mean blood glucose levels of the STZ, DRF45 and DHB45 treatment groups compared to the mean blood glucose of the control groups respectively.

Blood Glucose Levels (mmol/L)	
Water control vs STZ45	6.320 ± 0.07717 vs 26.17 ± 1.668***
Water control vs DRF45	6.320 ± 0.07717 vs 24.29 ± 3.196***
Water control vs DHB45	6.320 ± 0.07717 vs 21.87 ± 2.247***
RF control vs STZ45	6.610 ± 0.09597 vs 26.17 ± 1.668***
RF control vs DRF45	6.610 ± 0.09597 vs 24.29 ± 3.196***
RF control vs DHB45	6.610 ± 0.09597 vs 21.87 ± 2.247***
HB control vs STZ45	6.490 ± 0.1242 vs 26.17 ± 1.668***
HB control vs DRF45	6.490 ± 0.1242 vs 24.29 ± 3.196***
HB control vs DHB45	6.490 ± 0.1242 vs 21.87 ± 2.247***
VEH control vs STZ45	6.310 ± 0.1370 vs 26.17 ± 1.668***
VEH control vs DRF45	6.310 ± 0.1370 vs 24.29 ± 3.196***
VEH control vs DHB45	6.310 ± 0.1370 vs 21.87 ± 2.247***
STZ45 vs DRF45	26.17 ± 1.668 vs 24.29 ± 3.196
STZ45 vs DHB45	26.17 ± 1.668 vs 21.87 ± 2.247
DRF45 vs DHB45	24.29 ± 3.196 vs 21.87 ± 2.247

All data is expressed as ± SEM, (***) p < 0.0001, (**) p < 0.001, (*) p < 0.05), n = 10 per group.

3.3.5 IPGTT

The IPGTT of the animals was measured in the last week (week 6) of treatment. Following an intraperitoneal injection of 10 μ l glucose/g bw, The diabetic animals presented with significant increases in blood glucose levels over time (0 min, 3 min, 5 min, 20 min, 25 min, 30 min, 45 min, 60 min and 120 min) when compared to the blood glucose levels of the rooibos (RB), honeybush (HB) and the vehicle (VEH) control groups respectively (**Figure 3:1**).

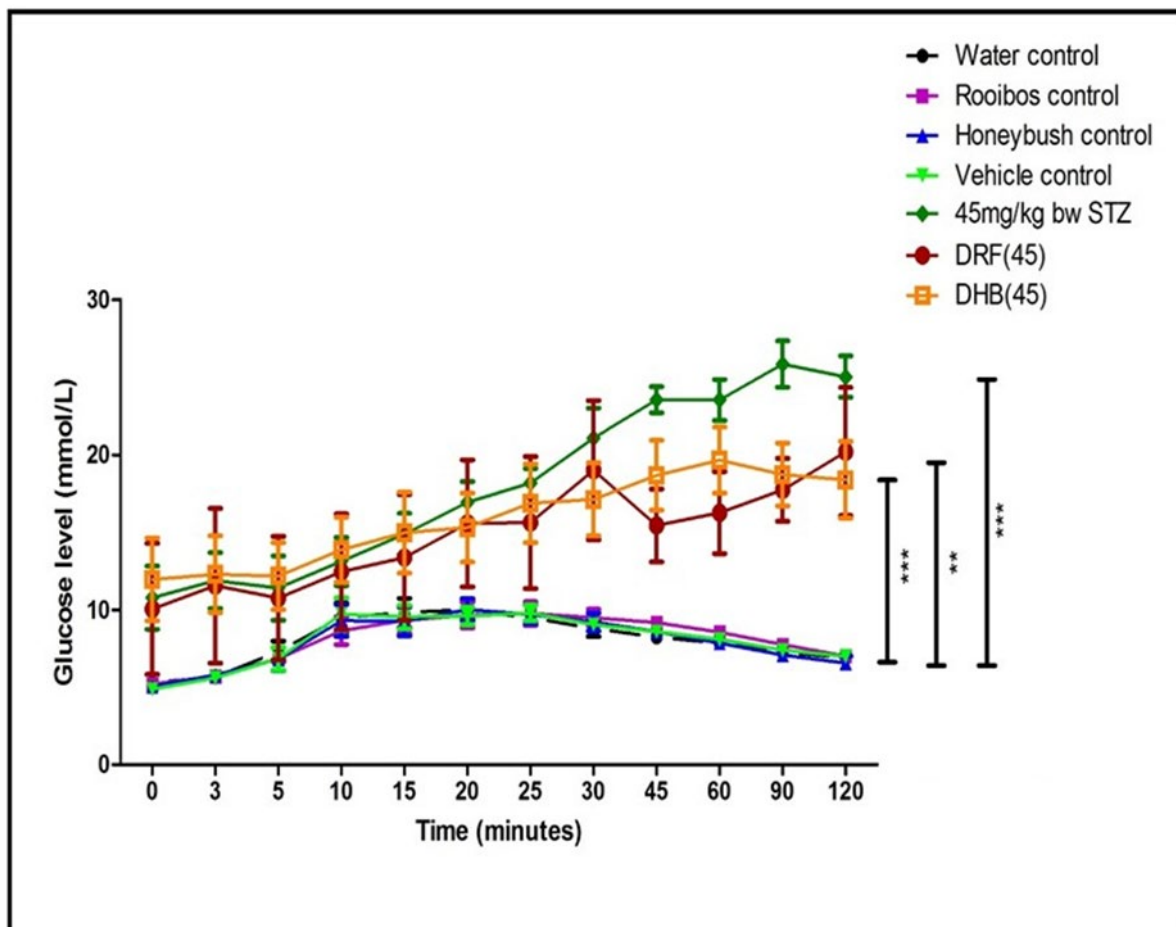


Figure 3:1 The IPGTT of the STZ45, DRF45 and DHB45 treatment groups vs control groups respectively measured at time 0 min, 3 min, 5 min, 20 min, 25 min, 30 min, 45 min, 60 min and 120 min, after the administration 10 μ l glucose/g bw, at week 6, *** $p < 0.0001$ diabetic groups (STZ45, DRF45 and DHB45) vs control groups (water, VEH, RF and HB) ($n = 4-10$ per group).

3.3.6 Relative Organ Weight (ROW) of the Kidney

The weight of each kidney was measured and calculated relative to the body weight of the animals. A significant increase in the ROW of all the diabetic (STZ45, DRF45 and DHB45) treatment groups was observed when compared to the ROW of the water, rooibos (RB), honeybush (HB) and the vehicle (VEH) control groups respectively (**Table 3:8**).

Furthermore, a significant decrease in the ROW of the diabetic honeybush treatment group (DHB45) was observed when compared to the STZ45 and DRF45 groups respectively (**Table 3:8**).

Table 3:8 Mean ROW of the kidney of the STZ, DRF45 and DHB45 treatment groups compared to the mean ROW of the kidney of the control groups respectively.

Mean ROW of the kidney (g)	
Water control vs STZ45	0.007 ± 0.000089 vs 0.01 ± 0.00023***
Water control vs DRF45	0.007 ± 0.000089 vs 0.01 ± 0.00034***
Water control vs DHB45	0.007 ± 0.000089 vs 0.009 ± 0.00029***
RF control vs STZ45	0.006 ± 0.00013 vs 0.01 ± 0.00023***
RF control vs DRF45	0.006 ± 0.00013 vs 0.01 ± 0.00034***
RF control vs DHB45	0.006 ± 0.00013 vs 0.009 ± 0.00029***
HB control vs STZ45	0.007 ± 0.00016 vs 0.01 ± 0.00023***
HB control vs DRF45	0.007 ± 0.00016 vs 0.01 ± 0.00034***
HB control vs DHB45	0.007 ± 0.00016 vs 0.009 ± 0.00029***
VEH control vs STZ45	0.007 ± 0.00014 vs 0.01 ± 0.00023***
VEH control vs DRF45	0.007 ± 0.00014 vs 0.01 ± 0.00034***
VEH control vs DHB45	0.007 ± 0.00014 vs 0.009 ± 0.00029***
STZ45 vs DRF45	0.01 ± 0.00023 vs 0.01 ± 0.00034
STZ45 vs DHB45	0.01 ± 0.00023 vs 0.009 ± 0.00029*
DRF45 vs DHB45	0.01 ± 0.00034 vs 0.009 ± 0.00029*

All data is expressed as ± SEM, (***) p < 0.0001, (**) p < 0.001, (*p < 0.05), n = 10 per group.

3.4 Blood Pressure

Blood pressure measurements were determined non-invasively once a week every second week throughout the 6-week treatment period. The CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific) monitors the systolic, diastolic and mean arterial pressure (MAP) blood pressure parameters of the animals by means of the tail cuff-occlusion method. By the end of the 6 weeks, no significance differences were observed in the mean systolic, mean diastolic, and MAP blood pressure measurements of all treatment groups and their age-matched controls (**Table 3:9**).

Table 3:9 Mean systolic, diastolic and arterial pressure of all treatment groups compared to the control groups.

	Mean Diastolic blood pressure (mm Hg)	Mean Systolic (mm Hg)	Mean Arterial Pressure (mm Hg)
Water (H ₂ O) control	98.52 ± 4.901	139.5 ± 4.767	111.9 ± 4.811
RF control	93.74 ± 5.175	133.3 ± 5.676	106.6 ± 5.337
HB control	96.82 ± 7.205	134.5 ± 7.003	109.0 ± 7.052
VEH control	113.4 ± 4.635	153.8 ± 5.130	126.5 ± 126.5
STZ45	106.9 ± 8.867	142.4 ± 12.42	120.4 ± 8.848
DRF45	114.0 ± 7.520	155.0 ± 8.617	127.3 ± 7.863
DHB45	110.6 ± 4.594	151.5 ± 4.830	123.9 ± 4.621

All data is expressed as ± SEM, no significant differences, n = 10 per group.

3.5 Aortic Contraction/Relaxation Studies

Aortic contraction/relaxation isometric tension studies were performed on aortas (3-4 mm) to assess vascular function. Cumulative concentrations of phenylephrine (Phe) followed by cumulative concentrations of acetylcholine (ACh) were used to induce aortic contraction and relaxation respectively.

3.5.1 Phenylephrine-induced Aortic Contraction

The STZ injury model showed a significant increase in aortic contractibility when compared to the VEH control ($p < 0.05$) (**Figure 3:2**). Similarly, the STZ injury model showed a significant increase in aortic contractibility when compared to the DRF45 and DHB45 treatment groups respectively ($p < 0.05$) (**Figure 3:3**). Furthermore, DHB45 showed a significant increase in aortic contractibility when compared to the DRF45 treatment group ($p < 0.05$) (**Figure 3:3**).

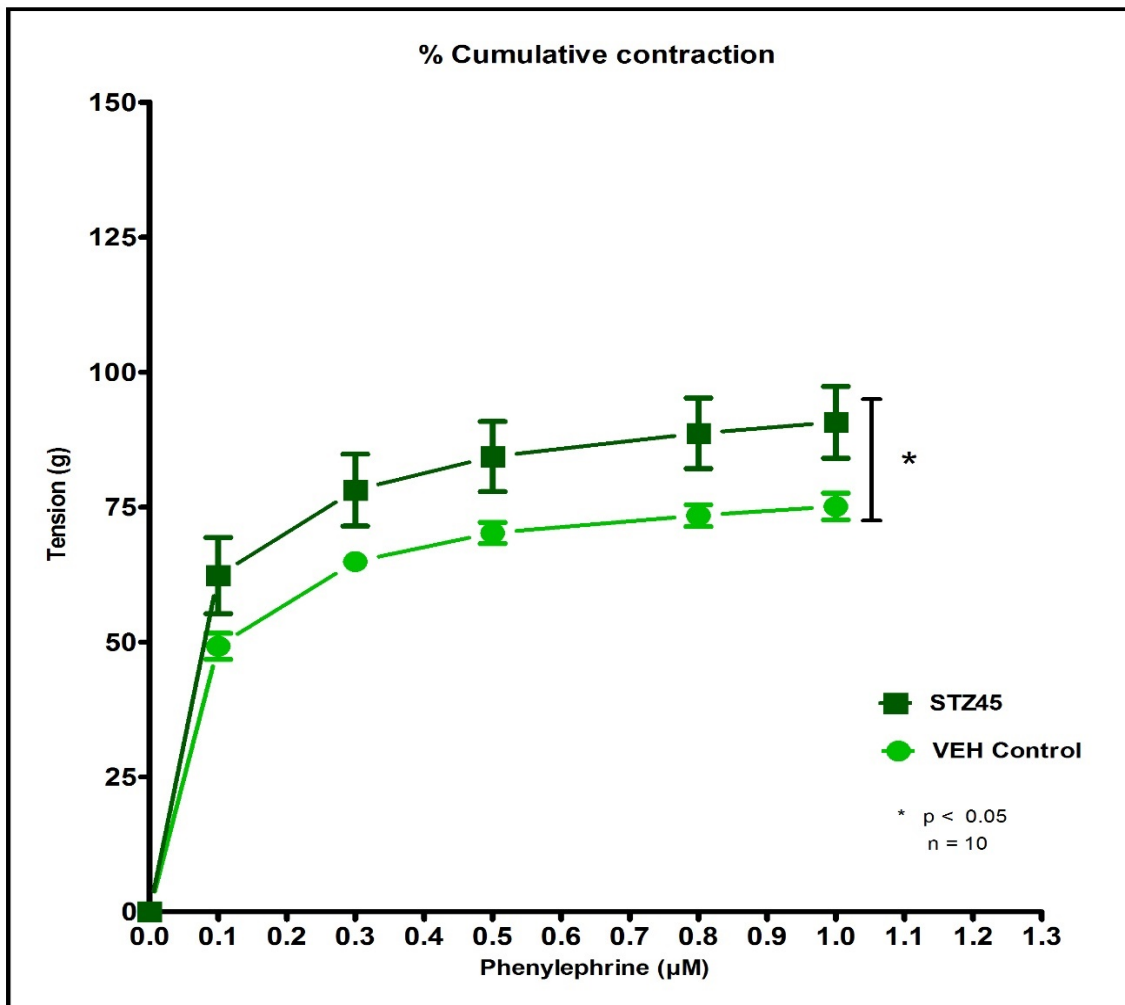


Figure 3:2 Cumulative Phenylephrine-induced aortic contraction of the STZ45 injury model compared to the VEH control. * $p < 0.05$, STZ45 vs VEH control ($n = 10$ per group).

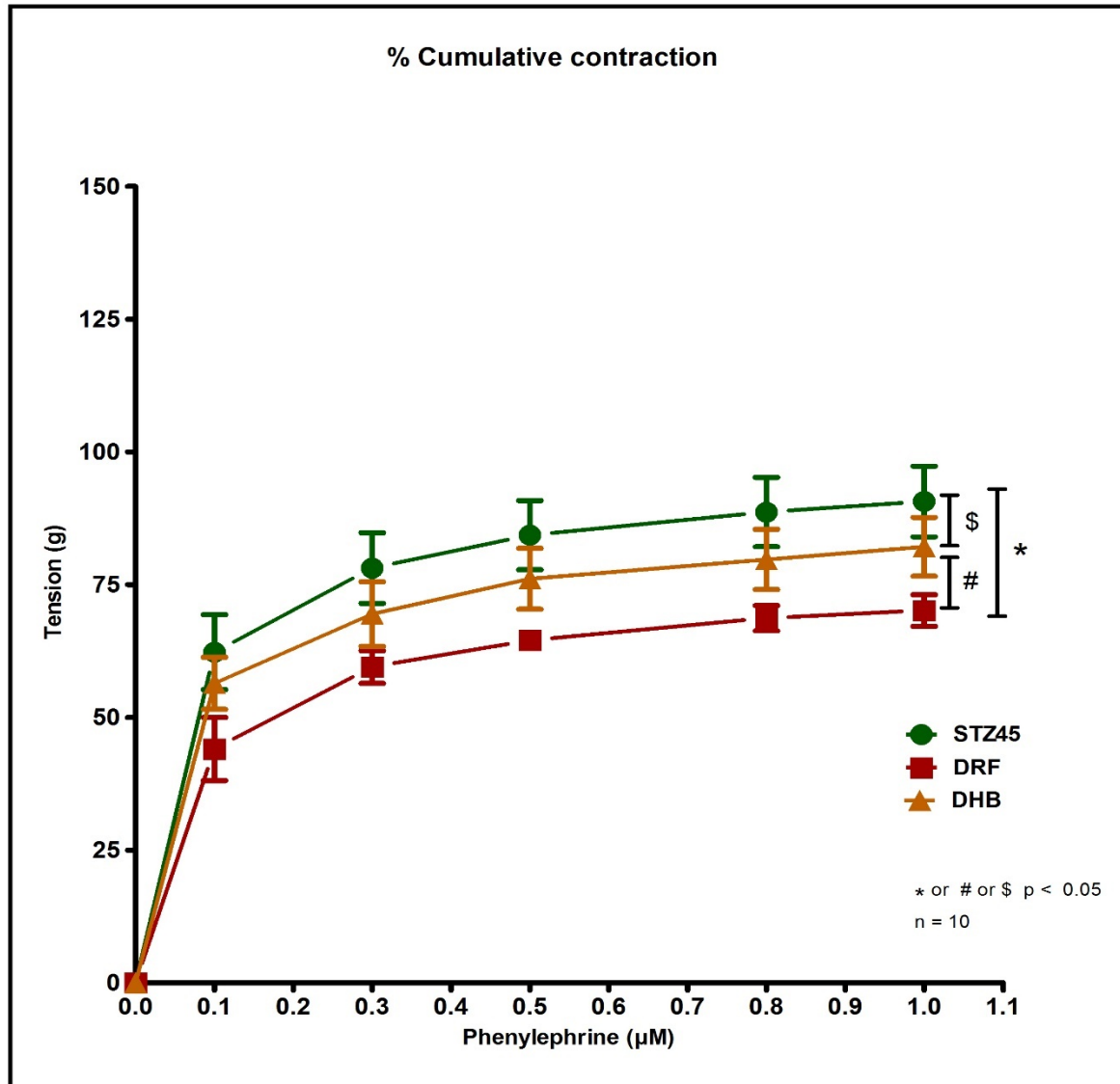


Figure 3:3 Cumulative phenylephrine-induced aortic contraction of the STZ45 injury model compared to DRF45 and DHB45 respectively. * $p < 0.05$, STZ45 vs DRF45, \$ $p < 0.05$, STZ45 vs DHB45 and # $p < 0.05$, DHB45 vs DRF45 ($n = 4 - 10$ per group).

3.5.2 Acetylcholine-induced Aortic Relaxation

The STZ injury model showed a significant decrease in aortic relaxation when compared to the VEH control ($p < 0.05$) (**Figure 3:4**). Similarly, the STZ injury model showed a significant decrease in aortic relaxation when compared to the DRF45 and DHB45 treatment groups respectively ($p < 0.05$) (**Figure 3:5**).

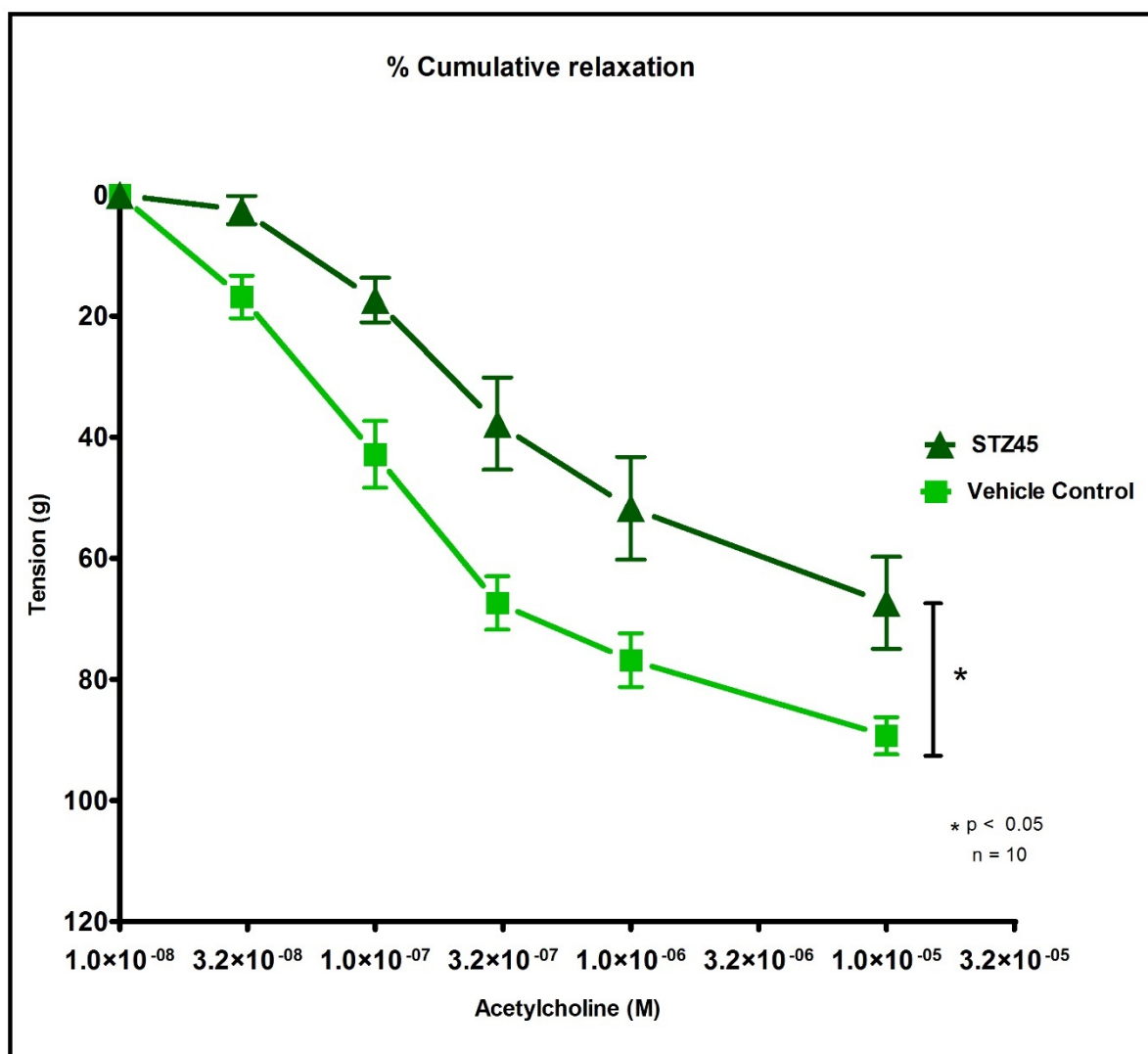


Figure 3:4 Cumulative acetylcholine -induced aortic relaxation of the STZ45 injury model compared to the VEH control. * $p < 0.05$, STZ45 vs VEH control ($n = 10$ per group).

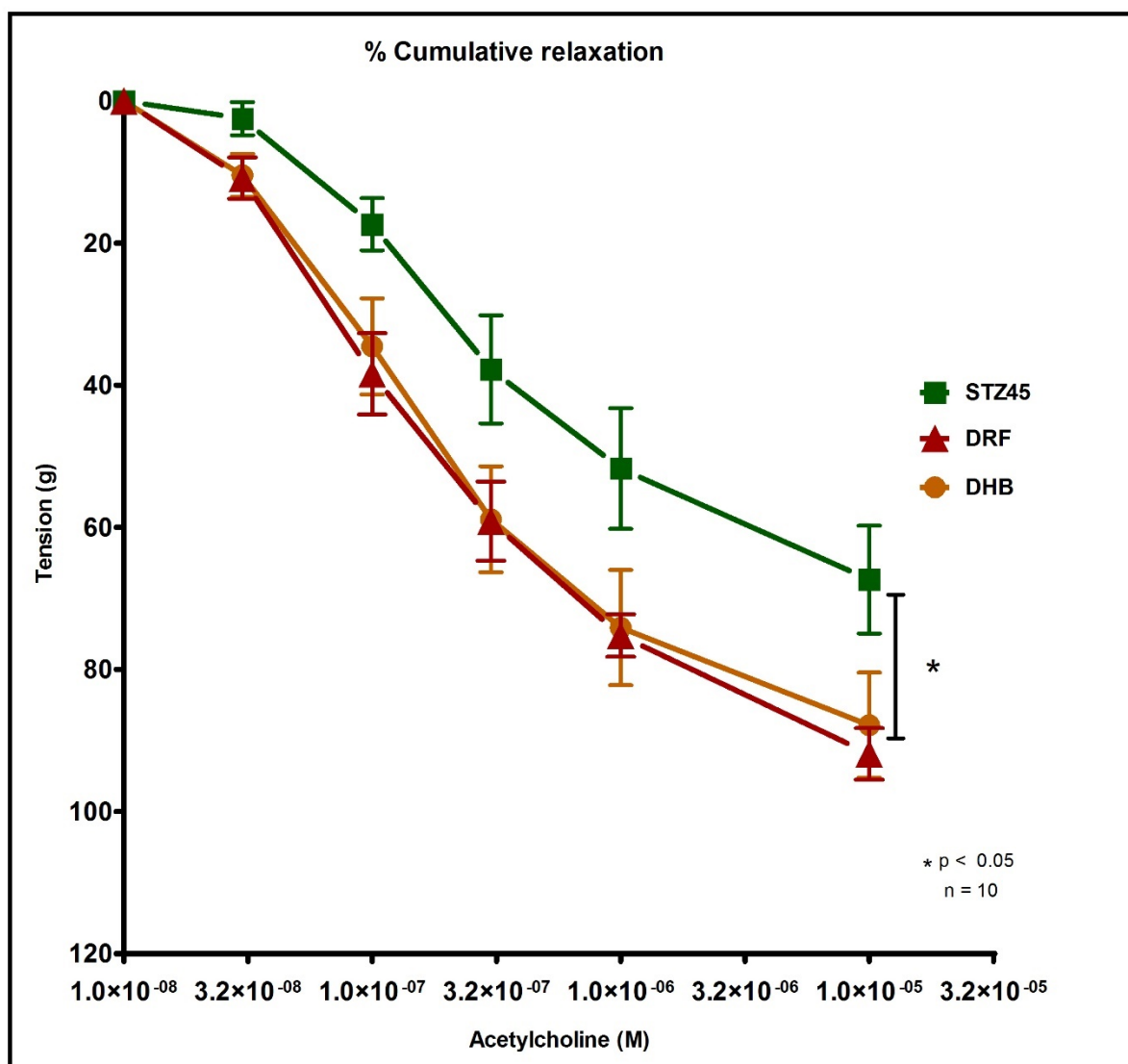


Figure 3:5 Cumulative acetylcholine - induced aortic relaxation of the STZ45 injury model compared to DRF45 and DHB45 respectively. * $p < 0.05$, STZ45 vs DRF45, * $p < 0.05$, STZ45 vs DHB45 (n = 10 per group).

3.6 Western Blot Analysis

Western blot analysis was performed on aortic tissue to assess the expression of the signalling proteins [eNOS (140 kDa) and PKB/AKT (63kDa)] involved in endothelial function. The expression of total and phosphorylated eNOS as well as that of total and phosphorylated PKB/AKT was determined. Furthermore, the ratio (phosphorylation over total) of each protein expression was determined.

3.6.1 Total (T) eNOS Expression

There were no significant differences in the T-eNOS expression of the treated diabetic animals (DRF45 and DHB45) when compared to the untreated STZ injury model. (**Figure 3:6**).

However, a significant reduction in T- eNOS expression was observed in diabetic animals (STZ, DRF45 and DHB45) and the VEH control when compared to the water, and the HB control groups respectively. Additionally, a significant reduction in T-eNOS expression was observed in the untreated STZ injury model when compared to the water, VEH, RB and HB control groups respectively (**Figure 3:6**)

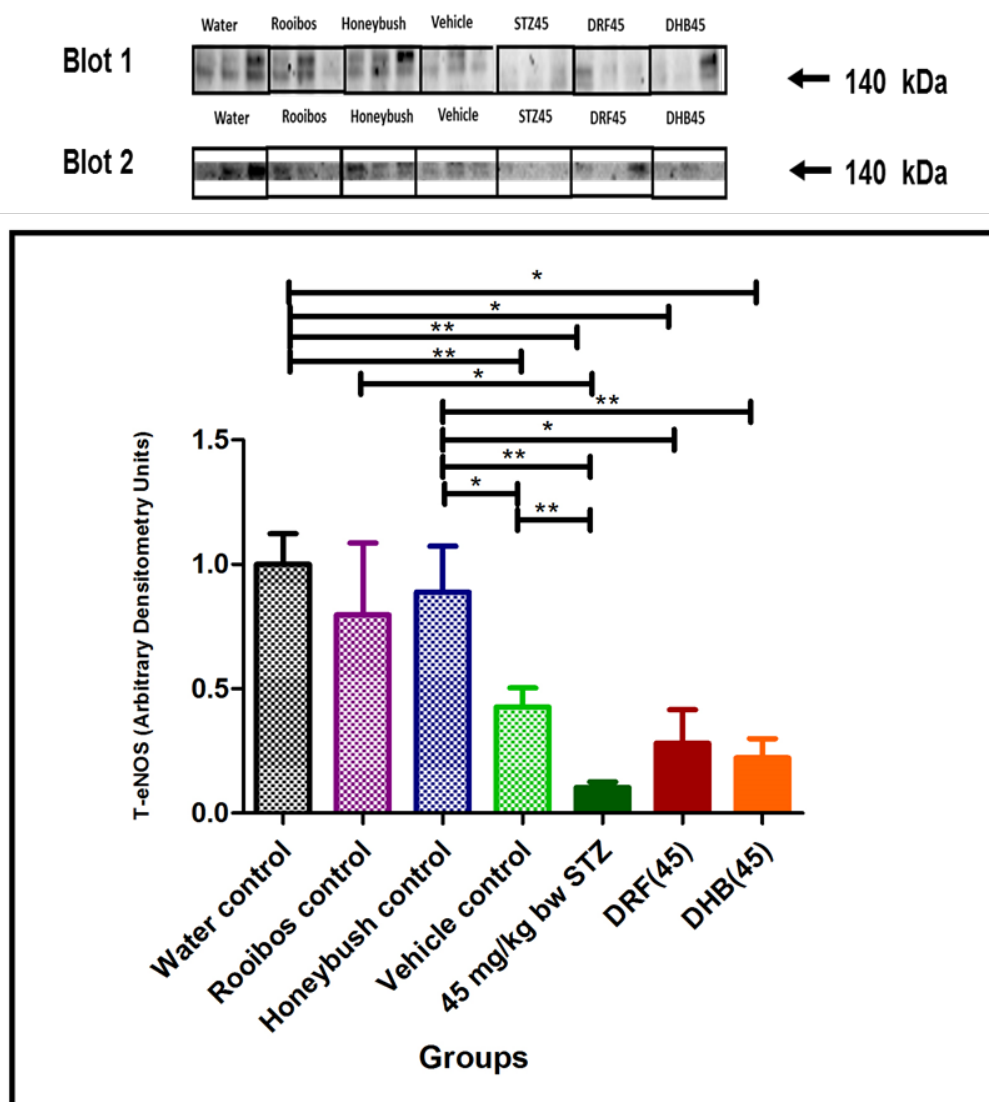


Figure 3:6 Total-eNOS expression in the aortic rings of the STZ45, DRF45 and DHB45 treatment groups compared to the control groups respectively. ** $p < 0.001$, * $p < 0.05$ ($n = 6$ per group).

3.6.2 Phosphorylated eNOS (P- eNOS) Expression

There were no significant differences in the P-eNOS expression of the treated diabetic animals (DRF45 and DHB45) when compared to the untreated STZ injury model. However, the STZ injury model induced a significant reduction in the P-eNOS expression of the untreated STZ group when compared to the VEH control group (STZ vs VEH: 0.37 ± 0.14 vs 1.13 ± 0.27 , $p < 0.05$) (**Figure 3:7**).

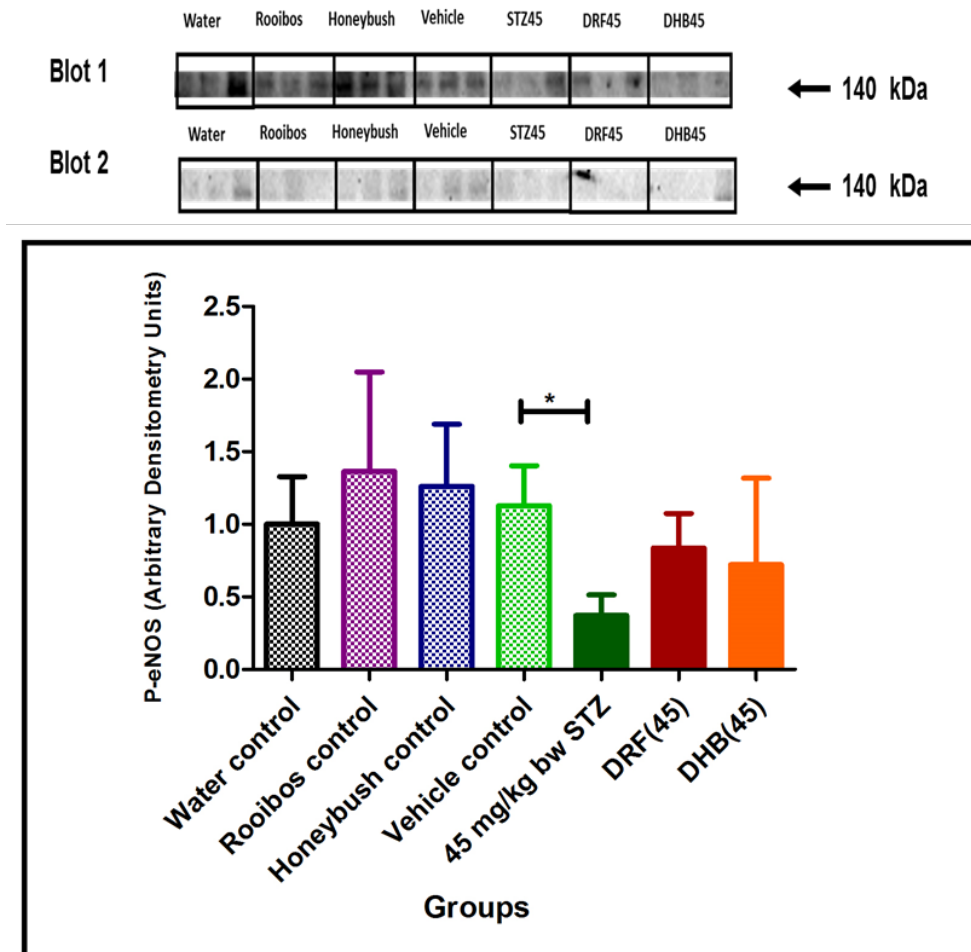


Figure 3:7 P: T eNOS expression in the aortic rings the STZ injury model compared to the VEH control. * $p < 0.05$, STZ45 vs VEH control ($n = 6$ per group).

3.6.3 P: T eNOS Ratio

There were no significant differences in the P: T eNOS ratio of the treated diabetic animals (DRF45 and DHB45) when compared to the untreated STZ injury model. However, a significant increase in the P: T eNOS ratio of the untreated STZ group was observed when compared to the water control (**Figure 3:8**).

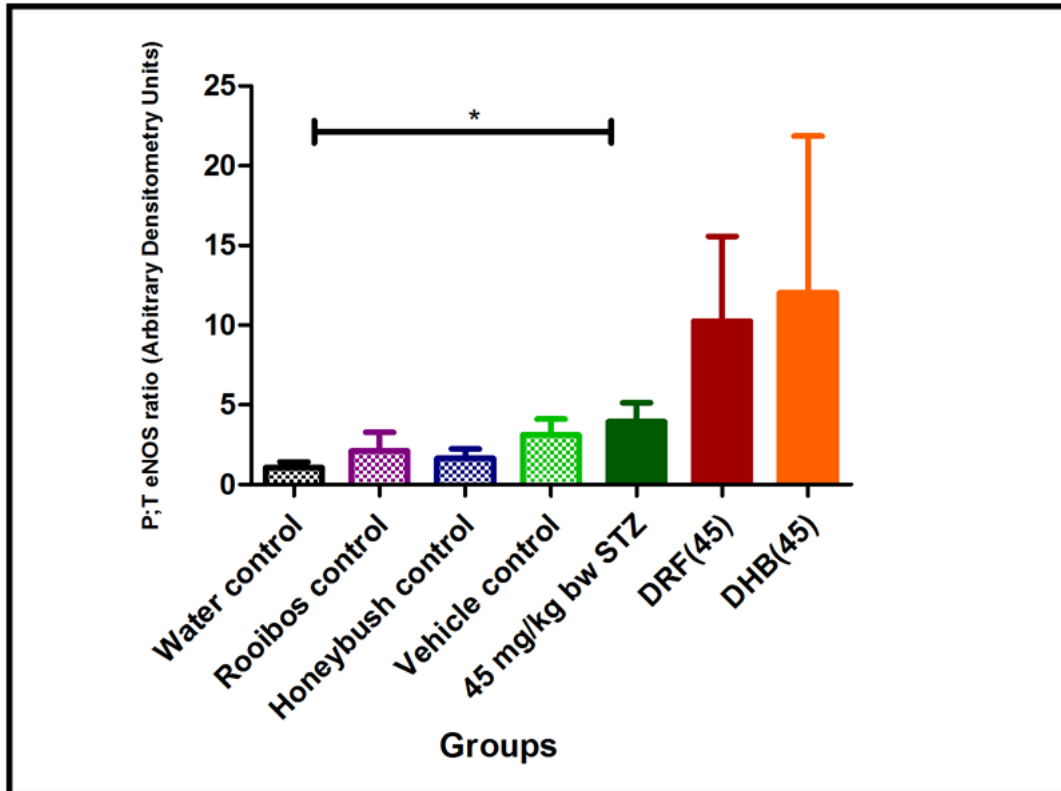


Figure 3:8 P: T eNOS ratio in the aortic rings of the untreated STZ injury model compared to the healthy water control. * $p < 0.05$ STZ45 vs water control, ($n = 6$ per group).

3.6.4 Total (T) PKB/AKT Expression

There were no significant changes observed in total PKB/AKT expression of the treated diabetic groups when compared to their respective control groups. However a significant decrease in T-PKB/AKT expression of the diabetic DRF45 and DHB45 groups was observed when compared to the VEH control respectively (VEH vs DRF45: 3.13 ± 0.52 vs 0.54 ± 0.30 , $p < 0.05$; VEH vs DHB45: 3.13 ± 0.52 vs 0.88 ± 0.53 , $p < 0.05$) (**Figure 3:9**).

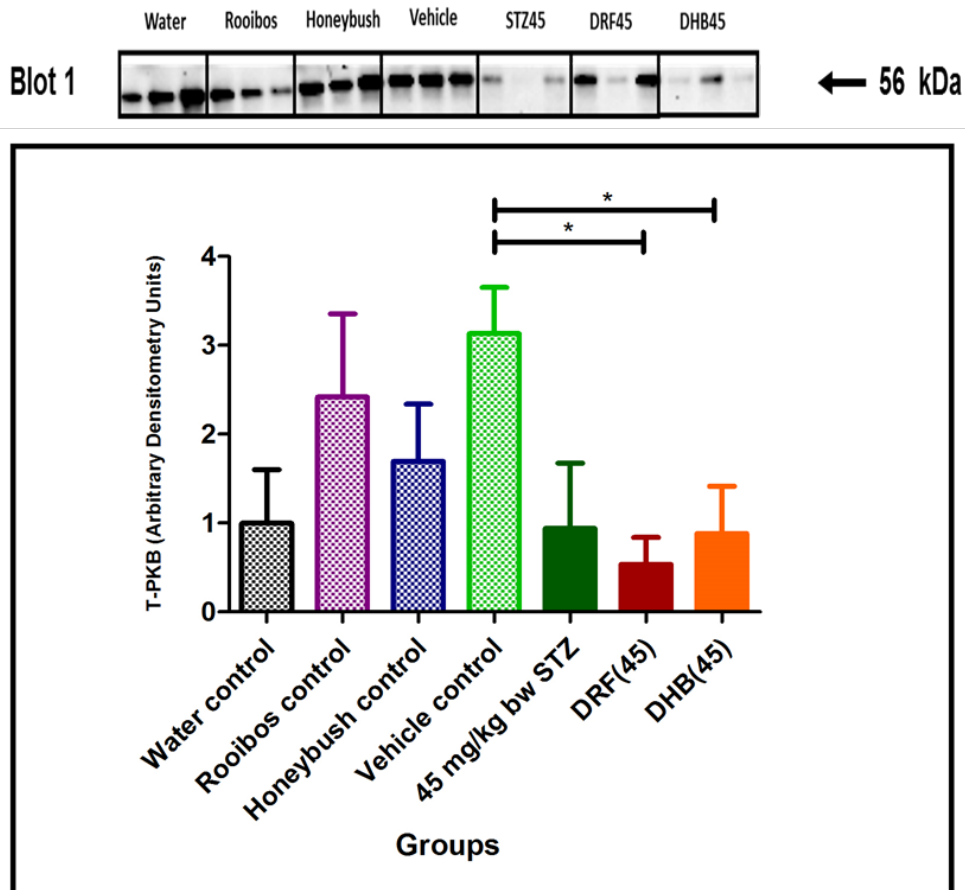


Figure 3:9 Total-PKB/AKT expression in the aortic rings of the DRF45 and DHB45 diabetic animals compared to the VEH control. * $p < 0.05$ VEH vs DRF45, * $p < 0.05$ VEH vs DHB45 ($n = 3$ per group).

3.6.5 Phosphorylated PKB/AKT (P- PKB/AKT) Expression

There were no significant changes observed P-PKB/AKT expression of the treated diabetic groups when compared to their respective control groups. However, the STZ injury model showed a significant reduction in phosphorylated PKB/AKT expression when compared to VEH control (STZ vs VEH: 0.14 ± 0.04 vs 0.80 ± 0.17 , $p < 0.05$) (**Figure 3:10**).

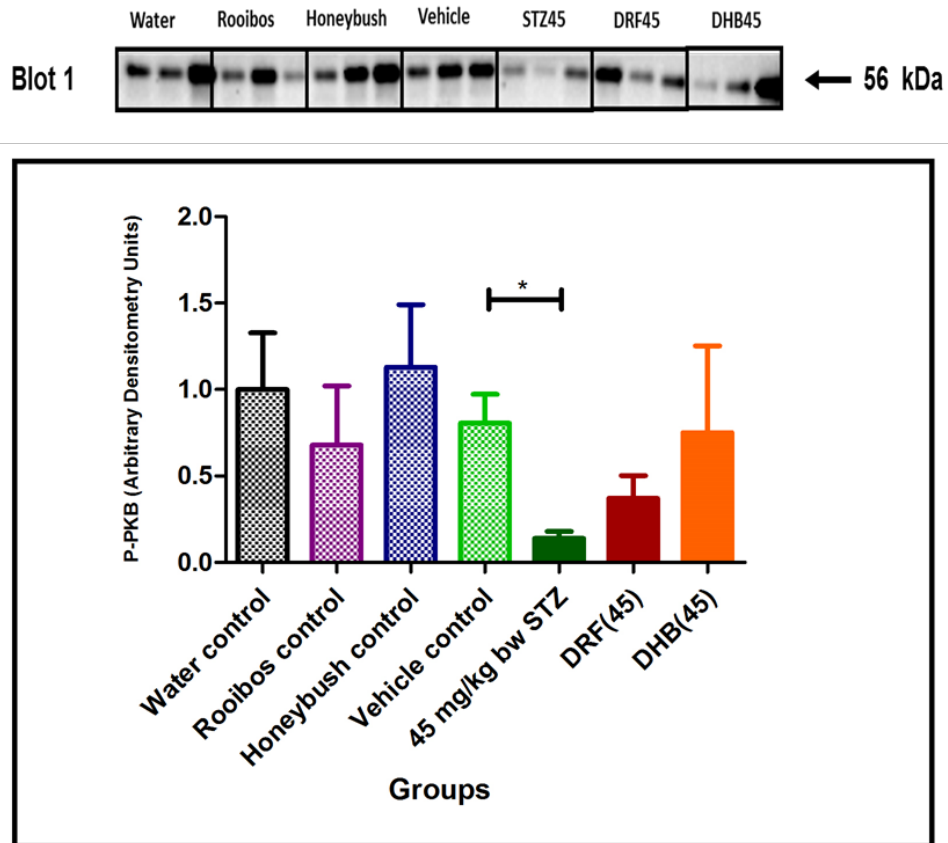


Figure 3:10 P-PKB/AKT expression in the aortic rings of the untreated STZ injury model compared to the VEH control group. * $p < 0.05$ STZ vs VEH ($n = 3$ per group).

3.6.6 P: T PKB/AKT Ratio

There were no significant differences in the P: T PKB/AKT ratio of the treatment and control groups (**Figure 3:11**).

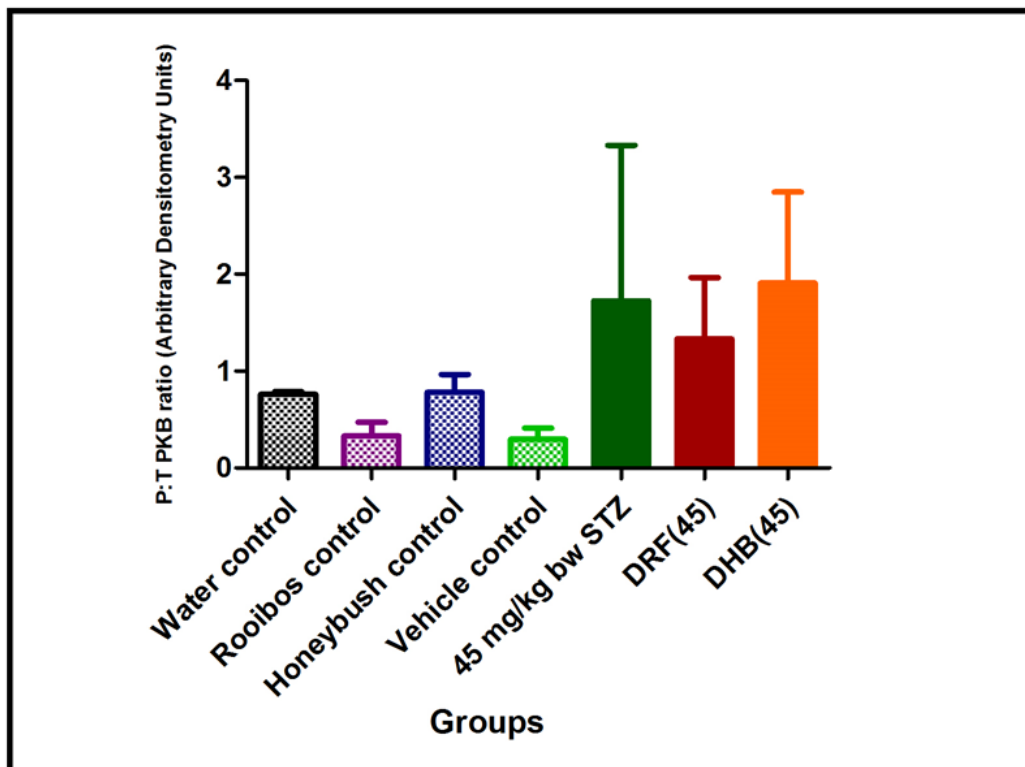


Figure 3:11 P: T PKB/AKT ratio in the aortic rings of the treatment and control groups. No significant differences ($n = 3$ per group).

3.7 Antioxidant Assays

The antioxidant activity of the catalase (CAT) and superoxide dismutase (SOD) enzyme of the animals was assessed in the kidney tissue. Additionally, the thiobarbituric acid reactive substances (TBARS) assay which measures lipid peroxidation in the kidney tissue was conducted to assess oxidative stress.

3.7.1 Catalase

There were no significant changes observed in the catalase activity of the treatment and control groups (**Figure 3:12**).

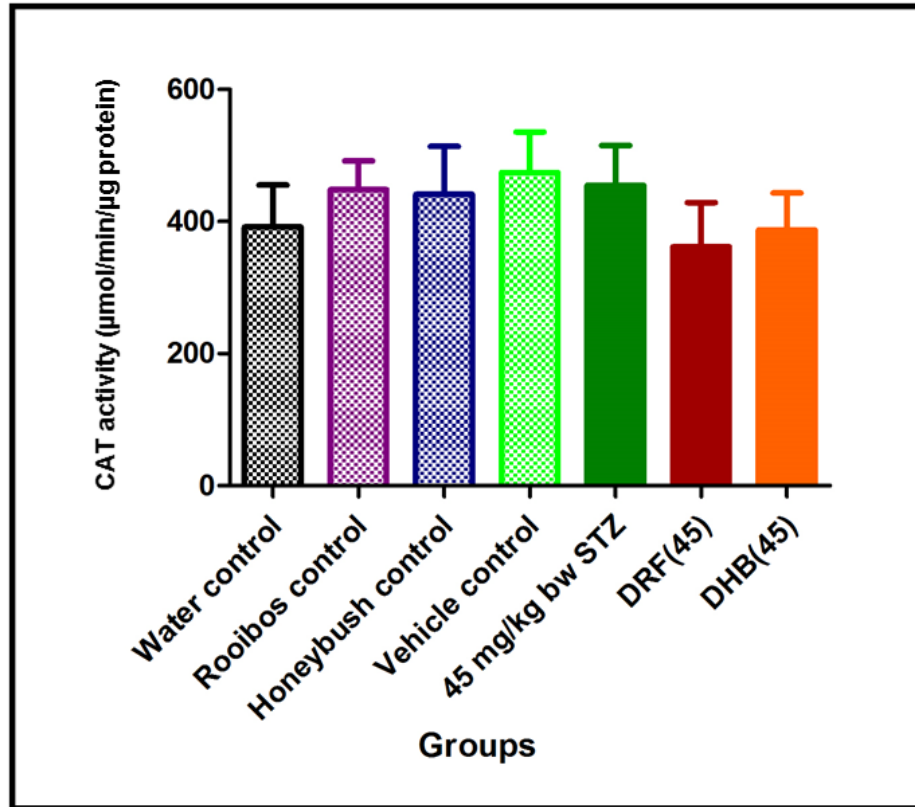


Figure 3:12 Catalase activity in the kidney tissue of the treatment and control groups. No significant differences ($n = 10$ per group).

3.7.2 SOD

There were no significant changes observed in the SOD activity of the treatment and control groups (**Figure 3:13**).

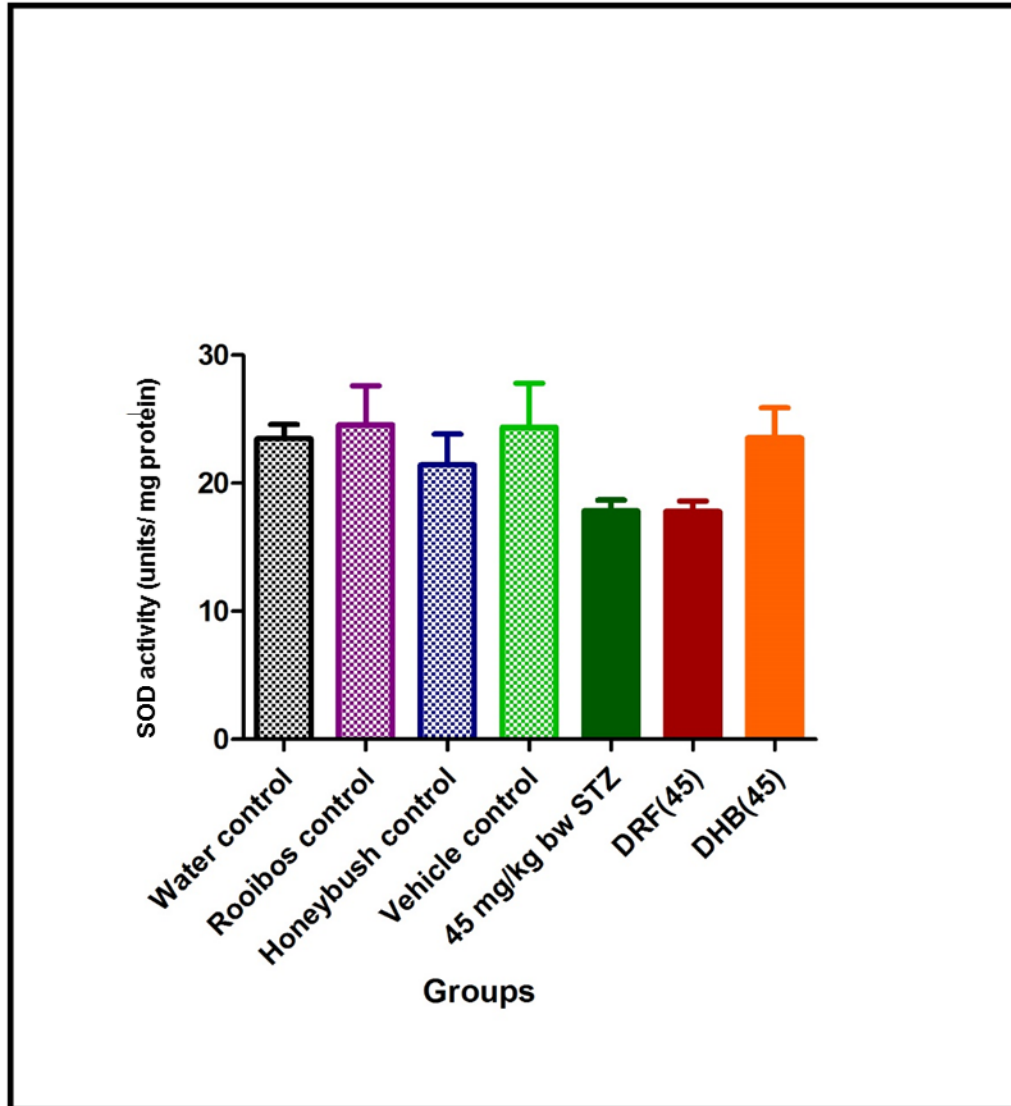


Figure 3:13 SOD activity in the kidney tissue of the treatment and control groups. No significant differences ($n = 10$ per group).

3.7.3 TBARS

There were no significant changes observed in the TBARS activity of the treatment and control groups (**Figure 3:14**).

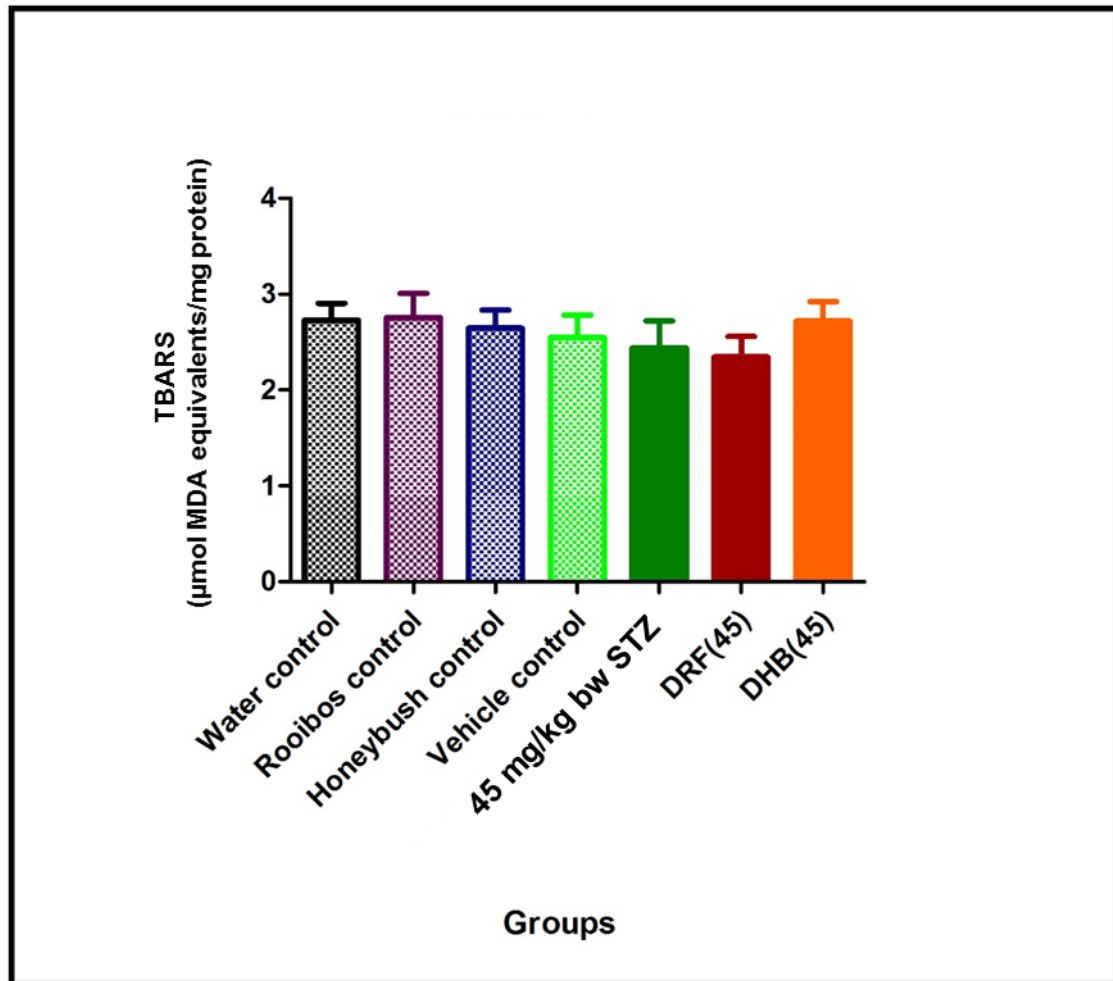


Figure 3:14 TBARS activity in the kidney tissue of the treatment and control groups. No significant differences ($n = 10$ per group).

CHAPTER 4 Discussion

In the present study, the aim of the study was to determine whether the 2% fermented RB and 4% fermented HB infusion have the potential to alleviate STZ diabetes mellitus-induced endothelial dysfunction, elevated blood pressure and oxidative stress. It is known that RB and HB are medicinal plants that are enriched with polyphenol- flavonoids that have antioxidant anti-diabetic properties.

4.1 Chemical Analyses

The 4% fermented HB infusion used in this study had a significantly higher soluble solids content than the 2% fermented RF infusion. However, total polyphenol content in both the infusions was not significantly different from each other (see section 3.2 chapter 3). It has been previously shown that the soluble solids content of *Aspalathus linearis* is different in every plantation harvested (Joubert *et al.*, 2008; Marnewick, 2009). Similarly, the total polyphenol content and phytochemical composition of the *Cyclopia species* is different in all harvests and is determined by the age of the plantation, the harvest type, the sample type and the area /origin of the plantation (Joubert *et al.*, 2008; Marnewick, 2009).

Aspalathus linearis and the *Cyclopia species* are both antioxidant-rich plants that have previously been documented to have similar antimutagenic, anti-inflammatory and anti-diabetic effects (McKay and Blumberg, 2007; Marnewick, Gelderblom and Joubert, 2000). Although they confer similar ameliorative effects in various disease models these medicinal plants differ in their phytochemical composition. *Aspalathus linearis* contains dihydrochalcones (aspalathin, aspalalinin, nothofagin) that that are unique to the plant species while the *Cyclopia species* contains xanthones (mangiferin, isomangiferin) (Joubert *et al.*, 2008; Ajuwon, Ayeleso and Adefolaju, 2018; Windvogel, 2019). Moreover, the *Aspalathus linearis* plant species contains a larger variety of flavones, flavonols and phenolic acids polyphenolic flavonoids while the *Cyclopia species* has a larger variation of flavonones (Joubert *et al.*, 2008). Despite the differences that exist in the phytochemical and nutrient composition, both *Aspalathus linearis* and the *Cyclopia species* are potent antioxidant agents that show promise as adjuvant therapies in the treatment of diabetes mellitus (Joubert *et al.*, 2008; Ajuwon, Ayeleso and Adefolaju, 2018; Windvogel, 2019). Furthermore, even though human studies that report back on the health benefits of *Aspalathus linearis* are few, and are

lacking in *Cyclopia species*, their pharmacognosy reports show that both these medicinal plants can be safely consumed as herbal beverages (McKay and Blumberg, 2007; Joubert *et al.*, 2008).

4.2 Biometric Measurements (Body Weight, Food Intake, Fluid Intake, Blood Glucose and IPGTT) of *Aspalathus linearis* (Rooibos) And *Cyclopia intermedia* (Honeybush)

4.2.1 Main Study

In the first pilot study, the healthy control rats successfully consumed the 2% fermented RB or 4% fermented HB infusions. However, the taste of the infusions was unpalatable to the diabetic animals thus resulting in their minimal consumption of the infusions (*see section 2.2.2 chapter 2*). As a result, in the main study, the diabetic animal groups (DRF45 and DHB45) were given 2% fermented rooibos (RB) or 4% fermented honeybush (HB) infusions as a pre-treatment for 1 week prior to STZ induction. Thereafter, animals received a single intraperitoneal injection of 45 mg/kg bw STZ and treatment followed for a total duration of 6 weeks. At the end of the treatment period, significant decreases in mean body weight coupled with significantly increased mean food intake, significantly increased mean fluid intake, significantly increased relative organ weight (ROW) gain, significantly increased blood glucose levels and significantly increased IPGTT measurements in the untreated diabetic (STZ45) control animals compared to the age-matched controls drinking tap water (water control) (*see section 3.3 chapter 3*) were observed. In diabetes mellitus, rapid weight loss, excessive thirst, heightened appetite and hyperglycaemia are the results of rapid tissue protein and muscle mass loss (Ayeleso, Brooks and Oguntibeju, 2014). Moreover, due to the excessively high blood glucose levels or hyperglycaemia, significant amounts of the excess glucose molecules are excreted via the urine thus causing a chronic state of glucosuria (Ayeleso, Brooks and Oguntibeju, 2014). To compensate for glucosuria, diabetic individuals experience excessive thirst and increased fluid intake. Moreover, the biochemical process of glucosuria is facilitated by the significant use of calories for every gram of glucose excreted. Thus, diabetic individuals experience significant increases in appetite and hunger. (Al-Qattan *et al.*, 2016; Ayeleso, Brooks and Oguntibeju, 2014). Therefore, the diabetogenic effects and symptoms experienced by the diabetic animals in our study are synonymous with

some of the early clinical symptoms that develop in humans with type 1 diabetes mellitus (Al-Qattan *et al.*, 2016; Guideline and Prevention, 2012).

In a clinical setting, patients with type 1 diabetes mellitus present with significant weight loss, hyperglycaemia, polydipsia, polyuria and severe ketoacidosis in the symptomatic stages of the disease (Guideline and Prevention, 2012). Moreover, similar to our findings, STZ-induced diabetes male Wistar rats (65 mg/kg STZ dose) developed significantly increased appetite, increased fluid intake, rapid weight loss and hyperglycaemia in another study that was conducted over a 24 week period (Wei *et al.*, 2003). Similarly, 45 mg/kg bw STZ-induced diabetic Wistar rats presented with similar characteristic signs of diabetes mellitus as those of the diabetic rats assessed in our study (Ulicna *et al.*, 2005). Thus, in our study, the 45 mg/kg bw STZ dose successfully established an *in vivo* model of streptozotocin diabetes mellitus characterized by significant weight loss, hyperglycaemia, increased appetite, impaired IPGTT and increased food intake and food consumption (see section 3.3 chapter 3).

4.2.2 *Aspalathus linearis*

Following the 1-week pre-treatment and the 6-week treatment, RB infusions, did not have any significant effects on the diabetogenic effects caused by the 45 mg/kg bw streptozotocin dose. Similar to the untreated diabetic animals (STZ45), the rooibos-treated diabetic rats (DRF45) experienced significant weight loss, significantly increased food consumption, significantly increased fluid intake, significantly increased kidney ROW gain, significantly increased blood glucose and impaired IPGTT levels that was not ameliorated by the daily consumption of 2% fermented RB infusions. (see section 3.3 chapter 3). These findings correlate with the results presented in two other observational studies that determined the impact that the fermented rooibos extract (FRE) tisanes had on the diabetic status (hyperglycaemia, glycated plasma haemoglobin and fructosamine) (Ayeleso, Brooks and Oguntibeju, 2014; Ulicna *et al.*, 2005). Similar to our findings, the daily consumption of 2.5% FRE tisane did not have any significant effects on the diabetic status of 45 mg/kg bw STZ-induced diabetic male Wistar rats (Ulicna *et al.*, 2005). In the same way, 2% FRE tisane administered to 50 mg/kg bw STZ-induced Wistar rats did not ameliorate the significant liver ROW gain and rapid body weight loss caused by the diabetic status (Ayeleso, Brooks and Oguntibeju, 2014). Furthermore, in a systematic review and

meta-analysis study of the effects of *Aspalathus linearis* and its associated polyphenols on diabetic animals, it was noted that FRE and green rooibos extract (GRE) treatments have little effects on elevated blood glucose levels or hyperglycaemia in STZ models of diabetes mellitus (Kamakura *et al.*, 2015; Sasaki, Nishida and Shimada, 2018; Ulicna *et al.*, 2005). However, diabetic animals treated with polyphenol-flavonoids such as phenylpyruvic acid glycoside (PPAG), isorientin or aspalathin (Asp), exhibited the most significant reductions in their blood glucose levels when compared to FRE, GRE and other associated polyphenols (Sasaki, Nishida and Shimada, 2018). In STZ-induced diabetes mellitus, diabetic mice treated with a daily oral gavage of 10 mg/kg bw PPAG exhibited significant increases in blood glucose levels that were restored to near normoglycaemia 4 days post-STZ injection (Himpe *et al.*, 2016). Similar significant reductions in elevated blood glucose levels were observed in STZ-induced (200 mg/kg bw STZ) diabetic mice treated with 10 mg/kg PPAG in the first 4 days after receiving an STZ-injection (Song *et al.*, 2017). Isorientin (15 mg/kg), a polyphenol isolated from *Gentiana olivieri* but also present in *Aspalathus linearis*, encouraged body weight gain and significantly improved blood glucose concentrations in STZ-induced (55 mg/kg bw) diabetic rats following a 25 day treatment (Sezik *et al.*, 2005). In male homozygous (*db/db*) mice, daily treatment with 130 mg/kg Asp for 6 weeks did not have any significant effects on the fasting blood glucose levels of the rats. Similarly, 130 mg/kg Asp administered daily for 6 weeks to *db/db* mice did not improve the elevated fasting glucose concentrations of the diabetic mice (Johnson *et al.*, 2017). However, in both observational studies, the 6 week Asp treatment significantly improved the oral glucose tolerance of the *db/db* mice (Dludla *et al.*, 2017; Johnson *et al.*, 2017).

All these findings suggest that the polyphenol antioxidants present in FRE such as PPAG and Asp may have a strong ROS and free-radical scavenging ability but a weak ability for facilitating blood glucose absorption into target tissues (Sasaki, Nishida and Shimada, 2018). However, when administered in isolation, the PPAG, isorientin and Asp flavonoids confer potent blood glucose lowering properties against hyperglycaemia by stimulating α -glucosidase inhibition and by suppressing sodium glucose co-transporter (SGLT) activity in the kidney to prevent blood glucose reabsorption (Sasaki, Nishida and Shimada, 2018). Therefore, even though the daily consumption of the 2% fermented RB infusions in our study and other similar studies

did not result in significant effects against the diabetic status (hyperglycaemia, hyperphagia, rapid weight loss, polydipsia and polyuria), the FRE tisanes significantly improved the oxygen radical absorbance capacity (ORAC) status and significantly reduced the amount of circulating advanced glycation end-products (AGEs) and malondialdehyde (MDA) levels in the blood plasma of STZ-induced diabetic Wistar rats (Ayeleso, Brooks and Oguntibeju, 2014; Ulicna *et al.*, 2005). Thus, in future investigations, oxidative stress markers such as plasma AGEs, MDA and ORAC measurements need to be taken in order to validate the preventative effects of RB infusions or FREs as suitable adjuvant therapies against hyperglycaemia-induced ROS and oxidative stress damage in the bloodstream and the vasculature of diabetic animals.

4.2.3 *Cyclopia intermedia*

Similar to the RB infusions, the daily consumption of 4% fermented honeybush (HB) infusion did not induce any significant effects against the diabetogenic effects of streptozotocin-induced diabetes mellitus. Following a 6-7 day pre-treatment and 6 week treatment, HB infusions administered daily to diabetic adult Wistar rats (DHB45) did not have any significant effects against the significant body weight loss, significantly increased food intake, significantly increased fluid intake, significantly increased ROW and the significantly increased blood glucose and impaired IPGTT levels of the honeybush-treated diabetic animals (DHB45) when compared to the untreated diabetic control group (STZ45) (see section 3.3 chapter 3). However, the 4% fermented HB significantly reduced the increased ROW of the kidney of the diabetic rats compared to RB (see section 3.3.6. chapter 3). Renal ROW is caused by STZ-induced nephrotoxicity, renal lesions, renal hypertrophy, increased glomerular volume and mesangial proliferation amongst other structural changes (Zafar and Naqvi, 2010). The underlying mechanisms involved in this positive effect of HB on increased ROW of the kidney are unknown. Thus, further investigations need to be conducted to determine the underlying mechanisms involved.

In STZ induced diabetes mellitus, a single dose of unfermented freeze-dried *Cyclopia intermedia* (*C. intermedia*) HB extract administered by oral gavage (50 mg/kg) significantly reduced the fasting glucose levels of the diabetic Wistar rats following a 6 hour observation period (Muller *et al.*, 2011). In the same study, daily consumption

of the unfermented *C. intermedia* HB infusions for 12 weeks by high-fat diet fed obese insulin-resistant (OBIR) rats significantly reduced their hyperglycaemic blood glucose concentrations to normoglycaemic levels (Muller *et al.*, 2011). Similarly, unfermented *Cyclopia maculata* (*C. maculata*) extract (30 mg/kg or 300 mg/kg) administered orally for 15 days before STZ-injection (40 mg/kg bw) and 6 days thereafter significantly improved the impaired glucose tolerance levels and the total serum triglyceride levels of the diabetic Wistar rats (Chellan *et al.*, 2014).

The hypoglycaemic effects and oxidative stress ameliorative properties of unfermented *Cyclopia species* is largely attributed to the bioactive polyphenol-flavonoids mangiferin and hesperidin present in the plant species (Jung *et al.*, 2004; Akiyama *et al.*, 2010; Pal, Sinha and Sil, 2014, Li *et al.*, 2018; Wang *et al.*, 2018). Mangiferin, a polyphenol xanthone extract found in the *Cyclopia species* was administered orally in water to STZ-induced (65 mg/kg bw) diabetic Wistar rats for 30 days. At the end of the 30 days, the mangiferin extract (40 mg/kg) significantly reduced plasma glucose levels and restored the kidney to body weight ratio of the diabetic rats to normal levels (Pal, Sinha and Sil, 2014). On the other hand, mangiferin (12.5, 25 or 50 mg/kg/day) administered by oral gavage to STZ-induced (53 mg/kg bw) diabetic Sprague Dawley rats did not induce any significant changes in their fasting blood glucose levels at the end of a 12 week treatment (Wang *et al.*, 2018).

Similarly, hesperidin, a naturally occurring polyphenol flavonone that is present in the *Cyclopia species* significantly reduced the elevated serum glucose levels and inhibited the significant decreases in body weight of STZ-induced (55 mg/kg bw) diabetic rats following a 21 day oral intake of 50 or 100 mg/kg hesperidin extract (Li *et al.*, 2018). In type 2 diabetic *db/db* mice, both 5 week 0.2 g/kg hesperidin and 0.2 g/kg naringin supplemented diets significantly reduced and restored the hyperglycaemic blood glucose levels of the *db/db* mice to normoglycaemic levels (Jung *et al.*, 2004). The highly effective hypoglycaemic effects of hesperidin and naringin are attributed to their ability to modulate the activities of hepatic glucose regulating enzymes (Jung *et al.*, 2004). Furthermore, following a biochemical analysis of the hepatic glycogen synthesis and hepatic glucokinase enzyme activity of *db/db* type 2 diabetic mice fed with hesperidin or naringin supplemented diets, a significant increase in hepatic glucokinase activity and hepatic glycogen synthesis was found compared to the untreated diabetic mice (Jung *et al.*, 2004). Furthermore, significant reductions in

glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) levels were observed in the naringin treated diabetic mice (Jung *et al.*, 2004). According to the investigators of the study, hesperidin and naringin possibly ameliorated hyperglycaemia in the *db/db* mice by acting as antioxidants that neutralize hyperglycaemia-induced oxidative stress in the pancreatic β -cells thus preserving the integrity of the intact, insulin positive and producing pancreatic β -cells (Jung *et al.*, 2004). Therefore, the increased bioavailability of insulin in the hesperidin and naringin treated diabetic rats facilitated insulin-stimulated inhibition of glycolysis, glycogen synthesis, increased hepatic glucose absorption and significant decreases in blood glucose levels and postprandial hyperglycaemia (Jung *et al.*, 2004). Similarly, the STZ-induced increase in G6P and decrease in hepatic glucokinase activity of the STZ-induced (70 mg/kg bw) diabetic rats fed with a diet supplemented with 1% hesperidin (10 g/kg) for 10 weeks, was restored to normal levels thus facilitating the significant decrease observed in the blood glucose levels of the diabetic rats (Akiyama *et al.*, 2010). Furthermore, it is reported that hesperidin and naringin extracts exhibit their hypoglycaemic effects in diabetes mellitus by increasing messenger RNA (mRNA) expression/activity of the glucose catabolic enzyme glucokinase and by reducing the expression and activity of the gluconeogenic G6P enzyme (Akiyama *et al.*, 2010).

In our study, the diabetic Wistar rats were treated with fermented HB *C. intermedia* infusions whereas in the previously conducted studies mentioned above, animals were fed unfermented/ green honeybush extracts. Currently, there aren't any published articles available that report back on the effects of fermented HB infusions in a diabetic setting as we investigated in our study. According to various research and review articles, fermented honeybush extract and tisanes undergoes a significant loss in its total antioxidant activity through the fermentation process (Ajuwon, Ayeleso and Adefolaju, 2018; McKay and Blumberg, 2007; Windvogel, 2019). This is as a result of the chemical, enzymatic and oxidative reactions that take place during the fermentation process. In an observational study that assessed the antioxidant capacity of 5 different *Cyclopia species* (*C. intermedia*, *C. sessiliflora*, *C. subternata*, *C. genistoides*, *C. maculata*), the fermented form of the *Cyclopia species* exhibited reduced superoxide radical scavenging capacity compared to the unfermented/green *Cyclopia species* form (Hubbe and Joubert. 2000). Furthermore, fermented *C. intermedia* and *C. maculata* exhibited the lowest superoxide scavenging capacity and

the lowest total polyphenol content (Hubbe and Joubert. 2000). Therefore, daily consumption of 4% fermented HB infusions may have not exhibited significant effects against the diabetic status in the 6-week treatment period due its low total polyphenol content. Thus, more research investigations, with a focus on the long-term treatment of fermented HB infusions, need to be done in order to elucidate the optimal duration of administration. Furthermore, it is imperative to continue investing fermented HB infusions as a possible treatment of hyperglycaemia and the diabetic status as honeybush is popularly and preferably consumed in its fermented form.

4.2.4 The effects of the palatability of *Aspalathus linearis* and *Cyclopia intermedia* on fluid intake

Honeybush drinking diabetic rats (DHB45) consumed a significantly lower volume of the 4% HB infusion compared to that of the rooibos (DRF45) drinking diabetic rats (see section 3.3.3 chapter 3). In the pilot study that was conducted prior to this main study, it was noted that the diabetic rats consuming HB infusions found the infusions to be unpalatable (see section 2.2.1 chapter 2). Compared to the RB infusion drinking rats, the HB infusion drinking diabetic rats consumed a significantly lower volume of the HB infusion that resulted in severe dehydration and removal of the rats from the pilot studying (see section 2.2.1 chapter 2). Consequently, in the main study, DHB45 and DRF45 treatment groups were introduced to the infusions as a pre-treatment for 6-7 days in order to acclimatize them to the taste of the infusions. Even though so, the DHB45 HB infusion drinking rats still had a significantly reduced fluid intake compared to the DRF45 RB infusion drinking rats. Both the *Cyclopia* species and *Aspalathus linearis* have a bitter medicinal/ herbal taste to the palate. However, the HB infusion might be more bitter as it seemed to be less palatable than RB infusions to the diabetic rats (see section 3.3.3 chapter 3).

4.3 Blood Pressure

4.3.1 Blood Pressure in Diabetes Mellitus

The blood pressure of the animals was measured in the diabetic animals and their age-matched controls by mean of the tail-cuff method. At the end of the 6-week treatment period, there were no significant differences between the mean systolic blood pressure, mean diastolic blood pressure and MAP of all the treatment groups

receiving rooibos (DRF45) or honeybush infusions (DHB45) and their healthy age-matched controls (see section 3.4 chapter 3). Throughout the 6-week treatment period, systolic blood pressure, diastolic blood pressure and mean arterial pressure (MAP) were measured once every second week using the CODA® Non-Invasive tail-cuff Blood Pressure acquisition system (Kent Scientific). The CODA® Non-Invasive tail-cuff Blood Pressure acquisition system measures blood pressure in rodents without the use of sedatives and surgical devices. Moreover, the validity of this blood pressure system has previously been established over a physiological range of various blood pressure points (Feng *et al.*, 2008).

In the present study, the STZ model of diabetes mellitus induced in rats by an intraperitoneal injection of 45 mg/kg bw STZ did not induce high blood pressure in the treated or untreated diabetic animals (see section 3.4 chapter 3). This is in contrast with reports from previously conducted studies where the moderate STZ doses of 45 mg/kg bw (Culshaw *et al.*, 2019; Raghunathan *et al.*, 2014) and 50 mg/kg bw (Bunag, Tomita and Sasaki, 1982) induced significant increases in the mean systolic blood pressure (Bunag, Tomita and Sasaki, 1982), the mean diastolic blood pressure (Culshaw *et al.*, 2019) and the mean arterial blood pressure (MAP) of the diabetic rats (Raghunathan *et al.*, 2014). However, in the studies mentioned above, the blood pressure parameters of the animals was measured either invasively using the carotid artery cannulation method (Raghunathan *et al.*, 2014) or radio telemetry devices (Culshaw *et al.*, 2019) or non-invasively by means of photoelectric sensors (Bunag, Tomita and Sasaki, 1982). While a different method (the non-invasive tail-cuff method) was used in the present study. Significant increases in mean systolic blood pressure as measured by means of the tail-cuff method has only been evident in animals induced with an STZ dose that is significantly higher than 45 mg/kg bw. In those studies, diabetes mellitus was induced with STZ doses 55 mg/kg bw (Majithiya and Balaraman, 2006), 60 mg/kg bw (Al-Qattan *et al.*, 2016; Erejuwa *et al.*, 2011) and 70 mg/kg bw (Tarhzaoui *et al.*, 2008). This suggests that the non-invasive tail-cuff method is highly effective in detecting blood pressure changes that occur in a severe disease state of STZ-induced diabetes mellitus but not in a moderate disease state. Thus, in future studies I recommend the use of an STZ dose higher than 45 mg/kg bw if the objective is to induce high blood pressure in an STZ diabetic model.

4.3.2 The effects of *Aspalathus linearis* on blood pressure

In the present study, the potential anti-hypertensive effects of the fermented 2% RB infusions were not observed in the 6-week treatment period. The RB infusion did not significantly change the mean systolic, diastolic and MAP of the diabetic animals (DRF45) when compared to the untreated (STZ45) and healthy controls (see section 3.4 chapter 3). The anti-hypertensive effects of *Aspalathus linearis* have been previously documented in various animal and human (*in vivo* and *in vitro*) studies (Persson *et al.*, 2006; Persson *et al.*, 2010; Persson, 2012). A significant decrease in the MAP of healthy rats was observed in the right carotid artery of rats that received intravenous injections of fermented rooibos extract (FRE) in a dose-dependent manner (10, 30, 100 mg/kg) (Khan and Gilani, 2006). Furthermore, a study that was conducted in our laboratories, green rooibos (GRT) extract administered for 16 weeks to high-fat diet induced obese rats significantly reduced the mean systolic, mean diastolic and MAP of the obese Wistar rats (Maqeda, 2018). However, in the present study, the 45 mg/kg STZ dose did not induce hypertension in the diabetic rats. Therefore, the 2% fermented RB infusion did not show its potential anti-hypertensive effects in the diabetic animals because the blood pressure of the all diabetic animals was normal and not significantly different from that of the healthy controls. This implies that the 45 mg/kg STZ dose was not high enough to induce high blood pressure as measured non-invasively in the 6-week duration of the study (see section 3.4 chapter 3).

4.3.3 The effects of *Cyclopia Species* on blood pressure

Currently, there are no published studies that have documented the effects of fermented HB infusions on blood pressure in experimental STZ-induced diabetes mellitus. However, in two previously conducted studies the preventative effects of the polyphenol-flavonoids, naringin, glucosyl-hesperidin and hesperidin were exhibited in stroke-prone spontaneously hypertensive rats (SHR) suggesting that the *Cyclopia species* has potential to treat or regulate high blood pressure (Amamoto, Uzuki and Ase, 2008; Ikemura *et al.*, 2012). However, in the present study, the daily consumption of fermented 4% HB infusions by the STZ-induced diabetic animals did not induce any significant changes in the blood pressure parameters of the diabetic rats (DHB45) when compared to that of their age-matched controls. Furthermore, the blood pressure

of the both the treated and untreated diabetic animals was normotensive throughout the study. This implies that the 45 mg/kg STZ dose used in our animals was not high enough to induce any increases in the blood pressure (as measured non-invasively) that could possibly have been ameliorated by the potential anti-hypertensive effects of the 4% fermented HB treatment (see *section 3.4 chapter 3*). Furthermore, although the STZ dose did induce sustained hyperglycaemia, it may not have caused a severe disease state. The STZ 45 mg/kg dose possibly didn't ablate or have severe detrimental effects to the pancreatic cells of the diabetic animals, which may have led to the development of associated conditions such as elevated blood pressure. Therefore, the 45mg/kg bw STZ dose possibly didn't cause changes in blood pressure because the sustained hyperglycaemia was manageable for the diabetic animals as some of the pancreatic cells of the animals were possibly still intact and functional.

4.4 Vascular Aortic Contraction/Relaxation Studies

4.4.1 Vascular Function in Diabetes Mellitus

In the present study, the thoracic aortas of the diabetic animals and their age-matched controls were harvested at the end of the 6-week treatment period. The aortic rings of all treatment groups were assessed by means of isometric tension studies by phenylephrine (Phe)-induced vascular contraction and acetylcholine (ACh)-induced vascular relaxation. The aortic rings of the untreated STZ animals (STZ45) had a significant pro-contractile response to Phe administration followed by a significantly reduced relaxation response to ACh when compared to the VEH control. Treatment with the fermented 2% RB infusions significantly reduced Phe-induced vascular contraction of the treated diabetic rats (DRF45) while significantly increasing vascular relaxation response of the DRF45 animals to ACh administration. Similarly, the fermented 4% HB infusions significantly reduced the vascular contraction response of the treated diabetic rats (DHB45) to Phe and significantly increased the vascular relaxation response of the DHB45 aortic rings to ACh. Furthermore, the diabetic animals treated with the RB infusions (DRF45) presented with a significantly decreased vascular contraction response to Phe administration compared to the diabetic rats treated with the HB infusions (DHB45) (see *section 3.5. chapter 3*).

One of the pathophysiological conditions that lead to impaired vascular function and the progression to ED and the consequent vascular injuries/diseases in diabetes

mellitus is reduced endothelial NO bioavailability and increased ROS production and oxidative stress (Deanfield, Halcox and Rabelink, 2007; Sena, Pereira and Seiça, 2013). The endothelium plays a pivotal role in maintaining vascular homeostasis. In response to shear stress and other chemical stimuli, the endothelium secretes endothelial derived NO which diffuses to the underlying vascular smooth muscle layer where it stimulates vasorelaxation via the cyclic GMP pathway amongst others (Deanfield, Halcox and Rabelink, 2007; Sena, Pereira and Seiça, 2013). However, in diabetes mellitus, the chronic exposure of the endothelium to hyperglycaemia leads to ED and reduced endothelial NO bioavailability through a number of hyperglycaemia induced mechanisms such as eNOS uncoupling, oxidative stress, glucose autoxidation, glucotoxicity, the increased production of AGEs and the expression of the receptor for AGEs (RAGEs) (Brownlee, M.D, 1995; Förstermann and Sessa, 2012; Maritim *et al.*, 2003). These reports imply that the anti-contractile and pro-relaxation effects that the STZ injury model had on the aortic tissues was caused by STZ-induced hyperglycaemia, ROS production and oxidative stress. Thereby, uncoupling eNOS and significantly reducing NO bioavailability and impairing NO-mediated vascular reactivity.

4.4.2 The Pro-Relaxation Effect of RB and HB Infusions

The vascular anti-contractile / pro-relaxation effect that the fermented 2% RB and 4% HB infusions had on the aortic rings of the treated diabetic rats is possibly due to the inherent antioxidant potential present in the polyphenol-flavonoids of the plants (Ajuwon, Marnewick and Davids, 2015). Flavonoid polyphenols have phenolic hydroxyl groups that act as nonenzymatic antioxidants, metal chelators, electron donors and reducing agents that scavenge ROS and unstable free radicals to stable molecules (Ajuwon, Marnewick and Davids, 2015). Thereby, eradicating the excess ROS molecules present in the vasculature of diabetic individuals. With reduced ROS and oxidative stress in the vasculature, eNOS uncoupling is potentially reversed, thereby increasing the activity of functional eNOS and NO production.

4.4.3 Effects of *Aspalathus linearis* on Vascular Function

The antioxidant potential of *Aspalathus linearis* in vascular reactivity has been largely attributed to two flavonoids, aspalathin and nothofagin (Ajuwon, Marnewick and Davids, 2015; Ku *et al.*, 2015). In a previous study, aspalathin and nothofagin had

inhibitory effects on NF- κ B pro-inflammatory signalling pathways, monocyte recruitment and hyperglycaemia-induced vascular permeability in the endothelium (Ajuwon, Marnewick and Davids, 2015). Furthermore, aspalathin and nothofagin exhibited an inhibitory effect against protein-leakage as a result of glucose-induced vascular permeability in endothelial cells that were isolated from mice (Ku *et al.*, 2015). Moreover, the pre-exposure of HUVECs to 50 μ M aspalathin or nothofagin reduced membrane disruption, hydrogen peroxide production and the expression of neutrophils and cell adhesion molecules (E-selectins, VCAM-1 and ICAM-1) (Ku *et al.*, 2015). Thus, these findings imply that in the present study, the anti-contractile and pro-relaxation effects that was evident in the aorta of the rooibos-treated diabetic rats could be due to the strong antioxidant potential of aspalathin and nothofagin (see section 3.5 chapter 3). Furthermore, more studies are however needed to verify the contribution of the individual polyphenols in our injury model.

4.4.4 Effects of *Cyclopia intermedia* on Vascular Function

Similarly, hesperidin and naringin are polyphenol flavonoids of the *Cyclopia species* that have been reported by various researchers to be largely responsible for the antioxidant and ameliorative effects of honeybush against hyperglycaemia-induced ED (Amamoto, Uzuki and Ase, 2008; Ikemura *et al.*, 2012). In two previously conducted studies, naringin, hesperidin and its soluble derivative G-hesperidin, significantly enhanced ACh-induced vasorelaxation in the aortic rings of SHR and SHRSP rats (Amamoto, Uzuki and Ase, 2008; Ikemura *et al.*, 2012). However, when tested on sodium nitroprusside-induced endothelial independent vasorelaxation, the polyphenols had no effects on the vasorelaxation response. Thereby, suggesting that hesperidin and G-hesperidin mediate their pro-vasorelaxation effect through NO endothelium-dependent mechanisms (Amamoto, Uzuki and Ase, 2008; Ikemura *et al.*, 2012). Thus, in the present study, the hesperidin and naringin polyphenols of 4% fermented HB may have mediated the anti-contractile and pro-relaxation effects observed in the vascular function of the aortas that were injured by the STZ injury model (see section 3.5 chapter 3)

4.5 Western Blot Analysis

4.5.1 Protein Expression in Diabetes Mellitus

In order to determine whether the anti-contractile and pro-relaxation effects of the RB and HB infusions on the vascular reactivity of the aortic rings is mediated via eNOS and PKB/Akt signalling, the total and phosphorylated protein expressions of eNOS and PKB/Akt were analysed in the aortic tissue. The STZ treatment significantly decreased total (T)-eNOS expression in the untreated diabetic STZ and treated diabetic DRF45 and DHB45 groups when compared to the healthy non-diabetic controls. The phosphorylated (P)-eNOS expression was significantly reduced in the untreated STZ group when compared to the VEH control. Additionally, a significant increase in the P: T-eNOS ratio of the untreated STZ group was observed in comparison to the water control. Furthermore, the STZ treatment induced a significant reduction in the total (T)-PKB and phosphorylated (P)-PKB expression of the untreated diabetic STZ and treated diabetic DRF45 and DHB45 groups when compared to the VEH control. However, there were no significant differences in the P: T-PKB ratio between the groups (*see section 3.6 chapter 3*).

PKB/Akt is one of the protein kinases that phosphorylates eNOS on Ser1177 in response to shear stress (Fleming and Busse, 2003; Förstermann and Sessa, 2012). eNOS phosphorylation on Ser1177 results in an influx of electrons from the reductase to the oxygenase domain of the enzyme and synergistically increases the sensitivity of the enzyme to calcium. Thereby, stimulating eNOS activation and the subsequent production of NO (Fleming and Busse, 2003; Förstermann and Sessa, 2012). Moreover, in the presence of insulin, PKB/Akt activation is enhanced (Du *et al.*, 2001). However, when insulin is deficient, hyperglycaemia inhibits eNOS activity by causing posttranslational modification at the PKB/Akt phosphorylation site (Du *et al.*, 2001). Thus, in the present study, the significantly elevated glucose levels that occurred in the STZ-induced diabetic rats may have caused the significantly reduced P-PKB and, P-eNOS and T-eNOS expression and impaired vasorelaxation in the aortic rings through hyperglycaemia-induced eNOS inhibition and STZ-induced insulin deficiency (*see section 3.6 chapter 3*).

4.5.2 Effects of *Aspalathus linearis* on protein expression

Furthermore, in the aortic rings of the diabetic rats of the present study, the fermented 2% RB infusion did not induce any significant effects against the significantly reduced eNOS and PKB expression of the rooibos drinking rats (DRF45) when compared that of the untreated STZ group. This is a novel finding and there is no other available literature that has previously reported the effects of fermented RB in an STZ model of diabetes mellitus. However, a decrease in eNOS and PKB expression that could not be ameliorated by treatment with an antioxidant-rich adjuvant like fermented rooibos is an indication of severe hyperglycaemia and oxidative stress-induced vascular damage (Du *et al.*, 2001). However, treatment with other forms of *Aspalathus linearis* that have a greater antioxidant potential such as green rooibos may be effective in ameliorating STZ-induced vascular injury (Maqeda, 2018).

4.5.3 Effects of *Cyclopia intermedia* on protein expression

Similarly, in the present study, the fermented 4% HB infusion did not have any significant effect on the significantly reduced eNOS and PKB expression due to STZ treatment. Currently there are no other published studies that report back on the effects of fermented HB infusions on aortic rings of STZ-induced diabetic animals. This is a novel study thus; further investigations need to be conducted in order to determine the signalling mechanisms involved in HB-mediated vasorelaxation of the aorta.

Furthermore, various technical issues were encountered in the laboratory that may have affected the final protein expression results of all treatment groups. The T-eNOS and P-eNOS protein expression results were performed on 2 separate blot membranes because the number of groups (7) and sample number (n = 5) per group exceeded the capacity that could be loaded onto one gel membrane. As a result, in each treatment group the first 3 samples (A, B, C) were loaded on blot 1 and the remaining 2 samples in addition to a repeat load of sample A (A, D, E) were loaded onto blot 2. However, the quality of protein loading onto blot 1 and blot 2 was unequal. This is evident in the protein expression of the repeat load (sample A) which occurred as a dark band in blot 1 and as a very faint band in blot 2 in all the treatment groups (see section 3.6 chapter 3 and appendix C). Furthermore, in both blots, the protein bands that represent T-eNOS and P-eNOS expression were very faint and difficult to distinguish from the other non-specific bands (see section 3.6.1-3.6.2 chapter 3 and

appendix C). In addition to that, the blots were overexposed and that interfered with the visibility of the relative protein bands expressed (see *section 3.6.1-3.6.2 chapter 3 and appendix C*). Similarly, the blots representing T-PKB and P-PKB protein expression were overexposed (see *section 3.6.4-3.6.5 chapter 3 and appendix C*). Moreover, the protein expression calculations of T-PKB and P-PKB was based on blot 1 only, thereby reducing the sample number to 3 per group ($n = 3$) instead of 5 (see *section 3.6.4-3.6.5 chapter 3 and appendix C*). Furthermore, the T-PKB and P-PKB results on blot 2 were not taken into consideration because the blot was damaged as a result of human handling (see *section 3.6.4-3.6.5 chapter 3 and appendix C*). Therefore, in future experiments the ratio of aortic tissue to the sample buffer must be optimised before conducting the main experiment. It is imperative to correctly determine the most suitable volume of sample buffer to avoid over diluting the aortic tissue. This way, the concentration of protein present in the aortic tissue samples will be more distinct and visible. Thereby, avoiding the need to overexpose the bands.

4.6 Antioxidant Activity Analysis

4.6.1 Antioxidant Enzyme Activity in Diabetes Mellitus

In the present study, the superoxide dismutase (SOD) and catalase (CAT) antioxidant enzyme activity of the animals was assessed in the kidney. At the end of the 6-week treatment period, there were no significant differences in the kidney SOD and CAT activity of the rooibos drinking diabetic (DRF45) and honeybush drinking diabetic (DHB45) rats when compared to their control groups respectively. Superoxide dismutase (SOD) and catalase (CAT) are primary antioxidant defences that facilitate the removal and neutralization of ROS and unstable and harmful oxygen free radicals thus eradicating oxidative stress. SOD dismutates superoxide anions ($2O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and catalase converts hydrogen peroxide to water (H_2O) and molecular oxygen (O_2) (Ayeleso, Brooks and Oguntibeju, 2014). Hyperglycaemia-induced biochemical reactions such as glucose autoxidation, the glycation of proteins and the activation of NF- κ B transcription factors amongst others lead to the overproduction of ROS and free radicals (e.g. superoxide anions, hydrogen peroxide, hydroxyl ions). Thereby, overwhelming the SOD and CAT enzymes, thereby decreasing or inactivating their activity (Maritim *et al.*, 2003; Naseeruddin *et al.*, 2009).

In previous studies of experimental models of STZ diabetes mellitus, it was shown that STZ-induced diabetes mellitus significantly decreases SOD and CAT activity of the kidney (Schmatz *et al.*, 2012; Babujanarthanam *et al.*, 2011; Kuo *et al.*, 2015; Mkhwanazi *et al.*, 2014). The decreased SOD and CAT activity observed in the kidneys of the diabetic rats assessed in the previously conducted studies can be attributed to the significantly higher STZ doses 50 mg/kg bw (Babujanarthanam *et al.*, 2011), 55 mg/kg bw (Schmatz *et al.*, 2012) and 60 mg/kg bw STZ (Mkhwanazi *et al.*, 2014; Kuo *et al.*, 2015) used to induce diabetes mellitus. However, in the present study, the STZ dose of 45 mg/kg bw did not induce any significant changes in the kidney SOD and CAT activity of any of the diabetic groups. It is possible that at a low dose of STZ, antioxidant enzyme activity is not significantly reduced in the kidney (see *section 3.7 chapter 3*). However, at doses higher than 45 mg/kg bw, STZ significantly reduces its antioxidant activity (Babujanarthanam *et al.*, 2011; Kuo *et al.*, 2015; Mkhwanazi *et al.*, 2014; Schmatz *et al.*, 2012). Furthermore, although the STZ dose did induce sustained hyperglycaemia, it may not have caused a severe disease state. The STZ 45 mg/kg dose possibly didn't ablate or have severe detrimental effects to the pancreatic cells of the diabetic animals which may have led to the development of associated conditions such as reduced endogenous antioxidant enzyme activity. Therefore, the 45mg/kg bw STZ dose possibly didn't cause changes in blood pressure and oxidative stress because the sustained hyperglycaemia was manageable for the diabetic animals as some of the pancreatic cells of the animals was possibly still intact and functional.

4.6.2 Antioxidant Effect of *Aspalathus linearis*

In the present study, the 2% fermented RB infusions did not have any significant effects on the SOD and CAT activity of the kidney in the diabetic adult Wistar rats (DRF45) when compared to their age-matched controls (see *section 3.7 chapter 3*). To date, there is no other study that has assessed the effects of 2% RB infusions on the antioxidant enzyme activity of the kidney in an STZ model of diabetes mellitus. The studies discussed below report on the effects that the RB infusions had in the livers of diabetic animals. The daily consumption of 2% RB infusion for 7 weeks in diabetic (50 mg/kg bw STZ) rats significantly increased liver SOD activity (Ayeleso, Brooks and Oguntibeju, 2014). In another study conducted by Canda *et al.*, 2014, opposing results were observed. Canda, Oguntibeju and Marnewick, 2014 argued that a decrease in

SOD activity following treatment with 2% fermented RB infusion is an indication of the ROS scavenging /neutralizing effects that rooibos flavonoids possibly confer to superoxide anions. Thereby, the compensatory upregulation of oxidative stress-induced SOD activity/expression was inhibited (Canda, Oguntibeju and Marnewick, 2014). However, in most previously conducted studies, treatment with an antioxidant rich extract increased SOD activity instead of decreasing it (Babujanarthanam *et al.*, 2011; Schmatz *et al.*, 2012; Ayeleso, Brooks and Oguntibeju, 2014; Mkhwanazi *et al.*, 2014; Kuo *et al.*, 2015). Furthermore, in the present study, the 2% RB infusions did not induce any significant changes in the CAT activity of the kidney in diabetic Wistar rats (DRF45). Similarly, the daily consumption of 2% RB infusions by diabetic (50 mg/kg bw STZ) rats for 7 weeks did not have any significant effects on the CAT activity in livers of the diabetic rats (Ayeleso, Brooks and Oguntibeju, 2014). In the same way, 2% RB infusions had no significant effects on the CAT activity in the livers of rats subjected to t-BHP-induced oxidative stress (in the last 2 weeks of treatment) when compared to their healthy age-matched controls (Canda, Oguntibeju and Marnewick, 2014).

In previously conducted studies, 2% RB infusions did improve the antioxidant capacity (AOC) of the liver and plasma samples of diabetic animals (Ulicna *et al.*, 2005; Ayeleso, Brooks and Oguntibeju, 2014; Canda, Oguntibeju and Marnewick, 2014). In our study, assays that quantify AOC were not conducted. From the findings gathered in the previously conducted studies, it is clear that that the fermented form of *Aspalathus linearis* does improve the antioxidant capacity of the body even though its antioxidant capacity may not be potent enough to induce significant changes in the expression or activity of endogenous antioxidant enzymes (Ayeleso, Brooks and Oguntibeju, 2014; Canda, Oguntibeju and Marnewick, 2014; Ulicna *et al.*, 2005). Thus, in future studies, I would recommend that other assays such as the oxygen radical absorbance capacity (ORAC) and the trolox equivalence antioxidant capacity (TEAC) assays be taken into consideration when assessing the antioxidant activity of herbal tisanes.

4.6.3 Antioxidant Effect of *Cyclopia intermedia*

In the present study, the 4% fermented HB infusions administered to STZ-induced diabetic adult male Wistar rats (DHB45) for 6 weeks did not induce any significant

changes in the SOD and CAT activity in the kidney tissue when compared to their age-matched controls (see section 3.7 chapter 3). To date, there is no other published study that reports back on the effects of fermented HB infusions on antioxidant enzyme activity and oxidative stress biomarkers in diabetes mellitus. However, the antioxidant effects of unfermented/green honeybush extracts has been investigated in two previous studies (Chellan *et al.*, 2014; van der Merwe *et al.*, 2017). Similar to the findings of the present study, the daily consumption of the unfermented honeybush extracts by healthy and diabetic rats did not induce any significant changes in the enzyme activity of SOD and CAT in the liver tissue of the animals (Chellan *et al.*, 2014; van der Merwe *et al.*, 2017). Furthermore, the unfermented *Cyclopia subternata* and *Cyclopia genistoides* polyphenol-enriched extracts (2.5 g/kg bw) were administered daily in rat cubes to healthy male Fischer rats for 28 days (van der Merwe *et al.*, 2017) while the unfermented *Cyclopia maculata* extracts (300 mg/kg bw) was administered to the STZ-induced (40 mg/kg bw) diabetic Wistar rats for 15 days as a pre-treatment followed by a 6 day treatment (Chellan *et al.*, 2014). Relying on a single method of determining antioxidant capacity is not enough to validate the antioxidant capacity of HB infusions and extracts. In addition to performing antioxidant enzyme assays, I would recommend that other measures of antioxidant capacity such as the ORAC and the TEAC plasma and tissue assays to be conducted in future studies.

4.7 Lipid Peroxidation

4.7.1 Lipid Peroxidation in Diabetes Mellitus

In the present study, lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) assay. At the end of the 6-week treatment period, there were no significant changes in the TBARS measurements of treated diabetic rats (DRF45 and DHB45) when compared their untreated diabetic (STZ45) and healthy age-matched controls (see section 3.7.3 chapter 3). Thiobarbituric acid reactive substances (TBARS) are by-products of lipid peroxidation that have been shown to be increased in STZ diabetes mellitus (Maritim *et al.*, 2003). Malondialdehydes (MDA), conjugated dienes and lipid hydroperoxides are other biomarkers of lipid peroxidation that have been previously assessed in other studies (Maritim *et al.*, 2003). Significant increases in TBARS, MDA, conjugated dienes and lipid hydroperoxides amongst other by-products of lipid peroxidation occur in diabetes mellitus due to the increased ROS

production, oxidative stress and the compromised antioxidant status of the affected individuals (Maritim *et al.*, 2003). In the past, various studies have documented lipid peroxidation that occurs in STZ-induced diabetes mellitus (Babujanarthanam *et al.*, 2011; Maritim *et al.*, 2003; Schmatz *et al.*, 2012). High levels of TBARS were quantified and detected in the lipid profiles of streptozotocin and alloxan induced diabetic male rats (Maritim *et al.*, 2003). Similarly, the expression of TBARS was significantly increased in STZ-induced (55 mg/kg bw) male Wistar rats 30 days post STZ injection (Schmatz *et al.*, 2012). Likewise, at 30 days post STZ injection, significantly high levels of TBARS were observed in untreated diabetic Wistar rats that received a single injection of 50 mg/kg STZ (Babujanarthanam *et al.*, 2011). However, in our present study, the STZ dose of 45 mg/kg bw did not induce lipid peroxidation in the 6 -week treatment period. This could imply that the dose of STZ used in our study may not have been high enough or of long enough duration to result in altered antioxidant enzyme activity or to induce lipid peroxidation. (Babujanarthanam *et al.*, 2011; Maritim *et al.*, 2003; Schmatz *et al.*, 2012).

4.7.2 Effects of *Aspalathus linearis* on Lipid Peroxidation

In the present study, the 2% fermented RB infusions administered for 6 weeks to diabetic rats (DRF45) and their age-match controls did not induce any significant change in the TBARS levels in their kidneys (see section 3.7.3 chapter 3). Similarly, the daily consumption of RB polyphenol-flavonoids did not induce any significant changes in TBARS expression in other studies (Hodgson *et al.*, 2002). However, when taking into consideration other measurements of lipid peroxidation such as conjugated dienes (Ayeleso, Brooks and Oguntibeju, 2014) and MDA levels (Ulicna *et al.*, 2005), the daily consumption of 2% RB infusions significantly reduced oxidative stress in other experimental models of STZ diabetes mellitus. This shows that the fermented form of RB does have ameliorative effects against oxidative stress (Ayeleso, Brooks and Oguntibeju, 2014; Ulicna *et al.*, 2005). However, in the present study, the potential beneficial effects of the fermented RB infusion were not shown because the STZ dose of 45 mg/kg bw used was not high enough to induce an oxidative stress insult in the kidney of the diabetic groups (see section 3.7.3 chapter 3).

4.7.3 Effects of *Cyclopia intermedia* on Lipid Peroxidation

Furthermore, diabetic Wistar rats (DHB45) and their healthy (HB control) and untreated diabetic (STZ45) age-matched controls were given fermented 4% HB infusions for 6 weeks. At the end of the treatment period, no significant differences were observed between the TBARS measurements of all the treatment groups (see *section 3.7.3 chapter 3*). To date, there are no published studies that report on the effects of fermented HB infusions on lipid peroxidation in an STZ model of diabetes mellitus. In other animal studies where animals were given unfermented/ green honeybush extracts, similar findings were observed (Chellan *et al.*, 2014; van der Merwe *et al.*, 2017). In one of the studies, unfermented *Cyclopia subternata* and *Cyclopia genistoides* polyphenol-enriched extracts (2.5 g/kg bw) that were given to healthy male Fischer rats for 28 days did not have any significant effects on the malondialdehyde (MDA) levels and the lipid peroxidation concentrations present in the liver of the healthy rats (van der Merwe *et al.*, 2017). Similarly, unfermented *Cyclopia maculata* extracts (300 mg/kg bw) did not induce any significant changes in the MDA levels and the lipid peroxidation concentrations present in the liver of STZ-induced (40 mg/kg bw) diabetic Wistar rats by the end of a 21 day treatment period (Chellan *et al.*, 2014). These reports imply that the *Cyclopia species* does not exhibit any beneficial effects against lipid peroxidation and oxidative stress markers in both STZ-diabetic and healthy rats (Chellan *et al.*, 2014; van der Merwe *et al.*, 2017).

4.8 Summary of the main finding and discussion

The aim of the study was to establish an *in vivo* model of STZ diabetes mellitus and to determine the modulating effects of fermented RB and fermented HB on the diabetogenic effects, blood pressure and the compromised antioxidant status that is observed in an animal model of diabetes mellitus. The STZ (45 mg/kg bw) injury model of diabetes mellitus used in the study successfully induced diabetes mellitus in the adult male Wistar rats. The animals developed hyperglycaemia, polydipsia, hyperphagia, increased ROW in the kidney and impaired IPGTT. Moreover, these symptoms were synonymous to the classic symptoms of human type 1 diabetes mellitus (Guideline and Prevention, 2012). However, the 2% fermented RB infusion and 4% fermented HB fusions did not ameliorate these diabetogenic effects in the 6 weeks treatment period. This is possibly due to the significantly lower AOC present

in the fermented form of RB and HB compared to AOC present in green/unfermented RB and HB extracts (Sasaki, Nishida and Shimada, 2018). Furthermore, a significant reduction in fluid intake and ROW gain was observed in the diabetic rats drinking HB when compared to that of the RB-treated diabetic rats and the untreated diabetic rats respectively. In the pilot study, the diabetic rats placed on the HB treatment did not consistently consume the HB infusion. This suggests that the HB infusion possibly had a stronger or a bitter medicinal taste compared to that of the RB infusion. Thereby, causing the HB-treated diabetic rats to drink less and present with a lower ROW gain and a lower fluid intake measurement when compared to the other diabetic groups. Furthermore, it is well-established that diabetes mellitus causes hyperglycaemia and oxidative stress induced vascular damage that leads to hypertension (Tarhzaoui *et al.*, 2008; Al-Qattan *et al.*, 2016; Erejuwa *et al.*, 2011; Majithiya and Balaraman, 2006). However, in the present study, the STZ model of diabetes mellitus did not induce any significant changes in the blood pressure parameters of the diabetic animals. However, increases in blood pressure were exhibited in previously conducted studies that used an STZ dose higher than 45 mg/kg bw (Al-Qattan *et al.*, 2016; Erejuwa *et al.*, 2011; Majithiya and Balaraman, 2006; Tarhzaoui *et al.*, 2008). This suggests that significant changes in blood pressure were not exhibited in the present study due to the low STZ dose of 45 mg/kg bw used to induce diabetes mellitus. Thus, the anti-hypertensive effects of 2% fermented RB and 4% fermented HB infusions were not exhibited in the diabetic animals because their blood pressure levels were normotensive throughout the study. Furthermore, the aortic rings of the STZ-induced diabetic rats exhibited a pro-contractile and an anti-relaxation response to Phe and ACh respectively when compared to the aortic rings of the VEH control. Treatment with 2% fermented RB and 4% fermented HB ameliorated this vascular damage by significantly reducing the pro-contractile and the anti-relaxation effect that was induced by the STZ injury model. Moreover, the 2% fermented RB infusion exerted a greater pro-vasorelaxation effect than the 4% fermented HB infusion. This suggests that fermented RB possibly has a stronger restorative effect and an inherent antioxidant potential than fermented HB. Moreover, the anti-contractile and pro-relaxation effects of the 2% fermented RB and 4% fermented HB in the vascular function of the aorta was possibly mediated by the aspalathin and nothofagin flavonoids of *Aspalathus linearis* (Ku *et al.*, 2015) and by hesperidin and naringin in the *Cyclopia* species (Amamoto, Uzuki and Ase, 2008; Ikemura *et al.*, 2012). Furthermore, total and

phosphorylated eNOS expression were significantly reduced in the aortic tissue of the all diabetic groups while the P; T eNOS ratio was increased and the total and phosphorylated PKB/Akt expression was significantly reduced in the untreated (STZ) diabetic group when compared to the VEH and the water control respectively. However, treatment with 2% fermented RB or 4% fermented HB did not ameliorate the reduced eNOS and PKB expression that was induced by STZ diabetes mellitus in the 6-week treatment period. Various technical issues such as human handling of the western blot membranes may have affected the protein expression results obtained in the aortic tissue. Additionally, it is well-documented that diabetes mellitus compromises the antioxidant status of the body through hyperglycaemia-induced ROS production and oxidative stress (Maritim *et al.*, 2003; Naseeruddin *et al.*, 2009). However, in the present study, there were no significant differences in the SOD and CAT activity of the kidney of the diabetic rats when compared to the healthy controls. Moreover, the 2% fermented RB and 4% fermented HB infusions did not have any significant effects on the antioxidant enzyme expression of all treatment groups. Furthermore, the TBARS levels detected in the kidney tissue of the diabetic rats was not significantly difference from that of the healthy controls. Moreover, treatment with 2% fermented RB or 4% fermented HB did not cause any significant changes in the TBARS levels of all treatment groups. Thus, the potential antioxidant and beneficial effects of both the 2% fermented RB and 4% fermented HB in antioxidant status could not be exhibited because the STZ dose of 45 mg/kg bw used in the study did not induce oxidative stress or any significant changes in AOC of the kidney tissue as measured by means of the antioxidant enzyme and TBARS assays.

CHAPTER 5 Conclusion

5.1 Final Conclusion

Streptozotocin-induced diabetes mellitus causes hyperglycaemia, weight loss, polydipsia, a heightened appetite and vascular damage in the aortic tissue. These detrimental effects are synonymous to some of the classical symptoms observed in type 1 diabetes mellitus. Furthermore, fermented rooibos and fermented honeybush was effective in restoring vascular function with rooibos exhibiting a stronger restorative effect than honeybush. Furthermore, the antioxidant potential of these medicinal plants is possibly translated to the vasculature in different mechanisms and signalling pathways such as eNOS activation and the subsequent release of endothelial-derived nitric oxide. In the present study we hypothesized that “streptozotocin (STZ)-induced diabetic adult male Wistar rats will develop hyperglycaemia, hypertension and oxidative stress that may impair aortic endothelial function and decrease their antioxidant status”. Furthermore, we hypothesized that “treatment with 2% fermented Rooibos (*Aspalathus linearis*) or 4% Honeybush (*Cyclopia intermedia*) infusion may ameliorate or prevent the development of aortic endothelial dysfunction, hyperglycaemia, hypertension, and oxidative stress in STZ-induced diabetic adult male Wistar rats”. By the end of the present study, the STZ-induced diabetic adult male Wistar rats developed hyperglycaemia and endothelial dysfunction that was characterized by a pro-contractile and an anti-relaxation response of the aorta to Phe-induced vasoconstriction and ACh-induced vasorelaxation. However, STZ treatment did not cause oxidative stress in the kidney or have any significant effects on the blood pressure of the animals. Furthermore, treatment with 2% fermented rooibos and 4% fermented honeybush infusion significantly improved vascular reactivity by reducing the STZ-induced vasoconstriction of the aorta and increasing its vasorelaxation. However, the rooibos and honeybush infusions did not ameliorate hyperglycaemia or have any significant effects on the blood pressure and antioxidant status of the kidney of the animals in the 6-week treatment period. Considering the research question “Does fermented rooibos (*Aspalathus linearis*) and fermented honeybush (*Cyclopia intermedia*) infusion alleviate hyperglycaemia, aortic endothelial dysfunction, elevated blood pressure and oxidative stress in STZ-induced diabetic Wistar rats? The present study has

successfully addressed the research question relating to endothelial dysfunction in STZ-induced diabetic Wistar rats, thus concluding that fermented rooibos and fermented honeybush infusion does alleviate endothelial dysfunction in the aorta. However, treatment with the rooibos and honeybush infusions did not alleviate hyperglycaemia or affect blood pressure and the antioxidant status of the diabetic rats as measured in the kidney in the 6-week treatment duration.

5.2 Limitations

- The western blot analysis of other endothelial proteins involved in vascular function and the glutathione peroxidase (GPx) antioxidant assay were not performed because of time and financial constraints.
- Sections of the aortas were used to conduct aortic vascular contraction/relaxation studies thus reducing the amount of aortic tissue used in the western blot analysis of proteins involved in endothelial function.
- The 45 mg/kg bw STZ dose did not induce hypertension in the 6-week treatment duration.

5.3 Further Directions

- An STZ dose that is higher than 45 mg/kg bw should be used to induce hypertension in the animal model, so that the potential anti-hypertensive effects of fermented RB and fermented HB can be exhibited.
- The total antioxidant status of the animals was measured with a single method of analysis. Other methods of measuring total antioxidant capacity and oxidative stress such as ORAC and the TEAC assays should be conducted.
- Determination of antioxidant enzyme activity in the liver, pancreas and blood serum.
- Determination of p22phox, cleaved caspase-3, NADPH-oxidase and AMPK protein expression in the aortic tissue of diabetic rats treated with fermented RB and fermented HB.
- Determination of the mechanisms involved in the effects of fermented RB and HB on vascular function and antioxidant activity through cultured aortic endothelial cells that have been pre-exposed to oxidative stress.

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Appendix A. Liquid Chromatography Mass Spectrometry (LCMS) Analysis Of Fermented Rooibos And Fermented Honeybush

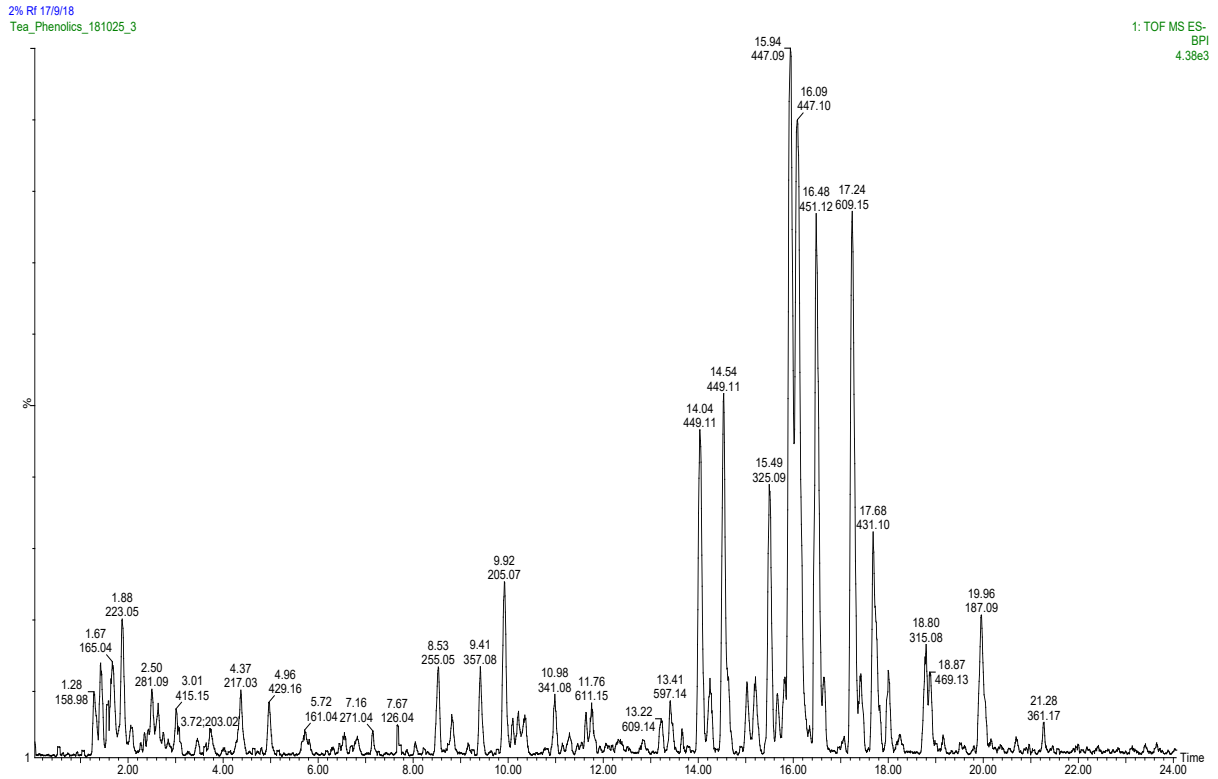


Figure A:1 LCMS determination of phenolic compounds present in the rooibos plant material used to prepare the 2% RF (fermented, 2 g/100ml) infusions consumed by the HB and DHB45 treatment groups

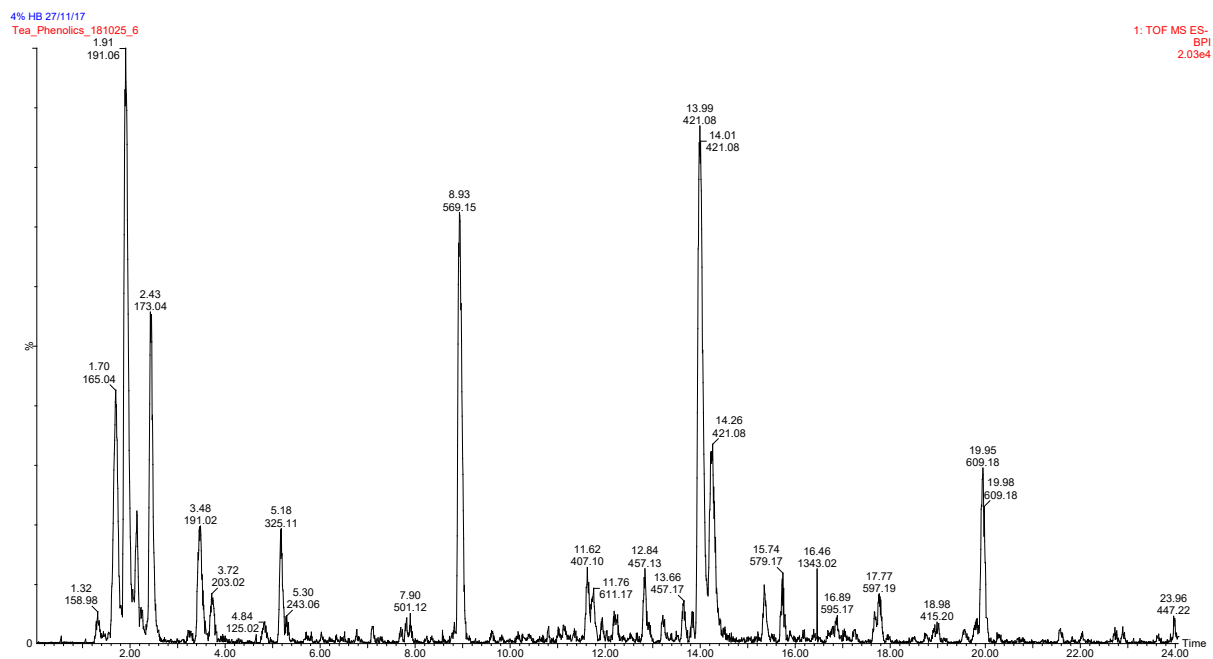


Figure A:2 LCMS determination of phenolic compounds present in the honeybush plant material used to prepare the 4% HB (fermented, 4 g/100ml) infusions consumed by the HB and DHB45 treatment groups.

Appendix B. Western Blot Gels

A. Total eNOS and Total PKB/Akt Protein Expression Gels

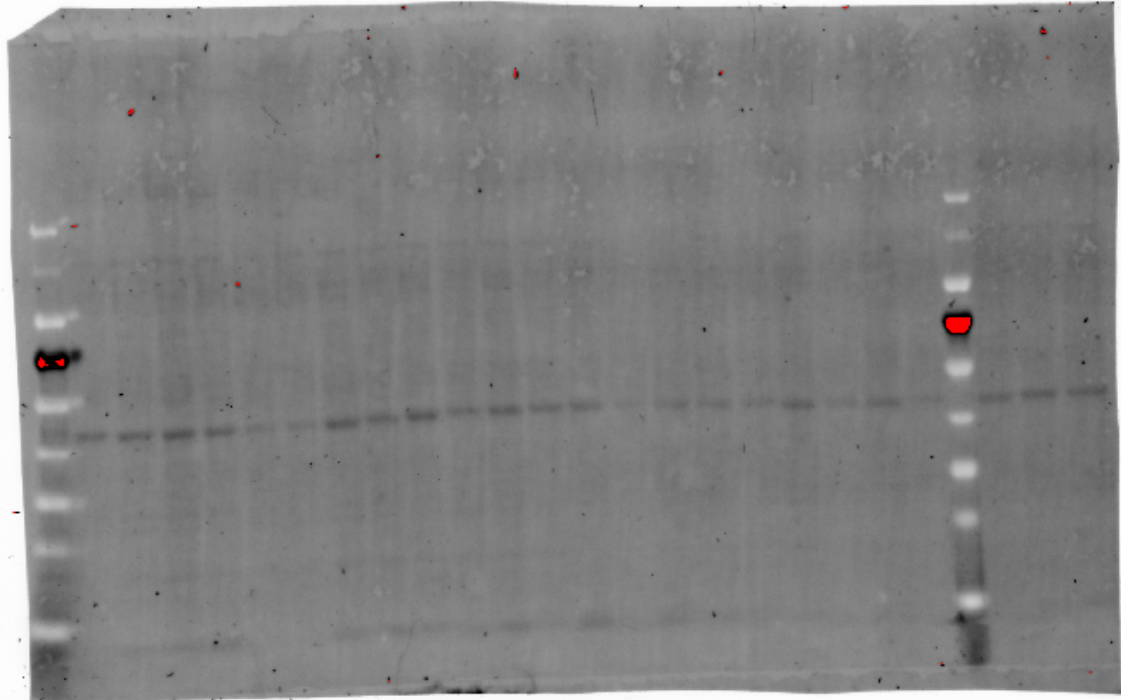


Figure B: 1 Gel 1 image of total eNOS and total PKB/Akt protein expression gel.

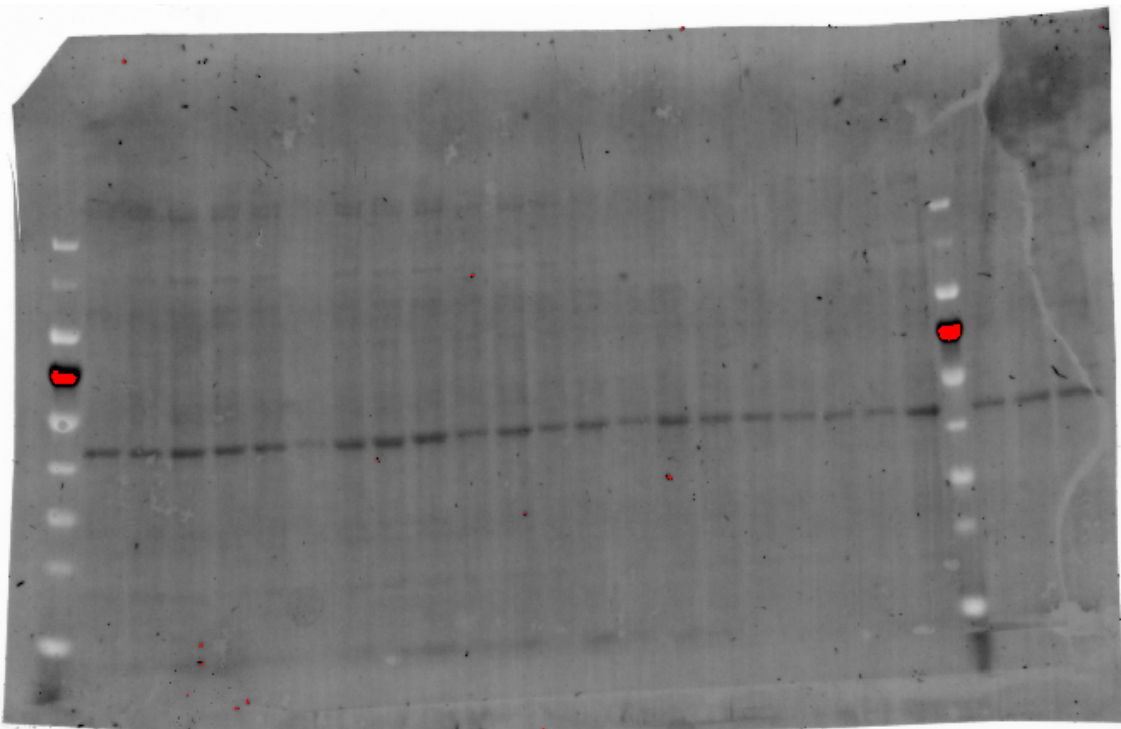


Figure B: 2 Gel 2 image of total eNOS and total PKB/Akt protein expression gel.

B. Phosphorylated eNOS and Phosphorylated PKB/Akt Protein Expression Gels

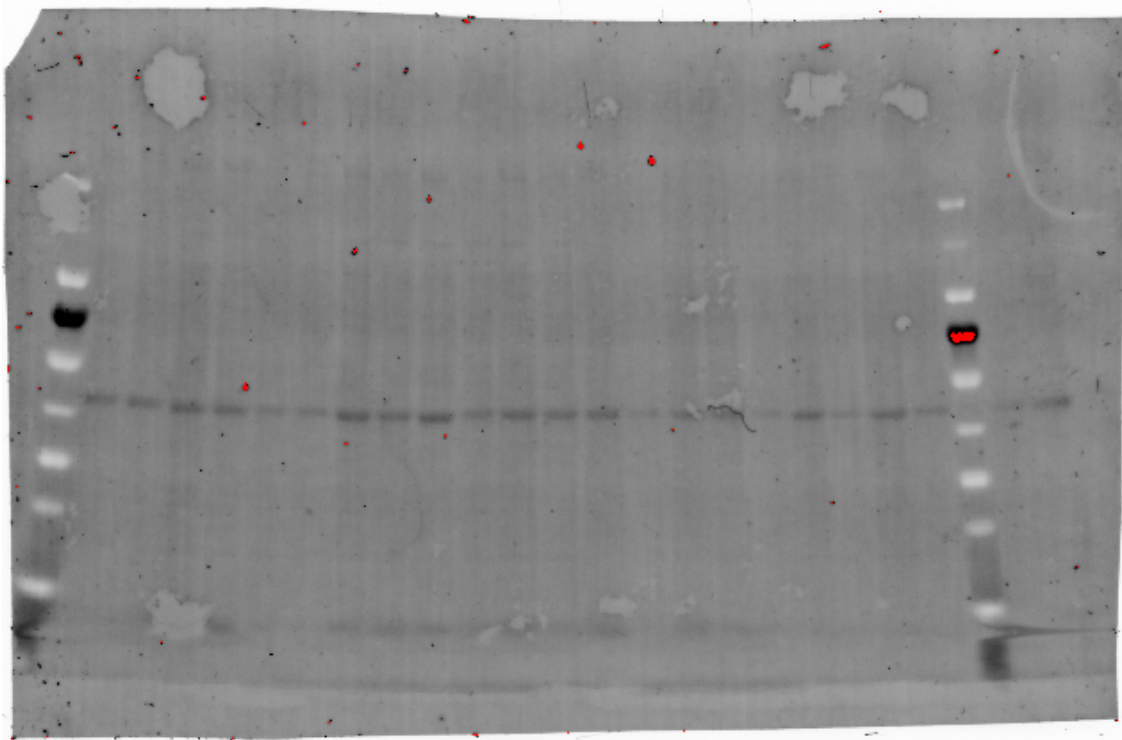


Figure B: 3 Gel 1 image of phosphorylated eNOS and phosphorylated PKB/Akt protein expression gel.

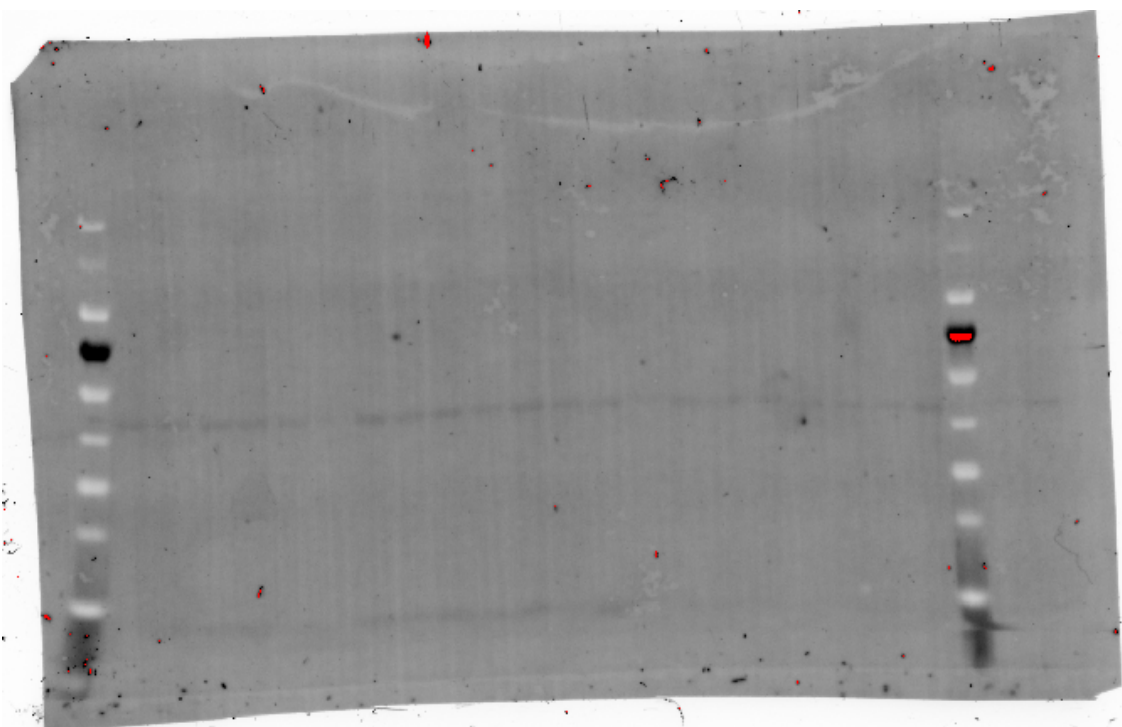


Figure B: 4 Gel 2 image of phosphorylated eNOS and phosphorylated PKB/Akt protein expression gel.

Appendix C. Western Blot Membranes

A. Total eNOS Membranes

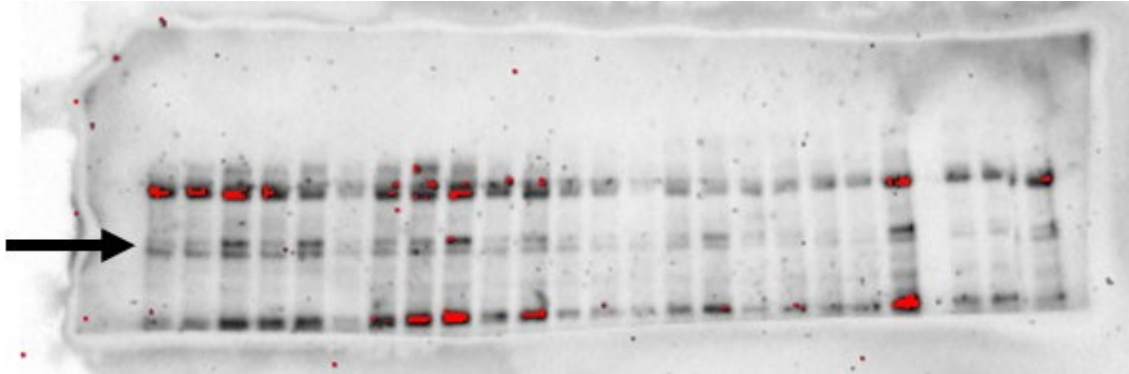


Figure C: 1 Blot 1 membrane of total eNOS protein expression.

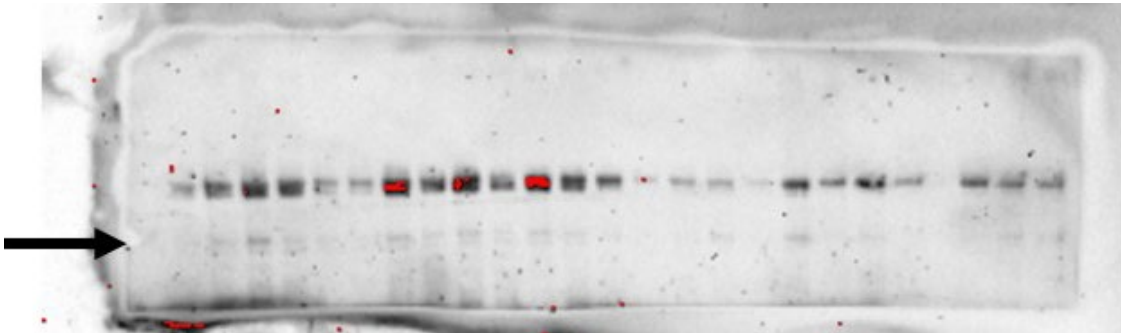


Figure C: 2 Blot 2 membrane of total eNOS protein expression.

B. Phosphorylated eNOS Membranes

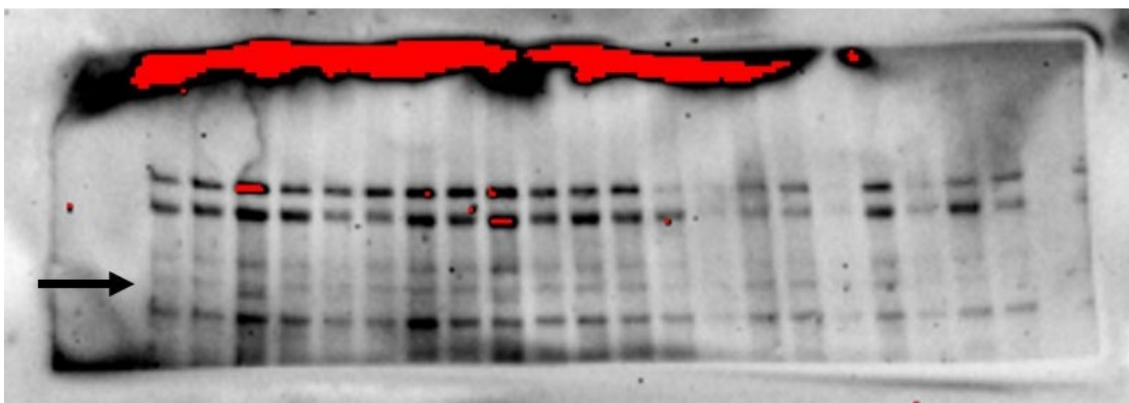


Figure C: 3 Blot 1 membrane of phosphorylated eNOS protein expression.

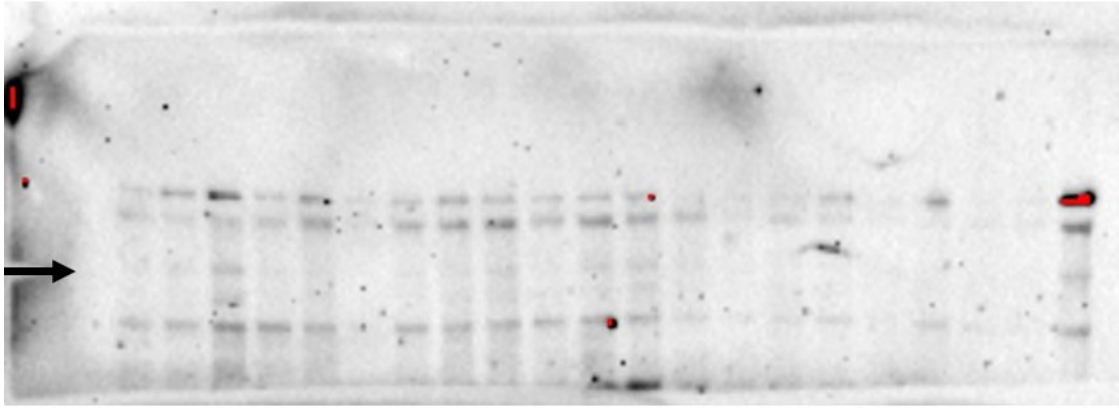


Figure C: 4 Blot 2 membrane of phosphorylated eNOS protein expression.

C. Total PKB/Akt Membranes

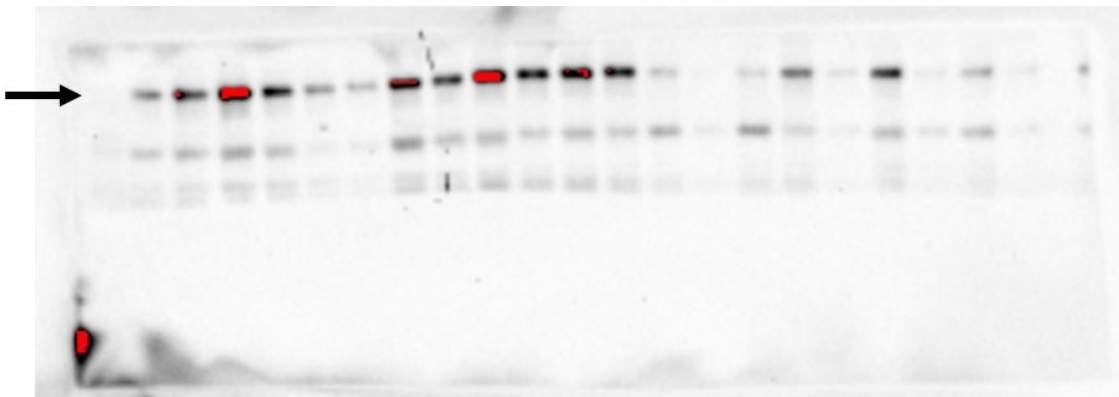


Figure C: 5 Blot 1 image of total PKB/Akt protein expression membrane.

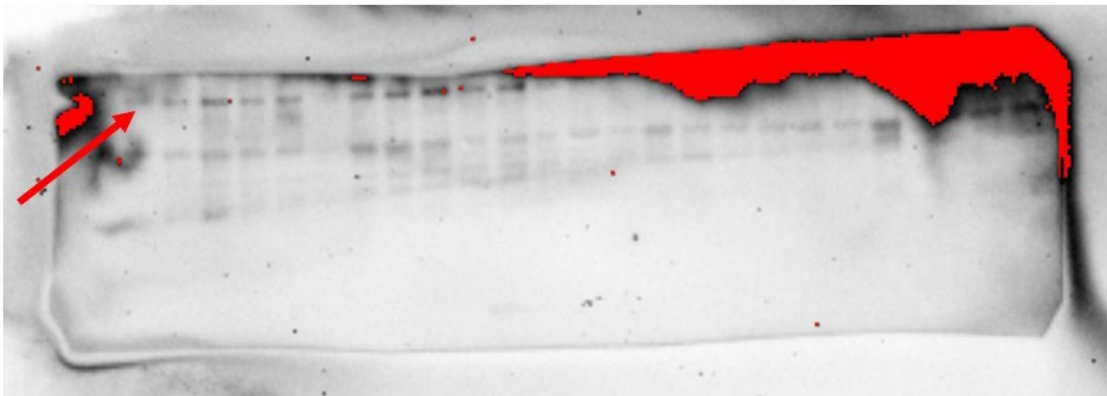


Figure C: 6 Blot 2 image of total PKB/Akt protein expression membrane (Damaged, not used in calculation of results).

D. Phosphorylated PKB/Akt Membranes

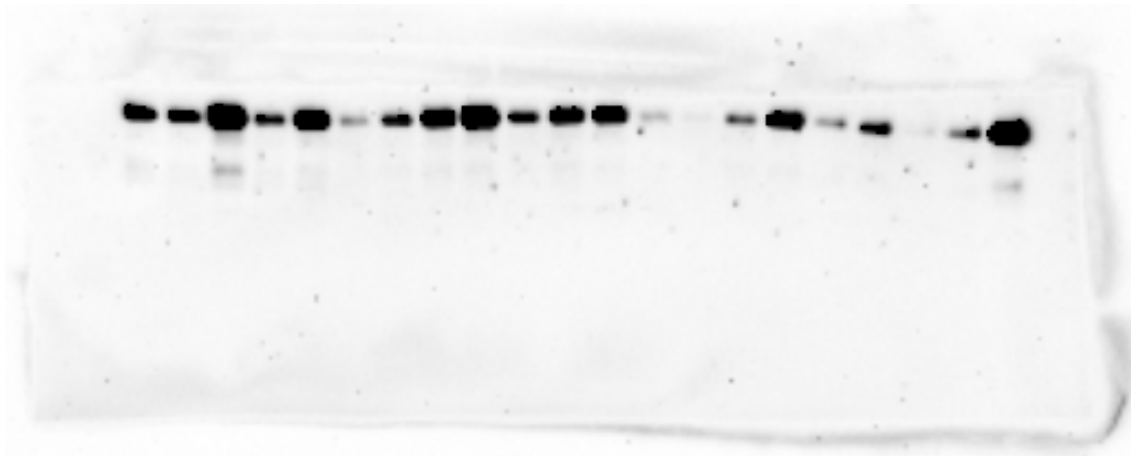


Figure C:7 Blot 1 image of phosphorylated PKB/Akt membrane.

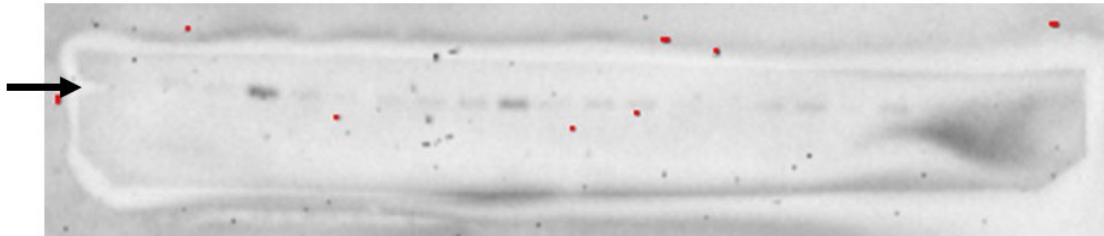


Figure C:8 Blot 2 image of phosphorylated PKB/Akt protein expression membrane (Damaged, not used in calculation of results).

Appendix D. Calculation of Total Polyphenol Daily Intake and Total Flavonol Daily Intake

The total daily polyphenol and total daily flavonol intake of the control and diabetic groups was calculated according to the formula below and as shown in the example given.

Data needed for the calculations:

Group	Soluble Solids (mg/ml)	Fluid Intake (ml/day)	Mean Body Weight (g)	Total Polyphenol Content (mg gallic acid/mg soluble solids)
RB control	5.18	48.41	347.4	0.17

The rooibos infusion has a total polyphenol content of 0.17 mg/mg soluble solids. Since there are 5.18 mg of soluble solids per ml of rooibos, firstly we need to multiply these values to know the amount of total polyphenols in 1 ml of infusion:

$$5.18 \text{ mg/ml} \times 0.17 \text{ mg/mg} = 0.88 \text{ mg/ml}$$

So, there are 0.88 mg of total polyphenols in 1 ml of rooibos. We also know their fluid intake per day, so we can calculate the total amount of polyphenols they took in per day:

$$0.88 \text{ mg/ml} \times 48.41 \text{ ml/day} = 42.6 \text{ mg/day}$$

That means, on average, a rat in the RB control group took in 42.6 mg total polyphenols per day. All we have to do now is convert that to an amount per 100 g of body weight, so that the values can be compared between the groups. For this we use the body weight of each animal.

$$(42.6 \text{ mg/day} / 347.4 \text{ g}) \times 100 \text{ g} = 12.26 \text{ mg/day}$$

For the RB control group:

Daily total phenolic intake (mg gallic acid equivalents/day / 100g BW) is 12.26.